JOURNAL OF BACTERIOLOGY, Nov. 1993, p. 7254–7259
0021-9193/93/227254-06$02.00/0

Vol. 175, No. 22

Requirement for the Polymerization and 5′→3′ Exonuclease Activities of DNA Polymerase I in Initiation of DNA Replication at *oriK* Sites in the Absence of RecA in *Escherichia coli rnhA* Mutants

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Received 6 July 1993/Accepted 21 September 1993

In previous studies, we found that the requirement for RecA protein in constitutive stable DNA replication (cSDR) can be bypassed by derepression of the LexA regulon and that DNA polymerase I (DNA PolI) is essential for this Rip (RecA-independent process) pathway of cSDR (Y. Cao, R. R. Rowland, and T. Kogoma, J. Bacteriol. 175:7247–7253, 1993). In this study, the role of DNA PolI in the Rip pathway was further examined. By using F′ plasmids carrying different parts of the *polA* gene, a series of complementation tests was carried out to investigate the requirement for the three enzymatic activities, polymerization, 3′→5′ exonuclease, and 5′→3′ exonuclease activities, of DNA PolI. The result indicated that both the 5′→3′ exonuclease and polymerization activities of DNA PolI are essential for bypassing the requirement for RecA in cSDR but that the 3′→5′ exonuclease activity can be dispensed with. Complementation experiments with rat DNA Polβ also supported the hypothesis that a nick translation activity is probably involved in cSDR in the absence of RecA. An analysis of DNA synthesis suggested that DNA PolI is involved in the initiation but not the elongation stage of cSDR. Moreover, the *dnaE293*(Ts) mutation was shown to render the bypass replication temperature sensitive despite the presence of active DNA PolI, suggesting that DNA PolIII is responsible for the elongation stage of the Rip pathway. A model which describes the possible roles of RecA in cSDR and the possible function of DNA PolI in the Rip pathway is proposed.

Escherichia coli rnhA mutants can dispense with DnaA protein, the *dnaA* gene product (11), which is essential for initiation of chromosome replication at *oriC* in *rnhA*$^+$ cells (13). It has been hypothesized that in the absence of RNase HI (encoded by *rnhA*), DNA-RNA hybrids are stabilized at certain sites (defined as *oriK* sites) on the chromosome, generating R-loops which can become sites for initiation of DNA replication (28). The initiation from *oriK* sites requires RecA protein (10). Thus, the growth of *rnhA recA*(Ts) (temperature-sensitive) cells is temperature sensitive when the *oriC* system is inactivated by a *dnaA*::Tn*10* mutation or deletion of the *oriC* site. The RecA requirement can be suppressed by activation of a bypass pathway, termed Rip, by introducing a *lexA*(Def) mutation (2). For example, *dnaA*(Ts) *rnhA recA*(Ts) *lexA*(Def)::Tn*5* strains are not temperature sensitive despite the presence of *dnaA*(Ts) and *recA*(Ts), which inactivate the *oriC* and *oriK* systems of initiation of DNA replication, respectively, at the restrictive temperature.

In our effort to elucidate the mechanism of the Rip pathway, in the accompanying paper (2) we have described the isolation of a transposon-insertion mutation that renders the Rip bypass pathway inoperative. The mutation, designated *polA25*::mini Tn*10spc*, maps in the *polA* gene, which encodes DNA polymerase I (DNA PolI). The *polA25*::miniTn*10spc* mutants are as sensitive to UV irradiation and methyl methanesulfonate as *polA1* mutants, which are known to be defective in excision repair. The combination of *recA*(Ts) and *polA25* (*polA25*::miniTn*10spc*) mutations renders cells temperature sensitive for growth, as does the *recA*(Ts) *polA1* combination. However, the temperature sensitivity of *recA*(Ts) *polA25* mutants can be suppressed by *lexA*(Def) as long as the *oriC* system is in operation. On the other hand, when cells depend on the Rip system for initiation of chromosome replication, as in *dnaA*::Tn*10 rnhA recA*(Ts) *lexA*(Def) strains, the *polA25* mutation inactivates the Rip pathway, rendering the cells temperature sensitive. Thus, *polA*$^+$ is required for the Rip pathway to operate in the absence of active RecA.

