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Roles of PriA Protein and Double-Strand DNA Break Repair Functions in UV-Induced Restriction Alleviation in *Escherichia coli*

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ABSTRACT

It has been widely considered that DNA modification protects the chromosome of bacteria *E. coli* K-12 against their own restriction–modification systems. Chromosomal DNA is protected from degradation by methylation of target sequences. However, when unmethylated target sequences are generated in the host chromosome, the endonuclease activity of the *Eco*KI restriction-modification enzyme is inactivated by the ClpXP protease and DNA is protected. This process is known as restriction alleviation (RA) and it can be induced by UV irradiation (UV-induced RA). It has been proposed that chromosomal unmethylated target sequences, a signal for the cell to protect its own DNA, can be generated by homologous recombination during the repair of damaged DNA. In this study, we wanted to further investigate the genetic requirements for recombination proteins involved in the generation of unmethylated target sequences. For this purpose, we monitored the alleviation of *Eco*KI restriction by measuring the survival of unmodified λ in UV-irradiated cells. Our genetic analysis showed that UV-induced RA is dependent on the excision repair protein UvrA, the RecA-loading activity of the RecBCD enzyme, and the primosome assembly activity of the PriA helicase and is partially dependent on RecFOR proteins. On the basis of our results, we propose that unmethylated target sequences are generated at the D-loop by the strand exchange of two hemi-methylated duplex DNAs and subsequent initiation of DNA replication.

THE restriction and modification (R–M) system of bacteria was widely considered a defense mechanism of the host against foreign genetic material (Arber 1971). According to this theory, host DNA is protected from the invasion of foreign DNA because it has a protective imprint (Murray 2000). Foreign DNA lacks this protective imprint and, therefore, is recognized and eventually cleaved. The imprint is a particular nucleotide sequence, known as the target sequence, which can be modified by methylation at specific adenine or cytosine residues. Such DNA is protected from cleavage by restriction enzymes while unmethylated DNA is not. If the target sequence is hemi-methylated after DNA replication, DNA is methylated before the next round of replication (Murray 2000).

Interestingly, it was recently found that modification of chromosomal DNA is not essential for discriminating self against foreign DNA (Makovets *et al.* 1999). Under a variety of conditions, unmethylated target sequences can be generated in the chromosomal DNA and, surprisingly, such DNA is not recognized as foreign DNA. The reason for this is an additional protection system in which the protease ClpXP prevents the cleavage of unmethylated chromosomal DNA. Thus, in the absence of ClpXP protease, the unmethylated bacterial chromosome is attacked by *Eco*KI and restricted. This protection by ClpXP protease is preferentially biased toward the bacterial chromosome and not toward incoming unmethylated phages (Doronina and Murray 2001).

The *Eco*KI enzyme, a member of the type IA R–M system, is encoded by three genes: *hsdR*, *hsdM*, and *hsdS*. It consists of two HsdR subunits, two HsdM subunits, and one HsdS subunit ($R_2M_2S_1$). The holoenzyme modifies hemi-methylated DNA via its methyltransferase activity, restricts unmethylated DNA, and leaves methylated DNA untouched. A smaller complex (M_2S_1) also exists, but it has only the methyltransferase activity. The HsdS subunit is responsible for target-site recognition, which is why both complexes and both activities respond to the same nucleotide sequence (Murray 2000). Restriction begins when *Eco*KI binds to an unmodified target sequence in the presence of *S*-adenosylmethionine and ATP. The target sequence is bipartite and asymmetric. In an ATP-dependent process, the enzyme moves the surrounding DNA toward itself while remaining bound to the target site. DNA cutting occurs far from the recognition site and is triggered when two *Eco*KI complexes collide (Studier and Bandyopadhyay 1988).

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The protease ClpXP acts on the *Eco*KI complex which has started restriction and it degrades the HsdR subunit. Degradation prevents further translocation and endonuclease activity of the *Eco*KI enzyme (Makovets *et al.* 1999; Doronina and Murray 2001). Specific degradation of the HsdR subunit also occurs when *Escherichia coli* acquires a new type I R–M system (Makovets *et al.* 1998). This means that the presence of unmodified target sequences on the chromosome makes the cell phenotypically restriction deficient, an effect known as restriction alleviation (RA).

Cells treated with agents that damage DNA, such as UV light, nalidixic acid, or 2-aminopurine, have all been shown to induce restriction alleviation (Bertani and Weigle 1953; Day 1977; Thoms and Wackernagel 1982, 1984; Hiom and Sedgwick 1992; Kelleher and Raleigh 1994). In addition, it was shown that some mutants like *dam*, *topA*, *mutD*, *rnhA*, and *recG* have permanently (constitutively) alleviated *Eco*KI restriction (Efimova *et al.* 1988; Kelleher and Raleigh 1994; Makovets *et al.* 1999; Blakely and Murray 2006). Since restriction alleviation was shown to be dependent on the ClpXP protease in all these cases (Makovets *et al.* 1999; Blakely and Murray 2006), it was proposed that DNA damage leads to generation of unmodified target sequences by different mechanisms. One is recombination dependent and occurs when the progress of DNA replication is blocked due to lesions or breaks in the DNA template. Regions of unmethylated DNA could be generated by recombining two hemimethylated DNA strands (Murray 2000). Alternatively, due to the mutagenic activity of 2-aminopurine, new unmodified target sequences could be occasionally created (Makovets *et al.* 1999). It has been recently proposed that initiation of a new replication fork at R-loops could also generate unmodified DNA (Blakely and Murray 2006).

Restriction alleviation was first observed and genetically best characterized in *E. coli* K-12 cells irradiated with UV light (Bertani and Weigle 1953; Day 1977). UV-induced RA is specific for the type IA restriction system (*Eco*KI) and does not affect type II (*Eco*RI) or type III (*Eco*PI) R–M systems (Thoms and Wackernagel 1982). As mentioned above, UV-induced RA is *clpXP* dependent (Makovets *et al.* 1999) but it also requires functional *recA*, *recBC*, and *recF* genes (Day 1977; Thoms and Wackernagel 1982, 1984), *de novo* protein synthesis, and sufficient time for expression (Thoms and Wackernagel 1982). Since UV-induced RA could not be induced in *lexA* (*lex-1*) and *recA* mutants (Day 1977), it was proposed that alleviation of restriction due to UV irradiation is another of the SOS functions. This hypothesis was dismissed later when it was shown that genetic control of RA differs from the regulation of SOS functions (Thoms and Wackernagel 1984). The SOS response is required for restriction alleviation after UV irradiation, but strains that have constitutive SOS induction such as *recA730* or *lexA55* do not alleviate restriction constitutively and express RA only after UV irradiation (Thoms and Wackernagel 1984; Hiom and Sedgwick 1992). Thus, DNA damage in the host DNA is critical for UV-induced RA. RA also depends on the *umuCD* genes, which are involved in mutagenic DNA repair (Hiom *et al.* 1991; Hiom and Sedgwick 1992). The necessity for DNA damage, recombination genes, and SOS response for UV-induced RA supports the proposed idea that unmethylated DNA can be generated by DNA repair via homologous recombination or by creating new unmethylated target sequences through increased frequency of mutations (Makovets *et al.* 1999; Murray 2000).

