Regulation of the switch from early to late bacteriophage λ DNA replication

Sylwia Barańska,¹ Magdalena Gabig,² Alicja Węgrzyn,²
 Grażyna Konopa,¹ Anna Herman-Antosiewicz,¹ Pablo Hernandez,³
 Jorge B. Schwartzman,³ Donald R. Helinski⁴ and Grzegorz Węgrzyn^{1,5}

Author for correspondence: Grzegorz Węgrzyn. Tel: +48 58 346 3014. Fax: +48 58 301 0072.
 e-mail: wegrzyn@biotech.univ.gda.pl

^{1,2} Department of Molecular Biology, University of Gdańsk¹ and Laboratory of Molecular Biology (affiliated with the University of Gdańsk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences², Kładki 24, 80-822 Gdańsk, Poland

³ Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006 Madrid, Spain

⁴ Center for Molecular Genetics and Department of Biology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

⁵ Marine Biology Center, Polish Academy of Sciences, Św. Wojciecha 5, 81-347 Gdynia, Poland

There are two modes of bacteriophage λ DNA replication following infection of its host, *Escherichia coli*. Early after infection, replication occurs according to the theta (θ or circle-to-circle) mode, and is later switched to the sigma (σ or rolling-circle) mode. It is not known how this switch, occurring at a specific time in the infection cycle, is regulated. Here it is demonstrated that in wild-type cells the replication starting from *ori* λ proceeds both bidirectionally and unidirectionally, whereas in bacteria devoid of a functional DnaA protein, replication from *ori* λ is predominantly unidirectional. The regulation of directionality of replication from *ori* λ is mediated by positive control of λ p_R promoter activity by DnaA, since the mode of replication of an artificial λ replicon bearing the p_{tet} promoter instead of p_R was found to be independent of DnaA function. These findings and results of density-shift experiments suggest that in *dnaA* mutants infected with λ , phage DNA replication proceeds predominantly according to the unidirectional θ mechanism and is switched early after infection to the σ mode. It is proposed that in wild-type *E. coli* cells infected with λ , phage DNA replication proceeds according to a bidirectional θ mechanism early after infection due to efficient transcriptional activation of *ori* λ , stimulated by the host DnaA protein. After a few rounds of this type of replication, the resulting increased copy number of λ genomic DNA may cause a depletion of free DnaA protein because of its interaction with the multiple DnaA-binding sites in λ DNA. It is proposed that this may lead to inefficient transcriptional activation of *ori* λ resulting in unidirectional θ replication followed by σ type replication.

Keywords: bacteriophage λ development, *Escherichia coli* DnaA protein, rolling-circle DNA replication, theta DNA replication, transcriptional activation of origin

INTRODUCTION

Bacteriophage λ has played a crucial role in the development of molecular biology (Thomas, 1993; Gottesman, 1999). It is a paradigm of molecular mechanisms of many general biological processes including DNA replication and it still serves as an important model system for molecular biology research. For example, the results obtained with λ replicons were successfully employed in studies on eukaryotic DNA replication (Stillman, 1994), and mechanisms of replication of some animal viruses (e.g. herpes simplex virus) resemble those found in λ (Kornberg & Baker, 1992; Subak-Sharpe & Dargan, 1998).

The genome of bacteriophage λ consists of 48 502 bp of double-stranded DNA (see Fig. 1). In the λ virion, this genetic material is packaged in a linear form in the head of the phage capsid. After adsorption to the surface of its host, *Escherichia coli*, and penetration of the phage genome into the bacterial cell, the linear DNA is immediately converted into the circular form due to single-stranded 5' extensions of 12 bases at both ends, which are complementary to each other. The ends are ligated by the host DNA ligase and, following the action of *E. coli* DNA gyrase, the phage genome becomes a negatively supercoiled structure (for recent reviews on λ DNA replication and phage development see Taylor & Węgrzyn, 1995, 1998).

Fig. 1. (a) A genetic map of bacteriophage λ with the replication region enlarged. Positions of certain important genes are marked. The replication region of the λ genome (present also in a typical λ plasmid) is presented in the lower part of the figure. All promoters and terminators present in the replication region are indicated. Actions of proteins forming the preprimosomal complex at *oriλ*, and repression and stimulation of the *p_R* promoter by Cro and DnaA, respectively, are indicated. (b) A map of λ plasmid pKB2 with marked λ genes and restriction sites used in this work during analysis of the directionality of replication by two-dimensional agarose gel electrophoresis and electron microscopy. pKB2 π is a pKB2 derivative bearing a single nucleotide substitution, C to A at nucleotide 422 of the *P* gene (Węgrzyn *et al.*, 1996).

