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The mechanism of the stringent control of λ plasmid DNA replication

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λ plasmid DNA replication is inhibited in amino acid-starved wild type *Escherichia coli* strains (stringent response) but not in amino acid-starved *rel*A mutants (relaxed response). This replication is perpetuated by the replication complex containing the λO protein (which is protected from proteases by other elements of the complex) and inherited by one of two daughter copies after a replication round. Since a fraction of stable λO protein was observed in *rel*A$^-$ and *rel*A$^+$ strains, and negative regulation by the λCro repressor does not seem to be important in the stringent or relaxed response of λ plasmid replication to amino acid starvation, the inhibition of λ plasmid replication in amino acid-starved wild type strains was investigated. λ plasmids were unable to replicate in amino acid-starved *rel*A$^-$ bacteria treated with rifampicin. Moreover, transcription from p_R, which produces mRNA for replication protein synthesis and serves as transcriptional activation of *ori*λ, was significantly decreased during the stringent response as well as in non-starved cells containing increased levels of ppGpp. However, it was little or totally not affected by the relaxed response. The replacement of p_R with p_{lac} (which is known to be uninhibited by ppGpp) in a λ plasmid resulted in its DNA replication during relaxed and stringent responses as well as during overproduction of ppGpp in unstarved bacteria. We conclude that ppGpp-mediated inhibition of transcriptional activation of *ori*λ is responsible for inhibition of λ plasmid DNA replication in amino acid-starved wild type strains. A model of stringent control of λ plasmid DNA replication is proposed and an analogy with eukaryotic DNA replication suggested.

Key words: DNA replication/lambda plasmids/replication complex/stringent control/transcriptional activation of origin

Introduction

The bacterial response to amino acid starvation has come to be known as the stringent response (for review see Cashel and Rudd, 1987). Guanosine 5′-diphosphate-3′-diphosphate (ppGpp) is the main effector of the stringent response. There are two pathways leading to ppGpp synthesis in *Escherichia coli* cells. Amino acid starvation results in the activation of the *rel*A gene product (ppGpp synthetase I) which catalyses indirectly the synthesis of ppGpp. During carbon source deprivation, ppGpp is produced in a *rel*A-independent reaction (Metzger *et al.*, 1989) which is dependent on *spoT* function (Hernandez and Bremer, 1991; Xiao *et al.*, 1991). Since SpoT protein is also a ppGpp 3′-pyrophosphohydrolase, it seems that it is a bifunctional enzyme capable of catalysing ppGpp synthesis (ppGpp synthetase II) or degradation (ppGpp-ase). During amino acid starvation of *rel*A mutants, the ppGpp synthetase II is inactivated (Lagosky and Chang, 1980; Hernandez and Bremer, 1991). Since there are also no active ppGpp synthetase I molecules in these bacteria, this phenomenon leads to a decrease in the ppGpp level in the cells. Such response of *rel*A mutants to amino acid starvation is called the relaxed response.

RNA polymerase is a target for ppGpp (Glass *et al.*, 1986; Tedin and Bremer, 1992; Vinella and D'Ari, 1994). An interaction of ppGpp with RNA polymerase results in a decreased level of transcription from many (but not all) promoters. However, it is still controversial whether this inhibition of RNA synthesis reflects an effect of ppGpp on promoter selection (Ryals *et al.*, 1982), or on transcriptional pausing during the synthesis of mRNA which makes RNA polymerase inactive (Hernandez and Bremer, 1993), or on a reduced rate of RNA polymerization which might sequester RNA polymerase molecules in the elongation phase and limit the number of free polymerases able to initiate new transcription (Sorensen *et al.*, 1994).

The stringent response causes many physiological changes in bacterial cells; it also affects DNA replication of several replicons (Herman *et al.*, 1994b). Among them, λ plasmid is one of the best investigated models. The stringent response causes an inhibition of λ plasmid DNA replication whereas the replication proceeds in amino acid-starved relaxed mutants (Węgrzyn *et al.*, 1991a,b). This replication is perpetuated by the replication complex which is inherited by one of two daughter copies after a replication round (Węgrzyn and Taylor, 1992). The replication complex contains the λO protein which is protected from proteases by other elements of this complex (Węgrzyn *et al.*, 1992). The λO initiator protein (the binding of this protein to *ori*λ sequence is the first step in assembly of the replication complex) is very rapidly degraded in *E.coli* cells when in a free form (Lipińska *et al.*, 1980; Gottesman *et al.*, 1981) and is not synthesized during the stringent and relaxed responses due to amino acid deprivation (Węgrzyn *et al.*, 1992). Thus, a new replication complex cannot be assembled during amino acid starvation. Since a stable fraction of λO protein was observed in both relaxed and stringent strains of *E.coli*

