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Formation of the preprimosome protects λ O from RNA transcription-dependent proteolysis by ClpP/ClpX

(ATP-dependent proteases/molecular chaperones/heat shock proteins)

MACIEJ ZYLICZ*†, KRZYSZTOF LIBEREK*, ALICJA WAWRZYNOW*, AND COSTA GEORGOPOULOS‡

*Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdansk, 80-822 Gdansk, Kladki 24, Poland; and ‡Department of Medical Biochemistry, Centre Medical Universitaire, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland

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ABSTRACT **Using the bacteriophage λ DNA replication system, composed entirely of purified proteins, we have tested the accessibility of the short-lived λ O protein to the ClpP/ClpX protease during the various stages of λ DNA replication. We find that binding of λ O protein to its *ori*λ DNA sequence, leading to the so-called "O-some" formation, largely inhibits its degradation. On the contrary, under conditions permissive for transcription, the λ O protein bound to the *ori*λ sequence becomes largely accessible to ClpP/ClpX-mediated proteolysis. However, when the λ O protein is part of the larger *ori*λ:O·P·DnaB preprimosomal complex, transcription does not significantly increase ClpP/ClpX-dependent λ O degradation. These results show that transcription can stimulate proteolysis of a protein that is required for the initiation of DNA replication.**

The replication of bacteriophage λ DNA depends on intricate interactions between the bacteriophage-encoded replication proteins and the bacterial host's replication machinery (for reviews, see refs. 1–4). After bacteriophage λ attachment, the linear double-stranded bacteriophage λ DNA is injected into the bacterial cell, where it is rapidly circularized and supercoiled. After early mRNA transcription and translation, λ DNA replication is initiated at a single site, *ori*λ, and proceeds bidirectionally according to the circle-to-circle, or θ, mode. Later, during the infection process, replication of λ DNA proceeds by a rolling-circle mechanism, the so-called σ mode.

Only two bacteriophage λ proteins, encoded by the *O* and *P* genes, appear to participate directly in the initiation and/or propagation of the replication forks. As a consequence, a small fragment of the λ genome, carrying only the *cro*, *O*, and *P* genes, the replication origin *ori*λ (located inside the *O* structural gene), and the p_R promoter can replicate autonomously as a plasmid, called λ*dv*. The p_R promoter is required not only for the expression of the *O* and *P* genes but also for the transcriptional activation of the *ori*λ sequence, an event known to regulate the frequency of λ DNA replication (for reviews, see refs. 2 and 5). It is believed that the early θ mode of bacteriophage λ DNA replication is mimicked by the λ*dv* plasmid.

The *in vitro* reconstitution of the λ*dv* plasmid DNA replication system using purified proteins has allowed the identification of intermediate reactions leading to the initiation of λ DNA replication (6–9). Four dimers of the λ O initiation protein bind to the *ori*λ sequence at the four repeating sequences (iterons), forming the large O-some nucleosome-like structure (10–15). The formation of the O-some complex changes the conformation of the *ori*λ sequence (16), thus helping to potentiate the loading of the DnaB helicase onto λ DNA adjacent to the *ori*λ sequence.

The second bacteriophage λ-encoded protein involved in DNA replication, λ P, is responsible for the sequestration of the bacterial DnaB helicase away from the host replication system. When the intracellular levels of the λ P initiation protein increase, λ P protein can compete efficiently with its host-encoded DnaC analogue for binding to DnaB (17). The λ P–DnaB complex formed as a result of this λ P increase interacts with the O-some structure to form the even larger *ori*λ:O·P·DnaB preprimosomal complex (7, 8, 12–14).

In vivo, λ O protein is extremely unstable (its half-life at 40°C is approximately 1.5 min; refs. 18 and 19). The ATP-dependent serine protease ClpP/ClpX was originally identified as a protease capable of efficiently degrading λ O *in vitro* (20, 21). The λ O protein, like several other DNA-binding replication proteins, has a tendency to aggregate (22). It was shown previously that purified ClpX, the ATP-dependent substrate-specificity component of the ClpP/ClpX protease, can protect λ O from aggregation and dissociate heat-induced λ O aggregates (22). This chaperone effect of ClpX enhances the specific binding of λ O to *ori*λ, thus indirectly leading to a stimulation of λ DNA replication *in vitro* (22). A regulatory mechanism responsible for the "decision" to either repair or destroy a protein substrate, based on the stability of the ClpX-protein substrate complex, recently has been proposed (23). The physiological role of ClpP/ClpX-dependent proteolysis in λ DNA replication is still not clear. In this paper, we show that specific proteolysis coupled with a transcriptional event can modulate the morphogenesis of the preprimosomal protein–DNA complex involved in the initiation of DNA replication.

