Acta Biochimica Polonica

Vol. 42 No. 2/1995

233–240

QUARTERLY

Inhibition of transcription starting from bacteriophage λ p_R promoter during the stringent response in *Escherichia coli*: implications for λ DNA replication*

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Received: 30 September, 1994; accepted: 15 March, 1995

Key words: stringent control, regulation of transcription, λDNA replication

Replication of λ plasmid DNA is halted in amino acid-starved wild type (stringent) strains whereas it proceeds in *relA* (relaxed) mutants. The only transcription which could be important in λ plasmid DNA replication in amino acid-starved *Escherichia coli* cells is that starting from the p_R promoter. Using a fusion which consists of the *lacZ* gene under the control of bacteriophage λ p_R promoter we found that transcription starting from this promoter was inhibited during the stringent, but not the relaxed, response in *E. coli*. We confirmed our conclusion by estimating the relative level of the p_R transcript by RNA-DNA hybridization. We propose that decreased transcription from the p_R promoter which serves as transcriptional activation of *ori*λ is responsible for inhibition of λ plasmid replication during the stringent response. The results presented in this paper, combined with our recent findings (published elsewhere), indicate that the transcriptional activation of *ori*λ may be a main regulatory process controlling λ DNA replication not only during the relaxed response but also in normal growth conditions.

λ Plasmids are replicons derived from bacteriophage λ. They contain a fragment of phage λ DNA comprising o_Rp_R operator/promoter region and *cro*, *cII*, *O* and *P* genes (Fig. 1). They replicate in *E. coli* as typical plasmids and are maintained in several copies per cell [1]. The products of the λ*O* and λ*P* genes are necessary for λ plasmid replication. Together with *E. coli* replication proteins they participate in the assembly of the replication complex at *ori*λ and its function (see [2] and [3] for review). Two other genes, *cII* and *cro*, do not participate directly in λ plasmid replication. The *cII* gene is totally dispensable in this process, however, Cro protein is an inhibitor of the p_R promoter. Transcription starting from this promoter has a double role in replication of λ plasmid. The first role is production of mRNA for synthesis of replication proteins (λO and λP). Nevertheless, even when replication proteins are present, the transcription at or near *ori*λ is necessary for initiation of λ DNA replication. This process, called transcriptional activation of *ori*λ, is known since long [4], however, its exact role is still poorly understood.

*A. S-P. is a holder of the fellowship from the Foundation for Polish Science in 1995. This work was supported by the UG grants BW-1190-5-0059-4 and BW-0010-5-0068-5.

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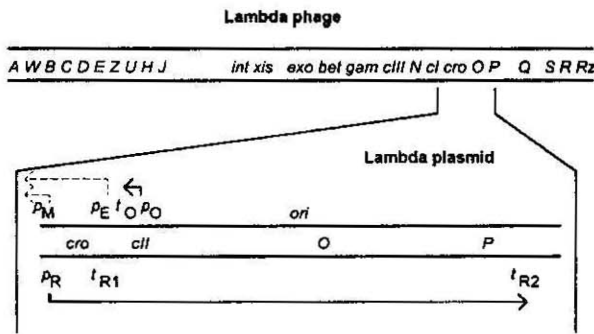

Fig. 1. A scheme of bacteriophage λ genome (Lambda phage) and its fragment present in the standard λ plasmid (Lambda plasmid).

The main genes are indicated in the genome of λ phage as well as in λ plasmid. The promoters (p_R, p_M, p_E and p_O) and terminators of transcription (t_{R1}, t_{R2} and t_O) present in the λ plasmid are marked. Particular transcripts are presented as arrows. The arrowheads of solid-line arrows indicate the ends of transcripts. In the case of dashed-line arrows, the ends of transcripts are not precisely defined as appropriate terminators are absent in the λ plasmid DNA. The origin of λ DNA replication (*ori*), located in the middle of the λ*O* gene, is indicated. The only transcription which is important for λ plasmid replication is that starting from the p_R promoter. It can stop quickly at the t_{R1} terminator (the appropriate transcript is indicated by thick line), however, this terminator is a weak one and a fraction of RNA polymerase molecules continues transcription through the *cII*, *O* and *P* genes before it stops at t_{R2}. The longer transcript (indicated by thin line) serves as mRNA for synthesis of the CII protein (which has no function in λ plasmid replication) and replication proteins λO and λP. The transcription starting from the p_R promoter and passing through or near the origin sequence is responsible for the so-called transcriptional activation of *ori*λ which is indispensable for the initiation of λ DNA replication.

