Composition of the λ plasmid heritable replication complex

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Previous studies indicated during replication of plasmids derived from bacteriophage λ (the so-called λ plasmids), that, once assembled, replication complex can be inherited by one of the two daughter plasmid copies after each replication round, and may function in subsequent replication rounds. It seems that similar processes occur during replication of other DNA molecules, including chromosomes of the yeast *Saccharomyces cerevisiae*. However, apart from some suggestions based on genetic experiments, composition of the λ heritable replication complex remains unknown. In amino acid-starved *Escherichia coli* *relA* mutants, replication of λ plasmid DNA is carried out exclusively

by the heritable replication complex as assembly of new complexes is impaired due to inhibition of protein synthesis. Here, using a procedure based on *in vivo* cross-linking, cell lysis, immunoprecipitation with specific sera, de-cross-linking and PCR analysis, we demonstrate that the λ heritable replication complex consists of O, P, DnaB and, perhaps surprisingly, DnaK proteins.

Key words: DNA helicase, molecular chaperone, replication complex inheritance, replication-initiation protein.

INTRODUCTION

Plasmids derived from bacteriophage λ , called λ plasmids, contain all the genes and regulatory sequences of the phage genome that are necessary for autonomous replication in *Escherichia coli* cells (for recent reviews, see [1–4]). It was demonstrated during replication of λ plasmids, that, once assembled, replication complex is not disassembled after a replication round, but rather it is inherited by one of the two daughter DNA copies and may function in following replication rounds [5,6]. Such a complex is a very stable structure that can operate for at least several cell generations [7]. According to this model (Figure 1), initiation of DNA synthesis from the origin of λ plasmid replication, *ori λ* , is not triggered by an assembly of the replication complex but rather by transcriptional activation of the origin [6,8]. Results obtained from recent studies suggest strongly that the process of

replication-complex inheritance is not restricted to λ but occurs also in other prokaryotic and eukaryotic replicons, including ARS (autonomously replicating sequence) regions of the yeast *Saccharomyces cerevisiae* [4,9,10].

Genetic studies [11] and *in vitro* experiments [12] suggested that functions of λ -encoded P protein (a λ replication protein) and bacterial DnaB protein may be necessary for stabilization of the λ O replication-initiator protein, which is otherwise degraded rapidly by the ClpP/ClpX protease. Stable O protein, protected from proteolysis by other components of the heritable replication complex, is an essential element of this structure [5,7,11]. Thus on the basis of results mentioned above, one might speculate that O, P and DnaB are components of the λ heritable replication complex. However, the exact composition of this complex *in vivo* remains unknown. Therefore, we aimed to elucidate the composition of the inherited replication complex, which should be an important step in understanding mechanisms of regulation of DNA replication.

EXPERIMENTAL

Bacterial strains and plasmids

E. coli mutants $\Delta relA251::kan$ (BM711) and *relA2* (CP79) were used as described previously [10,13]. The following λ plasmids were employed: pCB104 (wild-type) [14], pKB2 (wild-type) [15] and pGW1 [a pKB2-derived *O*(ts)524 mutant] [16]. For maps of these λ plasmids see Figure 2.

Culture media and amino acid starvation

LB medium [17] and a previously described [16] minimal medium 2 were used. Isoleucine starvation was provoked by addition of L-valine (final concentration, 1 mg · ml^{−1}) to a culture growing in minimal medium, as described previously [16].

Figure 1 A model for replication of λ plasmids in amino acid-starved *E. coli* *relA* mutants

After a round of replication of λ plasmid DNA (large circle), the replication complex (small black circle) is inherited by one of two daughter plasmid copies and may function in the following replication rounds. A new complex cannot be assembled due to inhibition of O-protein synthesis in the absence of amino acids, and to rapid degradation of this protein by the host-encoded ClpP/ClpX protease. Therefore, under these conditions only replication carried out by the heritable replication complex can occur (for recent reviews, see [4,33]).

Abbreviation used: ppGpp, guanosine 5'-diphosphate-3'-diphosphate.

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Figure 2 Plasmids and primers (oligonucleotides) used in this work

Plasmid pGW1 is a pKB2-derivative bearing the *O*(ts)524 mutation. Primer sequences are given in Table 1.