The λ -encoded replication proteins O and P are synthesized early after phage infection, since the O–P region is situated downstream of the *p_R* promoter and the weak *t_{R1}* terminator (Fig. 1). The O protein is a prototype initiator protein, which binds to the origin of replication, *oriλ*, and directs other viral and host replication proteins (including DnaB helicase) to this site in the process of replication complex assembly. Loading of DnaB helicase (liberated from P-mediated inhibition by DnaK, DnaJ and GrpE proteins) seems to be connected with the *p_R*-initiated transcriptional activation of *oriλ*, which is stimulated by the host DnaA function (A. Węgrzyn *et al.*, 1995a; G. Węgrzyn *et al.*, 1995a; Węgrzyn *et al.*, 1996; Szalewska-Pałasz *et al.*, 1998a). This allows for the establishment of bidirectional θ replication of the λ genome, which is observed at the early stages of infection (Schnos & Inman, 1970). In an *in vitro* system reconstituted from purified proteins (and lacking HU protein), λ DNA replication was shown to be independent of transcription (Alfano & McMacken, 1989a, b; Żylicz *et al.*, 1989) while proceeding unidirectionally (Mensa-Wilmot *et al.*, 1989). However, addition of RNA polymerase resulted in the appearance of a significant fraction of λ DNA molecules replicating bidirectionally (Learn *et al.*, 1993).

After five to six rounds of bidirectional θ replication, about 50 copies of the circular λ genome appear in the

infected host cell. Then, at about 15 min post-infection, a few of these molecules start to replicate according to the σ mode (Taylor & Węgrzyn, 1995, 1998). This leads to the production of long concatemers of λ DNA, up to about ten genome equivalents in length, which are cut at specific sites (called *cos* sites) and serve as the substrates for the phage packaging system. The mechanism of the switch from θ to σ replication has not been completely elucidated, but according to the predominant hypothesis, originally proposed by Dodson *et al.* (1986), σ may be preceded by one round of unidirectional θ replication initiated at *oriλ*, followed by displacement of the 5' end of the newly synthesized leading strand by its growing 3' end.

Although the mechanism of the switch from θ to σ replication has been proposed (see above), it remains unknown how this process is regulated and what triggers the change in the replication mode of λ DNA. We have found previously that the host *dnaA* gene function has a role in phage λ replication (G. Węgrzyn *et al.*, 1995b). The findings that (i) DnaA stimulates transcriptional activation of *oriλ* (G. Węgrzyn *et al.*, 1995a; Szalewska-Pałasz *et al.*, 1998a); (ii) transcription is necessary for bidirectional θ replication of λ DNA *in vitro* (Learn *et al.*, 1993); (iii) unidirectional θ replication is considered as a prerequisite for σ replication (Dodson *et al.*, 1986); and (iv) σ replication intermediates of the phage bearing

mutations in the *P* gene appear early after infection of the *dnaA* host (G. Węgrzyn *et al.*, 1995b; Konopa *et al.*, 2000), suggested that DnaA may be a crucial factor in triggering the switch from θ to σ replication of phage λ DNA in infected *E. coli* cells. Therefore, the aim of this work was to investigate the mechanism of regulation of this switch in wild-type λ phage and the role of the DnaA protein in this process.

METHODS

Bacterial strains, phages and plasmids. *Escherichia coli* wild-type strain MG1655 (Jensen, 1993) and its *dnaA46(ts) tnaA::Tn10* derivative (strain BM746), constructed by P1 transduction from strain BM215 (A. Węgrzyn *et al.*, 1995b), were used. An isogenic pair of *dnaA*⁺ and *dnaA::cm* (*dnaA* null) strains were also employed: this pair consists of the *dnaA::cm* strain TC3478 [*dnaA::cm araD139 Δ (ara-leu) Δ lacX74 galK galU hsdR rnh-373 rpsL thi*], which was described by Ingmer & Atlung (1992), and its *dnaA*⁺ *zid-3162::Tn10kan* derivative (BM3477), which was constructed by P1 transduction from strain CAG18558, described by Singer *et al.* (1989). Phages λ clb2 and λ red3cl857S7 (G. Węgrzyn *et al.*, 1995b) were used. λ plasmids pKB2 and pKB2 π have already been described by Kur *et al.* (1987). Plasmid pTC λ 1, an artificial λ plasmid bearing the *p*_{tet} promoter instead of *p*_R, was constructed by Herman-Antosiewicz *et al.* (1998a).

One-step growth experiments. Lytic development of bacteriophage λ was investigated by one-step growth experiments as described previously (G. Węgrzyn *et al.*, 1995b). Briefly, bacteria growing exponentially in LB medium at 30 °C were infected by phage λ clb2 in the presence of 3 mM NaN₃ (to prevent unsynchronized phage development) at the indicated m.o.i., and adsorption was carried out for 10 min at 43 °C. Following centrifugation, the bacterial pellet was resuspended in the same medium (with NaN₃) containing anti- λ serum and incubated for 5 min at 43 °C to neutralize unadsorbed phages. The suspension was then diluted 1000-fold with prewarmed (to 43 °C) medium (devoid of NaN₃) and aerated in a water-bath shaker. The number of 'infective centres' was estimated by plating samples taken during the first 10 min after dilution (time 0–10 min). In fact, the estimated number of 'infective centres' is the sum of the number of infected bacteria and the number of free, unadsorbed phages. However, as most of the unadsorbed phage particles were neutralized by anti- λ serum, the second value was ignored during the calculation of the burst size. The number of intracellular progeny phage was estimated by plating chloroform-treated samples of the infected culture withdrawn at different times, using strain MG1655 as a host. The burst size was calculated as the ratio of the number of progeny phages to the number of infective centres.