MATERIALS AND METHODS

Proteins and Plasmids. The ^{3}H-labeled λ O protein (1×10^5 units/mg) was purified to homogeneity as described (11, 14) by using the λ O- and λ P-overproducing plasmid (10). The pRLM216 plasmid, which overexpresses the mutant λ O(150–299) protein, was kindly provided by Roger McMacken (Johns Hopkins University, Baltimore). Its purification was carried out as described (11). The λ P protein (1.5×10^5 units/mg) was purified by the methods described in ref. 10. The *Escherichia coli* replication proteins and RNA polymerase enriched with σ^{70} were purified as described in refs. 8 and 24. The units of activities were defined by Zylicz *et al.* (25). Supercoiled plasmids containing the *ori*λ sequence (pRLM4) and *ori*φ82 (pRLM5) (26) were purified by using the alkaline lysis procedure, followed by cesium chloride-ethidium bromide gradient centrifugation.

Protease Assays. The standard protease assay reaction mixture (50 μl) containing ^{3}H-labeled λ O protein (250 ng,

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†To whom reprint requests should be addressed. e-mail: zylicz@biotech.univ.gda.pl.

40,000 cpm) was incubated in the presence or absence of supercoiled pRLM4 or pRLM5 DNA (750 ng or as shown in the various figure legends), λ P (350 ng), DnaB (350 ng), RNA polymerase (500 ng), and rNTPs (0.4 mM each) in 20 mM Hepes·KOH (pH 7.2), 10 mM $MgCl_2$, 5 mM ATP, 50 mM KCl, 25 mM NaCl, and 4 mg/ml BSA. After 4 min at 30°C, the ClpX (250 ng) and ClpP (1,000 ng) proteins were added, and incubation proceeded for an additional 5 min at 30°C. The reaction was stopped by the addition of ice-cold trichloroacetic acid (final concentration 10%). After centrifugation (5,000 × *g* at 4°C for 10 min), the radioactivity of the soluble fraction (supernatant) was estimated after the addition of toluene/Triton X-100 scintillation fluid. Each experiment was repeated six times, and the average value was estimated. In most cases the standard deviation was <15%.

Isolation of the λO–*ori*λ (O-some) DNA Complex. Size exclusion chromatography was performed essentially as described (14). The Sepharose 4B (Pharmacia) column (0.5 cm × 7.5 cm) was equilibrated with 40 mM Hepes·KOH (pH 7.6), 1 mM DTT, 10 mM $MgCl_2$, 100 mM KCl, 25 mM NaCl, and 0.5 mg/ml BSA. The 75-μl reaction mixture (in the same buffer) was supplemented with 2 μg of DNA, 1 μg of ^{3}H-labeled λ O and, as shown, ClpX (0.4 μg), ClpP (2 μg), RNA polymerase (0.5 μg), 5 mM ATP, and rNTPs (0.4 mM each), incubated for 30 min at 30°C, and loaded on a Sepharose 4B column. Four-drop fractions were collected directly into scintillation fluid, and the level of radioactivity was estimated by using a scintillation counter.

