Formation of linear plasmid multimers promoted by the phage lambda Red-system in *lon* mutants of *Escherichia coli*

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We report here the formation of plasmid linear multimers promoted by the Red-system of phage lambda using a multicopy plasmid comprised of lambda *redα* and *redβ* genes, under the control of the lambda *cI*857 repressor. Our observations have revealed that the multimerization of plasmid DNA is dependent on the *redβ* and *recA* genes, suggesting a concerted role for these functions in the formation of plasmid multimers. The formation of multimers occurred in a *recBCD*$^+$ *sbcB*$^+$ *xthA*$^+$ *lon* genetic background at a higher frequency than in the isogenic *lon*$^+$ host cells. The multimers comprised tandem repeats of monomer plasmid DNA. Treatment of purified plasmid DNA with exonuclease III revealed the presence of free double-chain ends in the molecules. Determination of the size of multimeric DNA, by pulse field gel electrophoresis, revealed that the bulk of the DNA was in the range 50–240 kb, representing approximately 5–24 unit lengths of monomeric plasmid DNA. We provide a conceptual framework for Red-system-promoted formation and enhanced accumulation of plasmid linear multimers in *lon* mutants of *E. coli*.

Introduction

Replication of the phage lambda chromosome is under temporal control: an early transitory phase characterized by the formation of circular monomers is followed by a robust second phase, the rolling circle mode of replication (Skalka, 1977). In the absence of normal replication DNA concatemers produced via recombination are required for the development of phage lambda, because they serve as intermediates in the packaging process (Stahl *et al.*, 1972; Stahl & Stahl, 1974). Genetic studies have illustrated linkage of recombination functions and DNA synthesis during phage maturation. Two activities, *gam* and *red*, specified by phage lambda are necessary for the rolling circle mode of replication (Enquist & Skalka, 1973). *gam* function is essential for the inhibition of host RecBCD nuclease and thereby renders protection to intermediates and products generated by replication (Unger & Clark, 1972; Karu *et al.*, 1975; Greenstein & Skalka, 1975). The *red* genes, *redα* and *redβ*, promote homologous genetic recombination but their role in the rolling circle mode of replication is unclear. Evidence for the role of *red* genes in growth and development of phage lambda is derived from two fronts: in a series of elegant studies, F. W. Stahl and his colleagues have shown that in an infection, when replication was inhibited, all the progeny molecules had undergone homologous recombination (Stahl *et al.*, 1972). A. Skalka and her colleagues showed that the packaging of phage lambda DNA was defective in *red gam* mutants plated on recombination-deficient *E. coli* strains (Enquist & Skalka, 1973).

Multimerization of plasmids has been used as a paradigm to study homologous genetic recombination in both pro- and eukaryotic systems (Bedbrook & Ausubel, 1976; Symington *et al.*, 1983; Harashima *et al.*, 1989). R. Kolodner and his colleagues have shown that oligomerization of plasmids and their subsequent resolution into monomers in bacteria is controlled by both *cis*- and *trans*-acting factors that participate in homologous recombination (James *et al.*, 1982, 1983; Doherty *et al.*, 1983). Most recently, A. Cohen and his colleagues discovered the occurrence of large amounts of linear multimers of plasmids in *recBCD*, *sbcB*, *sbcC* and *recBC sbcA* host strains and accordingly have proposed an hypothesis to account for the mutual dependence of plasmid multimer synthesis and recombination by the RecE-, RecF- and Red pathways (Cohen & Clark, 1986; Silberstein & Cohen, 1987; Nussbaum & Cohen, 1988; Berger & Cohen, 1989). Interestingly, however, the mechanism of linear multimer formation is different

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from that of normal plasmid replication and maintenance (Yarmolinsky & Sternberg, 1988; Niki *et al.*, 1990).

The role of phage lambda *red* genes, at the molecular level, in the formation of concatemers and thus in growth and development is unclear. The observation that RecE and RecF pathways play a prominent role in plasmid multimer synthesis (Berger & Cohen, 1989; Kusano *et al.*, 1989) and the functional similarity among RecE- and Red-pathways prompted us to seek physical evidence for the role of *redα* and *redβ* in plasmid multimer formation. In addition, we are interested in identifying gene(s) and their products involved in the regulation of homologous genetic recombination *in vivo*. To our knowledge such an approach has never been taken before. Our study on the possible role of *red* genes in multimer formation via recombination stems from a novel observation that the joining of terminal ends (*cos* to *cos* joining) of the phage lambda chromosome *in vitro* is promoted by *β* protein (Muniyappa & Radding, 1986). Results presented in this paper show that linear multimer formation is maximal in *lon* mutants of *E. coli* which are otherwise wild-type for the RecBCD, ExoI and ExoIII proteins, and we propose a model to account for their enhanced accumulation.

Methods

Bacterial strains, plasmids and growth conditions. E. coli strains used in this study are listed in Table 1. P1 lysates were prepared as described by Miller (1972) and transductions were done as described by Willets *et al.* (1969). The plasmid pSJS6, a gift from Drs S. J. Sandler and A. J. Clark of the University of California, Berkeley, CA, USA, is a derivative of pBR322 (Sandler & Clark, 1990). pSJS6 carries a fragment of lambda chromosome containing the *red* genes that encode the functions for general genetic recombination of phage lambda: exonuclease and *β* protein, and a *ninL4* deletion in the *gam* gene (Fig. 1). The expression of exonuclease and *β* protein is from the P_L promoter of phage lambda, under the control of the *cI*857 repressor.

Bacterial strains carrying plasmids were derived by transformation using the $CaCl_2$ method and stored at −20 °C in 50% (v/v) glycerol. Transformation of plasmid DNA into *E. coli* was carried out as described by Maniatis *et al.* (1982). Bacterial cells containing plasmids were grown in L-broth or on L-plates at the indicated temperatures (Luria & Burrous, 1957). Ampicillin and tetracycline were added to growth media at concentrations of 100 μg ml^{-1} and 25 μg ml^{-1}, respectively.