Density-shift experiments. These were performed according to G. Węgrzyn *et al.* (1995b). Briefly, in the first type of experiment, bacteria were grown in a 'light' minimal medium (Węgrzyn, G. *et al.*, 1995b) overnight at 30 °C, and after dilution (1:50, v/v) with fresh medium the growth was continued to an OD₅₀₀ of 0.2. The bacteria were pelleted, washed with TM buffer (10 mM Tris/HCl, pH 7.2, 10 mM MgSO₄) and suspended in 0.1 vol. of this buffer. After 60 min incubation at 43 °C, [³H]thymidine-labelled phage (8.3 × 10⁻⁵ c.p.m. p.f.u.⁻¹) was added to a m.o.i. of 10 and incubation was continued for 15 min. The suspension was sedimented, resuspended in the original volume of prewarmed (43 °C) 'heavy' minimal medium (containing ¹⁵NH₄Cl and

Fig. 2. Phage λ clb2 development in *E. coli* *dnaA*⁺ (MG1655; squares) and *dnaA46(ts)* (BM746; circles) (a), and *E. coli* *dnaA*⁺ (TC3478, squares) and *dnaA::cm* (BM3477, circles) (b) hosts at 43 °C. Phage burst size [p.f.u. per infective centre (p.f.u. IC⁻¹)] is presented.

[¹³C]glucose instead of NH₄Cl and glucose, respectively), and further incubation was performed at 43 °C. Samples of the infected culture were withdrawn at the indicated times, and total DNA was isolated and ultracentrifuged in a CsCl density gradient as described previously (G. Węgrzyn *et al.*, 1995b). Fractions were collected from the bottom of the tube and the radioactivity of each fraction was measured in a scintillation counter. In the second type of experiment, the procedure was the same as described above but bacteria growing in the 'light' medium were infected with unlabelled phage (m.o.i. of 10) and further incubation was continued in the 'heavy' medium containing 0.1 mCi [³H]thymidine ml⁻¹.

Measurement of total DNA synthesis. Synthesis of DNA in cells was investigated by measurement of incorporation of [³H]thymidine into trichloroacetic acid (TCA)-precipitable material according to the method described by Węgrzyn *et al.* (1991). Briefly, the bacterial cultures were labelled with 0.1 mCi [³H]thymidine ml⁻¹. Samples were withdrawn at the indicated times and transferred onto paper filters. The filters were placed immediately in 10% ice-cold TCA for 5 min, and transferred to 5% TCA for 5 min. The samples were then washed twice with 96% ethanol and dried at room temperature. The radioactivity of the samples was measured in a scintillation counter.

Two-dimensional agarose gel electrophoresis. Analysis of replication intermediates by two-dimensional agarose gel electrophoresis was performed according to Viguera *et al.* (1996), with modifications described by Śrutkowska *et al.* (1999).

Electron microscopy. Isolation of plasmid DNA was performed according to Viguera *et al.* (1996) and electron microscopy analysis of replicating plasmid DNA molecules was performed as described by Burkardt & Lurz (1984) and Śrutkowska *et al.* (1998).

RESULTS

Phage λ development in *E. coli* *dnaA*⁺ and *dnaA* hosts

Our previous studies on the role of the *dnaA* gene in bacteriophage λ development were performed with *E.*

Fig. 3. For legend see facing page.

Fig. 3. The fate of DNA of infecting λ phage in *E. coli* *dnaA*⁺ (MG1655) and *dnaA46(ts)* (BM746) (a), and *E. coli* *dnaA*⁺ (TC3478) and *dnaA::cm* (BM3477) (b) hosts at 43 °C. Bacteria growing in light medium at 30 °C were infected with phage λ red3cI857S7 (m.o.i. of 10) labelled with [³H]thymidine (8.3×10^{-5} c.p.m. p.f.u.⁻¹), and further incubation was performed in heavy medium (containing [¹³C]glucose and [¹⁵NH₄]Cl instead of glucose and NH₄Cl, respectively) at 43 °C. Samples were withdrawn at the indicated times, total DNA was isolated and centrifuged in a CsCl density gradient. Five-drop fractions were collected from the bottom of the tube and the radioactivity in each fraction was measured in a scintillation counter.

coli laboratory strains which were heavily mutagenized in their long history (G. Węgrzyn *et al.*, 1995b). Since such strains may also contain some as yet unidentified mutations (Glinkowska *et al.*, 1999), we decided to use in most experiments of the present work *E. coli* wild-type strain MG1655 and its derivatives. In accordance with previous results obtained with different *E. coli* K-12 hosts (G. Węgrzyn *et al.*, 1995b; Sutton & Kaguni, 1997; Szalewska-Pałasz *et al.*, 1998b) we found that the presence of the *dnaA46(ts)* mutation in the MG1655 genetic background allows for normal phage λ development at 43 °C (Fig. 2a). In these experiments we used phage λ cIb2, which is able only to grow by the lytic pathway, to avoid a possible influence of lysogenization on the results of our studies concerning the lytic pathway (note that phage DNA replication is also a part of this developmental mode). Analogous experiments performed with phage λ red3cI857S7 (used in the density-shift experiments; see below) gave very similar results, i.e. no considerable differences between the kinetics of intracellular phage development in the *dnaA*⁺ and *dnaA46(ts)* host at 43 °C (data not shown).

Since in the *dnaA46(ts)* host some residual activity of DnaA protein might be retained, we repeated the experiments described above except that a pair of isogenic *dnaA*⁺ and *dnaA* null (*dnaA::cm*) strains were used. Development of phage λ cIb2 (Fig. 2b) and phage λ red3cI857S7 (data not shown) was not affected by the absence of the *dnaA* function.

