

# Coupling of transcription and replication machineries in $\lambda$ DNA replication initiation: evidence for direct interaction of *Escherichia coli* RNA polymerase and the $\lambda$ O protein

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## ABSTRACT

Transcription proceeding downstream of the  $\lambda$  phage replication origin was previously shown to support initial steps of the  $\lambda$  primosome assembly *in vitro* and to regulate frequency and directionality of  $\lambda$  DNA replication *in vivo*. In this report, the data are presented indicating that the RNA polymerase  $\beta$  subunit makes a direct contact with the  $\lambda$ O protein, a replication initiator of  $\lambda$  phage. These results suggest that the role of RNA polymerase during the initiation of  $\lambda$  phage DNA replication may be more complex than solely influencing DNA topology. Results demonstrated in this study also show that gyrase supercoiling activity stimulates the formation of a complex between  $\lambda$ O and RNA polymerase, suggesting that the introduction of negative supercoils by DNA gyrase, besides lowering the energy required for DNA strand separation, may play an additional role in modeling protein–protein interactions at early steps of DNA replication initiation.

## INTRODUCTION

Bacteriophage  $\lambda$  DNA replication starts from binding of  $\lambda$ O replication initiator protein to four partially symmetrical iterons present at *ori $\lambda$*  (1). Interaction of  $\lambda$ O with the *origin* sequence and formation of higher-order nucleoprotein structure induces DNA helix destabilization within the AT-rich region positioned immediately to the right from  $\lambda$ O-binding sites (2,3). Subsequent steps, involving delivery of the host DnaB helicase to preprimosomal complex by the phage  $\lambda$ P protein (4,5) and liberation of

the helicase from inhibitory interaction with  $\lambda$ P (6,7) by the chaperone proteins DnaK, DnaJ and GrpE, are well characterized biochemically (5,8,9). After the rearrangement of the  $\lambda$ O– $\lambda$ P–DnaB complex by the heat shock proteins, replication starts in the presence of primase, DNA polymerase III, gyrase and SSB proteins (6,10).

*In vivo* and in a crude extract, initiation of replication of  $\lambda$  phage and plasmid DNA replication is dependent on transcription carried by bacterial RNA polymerase. In a system consisting of purified enzymes this dependence of  $\lambda$  DNA replication on ‘transcriptional activation’ is alleviated (11); however, it can be restored by the addition of bacterial histone-like protein, HU (12). HU blocks initiation of replication starting from *ori $\lambda$*  *in vitro* in the absence of RNA polymerase activity (12). In the case of wild-type  $\lambda$  sequence, activation of *ori $\lambda$*  is provided by transcription initiated at the *p<sub>R</sub>* promoter, located  $\sim$ 1000 bp upstream of the origin of replication. However, *in vivo* results demonstrated that replication of *ori $\lambda$* -containing plasmids was still efficient when the promoter was situated much closer to the replication initiation point or even downstream of it (13,14). Activation of *ori $\lambda$*  by the transcription proceeding in its vicinity plays a key role in the control of frequency of replication initiation and the switch from  $\theta$  to rolling circle mode during  $\lambda$  phage DNA replication (15–18).

Similar dependence of the replication initiation on the transcriptional activation was found in the case of the host chromosomal DNA replication (19). DnaA, a bacterial replication initiator protein, binds to specific DnaA boxes within *oriC* and unwinds the DNA duplex in an AT-rich region. This reaction is stimulated by HU protein and transcription process; however, as in the case of  $\lambda$  DNA replication, high concentrations of HU are inhibitory to DNA unwinding step, but RNA

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polymerase activity overcomes this negative influence (20). Allele-specific suppression of certain *dnaA* (ts) mutations by the changes in the *rpoB* gene, coding for the  $\beta$  subunit of RNA polymerase, suggested direct interaction between DnaA and RNA polymerase (21), which has been recently confirmed also *in vitro* (22). Observation that mutations in the *rpoB* gene suppress, in an allele-specific manner, the inability of some  $\lambda$  phage *P* mutants to plate on certain *dnaB* strains, implied also the possibility of direct protein-protein interactions between RNA polymerase and the  $\lambda$  replication complex (23).

$\lambda$ O replication initiator binds as a dimer to a partially symmetric sequence and induces a strong DNA bend (24,25). Interaction of  $\lambda$ O with four iterons within *ori $\lambda$*  results in the formation of a large nucleoprotein complex (O-some) and condensation of a DNA fragment encompassing all iterons and their intervening spacers (2). It was also proved that  $\lambda$ O, by binding to its recognition sequences, greatly stimulates transcription-coupled supercoiling of plasmid DNA by the gyrase. This property of  $\lambda$ O was attributed to its capability of inducing strong DNA bends (26). The process of transcription-dependent stimulation of supercoiling was proved to rely on the twin-domain model (27), according to which counter-rotation of the transcription machinery and template, as DNA is threaded through RNA polymerase active site, generates waves of supercoiling, positive in front of elongating enzyme, and negative behind it. This concept, originally postulated by Liu and Wang (28), became an attractive mechanism for regulation of local processes involving DNA transactions, by influencing DNA topology in a sequence- and context-dependent manner (29,30). Transcription was also shown to regulate chromatin structure and, thus, selection of replication origin sites and replication initiation timing during eukaryotic chromosomes doubling (31 and references therein). Therefore, studies on coordination of replication and transcription machineries seem to be of general importance.

In this report, we demonstrate evidence that the  $\lambda$ O protein, like the host replication initiator, DnaA, interacts directly with bacterial RNA polymerase. Moreover, the results of our studies indicate that the  $\beta$  subunit of RNA polymerase is a contact site for  $\lambda$ O. In addition, we present data suggesting that gyrase activity enhances this interaction.