The binding of the λO protein to *ori*λ sequence is the first step in the assembly of the replication complex. Since this protein is very unstable *in vivo* [5] its binding to *ori*λ was considered a rate-limiting step in the regulation of λ DNA replication. However, we have found that under conditions where the λO protein is not synthesized, i.e. during amino acid starvation, λ plasmid can replicate in *E. coli* strains bearing *relA* mutation [6, 7]. The replication is perpetuated by the replication complex assembled before the onset of amino acid starvation and inherited by one of two daughter copies after each replication round [8]. This complex contains the λO protein which is protected from proteases by other elements of the complex [9, 10]. The protected λO can be observed as a stable fraction of this protein during pulse-and-chase experiments. In spite of the existence of a stable fraction of the λO protein in relaxed as well as stringent strains, the replication is strongly inhibited during the stringent response [6–9].

The very quick and significant increase of the level of guanosine 5′-diphosphate-3′-diphosphate (ppGpp) in the starved cell is the first event of the stringent response (see [11] for review). This nucleotide interacts with RNA polymerase [12, 13] inducing inhibition or, rarely, activation of different promoters. *E. coli relA* mutants do not produce ppGpp during amino acid starvation, thus intracellular concentration of this nucleotide decreases in these strains.

As mentioned above, λ plasmids replicate during amino acid starvation of *relA* mutants, whereas the replication is inhibited during the stringent response [6–9]. It was suggested that ppGpp-mediated inhibition of RNA polymerase is responsible for halting of λ plasmid replication during the stringent response. According to this hypothesis, the transcription from the p_R promoter (which is the only promoter necessary for λ plasmid DNA replication) should decrease in *E. coli* cells containing high ppGpp concentrations, as during the stringent response. On the other hand, the transcription should be increased or stay unaffected during the relaxed response. To verify the above mentioned hypothesis we estimated the efficiency of transcription starting from the p_R promoter in amino acid-starved stringent and relaxed strains as well as under normal growth conditions.

MATERIALS AND METHODS

Bacterial strains. The following *E. coli* K-12 strains were used: CF1648, CF1652 (an isogenic pair of prototrophic *rel*$^+$ and Δ*relA*251::*kan* strains, respectively) [14] and BM65=72-12-6 (Δ(*lac-pro*) thi / F′ *lacI*^{q} *pro*$^+$) [15]. The Δ*relA* 251::*kan* mutation was transferred to BM65 by P1 transduction according to Silhavy *et al.* [16].

Plasmids. The λ plasmid pCB104 [8] was used. For construction of the pR-lacZ plasmid, the *Hin*dIII-*Bam*HI fragment (644 bp) from pHG276 [17] was cloned into the polylinker of pTL61T [18]. Therefore, the pR-lacZ plasmid contains the *lacZ* gene under the control of the p_R promoter. Plasmid pGW857 was con-

structed by ligation of *Eco*RI-*Sma*I fragment (2450 bp) from pGP1-2 [19], which contains the *cIts*857 allele coding for temperature-sensitive λ repressor and origin of replication derived from p15A, with *Eco*RI-*Pvu*II fragment (2066 bp) from pBR322 [20], containing the tetracycline-resistance gene. The plasmid pGW857 contains the *cIts*857 allele under the control of the p_{lac} promoter. All molecular cloning manipulations were carried out as described by Sambrook *et al.* [21].

Culture media and amino acid starvation. LB medium [21] was used during molecular cloning procedures. The minimal medium 3 [8] was used in all experiments. Isoleucine starvation of *E. coli* K-12 strains was induced by addition of L-valine to minimal medium to final concentration of 1 mg/ml.