Phage λ DNA replication in *E. coli* *dnaA*⁺ and *dnaA* hosts

To investigate phage λ DNA replication in infected cells we employed density-shift experiments. The cells growing in a light medium were mixed with phage lysate previously labelled with [³H]thymidine, and after adsorption the cultivation was continued in a heavy medium. We found that the adsorption efficiency of the phage on both *dnaA*⁺ and *dnaA46(ts)* hosts was very similar in these conditions, with a difference below 5 %. The fate of parental phage DNA was monitored by isolation of total DNA and ultracentrifugation in a caesium chloride density gradient. The recovery of labelled phage DNA was similar in both strains: 31 % in *dnaA*⁺ and 25 % in *dnaA46(ts)*, on average. In these experiments we used the λ red3 mutant to impair recombination between phage DNA molecules. When wild-type (MG1655; *dnaA*⁺) cells were infected, the phage DNA (originally all of it located at the full-light position, data not shown) was found in the full-light (non-replicated molecules) and heavy-light (molecules after at least one replication round) positions 5 and 15 min after infection (Fig. 3a). All radioactivity moved to the heavy-light fractions at 30 min, where it remained until the end of the experiment (60 min). Since we monitored the fate of parental phage DNA (labelled with [³H]thymidine), several rounds of replication proceeding by the θ mode would result in the shift of radioactivity only to the heavy-light position. At later times after infection σ replication intermediates should appear and if parental DNA molecules were to enter this mode of replication, a further shift towards full-heavy position should be observed (total length of σ intermediates is up to about ten λ genomes; thus, if such a molecule contained one strand of light ³H labelled DNA of the length of one unit of the genome and the rest of the duplex DNA structure were composed of heavy nucleotides, it should be located close to the full-heavy position). However, since on average only a few of about 50 λ DNA circles that appear due to θ replication enter rolling-circle replication (see Taylor & Węgrzyn, 1995), no significant shift towards the full-heavy position was observed (Fig. 3a). Different results were obtained when the *dnaA46(ts)* bacteria were infected at 43 °C. The shift towards the full-heavy position was already observed 15 min post-infection and it was much more significant at later times (Fig. 3a). This shift strongly suggests that a larger fraction of parental phage DNA molecules enter σ replication at this temperature.

The effect of the *dnaA::cm* allele on phage λ DNA replication was similar to that of the *dnaA46(ts)*

mutation at 43 °C (Fig. 3b). In these experiments, longer incubation times were employed due to considerably longer generation times of the *rnh dnaA⁺* and *rnh dnaA::cm* strains relative to MG1655-derived strains. Similar to experiments with the *dnaA46(ts)* mutant, both the efficiency of phage adsorption and the recovery of parental phage DNA were comparable in *dnaA⁺* and *dnaA* null hosts (data not shown).

Phage λ DNA synthesis in *E. coli dnaA⁺* and *dnaA46(ts)* hosts at 43 °C

The results presented in the preceding paragraphs suggested that contrary to the λ -infected wild-type host, the early round(s) of replication of phage λ DNA in the *dnaA* mutants may proceed predominantly by the σ mode. Therefore, on the basis of the previously proposed model concerning the mechanism of the switch from early to late λ DNA replication (Dodson *et al.*, 1986), one may speculate that early after infection of the host devoid of DnaA, λ DNA replicates according to the unidirectional θ mode, which is followed by σ replication. To test this hypothesis, we repeated the density-shift experiments except that a non-labelled phage was used. Bacteria growing in the light medium were infected with light phage and further incubation was continued in the heavy medium containing [3 H]thymidine. Several rounds of θ replication of the infecting phage genome should lead to the appearance of radioactive DNA at the full-heavy position relatively shortly after infection. Up to 30 min after the shift from light to heavy medium, one replication round of the host chromosome is possible at most, thus any radioactivity found in the full-heavy fraction must come from newly synthesized λ DNA. We determined that under the conditions used in these experiments, incorporation of [3 H]thymidine into TCA-precipitable material was effective during the first 15 min, and later the efficiency of incorporation dropped significantly (Fig. 4), most probably due to exhaustion of radioactive thymidine. As samples for DNA isolation and density-gradient centrifugation were withdrawn 15 and 30 min post-infection, radioactivity in the full-heavy fractions observed after the first time interval represented λ DNA molecules synthesized according to the θ mode, and that observed after the second time interval indicated the fate of λ DNA already synthesized during the first 15 min post-infection. We found considerable radioactivity in the full-heavy fractions in samples of the culture of the λ -infected *dnaA⁺* host withdrawn 15 min post-infection (Table 1), indicating that several rounds of θ replication had occurred. However, low activity was observed in analogous fractions in samples of the culture of the *dnaA46(ts)* host infected with λ at 43 °C (Table 1). Therefore, it may be suggested that at most one round of θ replication was possible in the case of a predominant number of infecting phage DNA molecules. Effective λ DNA replication in the *dnaA46(ts)* mutant during the first 15 min was confirmed by monitoring the distribution of radioactivity in samples withdrawn 30 min post-infection. Significant radioactivity in the full-heavy fractions was

Fig. 4. Incorporation of [3 H]thymidine (0.1 mCi ml $^{-1}$) into TCA-precipitable material in *E. coli dnaA⁺* (MG1655; squares) and *dnaA46(ts)* (BM746; circles) hosts growing in heavy medium at 43 °C.