From the data above it can be concluded that the restriction alleviation of unmodified λ-phage in UV-treated cells is due to inactivation of the restriction activity of the *Eco*KI enzyme if double-strand unmethylated target DNA is generated in the host chromosome. Therefore, we propose that restriction alleviation can be looked upon as an indirect measure of recombination and/or replication that generates unmethylated double-strand DNA (dsDNA). In other words, mutations in genes that block essential steps of DNA repair that are necessary for generation of unmethylated dsDNA would not alleviate restriction. The purpose of this study was to examine the effects of various *rec* mutations on the survival of unmodified λ-phage to elucidate the molecular mechanism of restriction alleviation after UV irradiation. Our data are consistent with a recent study, which demonstrated that RA is induced as a response to unmethylated target sequences produced by homologous recombination, R-loop formation, and DNA synthesis (Blakely and Murray 2006). Our results strongly suggest that recombination intermediates (D-loops), generated during the repair of dsDNA breaks (DSBs), and replication recovery after UV irradiation are critical for UV-induced RA.

MATERIALS AND METHODS

Bacterial strains and bacteriophages: The strain *E. coli* C600$r_{k^-}m_{k^-}$, which lacks the *Eco*KI restriction–modification system, was used for preparation of unmodified phage stock λ*virC* (designated as λ*vir*.0) (Salaj-Šmic *et al.* 1997). Phages λ*virC*, grown on the *E. coli* AB1157 ($r_{k^+}m_{k^+}$) strain, are modified and designated λ*vir*.K. The plating efficiency of unmodified phage λ was used to examine the activity of the *Eco*KI restriction system of the various bacterial strains presented in Table 1. λ-Phage stocks were prepared by the standard plate method. Mutant bacterial strains were made by P1*vir* transduction and selected for the appropriate antibiotic resistance (Miller 1992).

Media and growth conditions: High-salt Luria broth (LB) medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, and water was added to 1000 ml) was used for growth of cells and the plating of phage λ (Miller 1992). Solid media for plates was supplemented with 16 g of agar, or 8 g/liter for soft agar. All experiments were done with exponentially growing cells ($\sim$1–2 $\times$ 10^8 bacteria/ml, $OD_{650} = 0.4$) at 37° in LB medium.

TABLE 1

Strains used in this study

Bacterial strain	Relevant genotype	Source or reference
	Bacterial strains related to AB1157	
AB1157	F⁻ *thr-1 leuB6* Δ(*gpt-proA*)*62 hisG4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 supE44 rpsL31 kdgK51 rfbD1 mgl-51* λ⁻ *rac*⁻	Bachmann (1996)
RIK174	+ *recB1080*	Jockovich and Myers (2001)
RIK144	+ *recD1903*::Tn*10d(Tet)*	Jockovich and Myers (2001)
IRB103	+ *recO1504*::Tn*5*	Paškvan *et al.* (2001)
AM208	+ *recR256*::Tn*10-9*	Mahdi and Lloyd (1989)
WA576	+ *recF400*::Tn*5*	W. Wackernagel
IIB290	+ *recB1080 recD1903*::Tn*10d(Tet)*	P1.RIK144 × RIK174
IIB282	+ *recB1080 recO1504*::Tn*5*	P1.IRB103 × RIK174
WA618	+ *uvrA277*::Tn*10*	R. G. Lloyd
LMM1032	+ *recJ2052*::Tn*10kan*	D. Zahradka
IIB340	+ *recD1903*::Tn*10d(Tet) recJ2052*::Tn*10kan*	P1.LMM1032 × RIK144
SP254	+ *recN262*	R. G. Lloyd
N4454	+ Δ*ruvABC*::*Cm*	R. G. Lloyd
IIB354	+ Δ*recG263*::*Km*	P1.N4256 × AB1157
IIB356	+ Δ*ruvABC*::*Cm* Δ*recG263*::*Km*	P1.N4256 × IIB244
NK113	+ Δ*clpP1*::*cat*	Makovets *et al.* (1999)
SS96	+ *priA300*	Sandler (2000)
JC19009	+ *priA2*::*kan dnaC810 zjj-202*::Tn*10*	Sandler *et al.* (1996)
IIB417	+ *uvrA277*::Tn*10*	P1.WA618 × AB1157
IIB451	+ *sfiA11 priA2*::*kan*	P1.JC19009 × N5208
N5208	+ *sfiA11*	R. G. Lloyd
NK526	+ Δ*hsdR4*	N. Murray
IIB309	+ Δ*clpP1*:: *cat*	P1.NK113 × AB1157
IIB328	+ Δ*hsdR4* Δ*clpP*::*cat*	P1.IIB309 × NM526
IIB244	+ Δ*ruvABC*::*Cm*	P1.N4454 × AB1157
IV210	+ Δ*dam-16*::*kan*	P1.MK1 × AB1157
	Other	
C600$r_{k^-}m_{k^-}$	*thr-1 leuB6 lacY1 supE44 rfbD1 thi-1 tonA21* r_{k^-} m_{k^-}	M. Radman
N4256[a]	+Δ*recG263*::*Km*	R. G. Lloyd
MK1	*deoA21 lac624 lacY1* Cm^r Δ*dam-16*::*kan*	I. Matić

[a] MG1655 background (Bachmann 1996).

UV irradiation of cells: Ten milliliters of midlog phase cells were centrifuged (6000 × *g*, 6 min, 4°) and resuspended in the same volume of λ-buffer (10 mM Tris, 10 mM $MgSO_4$, pH 7.2). A 1-ml aliquot was taken to measure the zero-time value of RA (unirradiated sample) and the remaining culture was gently stirred during irradiation at room temperature by a 30-W Philips low-pressure Hg germicidal lamp at a distance of 1 m with the incident dose of 2.5 (J/m^2/sec). The thickness of the irradiated layer of suspension was <0.7 mm. After irradiation, cells were pelleted, resuspended in the same volume of fresh LB medium, and incubated with aeration for the duration of the time course (3 hr). A 0.1-ml sample was taken before and after UV irradiation, diluted, and plated immediately for cell survival (Miller 1992). Each strain was irradiated with a UV dose of 150 J/m^2. UV dose was determined with a VLX-3W UV dosimeter (Bioblock, Illkirch, France).

Restriction alleviation assay: UV-induced RA was measured as described previously (Čogelja-Čajo *et al.* 2001) with some modifications. During post-irradiation incubation, at appropriate times, 1-ml aliquots were taken, centrifuged, and resuspended in 0.2 ml of LB medium supplemented with 50 mM $MgSO_4$. Bacteria were infected with an unmodified virulent mutant of phage λ*vir*.0. The multiplicity of infection was <0.1 and adsorption of phages on bacteria was ~99.7%. After adsorption, 15 min at 37°, infected bacteria were plated for infective centers on an untreated overnight indicator strain AB1157. RA was expressed as the efficiency of plating unmodified phage λ onto UV-irradiated bacteria relative to that onto unirradiated cells. RA_0 value represents the initial plating efficiency of unmodified phage λ on unirradiated cells relative to phage titer on strain C600$r_{k^-}m_{k^-}$ at time 0, while RA_{max} value represents the maximal RA on UV-irradiated cells relative to phage titer strain C600$r_{k^-}m_{k^-}$ (usually reached after 2 hr of post-irradiation incubation).

