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Role of the *Escherichia coli* Nucleotide Excision Repair Proteins in DNA Replication

GERI F. MOOLENAAR, CELINE MOORMAN, AND NORA GOOSEN*

Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands

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DNA polymerase I (PolI) functions both in nucleotide excision repair (NER) and in the processing of Okazaki fragments that are generated on the lagging strand during DNA replication. *Escherichia coli* cells completely lacking the PolI enzyme are viable as long as they are grown on minimal medium. Here we show that viability is fully dependent on the presence of functional UvrA, UvrB, and UvrD (helicase II) proteins but does not require UvrC. In contrast, Δ*polA* cells grow even better when the *uvrC* gene has been deleted. Apparently UvrA, UvrB, and UvrD are needed in a replication backup system that replaces the PolI function, and UvrC interferes with this alternative replication pathway. With specific mutants of UvrC we could show that the inhibitory effect of this protein is related to its catalytic activity that on damaged DNA is responsible for the 3′ incision reaction. Specific mutants of UvrA and UvrB were also studied for their capacity to support the PolI-independent replication. Deletion of the UvrC-binding domain of UvrB resulted in a phenotype similar to that caused by deletion of the *uvrC* gene, showing that the inhibitory incision activity of UvrC is mediated via binding to UvrB. A mutation in the N-terminal zinc finger domain of UvrA does not affect NER in vivo or in vitro. The same mutation, however, does give inviability in combination with the Δ*polA* mutation. Apparently the N-terminal zinc-binding domain of UvrA has specifically evolved for a function outside DNA repair. A model for the function of the UvrA, UvrB, and UvrD proteins in the alternative replication pathway is discussed.

In *Escherichia coli*, nucleotide excision repair (NER) is initiated by the action of the UvrA, UvrB, and UvrC proteins. The UvrA protein loads UvrB onto a damaged site, after which UvrC binds to UvrB, resulting in the UvrBC-DNA incision complex. In this complex, first an incision is made at the fourth or fifth phosphodiester bond on the 3′ side of the damage, followed by incision at the eighth phosphodiester bond on the 5′ side of the damage. Both incisions are catalyzed by the UvrC protein, which contains two distinct active sites, one for each incision (20, 45). UvrD (helicase II) subsequently removes the damaged strand, and DNA polymerase I (PolI) fills in the resulting gap. Finally, the remaining nick is closed by DNA ligase (for reviews, see references 8 and 36).

Besides its function in NER, it is generally believed that the major role of PolI in the cell is the processing of the lagging strand during DNA replication (16). In *polA* mutant strains the joining of Okazaki fragments is severely retarded (31, 32, 42). The protein possesses three enzymatic activities, a 5′-3′ exonuclease activity located in the N-terminal part of the protein (the small domain) and a DNA polymerase activity which, together with a 3′-5′ exonuclease activity, is located in the C-terminal part of the protein (the Klenow domain) (5, 15). The combination of the 5′-3′ exonuclease and the polymerase activities results in the so-called nick translation activity, which is responsible for the removal of the RNA primers and the resynthesis of DNA in the lagging strand (16).

More than 25 years ago it was proposed that UvrB and UvrD might also be involved in DNA replication, since in vivo in the absence of DNA damage-inducing treatments, *uvrB* or *uvrD* mutations were found to be lethal in combination with a mutation in the *polA* gene (11, 29, 38, 39). Combining a deletion of the *uvrB* gene with either a *polA1* or a *polA12* mutation leads to inviability (38). *polA1* is an amber mutation introducing a stop codon at the position corresponding to residue 342, which results in a protein lacking the polymerase and proofreading activities but with a functional 5′-3′ exonuclease activity (14). *polA12* is an undefined mutation, resulting in thermosensitivity for all three activities of the PolI enzyme (14). The inviability of the *uvrB polA1* double mutant suggests that in the absence of the polymerase activity of PolI, DNA replication becomes dependent on the UvrB protein. Two different unidentified point mutations in *uvrD* are also lethal in combination with the *polA1* mutation, indicating a role for UvrD in replication as well (11, 39). In contrast with *uvrB* and *uvrD*, strains with point mutations in *uvrA* or *uvrC* (18, 25, 29, 37) in a *polA* mutant background have been reported to be viable, although the plating efficiency of a *uvrA6 polA12* strain was found to be reduced at 42°C (18, 37).

More recently it has been shown that *E. coli* cells in which the complete *polA* gene has been deleted are viable, although growth is restricted to synthetic media (13). Growth on rich media can be restored by introducing either the 5′-3′ exonuclease or the Klenow domain of PolI in this mutant strain. This implies that other enzymatic activities in the cell can substitute for the exonuclease and polymerase activities of PolI. To investigate the function of the Uvr proteins in these substituting activities, we have combined the *polA* deletion with defined deletions of the *uvrA*, *uvrB*, *uvrC*, and *uvrD* genes. We show that not only UvrB and UvrD but also UvrA are essential for the viability of a Δ*polA* strain. Using defined mutations in *uvrA* and *uvrB*, we have analyzed the involvement of the different functional domains of the UvrA and UvrB proteins in the PolI-independent replication system. In contrast to UvrA,