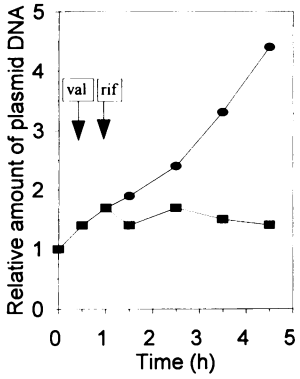

Fig. 1. Relative amount of λ plasmid (pAS1) DNA per ml of bacterial culture in the *rel*A$^-$ (CF1652) strain starved for isoleucine (circles), and in the same strain starved for isoleucine and treated with rifampicin (squares). Addition of L-valine (to 1 mg/ml), which induces isoleucine starvation, and rifampicin (to 25 μg/ml) are indicated by the arrows.

(Węgrzyn *et al.*, 1992) and negative regulation by the λCro repressor (which inhibits transcription from the p_R promoter) does not seem to be important in the stringent control of λ plasmid replication (Węgrzyn *et al.*, 1991b; Węgrzyn and Taylor, 1992), a reason for inhibition of λ plasmid replication during the stringent, but not relaxed, response remained unclear. Here we demonstrate that decreased transcription from the p_R promoter (which leads to the transcriptional activation of *ori*λ) caused by the high ppGpp level is responsible for the above mentioned phenomenon.

Results

λ plasmid DNA replication proceeding during the relaxed response is sensitive to rifampicin

It was previously reported that the replication of λ plasmid proceeded for several hours in amino acid-starved *rel*A mutants (Węgrzyn *et al.*, 1991b; Węgrzyn and Taylor, 1992). We investigated the plasmid DNA replication by estimation of the change in relative plasmid amount per ml of bacterial culture as a function of time. Thus, if the replication of plasmid DNA were stopped, the relative amount of plasmid DNA per ml of culture should stay constant. On the other hand, an increase in the amount of plasmid indicates continued plasmid DNA replication. We confirmed the replication of λ plasmid DNA during the relaxed response; moreover, we found that this replication is sensitive to rifampicin, an inhibitor of RNA polymerase (Figure 1).

Detection of the p_R transcripts

The results described above indicate the involvement of the RNA polymerase function in the replication of λ plasmid DNA during the relaxed response. The only transcription which is necessary for λ plasmid replication *in vivo* is that starting from the p_R promoter. The transcription can stop at the t_{R1} terminator, however, since this terminator is a weak one, a fraction of RNA polymerase molecules is able to continue the transcription through the t_{R1} terminator into the λ replication region. Thus, two transcripts appear as a result of the transcription from the p_R promoter: 311 nucleotides long and 2601 nucleotides long (Daniels *et al.*, 1983). The longer one is important for λ DNA replication because it functions as mRNA for synthesis of replication proteins (λO and λP) and a transcription leading to its appearance passes through the *ori*λ region and acts as a transcriptional activation of the *ori*λ. Therefore, we decided to investigate the level of this transcript in amino acid-starved stringent and relaxed strains. For detection of the transcript, we used a DIG-labelled DNA probe. In order to prepare the probe, single stranded DNA isolated from M13mp18λ*l* phage (containing a fragment of *l* strand of the λ replication region) was used as a template for the random priming reaction. Using such a probe, it should be possible to detect the p_R transcripts uncontaminated by any transcripts from the opposite (*l*) DNA strand. Indeed, by a hybridization with the probe described above, we found both p_R transcripts in the samples prepared from bacteria harbouring λ plasmid, but not from bacteria lacking such a plasmid (Figure 2A). Since the probe was prepared by the random priming reaction using a relatively long template, the longer transcript is much better visible than the shorter one after the hybridization. Moreover, we were not able to detect any p_R transcripts using a DIG-labelled DNA probe based on M13mp19λ*r* single stranded DNA (containing a fragment of *r* strand of the λ replication region) as a template (Figure 2B).