DNA PolI has three enzymatic activities, polymerization, 3′→5′ exonuclease, and 5′→3′ exonuclease, located in three distinct domains of the polypeptide (reviewed in reference 13) (see Fig. 1). Limited proteolysis of the protein yields two fragments of unequal sizes: the larger of the two fragments (the Klenow fragment) contains the 3′→5′ exonuclease and polymerization activities, and the smaller one contains the 5′→3′ exonuclease activity (7). Furthermore, the high-resolution structural analysis of the Klenow fragment has revealed that the 3′→5′ exonuclease and polymerization activities reside in two discrete domains within the fragment; the smaller N-terminal domain and the larger C-terminal domain, respectively (18). The cloning and nucleotide sequence analysis of the *polA25*::miniTn*10spc* allele has indicated that it is most likely to encode a truncated protein lacking the C-terminal 57 amino acid residues (2). In order to determine which of the three activities are missing in the mutant DNA PolI encoded by the *polA25*::miniTn*10spc* mutant allele, and to ascertain which activities of DNA PolI are essential for the Rip pathway, in this study we have carried out a series of complementation experiments using F′ factors that carry different parts of the *polA* gene.

MATERIALS AND METHODS

***E. coli* strains.** The *E. coli* strains used in this study are listed in Table 1. Some strain constructions are described elsewhere (2).

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype[a]					Other characteristic(s)	Source, reference, and/or construction information
	dnaA	*rnhA*	*recA*	*polA*	*lexA*		
AQ548	+	+	+	+	+	*dnaE293*(Ts)	This laboratory
AQ3476[b]	+	*102*	*200*	+	*71*::Tn*5*	*sfiA11*	Derivative of AQ377 (27)
AQ6386	+	+	+	+	+	Tn*10* at 4.75 min	CAG18436 (23)
AQ6466	+	+	+	+	+	Tn*10* at 87 min	CAG18495 (23)
AQ8747[b]	*5*	*102*	*200*	*25*::*spc*	*71*::Tn*5*	*sfiA11*	2
AQ8862	+	+	+	+	+	F′ *polA*$^+$, Cm^r	RM2425 (16)
AQ8863	+	+	+	+	+	F′ 5′→3′ exo,[c] Cm^r	RM2426 (16)
AQ8864	+	+	+	+	+	F′ Klenow, Cm^r	RM2427 (16)
AQ8865	+	+	+	+	+	F′ vector, Cm^r	RM2428 (16)
AQ8866	+	+	+	Δ	+	F′ D355A E357A	RM2429 (16)
AQ8937[b]	*5*	*102*	*200*	+	*71*::Tn*5*	*sfiA11*	2
AQ8939	+	+	+	+	+	*dnaE293*	P1.AQ6386 × AQ548 → Tc^r → temperature-sensitive growth
AQ8980[b]	+	*102*	*200*	+	*71*::Tn*5*	*dnaE293 sfiA11*	P1.AQ8939 × AQ3476 → Tc^r → temperature-sensitive growth
AQ9016	+	+	+	Δ	+	F′ D355A E357A	P1.AQ6466 × AQ8866 → Tc^r → Km^r and UV^s
AQ9045[b]	*5*	*102*	*200*	Δ	*71*::Tn*5*	*sfiA11*	P1.AQ9016 × AQ8937 → Tc^r on minimal medium plates → UV sensitive at 30°C, broth sensitive
AQ9194[b]	*5*	*102*	*200*	Δ	*71*::Tn*5*	*sfiA11*, pβL	AQ9045 transformed with pβL → Cm^r
AQ9286[b]	*5*	*102*	*200*	*25*::*spc*	*71*::Tn*5*	*sfiA11*, pβL	AQ8747 transformed with pβL → Cm^r

[a] + indicates a wild-type gene, a number indicates an allele, and Δ indicates a deletion.
[b] The remaining genotype is *metE90 trpA9605 thy-708 deo-29 lacZ118 lacI22.*
[c] exo, exonuclease.