RESULTS

UV-induced RA is dependent on the UvrA protein and primosome activity of PriA helicase: To examine the role of the *rec* gene products in UV-induced RA, we first determined experimental conditions for RA in wild-type cells. A simple test to measure restriction alleviation is based on measuring the plating efficiency of unmodified λ-phage on *E. coli* cells. The phage genome is a substrate for *Eco*KI cleavage, and unmodified

λ-phage show increased survival if plated on cells previously treated with DNA-damaging agents such as UV light. A typical curve showing a temporary increase of plating efficiency of unmodified λ-phage in UV-irradiated wild-type cells (*i.e.*, restriction alleviation) during 3 hr of post-irradiation incubation is shown in Figure 1. The optimal restriction alleviation under our conditions was observed at a UV dose of 150 J/m^2 for the wild-type strain, which is a stronger UV dose than used by others (Thoms and Wackernagel 1984; Kelleher and Raleigh 1994). We applied this UV dose in all experiments, except where mentioned, to expose each mutant to the same DNA damage; *i.e.*, the same UV dose ensures the same number of pyrimidine dimer formation in excision-proficient cells. In addition to λ*vir*.0, RA was determined in highly UV sensitive strains with modified phage λ*vir*.K. The plating efficiency of modified phage was always ~1 (data not shown), implying that the results obtained with unmodified phage λ*vir*.0 did not require correction for eventually reduced cell capacity for phage propagation. The maximal value of RA for wild-type cells was achieved after 120 min of post-UV incubation at 37°, which is similar to previous studies (Thoms and Wackernagel 1984; Čogelja-Čajo *et al.* 2001). For clarity, all results obtained in this study are summarized in Table 2. Table 2 contains data on the efficiency of plating λ*vir*.0 on uninduced cells relative to the phage titer on strain C600r$_k^-$m$_k^-$ at time 0 (RA$_0$); the efficiency of plating λ*vir*.0 on UV-induced cells, giving maximum RA relative to the phage titer on strain C600r$_k^-$m$_k^-$ (RA$_{max}$); the ratio of UV-induced RA$_{max}$

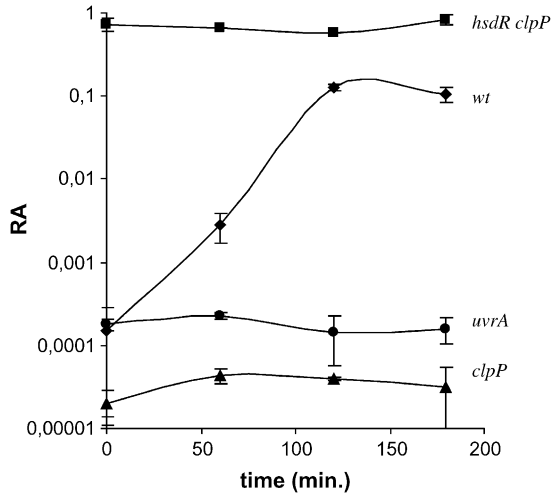

Figure 1.—UV-induced restriction alleviation is abolished in *clpP* and *uvrA* mutants during 3 hr of post-irradiation incubation. Strains wild type (*wt*; ♦), *clpP* (▲), *hsdR clpP* (■) and *uvrA* (●) were irradiated with a UV dose of 150 J/m^2. The values presented are the means of at least two independent experiments. Error bars represent standard deviation.

TABLE 2

Induction of restriction alleviation in *E. coli* mutants

Bacterial strain	Relevant genotype	RA$_0$	RA$_{max}$	RA	% cell survival[a]
AB1157	Wild type	0.00015 ± 0.00014	0.14 ± 0.011	~1000	4
RIK144	*recD*	0.00025 ± 0.00006	0.28 ± 0.09	>1000	4
RIK174	*recB1080*	0.00065 ± 0.00025	0.0031 ± 0.002	5	3
IIB290	*recB1080 recD*	0.00044 ± 0.0002	0.011 ± 0.0038	61	3
LMM1032	*recJ*	0.0004 ± 0.0002	0.12 ± 0.017	280	4
IIB340	*recD recJ*	0.0017 ± 0.00069	0.014 ± 0.0053	8	0.004
IV210	*dam-16*	0.0048 ± 0.0029	0.16 ± 0.09	33	0.5
IRB101	*recQ*	0.00025 ± 0.00016	0.157 ± 0.024	620	4
IRB103	*recO*	0.00015 ± 0.0001	0.00137 ± 0.00084	8	0.0045
AM208	*recR*	0.00025 ± 0.00014	0.0011 ± 0.00033	4	0.0061
WA576	*recF*	0.00023 ± 0.00016	0.0018 ± 0.0009	7.6	0.01
IIB282	*recB1080 recO*	0.00022 ± 0.000014	0.00021 ± 0.00002	~1	0.0009
SP254	*recN*	0.00025 ± 000041	0.12 ± 0.027	480	0.1
IIB354	Δ*recG*	0.0047 ± 0.0024	0.13 ± 0.035	29	0.067
IIB244	Δ*ruvABC*	0.00014 ± 0.000088	0.096 ± 0.05	640	0.002
IIB356	Δ*ruvABC* Δ*recG*	0.0029 ± 0.0011	0.022 ± 0.0035	8	0.000025
IIB309	Δ*clpP*	0.000019 ± 0.000015	0.00004 ± 0.00003	~2	0.3
IIB328	Δ*hsdR* Δ*clpP*	0.72 ± 0.13	0.84 ± 0.11	~1	2
IIB417	*uvrA*	0.00018 ± 0.000028	0.00016 ± 0.000056	~1	0.000016
N5208	*sfiA11*	0.00014 ± 0.000049	0.25 ± 0.14	>1000	1
IIB451	*sfiA11 priA2*	0.00013 ± 0.000014	0.00019 ± 0.000028	~1	<0.001
SS96	*priA300*	0.00017 ± 0.00013	0.0025 ± 0.0014	14	4
JC19018	*priA2 dnaC810*	0.0004 ± 0.0003	0.0035 ± 0.0016	8	2

RA$_0$, the efficiency of plating of λ*vir*.0 on uninduced cells relative to the phage titer on strain C600r$_k^-$m$_k^-$ at time 0; RA$_{max}$, the efficiency of plating of λ*vir*.0 on UV-induced cells, giving maximum RA relative to the phage titer on strain C600r$_k^-$m$_k^-$; RA, the efficiency of plating of λ*vir*.0 on UV-induced cells (RA$_{max}$) relative to that on uninduced cells (RA$_0$).

[a] Typical values.

relative to that on uninduced RA_0 (RA); and cell survival for all strains tested. As shown in Figure 1 and Table 2 for wild-type cells, $RA_0 = 0.00015$, $RA_{max} = 0.14$, and RA = ~1000. According to results obtained for wild-type and mutants used in this study, we considered that restriction alleviation following UV irradiation is induced if the RA_{max} value was ~>0.01 or RA was ~10. Taking into account the extent of standard deviation, we consider that the basal level of restriction alleviation is increased if the RA_0 value is at least four times higher compared to wild-type RA_0 value, but never reaches 0.01. The mutants that showed UV-induced $RA_{max} < 0.01$, and therefore a smaller RA value (~5–10), were said to have partially UV-induced RA (Table 2).