Table 1. The fate of phage λ DNA synthesized during infection of *E. coli dnaA⁺* and *dnaA46(ts)* hosts at 43 °C

Bacteria growing in light medium at 30 °C were infected with phage λ red3c1857S7 (m.o.i. of 10) and further incubation was performed in heavy medium (containing [13 C]glucose and $^{15}\text{NH}_4\text{Cl}$ instead of glucose and NH_4Cl , respectively) supplemented with [3 H]thymidine (0.1 mCi ml $^{-1}$) at 43 °C. Samples were withdrawn at the indicated times, total DNA was isolated and centrifuged in a CsCl density gradient. Five-drop fractions were collected from the bottom of the tube and the radioactivity in each fraction was measured in a scintillation counter. The radioactivity from full-heavy fractions is presented.

Host*	Radioactivity (c.p.m.)	
	15 min	30 min
<i>dnaA⁺</i>	32 668	32 929
<i>dnaA46</i>	2033	15 710

* *E. coli* MG1655 (*dnaA⁺*) and BM746 (*dnaA46*) strains were used as hosts.

found, perhaps representing σ replication intermediates (Table 1). Incorporation of radioactivity into DNA in the heavy–light fractions (mostly *E. coli* chromosomal DNA) was only slightly lower in the *dnaA46(ts)* host than in the wild-type strain (15 min post-infection the radioactivity in heavy–light fractions in the sample from the *dnaA46(ts)* bacteria was 71% of that found in the sample from the wild-type bacteria, and 30 min post-infection, the radioactivity in heavy–light fractions in

Fig. 5. (a) Theoretical schemes of expected pictures of two-dimensional agarose gel electrophoresis of λ plasmid (pKB2 or pKB2 π), digested with *Hind*III/*Bam*HI, replicating according to unidirectional leftward θ mode (Uni-L), unidirectional rightward θ mode (Uni-R), bidirectional θ mode (Bi) and combination of molecules replicating according to all these modes (All). (b) Results obtained after isolation of plasmid DNA from cultures of *dnaA*⁺ (MG1655) and *dnaA46* (BM746) hosts bearing pKB2 or pKB2 π and growing at different temperatures.

Fig. 6. Examples of electron microscopy analysis of λ plasmid (pKB2 or pKB2 π) replication intermediates. Plasmid molecules were isolated from *E. coli* cells, digested with *Hind*III/*Bam*HI, prepared for electron microscopy, and analysed by measuring lengths of replication bubbles and/or arms in bubble-containing and Y-shaped replication intermediates. For determination of the directionality of replication, the known position of *ori* λ was assumed as the only possible replication start point. Examples of bidirectionally replicating molecule (a) and unidirectionally replicating molecule (b) are presented (positions of *ori* λ s are indicated by arrows). Bars, 250 nm.

the sample from the *dnaA46*(ts) bacteria was 86% of that found in the sample from the wild-type bacteria). Moreover, we observed no shift of radioactivity to the full-heavy position in analogous samples from non-infected cultures (data not shown).

Despite the fact that some other possible (though, in our opinion, less likely) explanations of the data presented above could be provided, these results are still compatible with the hypothesis that in the absence of DnaA function λ DNA replicates predominantly according to the unidirectional θ mode, and after one round of such replication switches to the σ mode. Thus, the next step in our studies was to test the directionality of λ DNA replication in the presence and absence of DnaA function.

Directionality of θ replication of λ DNA analysed by two-dimensional agarose gel electrophoresis

Plasmids derived from bacteriophage λ (referred to as λ plasmids) contain all the genes and regulatory sequences necessary for autonomous replication in *E. coli*. These plasmids replicate in the host cells by the θ mode (for

reviews see Taylor & Węgrzyn, 1995, 1998). Therefore, we used the λ plasmids in studies on the directionality of θ replication of λ DNA in *dnaA*⁺ and *dnaA46*(ts) strains. Wild-type λ plasmids cannot be maintained in certain *dnaA*(ts) mutants (including *dnaA46*) even at 30 °C, but derivatives bearing a mutation of the π type in the λ *P* gene can (Kur *et al.*, 1987; Węgrzyn *et al.*, 1996). Thus, in experiments with a *dnaA46*(ts) host we used the π mutant plasmid. Since it was demonstrated previously that even $\lambda\pi$ plasmids can perform only one replication round in the *dnaA46*(ts) host after a shift from 30 °C to 43 °C (Kur *et al.*, 1987), we isolated plasmid DNA for two-dimensional agarose gel electrophoresis analysis 15 min after the temperature shift.