Table 1 Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5' to 3')
pCB1	ATT ATC AAG CAG CAA GGC GG
pCB2	TGG CTG ATG GTG CGA TAG TC
pCB3	CCG TAA GGC CGC AGA TGA G
pCB4	TGG TTA CTG TTG CGC CTG TT
pCB5	TTC AGG TTC ATC ATG CCG TC
pCB6	ATT TAA CGA CCC TGC CCT GA
pCB7	ACG TGC GTC CTC AAG CTG
pCB8	TCC GCA TAA ACG CTT CCA
pKB1	TAC GTT TTC ATG GAT ACA GGT TG
pKB2	AGT ATT TGG TGA AGG GAA CGA GT
pKB3	TTG CAC TGC CGG TAG AAC TC
pKB4	ACC TGC CAT CAC GAG ATT TC
pKB5	CCA ATA GCA GCC AGT CCC TTC
pKB6	TAT TCG GCT ATG ACT GGG CAC

Oligonucleotides and PCR amplification

Oligonucleotides used in this study are listed in Table 1. They were used to amplify certain λ plasmid regions as depicted in Figure 2. The PCR reactions were carried out as follows: 5 min of incubation at 94 °C, followed by 30 cycles at 94 °C for 30 s, 53.2 °C for 30 s and 72 °C for 1 min, and a final cycle at 72 °C for 5 min.

Table 2 Sensitivities of the sera used in this study

Sensitivity was defined as the smallest amount of the corresponding protein that was detected unambiguously by Western blotting with a given serum diluted 2500 times.

Serum	Sensitivity (ng)
Anti-O	3
Anti-P	5
Anti-DnaB	1
Anti-DnaK	1
Anti-DnaJ	1
Anti-GrpE	1
Anti-DnaG	5
Anti-DnaC	3

Antibodies

The following rabbit sera, whose production and evaluation of specificity were described previously, were used: anti-O [11], anti-P [18], anti-DnaB [19], anti-DnaK [20], anti-DnaJ [20], anti-GrpE [21], anti-DnaC (from Dr Kirsten Skarstad, Institute of Cancer Research, Oslo, Norway) and anti-DnaG (from Dr Roger McMacken, Johns Hopkins University, Baltimore, MA, U.S.A.). Sensitivity of sera was estimated by Western blotting, as described previously [11], using serial dilutions of purified protein samples of known concentrations. Purified O, P, DnaB, DnaK, DnaJ, GrpE, DnaC and DnaG proteins were provided generously by Dr Igor Konieczny, Dr Krzysztof Liberek and Dr Jarosław Marszałek (all of the Intercollegiate Faculty of Biotechnology, University of Gdańsk, and the Medical University of Gdańsk, Gdańsk, Poland). Sensitivities of all sera are presented in Table 2. In the immunoprecipitation experiments, the sera were diluted differentially (from 2000 to 10000 times) to achieve a similar sensitivity for each antibody sample.

Cross-linking/immunoprecipitation/PCR assay

The assay was based on a procedure developed previously [22–24]. A bacterial culture (10 ml; D_{575} , 0.2) was centrifuged (2000 g, 10 min) and the pellet was washed twice with 10 ml of 10 mM sodium phosphate buffer (pH 8.0). Next, the pellets were resuspended in 10 ml of the same buffer and formaldehyde was added to the final concentration of 1%. Thus treated, the cells were mixed and incubated at room temperature for 10 min, followed by 30 min of incubation on ice. The cells were then washed twice with 10 ml of ice-cold PBS. After the final wash the cells were suspended in 0.5 ml of solution A (10 mM Tris, pH 8/20% sucrose/50 mM NaCl/10 mM EDTA) containing lysozyme (5 mg · ml⁻¹) and incubated at 37 °C for 30 min. Next, 0.5 ml of 2 × IP buffer (where 2 × IP buffer is 100 mM Tris, pH 7/300 mM NaCl/2% Triton X-100/10 mM EDTA) with PMSF (final concentration, 1 mM) and RNase A (final concentration, 50 µg · ml⁻¹) was added and the mixture was incubated at 37 °C for 10 min. The mixture was sonicated using a Bioblock sonicator (2.5 min with 9.9 s pulses and 9.9 s intervals, 15% amplitude). Thus obtained, cell lysates were centrifuged for 10 min at 13 500 g. The supernatant was then transferred into clean 1.5 ml tubes and 75 µl was removed to be used as a total DNA control (input). To the remaining supernatant appropriate sera were added and the mixture was incubated on a rocker for 1 h. A sample to which no antibodies had been added was treated as a control. Next, 30 µl of a 50% Protein A–Sepharose (Pharmacia) slurry was added, followed by incubation on a rocker for 1 h. The protein–DNA complexes were then collected by centrifugation at 5000 g for