Chemicals and radioisotopes. [*methyl*-^{3}H]thymidine (20 Ci/mmol) was from Dupont Company (Boston, Mass.). Other chemicals used were described previously (2).

Media and growth conditions. All media used are described in the accompanying paper (2). All strains were grown in Luria broth (LB), with the exception of Δ*polA* derivatives, which were grown in M9 glucose minimal medium supplemented with Casamino Acids (CAA medium) (2).

Complementation test. The strains harboring F′ episomes carrying different parts of the *polA* gene and a Cm^r marker were obtained from R. Maurer (16). F′ D355A E357A is an F′ episome which carries a *polA* gene with two mutations that specifically affect the 3′→5′ exonuclease activity without inactivating the other two activities of the DNA PolI. These F′ strains were mated with recipient strain AQ8747 in LB and with recipient strain AQ9045 in CAA medium at 30°C for 1 h. The exconjugants were selected for Cm^r, Tc^r, and Km^r on LB or CAA plates. Complementation with rat Polβ was carried out by introducing plasmid pβL into AQ8747 and AQ9045 by transformation, selecting for Cm^r. The pβL plasmid, which was constructed by J. Sweasy and obtained via R. Maurer (16), is a pHSG576 derivative bearing cDNA encoding rat DNA Polβ placed under the *lac* promoter.

Southern blot hybridization. Total DNA was extracted from overnight cultures according to K. Wilson's method (30). The isolated DNA was then digested with *Eco*RI, electrophoresed in a 1% agarose gel, and blotted onto a nylon transfer membrane (pore size, 0.45 μM; Amersham Corporation, Arlington Heights, Ill.). The probe was prepared by labeling the *Sst*I-*Eco*RV fragment of pRRR25 (2) (~500 bp) with α-[^{32}P]dCTP (ICN Biomedicals, Inc., Irvine, Calif.) by using the Random Primers Labeling kit from BRL Life Technologies, Inc. (Gaithersburg, Md.). Hybridization was performed as described by Sambrook et al. (22).

Determination of DNA synthesis rate on plates. The assay to determine the rate of DNA synthesis was based on the method of Lieberman and Witkin (15) with a few modifications. Cells were cultured in nutrient broth to log phase (~1.5 × 10^8 cells per ml). Next, 0.03-ml volumes of the cultures were plated on two sets of nutrient agar plates. After a 60-min incubation at 30°C, one set was shifted to 42°C and the other remained at 30°C. Immediately, and every hour thereafter, cells were pulse labeled by quickly spreading 0.03 ml of prewarmed [^{3}H]thymidine (0.1 mCi/ml) on duplicate plates from each of the 30 and 42°C incubators. The plates were then returned to incubation at their respective temperatures. After 10 min, the labeling was stopped by washing the surfaces of the plates with 0.3 ml of cold M9 glucose medium supplemented with thymine (50 μg/ml). The washes from duplicate plates were combined and chilled on ice for 15 min. A 0.1-ml sample of the wash was loaded onto a piece of Whatman 3MM filter paper. The filters were soaked in 15% cold trichloroacetic acid for 15 min, 5% cold trichloroacetic acid for 15 min, 50% ethanol for 10 min, and 95% ethanol for 10 min. The filters were then air dried, and radioactivity was determined by liquid scintillation counting. Under these conditions, DNA synthesis in a *dnaG3*(Ts) strain (a primase mutant) immediately ceased at the restrictive temperature (data not shown).

RESULTS

Complementation with F′ plasmids carrying various parts of the *polA* gene. DNA PolI has three enzymatic activities located in three distinct domains of the polypeptide (Fig. 1). To determine which of the three activities, polymerization, 3′→5′ exonuclease, and 5′→3′ exonuclease, is required for the Rip pathway, complementation experiments were carried out with F′ plasmids carrying one or more of the activities (Table 2). AQ8747 [*dnaA*(Ts) *rnhA recA*(Ts) *polA25*::mini*Tn10spc lexA*(Def)] is temperature sensitive for growth. This is because at high temperatures, the *oriC* system does not operate because of *dnaA*(Ts) and the *oriK* system is inoperative because of *recA*(Ts). Also, the Rip system fails to suppress the RecA defect because of the presence of the *polA* mutation, as shown