Estimation of the activity of a promoter fused upstream to the lacZ gene under conditions that mimic the stringent or relaxed control. Bacteria harboring the *lacZ* gene under the control of investigated promoter were grown in minimal medium to A_{500} = 0.2 under conditions ensuring repression of the particular promoter. In order to induce isoleucine starvation, L-valine was added to final concentration of 1 mg/ml. Following 15 min starvation, picolinic acid and Casamino acids were added to final concentrations of 1.5 mM and 1%, respectively. Then the investigated promoter was derepressed by temperature shift from 30°C to 37°C or by medium exchange (see Results for details). β-Galactosidase activity was measured 90 min after promoter derepression.

Measurement of β-galactosidase activity. The intracellular level of β-galactosidase activity was measured as described by Miller [22].

Isolation of total RNA from bacteria. A sample of bacterial culture (1.4 A_{500} units) growing in minimal medium was withdrawn, the volume of the sample was adjusted to 3.5 ml with the same medium, and the sample was transferred immediately to an equal volume of boiling lysis buffer (1% SDS, 100 mM NaCl, 8 mM EDTA, 0.1% diethyl pyrocarbonate, pH = 7.0) for 1 min. Then the tube was transferred to ice-bath for 2 min. Following two cycles of extraction with an equal volume of phenol-chloroform (1:1, v/v), sodium acetate (to 0.3 M) and a double volume of 96% ethanol were added to the upper (water) phase. The precipitation was carried out at –20°C. After centrifugation (9000 × *g*, 30 min, 4°C) the pellet was washed with 70% ethanol and dried. Formamide (0.1 ml) was added to the pellet and the sample was incubated at 60°C for 5 min and then transferred to ice-bath for 1 min. The RNA sample dissolved in formamide was kept at –20°C.

RNA gel electrophoresis and hybridization. Twenty µl of RNA solution in formamide were mixed (5:1, v/v) with loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol, 0.1% diethyl pyrocarbonate), ethidium bromide was added (to 20 µg/ml) and the sample was loaded into the well of 1.5% agarose gel (SEA-KEM Agarose, FMC BioProducts) prepared in the buffer containing 20 mM Mops, pH = 7.0, 5 mM sodium acetate, 1 mM EDTA, 0.1% diethyl pyrocarbonate and 2.2 M formaldehyde. The electrophoresis was run in the same buffer except that formaldehyde was omitted. The gel was run at 5 V/cm and photographed over a transilluminator (312 nm wavelength) to visualize ribosomal RNA (internal RNA control). RNA was transferred to positively charged nylon filter as described by Sambrook *et al.* [21]. The hybridization with single stranded DNA probes labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate was performed by using DIG DNA Labeling and Detection Kit (Boehringer Mannheim) and according to the producer's instruction. The template for random priming reaction consisted of the previously denatured *Hin*dIII-*Bam*HI fragment (4273 bp) of pKB2 plasmid [23], containing a replication region of λ DNA. The relative amount of RNA was estimated by densitometry. The intensity of the band after the hybridization was proportional to the amount of RNA loaded on the gel (not shown).

Measurement of the plasmid DNA amount. The relative amount of plasmid DNA in *E. coli* strains was measured as described previously [24, 25].

RESULTS

Measurement of β-galactosidase activity in the cells harboring the *lacZ* gene under the control of investigated promoter is one of the common methods used for estimation of the activity of promoters in particular conditions.