20 s and washed five times with 1 ml of $1 \times$ IP buffer and twice with 1 ml of TE buffer (10 mM Tris/HCl, pH 8.0/1 mM EDTA). Finally, the complexes were suspended in 200 μ l of TE buffer and incubated at 65 °C overnight to reverse cross-links. The DNA was purified using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH). DNA solutions were then subjected to serial dilution, and 2.5 μ l of 80-fold-diluted immunoprecipitate and 200-fold diluted input were taken for the PCR reaction (final volume, 25 μ l). Products of PCR reactions were subjected to 1.6% agarose gel electrophoresis.

Measurement of the O protein stability

Stability of the λ -encoded O protein in *E. coli* cells harbouring a λ plasmid was investigated in pulse-and-chase experiments followed by immunoprecipitation of cell lysates with anti-O serum, SDS/PAGE, autoradiography and densitometry of appropriate bands on autoradiograms as described previously [11].

RESULTS

Two facts were very helpful in elucidating the composition of the λ heritable replication complex. First, all components necessary for λ DNA replication and the order of their assembly have been determined in studies *in vitro* [25,26]. Binding of the O protein to *ori* λ initiates the assembly of the replication complex. The P protein delivers DnaB helicase to the *ori* λ -O structure (called the 'O-some'), forming a nucleoprotein complex (*ori* λ -O-P-DnaB) called a 'preprimosome'. Since the P protein inhibits activity of DnaB, the action of the heat-shock proteins DnaK, DnaJ and GrpE is necessary for remodelling of the preprimosome in order to liberate DnaB helicase from P-mediated inhibition. Then, DNA polymerase III holoenzyme together with accessory proteins can initiate replication, providing that transcriptional activation of *ori* λ (see below) triggers this process [6,27].

The second helpful fact is that under conditions of amino acid starvation of *E. coli relA* mutants, replication of λ plasmid DNA proceeds solely due to activity of the heritable replication complex [4–6] (Figure 1). This is because the λ replication complex contains the O protein, which is extremely unstable in its free form in *E. coli* cells but stabilized in this complex [28]. In amino acid-starved cells, synthesis of the O protein is severely impaired due to lack of amino acids, and thus assembly of new replication complexes is inhibited [28]. Moreover, initiation of λ plasmid replication requires transcription proceeding near *ori* λ , the so-called transcriptional activation of the origin. This transcription starts from the *p_R* promoter, whose activity is significantly decreased in the presence of guanosine 5'-diphosphate-3'-diphosphate (ppGpp), the alarmone of the stringent response (a bacterial response to amino acid starvation) [6,29]. Mutants of the *relA* gene do not produce ppGpp in amino acid-starved cells [30], and under such conditions λ plasmid replication may be initiated and carried out by the heritable replication complex.

Our first attempts to characterize components of the λ heritable replication complex were based on an assumption that isolation of a stable replication complex bound to DNA from untreated cells is possible. We proved, however, that any chemical or physical procedures leading to cell lysis (e.g. sonication) caused disassembly or destruction of the λ replication complex, as we were not able to detect the O protein bound to DNA (results not shown). The only positive signal of the O protein- λ DNA complexes was obtained when a gene coding for the E protein of bacteriophage Φ X174 was overexpressed from a multicopy plasmid co-existing in the host cells with a λ plasmid. The E protein provokes formation of large holes in cell membranes,

Figure 3 Detection of PCR products after cross-linking and immunoprecipitation, performed in different experimental systems