* Corresponding author. Mailing address: Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. Phone: (31) 715274773. Fax: (31)715274537 E-mail: N.Goosen@chem.leidenuniv.nl.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or marker(s)	Source or reference
E. coli strains		
JC7620	*recB21 recC22 sbcB12*	17
CS4985	Δ*uvrA*::Cm	4
CS5316	JC 7620 Δ*uvrB*::Cm	This paper
CS5388	JC 7620 Δ*uvrC*::Cm	This paper
SY124	AB1157 *uvrC*::Tn*10*	P. Strike
GE1752	Δ*uvrD*::Tc	7
S90C	Δ(*lac-pro*)	33
HP3430	S90C Δ(*bio-uvrB261*)	33
CS5531	S90C Δ*uvrD*::Tc	This paper
KMBL1001	No known mutations (F^- derivative of W1485)	R. Devoret
CS5428	KMBL1001 Δ*uvrA*::Cm	This paper
CS5429	KMBL1001 Δ*uvrB*::Cm	This paper
CS5430	KMBL1001 Δ*uvrC*::Cm	This paper
CS5431	KMBL1001 Δ*uvrD*::Tc	This paper
CS5432	KMBL1001 Δ*uvrC*::Cm Δ*uvrD*::Tc	This paper
CS5458	KMBL1001 *uvrC*::Tn*10* Δ*uvrA*::Cm	This paper
CS5530	KMBL1001 *uvrC*::Tn*10* Δ*uvrB*::Cm	This paper
CJ225	CM4722 Δ*pol*::Km, pCJ100 (F′ *polA*$^+$, Cm)	13
CJ231	CM4722 Δ*pol*::Km, pCJ102 (F′ 5′exo, Cm)	13
CJ233	CM4722 Δ*pol*::Km, pCJ103 (F′ Klenow, Cm)	13
Plasmids		
pUvr-A7	*uvrA*, Ap	1
pNP12	*uvrB*, Km	43
pNP50	*uvrB*, Km (pNP12 without *dnaA* box)	Our laboratory, unpublished
pCA32	*uvrC*, Tc	44
pBL12	*uvrC*, Ap	49
pSC101	Tc	3
pCL1920	Sm, ori pSC101	19
pUC4-KSAC	Ap, Km	Pharmacia
pJA87-1	*uvrA* with TAB linker in ATPase 1, Km	4
pJA87-6	*uvrA* with TAB linker in ATPase 2, Km	4
pJA87-C253S	*uvrA*(C253S), mutation in Zn finger 1, Km	46
pJA87-C763S	*uvrA*(C763S), mutation in Zn finger 2, Km	46
pNP77-B430	*uvrB430*, Km	26
pNP77-B(R544H)	*uvrB*(R544H), Km	26
pNP78-B(G509S)	*uvrB*(G509S), Km	26
pNP97	*uvrB630*, Km	27
pDR3274	*uvrC*(D466A), Tc	20
pCA161	*uvrC*(R42A), Ap	45
pWU1	Km Tc, ori pSC101	This paper
pNP120	*uvrA*, Tc, ori pSC101	This paper
pNP121	*uvrB*, Tc, ori pSC101	This paper
pNP122	*uvrC*, Tc, ori pSC101	This paper
pNP136	*uvrA*, Sm, ori pSC101 (Sm in pNP120)	This paper
pNP137	pNP136, *uvrA*(ATP1) (from pJA87-1), Sm	This paper
pNP138	pNP136, *uvrA*(ATP2) (from pJA87-6), Sm	This paper
pNP139	pNP136, *uvrA*(Zn1) (from pJA87-C253S), Sm	This paper
pNP140	pNP136, *uvrA*(Zn2) (from pJA87-C763S), Sm	This paper
pNP123	pNP121, *uvrB430* (from pNP77-B430), Tc	This paper
pNP129	pNP121, *uvrB630* (from pNP97), Tc	This paper
pNP132	pNP121, *uvrB*(R544H) [from pNP77-B(R544H)], Tc	This paper
pNP133	pNP121, *uvrB*(G509S) [from pNP77-B(G509S)], Tc	This paper
pCA154	pNP122, *uvrC*(D466A) (from pDR3274), Tc	This paper
pCA179	pNP122, *uvrC*(R42A) (from pCA161), Tc	This paper

UvrB and UvrD the presence of the UvrC protein appear to have a negative effect on the viability of the Δ*polA* cells, and we show that this negative effect is the result of its incision activity.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. For the construction of the chromosomal deletion of the *uvrB* gene, plasmid pNP12 was digested with *Eco*RI and *Stu*I, thereby deleting the *uvrB* gene. The remaining flanking DNA was treated with Klenow polymerase, and *Xba*I linkers were ligated to the blunt ends. Next, an *Xba*I fragment containing the chloramphenicol resistance (Cm^r) gene was ligated to the *Xba*I sites. The resulting plasmid was digested with *Pst*I and *Bam*HI, and the linear DNA containing the Cm^r gene was introduced into JC7620. Homologous recombination with the chromosome of this strain resulted in a Cm^r strain in which the *uvrB* gene has been deleted. In a similar way, a chromosomal deletion of the *uvrC* gene was constructed. Plasmid pCA32 was digested with *Bgl*II and ligated with a *Bgl*II-*Bam*HI fragment containing the Cm^r gene, thereby replacing the *uvrC* gene with the Cm^r gene. The resulting plasmid was linearized with *Pst*I and allowed to recombine with the chromosome of JC7620. The presence of the Δ*uvrB*::Cm and Δ*uvrC*::Cm mutations was confirmed both by Southern blotting and by PCR using oligonucleotides flanking the deleted and replaced region. The PCR product was analyzed on a gel for its size and restriction pattern (results not shown). Finally, it was

shown that the Δ*uvr* strains were UV sensitive and that this sensitivity could be complemented by the appropriate *uvr* gene located on a plasmid (not shown). The Δ*uvrA*::Cm, Δ*uvrB*::Cm, Δ*uvrC*::Cm, and Δ*uvrD*::Tc mutations were transferred to KMBL1001 by P1 transduction, and transductants were selected on Luria-Bertani (LB) medium containing 2.5 mM sodium citrate and the appropriate antibiotic. The presence of the mutation was verified by testing for UV sensitivity. For the construction of double mutants, the Δ*uvrA*::Cm and Δ*uvrB*::Cm mutations were transferred to KMBL1001 *uvrC*::Tn10, and the Δ*uvrD*::Tc mutation was transferred to KMBL1001 Δ*uvrC*::Cm.

Media. LB medium and plates were made as described previously (23). Minimal medium contained, per liter, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid, 10 g of K_2HPO_4, 3.5 g of $Na(NH_4)HPO_4 \cdot 4H_2O$, 4 g of glucose, and 10 mg of thiamine. For derivatives of strain C90S, the minimal medium was supplemented with proline (50 μg/ml) and biotin (0.5 μg/ml). Strains containing the F plasmids with the *polA* gene or fragments thereof were plated on medium with 120 μg/ml IPTG (isopropyl-β-D-thiogalactopyranoside) to allow optimal expression of the (truncated) PolI proteins. Antibiotics were used in the following concentrations: chloramphenicol, 12.5 μg/ml; tetracycline, 25 μg/ml; streptomycin, 25 μg/ml; and kanamycin, 25 μg/ml.

Transduction of the Δ*polA* mutation. Strains were grown in LB medium containing the appropriate antibiotics at 37°C to an optical density at 600 nm of 0.4. Log-phase cells were spun down and resuspended in LB medium containing 2.5 mM $CaCl_2$ and 5 mM $MgSO_4$ at a concentration of 10^9 cells/ml. To 1 ml of cells, 10 μl of a P1 lysate from CJ225 was added (resulting in 0.1 phage per bacterial cell). The phage were allowed to infect the cells for 20 min at 37°C. The cells were centrifuged and resuspended in 2 ml of minimal medium, after which they were incubated for another hour at 37°C. The cells were washed with minimal medium, and finally 100 μl of the cells was plated on minimal medium plates or LB plates containing 2.5 mM sodium citrate and supplemented with the appropriate antibiotics.