Table 1. *E. coli strains used*

Strain	Genotype	Source
AB1157	F$^-$ *thr-1 ara-14 leuB6 Δ(gpt–proA)62 lacY1 tsx33 supE44 galK2 hisG4 rspL31 kdgK51 xyl-5 mtl-5 argE3 thi-1*	Laboratory collection
JC12578	F$^-$ *thr-1 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-5 proA2 his4 rspL31 tsx33 supE44 Δ(srlR–recA)306*::Tn*10 recF146 uvrA6 sfiB103*	A. J. Clark
JK7	As AB1157 but *recA56 srl-300* Tn*10*	R. Jayaraman
KM102	As AB1157 but *Δlon non*::Tn*10*	This Study

Test for cell viability. Bacterial cells from a fresh transformant colony were grown in L-broth, containing antibiotic, to mid-exponential phase at 30 °C and then at 42 °C for 3 h. At regular time intervals aliquots were taken, diluted appropriately and plated on L-plates to determine the number of viable cells. Cell number (density) was determined by monitoring the optical density at 600 nm.

Purification of total plasmid DNA. Bacterial cells were grown in 3 ml cultures at 30 °C to mid-exponential phase and then transferred to 42 °C for 3 h to facilitate the derepression of *red* genes. Total plasmid DNA was isolated from the cell-free lysates as described by Clark & Cohen (1986) and Silberstein *et al.* (1990). Briefly, the lysed cell suspension was centrifuged to pellet the cell debris and the supernatant was treated with RNAase (50 μg) for 30 min. The sample was then extracted with phenol/chloroform/isoamyl alcohol solution (25:24:1, by vol.). The DNA in the aqueous phase was precipitated with ethanol.

Plasmid DNA was also prepared from cell-free lysates by CsCl–ethidium bromide density centrifugation (Maniatis *et al.*, 1982). Linear plasmid DNA was present exclusively in the upper band in this gradient system. A major fraction of total plasmid DNA from *lon* mutant strains was present in the upper band. Samples of the plasmid DNA preparation were loaded onto a 0·5% agarose gel and electrophoresed in 89 mM-Tris/borate, 2 mM-EDTA (pH 8·3) at 2 V cm^{-1} for 20 h. The gel was stained with ethidium bromide and photographed.

Southern blotting. Total plasmid DNA preparations electrophoresed on agarose gels were transferred to Nytran membrane as described by Southern (1975). The membrane was probed with a radiolabelled *Eco*RI fragment containing lambda DNA (Fig. 1). For estimation of relative amounts of multimeric DNA the autoradiograms were scanned with an LKB Ultroscan XL densitometer and the data analysed using an LKB Gelscan XL.

Restriction enzyme digestion. Total plasmid DNA was digested in a standard assay buffer with 2 units of *Sal*I, *Eco*RI or *Xho*I, where indicated, as suggested by the manufacturer. The nicking of pSJS6 DNA by *Sal*I and pBR322 DNA by *Eco*RI was done under standard assay conditions in the presence of 0·1 μg ethidium bromide ml^{-1} and the reaction was carried out in dark (Rould *et al.*, 1992).

Exonuclease III digestion. Total plasmid DNA obtained from JK7 (*recA56 lon*$^+$) and KM102 (*rec*$^+$ *lon*) strains harbouring pSJS6 was purified by CsCl density centrifugation and treated with 20 units of *E. coli* exonuclease III at 37 °C for 15 min in a buffer containing 60 mM-Tris/HCl, 0·6 mM-$MgCl_2$ and 1 mM-dithiothreitol (pH 8·0). The reaction was terminated by adding EDTA to a final concentration of 25 mM. Individual samples were electrophoresed on a 0·5% agarose gel as described above.

Pulse field gel electrophoresis. Total plasmid DNA extracted from the *lon* mutant (KM102), harbouring pSJS6, which had been subjected to the temperature shift from 30 °C to 42 °C for 3 h, was isolated by CsCl density gradient centrifugation. A 2 μg sample of this DNA was suspended in molten agarose, and a solidified agarose block was placed in a 0·9% agarose gel. Electrophoresis was done in a LKB Pulsaphor electrophoresis unit with hexagonal electrodes, in a buffer containing 45 mM-Tris/borate and 1 mM-EDTA (pH 8·3), at a constant voltage of 6 V cm^{-1} at 20 °C for 24 h. The pulse time was as follows: 20 s east-to-west and 20 s north-to-south. Multimers of full-length lambda DNA was used as a standard marker. The DNA was transferred to a Nytran membrane and analysed by Southern hybridization as described above.

Preparation of deletion mutants by Bal31 nuclease digestion. pSJS6 DNA (5 μg) linearized by *Sal*I was incubated at 30 °C with Bal31 nuclease (10 units) in a reaction mixture (50 μl) containing 50 mM-Tris/HCl (pH 7·5), 10 mM-$CaCl_2$, 10 mM-$MgCl_2$, 0·5 M-NaCl and 50 μg bovine serum albumin ml^{-1}. At different time intervals aliquots of DNA were removed from the reaction mixture and heated at 75 °C for

10 min. The DNA was precipitated by ethanol. The resuspended DNA was treated with Klenow and NTPs to create blunt ends and recircularized as described by Maniatis *et al.* (1982). The recircularized plasmid DNA was transformed into *E. coli* as described above. DNA was isolated from individual colonies and then digested with *Bam*HI to map the extent of the deletion.

Results

Red-system-promoted formation of plasmid linear multimers

Previous studies have revealed that the synthesis of plasmid multimers is dependent on the cellular recombination machinery (reviewed in: Smith, 1988; Clark & Low, 1988; Mahajan, 1988). This prediction has been substantiated by a number of studies using recombination-defective strains of *E. coli* (Cohen & Clark, 1986; Kusano *et al.*, 1989). To elucidate the possible role of recombination enzymes of the Red-system of phage lambda in plasmid multimerization, we used plasmid pSJS6 which contains the replicon of the plasmid pBR322 that codes for ampicillin resistance (Fig. 1). This plasmid also contains the *red* genes, *redα* (*exo*) and *redβ* (*bet*), expressed from the lambda P_L promoter, under the control of the *cI*857 repressor. The expression of *red* genes was accomplished by transferring the cultures harbouring pSJS6 from 30 °C to 42 °C. Such a treatment results in transient expression of Lon protease, which has been shown to regulate the stability of a number of proteins specified by the host and lambda phage (Gottesman, 1989).