I, input; C, negative control (without any serum); O, P, DnaB, DnaK, DnaJ and GrpE, experiments with anti-O, anti-P, anti-DnaB, anti-DnaK, anti-DnaJ and anti-GrpE sera, respectively; M, molecular-mass markers (1 kb ladder, Fermentas; bands of 250 and 500 bp are visible). (A) Experiments with non-starved *E. coli* Δ *relA251::kan* bacteria bearing a λ plasmid (pCB104); pairs of primers used in particular PCR reactions are indicated above the lanes. (B) Experiments with isoleucine-starved *E. coli* Δ *relA251::kan* bacteria bearing a λ plasmid (pCB104); primers pCB1 and pCB2 were used. (C) Experiments with non-starved *E. coli* Δ *relA251::kan* bacteria bearing a λ plasmid (pCB104); primers pCB1 and pCB2 were used. (D) Experiments with non-starved *E. coli relA2* bacteria bearing a λ Q(ts)524 plasmid (pGW1). Samples of bacterial cultures growing at 30 °C (marked as 30°) and 1 h after a shift to 43 °C (marked as 43°) were withdrawn; primers pCB1 and pCB2 were used. For descriptions of the primers used see Table 1 and Figure 2.

and thus plasmid DNA molecules could be liberated from cells. Following ultracentrifugation of the extracellular material in a sucrose gradient, collection of fractions containing plasmid DNA, agarose gel electrophoresis, transfer to a nitrocellulose membrane and Western blotting with anti-O serum, a signal was detected at a position corresponding to plasmid DNA (results not shown). Although these results indicated that stable complexes consisting of λ plasmid DNA and proteins, including the O protein, indeed exist in *E. coli* cells, the signals observed were too weak for more detailed studies on the composition of the heritable replication complex (results not shown).

Due to the problems described above, we decided to use a less direct but more useful method. We employed a technique described previously [22–24] allowing for detection of DNA-bound proteins *in vivo* (see the Experimental section for details). *E. coli* cells bearing λ plasmids (pKB2 and pCB104 or their derivatives, Figure 2) were cultivated under different conditions. In cells, cross-links between different protein molecules and between proteins and DNA were introduced by using formaldehyde. Following cell lysis, λ plasmid DNA was isolated and subjected to fragmentation by sonication, under conditions

Table 3 Detection of PCR products after the cross-linking and immunoprecipitation procedures performed with unstarved and amino acid-starved *E. coli* $\Delta relA251::kan$ bacteria bearing a λ plasmid (pCB104)

None denotes negative controls, when experiments were performed without any antibodies. Input denotes positive controls, when isolated DNA without immunoprecipitation was added as a template for PCR reactions. Bacteria were grown in a minimal medium and samples were withdrawn either before or 1 h after onset of isoleucine starvation. In both cases the results were the same. Symbols: + denotes detection of specific PCR products on an electrophoregram, and — denotes a lack of such a signal.

Serum	Primer pair ...	PCR result			
		pCB1 and pCB2	pCB3 and pCB4	pCB5 and pCB6	pCB7 and pCB8
None		—	—	—	—
Anti-O		+	—	—	—
Anti-P		+	—	—	—
Anti-DnaB		+	—	—	—
Anti-DnaK		+	—	—	—
Anti-DnaJ		—	—	—	—
Anti-GrpE		—	—	—	—
Anti-DnaG		—	—	—	—
Anti-DnaC		—	—	—	—
Input		+	+	+	+

yielding fragments, on average, of 1000 bp. Then immunoprecipitation reactions with sera against certain proteins were performed. Therefore, we were able to collect specifically cross-linked complexes of proteins and DNA fragments. The cross-links were reversed by overnight incubation at 65 °C. To detect specific DNA fragments to which a given protein was bound, we performed PCR reactions with pairs of primers distributed throughout a plasmid molecule (Figure 2). Positive results of amplification were expected only when a particular DNA fragment was cross-linked (directly or through another protein) to a protein for which a specific serum was used for immunoprecipitation.