In addition to wild-type cells, we wanted to confirm the earlier observation that RA in UV-irradiated cells is protease ClpXP dependent and to examine whether UV-induced RA is dependent on presence of the HsdR subunit of the *Eco*KI restriction enzyme. Namely, it is known that RA induction is dependent on the ClpXP protease, which is necessary for the degradation of the HsdR subunit and subsequent restriction-deficient phenotype (Makovets *et al.* 1999). Until now, it has not been shown that the HsdR subunit of *Eco*KI is involved in UV-induced RA. Our results are presented in Figure 1. As expected, cells mutated in *clpXP* genes did not alleviate restriction; *i.e.*, maximal restriction was observed. Figure 1 shows low plating efficiency of unmodified λ in UV-irradiated *clpP* or *clpX* strains through 3 hr of post-irradiation incubation ($RA_{max} \sim 0.00004$; and data not shown for *clpX* mutant). This result confirms the requirement for ClpXP protease in UV-induced RA. In contrast, the maximal plating efficiency of unmodified λ*vir*0 or maximal RA was observed in a *hsdR clpP* mutant where the HsdR subunit is missing ($RA_{max} \sim 1$; Figure 1; Table 2), indicating its involvement in UV-induced RA.

It is known that the RecBCD enzyme is involved in the UV-induced RA phenomenon (Thoms and Wackernagel 1984), implying that unmethylated target sequences could be produced by recombination. Since the RecBCD enzyme must bind to free dsDNA ends that are blunt or nearly blunt to initiate recombination, we wanted to test whether UV-induced RA depends on DSBs produced during excision repair of pyrimidine dimers. DSBs can be introduced by excision repair of two closely spaced photoproducts on opposite DNA strands (Bonura and Smith 1975; Sedgwick 1975). Alternatively, the encounter of a replication fork with nicks created by excision repair in the DNA template can also result in blunt-end DSBs (Hanawalt 1966).

To test this possibility, we measured the survival of unmodified λ-phage in a *uvrA* mutant. The UvrA protein loads UvrB protein onto a damaged DNA site after which UvrC binds to UvrB, resulting in a UvrBC-incision complex. Therefore, mutation in the *uvrA* gene blocks the incision step of the excision repair of UV-induced lesions and other DNA damage (Van Houten 1990). In

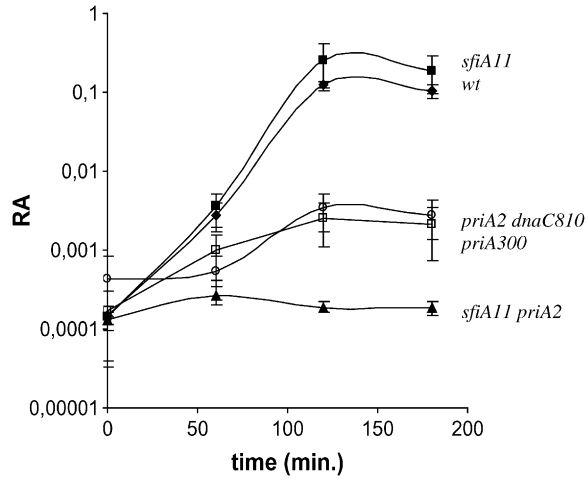

Figure 2.—UV-induced restriction alleviation is dependent on PriA protein. Strains *wt* (♦), *sfiA11* (■), *sfiA11 priA2* (▲), *priA300* (□), and *priA2 dnaC810* (○) were irradiated with a UV dose of 150 J/m² and incubated for 3 hr. The values presented are the means of at least three independent experiments. Error bars represent standard deviation.

contrast to a previous result where a modest effect was observed (RA ~ 60) (Thoms and Wackernagel 1982), UV-induced RA was completely abolished in a *uvrA* mutant ($RA_{max} = 0.00016$; RA ~ 1). This result suggests that generation of unmethylated dsDNA is dependent on the excision repair protein UvrA (Figure 1; Table 2). The reason for this difference could be due to either different *uvrA* alleles or different UV doses used. The same experiment was repeated by using more appropriate UV doses for excision-repair-deficient mutants: 3, 10, 30, and 60 J/m². However, with each UV dose we observed the same effect: no UV-induced restriction alleviation (data not shown). Since we used a *uvrA* mutant inactivated with a Tn*10* insertion, there is the possibility that *uvrA1* is not a null mutant in all enzymatic activities of the UvrA protein (see discussion).

The introduction of DSBs during UV-induced RA could be the result of both incision and replication of DNA. Broken replication forks (indirectly induced DSBs) are known to be repaired by RecBCD and RecA via a recombination intermediate D-loop, onto which the PriA helicase can bind and load the DnaB helicase and the remainder of the replisome (Sandler and Marians 2000). The PriA protein possesses three biochemical activities: 3′ → 5′ DNA helicase, ATPase, and the specific activity for primosome assembly. If DSBs are indeed processed via formation of D-loops, then PriA would be required for UV-induced RA.

To test whether *priA* is needed for UV-induced RA, we used a *sfiA11 priA2* double mutant because the *sfiA* mutation reduces the filamentous phenotype of a *priA2 null* mutant (Nurse *et al.* 1999). As expected, UV-induced RA was very low in a *sfiA priA2* double mutant ($RA_{max} = 0.00019$; RA ~ 1), compared to the *sfiA* control (RA > 1000) (Figure 2; Table 2). We next tested

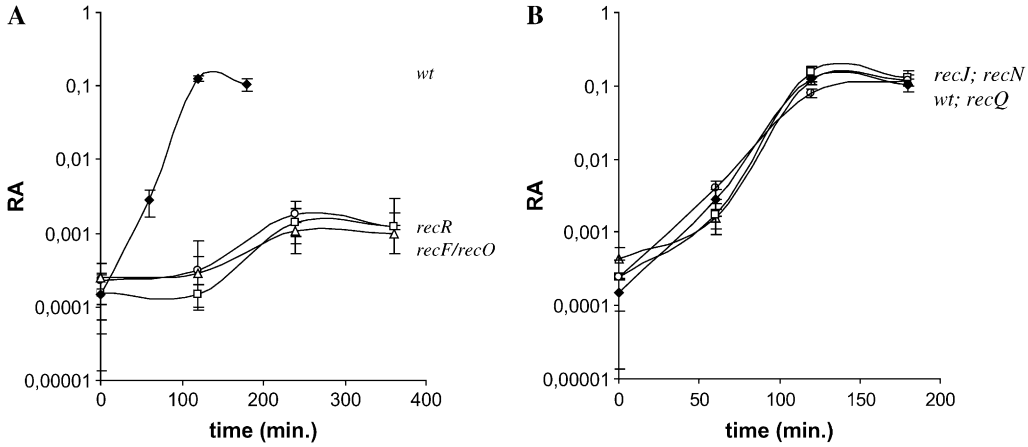

FIGURE 3.—UV-induced restriction alleviation in *recFOR* pathway mutants. (A) Strains *wt* (♦), *recO* (□), *recF* (○), and *recR* (Δ) were irradiated with a UV dose of 150 J/m^2 and incubated for 6 hr. (B) Strains *wt* (♦), *recJ* (Δ), *recQ* (□), and *recN* (○) were irradiated with a UV dose of 150 J/m^2 and incubated for 3 hr. The values presented are the means of at least three independent experiments. Error bars represent standard deviation.

which activity of the PriA is required for UV-induced RA. We repeated the same experiment using the *priA300* mutant that lacks ATPase and helicase activities but is capable of catalyzing primosome assembly *in vitro*, and the *priA2 dnaC810* double mutant. The latter mutant has a suppressor mutation in *dnaC810*, which enables DnaB loading directly onto replication forks without PriA (Liu *et al.* 1999). UV-induced RA was restored in the *priA300* mutant but to a reduced level (RA_{max} = 0.0025; RA = 14; Figure 2; Table 2). A similar result was obtained with the *priA2 dnaC810* mutant (RA_{max} = 0.0035; RA = 8). These results are consistent with our idea that PriA is required for UV-induced RA, particularly for its primosome assembly activity. The helicase activity of PriA is also needed for maximal effect on UV-induced RA induction, while DnaC was not as efficient for substituting PriA in DnaB-loading activity. Perhaps inappropriate loading of the DnaB helicase on a D-loop (Xu and Marians 2003) is the reason for the less efficient RA induction observed when the suppressor mutation *dnaC810* is present.