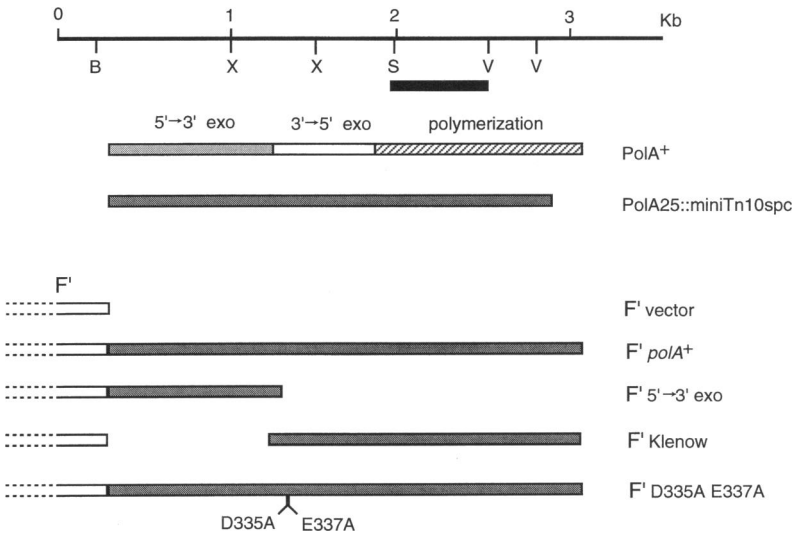

FIG. 1. The structures of PolA$^+$ and PolA25 and of F′ episomes carrying different parts of the *polA* gene. The restriction map of the *polA* gene is shown relative to the three domains of PolA$^+$ (DNA PolI) (adapted from references 12 and 13) and PolA25. B, *Bgl*II; X, *Xho*I; S, *Sst*I; V, *Eco*RV. The solid bar indicates the fragment that was used to detect F′ Klenow (see the text). The F′ episomes carrying different parts of *polA* are described elsewhere (4).

in the accompanying paper (2). When the F′ plasmid carrying a whole *polA* gene with or without the 3′→5′ exonuclease activity or carrying the Klenow fragment (F′ Klenow) (the polymerization and 3′→5′ exonuclease activities) was introduced, the strain could grow at 42°C. This indicated that those plasmids could complement the *polA25*::miniTn*10spc* defect. On the other hand, the F′ plasmid carrying the 5′→3′ exonuclease domain only did not complement the defect (Table 2). The results indicated that at least the polymerization activity of DNA PolI is missing in PolA25 polymerase, and this activity is essential for the Rip pathway.

Since it was not known exactly which activities were missing in the PolA25 polymerase, the complementation experiment described above did not allow us to determine whether either or both of the exonuclease activities are required. Therefore, we constructed a strain identical to AQ8747 except for substitution of the Δ*polA* allele in place of *polA25*::miniTn*10spc* and repeated the complementation experiment with this strain (AQ9045). The F′ episome that carried both the 5′→3′ exonuclease and polymerization activities (F′ D355A E357A) could complement the Δ*polA* defect (Table 2). In contrast, F′ Klenow failed to complement the defect. The presence of F′ Klenow in the strain was ascertained by detection of the sequence which corresponds to the polymerization domain of the *polA* gene (data not shown). Similarly, the F′ plasmid that carried only the 5′→3′ exonuclease activity did not complement the defect (Table 2). These results indicated that the Rip bypass pathway requires both the 5′→3′ exonuclease and polymerization activities of DNA PolI.