## MATERIALS AND METHODS

### Strains

*Escherichia coli*, strain C600 (*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*) (32), was used for plasmid purification and strain MM294 (*supE44 hsdR endA1 pro thi*) (33) was used for the  $\lambda$ O and  $\lambda$ P protein overexpression.

### Plasmids

The following plasmids were employed: pRLM4 (*ori $\lambda$ , cro(ts), kan<sup>R</sup>*) was described previously (34); pLamberA (*ori $\lambda$ , colE1, amp<sup>R</sup>*) was constructed by removing the 916-bp HindIII-NcoI fragment from a plasmid pLamber

(35); plasmids: pGP1-2 (T7 RNA polymerase gene under the control of  $\lambda$  repressor CI 857(ts); kan<sup>R</sup>) (36) and pEW1 (*O* and *P* genes under the control of a T7 phage promoter) (37) were used for overproduction of the  $\lambda$ O and  $\lambda$ P proteins.

### Proteins, antibodies and antibiotics

Both  $\lambda$ O and  $\lambda$ P proteins were prepared from *E. coli* strain MM294 bearing plasmids pGP1-2 and pEW1. Proteins were purified according to a method described previously (38). Other proteins and antibiotics were from the following sources: *E. coli* DNA gyrase was obtained from New England BioLabs. Bovine serum albumin (BSA) and novobiocin were from Sigma and *E. coli* RNA polymerase holoenzyme was provided by Eppicentre.

The following antibodies were used in this study: a polyclonal rabbit antibody against the  $\lambda$ O protein (39) and monoclonal mouse antibodies, which were specific for  $\alpha$ ,  $\sigma$ ,  $\beta$  and  $\beta'$  subunits of *E. coli* RNA polymerase, were obtained from Neoclone; secondary HRP-conjugated anti-rabbit and anti-mouse antibodies were obtained from Sigma.

### Surface plasmon resonance

To analyze a direct interaction between  $\lambda$ O protein and *E. coli* RNA polymerase, surface plasmon resonance (SPR) analyses were performed using a double-stranded 141-bp linear DNA fragment containing the four iterons from *ori $\lambda$* . The DNA fragment was obtained by polymerase chain reaction (PCR) amplification with following program: initial denaturation at 94°C for 3 min; 36 cycles of denaturation at 94°C for 20 s; annealing at 55°C for 30 s and extension at 72°C for 30 s. The following primers were used: forward: 5' - biotin - TCA AGC AGC AAG GCG GCA TGT TTG G-3' and reversed: 5' TGT CCC CCT GTT TTG AGG GAT AG-3'. Then, the DNA fragment in TNE buffer (10 mM Tris pH 7.6; 300 mM sodium chloride and 1 mM EDTA) was immobilized on a streptavidin matrix-coated Sensor Chip S.A. (Biacore) by biotin covalent linkage, following the manufacturer's instructions. Experiments were carried out on a Biacore 2000 instrument by a 5-min injection of  $\lambda$ O protein (50 nM) in HBS buffer (10 mM HEPES- KOH pH 7.4; 150 mM sodium chloride; 3 mM EDTA and 0.005% surfactant P20), followed with a 5-min injection of *E. coli* RNA polymerase (25 nM) and BSA (BSA; 25 nM) in binding buffer (10 mM HEPES pH 7.6; 10 mM MgCl<sub>2</sub>; 20 mM KCl; 0.5 mM DTT and 0.1 mM EDTA). Next, the HBS buffer was injected over the chip surface and the dissociation phase was recorded. The analyses were performed at 15  $\mu$ l/min flow speed. Following the completion of each protein co-injection, the surface of the chip was regenerated by applying 0.2% sodium dodecyl sulfate (SDS), which releases all bound protein without affecting the binding capacity of the immobilized DNA. Data were evaluated using Biacore AB's BIA evaluation software. The results are presented as the sensogram, obtained after subtraction of the background response signal and correction of the buffer effect.

As a control of nonspecific interactions, an empty reference cell was used.

#### Detection of protein complexes by disuccinimidyl glutarate cross-linking

Reactions were performed in the buffer containing: 10 mM HEPES-KOH pH 7.6; 10 mM MgCl<sub>2</sub>; 20 mM KCl; 0.5 mM DTT, 0.1 mM EDTA and 50 µg/l poly (dI:dC). Supercoiled λ plasmid DNA (2.8 nM) (purified by ultracentrifugation in a cesium chloride/ethidium bromide gradient) was mixed with λO protein (0.5 µM) and after a 10-min incubation at 30°C reaction was applied onto a 1-ml Sepharose 4B-CL column, equilibrated with a reaction buffer. DNA-λO complexes were separated from unbound protein fraction. Next, to the reaction mixture RNA polymerase (22 nM) and NTPs (0.5 mM each) were added. After a 10-min incubation at 30°C, protein complexes were cross-linked with disuccinimidyl glutarate (DSG) reagent (a final concentration of 0.02 µM) and incubation was prolonged for an hour at 30°C. Reactions were quenched by addition of Tris-HCl pH 7.5 to 50 mM and incubation for next 10 min at 30°C. Subsequently, the cross-linked complexes were separated electrophoretically in 7.5% SDS-polyacrylamide gels, transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore) and detected by immunoblot analysis using anti-λO polyclonal antibodies or monoclonal antibodies specific for various subunits of *E. coli* RNA polymerase. An ECL-Western Blotting Substrate Kit (Pierce) was used to detect antibody-antigen complexes.