We used *E. coli dnaA*⁺ and *dnaA46*(ts) hosts bearing plasmids pKB2 and pKB2 π , respectively. Following isolation, these plasmids were digested with different restriction enzymes (shown in Fig. 1b) and analysed by two-dimensional agarose gel electrophoresis. Theoretical patterns of λ plasmid replication intermediates in the case of bidirectional and unidirectional (leftward and rightward) replication in samples digested with *Hind*III and *Bam*HI, predicted by a computer method assuming that replication forks initiate synchronously and travel at the same rate (Viguera *et al.*, 1998), are presented in Fig. 5(a). The results obtained and their interpretation are presented in Fig. 5(b). In the *dnaA*⁺ host, replication of wild-type λ plasmid (pKB2) proceeds both bidirectionally and unidirectionally. Bidirectional replication is represented by the characteristic shape of the bubble arc and the intensive descending arm of the simple-Y arc. The dots at the top and at the ascending arm of the simple-Y arc suggest rightward and leftward unidirectional replication, respectively. No significant differences in the pattern of replication intermediates were observed between DNA samples isolated from *dnaA*⁺/pKB2 bacteria growing at different temperatures: 30 °C, 37 °C or 43 °C (data not shown). Similar to the wild-type plasmid in the *dnaA*⁺ host, replication of pKB2 π in *dnaA46* cells at 30 °C proceeds both bidirectionally and unidirectionally. In the case of unidirectional replication, the intermediates of both leftward and rightward replication were detected. Thus, the presence of the π mutation in the plasmid and *dnaA46*(ts) mutation in the host genome has no significant influence on the directionality of plasmid replication at 30 °C. However, analysis of pKB2 π replication intermediates isolated from the *dnaA46*(ts) mutant growing at 43 °C revealed that bidirectional replication was less frequent relative to that observed at 30 °C as the descending arm of the simple-Y arc is considerably less intensive.

In conclusion, replication of λ plasmids in *E. coli* cells proceeds both bidirectionally and unidirectionally, but in the absence of *dnaA* function (*dnaA46* mutant at 43 °C) the bidirectional replication seems to be less frequent. This was also confirmed during the analysis of replication intermediates after digestion of plasmid DNA with other restriction enzymes: *Bgl*II and *Sal*I, *Eco*RV and *Sal*I, and *Nde*I (data not shown).

Table 2. Electron microscopy analysis of the directionality of λ plasmid replication

E. coli MG1655 (*dnaA*⁺) and BM746 (*dnaA46*) strains were used as hosts. Plasmids pKB2 and pKB2 π (in the *dnaA46* host) were investigated. About 100 Y-shaped or bubble-containing plasmid fragments (*Hind*III–*Bam*HI fragments bearing *ori* λ) were analysed in each sample. Only the molecules of the expected lengths were considered. Since in a population of λ plasmid molecules isolated from *E. coli* cells there are both monomers and multimers, in the latter case replication initiated from one origin enters another replication region and may give ambiguous results. Therefore, only DNA fragments revealing clear features of unidirectional or bidirectional replication initiated from *ori* λ present in the same fragment were considered. Among unidirectionally replicating molecules, in all cases the distribution between rightward and leftward replication intermediates was roughly equal (data not shown).

Replication mode	Replication intermediates in <i>E. coli</i> hosts (%)			
	<i>dnaA</i> ⁺ (30 °C)	<i>dnaA</i> ⁺ (43 °C)	<i>dnaA46</i> (30 °C)	<i>dnaA46</i> (43 °C)
Bidirectional θ	36	34	40	11
Unidirectional θ	64	66	60	89

Electron microscopic analysis of the directionality of θ replication of λ DNA

Two-dimensional agarose gel electrophoresis demonstrated that an impaired DnaA function may result in less frequent bidirectional replication of λ plasmid DNA. To obtain quantitative data, we analysed plasmid molecules (isolated as for two-dimensional agarose gel electrophoresis) using electron microscopic techniques. Plasmid DNA molecules isolated from *dnaA*⁺ or *dnaA46*(ts) bacteria were cut with *Hind*III and *Bam*HI (see Fig. 1b) and analysed by electron microscopy. Fragments of molecules containing bubbles as well as Y-shaped DNA fragments were identified and the lengths of appropriate arms were measured. For determination of the directionality of replication, the known position of *ori* λ was assumed to be the only possible replication start point (Fig. 6). Thus, we could determine the fraction of bidirectionally and unidirectionally replicating plasmids. We found that in the *dnaA*⁺ host about 40% of λ plasmid molecules replicate bidirectionally irrespective of temperature (Table 2). A similar distribution of plasmid replication intermediates was found in the *dnaA46*(ts) mutant growing at 30 °C, confirming the conclusion based on two-dimensional agarose gel analysis that combination of π and *dnaA46*(ts) mutations has little effect on directionality of λ plasmid replication at this temperature. However, among all replication intermediates found in *dnaA46*(ts) bacteria at 43 °C, only about 10% were derived from bidirectionally replicating plasmids (Table 2). These results are in agreement with our conclusion, based on data from two-dimensional agarose gel electrophoresis, that bi-

Table 3. Electron microscopic analysis of directionality of pTC λ 1 plasmid replication in *E. coli dnaA46*(ts) mutant

Replication mode	Replication intermediates (%) [*]	
	30 °C	43 °C
Bidirectional θ	46	40
Unidirectional θ	54	60

^{*}For experimental details see legend to Table 2. Among unidirectionally replicating molecules, in all cases the distribution between rightward and leftward replication intermediates was roughly equal (data not shown).

directional DNA replication from *ori* λ is impaired in the absence of functional DnaA.