We tested the role of *lon* function in the production of plasmid linear multimers promoted by *red* genes in the isogenic *E. coli* strains AB1157 and KM102; the latter was obtained by transduction of AB1157 by bacteriophage P1. We used pSJS6 Form I DNA prepared from a

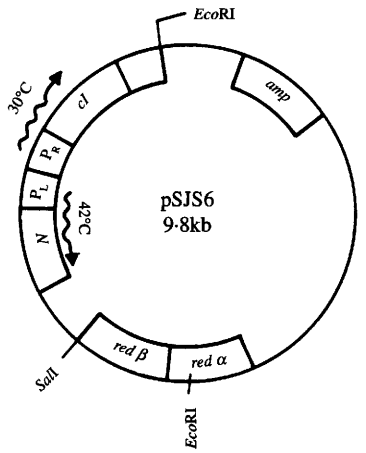

Fig. 1. Structure of plasmid pSJS6. The double line indicates genes that are relevant to this study and the region marked *amp* is the gene for ampicillin resistance. For other details see Methods.

recA host to transform strains AB1157 and KM102. Single colony isolates containing plasmids were selected by plating the transformed cells in the presence of ampicillin and tetracycline, where required. For the preparation of DNA they were grown in L-broth, containing the above antibiotics, at 30 °C to mid-exponential phase and then transferred to 42 °C for 3 h to facilitate derepression of *red* genes. The plasmid DNA was isolated from several independent transformants and analysed by agarose gel electrophoresis. We detected large amounts of abnormally high molecular mass (hmm) DNA on agarose gels. The pattern of one such DNA preparation obtained from identical density of cells, is illustrated in Fig. 2. In addition to the expected topological forms of DNA, such as Form I, Form II and Form III, these preparations contained several DNA species of larger size. To exclude the possibility that hmm DNA represents different topological forms of monomeric DNA, we treated total pSJS6 plasmid DNA with *Sal*I in the presence and in the absence of ethidium bromide. Digestion of duplex DNA with a restriction endonuclease, in the presence of ethidium bromide, results in nicking of DNA (Rould *et al.*, 1992). pSJS6 plasmid DNA has a single recognition site for *Sal*I and introduction of a nick by *Sal*I should convert the supercoiled forms into an open circular form. Treatment of pSJS6 DNA obtained from *recA*$^+$ *lon*$^+$ (AB1157) and *recA*$^+$ *lon*$^-$ (KM102) hosts with *Sal*I in the presence of ethidium bromide partially converted Form I DNA into Form II DNA. Nonetheless, the slow-migrating bands of plasmid DNA which persisted do not represent topoisomers of Form I DNA (see below). The absence of such a conversion of slow-migrating plasmid DNA suggested that these molecules are multimers of pSJS6 monomeric DNA. This conclusion is supported by the cleavage of pSJS6 plasmid DNA by *Sal*I, which produced a single species of Form III DNA (Fig. 2). These data, therefore, indicate that the hmm DNA contains tandem repetitions of the monomer unit of pSJS6 plasmid DNA.

To confirm that the hmm DNA is indeed pSJS6 plasmid DNA, and not chromosomal DNA, the identity of the DNA was determined by Southern blotting and hybridization using a radiolabelled *Eco*RI DNA fragment derived from pSJS6 plasmid DNA (Fig. 1). As shown in Fig. 2(*b*), the hybridization pattern clearly corresponded with that of the gel stained with ethidium bromide. The relative amounts of multimers produced by the Red system was dependent on the host *lon* function. For instance, mutations in *lon* increased the extent of accumulation of plasmid multimers (Fig. 2). To test whether the *recA*$^+$ function was essential for the Red-system-promoted plasmid multimerization, we transformed pSJS6 plasmid DNA into JC12578, a *recA* null strain derived from AB1157. The extent of formation of