#### Gel filtration

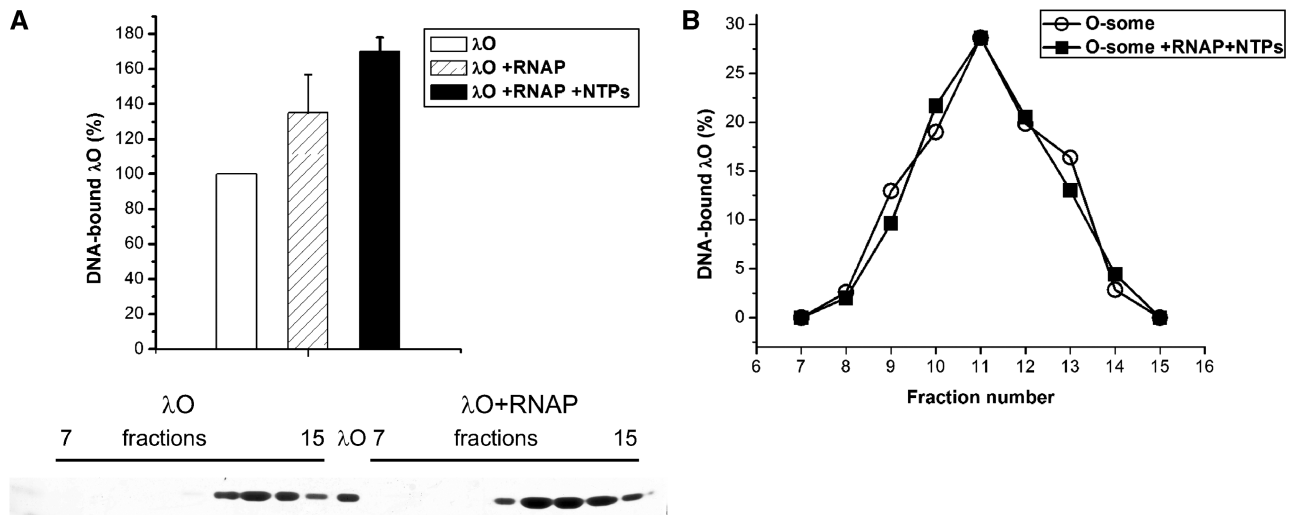
Size-exclusion chromatography was performed to investigate the influence of transcription on pre-formed O-some complexes. The Sepharose 4B-CL (Sigma) column (0.5 × 8 cm) was equilibrated with a buffer containing: 10 mM Tris-HCl pH 7.6; 10 mM MgCl<sub>2</sub>; 20 mM KCl; 0.5 mM DTT; 0.1 mM EDTA. The 120-µl reaction mixture (in the same buffer) supplemented with 10 µg of the supercoiled pRLM4 plasmid and 6 µg of λO protein was incubated for 10 min at 30°C and loaded on a Sepharose 4B-CL column at room temperature. Fractions containing O-some were collected. The presence of plasmid DNA in the O-some fractions was initially confirmed by ethidium bromide staining and visualization under ultraviolet (UV) light. During subsequent repetitions of the experiment, the presence of λO in the void volume fraction was confirmed by amide black staining; fractions containing λO were pooled and divided into two parts. Subsequently, to one of the O-some-containing samples (~100–120 µl) 2.5 µg of RNAP polymerase was added. After 10 min at 30°C, rNTPs (2 mM each) were added to the mixture and incubation was continued for the next 10 min at 30°C. The reaction mixture containing only O-some was also incubated at 30°C. Finally, the two mixtures (separately) were loaded on a Sepharose 4B column. Two-drop fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by silver staining.

Each fraction content was quantitated densitometrically and compared to a sample containing known amount of the λO protein.

## RESULTS

### RNA polymerase activity facilitates λO protein-DNA interaction

λO protein binding to its DNA recognition sequences is a primary step in the initiation of DNA replication. Nucleoprotein structure called O-some, formed due to λO oligomerization and its DNA-bending properties, serves as a landing pad for other proteins involved in the initiation of replication. As RNA polymerase activity may protect λ replication complex from disassembly during unfavorable conditions (40), we wanted to test whether RNA polymerase binding to promoter regions present in λ plasmid DNA (mainly to the strongest p<sub>R</sub> promoter) and transcription activity may influence O-some stability or the affinity of λO protein binding to the *oriλ*-containing plasmid DNA. To study this possibility closer, we performed reactions, using supercoiled λ plasmid DNA, and purified proteins: λO and RNA polymerase. λO-DNA complexes, formed in the presence and absence of active RNA polymerase, were resolved by gel filtration (Sepharose 4B-CL). To test whether efficient transcription occurred under the conditions we used, total RNA synthesis was tested by measuring amount of [ $\alpha$ -<sup>32</sup>P]UTP incorporated into acid-insoluble material (data not shown). Subsequent analysis of fractions, containing λO bound to DNA sequences, demonstrated that the formation of such a nucleoprotein structure is enhanced by the transcription process. Assembly of O-some was stimulated by 70% in fractions containing RNA polymerase and nucleotides, in comparison to those where only λO and DNA were present (Figure 1A). In addition, the formation of nucleoprotein complexes by the O-protein was facilitated by RNA polymerase even in the absence of transcription process, although this effect was less pronounced than the one observed in the reactions where RNA polymerase was allowed to transcribe (Figure 1A). This result suggested that RNA polymerase could facilitate λO protein binding to the *oriλ*-containing plasmid. However, this result could also mean that, under the conditions we used, RNA polymerase activity enhanced stability of the formed O-some structure. To verify the latter hypothesis, we performed analogous gel filtration experiment in which nucleoprotein complexes of the λO protein were formed without RNA polymerase and separated by size-exclusion chromatography from unbound protein. Subsequently O-some containing fractions were collected and divided into equal portions. One of the samples was supplemented with RNAP and NTPs and both were subjected to a second round of gel filtration on separate columns. The amount of λO-containing nucleoprotein complexes was similar in both cases (Figure 1B), indicating that RNA polymerase may play a role in λO binding to DNA, rather than in stabilization of O-some structure during chromatography. Additionally, an experiment was performed in which