DnaA affects the directionality of replication from *ori* λ by acting on the *p*_R promoter

The results described above indicated that the *dnaA* gene product affects the directionality of replication from *ori* λ directly or indirectly. Since it was demonstrated previously that DnaA directly stimulates transcription from the λ *p*_R promoter (Szalewska-Palasz *et al.*, 1998a), whose activity is necessary for transcriptional activation of *ori* λ , we asked whether this direct DnaA-mediated regulation is responsible for the observed effects of the *dnaA46*(ts) mutation on the replication from *ori* λ . Thus, we investigated replication of an artificial λ plasmid called pTC λ 1, which bears the *p*_{tet} promoter instead of *p*_R (Herman-Antosiewicz *et al.*, 1998a). DnaA-independence of activity of the *p*_{tet} promoter has been demonstrated previously (Herman-Antosiewicz *et al.*, 1998b). Using both two-dimensional agarose gel electrophoresis (data not shown) and electron microscopy (Table 3) we found that inactivation of the *dnaA* gene product has little effect on the directionality of pTC λ 1 replication (Table 3). Since the only considerable difference between pTC λ 1 and standard λ plasmid replicons is in the kind of promoter located upstream of *ori* λ , we conclude that DnaA affects the directionality of natural λ replicons by acting on the *p*_R promoter.

DISCUSSION

A role for the host-encoded DnaA protein in the regulation of the switch from θ to σ DNA replication of bacteriophage λ

Upon infection of its *E. coli* host, replication of bacteriophage λ DNA proceeds according to the θ mode and is then switched to σ mode (reviewed by Taylor & Węgrzyn, 1995, 1998). Dodson *et al.* (1986) proposed that σ replication may be preceded by one round of unidirectional θ replication initiated at *ori* λ followed by displacement of the 5' end of the newly synthesized

Fig. 7. A model for the regulation of the switch from θ to σ replication of phage λ genome in wild-type bacteria (left panel) and in *dnaA* mutants (right panel). In a wild-type host, DnaA-mediated stimulation of transcriptional activation of *oriλ* allows for initiation of bidirectional θ replication of λ DNA. After five to six rounds of replication, about 50 copies of the λ genome appear. Due to presence of many DnaA binding sequences in λ DNA, cellular DnaA protein is titrated out and transcriptional activation of *oriλ* becomes significantly less efficient. This leads to the initiation of unidirectional θ replication, which after one round switches to the σ mode according to the mechanism previously described by Dodson *et al.* (1986), who proposed that a round of unidirectional θ replication initiated at *oriλ* is followed by displacement of the 5' end (dot) of the newly synthesized leading strand by its growing 3' end (arrow). In a λ -infected *dnaA* mutant, due to impaired stimulation of the p_R promoter, unidirectional θ replication starts shortly after infection which results in the appearance of σ replication intermediates considerably earlier than in the wild-type host. For simplification of the diagram (in both panels), at the switch from θ to σ replication only the fate of the parental strand directing the synthesis of the leading strand is shown. However, one should note that there is still another copy of λ DNA after a round of replication (the copy formed due to synthesis of the lagging strand that is not shown in the diagram), which can initiate following replication round(s).

leading strand by its growing 3' end (Fig. 7). However, the precise mechanism regulating this switch is still not known. To find factor(s) responsible for this regulation we focused our attention on proteins that could potentially be involved in the control of directionality of DNA replication initiated at *oriλ*. It was previously demonstrated using an *in vitro* system reconstituted from purified proteins that replication of λ DNA proceeds unidirectionally in the absence of transcription, but a significant fraction of molecules replicate bidirectionally after the addition of RNA polymerase (Learn *et al.*, 1993). The only transcription which is

absolutely necessary for λ DNA replication *in vivo* and can be involved in λ DNA replication *in vitro* seems to be that initiated at the p_R promoter. This promoter is stimulated by the host DnaA protein (G. Węgrzyn *et al.*, 1995a, Szalewska-Pałasz *et al.*, 1998a). Therefore, we investigated replication of phage λ DNA in *dnaA* mutants.

Our density-shift experiments suggested that in the absence of functional DnaA, a large fraction of parental phage λ DNA molecules enters σ replication shortly after infection. These results confirmed the previously reported electron microscopic observations that σ replication intermediates appear in λ -infected *dnaA* mutant cells as soon as 5 min post-infection (G. Węgrzyn *et al.*, 1995b; Konopa *et al.*, 2000). In those studies, a λ phage bearing the mutated *P* gene and *E. coli* hosts bearing multiple mutations (apart from *dnaA46*) were used (G. Węgrzyn *et al.*, 1995b; Konopa *et al.*, 2000). Here, we demonstrate that our previous results are very similar to those obtained with a host with a wild-type genetic background and λP^+ phages. In fact, using electron microscopy we were able to observe σ replication intermediates early after infection of the strain BM764 (MG1655-derived *dnaA46* mutant) by λP^+ phage, similar to the results reported previously with more complex genetic backgrounds (G. Węgrzyn *et al.*, 1995b; Konopa *et al.*, 2000 and our unpublished results).

By analysis of replication initiated at *oriλ*, performed using two dimensional agarose gel electrophoresis and electron microscopy, we demonstrated that while in the wild-type host and in the *dnaA46(ts)* mutant at 30 °C bidirectional replication is frequent, in the *dnaA46(ts)* host at 43 °C it is predominantly unidirectional. Since replacement of the p_R promoter with p_{tet} (whose activity is not dependent on DnaA) resulted in abolition of the effect of the *dnaA* mutation on the replication directionality, we conclude that DnaA acts in the control of directionality of replication from *oriλ* by regulation of transcription initiated at p_R .