Purified *in Vitro* DNA Replication System. The replication reaction was carried out essentially as described (8). The premixture reaction (125 μl) consisted of 40 mM Hepes·KOH (pH 7.2), 7.2 mM magnesium acetate, 4 mM ATP, 3 μg of pRLM4 supercoiled λ DNA, and 2 μg of λ O (in the case of O-some formation) or 2 μg of λ O, 1.5 μg of λ P, and 1.5 μg of DnaB (in the case of preprimosome-complex formation). After 4 min at 30°C, the 25-μl premixture, containing 1 μg of RNA polymerase, 0.75 μg of ClpX, and 3 μg of ClpP and rNTPs (final concentration 0.2 mM each) was added. After 10 min at 30°C, 1.5 μg of λ P and 1.5 μg of DnaB (in the case of O-some formation) was added. Both reactions were supplemented with the *E. coli* replication-protein mixture (100 μl) containing 40 mM Hepes·KOH (pH 7.2), 7.2 mM magnesium acetate, 2 mM ATP, 8 μg of single-stranded DNA binding protein, 2 μg of GyrA, 0.9 μg of GyrB, 0.1 μg of DnaJ, 5 μg of DnaK, 2.5 μg of GrpE, 1 μg of DnaG, 2 μg of DNA polymerase III, 250 μM each dATP, dCTP, dTTP, dGTP, [*methyl*-^{3}H]dTTP (50 cpm/pmol of total deoxynucleotides), and 0.2 mM each rNTP. After the indicated times at 30°C, 25-μl aliquots were precipitated with 10% trichloroacetic acid in the presence of carrier calf thymus DNA (500 μg) and 50 μl of saturated sodium pyrophosphate, and the incorporation of [^{3}H]dTMP into DNA was measured as described (27).

RESULTS

It has previously been shown that the ClpP/ClpX protease efficiently degrades the λ O replication protein (20, 21). Here, we extend these studies by asking whether ClpP/ClpX is able to hydrolyze the λ O protein when bound to *ori*λ DNA. We found that increasing concentrations of *ori*λ-containing DNA significantly inhibit the ClpP/ClpX-dependent proteolysis of λ O in the purified component system (Fig. 1). Such an effect was not observed when *ori*ϕ82 plasmid DNA was used instead of *ori*λ (Fig. 1; it is known that the λ O protein does not bind specifically to the *ori*ϕ82 DNA, see ref. 26). The kinetic analysis shows that binding of λ O to *ori*λ DNA significantly decreases its rate of hydrolysis. Wickner *et al.* (28) have made analogous findings with the bacteriophage P1 RepA protein and its degradation by the ClpP/ClpA protease.

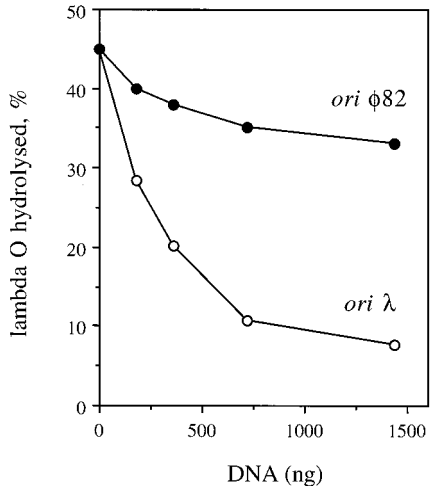

FIG. 1. Accessibility of λ O protein in the O-some structure to ClpP/ClpX-dependent proteolysis. Increasing amounts of *ori*λ-containing DNA (○) or *ori*ϕ82-containig DNA (●) were added to the standard protease assay reaction mixture containing ^{3}H-labeled λ O. After a 3-min preincubation at 30°C, ClpX and ClpP proteins were added. Incubation proceeded for an additional 5 min at 30°C, the reaction was stopped, and the amount of ^{3}H-labeled λ O hydrolyzed was estimated as described in *Materials and Methods*.

The formation of the *ori*λ:O·P·DnaB preprimosomal complex further stabilizes the λ O protein from ClpP/ClpX-promoted proteolysis (Fig. 2). In a control experiment, we found that the presence of *ori*λ-containing plasmid DNA does not change the kinetics of degradation of the λ O truncation mutant, λ O 150–299, which does not contain the DNA-binding motif. This result, in conjunction with the ϕ82 experiments, strongly suggests that the specific binding of λ O to the *ori*λ sequence is responsible for the observed inhibition of ClpP/ClpX-dependent λ O proteolysis in the presence of λ DNA.