TABLE 2. Complementation of *polA25*::miniTn*10spc* and Δ*polA* strains with F′ plasmids carrying different parts of DNA PolI

Strain and plasmid	DNA PolI activity[a] on F′ plasmid			Colony formation[b] at:	
	5′→3′ exo[c]	3′→5′ exo[c]	Polymerase	30°C	42°C
AQ8747					
F$^-$				+	−
F′ *polA*$^+$	+	+	+	+	+
F′ vector	−	−	−	+	−
F′ 5′→3′ exo[c]	+	−	−	+	−
F′ Klenow	−	+	+	+	+
F′ D355A E357A	+	−	+	+	+
AQ9045					
F$^-$				+	−
F′ *polA*$^+$	+	+	+	+	+
F′ vector	−	−	−	+	−
F′ 5′→3′ exo[c]	+	−	−	+	−
F′ Klenow	−	+	+	+	−
F′ D355A E357A	+	−	+	+	+

[a] +, activity present on plasmid; −, activity not present on plasmid.
[b] +, colonies formed; −, no colonies formed.
[c] exo, exonuclease.

Complementation with rat DNA *Pol*β. cDNA coding for rat DNA Polβ has been cloned and expressed in *E. coli*, and the expressed Polβ can complement the temperature sensitivity defect of *polA12*(Ts) of *E. coli* (25). pβL is a plasmid derived from pHSG576 (with a pSC101 replication origin) that carries rat Polβ cDNA fused to the *lac* promoter (16). The plasmid was introduced by transformation into AQ8747 and AQ9045 to test whether rat Polβ can complement the defects of *polA25*::miniTn*10* and Δ*polA* in the Rip pathway function. The plating efficiency of the derivatives of AQ9045 at 42°C did not significantly improve in the presence of the plasmid (Table 3). On the other hand, the plating efficiencies of the AQ8747 derivatives were 100- and 500-fold better than those of the parental strain at 30 and 42°C, respectively (Table 3). Although the strains had a *lacI* mutation which allows constitutive expression from the *lac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG), an inducer of the *lac* promoter, was added at various concentrations to ensure full expression of the gene (Table 3). The presence of the inducer made only a slight difference. Thus, Polβ can partially complement *polA25*::miniTn*10spc*, but not Δ*polA*, for the function in the Rip pathway.

DNA synthesis. The effects of the *polA25*::miniTn*10spc* mutation on the Rip system and the complementation by F′

TABLE 3. Complementation with pβL plasmid

Strain	Relevant genotype	Presence of pβL plasmid[a]	Plating efficiency[b] at:		
			30°C	42°C	
				Without IPTG	With IPTG[c]
AQ8747	*polA25*	−	$6.25 \pm 3.51 \times 10^{-3}$	$7.94 \pm 3.06 \times 10^{-6}$	
AQ9286	*polA25*	+	$6.04 \pm 0.38 \times 10^{-1}$	$3.58 \pm 2.56 \times 10^{-3}$	$8.28 \pm 3.21 \times 10^{-3}$
AQ9045	Δ*polA*	−	$4.61 \pm 1.29 \times 10^{-1}$	$5.23 \pm 3.19 \times 10^{-6}$	
AQ9194	Δ*polA*	+	$4.88 \pm 1.44 \times 10^{-1}$	$9.39 \pm 2.01 \times 10^{-6}$	$4.40 \pm 1.41 \times 10^{-5}$

[a] +, plasmid present; −, plasmid not present.
[b] The plating efficiency for AQ8747 and AQ9286 was determined on LB plates, and the plating efficiency for AQ9045 and AQ9194 was determined on CAA plates, as described previously (2). No IPTG was added at 30°C. The values are averages (± standard errors of the means) of two to four independent determinations.
[c] Values are averages of four determinations at concentrations of IPTG between 1×10^{-4} and 5×10^{-3} M.

Klenow were examined at the DNA synthesis level. The initial analysis of DNA synthesis with cells growing in liquid medium showed that there was significant degradation of DNA in some strains at the restrictive temperature (data not shown). Therefore, we adopted the procedure developed by Lieberman and Witkin (15), which allows determination of the rates of DNA synthesis in cells growing on nutrient agar plates, conditions similar to those in which the complementation analysis for viability was carried out as described above. The results summarized in Fig. 2 show that the DNA synthesis rate in AQ8747 [*dnaA*(Ts) *rnhA recA*(Ts) *lexA*(Def) *polA25*::miniTn*10spc*] decreased slowly to zero at the restrictive temperature. On the other hand, the *polA25*::miniTn*10spc* mutant carrying F′ Klenow continued DNA synthesis at a rate as high as that attained by the *polA*$^+$ counterpart at 42°C. Thus, the DNA synthesis defect of the *polA25*::miniTn*10spc* mutant in the Rip pathway was completely mitigated by the presence of F′ Klenow.