Transcription from the p_R promoter is inhibited by high ppGpp concentrations

Because of differences in λ plasmid DNA replication in different growth conditions and in different strains (see next subsection of Results), we estimated λ plasmid DNA amounts in bacterial cells at the times when the samples for RNA isolation were withdrawn and calculated the relative level of the p_R transcript per λ plasmid (i.e. template DNA) dose. We found that the level of the p_R transcript was several fold lower in an isoleucine-starved *rel*A$^+$ strain harbouring the λ plasmid than in unstarved bacteria, however, it was almost unchanged in an isoleucine-starved *rel*A$^-$ mutant (Figure 3). Since the mRNA decays during the starvation period (the half-life is as short as a few minutes) and as we estimated a level of the p_R transcript a relatively long time after the onset of amino acid starvation, we considered that these results indicated a decreased transcription from the p_R promoter which was caused by high ppGpp levels. However, it was proposed that the efficiency of mRNA synthesis during the stringent and relaxed responses may be affected by a highly decreased rate of translation caused by amino acid deprivation when both processes are coupled (Vogel *et al.*, 1992). In contrast to previous suggestions (Svitil *et al.*, 1993), it was demonstrated recently that ppGpp has little or no direct effect on translation rate or fidelity (Sorensen *et al.*, 1994). Therefore, in order to examine whether the inhibition of the transcription from the p_R promoter is caused by ppGpp, we estimated the p_R transcript level in unstarved wild type bacteria harbouring a λ plasmid and a plasmid containing the *rel*A gene under the control of an inducible promoter. The pALS10 plasmid contains the *rel*A gene under the control of the p_{tac} promoter, therefore, addition of IPTG to bacterial culture causes overexpression of the *rel*A gene and production of large amounts of ppGpp in the cells (Svitil *et al.*, 1993). The pALS13

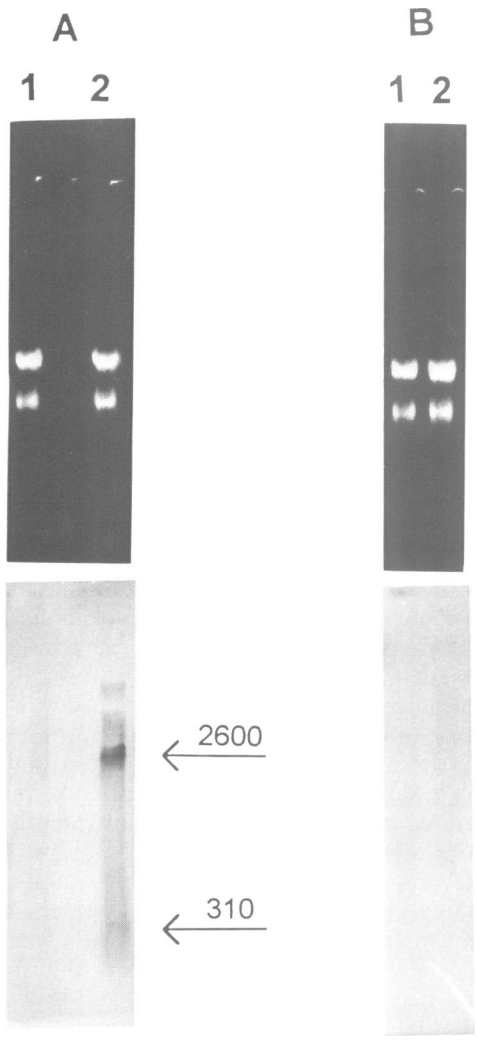

Fig. 2. An example of the detection of the p_R transcripts (311 and 2601 nucleotides long, respectively) by hybridization with the DIG-labelled DNA probe prepared by using M13mp18λ*I* single stranded DNA (**A**) or M13mp19λ*r* single stranded DNA (**B**) as a template for the labelling reaction. Upper panels: the gels photographed over a transilluminator (312 nm wavelength) showing ribosomal RNAs (internal controls); bottom panels: nitrocellulose membranes after hybridization. Lanes 1: samples isolated from the MG1655 strain; lanes 2: samples isolated from the MG1655 strain harbouring the λ plasmid, pKB2. The positions corresponding to RNA of ~310 nucleotides and 2600 nucleotides are indicated by the arrows.

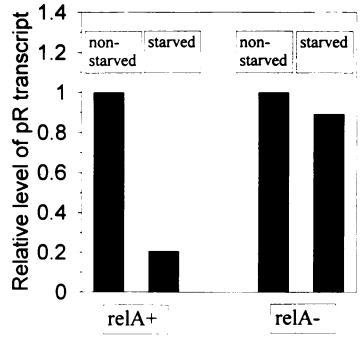

Fig. 3. Relative level of the p_R transcript (2601 nucleotides long) per λ plasmid DNA amount in the *rel*A$^+$ (CP78) and *rel*A$^-$ (CP79) strains harbouring the λ plasmid (pKB2), which were, or were not, starved for isoleucine for 45 min. The values obtained for the non-starved bacteria were assumed to be 1.