UV-induced RA in *recF* pathway mutants: The replication fork might encounter an unrepaired pyrimidine dimer, leaving the lesion in a single-strand gap (SSG) at the stalled fork. After some period of time, DNA synthesis resumes and the bacteria continues to divide. Resumption of replication following UV irradiation is dependent upon the RecFOR pathway (Courcelle *et al.* 1997, 1999). In wild-type cells, the RecFOR complex, the product of *recF*, *recR*, and *recO* genes, is involved in replacement of the single-stranded DNA binding (SSB) protein coating single-strand DNA (ssDNA) with the RecA protein and in stabilization of the RecA filaments at an arrested replication fork (Courcelle *et al.* 1999, 2003; Courcelle and Hanawalt 2001; Morimatsu and Kowalczykowski 2003; Chow and Courcelle 2004). Other proteins in the RecFOR pathway (RecQ helicase and RecJ nuclease) are shown to degrade nascent lagging strands, producing more ssDNA from the original SSG (Courcelle and Hanawalt 1999; Courcelle *et al.* 2003). The RecQ protein, a 3′ → 5′ helicase, and RecJ, a 5′ → 3′ exonuclease, act together to process a dsDNA break or gap to generate the 3′ single strand (Harmon and Kowalczykowski 1998). The biochemical activity of another protein of the RecF pathway, RecN, is unknown but it is probably required for RecBCD-dependent repair of dsDNA breaks (Lloyd *et al.* 1983; Wang and Smith 1986).

Since the RecFOR complex is involved in replication and recombination processes, we wanted to confirm its role in UV-induced RA. A previous study has shown that UV-induced RA is absent in a *recF* mutant after 90 min of post-irradiation incubation (Thoms and Wackernagel 1984; Figure 3A). Since the induction of the SOS response is delayed in *recF/O/R* mutants (Thoms and Wackernagel 1987; Hegde *et al.* 1995; Whitby and Lloyd 1995), we asked whether a longer post-irradiation incubation of 4 hr would induce RA in these mutants. Figure 3A and Table 2 show that the maximal expression of RA was achieved after 4 hr instead of the usual 2 hr of post-irradiation incubation, but the effect was rather small: *recF* (RA_{max} = 0.0018; RA ~ 8), *recR* (RA_{max} = 0.0011; RA = 4), and *recO* (RA_{max} = 0.00137; RA = 8). This result shows that reduced UV-induced RA can be expressed in *recF/O/R* mutants, but it is delayed in a manner similar to the SOS response. It can be concluded that UV-induced RA is partially dependent on RecFOR proteins.

We next characterized UV-induced RA in other *recF* pathway genes: *recJ*, *recQ*, and *recN*. As shown in Figure 3B and Table 2, UV-induced restriction alleviation was normally expressed in these mutants and RA_{max} was >0.1.

RecA-loading activity of the RecBCD enzyme is required for UV-induced RA: Since a functional RecBCD enzyme is required for UV-induced RA, it was of interest to test which of the RecBCD enzyme activities are required for RA induction. RecBCD enzyme is a heterotrimer composed of RecB, RecC, and RecD subunits. It plays a central role in the major pathway of recombination and DNA repair of double-strand breaks in *E. coli*. It is a multifunctional enzyme regulated by the octamer sequence Chi, which stimulates recombination

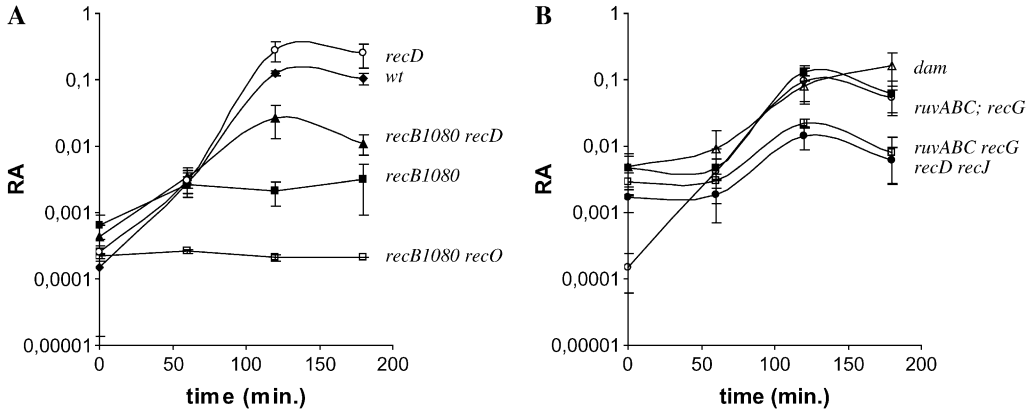

FIGURE 4.—UV-induced restriction alleviation is abolished when RecA-loading activity is inactivated (A). Strains (A) *wt* (♦), *recB1080* (■), *recB1080 recO* (□), *recB1080 recD* (▲), and *recD* (○) were irradiated with a UV dose of 150 J/m^2 and incubated for 3 hr. (B) Constitutive RA and UV-induced RA are expressed in *recG* (■), *ruv recG* (□), *dam* (Δ), and *recD recJ* (●) mutants, but not in *ruvABC* (○), where only UV-induced RA is expressed. Cells were irradiated with a UV dose of 150 J/m^2 and incubated for 3 hr. The values presented are the means of at least three independent experiments. Error bars represent standard deviation.

(for a review see KOWALCZYKOWSKI *et al.* 1994). Three biochemical activities of the RecBCD enzyme are essential for recombination: 5′ → 3′ exonuclease, helicase, and RecA-loading activity (AMUNDSEN and SMITH 2003; IVANČIĆ-BAĆE *et al.* 2003). In a previous study, we have shown that RA is normally induced in a *recD* mutant and have concluded that the helicase activity of the RecBCD enzyme is required for UV-induced RA (ČOGELJA-ČAJO *et al.* 2001). To test specifically for the requirement of RecA-loading activity for UV-induced RA, we used the *recB1080* mutant (ANDERSON *et al.* 1999; JOCKOVICH and MYERS 2001). This mutant has a point mutation in the C-terminal portion of the RecB subunit. Consequently, the RecB1080CD form of the enzyme is nuclease deficient and is unable to load RecA protein onto ssDNA, while it retains functional helicase activity (YU *et al.* 1998a,b; ANDERSON *et al.* 1999; WANG *et al.* 2000). In this mutant, it is possible to distinguish RecA-loading activity from the helicase activity of RecBCD enzyme.