Plasmids. The plasmids used in this study are listed in Table 1. Plasmid pWU1 was used to clone the *uvr* genes on a vector containing a pSC101 origin (which does not require PolI for initiation of replication), and was constructed by insertion of the *Eco*RI fragment containing the kanamycin resistance (Km^r) gene from pUC4-KSAC (Pharmacia) into the *Eco*RI site of pSC101. Plasmid pNP120 was constructed by digestion of pUvr-A7 with *Hin*dIII and filling in of the ends with Klenow polymerase. Next, after digestion with *Pst*I, the *Pst*I-blunt-end fragment containing the *uvrA* gene was inserted into the *Pst*I and *Pvu*II sites of pWU1. Plasmid pNP121 was constructed by inserting the *Pst*I-*Stu*I fragment from pNP50 containing the *uvrB* gene into the *Pst*I and *Pvu*II sites of pWU1. Plasmid pNP122 was constructed by inserting the *Pvu*II-*Pst*I fragment from pCA32 containing the *uvrC* gene into the *Pvu*II and *Pst*I sites of pWU1. Plasmids expressing mutant *uvr* genes were constructed by restriction fragment exchange between previously isolated *uvrA*, *uvrB*, and *uvrC* mutants and plasmids pNP120, pNP121, and pNP122. Since overproduction of UvrA turned out to be lethal in a Δ*polA* strain, the different *uvrA* plasmids had to be introduced into the double mutant KMBL1001 Δ*uvrA*::Cm *uvrC*::Tn10. To be able to do this, the tetracycline resistance (Tc^r) genes of the pNP120 derivatives had to be replaced by another resistance gene. This was done by insertion of a *Hin*dIII fragment containing the streptomycin resistance (Sm^r) gene into the *Hin*dIII sites located in the Tc^r genes of the pNP120 derivatives.

RESULTS

UvrA, UvrB, and UvrD proteins are essential in a Δ*polA* background. In the past, the viability of *polA* mutants has been tested with strains carrying a point mutation in the *polA* gene, which still might produce a partially functional PolI enzyme. To test the requirement for the different Uvr proteins in the complete absence of the PolI enzyme, we made use of a Δ*polA*::Km mutation, in which the *polA* gene has been removed and replaced by a kanamycin resistance gene. First we constructed isogenic strains in which the *uvrA*, *uvrB*, or *uvrC* gene has been deleted and replaced by a chloramphenicol resistance gene and the *uvrD* gene has been replaced by a tetracycline resistance gene. These strains were infected with a P1 lysate that was made on the Δ*polA*::Km strain, and transductants were selected on minimal medium with kanamycin at 30 and 37°C. The wild-type strain KMBL1001 yielded Δ*polA* transductants at 30°C but not at 37°C (Fig. 1). Apparently, in our genetic background the Δ*polA* strain is viable on minimal medium at low temperature only. The Δ*uvrA*, Δ*uvrB*, and Δ*uvrD* strains did not give rise to kanamycin-resistant transductants (Fig. 1 and Table 2), even after prolonged incubation at 30°C. Surprisingly, not only did the Δ*uvrC* strain produce Δ*polA* transductants at 30°C, but these colonies were larger than the transductants of the isogenic wild-type strain (Fig. 1A), suggesting that the presence of the UvrC protein has a negative effect on the growth of a Δ*polA* strain. This effect was even more clear at 37°C, where the wild-type strain yielded no

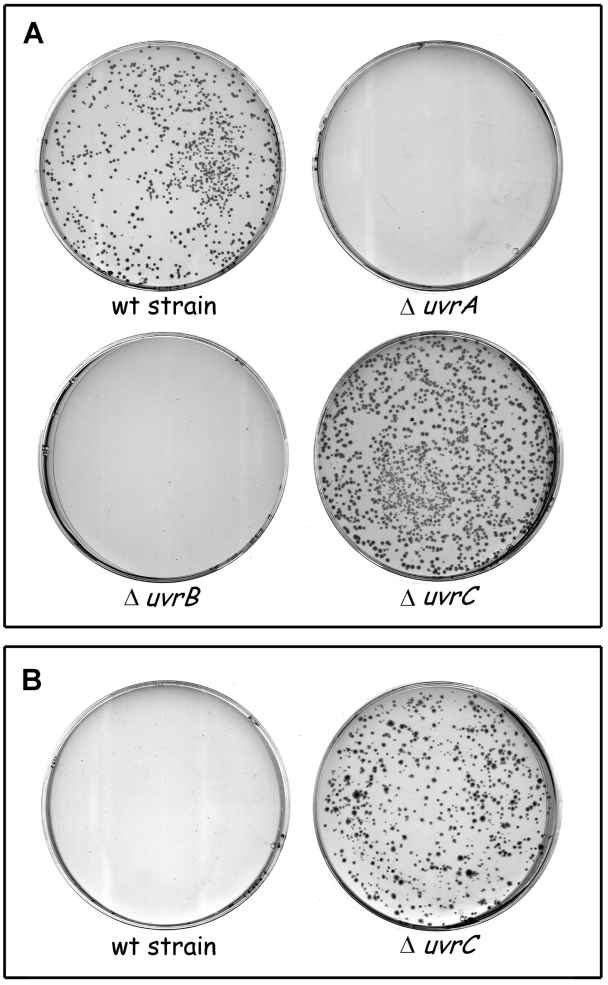

FIG. 1. Transduction of the Δ*polA*::Km mutation into different genetic backgrounds. After infection with a P1 lysate grown on CJ225, the cells were plated on minimal medium containing kanamycin and the plates were incubated for 60 h at 30°C (A) or 37°C (B). Shown are the results with KMBL1001 (wild-type [wt] strain) and the isogenic Δ*uvrA*, Δ*uvrB*, and Δ*uvrC* derivatives. The Δ*uvrD* mutant strain gave results identical to those with the Δ*uvrA* and Δ*uvrB* mutant strains (not shown).

TABLE 2. Transduction of the Δ*pol*::Km mutation to Δ*uvr* strains

Strain	Colonies formed on minimal medium at[a]:	
	30°C	37°C
KMBL1001	+	−
KMBL1001 Δ*uvrA*	−	−
KMBL1001 Δ*uvrB*	−	−
KMBL1001 Δ*uvrC*	++	++
KMBL1001 Δ*uvrD*	−	−
KMBL1001 Δ*uvrA uvrC*::Tn*10*	−	−
KMBL1001 Δ*uvrB uvrC*::Tn*10*	−	−
KMBL1001 Δ*uvrC* Δ*uvrD*	−	−

[a] −, no colonies; +, small colonies; ++, larger colonies.