In order to investigate the p_R promoter activity we constructed the pR-lacZ plasmid which contains the *lacZ* gene under the control of this promoter. However, the classical method described above could not be used when comparing stringent and relaxed conditions. In the case of both stringent and relaxed response the cells are starved for a particular amino acid. Therefore, the synthesis of proteins, and thus also the synthesis of β-galactosidase, is decreased. Nevertheless, we found experimental conditions allowing to investigate the activity of a particular promoter fused upstream to the *lacZ* gene under conditions that mimic stringent and relaxed response. We provoked amino acid starvation by standard methods. Amino acid starvation of wild type (rel^+) strains results in quick and efficient synthesis of ppGpp — an effector of the stringent control. The ppGpp concentration reaches the maximum value several minutes after the onset of amino acid starvation. In order to estimate the increase of β-galactosidase activity in the cells containing a high ppGpp concentration we had to add amino acids to the culture of starved bacteria. However, the addition of amino acids means the stop of starvation and halts the production of ppGpp. Since ppGpp is rapidly degraded in bacterial cells by the product of the *spoT* gene, the concentration of this nucleotide drops dramatically. Picolinic acid is an inhibitor of the SpoT protein [26]. Thus, we added the picolinic acid to the bacterial culture together with amino acids 15 min after the onset of amino acid starvation. Hence, ppGpp was not degraded and its concentration remained high in non-starved bacteria. ppGpp is not synthesized in amino acid-starved *relA* mutants. Therefore, all manipulations described above allowed to keep the low concentration of ppGpp in relaxed mutants. Since β-galactosidase is a stable enzyme, it was necessary to repress the p_R promoter before the induction of amino acid starvation. We used the plasmid (pGW857) harboring the *cI*857 allele which codes for temperature-sensitive repressor of the p_R promoter. Increasing of temperature of bacterial culture from 30°C to 37°C resulted in derepression of the p_R promoter and production of β-galactosidase. Control experiments with the p_{lac} promoter were performed in an analogous system except that we have investigated the chromosomal lactose operon. The promoter was repressed by cultivating bacteria in minimal medium containing glucose, and derepression of the p_{lac} promoter was reached by replacing glucose with lactose in the minimal medium (the culture was centrifuged at 2000 × *g* for 10 min, washed twice and resuspended in minimal medium containing lactose instead of glucose).

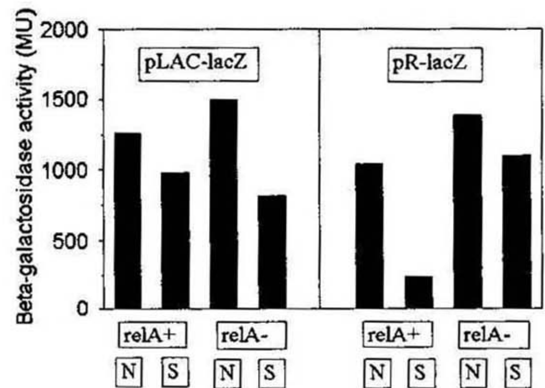

Fig. 2. β-Galactosidase activity (in Miller units, MU) in $relA^+$ and $relA^-$ strains non-starved (N) or starved for isoleucine (S).

The strains containing the *lacZ* gene under the control of the p_{lac} (CF1648 and CF1652) or p_R promoter (BM65/pR-lacZ /pGW857 and BM65*relA* /pR-lacZ / pGW857) were investigated. The experiments were performed as described in Materials and Methods; see also text for details. The β-galactosidase activity measured in non-starved bacteria before the derepression of the p_{lac} or p_R promoter was lower than 2 Miller units.

In the control experiments we found that conditions which mimicked the stringent response did not affect significantly the increase of β-ga-

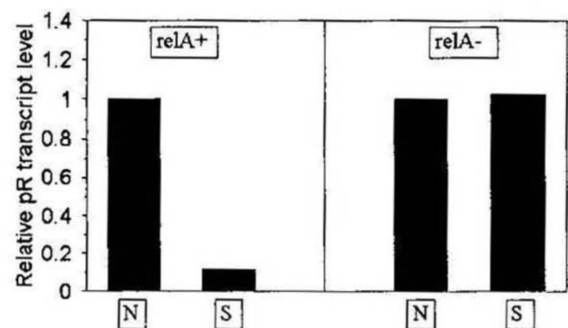

Fig. 3. Relative level of the longer p_R transcript (see Fig. 1 for description of the p_R transcripts) per amount of λ plasmid in non-starved (N) and isoleucine-starved for 60 min (S) $relA^+$ (BM65/pCB104) and $relA^-$ (BM65relA/ pCB104) strains.