The *recB1080* single mutant showed a reduced level of UV-induced RA ($RA_{max} < 0.01$; RA ~ 5), indicating that UV RA is partially induced and that only fully functional RecBCD is required for efficient RA. When both mechanisms of RecA loading were abolished (IVANČIĆ-BAĆE *et al.* 2003), as was the case in the *recB1080 recO* double mutant, there was almost no UV-induced RA ($RA_{max} = 0.0002$; RA ~ 1; Figure 4A). As expected, the RecA-loading activity is required for UV-induced RA. To further confirm that RecA-loading activity is required for UV-induced RA, we measured the plating efficiency of unmodified λ-phage in a *recB1080 recD* double mutant. It is known that RecB1080C(D$^-$), an enzyme produced by *recB1080 recD* cells, possesses RecA-loading activity due to inactivation of the RecD subunit, an inhibitor of RecA loading (AMUNDSEN *et al.* 2000). As a control, the results for a *recD* single mutant are included in Figure 4A. RA following UV irradiation was induced in both the *recD* ($RA_{max} = 0.28$; RA > 1000) and the *recB1080 recD* mutant ($RA_{max} = 0.011$; RA ~ 60) (Figure 4A). Taken together, these results indicate that RecA-loading activity by the RecBCD enzyme is required for UV-induced RA.

Constitutive RA: In our previous experiments, it was shown that RA can be induced in response to DNA damage by UV light. However, better survival of unmodified phage λ*vir*.0 at the zero time of the experiment (increased RA_0 value without UV irradiation) can be seen in some mutants. This so-called constitutive RA was reported for type I *Eco*KI, but not for a type II system, and may occur for type III systems (EFIMOVA *et al.* 1988). RA was also observed in *dam*, *topA*, *mutD*, *rnhA*, and *recG* mutants (Figure 2; Table 2; EFIMOVA *et al.* 1988; KELLEHER and RALEIGH 1994; MAKOVETS *et al.* 1999; BLAKELY and MURRAY 2006). These mutants accumulate DSBs (*dam* and *topA*) (WANG and SMITH 1986; KOUZMINOVA *et al.* 2004), have increased frequency of mismatches (*mutD*) (ECHOLS *et al.* 1983), or enable R-loops to persist and initiate DNA replication (*rnhA* and *recG*) (KOGOMA 1997). Constitutive RA is suppressed in all these mutants by the *clpXP* mutation (MAKOVETS *et al.* 1999; BLAKELY and MURRAY 2006). These mutants also share other common features, such as (i) constitutively induced SOS response (PETERSON *et al.* 1985; LLOYD and BUCKMAN 1991; KOGOMA *et al.* 1993; SLATER *et al.* 1994; KOUZMINOVA *et al.* 2004), (ii) unscheduled initiation of chromosomal replication (KOGOMA 1997; KOUZMINOVA *et al.* 2004), and (iii) recombination proficiency (*i.e.*, they have functional RecBCD and RecFOR pathways of recombination). The reason for constitutive RA in these mutants is most likely due to modulated initiation of replication on either R-loops (*rnhA* and *recG* mutants) or *oriC* (*dam* mutant) (BAKKER and SMITH 1989; KOGOMA 1997; BLAKELY and MURRAY 2006) than to accumulation of DSBs. This conclusion is supported by the observation that a *dam mutH* double mutant that does not accumulate DSBs still exhibits constitutive RA (EFIMOVA *et al.* 1988; KELLEHER and RALEIGH 1994).

In this study, we found a new class of mutants that show constitutive RA activity; *i.e.*, the basal level of restriction alleviation (RA_0) is at least four times greater compared

to the wild-type RA_0 value. As shown in Figure 4A and Table 2, constitutive RA was observed in a *recB1080* mutant ($RA_0 = 0.00065$), but not in a *recD* mutant, which is also nuclease deficient. It is known that recombination in *recD* mutants, which lacks the nuclease activity in RecBCD, is partially dependent on the RecJ nuclease (Lloyd *et al.* 1988; Lovett *et al.* 1988; Lloyd and Buckman 1991; Ivančić-Baće *et al.* 2005), so we were interested in testing whether the lack of 5′ → 3′ nuclease activity of this enzyme would enhance constitutive RA in a *recD* background. The results of constitutive and UV-induced RA in the single *recD* or the double-mutant *recD recJ* are presented in Figure 4, A and B. In agreement with our expectations, a *recD recJ* mutant exhibited a higher RA_0 value (0.0017) compared to wild type (0.00015) and *recD* (0.00025). Due to constitutive RA expression, *recD recJ* showed modest UV-induced RA (RA = 8), although its RA_{max} is relatively high (0.014). A *recD* single mutant had a lower RA_0 value probably due to preservation of residual 5′ → 3′ exonuclease activity of the RecBCD enzyme and the presence of a functional RecJ nuclease. As expected, the single *recJ* mutant did not show constitutive RA due to functional nuclease activity of the RecBCD enzyme (Figure 3B). However, the nuclease-deficient *recB1080 recJ* double mutant also did not show constitutive restriction alleviation (data not shown). The reason for this difference is probably due to lack of a constitutive SOS response in the *recB1080 recJ* double mutant (Ivančić-Baće *at al.* 2006).

To complete the genetic requirements for constitutive and induced restriction alleviation after UV irradiation, we have also done experiments with the mutants *recG*, *ruvABC recG*, *ruvABC*, and *dam* (Table 2; Figure 4B). The RuvAB and RecG proteins are helicases that catalyze branch migration, and the RuvC protein is a nuclease that resolves Holliday junctions in the late stages of homologous recombination (West 1996). RecG is also a junction-specific RNA/DNA helicase, which dissociates the R-loop and catalyzes branch migration of the Holliday junction in the reverse direction (Whitby *et al.* 1993; Hong *et al.* 1995; Vincent *et al.* 1996). Dam methyltransferase (DamMT), encoded by the *dam* gene, is an enzyme that methylates GATC sequences in *E. coli* DNA (for a review see Løbner-Olesen *et al.* 2005). As expected and in agreement with data from the literature (Efimova *et al.* 1988; Kelleher and Raleigh 1994; Blakely and Murray 2006; Table 2), constitutive RA was observed in *recG* (0.0047), *ruvABC recG* (0.0029), and *dam* (0.0048) mutants, and not in a *ruvABC* mutant (0.00014). Restriction alleviation was induced in all these mutants after UV irradiation (Figure 4B), since RA_{max} value was > 0.01 (Table 2).

DISCUSSION

A current interpretation for RA induction is that DNA damage induces loss of restriction activity due to generation of unmethylated target sequences within the bacterial chromosome (Makovets *et al.* 1999). It was proposed that unmethylated target sequences can be generated from replication associated with homologous recombination following DNA damage (Makovets *et al.* 1999; Murray 2000; Blakely and Murray 2006). In agreement, we provide evidence that a similar model can be applied to bacteria damaged by UV irradiation. In addition to a requirement for ClpXP, RecA, RecBCD, and induction of the SOS response, we show that UV-induced RA is also dependent on the excision repair protein UvrA, the RecA-loading activity of the RecBCD enzyme, the primosome activity of PriA, and is partially dependent on RecFOR proteins. We also show that RA is not dependent on *recN*, *recJ*, *recQ*, *recG*, and *ruvABC*.

The RecBC(D) enzyme was shown to be essential at an early stage during signal generation for alleviation of restriction, but it was not clear if the enzyme was also directly involved in the RA process due to experimental limitations (Thoms and Wackernagel 1984). It was shown that restriction alleviation after UV irradiation was blocked in a *recB* mutant, and also in an extragenic suppressor strain of the *recBC* defect (*recB21C22 sbcB12* strain), which is recombination proficient, or in the double-mutant *lexA55 recB*, which has a constitutive SOS response. On the basis of these results it was concluded that the RecBCD enzyme is essential for *Eco*KI restriction alleviation (Thoms and Wackernagel 1984). However, RecBCD also has a destructive role because it degrades λDNA fragments to acid-soluble products after *Eco*KI cleavage (Simmon and Lederberg 1972). It also preserves the integrity of the bacterial chromosome after the cleavage of host DNA by a type I R–M system in the absence of efficient RA (Makovets *et al.* 2004). Similarly, it was proposed that RecBCD defends the host chromosome against restriction by the type II R–M system (Handa *et al.* 2000).