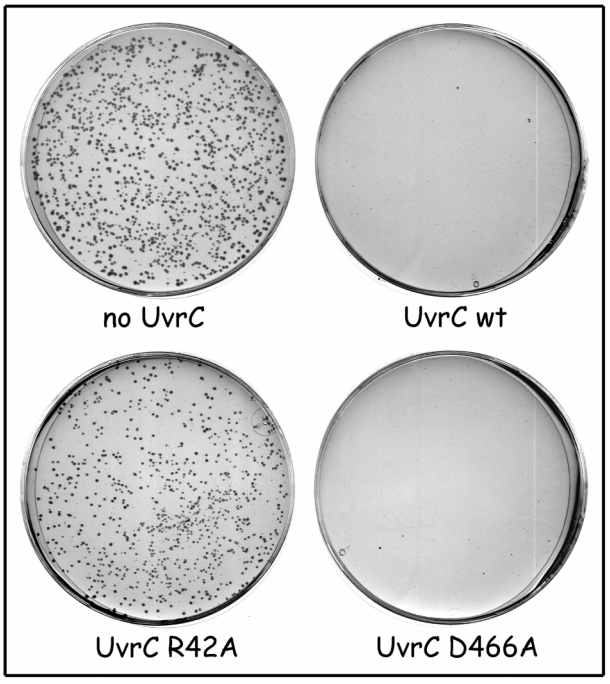

FIG. 2. Effect of *uvrC* mutations on transduction of the Δ*polA*::Km mutation. KMBL1001 Δ*uvrC* with pSC101 (no UvrC), pNP122 (wild-type [wt] UvrC), pCA154 (R42A), and pCA179 (D466A) was infected with a P1 lysate grown on CJ225. After infection, the cells were plated on minimal medium with kanamycin and the plates were incubated at 30°C for 60 h.

TABLE 3. Effect of *uvr* mutation on the Δ*pol*::Km transduction

Mutation, strain, and plasmid	Colonies formed on minimal medium at[a]:	
	30°C	37°C
uvrC mutations		
KMBL1001 + pNP122 (wt[b] UvrC)	−	−
KMBL1001 Δ*uvrC* + pSC101 (no UvrC)	++	++
KMBL1001 Δ*uvrC* + pNP122 (wt UvrC)	−	−
KMBL1001 Δ*uvrC* + pCA154 [UvrC(D466A)]	−	−
KMBL1001 Δ*uvrC* + pCA179 [UvrC(R42A)]	+	+
uvrB mutations		
KMBL1001 + pNP121 (wt UvrB)	+	−
KMBL1001 Δ*uvrB* + pSC101 (no UvrB)	−	−
KMBL1001 Δ*uvrB* + pNP121 (wt UvrB)	+	−
KMBL1001 Δ*uvrB* + pNP129 (UvrB630)	++	++
KMBL1001 Δ*uvrB* + pNP123 (UvrB430)	−	−
KMBL1001 Δ*uvrB* + pNP133 [UvrB(G509S)]	−	−
KMBL1001 Δ*uvrB* + pNP132 [UvrB(R544H)]	−	−
uvrA mutations		
KMBL1001 + pNP120 (wt UvrA)	−	−
KMBL1001 Δ*uvrA* + pNP120 (wt UvrA)	−	−
KMBL1001 Δ*uvrC* Δ*uvrA* + pCL1920 (no UvrA)	−	−
KMBL1001 Δ*uvrC* Δ*uvrA* + pNP136 (wt UvrA)	++	++
KMBL1001 Δ*uvrC* Δ*uvrA* + pNP137 [UvrA(ATP1)]	−	−
KMBL1001 Δ*uvrC* Δ*uvrA* + pNP138 [UvrA(ATP2)]	−	−
KMBL1001 Δ*uvrC* Δ*uvrA* + pNP139 [UvrA(Zn1)]	−	−
KMBL1001 Δ*uvrC* Δ*uvrA* + pNP140 [UvrA(Zn2)]	−	−

[a] −, no colonies; +, small colonies; ++, larger colonies.
[b] wt, wild type.

Δ*polA* transductants whereas the Δ*uvrC* strain did (Fig. 1B). Double mutants carrying Δ*uvrA uvrC*::Tn*10*, Δ*uvrB uvrC*::Tn*10*, or Δ*uvrD* Δ*uvrC* were also inviable in combination with the Δ*polA* mutation (Table 2), showing that the requirement for the UvrA, UvrB, and UvrD proteins cannot be ascribed to a squelching of the negative effect of UvrC by these proteins. To demonstrate that the inability to obtain Δ*polA* transductants in the Δ*uvrA*, Δ*uvrB*, and Δ*uvrD* strains was not due to a general transduction deficiency of these strains, we also did the reciprocal experiment by transferring the Δ*uvr* mutations to KMBL1001 Δ*polA*. As expected, the Δ*uvrC*::Cm mutation could be successfully introduced into the Δ*polA* strain, whereas no transductants of the Δ*uvrA*::Cm, Δ*uvrB*::Cm, or Δ*uvrD*::Tc mutations were found. Taken together, the results show that the UvrA, UvrB, and UvrD proteins are essential for a process that substitutes for PolI function, whereas the UvrC protein seems to interfere with this process.

The 3′ incision activity of UvrC interferes with the PolI-independent replication process. Plasmid pNP122 contains the wild-type *uvrC* gene inserted in a pSC101 derivative, a vector that does not require PolI for its replication initiation. Introduction of pNP122 into a wild-type strain (KMBL1001) or a Δ*uvrC* strain abolishes the generation of Δ*polA* transductants, even at 30°C (Fig. 2 and Table 3). Apparently, a higher level of UvrC totally blocks the PolI-independent replication pathway. The UvrC protein has two catalytic sites for cleavage of the DNA during NER. The N-terminal half of the protein contains the active site for incision of the DNA at the 3′ side of the damage (45), and the C-terminal half contains the site for incision at the 5′ side of the damage (20). Mutations in *uvrC* that selectively inactivate one of the catalytic sites have been constructed. Mutant UvrC(R42A) is no longer capable of inducing the 3′ incision (45), and in UvrC(D466A) the 5′ incision is impaired (20). Each of the catalytic-site mutations was introduced in pNP122, and the resulting plasmids were tested for their capacity to allow transduction of the Δ*polA* mutation (Fig. 2 and Table 3). Like wild-type UvrC, mutant UvrC (D466A) abolished the generation of Δ*polA* transductants. Expression of the UvrC(R42A) mutant, however, was not lethal in combination with the Δ*polA* mutation, although the colonies were somewhat smaller (Fig. 2; Table 3). Apparently it is the DNA incision activity by the 3′ catalytic site of UvrC that causes the lethality of the overproduction of the UvrC protein in a Δ*polA* strain.