First, we checked whether this method works for the λ replication initiator protein, O, which is known to bind specifically to the *ori λ* region. As shown in Figure 3(A), binding of the O protein to λ plasmid was found only in the case of the DNA fragment encompassing *ori λ* . Therefore, we concluded that this method can be used for detection of components of the heritable replication complex. Since in amino acid-starved *relA* mutants λ plasmid replication is carried out solely by the heritable stable replication complex, we investigated the *relA* bacteria bearing a λ plasmid. Samples of cultures were withdrawn 1 h after onset of amino acid starvation. Then the procedure was performed as described above but with different sera, against proteins known to be (i) involved in formation of the λ replication complex (O, P, DnaB, DnaK, DnaJ, GrpE), (ii) active during DNA replication elongation (DnaG) and (iii) dispensable for λ DNA replication (DnaC). Results presented in Figure 3(B) and Table 3 indicate that we were able to detect interactions of some proteins only with a DNA fragment encompassing *ori λ* . This is an indication that the method used in these studies did allow us to detect interactions between the replication complex and *ori λ* region but, fortunately, its sensitivity was low enough to avoid detecting both unspecific interactions (control experiments with anti-DnaC serum) and interactions between replication proteins and DNA at the stage of replication elongation (control experiments with anti-DnaG serum). Most probably, DnaG–DNA complexes were not detected because the fraction of replicating DNA molecules at a given moment in a population of unsynchronized bacterial cells is very low. On the other hand, it seems that the heritable replication complex is bound to the *ori λ* region throughout the whole cell cycle awaiting a signal (most

Figure 4 Stability of the O protein in *E. coli* cells bearing a wild-type plasmid derived from bacteriophage λ , pKB2 (●), and its *O*(ts)524 derivative, pGW1 (■) at 43 °C

Bacteria were cultivated at 30 °C and shifted to 43 °C 1 h before labelling. The cells were labelled with [35 S]methionine (5 μ Ci $\cdot$ ml $^{-1}$) for 5 min, and then an excess of L-methionine was added (up to 1 mg $\cdot$ ml $^{-1}$). Samples were withdrawn at the indicated times after chasing, and, following cell lysis, immunoprecipitation with anti-O serum, SDS/PAGE and autoradiography, the quantification of the labelled O protein remaining after different chase times was performed by densitometry.

probably transcriptional activation of the origin) for triggering initiation of replication, the process that is finished within a few seconds [4].

Results presented in Figure 3(B) and Table 3 strongly suggest that the O, P, DnaB and DnaK proteins (but not DnaJ, GrpE, DnaG and DnaC) form a complex bound to λ DNA in the region of *ori λ* . It is known that P, DnaB and DnaK proteins do not bind to specific DNA sequences. Therefore, we conclude that these proteins are components of the O protein-containing λ replication complex.

The heritable replication complex exists not only in amino acid-starved cells but also under normal growth conditions [7], although in growing bacterial cells new replication complexes are formed continuously. Nevertheless, we repeated the experiments described above using cells growing under normal conditions. The same results were obtained (Figure 3C), suggesting that the

Table 4 Detection of PCR products after the cross-linking and immunoprecipitation procedures performed with unstarved and amino acid-starved *E. coli relA2* bacteria bearing a wild-type λ plasmid (pKB2) or its *O*(ts)524 derivative (pGW1)

Bacteria were grown in a minimal medium at 30 °C and samples were withdrawn either before or 1 h after onset of isoleucine starvation and/or a temperature shift to 43 °C. Results with the pKB2 plasmid (at both temperatures) were the same as those with pGW1 at 30 °C (results not shown). In most experiments the results were the same for both starved and non-starved cells, except those performed with pGW1-bearing cells and anti-O serum at 43 °C. In this case the signal was detected in non-starved but not in starved cells (denoted as +/– in the Table). For other symbols and explanations see Table 3. Only results with pCB1 and pCB2 primers are shown because, apart from input controls, no signal was detected with other pairs of primers (results not shown).

Serum	PCR result	
	pGW1 (30 °C)	pGW1 (43 °C)
None	–	–
Anti-O	+	+/–
Anti-P	+	–
Anti-DnaB	+	–
Anti-DnaK	+	–
Anti-DnaJ	–	–
Anti-GrpE	–	–
Anti-DnaG	–	–
Anti-DnaC	–	–
Input	+	+

heritable and newly assembled λ replication complexes are equivalent.