**Figure 1.** RNA polymerase and transcription process enhance  $\lambda$ O binding to *oriλ*. (A) Formation of O-some in the presence or absence of RNA polymerase activity was analyzed by gel filtration. Nucleoprotein complexes were formed in the presence of supercoiled  $\lambda$  plasmid DNA (10  $\mu$ g) and  $\lambda$ O protein (1.5  $\mu$ M) and RNA polymerase (2.5  $\mu$ g) and nucleotides (2 mM each) (as indicated). When the influence of either RNA polymerase or transcription process on O-some formation was assessed, RNA polymerase was first incubated with plasmid DNA and nucleotides (as indicated) for 10 min at 30°C, and then the  $\lambda$ O protein was added and incubation was prolonged for another 10 min. Samples were loaded on a Sepharose 4B column; fractions were collected and subjected to SDS-PAGE, followed by silver staining and densitometry. The panel below the graph shows an example of SDS-PAGE separation of fractions obtained after gel filtration of the  $\lambda$ O-DNA complexes formed in the presence or absence of RNA polymerase (as indicated). (B) Stability of  $\lambda$ O-DNA complex, formed and isolated as described above, was assessed in the presence and absence of transcription. Fractions containing O-some obtained after first round of gel filtration were pooled and divided into half. One of the samples was supplemented with RNA polymerase (2.5  $\mu$ g) and NTPs (2 mM). Both samples were subsequently (separately) subjected to a second round of gel filtration. Fractions containing O-some were collected and analyzed by SDS-PAGE as described earlier. The quantity of  $\lambda$ O protein present in the sample where RNA polymerase and nucleotides were absent was assumed as 100%, and elution profile of both samples was presented according to this value.

pre-formed O-some complex were challenged with an excess of biotinylated DNA competitor (containing iteron sequences) in the presence and absence of RNA polymerase and transcription process. The amount of  $\lambda$ O protein bound to competitor DNA was monitored by the separation of biotinylated DNA using magnetic beads, SDS-PAGE and blotting. The results of this experiment confirmed that RNA polymerase and transcription process do not substantially influence O-some stability (for details see Figure S1 in Supplementary Data). Stimulation of the  $\lambda$ O protein binding to iterons by the presence of RNA polymerase and also by transcription process was further confirmed by electrophoretic mobility shift assay (EMSA) performed with supercoiled  $\lambda$  plasmid (see Figure S2 Supplementary Data).

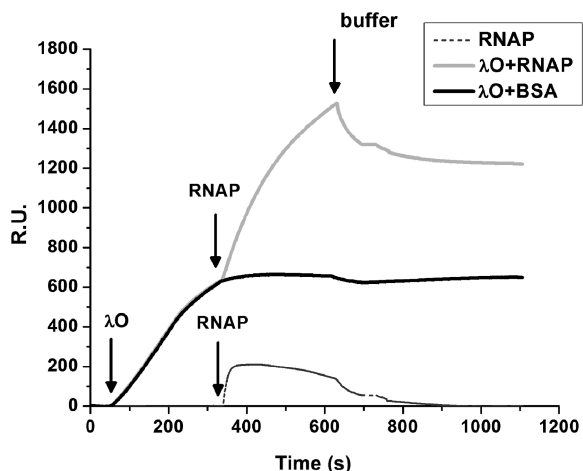
#### $\lambda$ O initiator protein interacts with the host RNA polymerase

During experiments testing the influence of RNA polymerase activity on  $\lambda$ O protein binding to *oriλ*, we obtained evidence suggesting that the  $\lambda$ O initiator may interact directly with the host transcription machinery. In addition, similarity of the initial steps of replication at the *E. coli* and phage  $\lambda$  origin with respect to their dependence on transcriptional activation and recent confirmation of the direct contact between the host initiator protein DnaA and RNA polymerase (RNAP) (22), as well as genetic data suggesting that transcriptional machinery may directly associate with the components of the phage

replication complex (23), encouraged us to follow that trail. Therefore, we analyzed the formation of the putative complex between RNAP and the  $\lambda$ O protein bound at the iterons, using SPR technique. A DNA fragment encompassing all four  $\lambda$ O binding sites of *oriλ* was linked to the sensor chip.  $\lambda$ O and RNAP were injected sequentially over the sensor matrix and the signal, reflecting a change of mass on the immobilized DNA fragment, was measured. Analysis of the sensograms showed that  $\lambda$ O bound efficiently to the sensor-attached DNA. Subsequent injection of RNAP resulted in detection of RNA polymerase binding to the pre-formed  $\lambda$ O-DNA complex (Figure 2). The response signal, seen after injection of RNAP, was relatively high, indicating that the complex between the host and phage protein is formed with high binding affinity. As RNA polymerase displays some unspecific binding affinity to double-stranded DNA, additional control was made to assess the enzyme binding to sensor-attached DNA, containing *oriλ* sequence. In the absence of the  $\lambda$ O protein, RNA polymerase bound to the DNA fragment; however, this interaction was considerably weaker than that observed with O-containing nucleoprotein complex (Figure 2).