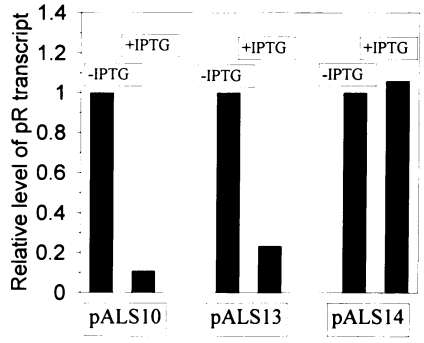

Fig. 4. Relative level of the p_R transcript (2601 nucleotides long) per λ plasmid DNA amount in the wild type (MG1655) strain harbouring the λ plasmid (pKB2) and containing, in addition, pALS10, pALS13 or pALS14 plasmid, which was, or was not, treated with IPTG for 2 h. The addition of IPTG (to 2 mM) induces ppGpp accumulation in the cells containing pALS10 or pALS13, but not pALS14, plasmid. The values obtained for the bacteria non-induced by IPTG were assumed to be 1.

plasmid is the same as pALS10 but contains the truncated *rel*A gene which codes for a truncated protein, metabolically unstable, but active in ppGpp production. The pALS10- and pALS13-mediated overproduction of ppGpp has been proved in the MG1655 strain which was used in our experiments. The pALS14 plasmid contains the truncated *rel*A gene which codes for non-active RelA protein. This plasmid was used in the control experiments. We found that the high ppGpp concentration caused by overexpression of the active *rel*A gene (in the case of pALS10 and pALS13 plasmids) resulted in significantly decreased p_R transcript level whereas the overproduction of the non-active RelA protein (in the case of pALS14 plasmid) did not affect the relative level of this transcript in the cell (Figure 4). Therefore, we conclude that a high ppGpp level during the stringent response is responsible for decreased transcription from the p_R promoter. Moreover, since the λO initiator protein cannot be synthesized in both stringent and relaxed strains during amino acid starvation, we propose that ppGpp-mediated inhibition of transcriptional activation of *ori*λ is responsible for the inhibition of λ plasmid DNA replication during the stringent response.

Decreased transcription from the p_R promoter is responsible for the inhibition of λ plasmid DNA replication during the stringent response

If the hypothesis presented above were true, the replacement of the p_R promoter with a promoter insensitive to ppGpp in a λ plasmid should result in its replication in amino acid-starved *rel*A$^-$ as well as in *rel*A$^+$ strains. It was demonstrated previously that the p_{lac} promoter is not inhibited by ppGpp (Glass *et al.*, 1987; Vogel *et al.*, 1992). Thus, we have used the pAS3 plasmid—a λ plasmid containing the p_{lac} promoter instead of the p_R promoter (Szalewska *et al.*, 1994). In the control experiment, we used the parental λ plasmid, pKB2. We found that while the replication of plasmid pKB2 was inhibited in an isoleucine-starved *rel*A$^+$ strain, the plasmid pAS3 replicated during the relaxed as well as stringent responses

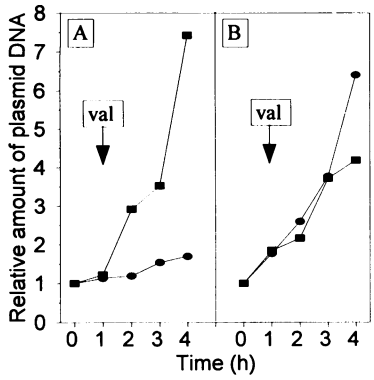

Fig. 5. Relative amount of λ plasmid, pKB2 (**A**) or pAS3 (**B**), DNA per ml of the bacterial culture in the *rel*A$^+$ (CP78) (circles) and *rel*A$^-$ (CP79) (squares) strains starved for isoleucine. The addition of L-valine (to 1 mg/ml) which induces isoleucine starvation is indicated by the arrow.

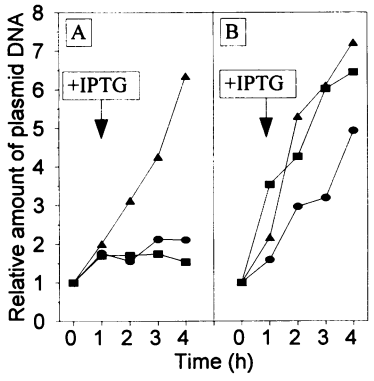

Fig. 6. Relative amount of λ plasmid, pKB2 (**A**) or pAS3 (**B**), DNA per ml of bacterial culture in the wild type (MG1655) strain containing, in addition, pALS10 (circles), pALS13 (squares) or pALS14 (triangles) plasmid. The addition of IPTG (to 2 mM), indicated by the arrow, induces ppGpp accumulation in cells containing pALS10 or pALS13, but not pALS14, plasmid.