In contrast to the results described above with the purified component system, we previously showed that when λ O proteolysis is investigated (in the absence of λ P) in a crude

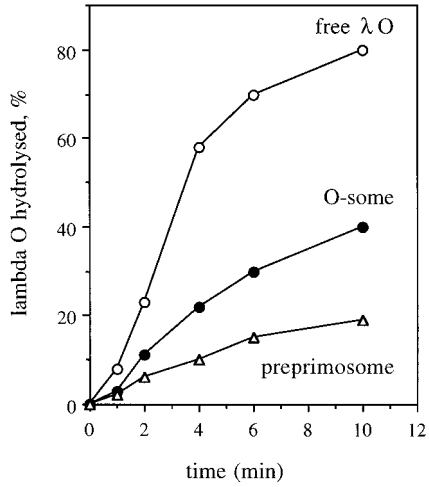

FIG. 2. Stability of λ O protein during the prepriming steps of λ DNA replication. The ^{3}H-labeled λ O protein was incubated either alone (○) or with 400 ng *ori*λ DNA (●) or in the presence of *ori*λ DNA, λ P, and DnaB (△). After a 3-min preincubation at 30°C, the ClpX and ClpP proteins were added. The reaction was stopped at the indicated time points, and the amount of ^{3}H-labeled λ O hydrolyzed was estimated as described in *Materials and Methods*. The experiments were repeated with a greater amount of DNA (750 ng). The kinetics were similar except that the difference between O-some and preprimosome was not as prominent (result not shown).

enzymatic fraction capable of supporting *in vitro* λ DNA replication, the presence of *ori*λ-containing DNA does not influence the kinetics of λ O degradation (20). This result suggests that in crude *E. coli* extracts, λ O may be degraded either by different proteases and/or that an additional activity exists that makes λ O more amenable to ClpP/ClpX proteolysis. While investigating these differences, we found that preincubation of the crude *E. coli* extracts with rifampicin (an antibiotic that blocks initiation of RNA transcription) severely diminishes degradation of λ O bound to *ori*λ DNA (result not shown). Previous studies have clearly established that transcription is needed for λ DNA replication *in vivo* as well as in crude enzymatic fractions (for reviews, see refs. 3 and 5). Therefore, we tested whether transcription modulates the accessibility of the λ O protein to ClpP/ClpX-dependent proteolysis. We found that the presence of highly purified RNA polymerase and rNTPs indeed overcomes the inhibition of λ O degradation caused by its binding to *ori*λ DNA (Fig. 3). As shown in a control experiment, the presence of RNA polymerase alone (in the absence of rNTPs) does not exert a significant effect on λ O degradation (Fig. 3).

By using an established procedure for size exclusion chromatography on a Sepharose 4B column, we were able to isolate an ^{3}H-labeled λO–*ori*λ DNA complex and show that λ O bound to DNA is largely resistant to ClpP/ClpX-dependent degradation (Fig. 4). In the presence of RNA polymerase and rNTPs (but in the absence of the ClpP/ClpX protease), we observed a significant amount of λ O protein in complex with λ DNA, suggesting that even though λ O is efficiently released under these conditions, it can quickly reassociate with its *ori*λ DNA sequence. Only when both transcription and ClpP/ClpX are simultaneously present is most of the λ O protein degraded (Fig. 4).

It has been shown that the O-some structure attracts the λ P–DnaB complex, leading to the formation of a very stable *ori*λ:O·P·DnaB preprimosomal complex (7, 8, 12–14, 29). Interestingly, transcription does not alter the relative resistance of λ O present in the *ori*λ:O·P·DnaB preprimosomal complex to ClpP/ClpX proteolysis (Fig. 3). The inhibition of λ O degradation in the presence of RNA polymerase depends on the presence of both DnaB and λ P protein (Fig. 5),