Strains that accumulate DSBs such as *dam*, *rnhA*, and *topA* express constitutive SOS induction and constitutive restriction alleviation. However, mutations that prevent repair-mediated breaks (*mutH*) or constitutive SOS induction (*recA430*) in a *dam* mutant do not suppress constitutive RA (Efimova *et al.* 1988; Kelleher and Raleigh 1994). On the other hand, a *recG* mutant does not accumulate DSBs but does express constitutive SOS induction and constitutive RA by *Eco*KI (Lloyd and Buckman 1991; McCool *et al.* 2004; Blakely and Murray 2006). Another interesting example is a *priA* mutant that has constitutive SOS induction but does not induce constitutive RA (Table 2; Figure 2). Therefore, in agreement with previous studies (Thoms and Wackernagel 1984; Hiom and Sedgwick 1992), we conclude that SOS induction itself is required to alleviate restriction but is not sufficient to induce RA. SOS induction possibly indicates that DNA replication is blocked, *i.e.*, that

TABLE 3

Requirements for constitutive and UV-induced RA in *E. coli*

Genotype	Replication recovery after UV irradiation[a]	Constitutive SOS response[b]	dsDNA break repair[d]	Constitutive RA[e]	UV-induced RA[f]
Wild type	+	−	+	−	+
recA	−	−	+	−	−
recB	+	−	−	−	−
recF/O/R	−	−	+	−	±
uvrA	−	−	+	−	−
priA	−	+	+	−	−
recG	+	+	+	+	+
ruv recG	+	+	+	+	+
dam	+ (ND)[c]	+	+	+	+
recB1080	+ (ND)[c]	+	±	+	±
recD recJ	+	−	±	+	+
recN	+	+	+	−	+
ruv	+	+	+	−	+

[a] The results are presented in Kogoma (1997), Courcelle *et al.* (1997, 1999), Chow and Courcelle (2003), and Donaldson *et al.* (2004).

[b] The results are presented in Peterson *et al.* (1985), Lloyd and Buckman (1991), Asai and Kogoma (1994), Nurse *et al.* (2001), O'Reilly and Kreuzer (2004), McCool *et al.* (2004), and Ivančić-Baće *et al.* (2006).

[c] ND, not determined, but we assume that replication recovery takes place in these mutants due to the RA induction phenotype.

[d] The presence of functional RecBCD is indicated by a "+," and the presence of a partially functional RecBCD enzyme is indicated by a "±."

[e] Based on our results and previous studies by Efimova *et al.* (1988), Kelleher and Raleigh (1994), Makovets *et al.* (1999), and Blakely and Murray (2006).

[f] Based on our results and previous studies by Day (1977), Thoms and Wackernagel (1982, 1984), and Kelleher and Raleigh (1994).

single-strand gaps are generated at stalled replication forks.

It is known that DNA damage is also essential for restriction alleviation. There are at least two major types of DNA damage. If the fork encounters a DNA single-strand nick or gap, the replication fork will collapse, creating a DSB. If an unrepaired lesion is encountered, the lesion is left in a DNA gap at the stalled fork. Numerous studies have demonstrated that replication restart can proceed in a number of ways that are completely dependent upon several genes, mostly associated with homologous recombination (for a review see Kuzminov 1999). When a cell is subjected to high doses of UV light or a chemical mutagen, DNA lesions transiently block replication, which causes an induction of the SOS response (Setlow *et al.* 1963; Sassanfar and Roberts 1990). At a UV dose of 50 J/m^2, replication restart (recovery) is seen 30 min after the DNA damage is introduced (Courcelle *et al.* 2003). The recovery of replication requires RecA, RecF, RecO, RecR, UvrA, and PriA proteins (Masai *et al.* 1994; Courcelle *et al.* 1997, 1999; Rangarajan *et al.* 2002). Interestingly, none of the mutants in these genes is able to alleviate restriction following UV irradiation. On the other hand, RecBCD is not required for replication recovery but is essential for restriction alleviation as mentioned above, probably due to its major role in DSB repair. On the basis of these observations, we summarized all the data and listed the requirements for constitutive RA and restriction alleviation following UV irradiation in various mutants in Table 3. Table 3 shows that both replication recovery and functional DSB repair are required for UV-induced RA. In this regard, we also note that constitutive RA requires constitutive SOS induction in addition to functional replication recovery and DSB repair. The constitutive SOS response probably reflects abundant endogenous lesions in these mutants.

A *uvrA* mutant is not able to excise and remove UV-induced lesions, so it fails to recover replication although it shows elevated levels of strand exchange (Courcelle *et al.* 2003). This result indicates that one of the reasons for the absence of restriction alleviation in a *uvrA* mutant is the lack of replication recovery after UV irradiation. This could explain the different results obtained by us and a previous study regarding the *uvrA* effect (Thoms and Wackernagel 1982). Recovery of DNA synthesis was reported to occur in a *uvrA6* mutant that is excision repair deficient (Kogoma 1997 and references therein), but not in a *uvrA*::Tn*10* mutant (Courcelle *et al.* 1999). In a previous study (Thoms and Wackernagel 1982), the *uvrA1* mutant was used (*i.e.*, *uvrA6* mutant), whereas in our research we used the *uvrA*::Tn*10* mutant. However, it should be stressed that a second role of excision repair in UV-induced RA is in the appearance of dsDNA ends, which are the result of replication fork collapse.

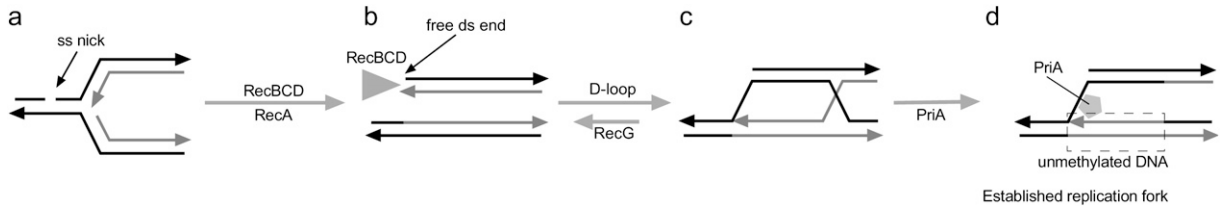

FIGURE 5.—A model for the generation of unmethylated dsDNA in UV-irradiated cells. dsDNA ends can be created either directly by excision repair proteins or after replication of a nicked template (a). Two recombining DNA molecules involved in recombinational repair by the RecBCD enzyme are hemi-methylated (b). Creation of a D-loop generates an unmethylated dsDNA (c) that is preserved after initiation of replication by PriA (d).