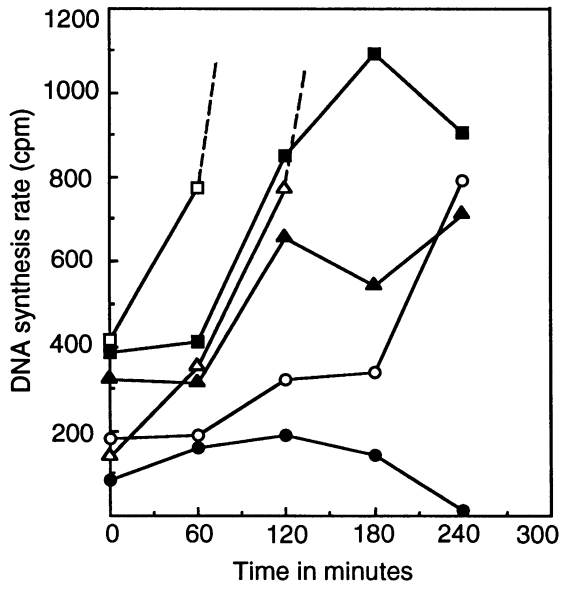

FIG. 2. Rates of DNA synthesis in *recA*(Ts) *polA25*::miniTn*10spc* strains at 30 and 42°C. AQ8747 (circles), AQ8747(F′ *polA*$^+$) (triangles), and AQ8747(F′ Klenow) (squares) cells were grown in nutrient broth at 30°C to 1 to 2×10^8 cells per ml, transferred onto minidishes, and incubated at 30°C (open symbols) and 42°C (shaded symbols). At intervals, the cells were pulse labeled with [^{3}H]thymidine as described in Materials and Methods. For comparison, the DNA synthesis rates (counts per minute per 10-min pulse) are shown after correction for the differences in cell densities at time zero. The broken lines indicate that AQ8747(F′ *polA*$^+$) at 180 min and AQ8747(F′ Klenow) at 120 min had rates of 2,602 and 2,041 cpm, respectively. The estimated standard error of the mean for these experiments was 12%.

Effects of a *dnaE*(Ts) mutation. The *dnaE293*(Ts) allele encodes a DnaE protein, the α subunit of DNA PolIII, which is temperature sensitive for DNA replication that originates from *oriC* (13). Introduction of the *dnaE*(Ts) mutation into the *recA*(Ts) *polA*$^+$ *lexA*(Def) mutant rendered the cells temperature sensitive, indicating that DNA replication from both *oriC* and *oriK* sites depends on an active DnaE protein (data not shown). This implies that DNA PolIII replisome is also responsible for the replication that is initiated at *oriK* sites by DNA PolI, despite the absence of otherwise essential RecA protein.

DISCUSSION

The polymerization, 5′→3′ exonuclease, and 3′→5′ exonuclease activities of DNA PolI reside in three discrete domains of the enzyme (see the introduction) (Fig. 1). The mutant DNA PolI encoded by the *polA25*::miniTn*10spc* allele is expected to lack the C-terminal 57 amino acid residues (2). This portion of the enzyme includes the last α helix and three β sheets which contain at least two residues crucial for the polymerization activity, as revealed by site-directed mutagenesis (19). Thus, it is very likely that our mutant DNA PolI is lacking at least the polymerization activity. This is consistent with the extreme sensitivity of *polA25*::miniTn*10spc* mutants to methyl methane sulfonate and UV irradiation (2), indicating a defect in excision repair which requires both the 5′→3′ exonuclease and polymerization activities (4, 29). We can rule out the possibility that the mutant polymerase also lacks the 5′→3′ exonuclease activity, because the Rip pathway requires both the polymerization and the exonuclease activities yet the Klenow fragment can complement the *polA25*::miniTn*10spc* defect for the same activity (Table 2). We do not know whether the *polA25*::miniTn*10spc* mutation also affects the 3′→5′ exonuclease activity. Point mutations that specifically reduce the polymerization activity without affecting the 3′→5′ exonuclease activity have been constructed (19), suggesting structural independence of the two domains of the enzyme. Previously, we observed that the *polA25*::miniTn*10spc* mutation is compatible with *lexA*(Def) whereas *polA1*, which inactivates both activities, is not (2). This suggests that the mutant polymerase probably has at least some 3′→5′ exonuclease activity.