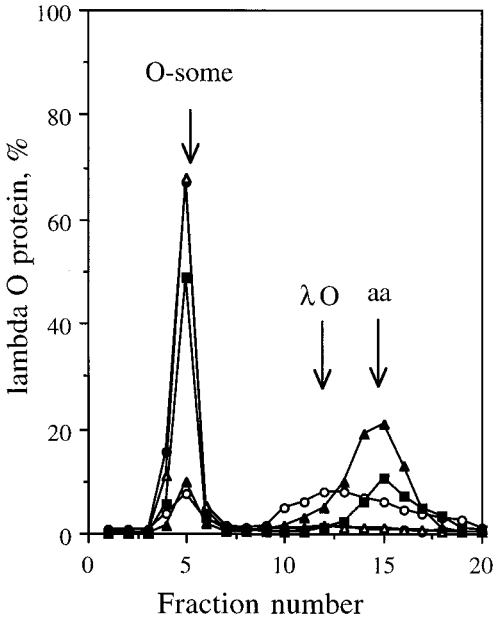

FIG. 4. The simultaneous presence of ClpP/ClpX and transcription leads to λ O degradation in the O-some structure. ^{3}H-labeled λ O protein was preincubated with *ori*λ DNA. After a 3-min preincubation at 30°C, RNA polymerase supplemented with rNTPs (△) or ClpP/ClpX protease supplemented with 5 mM ATP (■) was added, and incubation continued for an additional 10 min at 30°C. In a separate experiment, after preincubation of ^{3}H-labeled λ O with *ori*λ DNA, RNA polymerase and ClpP/ClpX were added at the same time, and the reaction continued for an additional 10 min in the presence of 5 mM ATP and 0.2 mM each UTP, CTP, and GTP (▲). The reaction mixtures were passed through a Sepharose 4B column, and the level of radioactivity in each fraction was quantified as described in *Materials and Methods*. In control experiments, ^{3}H-labeled λ O protein alone (○) or in the presence of *ori*λ DNA (●) was also applied onto a Sepharose 4B column. See *Materials and Methods* for more details, including protein concentrations. aa, free amino acids or small peptides.

suggesting that the formation of the *ori*λ:O·P·DnaB preprimosomal complex is responsible for this effect.

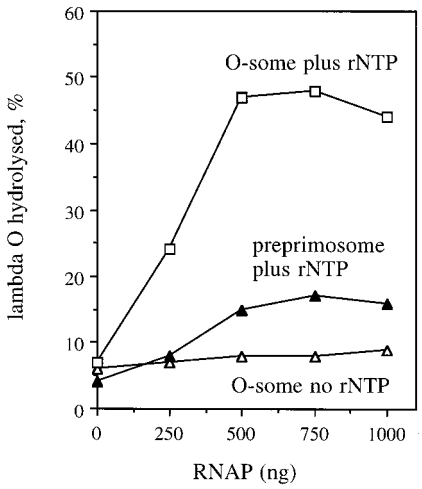

FIG. 3. λ O hydrolysis during transcription of the *ori*λ DNA region. The ^{3}H-labeled λ O protein was preincubated with *ori*λ DNA (△), *ori*λ DNA and rNTPs (□), or *ori*λ DNA, rNTPs, λ P and DnaB (▲). After a 3-min preincubation at 30°C, increasing amounts of RNA polymerase (supplemented with rNTPs) and ClpP/ClpX protease (supplemented with 5 mM ATP) were added. Following a 5-min incubation at 30°C. reactions were stopped and the amount of λ O hydrolyzed was estimated as described in *Materials and Methods*.

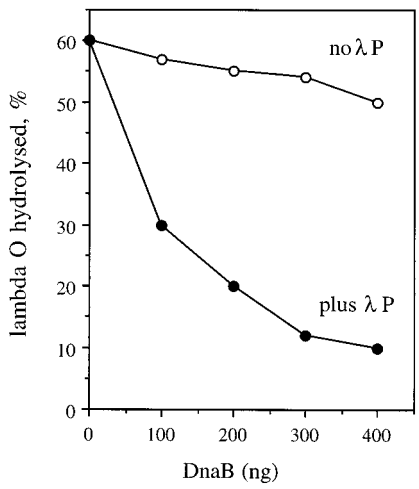

FIG. 5. Both DnaB and λ P are required for the stabilization of λ O when present in the preprimosomal complex. Increasing amounts of DnaB helicase were added to the ^{3}H-labeled λ O and *ori*λ DNA in the presence (●) or absence (○) of λ P. After a 3-min preincubation at 30°C, ClpP/ClpX protease, RNA polymerase, and rNTPs were added. Following a 5-min incubation at 30°C, the reactions were stopped, and the amount of λ O hydrolyzed was estimated as described in *Materials and Methods*.