To check whether interactions described above can indeed be ascribed to the heritable replication complex (and not to any putative unknown interactions between DNA and P, DnaB or DnaK proteins), we used a λ plasmid with a temperature-sensitive mutation in the *O* gene, *O*(ts)524. The function of the *O* gene is absolutely necessary for activity of the heritable replication complex [5,16]. One indication of the existence of the λ heritable replication complex is the presence of a stable fraction of the *O* protein [5,7,11]. We found that, contrary to the wild-type λ plasmid, no stable *O* protein fraction was present in cells harbouring the *O*(ts)524 plasmid at 43 °C (Figure 4). Hence, if P, DnaB and DnaK were components of the heritable replication complex we should not be able to detect their interactions with λ DNA in cells bearing the *O*(ts)524 plasmid and cultivated at 43 °C. We found that in cells growing at both 30 °C and 43 °C the *O*(ts)524 gene product can interact with the *ori* λ region (Figure 3D). However, formation of the replication complex was abolished at 43 °C, as no signal was observed when immunoprecipitation was performed with anti-P, anti-DnaB and anti-DnaK antibodies (Figure 3D, Table 4). Thus it seems that the *O*(ts)524 protein can bind to DNA but is defective in further steps of the replication complex assembly. At 30 °C the results were the same as for the wild-type plasmid (Table 3). Furthermore, when experiments with the *O*(ts)524 plasmid mutant were repeated in amino acid-starved cells the same results were obtained, but in the case of samples withdrawn from the culture growing at 43 °C and immunoprecipitated with the anti-O serum PCR amplification was impaired (Table 4). These results confirm that *O* activity is indispensable for assembly and function of the λ heritable replication complex, and indicate that interactions of P, DnaB and DnaK proteins with the λ DNA region near *ori* λ are due to their presence in this complex rather than to any direct, unpredicted protein–DNA interactions.

DISCUSSION

The process of λ replication-complex inheritance by one of the two daughter plasmid copies after each round of replication has been demonstrated previously (for a review, see [4]), but the composition of this structure has remained largely unknown. On the other hand, its composition should have general biological meaning, as inheritance of the replication complex seems to be a general process rather than a phenomenon restricted to λ [4].

In this report we present results which suggest strongly that O, P, DnaB and DnaK proteins are components of the multiprotein structure that remains bound to one copy of the *ori* λ after a round of replication. Results of previous genetic studies [11] and *in vitro* experiments [12] led to speculations about the presence of O, P and DnaB proteins in the λ heritable replication complex. Namely, a stable fraction of the *O* protein (the fraction that corresponds to *O* protein molecules present in the heritable replication complex) could not be detected in *E. coli dnaB* mutants and when the λ *P* gene was disrupted [11], and formation of the *ori* λ -bound O–P–DnaB preprimosome resulted in protection of the *O* protein from ClpP/ClpX-mediated proteolysis *in vitro* [12]. On the other hand, the presence of DnaK in the λ heritable replication complex is rather unexpected. Our results cannot be explained by a possibility of DnaK detection in the complex at the moment of its action to remodel the preprimosome, as other proteins involved in this remodelling, DnaJ and GrpE, were not detected by the method used in this study. Although one could argue that these negative results might be explained by the failure of the respective antibodies to bind their targets because of occlusion of the epitopes in DnaJ and GrpE, such a possibility seems to be unlikely as polyclonal antibodies were used. Thus we expect that at least some epitopes of each protein should still be available for interactions with polyclonal antibodies.

The DnaK protein is a molecular chaperone and it was thought to exist in an unbound form rather than to be a component of a stable protein complex. On the other hand, it was reported previously that a stable fraction of the *O* protein (the fraction that corresponds to *O* protein molecules present in the heritable replication complex) could not be detected in *E. coli dnaK* mutants [11]. Moreover, a stable nucleoprotein complex consisting of *ori* λ , O, P, DnaB and DnaK proteins was detected *in vitro* [31,32], but DnaK was not considered a component of the stable λ replication complex. Therefore, the results mentioned above are compatible with the results presented in this report, indicating that DnaK may indeed be included in the heritable replication complex.

One could argue that a small portion of preprimosomes (O–P–DnaB complexes) assembled at *ori* λ are not activated properly by DnaK, DnaJ and GrpE proteins to liberate the DnaB helicase from P-mediated inhibition. Thus the isolated complex might not be the active heritable λ replication complex but an inactive preprimosome. However, such a hypothesis is unlikely. Namely, it was demonstrated that the frequency of λ plasmid replication initiation under conditions of amino acid starvation of *relA* mutants is very similar to that observed under normal growth conditions [5,7]. This suggests strongly that all (or almost all) inherited λ replication complexes are active. Moreover, studies *in vitro* revealed that DNA-bound preprimosomes may be isolated by filtration and that they can direct DNA replication [25,32], indicating again that inactive preprimosomes are very rare, if they exist at all.

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