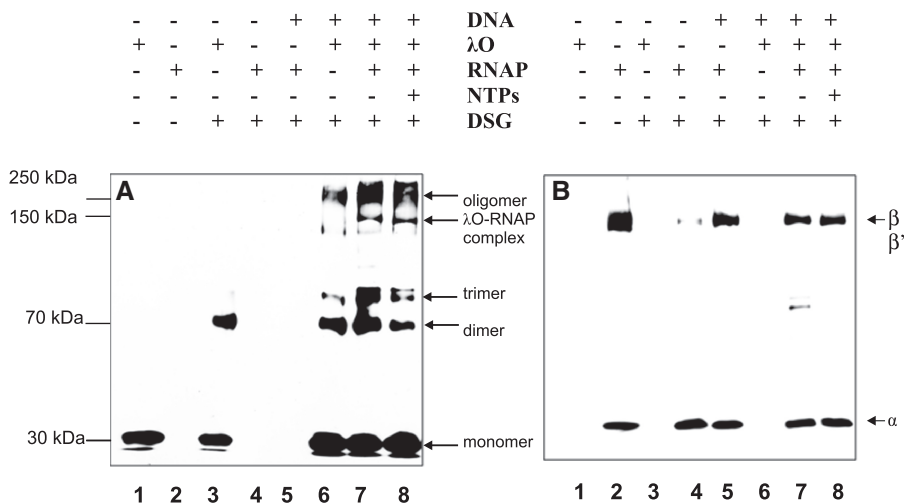
To further confirm the interaction between  $\lambda$ O and RNA polymerase *in vitro*, cross-linking experiments with DSG were performed. DSG is a homobifunctional protein cross-linking reagent with amine-reactive NHS esters at both ends of the 7.7-Å long arm. The  $\lambda$ O protein was incubated together with the supercoiled  $\lambda$  plasmid DNA and, subsequently, the fractions containing 'O-some' were

separated by gel filtration from the unbound protein. The plasmid contained all  $\lambda$ O-binding sites and  $\lambda$  phage promoters in their native orientation and relative position. 'O-some' fractions were collected and divided into individual reactions, supplemented with RNAP and nucleotides before adding DSG. The cross-linked complexes were separated in SDS-PAGE gels, blotted and probed with



**Figure 2.** RNA polymerase forms a complex with the DNA-bound  $\lambda$ O protein. Association of the host RNAP holoenzyme with  $\lambda$ O nucleoprotein complex was estimated by SPR technique. Biotinylated DNA containing four iterons present at *ori $\lambda$*  was immobilized on a sensor chip. Subsequently, a 5-min injection of  $\lambda$ O protein (50 nM) was performed followed by a 5-min injection of RNAP (25 nM) or BSA and a change of protein mass on the sensor was monitored. In the next step, dissociation was monitored by running a buffer over the sensor chip surface (15  $\mu$ l/min flow speed). Data were evaluated using Biacore AB's BIA evaluation software. The presented sensogram reflects results obtained after subtraction of the background response signal and correction of the buffer effect.

antibodies against  $\lambda$ O or RNA polymerase (specific against subunits  $\alpha$ ,  $\sigma$ ,  $\beta$  and  $\beta'$ ) (Figure 3A and B). Monomeric form of the  $\lambda$ O protein (32 kDa) was observed when no DSG was present in the reaction (Figure 3A, lane 1). In a control reaction without DNA, large fraction of  $\lambda$ O migrated, as expected, at the dimer position in the presence of the cross-linker (Figure 3A, lane 3). DNA-bound  $\lambda$ O protein cross-linked, forming several species of increasing molecular mass, representing  $\lambda$ O oligomers (lane 6). When a mixture of 'O-some' and RNAP was subjected to cross-linking, the formation of a new complex was observed, absent from the sample containing only  $\lambda$ O and DNA. This complex migrated at the position similar to RNAP  $\beta$  subunit and was also detected in a sample containing nucleotides (Figure 3A and B; lanes 7 and 8). Under these conditions, no discrete cross-linked complex of RNAP subunits was detected on a blot subjected to anti-RNAP antibodies, in the reactions where only RNAP was present, regardless of the presence or absence of plasmid DNA (Figure 3B, lanes 4 and 5). Instead, a portion of RNAP formed large cross-linked complexes that did not enter the gel. In addition, an increase in the efficiency of formation of the  $\lambda$ O dimer and trimer was observed in the presence of DSG and RNA polymerase, in comparison to the sample where only 'O-some' was subjected to cross-linking (Figure 3A compare lanes 6 and 7). Moreover, much more of a large oligomeric form of the  $\lambda$ O protein was also detected in the presence of RNA polymerase and transcription process (Figure 3A, compare lanes 6, 7 and 8). This cross-linked oligomer had an apparent molecular weight of about 250 kDa, matching the molecular weight of four  $\lambda$ O protein dimers, present in the O-some structure. These results may suggest that some changes in the



**Figure 3.** RNA polymerase interacts with the O-some structure. The  $\lambda$ O protein (0.5  $\mu$ M) was bound to *ori $\lambda$* -containing plasmid (pLamberA; 2.8 nM) and separated from unbound protein fraction by size-exclusion chromatography. Subsequently, reaction was supplemented with RNA polymerase (22 nM) and subjected to DSG cross-linking in the presence (at a final concentration of 0.05 mM) or absence of nucleotides (lanes 6–8).  $\lambda$ O and RNA polymerase were subjected to DSG cross-linking without the presence of DNA (lanes 3 and 4, respectively), or RNA polymerase alone was cross-linked in the presence of plasmid DNA (lane 5). Protein complexes were resolved by SDS-PAGE and detected by immunoblotting, performed with polyclonal antibodies specific against  $\lambda$ O (A) or a mixture of monoclonal antibodies against  $\alpha$ ,  $\beta$  and  $\beta'$  subunits of RNAP (B). The presence or absence of each reaction component is indicated.  $\lambda$ O-RNA polymerase complex was depicted by an arrow. Position of monomeric and oligomeric forms of the  $\lambda$ O protein was marked.