(Figure 5). We have also demonstrated that high ppGpp levels in unstarved bacterial cells, caused by overexpression of active *rel*A genes, resulted in inhibition of pKB2 plasmid DNA replication, whereas the pAS3 plasmid did replicate in these conditions (Figure 6). The overexpression of the inactive *rel*A gene did not affect the replication of either pKB2 or pAS3 plasmid (Figure 6). These results indicate that the hypothesis which concerns a ppGpp-mediated inhibition of transcriptional activation of *ori*λ as a reason for inhibition of λ plasmid DNA replication during the stringent response, seems to be true.

Discussion

The plasmids derived from coliphage λ, so called λ plasmids, contain the replication region from λ phage DNA which consists of o_R/p_R operator/promoter, and λ*cro*, λ*O* and λ*P* genes; the origin of DNA replication of these plasmids (*ori*λ) is located in the middle of the λ*O* gene. The mechanism of initiation of λ plasmid DNA replication has been extensively investigated, mainly *in vitro*. Using the *in vitro* replication system reconstituted with purified lambda and *E.coli* proteins, the sequence of events leading to the assembly of the replication complex at *ori*λ has been demonstrated. The first step is the binding of the λO initiator protein to the *ori*λ and the forming of the so called O-some (Dodson *et al.*, 1986; Alfano and McMacken 1989a). The complex of two proteins λP and DnaB interacts with the O-some forming a stable pre-primosomal complex *ori*λ−λO−λP-DnaB (Alfano and McMacken, 1989a). Since the λP protein is an inhibitor of DnaB helicase (Biswas and Biswas, 1987), this complex cannot promote directly a start of DNA replication (Dodson *et al.*, 1989). The action of heat shock proteins DnaJ, DnaK and GrpE, is necessary for a rearrangement of the pre-primosomal complex in such way that an inhibitory effect of λP is no longer observed (Liberek *et al.*, 1988; Alfano and McMacken, 1989b; Żylicz *et al.*, 1989; Żylicz, 1993). Then, the DnaB helicase begins to unwind the DNA near the *ori*λ sequence. The DnaG primase recognizes the DnaB−single stranded DNA complex and proceeds to make RNA primers. Finally, the DNA polymerase III holoenzyme extends these RNA primers into DNA (Żylicz *et al.*, 1988). For λ plasmid DNA replication *in vivo*, the function of RNA polymerase is necessary not only for the production of mRNA for λO and λP protein synthesis. Transcription at or near the *ori*λ, which normally starts from the p_R promoter and is called the transcriptional activation of *ori*λ, is also indispensable. It is also necessary for λ plasmid replication *in vitro* reconstituted with purified λO and λP proteins and a crude extract from *E.coli* cells called Fraction II. On the other hand, RNA polymerase function is dispensable for the replication reconstituted with purified lambda and *E.coli* proteins (Mensa-Wilmot *et al.*, 1989). It was suggested that the binding of histone-like HU proteins to the template DNA in the region surrounding *ori*λ inhibits λ DNA replication, and when the *ori*λ template is transcribed by RNA polymerase, HU-mediated inhibition of the replication is abolished as a result of the removal of HU proteins (Mensa-Wilmot *et al.*, 1989). λO protein was known to be very rapidly degraded *in vivo* (Lipińska *et al.*, 1980; Gottesman *et al.*, 1981). Thus in the model of λ plasmid DNA replication *in vivo* (Matsubara, 1981), it was assumed that the binding of the unstable λO initiator protein to the *ori*λ sequence leads to only one initiation event after which the pre-primosomal complex disassembles. The next replication round would be an effect of a dilution of the λCro protein, which is a repressor of the p_R promoter, when the volume of a bacterial cell would increase during its growth. The transcription caused by Cro derepression would allow production of new portion of the λO protein and serve as transcriptional activation of *ori*λ. Since the λ*cro* gene belongs to the same operon as λ*O* and λ*P*, which is under the control of the p_R promoter, a round of λ plasmid DNA replication also results in an increase of the λ*cro* gene dosage, thus the p_R promoter would be repressed again by an increased amount of λCro protein.