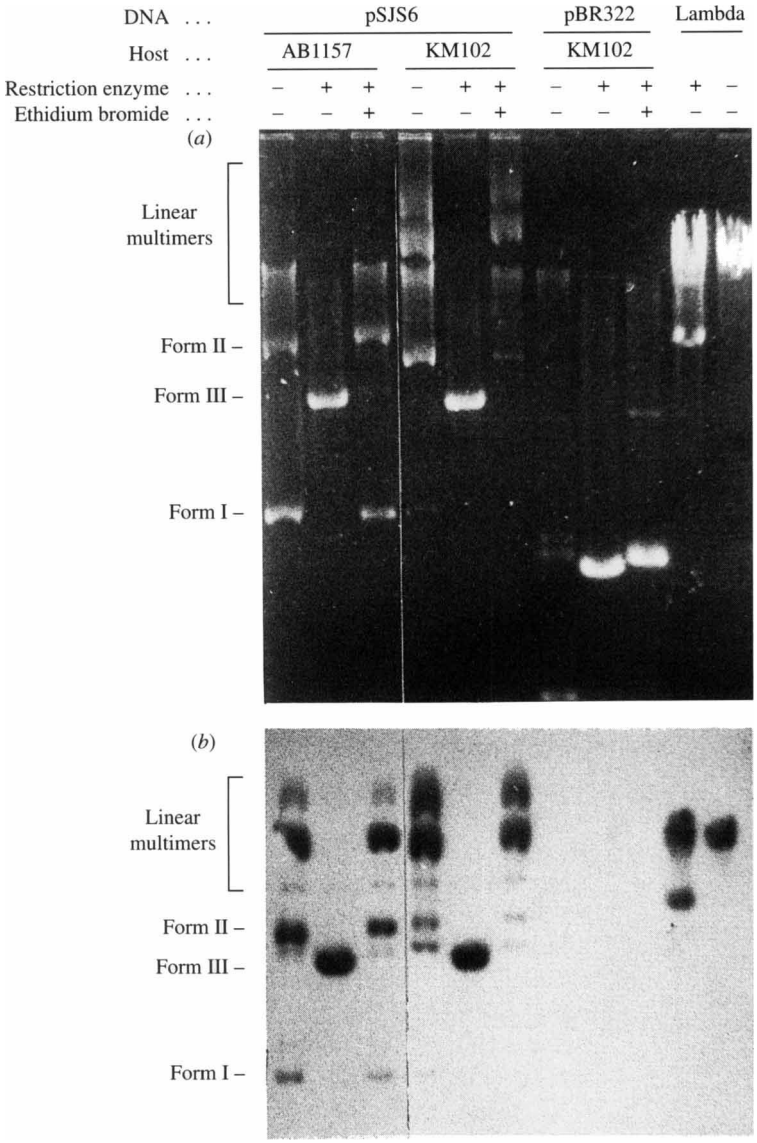

Fig. 2. Accumulation of plasmid multimers is maximal in *lon* mutants of *E. coli*. (*a*) Electrophoretic analysis of plasmid DNA. Total plasmid DNA was purified from the designated genotypes harbouring pSJS6 and pBR322 and analysed on an agarose gel as described in Methods. The gel was stained with ethidium bromide and photographed. (*b*) The DNA in the above gel was transferred to a Nytran membrane and visualized by Southern hybridization with a radiolabelled *Eco*RI fragment as a probe (Fig. 1). The location of different topological forms of plasmid monomeric DNA is indicated: Form I DNA, negatively superhelical DNA; Form II, nicked closed circular DNA; Form III, linear duplex DNA. The lambda chromosome was digested with *Xho*I.

hmm DNA was 100-fold less than that obtained in *recA*$^+$ host cells, suggesting a requirement for RecA protein (data not shown). These studies on the formation of hmm DNA *in vivo* corroborate an earlier finding of the concerted action of the RecA and β proteins in the formation of joint molecules *in vitro* (Muniyappa & Radding, 1986). More importantly, we observed that the accumulation of hmm DNA of pSJS6 was maximal in the *lon* mutant in a *recBCD*$^+$ *exo*I$^+$ *xthA*$^+$ genetic background.

To further investigate our finding of enhanced accumulation of hmm DNA in a *lon* mutant of *E. coli*, we transformed pBR322 plasmid DNA into a *lon* mutant. DNA was prepared from these cells as described above. Analysis of pBR322 plasmid DNA on agarose gels showed low amounts of plasmid oligomers (Fig. 2). Treatment of pBR322 DNA with *Eco*RI in the presence of ethidium bromide did indeed convert Form I DNA into Form II DNA; the slow-migrating bands persisted however (compare lanes in the absence and presence of *Eco*RI and ethidium bromide). To exclude the possibility of selective DNA extraction and to ascertain that the same cell density corresponds to cell number, we extracted and analysed chromosomal DNA from various hosts, harbouring pSJS6, and found that cell density correlates with the amounts of chromosomal DNA in all the strains (data not shown).

Time-dependent synthesis of plasmid multimers

Total plasmid DNA preparation for the experiment in Fig. 2 was obtained from cultures 3 h after thermal derepression of *red* genes. It is, however, possible that the reduced accumulation of plasmid multimers in *recA*$^+$

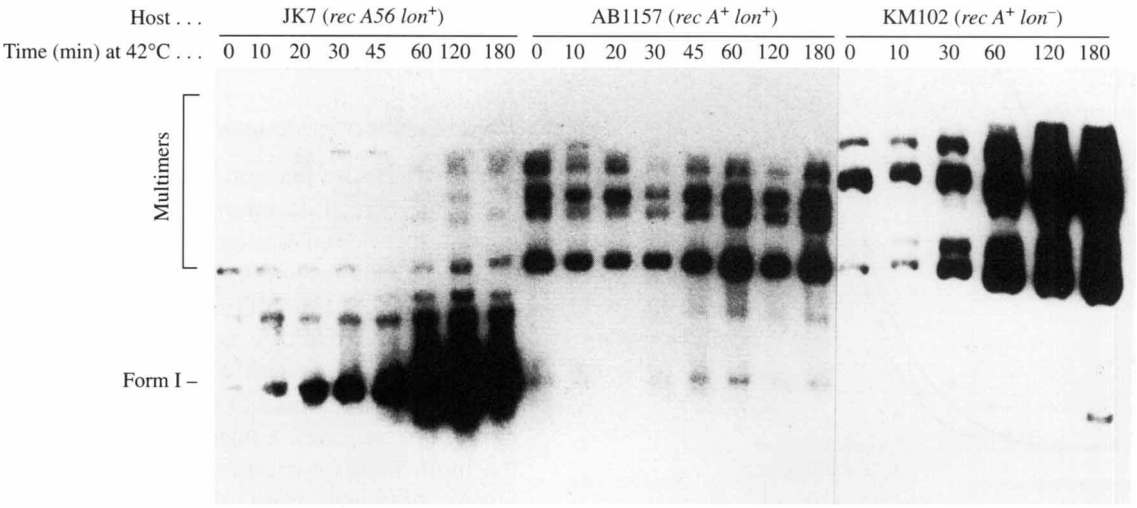

Fig. 3. Time-dependent accumulation of plasmid linear multimers at 42 °C. Exponential phase cells of the indicated genotype, harbouring pSJS6 plasmid DNA, were shifted from 30 °C to 42 °C. Total plasmid DNA preparations, obtained from cultures 3 h after thermal derepression of *red* genes, and taken at the indicated time intervals, was analysed on a 0·5% agarose gel as described in Methods.

lon$^+$ (AB1157) cells may reflect increased degradation of hmm DNA at earlier times during induction. To test this hypothesis, we compared the kinetics of accumulation of hmm DNA in a set of isogenic strains: *recA56 lon*$^+$ (JK7), *recA*$^+$ *lon*$^+$ (AB1157) and *recA*$^+$ *lon*$^-$ (KM102). Aliquots of identical numbers of cells were taken after derepression of *red* genes at various time intervals. Total plasmid DNA was analysed on agarose gels as described above. As illustrated in Fig. 3, the kinetics of accumulation of pSJS6 Form I DNA in the *recA56 lon*$^+$ strain increased until about 2 h. During the initial 60 min time period, there was virtually no indication of the production of plasmid multimers; however, traces of multimers were detectable after 2 h. The basis for the production of large amounts of Form I DNA in this strain, at present, is unknown. By contrast, a time-dependent increase, albeit low, in the amount of multimeric DNA was observed in the *recA*$^+$ *lon*$^+$ genetic background, while the *recA*$^+$ *lon*$^-$ strain showed a continuous increase of plasmid multimers during the same time period. The persistence of Form I DNA, although not in substantial amounts, argues that the *de novo* synthesis of plasmid multimers proceeds through the monomeric form of plasmid DNA. However, it is possible that initial production of multimers may arise from monomers and that further higher multimer synthesis results from recombination between multimers. The presence of large amounts of multimeric DNA prior to the derepression of *red* genes is intriguing. However, it is not due to the nature of plasmid DNA used for transformation of these strains (see below). The DNA sample(s) used for transformation was isolated from *recA* cells, and consisted of Form I DNA as confirmed by agarose gel electrophoresis. The occurrence of plasmid multimers implies that plasmid recombination occurs during the exponential phase of growth prior to the induction of *red* genes. Insight into this phenomenon was gained during the course of analysis of proteins in cell-free extracts obtained from these strains from various stages of growth. We found that substantial amounts of the products of *redα* and *redβ* were present in these extracts prior to their induction (data not shown). We propose, therefore, that the presence of multimers in these strains may be a consequence of poor repression of the P_L promoter in a multicopy plasmid thereby resulting in the expression of *red* genes. Nonetheless, the production of plasmid multimers, following the induction of *red* genes, appears to be a function of incubation at 42 °C.