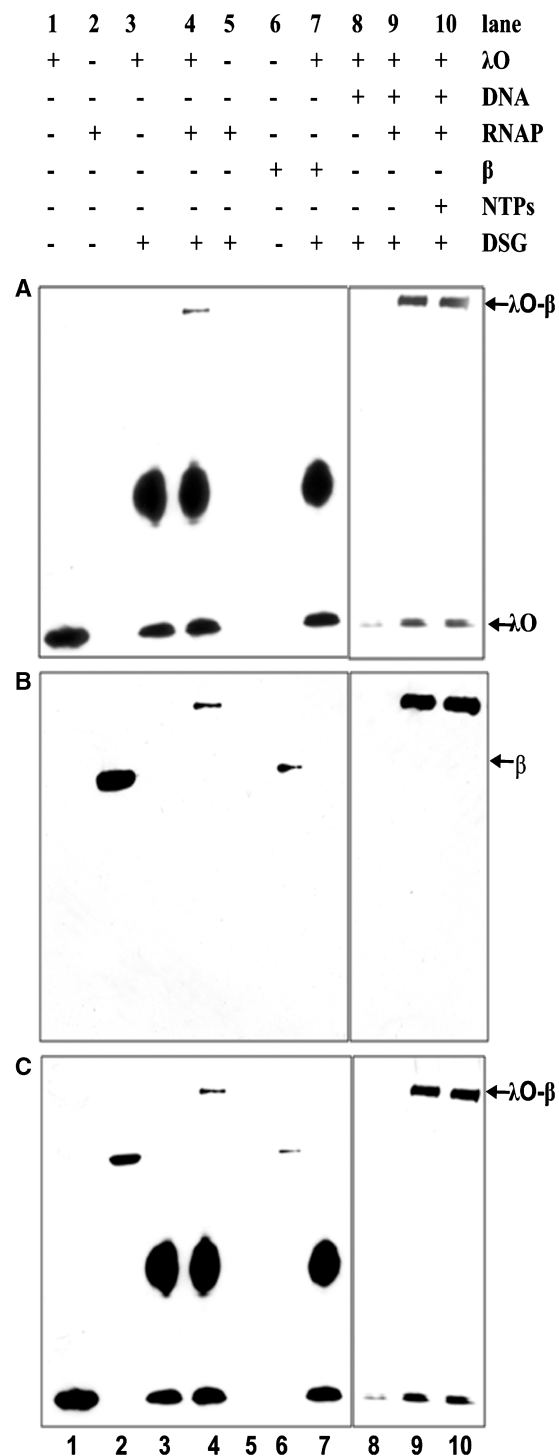
conformation of O-some structure occur in the presence of RNA polymerase activity.

#### Identification of the RNA polymerase $\beta$ subunit as a contact site for the interaction with the $\lambda$ O initiator protein

Due to complexity of the cross-linking pattern obtained with the multisubunit RNA polymerase and  $\lambda$ O and the difficulty to distinguish individual complexes after reaction with DSG, we performed that experiment using varying concentrations of the cross-linking reagent. When the efficiency of cross-linking reaction was sufficiently high, a large portion of reacting proteins was lost from the final picture as a result of the formation of high molecular mass complexes, unable to enter the gel. However, under such conditions, we were also able to identify two products of the cross-linking reaction:  $\lambda$ O dimer and the second one, resulting from the interaction between  $\lambda$ O and RNA polymerase (>250 kDa), (Figure 4A–C). Subsequent analysis of cross-linked species by immunoblotting, performed with purified monoclonal antibodies specific for each of the subunits of RNA polymerase (Figure S3 Supplementary Data), revealed that the high molecular mass cross-linked product cross-reacted with antibodies directed against both  $\lambda$ O and RNAP  $\beta$  subunit and most probably represents  $\lambda$ O tetramer cross-linked to the  $\beta$  subunit. Moreover, we observed the formation of such a complex even in the absence of DNA, which suggest that both proteins are able to interact in solution, even in the absence of specific DNA sequences recognized by these proteins, although with a lower efficiency than in the case of DNA-bound molecules (Figure 4A–C, lane 4). RNAP  $\beta$  subunit alone could form no complex with the  $\lambda$ O protein, detected after cross-linking (Figure 4A–C, lane 7). This is most probably due to altered conformation of the free  $\beta$  subunit in comparison to that observed when  $\beta$  is embedded in the RNAP core structure. These results corroborate conclusions from the SPR experiment that the  $\lambda$ O initiator protein is able to interact directly with the host RNA polymerase *in vitro* and indicate that the  $\beta$  subunit of RNA polymerase is a target for interaction with  $\lambda$ O.