The C-terminal domain of UvrB contains an important binding domain for UvrC (27, 40). A truncated UvrB protein lacking this domain (UvrB630) no longer stabilizes the binding of UvrC to the UvrB-DNA preincision complex during the repair reaction, and as a result the incision at the 3′ side of the damage is severely reduced (27). We have tested whether the same truncated UvrB protein does support DNA replication in the absence of a functional PolI enzyme. Table 3 shows that a Δ*uvrB* strain with a pSC101 plasmid that expresses either the wild-type UvrB protein (pNP121) or the truncated UvrB630 (pNP129) does allow the formation of Δ*polA* transductants at 30°C. This means that the UvrC-binding domain of UvrB is dispensable for its role in PolI-independent replication. The pNP129-containing strain even allowed formation of Δ*polA* transductants at 37°C, whereas the pNP121-containing strain did not (Table 3). This difference in growth is comparable to the difference found between the strain lacking the *uvrC* gene

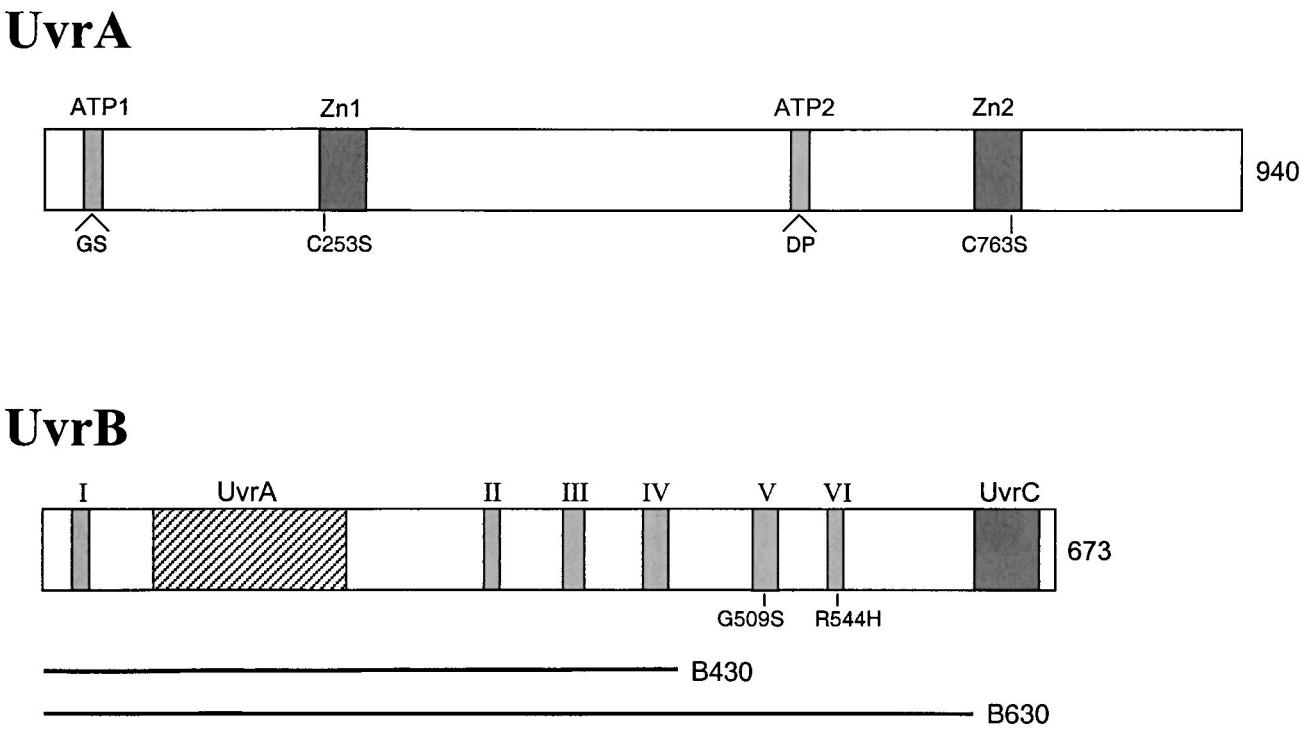

FIG. 3. Schematic representation of the UvrA and UvrB proteins. The UvrA protein contains two ATPase sites and two zinc-binding motifs, and the mutations in these domains used in this study are indicated. The UvrB protein contains UvrA- and UvrC-binding domains and six ATPase-helicase motifs (I to VI). The positions of the substitutions in motifs V and VI are shown. The lengths of the truncated UvrB proteins UvrB430 and UvrB630 are indicated.

and the wild-type strain (KMBL1001) (Fig. 1; Table 2), suggesting that in the absence of the UvrC-binding domain of UvrB, the UvrC protein no longer exerts its negative effect in the PolI-independent replication pathway.

We have also inserted the *uvrA* gene in a pSC101 vector (pNP120). Surprisingly, when this plasmid was introduced in either a wild-type strain (KMBL1001) or a Δ*uvrA* strain, no Δ*polA* transductants could be obtained (Table 3). Apparently, although the UvrA protein is essential, a higher level of the protein is unfavorable for *E. coli* lacking the PolI enzyme. The same plasmid, however, did allow deletion of the *polA* gene in a *uvrC*::Tn10 Δ*uvrA* double mutant (Table 3), indicating that a higher level of UvrA results in more deleterious incisions by the UvrC protein.

Role of functional domains of the UvrA and UvrB proteins in PolI-independent replication. From structural and mutational studies (see reference 8 for a review), different functional domains in UvrA and UvrB can be indicated (Fig. 3). The UvrA protein contains two ATP-binding sites and two zinc-binding sites. The UvrB protein contains six so-called helicase motifs (I to VI) which are involved in ATPase and DNA-unwinding activity. In addition, an important UvrC-binding domain is located in the C-terminal part of the protein, and a putative UvrA-binding domain is present between motifs I and II.

As shown above, a UvrB protein lacking the C-binding domain still supports transduction of the Δ*polA* mutation. We have also tested UvrB with a larger deletion (UvrB430), which lacks 243 amino acids from the C terminus, including helicase motifs V and VI. This truncated protein has been shown to form damage-specific $UvrA_2B$-DNA complexes, but it can no longer form the UvrB-DNA preincision complex (our laboratory, unpublished data). Expression of the truncated UvrB protein in a Δ*uvrB* background does not allow formation of Δ*polA* transductants (Table 3), suggesting that the helicase motifs are important for the activity of UvrB in PolI-independent replication. This was further tested with two UvrB mutant proteins having an amino acid substitution in helicase motif V (G509S) or VI (R544H). Both mutant proteins have been shown to bind UvrA and to bind to a damage site in the $UvrA_2B$ complex, but they are disturbed in ATPase and DNA-unwinding activity and as a result can no longer form the UvrB-DNA preincision complex (26). Like in the complete absence of the UvrB protein, no Δ*polA* transductants were found upon expression of the point mutants, suggesting that the action that is required by the Uvr proteins for PolI-independent replication involves ATPase-induced conformational changes of the UvrB protein similar to those for formation of the preincision complex.

The two ATPase sites of UvrA have shown to be essential for the NER reaction (4, 41). The N-terminal ATPase site (ATP1) seems to be important for dimerization of UvrA (22, 30), and the C-terminal site (ATP2) is thought to be involved in the dissociation of UvrA from undamaged DNA (41). Mutant UvrA proteins with two amino acid insertions within ATP1 or ATP2 have been constructed and purified in the past (4). Both proteins displayed 50% of the ATPase activity of wild-type UvrA, and they were both defective in NER. We have inserted the *uvrA* genes with the corresponding mutations in pSC101 and introduced these plasmids in the double *uvrC*::Tn10 Δ*uvrA* double mutant. Table 3 shows that neither of the mutant UvrA proteins supported the PolI-independent replication, indicating that the two ATP sites are also important for this process.