In the light of this elegant model based on the λCro autoregulatory loop, the finding that λ plasmid DNA replication proceeds in amino acid-starved *rel*A mutants, when the λO protein should not be synthesized, was surprising (Węgrzyn *et al.*, 1991b). Further experiments confirmed that the λO protein is not synthesized during the relaxed response (Węgrzyn *et al.*, 1992). However, a stable fraction of the λO protein, which is most probably identical with the λO enclosed in the replication complex, has been demonstrated (Węgrzyn *et al.*, 1992; Pawłowicz

et al., 1993). Then it was proposed that λ plasmid DNA replication proceeding during the relaxed response is perpetuated by the replication complex which is not disassembled after a replication round but is inherited by one of two daughter plasmids (Węgrzyn and Taylor, 1992). Indeed, it was demonstrated that from two daughter plasmid copies synthesized after the onset of amino acid starvation only one is able to enter into the next round of replication (Węgrzyn and Taylor, 1992). Nevertheless, the existence of a stable fraction of the λO protein in relaxed and stringent strains (Węgrzyn *et al.*, 1992) raised the following question: what factor inhibits λ plasmid DNA replication during the stringent response?

Our experiments demonstrated that RNA polymerase function is indispensable for λ plasmid replication in an amino acid-starved relaxed mutant (Figure 1). They suggested that an inhibition of this function by ppGpp during the stringent response may result in the phenomenon observed. Indeed, we found that the p_R promoter is sensitive to ppGpp (Figures 3 and 4). In accordance with the above mentioned suggestion, the replacement of the p_R promoter with one non-sensitive to ppGpp restored the λ plasmid's ability to replicate even in high ppGpp concentrations (Figures 5 and 6). Since the only known function of transcription proceeding through the replication region of λ plasmid which could carry weight during the stringent or relaxed response (when the λO initiator protein is not synthesized due to amino acid deprivation) is the transcriptional activation of *ori*λ, we consider that this process is crucial in the control of λ plasmid DNA replication in amino acid-starved *E.coli* strains.

We propose a model for the stringent control of λ plasmid replication (Figure 7). During the relaxed response, a new replication complex cannot be assembled due to amino acid deprivation and the instability of free λO molecules. However, the replication may be driven by the replication complex inherited by one of two daughter plasmids after the previous replication round. The replication would be triggered by the transcriptional activation of *ori*λ and perpetuated by an RNA polymerase unaffected by ppGpp. In amino acid-starved wild type strains (the stringent response), the formation of a new replication complex similarly to that in the relaxed response is impossible. However, unlike the relaxed response, the action of ppGpp on RNA polymerase abolishes the transcriptional activation of *ori*λ in this case. Thus, λ plasmid is not able to replicate during the stringent response. In normal growth conditions, the replication complex may be assembled, moreover, the 'old' replication complex can function again in the next replication round. Also, transcriptional activation can proceed normally.

Our model suggests an additional role for transcriptional activation of *ori*λ, beside cleaning the DNA template of histone-like, HU, proteins in order to facilitate replication complex assembly (Mensa-Wilmot *et al.*, 1989). The transcriptional activation might, for example, be crucial in the adequate positioning of the replication complex at *ori*λ, as was recently suggested (Szalewska *et al.*, 1994). One may presume that when a new replication complex is assembling, both functions of the transcriptional activation are realized during one transcription event. When replication starts from the 'old' replication complex, only the second role is important. In accordance with our