Model for regulation of the switch from θ to σ DNA replication

Transcription initiated at the p_R promoter and proceeding near or through *oriλ* is called transcriptional activation of *oriλ*. This process has been known for a long time (Dove *et al.*, 1969; Nijkamp *et al.*, 1971) to be necessary for λ DNA replication *in vivo*, but its exact role was unclear until recently. The results presented in this paper, together with previously reported observations (Learn *et al.*, 1993; G. Węgrzyn *et al.*, 1995b, 1996), indicate that transcriptional activation of *oriλ* is necessary for initiation of bidirectional replication from this region. This led us to propose a mechanism of regulation of the switch from θ to σ replication of bacteriophage λ DNA. Since DnaA positively regulates transcription from the p_R promoter (Szalewska-Pałasz *et al.*, 1998a), its activity is necessary for frequent initiation of bidirectional replication from *oriλ* in *E. coli*. Early after infection, in spite of the presence of many

DnaA boxes in the *E. coli* chromosome, there is a sufficient concentration of free DnaA molecules in the cell to stimulate bidirectional θ replication, as maximal activation of p_R by DnaA occurs at relatively low concentrations of this protein (Szalewska-Pałasz *et al.*, 1998a). However, after a few rounds of bidirectional θ replication, many copies of the λ genome appear (about 50 copies after five to six replication rounds). Since it was previously demonstrated by Szalewska-Pałasz *et al.* (1998c) that there are many DnaA binding sites in λ DNA, the DnaA protein may be titrated out. This should lead to an inefficient transcriptional activation of *ori λ* resulting in unidirectional θ replication followed by the σ replication mode. In *dnaA* mutants, weak transcription from the p_R promoter allows predominantly unidirectional θ replication and a switch to σ replication shortly after infection. This model is presented schematically in Fig. 7. If this model is correct, the DnaA box sequences responsible for titrating out DnaA molecules after several rounds of bidirectional θ replication of the λ genome in the wild-type host should be of weak affinity to this protein. This would allow efficient transcriptional activation of *ori λ* and bidirectional θ replication at the beginning of the infection cycle. In accordance with this prediction, it was reported that most DnaA boxes present in the λ genome are weak ones (Szalewska-Pałasz *et al.*, 1998c).

Better & Freifelder (1983) reported that σ replication intermediates can be also occasionally found at early times after infection of wild-type hosts by λ phage. They found that θ replication is predominant, but not exclusive, at early stages of infection and σ replication is predominant, but not exclusive, at late stages of infection. This is also compatible with the results presented in this report and with our model of the switch from θ to σ replication, as we have demonstrated that in the presence of DnaA function the replication of λ plasmids proceeds bidirectionally and unidirectionally, and in the absence of DnaA function the replication is predominantly, but not exclusively, unidirectional. In fact, after inactivation of DnaA we observed (using electron microscopy) a decrease in the fraction of bidirectionally replicating λ DNA from 40% to 10%, whereas the *dnaA46(ts)* mutation at 43 °C was responsible for a huge increase in σ -type replication intermediates as revealed by density-shift experiments. However, one should note that the electron microscopy studies were performed with plasmids occurring in many copies per cell, whereas in the density shift experiments only a few phages infected one cell. Moreover, whole population of λ plasmid molecules was investigated by electron microscopy, whereas the fate of only parental phage λ DNA molecules was monitored in density-shift experiments.

A physiological role for the switch from θ to σ DNA replication

It is also interesting to consider a physiological role for a DnaA-regulated switch from θ to σ replication of phage λ DNA. Since efficient growth of λ phage was

observed in the *dnaA46(ts)* mutant at 43 °C, i.e. under conditions in which σ replication starts early after infection, it raises the question as why should θ replication occur at early stages of infection in a wild-type host? The physiological role of θ replication should be the production of many copies of λ DNA to be used as templates for high level of expression of phage genes employed during lytic development. If this is true, the amount of λ DNA produced by σ replication is high enough for expression of phage proteins in amounts sufficient to support normal lytic development. However, it is worth noting that studies on λ development, which demonstrated efficient production of phage progeny in *dnaA* mutants (G. Węgrzyn *et al.*, 1995b; Sutton & Kaguni, 1997; Szalewska-Pałasz *et al.*, 1998b; Glinkowska *et al.*, 1999; this work), were carried out under standard laboratory conditions (i.e. in LB medium with good aeration, etc.) that support high *E. coli* growth rates. It was demonstrated recently that under these conditions, in a wild-type host, phage λ produces at least some proteins in excess of those needed for its effective propagation: thus efficient growth of the phage may be achieved even under less favourable conditions (Gabig *et al.*, 1998). This phage developmental strategy may be responsible for efficient λ growth in *dnaA* mutants, in which expression of phage genes is expected to be decreased relative to the wild-type host. If this is true, one might expect an inhibition of λ lytic development in a *dnaA* mutant growing in poor media. Indeed, it has been reported that phage λ lytic growth is less efficient in wild-type cells cultivated in minimal media, and it may be completely inhibited in slowly growing *dnaA46(ts)* mutants at 43 °C (Węgrzyn *et al.*, 2000).

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