To investigate this effect further, we preincubated λ O with *ori*λ DNA or preincubated λ O with DnaB, λ P, and *ori*λ DNA for 4 min at 30°C to allow the formation of the O-some and the *ori*λ:O·P·DnaB preprimosomal structures, respectively. Subsequently, RNA polymerase, rNTPs, and ClpP/ClpX protease were added. After a further 10-min incubation at 30°C, the rest of the required replication proteins supplemented with dNTPs (including [^{3}H]dTTP) were added. Replication was stopped at appropriate time points, and DNA synthesis was measured by the incorporation of [^{3}H]dTMP in λ DNA. As expected, transcription makes the λ O present in the O-some structure completely accessible to ClpP/ClpX-dependent proteolysis, resulting in an almost complete inhibition of DNA replication. The situation is different when the initiation reaction proceeds to preprimosomal complex formation, in which case the presence of RNA polymerase does not significantly influence the rate of initiation of λ DNA replication (Fig. 6).

DISCUSSION

The *in vitro* reconstitution of the λ DNA replication system using purified proteins has allowed the dissection of the various intermediate reactions that lead to the initiation of λ DNA replication (7, 8, 30). A detailed model of the initial steps leading to λ DNA replication is presented in Fig. 7. Transcriptional activation by RNA polymerase, initiated at the p_R promoter, is required for the initiation of DNA replication from the *ori*λ:O·P·DnaB preprimosomal complex (5, 27, 31, 32). It also is known that transcription initiated at the p_R promoter stops at the already-assembled *ori*λ:O·P·DnaB preprimosomal complex (5, 32).

In this work, we show that transcription enables the ClpP/ClpX protease to degrade λ O when present alone in the O-some structure but not in the *ori*λ:O·P·DnaB preprimosomal complex. These findings agree with the *in vivo* results, according to which λ P and DnaB are required for the stabilization of a subset of λ O protein molecules (33). Work from the same laboratory showed that this stable fraction of λ O protein accumulates in the *dnaJ259* or *grpE280* genetic backgrounds (34). Previous data have shown that the DnaJ and GrpE heat-shock proteins, in concert with DnaK, are necessary for the partial disassembly of the preprimosomal complex (9, 14, 29, 30). Therefore, it is highly probable that in the case of the *dnaJ259* or *grpE280* mutants, the preprimosomal complex is not disassembled, leading to the stabilization of its λ O protein component.

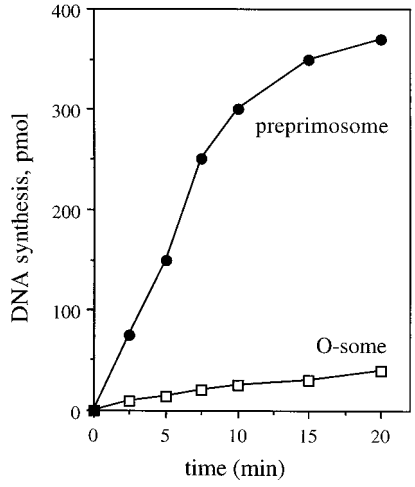

FIG. 6. Kinetics of λ DNA synthesis. The λ DNA replication reaction was performed essentially as described (8). After O-some (□) or preprimosome (●) formation, the ClpP/ClpX protease and RNA polymerase with rNTPs (ATP supplemented to 5 mM) were added. After a 10-min preincubation at 30°C, the O-some reaction was supplemented by λ P and DnaB helicase, and the replication was initiated by the addition of the remaining *E. coli* replication proteins (see *Materials and Methods* for concentrations and details). The replication reaction was stopped at various time points, and the amount of [^{3}H]dTMP incorporated into DNA was estimated as described in *Materials and Methods*.