Amino acid substitutions in the two zinc-binding domains have also been constructed. Substitution C763F (47) or C763S

TABLE 4. Effect of the PolI domains on Δ*pol*::Km transduction

Strain and plasmid	Colonies formed on[a]:			
	LB medium		Minimal medium	
	30°C	37°C	30°C	37°C
S90C	−	−	+	+
S90C + FPolA	++	++	++	++
S90C + FKlenow	++	++	++	++
S90C + FExo	+	+	++	++
S90C Δ*uvrB261*	−	−	−	−
S90C Δ*uvrB261* + FPolA	++	++	++	++
S90C Δ*uvrB261* + FKlenow	−	−	++	++
S90C Δ*uvrB261* + FExo	−	−	−	−
S90C Δ*uvrD*	−	−	−	−
S90C Δ*uvrD* + FPolA	++	++	++	++
S90C Δ*uvrD* + FKlenow	−	−	−	−
S90C Δ*uvrD* + FExo	−	−	−	−

[a] −, no colonies; +, small colonies; ++, larger colonies.

(46) in the C-terminal zinc-binding motif (Zn2), resulted in a UvrA protein that is completely defective in NER. In contrast, substitution C253S or C256S in the N-terminal zinc-binding domain (Zn1), although resulting in the loss of zinc coordination, did not lead to any defect in the repair reaction (46). We have tested the C763S and C253S mutations for their effect on the PolI-independent replication. In contrast to the differential effect on NER, both mutations now prevented the transduction of the Δ*polA* mutation (Table 3), which means that both zinc-binding motifs are essential for the UvrA-mediated replication pathway. To test whether the C253S protein was properly expressed in the *uvrC*::Tn10 Δ*uvrA* strain, we also introduced pBL12, expressing the wild-type UvrC protein in the cells. The resulting strain containing both the *uvrC* and the *uvrA*(C253S) plasmids appeared to be UV resistant, whereas the same strain with only the *uvrC* or *uvrA*(C253S) plasmid was UV sensitive. This confirms not only that UvrA(C253S) is expressed but also that the mutant protein is indeed active in NER. The fact that the N-terminal zinc-binding domain is essential for PolI-independent replication but not for repair suggests that this domain has specifically evolved in UvrA for its function in replication.

Importance of the polymerase and exonuclease activities of PolI. The PolI enzyme has two important enzymatic activities for the processing of the Okazaki fragments that are generated on the lagging strand during DNA replication. The polymerase activity (together with the 3′-5′ proofreading activity located in the Klenow fragment) extends the 3′ end of an Okazaki fragment, and the 5′-3′ exonuclease activity removes the RNA primers. *E. coli* strains with a deletion in the chromosomal *polA* gene are viable on minimal medium only, but expression of either the 5′-3′ exonuclease or the Klenow fragment portion of the enzyme is sufficient to allow growth on rich medium (13). This means that there must be alternative pathways for each of the two functions of PolI. To test which of these pathways involve the Uvr(A)B and UvrD enzymes we introduced F′ plasmids expressing either the complete PolI enzyme or only one of the functional domains in a strain lacking the *uvrB* (HP3430) or *uvrD* (CS5531) gene and repeated the Δ*polA* transduction experiments.

As expected, the wild-type strain S90C gave rise to Δ*polA* transductants only on minimal medium and not on LB medium (Table 4). In contrast to the case for KMBL1001, transductants were also found at 37°C, confirming that viability of the Δ*polA* transductants is strongly dependent on the genetic background. In the presence of the F plasmid expressing either the complete PolI enzyme or the functional fragments, transductants were found on both LB and minimal media. The colonies on rich medium with the FExo plasmid were smaller than those observed with the other two plasmids. Possibly the expression or stability of the exonuclease part of the protein in our genetic background is somewhat reduced. The strain lacking the *uvrB* gene gave transductants on LB only in the presence of the complete PolI enzyme and not with either of the fragments. On minimal medium, however, normal transductants were found with both FPolA and FKlenow but not with FExo (Table 4). Apparently, under slow-growth conditions, UvrB is needed only when the polymerase activity is missing, but under fast-growth conditions, it is required to substitute for both the polymerase and the exonuclease activities.

The isogenic strains lacking the *uvrD* gene gave a different result (Table 4). Now transductants could be found on both types of media only with the complete PolI enzyme and not with either of the truncations. This shows that the UvrD protein is essential for both alternative activities that replace polymerase and exonuclease activities.

DISCUSSION

We have shown that the UvrA, UvrB, and UvrD (helicase II) proteins are essential for the viability of *E. coli* cells lacking the *polA* gene, indicating that they play a crucial role in alternative pathways that substitute for the polymerase and exonuclease functions of the PolI enzyme. The UvrD protein appears to be essential for both substituting activities, since expression of the polymerase or exonuclease activity alone is not sufficient for survival of a Δ*uvrD* strain. Being a very efficient DNA helicase, the most likely function of UvrD is to unwind the DNA-RNA hybrids in the Okazaki fragments. Not only would this unwinding facilitate the removal of the RNA primers by exonucleases or RNAses, but extension of the unwinding into the DNA-DNA hybrid would also result in larger gaps, which might facilitate the entry of an alternative polymerase like PolIII. The UvrD protein has been shown to be able to unwind DNA from a nick (34), but the initiation of this reaction requires very high UvrD concentrations (35). From the nick UvrD can unwind the DNA in both directions (34). The UvrD protein has a helicase activity with a 3′-to-5′ polarity (21), which means that the protein can be loaded on the nicked DNA in two different ways, either on the nicked strand or on the continuous strand. With respect to the processing of the lagging strand, loading of UvrD on the continuous strand would result in displacement of the RNA primer (Fig. 4A), which would account for the function of UvrD in the alternative replication pathway as described above. Loading of UvrD on the opposite strand, however, would displace the DNA end that needs to be elongated (Fig. 4A). Such a displacement is expected to interfere with the action of any alternative polymerase. It is therefore conceivable that in *E. coli* there is a mechanism to load UvrD onto the appropriate strand, so that it unwinds in the proper direction.