To relate the dependence of the formation of hmm DNA to the availability of *red* functions, we sought to inhibit *de novo* protein synthesis. Accordingly, we added chloramphenicol (100 μg ml^{-1}) to a *lon* mutant harbouring pSJS6, immediately after thermal derepression of *red* genes. Total plasmid DNA preparations obtained from identical numbers of cells were resolved on agarose gels and analysed by Southern hybridization. In the absence of chloramphenicol, the production of multimeric DNA increased with time and when the process of translation was inhibited, the relative amount of hmm DNA uniformly decreased by approximately 2·5-fold (data not shown). Taken collectively, these results suggest that expression of *red* genes sets the mode of replication from theta to the production of multimers.

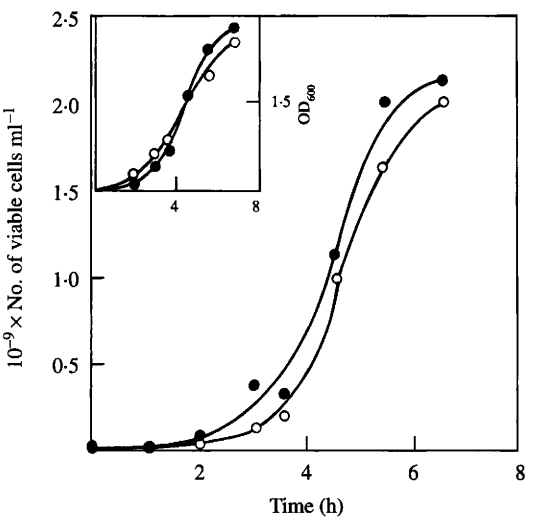

Fig. 4. Lack of association of cell lethality with the formation of plasmid linear multimers promoted by the Red-system of phage lambda. Cultures of exponentially growing cells harbouring pSJS6 were transferred from 30 °C to 42 °C. At the indicated time intervals aliquots were taken and diluted appropriately and the number of viable cells was determined by plating on L-plates containing the appropriate antibiotics. ○, KM102 (*recA*$^+$ *lon*$^-$); ●, AB1157 (*recA*$^+$ *lon*$^+$). Inset, Determination of the total number of cells as monitored by the optical density at 600 nm. ● ○, As main figure.

Reduced multimer formation in a recA$^+$ *lon*$^+$ *strain is not attributable to cell lethality*

It has been established that plasmids such as pBR322 and other derivatives from the ColE1 origin lineage are unstable in certain host strains, consequently causing cell death. Most pertinent to this report, however, are the observations dealing with population analyses of bacterial cells producing plasmid multimers resulting in cell lethality (Kusano *et al.*, 1989). Since the magnitude of synthesis of plasmid multimers differs between *recA*$^+$ *lon*$^+$ and *recA*$^+$ *lon*$^-$ genetic backgrounds, it is plausible that the determining factor, following induction of *red* genes, is the continued viability of cells. Therefore, to gain an insight into the inability of *recA*$^+$ *lon*$^+$ cells to accumulate hmm DNA, we monitored the growth and viability of cells. In a typical experiment a single-colony transformant of *recA*$^+$ *lon*$^+$ (AB1157) and *recA*$^+$ *lon*$^-$ (KM102) strains, harbouring pSJS6 were grown in L-broth at 30 °C. When the growth reached mid-exponential phase the cultures were shifted to 42 °C to facilitate the expression of *red* genes. Aliquots were removed at different time intervals and total numbers of viable cells were determined on L-plates. As illustrated in Fig. 4, the doubling time as monitored by measuring the density as OD_{600} (inset) and plating efficiency was identical between *recA*$^+$ *lon*$^+$ and *recA*$^+$ *lon*$^-$ strains harbouring pSJS6. These results, therefore, argue that the reduced production of multimeric DNA in the *recA*$^+$ *lon*$^+$ strain is attributable to the negative effect of *lon* function and not related to plasmid-mediated host cell lethality.

Further characterization of high molecular mass DNA

Earlier studies on plasmid recombination have shown an excellent correlation among genetic recombination and generation of circular oligomers of plasmid DNA (Fishel *et al.*, 1981; James *et al.*, 1982). To distinguish between circular and linear multimeric DNA produced by the Red-pathway of phage lambda, purified total plasmid DNA was treated with *E. coli* exonuclease III, which degrades linear duplex DNA from the 3′ ends releasing 5′-mononucleotides. Consequently, as shown in Fig. 5, the hmm bands corresponding to multimeric forms of DNA, obtained from the *recA*$^+$ *lon*$^-$ strain, were significantly reduced in intensity after digestion with exonuclease III, suggesting that the hmm DNA consists of linear multimeric forms. Under these conditions, Form I DNA, which is the major species of DNA present in *recA56 lon*$^+$ cells, resisted digestion by exonuclease III (data not shown). Consistent with these results, Silberstein *et al.* (1990) have also observed by electron microscopy the occurrence of plasmid multimers with free ends, produced by the *red* genes.