#### DNA gyrase activity stimulates interaction between RNA polymerase and the $\lambda$ O protein

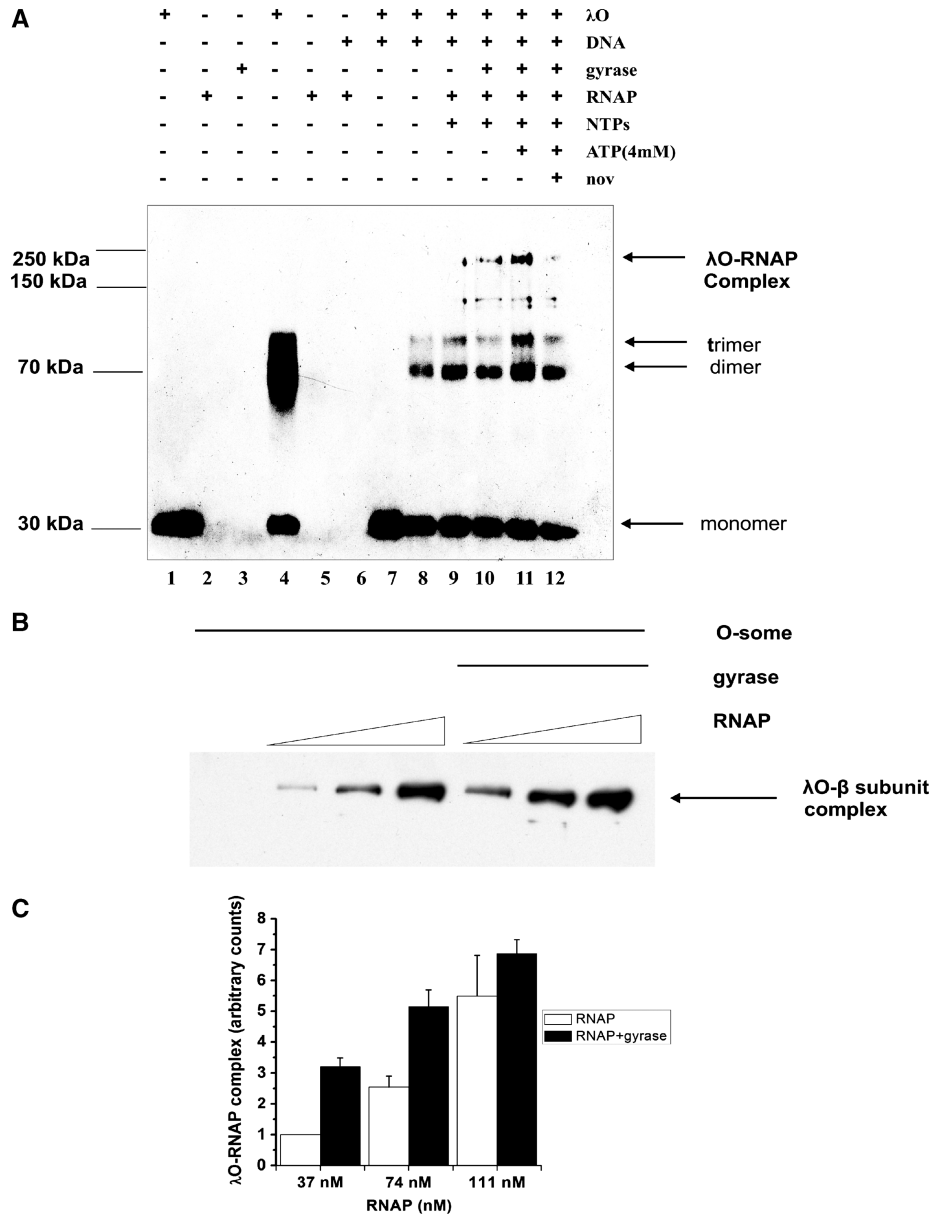
Results of the experiments presented to this point suggest that  $\lambda$  phage replication initiator protein,  $\lambda$ O, interacts with the host RNA polymerase. This conclusion may have important implications for the role of transcription in activating replication initiated by the  $\lambda$  phage proteins. During transcription elongation, RNA polymerase introduces topological changes into DNA template, positive supercoils are generated ahead of the transcription machinery while negative are left in its wake (28). Transient waves of supercoiling produced by RNAP can be stabilized by the action of DNA topoisomerases. It was demonstrated that DNA gyrase rapidly converts transcription-induced positive supercoils into the negative ones (41,42). Moreover,  $\lambda$ O protein was shown to greatly enhance transcription-coupled negative supercoiling of plasmid DNA by the gyrase in *in vitro*



**Figure 4.**  $\beta$  Subunit of RNA polymerase makes a direct contact with both iteron-bound  $\lambda$ O and a free form of the  $\lambda$ O protein.  $\lambda$ O (0.5  $\mu$ M) was bound to *ori $\lambda$* -containing plasmid (2.8 nM) and separated from unbound protein fraction by gel filtration. Subsequently, reaction was supplemented with RNA polymerase (22 nM) and subjected to DSG cross-linking in the presence or absence of nucleotides (500  $\mu$ M). Free  $\lambda$ O protein was also subjected to DSG cross-linking with RNA polymerase. Subsequently, protein complexes were resolved by SDS-PAGE and detected by immunoblotting, performed with polyclonal antibodies specific against  $\lambda$ O (A) or monoclonal antibodies against  $\beta$  subunit of RNAP (B) or simultaneously against both proteins (C). The presence or absence of each reaction component is indicated.  $\lambda$ O–RNAP polymerase complex was depicted by an arrow.

reaction (26). Therefore, we aimed to test whether gyrase activity may influence the formation of the  $\lambda$ O–RNA polymerase complex in a reaction consisting of purified proteins and a supercoiled  $\lambda$  plasmid DNA. To assess this possibility,  $\lambda$ O–plasmid DNA nucleoprotein

complexes were isolated and supplemented with RNAP and/or *E. coli* gyrase and nucleotides. The formation of the complex between RNAP  $\beta$  subunit and the  $\lambda$ O protein was analyzed by cross-linking with DSG and subsequent immunoblotting (Figure 5A). In the presence of the active



**Figure 5.** Gyrase activity stimulates the formation of a complex between RNA polymerase and the  $\lambda$ O protein in an ATP-dependent manner. (A) In the presence of  $\lambda$ O (0.5  $\mu$ M) and the supercoiled  $\lambda$  plasmid DNA (2.8 nM) O-some structure was formed and separated from unbound protein fraction by gel filtration. Next, one of the samples was supplemented with additional ATP (final concentration of 4 mM) and novobiocin was added to the reaction mixture to a final concentration of 100 nM. Subsequently, *E. coli* RNA polymerase (22 nM), gyrase (9 nM) and NTPs (0.5 mM) (as indicated) were added and after a 10-min incubation at 30°C protein complexes were cross-linked with DSG. Complexes were detected by SDS-PAGE followed by immunoblotting with antibodies against  $\lambda$ O.  $\lambda$ O–RNAP polymerase complex was depicted by an arrow as well as oligomeric forms of the  $\lambda$ O protein. The presence or absence of each reaction component was indicated. Lanes 1–3 and 6 contain uncross-linked  $\lambda$ O, RNAP and gyrase and O-some, respectively. (B) Experiment was performed analogously as in point A, but higher concentration of the cross-linking reagent was added (1  $\mu$ M) and various ratios of RNAP to  $\lambda$ O were used, as indicated, in the presence of constant concentration of gyrase (8.3 nM), NTPs (500  $\mu$ M) and ATP (4 mM). (C)  $\lambda$ O–RNAP polymerase complex formation in the presence of gyrase activity was measured as described in point B in three independent experiments. Relative amount of  $\lambda$ O– $\beta$  subunit complex formed in the presence and absence of gyrase activity was measured densitometrically and presented as a function of RNA polymerase concentration with error bars representing SD. Amount of the complex observed in the sample where the lowest concentration of RNA polymerase was present and no gyrase was added was assumed as 1 and other values reflect this value.