We would like to propose that the UvrA and UvrB proteins orient the UvrD protein, by binding at or near the entry site of the helicase. Several arguments for such a model can be given. (i) In NER the UvrD protein removes the damage-containing oligonucleotide that results from the two incisions made in the UvrBC-DNA complex. For this action the UvrD protein also needs to initiate unwinding from a nick. From each of the two nicks that are present, UvrD can potentially start to unwind the DNA in two directions: towards the DNA damage, thereby releasing the oligonucleotide, or in the opposite direction,

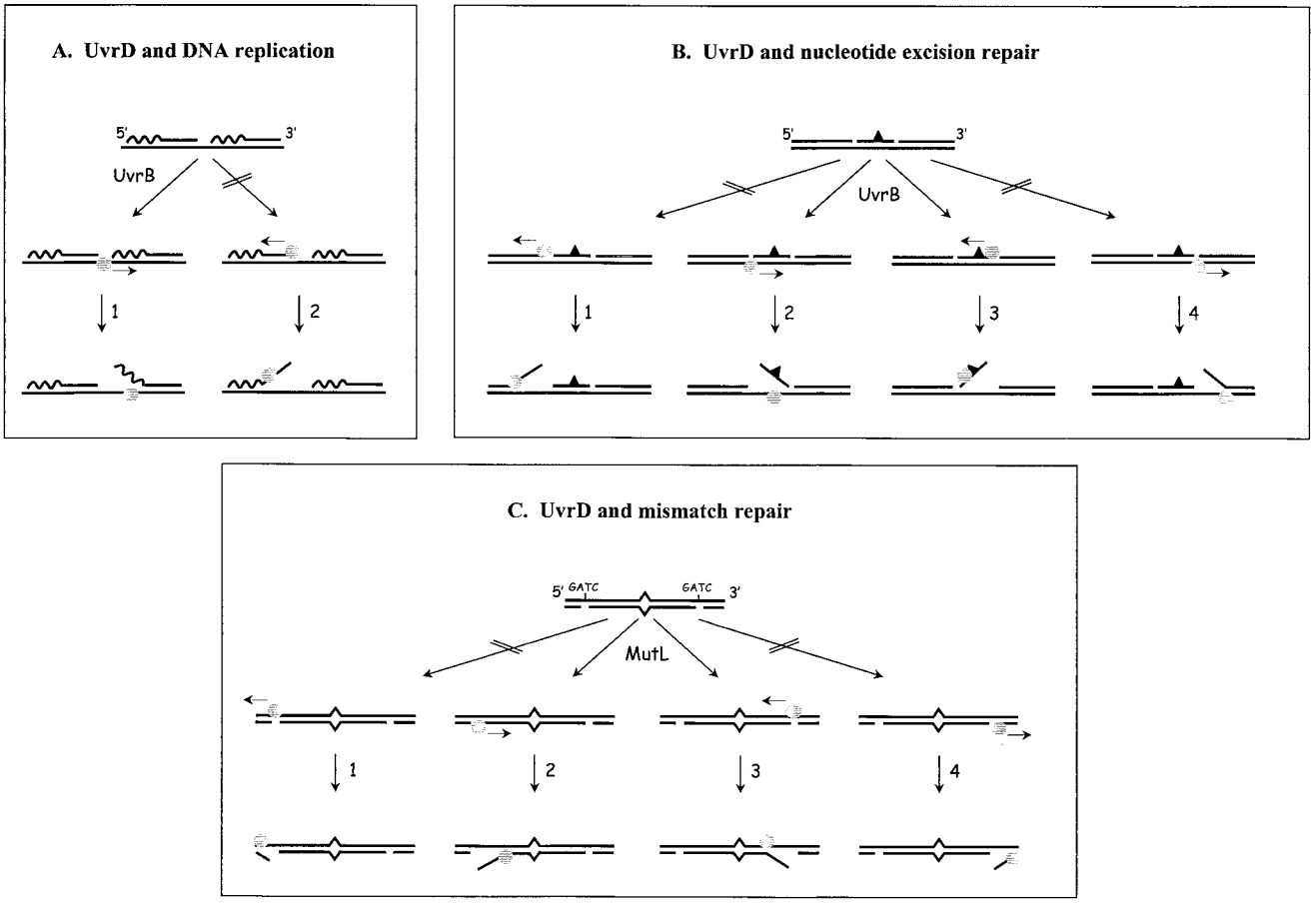

FIG. 4. Models for protein-mediated orientation of the UvrD helicase (circles). (A) DNA replication. The lagging strand is shown. The wavy line represents the RNA. Loading of UvrD onto the continuous strand will unwind the RNA-DNA hybrid (1) and loading on the opposite strand will displace the 3′ end that needs to be elongated by DNA polymerase (2). An interaction of UvrD with UvrB orients UvrD in the proper direction (1). (B) Nucleotide excision repair. DNA with a damage (triangle) after incision by UvrC is shown. Loading of UvrD on the 3′ nick (3) or opposite the 5′ nick (2) will lead to removal of the damaged oligonucleotide. Loading of UvrD onto the 5′ nick (1) or opposite the 3′ nick (4) will lead to unwinding in the opposite direction. An interaction between UvrB and UvrD directs UvrD (2 or 3). (C) Mismatch repair. DNA with a mismatch and two nicked GATC sites is shown. For unwinding of the DNA by UvrD in the direction of the mismatch, the helicase needs to bind to the nicked strand when the GATC is located at the 5′ side of the mismatch (2) or to the continuous strand when the GATC is at the 3′ side (3). The other orientations will direct the helicase away from the mismatch (1 and 4). The interaction of UvrD with MutL orients the protein in the proper direction (2 and 3).

which will not result in oligonucleotide removal (Fig. 4B). Possibly the UvrBC proteins shield one of the nicks, but efficient release of the damage-containing oligonucleotide still requires that the UvrD protein is properly oriented on the other nick. A possible physical interaction between UvrB and UvrD not only would account for such a directed DNA binding, but simultaneously it could stimulate the initiation of the helicase activity, which on a nicked DNA substrate in the absence of other proteins is very slow (35). The fact that the homologous Rep helicase can not substitute for UvrD in NER (12) supports the proposed specific interaction between UvrB and UvrD. For the PolI-independent replication, the same interaction between UvrB and UvrD on the lagging strand might stimulate and direct UvrD towards the unwinding of the DNA-RNA hybrid. (ii) In addition to its role in NER, UvrD is also an important factor in mismatch repair. In this process MutS and MutL bind to a mismatched base, and the MutH protein generates a nick at a nearby GATC sequence (for a review, see reference 24). UvrD, together with one of several nucleases, will remove the mismatch-containing strand, starting at the nicked GATC site. Since this nick can be located either 3′ or 5′ to the mismatch, the UvrD protein needs to be loaded onto the nicked or continuous strand, depending on the location of the GATC site (Fig. 4C). It has been shown that MutS and MutL not only activate the unwinding by UvrD but also bias the unwinding in the direction of the mismatch (6). A physical interaction between MutL and UvrD has been shown (10), and it is likely that this interaction serves to load the helicase on the proper strand. Such a MutL-mediated activation and orientation of UvrD is very similar to our proposed model for the UvrB-mediated activation and orientation of this helicase.