The results of the complementation experiments with the F′ episomes indicate that both the polymerization and the 5′→3′ exonuclease activities are required for the Rip pathway. This suggests that the Rip bypass pathway involves a nick transla-

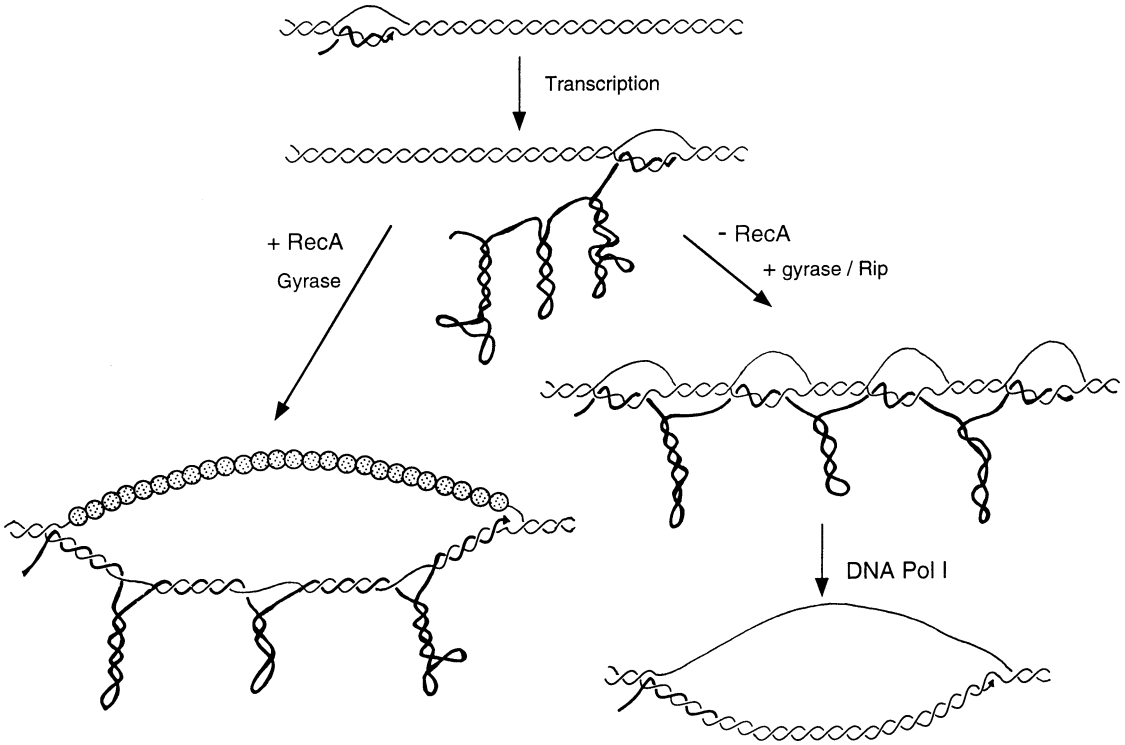

FIG. 3. The R-loop model and possible role of DNA PolI in the Rip pathway. The thin and thick lines indicate DNA and RNA strands, respectively. The arrows indicate the 3′ OH ends. The stippled circles represent RecA protein, which is here hypothesized to bind the single-stranded DNA in the R-loop on the basis of the known properties of RecA (5, 6). The possible involvement of a helicase and a single-stranded DNA binding protein is ignored. (See the text for details.)