The values obtained for non-starved bacteria were assumed as 1.

lactosidase activity when the *lacZ* gene was under the control of the p_{lac} promoter (Fig. 2). These results agree with previous reports showing that ppGpp does not inhibit the activity of the p_{lac} promoter [27–29]. However, we found that in strains harboring the *lacZ* gene under the control of the p_R promoter the increase of β-galactosidase activity was significantly lower under conditions which mimicked the stringent response than during normal growth conditions, whereas it was practically unaffected in the *relA*⁻ mutant (Fig. 2). These results suggest that the p_R promoter is under the stringent control.

This conclusion was confirmed by another experiment. We isolated total RNA from amino acid-starved as well as non-starved *relA*$^+$ and *relA*$^-$ bacteria harboring the λ plasmid, pCB104. After agarose gel electrophoresis RNA was transferred to positively charged nylon filter and hybridization with digoxigenin-labelled DNA probe containing λ replication region was performed. The relative amount of the RNA was measured densitometrically. Since λ plasmid replication differs in amino acid-starved stringent and relaxed bacteria, the amount of DNA template was different in investigated *relA*$^+$ and *relA*$^-$ strains. Therefore, we calculated the relative level of particular RNA transcripts per λ plasmid, i.e. per amount of DNA template. We estimated that the level of the longer p_R transcript (see Fig. 1) was several-fold lower in amino acid-starved *relA*$^+$ strain than in unstarved bacteria (Fig. 3) whereas it was very similar in isoleucine-starved and non-starved *relA*$^-$ mutant.

DISCUSSION

Replication of λ plasmid DNA proceeds in amino acid-starved *relA* mutants of *E. coli* whereas it is inhibited during the stringent response [6–9]. It was suggested earlier that this inhibition could be due to a ppGpp-mediated decrease of transcription [7]. We found that in *relA*$^+$ strain harboring the *lacZ* gene under the control of the p_R promoter the activity of β-galactosidase was lower under conditions which mimicked the stringent response than under normal growth conditions. In the control experiments we demonstrated that the activity of β-galactosidase was not affected by stringent conditions when the *lacZ* gene was under the control of the p_{lac} promoter, known to be insensitive to ppGpp. Under the conditions of the control experiments we have observed slightly lower activity of β-galactosidase in previously starved *relA*$^-$ strain in comparison with non-starved bacteria. Most probably these results reflect the phenomenon of premature termination of transcription starting from the p_{lac} promoter in amino acid-starved relaxed mutants, which was recently described [29]. All these results suggest that the transcription starting from the p_R promoter is inhibited by the high ppGpp concentration. Moreover, we demonstrated that the relative level of the p_R transcript was significantly decreased during the stringent, but not the relaxed, response. Therefore, it seems that the hypothesis concerning ppGpp-mediated inhibition of transcription from the p_R promoter as a key process in the stringent control of λ plasmid replication is fully substantiated. In accordance with this hypothesis, we have recently demonstrated that λ plasmid replication in amino acid-starved relaxed mutants is sensitive to rifampicin — an inhibitor of RNA polymerase [30]. Moreover, the replacement of the p_R promoter with the p_{lac} promoter in a λ plasmid resulted in its DNA replication during relaxed and stringent responses [30].

The p_R is the only promoter which could be of importance in the λ plasmid replication. Since during the relaxed response no protein synthesis is necessary for λ DNA replication [6], the transcription starting from the p_R promoter is probably indispensable only for transcriptional activation of *ori*λ. Until recently, the binding of the λO protein to *ori*λ was considered a rate-limiting step in the regulation of λ DNA replication. However, we demonstrated that neither absence nor excess of λO-digesting protease affected λ plasmid or λ phage replication in *E. coli* [31]. We have recently suggested that the transcriptional activation of *ori*λ is not only indispensable for λ DNA replication but also may play an important role in the control of the initiation of this process [32]. It was demonstrated previously that λ plasmids could not replicate in *E. coli* cells lacking the DnaA function [23]. We found that the transcriptional activation of *ori*λ was positively regulated by DnaA [33]. Taking into account of these data it may be concluded that the transcriptional acti-

vation of *ori*λ is a main regulatory process in the control of λ DNA replication not only in amino acid-starved relaxed mutants but also under normal growth conditions.

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