Our proposed model implies that UvrB specifically binds to the lagging strand at or near the junctions between the Okazaki fragments. The requirement for UvrA indicates that such a binding should be mediated via the same $UvrA_2B$ complex that in NER recognizes structural changes in the DNA as a result of a DNA damage. In the lagging strand, however, a different kind of DNA structure has to be recognized, since it is very unlikely that DNA damages play a role in the PolI-independent replication pathway. Possibly the $UvrA_2B$ complex is capable of recognizing the non-B conformation of RNA-DNA hybrids. The presence of nicks or small gaps might also be important for the recognition. The $UvrA_2B$-DNA com-

plex formed in the lagging strand could subsequently interact with UvrD, thereby directing its helicase activity. Alternatively, in analogy to the sequential reactions during NER, the UvrA protein could first dissociate from the complex and then UvrD could bind to the resulting UvrB-DNA complex. The requirements for functional ATPase sites in UvrA and ATPase-helicase motifs in UvrB indicate that for the proposed binding of $UvrA_2B$ or UvrB in the lagging strand, similar ATPase-induced conformational changes are required, which during NER lead to formation of the preincision complex.

Our finding that the N-terminal zinc-binding motif of UvrA is essential for the alternative replication pathway but not for NER suggests that this domain has specifically evolved for the role of $UvrA_2B$ in replication. In many cases zinc-binding domains have been found to participate in DNA interactions (2). Possibly the N-terminal zinc-binding domain is involved in the proposed specific binding of $UvrA_2B$ in the lagging strand, as discussed above. The C-terminal zinc-binding domain has been shown to be important for the binding of UvrA to damaged and nondamaged DNA (47). The fact that a mutation in the C-terminal zinc-binding domain of UvrA obstructs both DNA repair and PolI-independent replication suggests that this DNA-binding domain is important for both processes. On the other hand it cannot be excluded that the particular mutation not only affects the structure not only of the zinc-binding motif but also of other domains of the protein, thereby indirectly influencing the activity of UvrA in the two processes.

Unlike UvrD, the UvrB protein seems less important for the cells when the Klenow fragment of PolI is present. Δ*polA* transductants of a *uvrB* strain expressing this polymerase domain can be found on minimal medium. If indeed the role of UvrB is to orient the UvrD protein, a possible explanation for the effect of the Klenow fragment could be that binding of the polymerase domain to the 3′ end of an Okazaki fragment prevents DNA unwinding from this 3′ end. As a consequence, the Klenow fragment itself would direct the helicase towards unwinding of the DNA-RNA hybrid. At higher growth rates (i.e., on LB medium) the amount of Klenow fragment probably becomes limiting, and therefore under these conditions, the UvrB protein is essential again.

A striking observation in this study is the fact that the UvrC protein has a strong negative effect on the alternative replication pathway. In the absence of UvrC, Δ*polA* transductants grow much better, and overproduction of UvrC is lethal in a Δ*polA* strain. UvrC contains two catalytic sites for incision of damaged DNA. The N-terminal part of the protein contains the active site for incision at the 3′ side of the damage, and the active site for 5′ incision is located in the C-terminal part. DNA incision by the N-terminal active site appeared to be mainly responsible for the negative effect of UvrC in a Δ*polA* strain. A UvrB mutant lacking the UvrC-binding domain could counteract the negative effect of the presence of UvrC. This strongly suggests that UvrC induces strand incisions by binding to UvrB at a specific DNA target. In what way could such single-strand incisions influence the viability of a Δ*polA* strain? As discussed above, UvrB or $UvrA_2B$ might bind specifically at or near the junction of an Okazaki fragment. A subsequent binding of UvrC, followed by a strand incision in the Okazaki fragment, would not obviously be deleterious. On the contrary, such an incision is expected to be advantageous, since it would help to remove the RNA primer. If, however, the orientation of the $UvrA_2B$-DNA or UvrB-DNA complex in the lagging strand would lead to UvrC incision in the opposite (template) strand, a double-strand break would be generated, which, if not repaired, is lethal for the cell.

Expression of the UvrC mutant with a base substitution in the 3′ catalytic site (R42A) in a Δ*polA* strain was not lethal, but the resulting colonies were clearly smaller than those of a Δ*polA* strain without any UvrC. This could mean that the R42A mutant is somewhat leaky and that a limited number of incisions are still induced. Alternatively the R42A mutant could interfere with the PolI-independent replication just by binding to UvrB without inducing incisions, thereby hindering the proposed interaction of UvrD with UvrB.

Overexpression of the UvrA protein in a Δ*polA* strain appeared to be lethal as well, whereas overexpression of the same protein in a Δ*polA* Δ*uvrC* double mutant is not. Apparently a higher level of UvrA leads to more deleterious incisions by UvrC. A higher level of UvrB protein does not show this effect, suggesting that the UvrA concentration in the cell is limiting. Increasing the level of UvrA by the introduction of a multicopy plasmid will result in the formation of more $UvrA_2B$ complexes and subsequently the binding of more of these complexes to the proposed sites in the lagging strand. As a result, more targets for incision by UvrC are formed, finally leading to the death of the cells.

The viability of a Δ*polA* strain in the presence of UvrA, UvrB, and UvrC strongly depends on the genetic background of the strain. Strain KMBL1001 (which does not have any known chromosomal mutations) with the Δ*polA* mutation could survive on minimal medium only at 30°C, whereas strain S90C with the same mutation was viable on minimal medium at 30 and 37°C. The influence of the strain background on the severity of the Δ*polA* mutation has been described before (14). A particular *E. coli* strain (SY203) carrying a *polA* deletion was shown to be nonviable on minimal medium at 37°C, although the authors did not report whether the strain could survive at lower temperatures. In this case also, the inviability could not be ascribed to a specific chromosomal mutation (14).

Additional deletion of the *uvrC* gene allowed KMBL1001 to grow at 37°C as well. Possibly the effect of the strain backgrounds is related to differences in *uvrC* expression in the different strains. A higher level of UvrC will lead to more harmful incisions, which need to be repaired for survival of the cell. At lower growth rates the cell has more time for repair, and therefore strains that contain more UvrC protein can survive at lower temperatures but not at higher temperatures.

The results in this paper show that the 3′ catalytic site of UvrC induces incisions in nondamaged DNA in vivo, causing a negative effect when the cell is dependent on the PolI-independent replication pathway. It is not clear from our experiments whether the same incision activity on nondamaged DNA has a function in other processes in the cell. DNA incision by a complex of UvrB and UvrC in the absence of DNA damage has also been shown in vitro (9, 28, 48). This incision, however, which takes place seven nucleotides from a single strand-double strand junction, is induced by the catalytic site that on damaged DNA makes the 5′ nick and is independent of UvrA (28). For this UvrC-induced incision also, a clear in vivo function has not yet been found. The determination of potential functions of the two types of damage-independent incision awaits a further characterization of substrates that are incised by Uvr(A)BC.

In summary, we have shown that UvrA, UvrB, and UvrD, together with other, as-yet-unidentified proteins like polymerase(s) and exonuclease(s), can take over the function of the PolI enzyme in DNA replication. The existence of such backup systems can be very important for the cell, since it provides flexibility, both on short- and long-term scales. On a short-term scale, backup systems can ensure the survival of cells in which, as a result of internal or external variations, the level of a specific protein drops below a critical level. On a

long-term scale, backup systems allow proteins to evolve into having other functions, even if this results in the eventual loss of their original functions.

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