Further physical characterization was done to estimate the size of the multimeric DNA by pulse field gel electrophoresis. The size of plasmid multimers produced by the products of *red* genes was analysed with a lambda ladder DNA as standard markers (Fig. 6). The bulk of linear plasmid multimers were located between 50 and 240 kb with a large population of DNA molecules of 100–200 kb length, which corresponded to more than 10–20 plasmid unit lengths. The electron microscopic analysis of the length of plasmid multimers mediated by the *red* genes yielded five plasmid unit lengths in the presence of *gam* function, and when mediated by *red* genes alone, they were longer than two unit lengths (Silberstein *et al.*, 1990). The reason for this difference in unit length of plasmid multimers, we believe, is a consequence of increased stability, due to the loss of *lon* function, of enzymes and proteins that promote the formation of multimers.

Production of linear plasmid multimers is abolished in a redβ mutant

The foregoing observations have underscored the role of *recA* in augmenting the Red-system-promoted synthesis of plasmid multimers. To test whether *redβ* is acting in concert with *recA* (Muniyappa & Radding, 1986), we constructed a series of nested deletions in *redβ*. For this purpose, we cleaved pSJS6 plasmid DNA, isolated from a *recA lon*$^+$ host with *Sal*I (Fig. 1). The linearized DNA

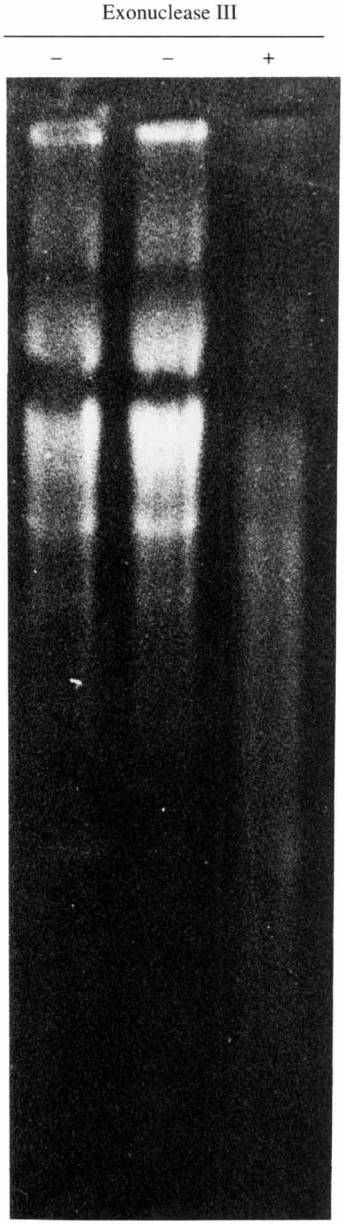

Fig. 5. Plasmid linear multimers produced by the Red-system contain free double-chain ends. Total plasmid pSJS6 DNA, obtained from strain KM102 following induction of *red* genes, was purified by CsCl density gradient centrifugation. The DNA was digested by exonuclease III as described in Methods. The samples were resolved on a 0·5% agarose gel, stained with ethidium bromide and photographed.

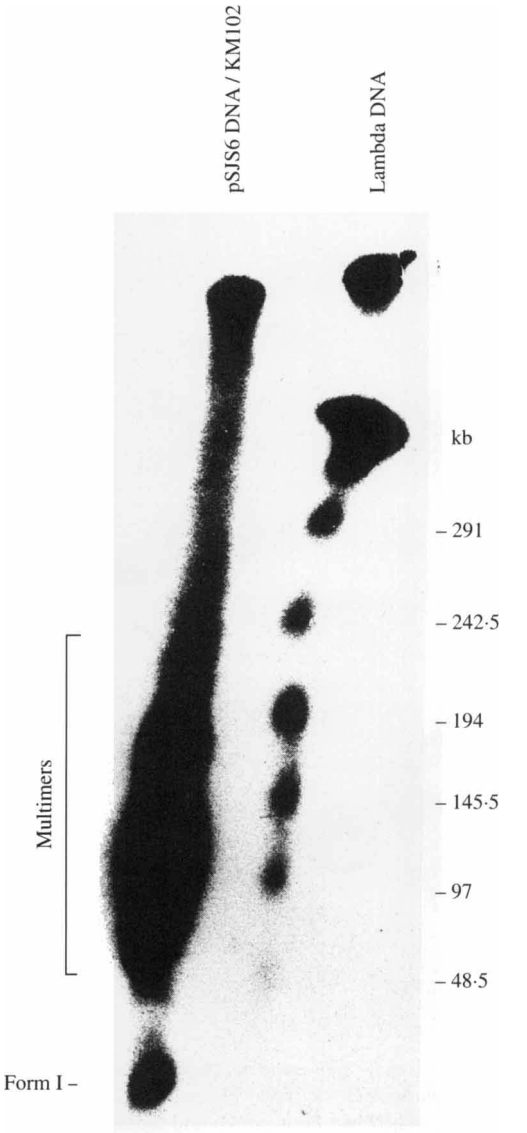

Fig. 6. Determination of the size of plasmid multimers produced by the *red* genes. Total plasmid DNA was extracted from strain KM102 harbouring pSJS6 and purified by CsCl density gradient centrifugation. A 2 μg amount of purified DNA was used for pulse field gel electrophoresis as described in Methods. The size of the standard markers is indicated on the right.

was then digested by Bal31 nuclease, for various time periods, and then treated with Klenow to create blunt ends. The DNA was recircularized by ligation and used for transformation of a *recA* strain. Two clones, pKM127.2 and pKM132.9 with deletions of 500 bp and 850 bp in *redβ*, were transformed into the *recA*$^+$ *lon*$^-$ (KM102) strain. Total plasmid DNA preparations collected from these strains, harbouring the respective constructs, were analysed by electrophoresis and Southern hybridization as described above. A comparison of the extent of accumulation of plasmid DNA revealed that the wild-type *redβ* produced maximal amounts of multimeric DNA. On the other hand, one of the subsequently constructed deletions in *redβ* (pKM127.2) produced basal levels of multimers of plasmid DNA, while in the DNA preparation obtained from the same strain harbouring pKM132.9, the multimers were completely absent (Fig. 7). These results, therefore, suggest that the production of multimers of plasmid DNA is mediated by recombination and requires the functional *redβ* gene product.