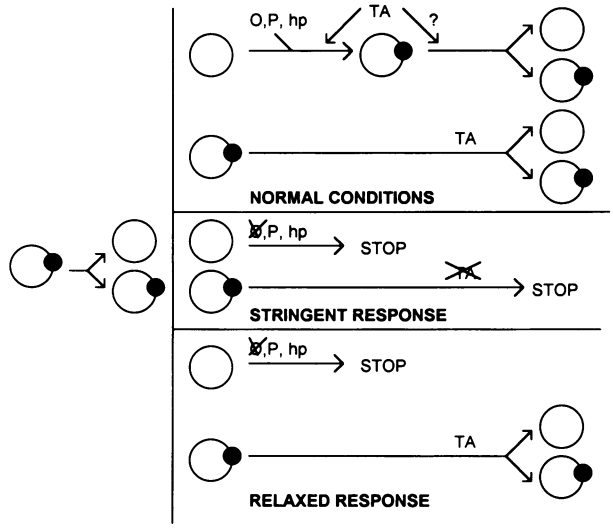

Fig. 7. A model of λ plasmid DNA replication in *E.coli* cells growing in normal growth conditions (NORMAL CONDITIONS), during the stringent response (STRINGENT RESPONSE), and during the relaxed response (RELAXED RESPONSE). A replication round (left panel) results in the appearance of two daughter plasmid copies (open circles), one of which inherits the replication complex (filled, small circle). In normal growth conditions (upper panel), a new replication complex is assembled at *ori*λ with λO, λP and host proteins (hp) on the daughter copy devoid of the 'old' replication complex. The transcriptional activation of the *ori*λ (TA) may have a double role: removal of HU proteins from the DNA template which allows the asembly of the replication complex at the *ori*λ sequence, and the triggering of the DNA replication by, for example, an adequate positioning of the replication complex at *ori*λ. Both these functions may be (but also may not be) an effect of the same transcription event. In the case of the plasmid bearing an 'old' replication complex, only the second function of transcriptional activation is important. During the stringent response (middle panel), a new replication complex cannot be assembled due to amino acid deprivation and a lack of λO protein synthesis. Replication also cannot start from the plasmid bearing an 'old' replication complex because of ppGpp-mediated inhibition of transcriptional activation of *ori*λ. Although synthesis of the λO protein, and assembly of a new replication complex is also not possible during the relaxed response (bottom panel), the replication complex assembled before the onset of amino acid starvation can drive the replication of λ plasmid DNA, as transcriptional activation of *ori*λ can proceed normally in these conditions.

model, the results were presented suggesting that λ plasmid DNA replication driven by the 'old' replication complex is not restricted to amino acid-starved *E.coli relA*$^-$ strains, but that in λ plasmid-harbouring bacteria growing in nutrient medium, every second plasmid circle bears a replication complex that originates from the preceding round of replication (Węgrzyn and Taylor, 1992).

It is worth mentioning that there are many analogies between the mechanisms of initiation of DNA replication in λ and in eukaryotes (recently reviewed by Stillman, 1994). It was recently postulated, that the origin recognition complex (ORC) might be bound to the ARS1 locus (the origin sequence) at most, and perhaps all, stages of the cell cycle (Bell and Stillman, 1992; Diffley and Cocker, 1992) in the yeast *Saccharomyces cerevisiae*. The existence of the λ replication complex bound to the λ DNA, presented in our model, might also be a good analogy to the above mentioned phenomenon. Of course, in such a case, there should be a factor which could trigger the replication process. An existence of a factor (so called

Table I. *Escherichia coli* K12 strains, plasmids and bacteriophages

Strain/plasmid/ bacteriophage	Genotype/properties	Source/reference
E.coli strains		
CP78	*leu arg thr his thi*	Fiil and Friesen (1968)
CP79	*leu arg thr his thi relA relX*	Fiil and Friesen (1968)
MG1655	Wild type	B.Bachmann
CF1652	As MG1655 but Δ*rel*A251::*kan*	Xiao *et al.* (1991)
Plasmids		
pKB2	λ plasmid, kan^R	Kur *et al.* (1987)
pAS1	λ plasmid, tet^R	Szalewska *et al.* (1994)
pAS3	λ plasmid, the p_R promoter replaced with the p_{lac} one	Szalewska *et al.* (1994)
pALS10	pBR322-derived replicon containing the *rel*A gene under the control of the p_{tac} promoter, amp^R	Svitil *et al.* (1993)
pALS13	As pALS10 but containing a truncated *rel*A gene coding for the active ppGpp synthetase I	Svitil *et al.* (1993)
pALS14	As pALS10 but containing a truncated *rel*A gene coding for a non-active protein	Svitil *et al.* (1993)
Bacteriophages		
M13mp18	Modified M13 phage containing a polylinker sequence for cloning purposes	Yanisch-Perron *et al.* (1985)
M13mp19	As M13mp18 but containing a polylinker sequence in an opposite orientation	Yanisch-Perron *et al.* (1985)
M13mp18λ*l*	As M13mp18 but containing the *Hin*dIII–*Eco*RI fragment of the *l* strand of λ DNA (a part of the replication region) cloned into the polylinker	This work
M13mp19λ*r*	As M13mp19 but containing the *Hin*dIII–*Eco*RI fragment of the r strand of λ DNA (a part of the replication region) cloned into the polylinker	This work

licensing factor) that functions to permit DNA replication at the appropriate time in the S phase has been proposed (Leno *et al.*, 1992). If our model were true, the binding of the initiator protein (λO) to the origin sequence (*ori*λ) could not be the main regulatory event, similarly to the regulation of initiation of eukaryotic DNA replication and contrary to previous suggestions (Matsubara, 1981). Recently we found that neither absence nor excess of the ClpXP protease, which is responsible for λO protein degradation *in vivo* (Bejarano *et al.*, 1993; Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993), affects λ plasmid or λ phage replication (Szalewska *et al.*, 1994). Hence, the stabilization of λO protein has no effect on the regulation of λ plasmid replication, which is in strong contrast to previous models. Thus, it is tempting to speculate that transcriptional activation of *ori*λ plays a crucial regulatory role in the initiation of λ plasmid DNA replication in *E.coli* cells.