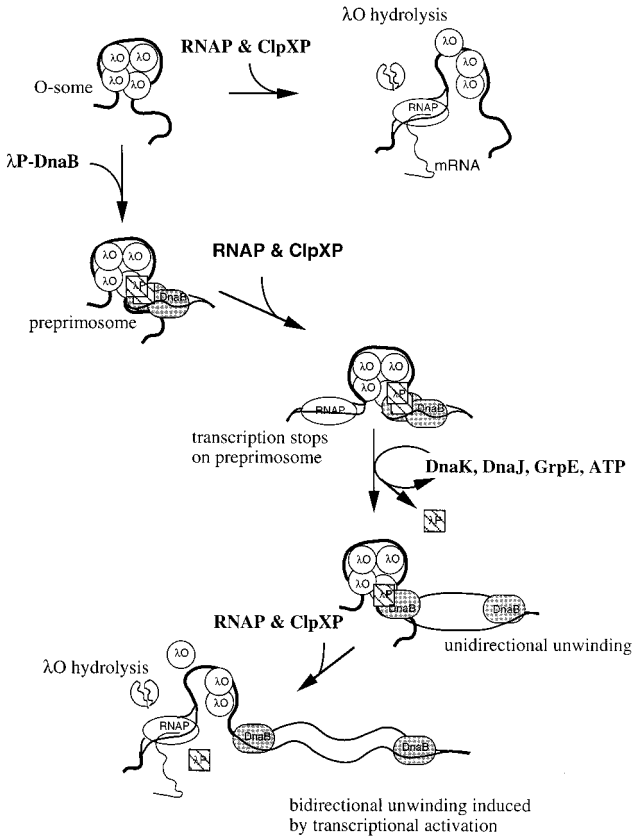

FIG. 7. A model for bidirectional replication of *ori*λ DNA. Following O-some formation, the simultaneous presence of transcribing RNA polymerase and ClpP/ClpX protease leads to the hydrolysis of λ O protein. In contrast, when the preprimosome complex (*ori*λ:O·P·DnaB) has been assembled, transcription by RNA polymerase does not result in λ O hydrolysis by the ClpP/ClpX protease. Apparently, transcription stops at the assembled preprimosome complex. Following DnaK/DnaJ/GrpE molecular chaperone machine action, the λ P protein dissociates from the preprimosome complex, thus allowing DnaB helicase to unwind DNA in the left-to-right direction only. We suggest that the additional presence of the ClpP/ClpX protease and transcription at this stage results in efficient hydrolysis of λ O, thus allowing the DnaB helicase to unwind DNA in the right-to-left direction as well, thus resulting in bidirectional λ DNA replication.

The results presented in this paper suggest that transcription proceeding through the *ori*λ sequence may attenuate the initiation of λ DNA replication at the stage of the O-some complex formation. This phenomenon could have several important biological implications. First, this mechanism could play an important role in the suppression of nonspecific initiation of λ DNA replication. For example, it is known that the λ O protein can interact nonspecifically with double- and/or single-stranded λ DNA, thus promoting initiation of λ DNA replication at sites other than *ori*λ (ref. 35; M.Z., unpublished results). Transcription coupled with the ClpP/ClpX-dependent proteolysis could be involved in the degradation of λ O complexed with DNA at such nonspecific sites. Only λ O present in the *ori*λ:O·P·DnaB preprimosomal complex would survive ClpP/ClpX-promoted proteolysis. Second, the attenuation of λ DNA replication at the stage of the O-some structure would obviously suppress further preprimo-

somal complex formation. This would be important if other elements of the preprimosome (namely λ P and/or DnaB) were not available.

The ClpP/ClpX protease in concert with transcription may be involved in the transition from the uni- to bidirectional λ DNA replication mode. The removal of λ O should allow the DnaB helicase to unwind the *ori*λ DNA structure in the right-to-left direction, thus leading to the establishment of the bidirectional DNA replication mode (Fig. 7). McMacken and colleagues (5, 32) postulated that the assembled O-some structure may cause a physical barrier for the passage of DnaB helicase in the right-to-left direction, thus necessitating the rearrangement of the O-some structure by RNA transcription if DNA replication were to proceed in that direction. In support of this suggestion, we have shown that after DnaK/DnaJ/GrpE-dependent activation of the preprimosomal complex, the λ O protein is efficiently degraded (unpublished results).

Under stress conditions, such as those produced during a heat shock or infection with bacteriophage λ, the involvement of ClpX in other metabolic processes (e.g., proteolysis of host proteins, molecular chaperone action, etc.) may result in the transient stabilization of λ O, thus favoring unidirectional λ DNA replication. It was previously suggested that the unidirectional (θ) mode of λ DNA replication could be an intermediate leading to the rolling-circle (σ) mode of DNA replication (13).

It has been demonstrated that inactivation of the *clpX* gene has a minor effect on λ bacteriophage growth (21, 36). However, inactivation of ClpX may be compensated for by other chaperones (4) and/or other proteases, because at least *in vitro* it is possible to isolate two different enzymatic activities (distinct from ClpX/ClpP) that efficiently hydrolyze the λ O protein (20).

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