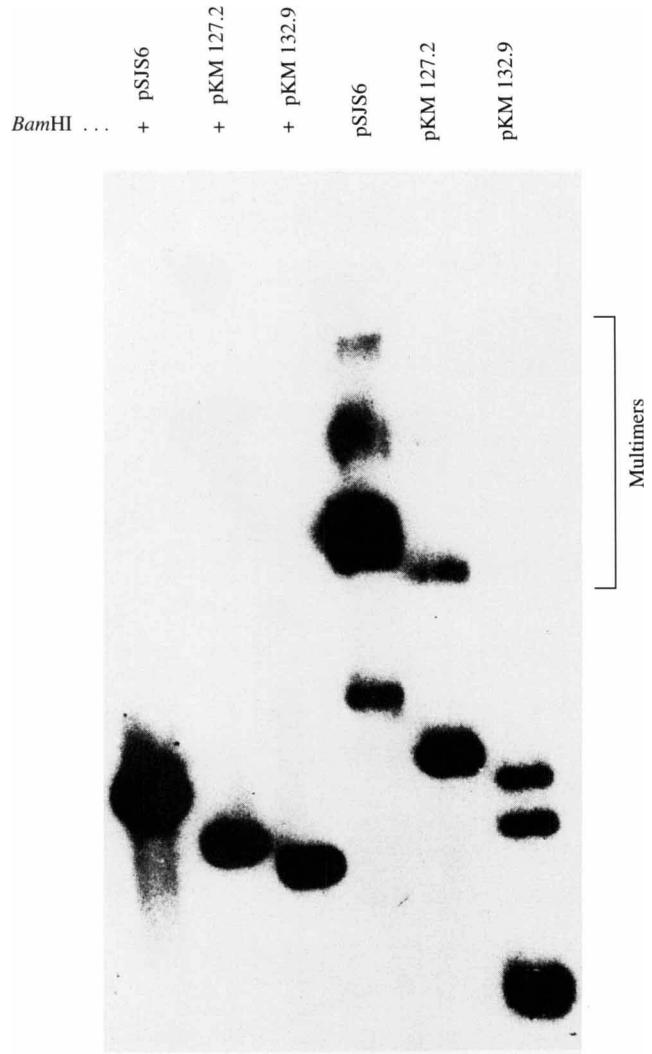

Fig. 7. A functional *redβ* gene is required for the formation of multimers of plasmid DNA. Total plasmid DNA was obtained from strain KM102, which had been grown and induced as described, and which harboured the indicated constructs. A portion of total plasmid DNA from each preparation was linearized by *Bam*HI under reaction conditions as described in Methods. The remaining sample of total plasmid DNA was loaded onto an agarose gel and analysed by Southern hybridization.

Discussion

Action of the RecBC and RecF pathways

The small circular genomes of bacteriophages and plasmids have provided convenient molecular models for elucidating the genetic requirements and various aspects of the mechanism(s) of homologous genetic recombination. A large number of studies have shown that the oligomerization of circular plasmids in *E. coli* is controlled by host recombination machinery (reviewed in: Smith, 1988; Clark & Low, 1988; Mahajan, 1988). Studies on the isolated oligomeric forms of DNA have revealed interesting structures such as 'figure-of-eight', the recombination intermediate predicted to occur with circular substrates in both gene conversion and reciprocal exchanges (Fishel *et al.*, 1981; McCarthy, 1982).

The recombination of bacterial plasmid DNA has been studied intensively in various genetic backgrounds in *E. coli* (Cohen & Clark, 1986; Niki *et al.*, 1990). These studies have shown that plasmids, in wild-type *E. coli*, recombine by a pathway that differs from that of conjugational recombination (Cohen & Clark, 1986). Mutations in *recB* and *recC* alleles which reduce conjugational recombination have no apparent effect on plasmid recombination (Kolodner, 1980). On the other hand, plasmid recombination depends on the functions of *recF*, *recJ* and *recO* which participate in *recF* conjugational recombination (Kolodner *et al.*, 1985). This pathway becomes pronounced in *recB recC sbcB sbcC* mutants. The other pathway, the RecE pathway, is activated in *E. coli* cells carrying an *sbcA* mutation (Clark & Low, 1988). The RecE pathway is partially coded for by a cryptic lambdoid prophage. Plasmid recombination in these strains occurs with a high efficiency and does not require the *recA* and *recF* gene products (Clark & Low, 1988). All this information was generated while establishing a correlation among genetic recombination and the formation of circular oligomers and, subsequently, their interconversion into monomers. The exact relationship among the pathways of conjugational recombination and plasmid recombination is unknown. Nonetheless, the studies of James *et al.* (1982) have revealed that the RecBC recombination pathway produces circular oligomers whereas the RecF pathway converts them into monomers.

Generation of linear multimers

A. Cohen and his colleagues discovered yet another mode of plasmid recombination that results in the formation of linear multimers (Cohen & Clark, 1986; Silberstein & Cohen, 1987). In wild-type *E. coli*, inactivation of RecBCD enzyme either by phage lambda *gam* protein or by mutations in *recB* and *recC* alleles switches the mode of synthesis of plasmids from circular monomers to linear multimers. The production of linear multimers requires not only the activities of the RecF pathway, such as *recF*, *recJ*, *recN*, *recO*, *recQ* and *ruv* but also the product of the *recA* gene (James *et al.*, 1982; Kolodner *et al.*, 1985; Kusano *et al.*, 1989). A corollary to this phenomenon exists in recombination-aided replication of the phage lambda chromosome (Skalka, 1977). In wild-type cells, in the presence of a potent RecBCD nuclease, the predominant form of replication is theta-type. Inactivation of RecBCD nuclease diverts phage lambda chromosome replication towards the production of linear multimers by the rolling-circle mode

of replication. The production of viable phage particles is greatly facilitated with the concomitant activation of Rac prophage, suggesting that DNA replication and recombination are tightly interrelated (Kushner *et al.*, 1974; Joseph & Kolodner, 1983; Clark & Low, 1988).

It has been shown by Berger & Cohen (1987) and Nussbaum & Cohen (1988) that Red-combination machinery promotes plasmid recombination in *recB recC sbcB* cells in a *recA*-independent manner. These studies involved intrachromosomal recombination among heteroalleles of a luciferase gene. Interestingly, those authors have also noted the inability of *red* functions to promote intraplasmidic recombination in RecBCD$^+$ ExoI$^+$ *E. coli* strains.