Materials and methods

Bacterial strains, plasmids and phages

The *E.coli* K12 strains, plasmids and bacteriophages are listed in Table I. For the construction of M13mp18λ*l* and M13mp19λ*r* phages, the *Eco*RI–*Hin*dIII fragment from plasmid pKB2 (containing a part of the replication region) was inserted into a polylinker of M13mp18 and M13mp19 RF DNA respectively. Thus, the hybrid M13mp18λ*l* phage contains a fragment of the *l* strand of λ DNA and M13mp19λ*r* phage contains a fragment of the *r* strand of λ DNA. All DNA manipulations were carried out according to Sambrook *et al.* (1989).

Culture media and amino acid starvation

The minimal media 1, 2 and 3 have been already described (Węgrzyn *et al.*, 1991b; Węgrzyn and Taylor, 1992). These media were used in all experiments. Isoleucine starvation of bacteria growing in a minimal medium was induced by addition of 1 mg/ml L-valine to the culture.

Overexpression of the relA gene and overproduction of ppGpp in the unstarved cells

The overexpression of different forms of the *rel*A gene (cloned on pALS10, pALS13 and PALS14 plasmids) was induced by addition of IPTG (to 2 mM) to appropriate bacterial culture. In the cases of pALS10 and pALS13, but not pALS14, the IPTG induction causes ppGpp accumulation (Svitil *et al.*, 1993). It has been proved in MG1655 strain which was used in this study. We found that the overexpression of the active *rel*A gene, but not the inactive *rel*A gene, caused an inhibition of bacterial growth and an inhibition of pALS plasmid replication (data not shown). Therefore, we estimated that the *rel*A gene dosage was not significantly affected by the IPTG induction in the bacteria harbouring pALS10, pALS13 as well as pALS14 plasmids (data not shown).

Isolation of total RNA from bacteria

A sample of bacterial culture (1.4 OD 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 twice extraction with equal volume of phenol-chloroform (1:1, v/v), sodium acetate (to 0.3 M) and 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) a pellet was washed with 70% ethanol and dried. 0.1 ml of formamide were added to the pellet and the sample was incubated at 60°C for 5 min and then transferred to an ice-bath for 1 min. The RNA sample dissolved in formamide was kept at −20°C.

RNA gel electrophoresis and hybridization

20 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 above described buffer but lacking formaldehyde. After running at 5 V/cm the gel was photographed over a transilluminator (312 nm wavelength) and a relative amount of ribosomal RNA (internal RNA control) was estimated using UVP EASY densitometry system. The values obtained were taken into consideration in calculation of the relative amount of the p_R transcript, except in the experiments where the stringent and relaxed responses were compared. RNA was transferred to nitrocellulose as described by Sambrook *et al.* (1989). The hybridization with single stranded DNA probes labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate was performed by using DIG DNA labelling and detection kit (Boehringer Mannheim) and according to the instructions. The relative amount of RNA was estimated by densitometry. The proportionality of the amount of RNA loaded on the gel to the intensity of the band after the hybridization has been proved (data not shown).

Measurement of plasmid DNA replication

Since radioactive thymidine incorporation may be inadequate in the study of DNA replication when comparing the stringent and relaxed

response (Węgrzyn *et al.*, 1991b; Herman *et al.*, 1994b), we investigated the replication of plasmids on the basis of estimation of the change in relative plasmid amount per ml of the bacterial culture with time. The relative amount of plasmid DNA was measured according to Herman *et al.* (1994a). Briefly, a sample (5 ml) of bacterial culture was centrifuged (6000 *g*, 5 min) and the pellet was washed with 1 ml of 0.9% NaCl, frozen and kept at −20°C. After thawing, lysis by alkali was carried out according to Sambrook *et al.* (1989). Agarose gel electrophoresis (Sambrook *et al.*, 1989) was then performed and the relative amount of plasmid DNA in the bands on an electrophoregram was estimated using UVP EASY densitometry system.

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