tion activity. Alternatively, since polymerization activity is stimulated by the presence of 5′→3′ exonuclease activity (14), it is possible that the requirement for the exonuclease activity may only reflect a need for a high level of polymerization activity. Rat DNA Polβ expressed in *E. coli* has been demonstrated to complement the defect of *polA12*(Ts) in the *recA polA* lethality (25) and to compensate for the need for a high level of DNA PolI activity in *dnaQ* mutants (16). This eukaryotic polymerase completely lacks the exonuclease activity (26). Thus, our observation that rat Polβ can complement, albeit partially, the PolA25 polymerase defect and yet fails to do so for Δ*polA* strains (Table 3) is consistent with the possibility that both activities are involved in the Rip bypass pathway.

In the accompanying paper, we concluded that DNA PolI is specifically required at the initiation step of the *oriK* system in the absence of RecA (2). This conclusion is further supported by the additional observations presented in this report, i.e., the slow stopping of DNA synthesis upon a shift to the restrictive condition and the dependence on DNA PolIII. The latter observation indicates that DNA PolI does not substitute for DNA PolIII in the elongation stage.

How does RecA protein facilitate R-loop formation, and what is the role of DNA PolI in the Rip bypass pathway? We postulate that transcripts in selected transcription units hybridize to the template DNA strands. Evidence indicating that RNA polymerase has a property by which it influences formation of such hybrids has been presented (8, 20). RecA protein may catalyze this hybrid formation and stabilize it (Fig. 3). It has recently been demonstrated that RecA protein effectively promotes DNA-RNA hybrid duplex formation in vitro (5, 6). In addition, a high level of negative supercoiling generated by gyrase may be required for efficient hybrid formation in vivo. Because of the tendency of transcripts to form stable secondary structures, hybrid formation could be segmental, disrupted by unpaired regions (Fig. 3). It is not unreasonable to expect RecA protein to facilitate formation of such interrupted hybrids because RecA can tolerate a large segment of nonhomology and insertions in the DNA strand assimilation process (1, 21). The resulting R-loop could allow replisome assembly for DNA synthesis, as discussed previously (10, 28).

In the absence of RecA, a large R-loop may not be formed. In this case, segmented hybrids may possibly be interrupted by regions of DNA duplex (Fig. 3). This step may involve the product of the hypothetical gene (the *rip* gene) which is under LexA control. DNA PolI could recognize the 5′ end of the transcript and introduce a nick on the hybridized RNA strand. Such a structure-specific RNase H activity of bacterial DNA polymerases, including DNA PolI, on similar substrates has been demonstrated in vitro (17). DNA PolI could then extend the 3′ OH end of the cleaved transcript, simultaneously digesting the RNA (nick translation) so as to enlarge the R-loop (Fig. 3). The displaced DNA strand in the enlarged R-loop (i.e., now a D-loop) could facilitate the initiation of lagging strand synthesis. The role of DNA PolI in this process could be similar to its role in the initiation of ColE1-type plasmid replication in the absence of RNase HI: DNA PolI extends the 3′ OH end of the primer RNA that is hybridized to the template DNA strand (3). This is consistent with the observation that ColE1-type plasmid replication in *rnhA* mutants does not depend on *recA*$^+$ (24). One significant difference is that the Rip system involves an additional factor (Rip$^+$) which is derepressed by *lexA*(Def). In addition, the nick

translation activity of DNA PolI could also be needed for the removal of persisting R-loop, as previously proposed (9). A better understanding of the exact mechanism of the Rip bypass pathway would require identification of the factor(s) that is necessary to be derepressed by the *lexA*(Def) mutation.

ACKNOWLEDGMENTS

We are grateful to J. Sweasy and L. Loeb for plasmid pβL; to C. Joyce, N. Grindley, and R. Maurer for F′ *polA* episomes; and to Evelyn Witkin for advice on DNA synthesis rate determination on plates. We thank Tsuneaki Asai, Tom Magee, Erik Boye, and Evelyn Witkin for critically reading the manuscript.

This work was supported by Public Health Service grant GM22092 from the National Institutes of Health.

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