RNA polymerase, a band corresponding to  $\lambda$ O–RNAP cross-linked product was observed (Figure 5A, lane 6). Addition of DNA gyrase to the reaction, stimulated interaction between RNA polymerase and  $\lambda$ O in a manner dependent on adenosine triphosphates (ATP) concentration (Figure 5A, lanes 7 and 8). Novobiocin, in turn, reduced this stimulatory effect (Figure 5A lane 9). This aminocoumarin antibiotic, which is a competitive inhibitor of the gyrase ATP-ase activity, also inhibits supercoiling activity of gyrase, which is exerted at the expense of ATP. We also tested  $\lambda$ O– $\beta$  subunit complex formation in the presence of transcription and gyrase activity at various ratios of RNAP to the  $\lambda$ O protein and at higher concentration of cross-linking reagent, yielding higher molecular mass cross-linked product (Figure 5B). In this case, we also observed the enhancement of the  $\lambda$ O– $\beta$  subunit conjugation by DSG, in the reactions where gyrase was present, in comparison to the ones where transcription was allowed to proceed in the presence of the  $\lambda$ O protein only. Stimulation of the  $\lambda$ O– $\beta$  complex formation was assessed densitometrically and summarized in the Figure 5C. These results indicate that ATP-dependent gyrase activity may facilitate the contact between the  $\lambda$ O replication initiator and the host RNA polymerase.

## DISCUSSION

Transcription was shown to play a pivotal role in the regulation of bacteriophage  $\lambda$  replication. *In vitro*, transcription was necessary in the step preceding preprimosome rearrangement by the heat shock proteins and this process was also shown to be able to overcome inhibitory action of the histone-like HU protein (12,43). Both *in vivo* and *in vitro*, activity of RNA polymerase stimulated bidirectional replication starting at the  $\lambda$  origin (16,44). In addition, it was demonstrated that efficient transcription process was required for the  $\lambda$  replication complex to survive heat shock-provoked disassembly by GroEL proteins after transient DNA relaxation by gyrase (40). These results suggested that transcription stabilizes replication complex under unfavorable conditions.

Results presented in this work strongly indicate that the  $\lambda$ O protein interacts directly with bacterial RNA polymerase (Figures 2 and 3). On RNAP part,  $\beta$  subunit seems to be responsible for the contact with  $\lambda$  phage replication initiator (Figure 4), similarly as it was suggested for the host initiator DnaA protein (21,22). This interaction seems to facilitate  $\lambda$ O protein binding to iteron-containing DNA as we observed enhanced formation of  $\lambda$ O nucleoprotein complex even in the absence of nucleotides (data not shown) and transcription process further supported association of  $\lambda$ O with *ori $\lambda$*  (Figure 1). It is unlikely that this stimulation plays a primary role in the transcriptional activation process; however, it may be helpful under conditions of limiting  $\lambda$ O protein. Although we used under mild conditions, O-some structure is very stable and therefore we did not observe any enhancement of this nucleoprotein complex stability by transcription (Figure 1B); it cannot be excluded that direct interaction of RNA polymerase and  $\lambda$ O may support replication complex survival

under more harsh circumstances.  $\lambda$ O–RNAP association may also help in organizing replication hyperstructure consisting of phage and host proteins necessary for phage DNA replication and progeny production, in keeping with the model of a ‘factory’ where many proteins are engaged in various DNA transactions (45).

It was demonstrated that  $\lambda$ O protein, most probably due to its DNA-bending properties, stimulates transcription-coupled DNA supercoiling by gyrase *in vitro* (26,27). Here, we show that gyrase activity—in turn—stimulates formation of a complex between RNA polymerase  $\beta$  subunit and the  $\lambda$ O protein. We have not determined whether this stimulatory influence of gyrase relies on, connected with supercoiling, increase in probability of juxtaposition of RNA polymerase and  $\lambda$ O position on DNA. Supercoiling could also actively affect conformation of the  $\lambda$ O complex in a way favoring the interaction. Topological state of the loop formed by Lac repressor bound to adjacent operator sites was also shown to influence repressor conformation (46). Similarly, DNA supercoiling was implicated in assisting in octamerization of the  $\lambda$  CI protein and, hence, facilitating autoregulatory repression of  $p_{RM}$  promoter by aiding CI in binding to distant  $O_{L3}$  and  $O_{R3}$  operators and DNA looping (47,48). In addition, it was demonstrated that several promoters are sensitive to the supercoiling level (49), so the stimulatory effect of gyrase activity on the formation of RNA polymerase– $\lambda$ O complex may result from altered transcriptional activity of promoters located on plasmid DNA. Whatever mechanism operates in gyrase-mediated stimulation of  $\lambda$ O and RNA polymerase interaction, this observation emphasizes the importance of DNA supercoiling, not only in lowering the energy necessary for DNA strand separation but also in modeling of protein–DNA and protein–protein interactions.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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