Role of red genes in formation of plasmid multimers

We found that expressions of *red* genes in RecBCD$^+$ ExoI$^+$ ExoIII$^+$ host cells led to the synthesis of plasmid multimers. Under these conditions, multimer production promoted by the Red-system was higher in *recA*$^+$ than in *recA* mutant strains, which are otherwise wild-type for *recB*, *recC*, *sbcB*, *sbcC* genes. Recently, Silberstein *et al.* (1990) have shown that in wild-type strains the production of maximal amounts of plasmid DNA promoted by the Red-system requires *gam* function. The source of this seemingly conflicting data is unknown at present. Several lines of evidence, however, argue that it is not due to the increased degradation of multimers by RecBCD nuclease. It has been suggested that degradation of DNA substrates and intermediates is not sufficient to cause the return of rolling circle DNA replication to theta-mode of replication (Skalka, 1977). Correspondingly, Kusano *et al.* (1989) have shown that in *recA* strains there was 100-fold reduction in the extent of synthesis of multimeric DNA in the absence of *recB*, *recC*, *sbcB*, *sbcC* gene products.

The induction of *red* genes requires the transfer of cultures from 30 °C to 42 °C and such a shift also results in expression of the host Lon protease. We reasoned that Lon protease might play a dominant role by cleaving enzymic machinery that participates in the initiation of plasmid recombination as well as those components that act on the putative recombination intermediates. Consistent with this hypothesis, we observed that the formation of linear multimers, after the induction of *red* genes, was at least 3-fold higher in *lon* mutants than in the isogenic *lon*$^+$ strains. It is likely, therefore, that Lon protease rapidly destroys the protein components that are processing the labile DNA substrates and intermediates. Furthermore, the finding that the magnitude of Red-promoted plasmid multimer synthesis was higher in the *recA*$^+$ strain is compatible with our earlier observations that β protein acts in concert with RecA protein in

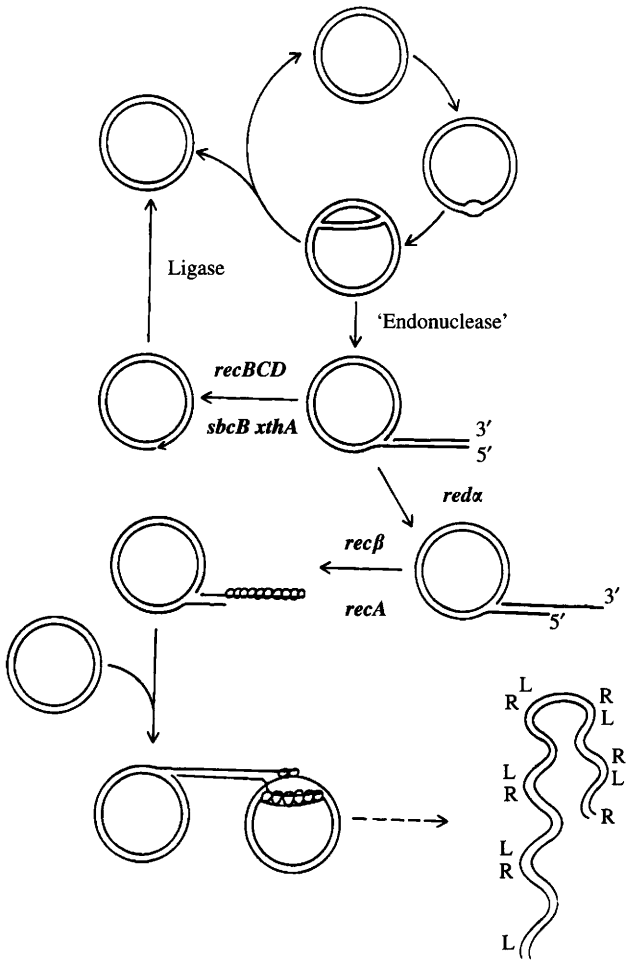

Fig. 8. Diagram depicting a hypothetical path to the formation of plasmid linear multimers promoted by the Red-pathway of phage lambda. For details see Discussion.

the formation of joint molecules (Muniyappa & Radding, 1986). Our genetic analysis of the negative effect of Lon protease on the formation of plasmid multimers provides intriguing clues as to the interaction of other cellular enzymic activities that regulate homologous recombination and perhaps other processes related to DNA metabolism.

A conceptual framework for the Red-pathway

To think about how the Red-pathway of phage lambda facilitates the formation and enhanced accumulation of plasmid linear multimers in wild-type as well as in *lon* mutants of *E. coli*, it is important to consider a conceptual framework that could account for their production (Fig. 8). There appears to be a general agreement that the formation of linear multimers or concatemers is sensitive to the action of *recBCD xthA sbcB sbcC* gene products. The negative effect of *lon*

function is partly attributable to its ability to degrade the enzymes and proteins processing the labile DNA substrates and recombination intermediates. There are at least two concerted mechanisms by which such substrates and intermediates could contribute to the formation of linear multimers. The first possibility is the rolling circle replication: we presume that an intermediate in the monomer circle replication is converted into an open circular form by the action of an 'endonuclease'. Mechanisms of the second category include the recombination-dependent or the bubble-migration mode (Formosa & Alberts, 1986). In such a situation, a 3′ single-stranded tail is produced by the action of *redα*, and serves as a substrate for the binding of *redβ*. The nucleoprotein filament (Muniyappa & Radding, 1986) thus formed invades a homologous sequence on the circular template. This interpretation parallels in several aspects the production of concatemers during late replication in phage-infected bacterial cells (Skalka, 1977; Stahl *et al.*, 1973). Recently, Silberstein *et al.* (1990) have provided complementary evidence, by electron microscopy, for the occurrence of two types of molecular structures: sigma-shaped and linear molecules, believed to be the intermediates in the path to the formation of plasmid linear multimers.

In summary, all the experimental evidence and the framework that we have provided is compatible with the notion that double-stranded ends provoke recombination promoted by the Red-pathway (Stahl, 1986; Thaler *et al.*, 1987; Takahashi & Kobayashi, 1990). Taken collectively, our genetic analysis has revealed that the formation of plasmid multimers is maximal in *lon* mutants in RecBCD$^+$ ExoI$^+$ ExoIII$^+$ proficient host cells. In addition, or more importantly, these new concepts initiate an understanding of the interplay among cellular enzymic machinery related to DNA metabolism and protein degradation in plasmid recombination.

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