The RecFOR complex is known to facilitate the loading of RecA protein onto SSB-coated gaps (MORIMATSU and KOWALCZYKOWSKI 2003). It was shown that replication fails to recover in mutants lacking *recF*, *recO*, or *recR* gene products (COURCELLE *et al.* 1999; RANGARAJAN *et al.* 2002). In these mutants, the DNA lesions are removed by excision repair but nascent strands of the disrupted replication fork are not protected and are degraded by the action of RecQ helicase and RecJ nuclease (COURCELLE *et al.* 1997; COURCELLE and HANAWALT 1999). We observed small and delayed induction levels of UV-induced RA in *recF/O/R* mutants, which are probably dependent on the RecBCD function, similarly to SOS induction following UV irradiation. Taken together, these results indicate that *recF/O/R* mutations affect both replication recovery and activation of RecA for SOS induction following UV irradiation (RANGARAJAN *et al.* 2002). Both of these functions are required for successful UV-induced RA, which explains the small and delayed restriction alleviation.

Another mutant that fails to recover replication after UV irradiation is *recA* (RANGARAJAN *et al.* 2002). Since RecA protein is involved in the central steps of any recombination process and SOS induction, this result is not surprising and it is obvious that RA following UV irradiation cannot be induced (DAY 1977).

The PriA protein is a 3′ → 5′ DNA helicase and the specificity protein for primosome assembly (XU and MARIANS 2003 and references therein). It was demonstrated that *priA* mutants are defective in homologous recombination (KOGOMA *et al.* 1996; SANDLER *et al.* 1996), DSB repair (KOGOMA *et al.* 1996), and both forms of stable DNA replication (MASAI *et al.* 1994). This is why *priA null* mutants have reduced cell viability, defective cell division, and increased sensitivity to DNA damage, constitutive SOS induction, and recombination deficiency (NURSE *et al.* 1991; KOGOMA *et al.* 1996). In agreement with our observation, the lack of replication recovery and recombination deficiency are strong arguments for the absence of RA induction following UV irradiation.

On the other hand, the second group of mutants listed in Table 3 are those that have constitutive RA (*dam*, *recG*, *ruv recG*, *recB1080*, and *recD recJ*). Among these mutants, only the *recD recJ* double mutant does not express constitutive SOS. The simplest explanation for constitutive RA in this mutant would be that less nascent ssDNA is degraded during the processing of dsDNA ends. Therefore, creation of 3′ ssDNA recombinogenic filaments occurs more frequently. Accordingly, a *recD* single mutant had a lower RA_0 value probably due to the action of the RecJ nuclease (Table 2).

Finally, *recN* and *ruvABC* represent a third group of mutants (Table 3). These mutants express a constitutive SOS response (ASAI and KOGOMA 1994; O'REILLY and KREUZER 2004), but do not show constitutive RA. This indicates that unmethylated dsDNA does not accumulate in these mutants, supporting the finding that processing of Holliday junctions is not required for restriction alleviation (BLAKELY and MURRAY 2006).

On the basis of these and previous results, we provide arguments that replication restart at a D-loop is the mechanism for generation of unmethylated dsDNA that might contain the *Eco*KI target sequence following UV irradiation, as shown in our model in Figure 5. The strongest argument is the simultaneous requirement for DNA damage (SOS induction), replication recovery, and DSB repair (or functional RecBCD) for UV-induced RA (Table 3). Invasion of unmethylated ssDNA (created during DSB repair) into a homologous hemi-methylated dsDNA region would generate unmethylated dsDNA at D-loops (MURRAY 2000; BLAKELY and MURRAY 2006; Figure 5) stabilized by PriA activity. Binding of PriA to D-loops promotes replication fork assembly and replication recovery as shown by biochemical and *in vivo* studies (MCGLYNN *et al.* 1997; LIU and MARIANS 1999; LIU *et al.* 1999; RANGARAJAN *et al.* 2002). RecG was shown to dissociate junctions, *i.e.*, to disrupt D-loop structures and destabilize R-loops by removing RNA (WHITBY *et al.* 1993; HONG *et al.* 1995; VINCENT *et al.* 1996). If unmethylated dsDNA is formed at a D-loop, then generation of unmethylated dsDNA would be decreased by RecG, which disrupts D-loop and R-loop formation. The high RA_0 value in the *recG* mutant supports this prediction (Figure 4B; BLAKELY and MURRAY 2006). The need for RecA-loading activity of the RecBCD enzyme also argues that a recombinogenic filament is required for UV-induced RA.

Another alternative explanation for the mechanism of restriction alleviation has been recently proposed for

type III and partially type I restriction systems. By using single-infecting phage conditions, it was shown that type I and type III, but not type II, restriction was alleviated by homologous recombination functions of a Rac prophage, *i.e.*, by RecE and RecT proteins (Handa and Kobayashi 2005). It has been proposed that DNA replication of infecting phage could take place before a type III (or type I) restriction enzyme complex meets another enzyme to cleave DNA. If two daughter copies of the phage genome carry breaks at different loci, then RecET-mediated homologous recombination would be able to reconstitute one intact copy from them (Handa and Kobayashi 2005). However, further work is necessary to test the impact of RecET-mediated recombination on UV-induced RA, since the RecET effect is seen only when RecBCD nuclease is inactivated and in the presence of Rac prophage. These genetic requirements are different from those for UV-induced RA.

Before we proceed to outline our model for generation of unmethylated dsDNA, we would like to stress that only a small fraction of λ infections may undergo restriction alleviation. Up to 75% of the infecting nonmodified λDNA is converted to acid-soluble material (Thoms and Wackernagel 1982). The reason for the small fraction of λ survival could be that unmethylated dsDNA that would stimulate alleviation of restriction occurs rarely as the result of DNA repair on the chromosome, in a small fraction of UV-irradiated cells. Interestingly, it has been recently demonstrated that higher order DNA structure has an enormous effect on the activity of the type I restriction–modification enzymes (Keatch *et al.* 2004). DNA in the nucleoid is condensed and coated with nonsequence-specific ligands whereas foreign DNA is relatively naked and in a random coil conformation. The naked form of DNA is a good substrate for translocation and cleavage, while translocation on the nucleoid DNA is inefficient and the ClpXP protease can inactivate the enzyme. Thus, the difference in DNA conformation can explain why unmodified target sites in phage λ do not stimulate the RA response.

We propose that the replication fork breaks when it encounters a nick generated during the excision repair in one of the template strands or directly by the excision repair of two closely spaced photoproducts on opposite DNA strands in the chromosome. This dsDNA end has to be processed by the RecBCD and RecA proteins to promote homologous pairing and strand exchange with an intact sister hemi-methylated duplex. Strand exchange creates a D-loop where two newly synthesized unmethylated DNA strands anneal, thus creating unmethylated dsDNA that might contain the target sequence for the *Eco*KI enzyme. The PriA protein then targets this D-loop and replication is possibly stimulated by the RecFOR proteins (McGlynn *et al.* 1997; Nurse *et al.* 1999; Xu and Marians 2003). Subsequent resolution of the Holliday junction at the D-loop by the RuvABC resolvase would restore a replication fork and fix the unmethylated dsDNA fragment. A similar model for generation of unmethylated dsDNA during the repair of broken replication fork was proposed by Foster (1998) and Blakely and Murray (2006). Finally, a form of DNA replication called inducible stable DNA replication (iSDR), which is suggested to occur during DSB repair, also partially requires RecFOR and strongly requires RecBC (Kogoma 1997). Initiation of iSDR requires DNA damage, D-loop formation by RecA and RecBC, the primosome assembly activity of PriA, and SOS induction (Kogoma 1997), which are similar to the requirements for UV-induced RA. On the other hand, constitutive stable DNA replication that is induced in *rnhA* and *recG* mutants is similar to constitutive RA (Blakely and Murray 2006). SOS induction is dispensable for iSDR in *recD* mutants (Kogoma 1997), which explains the constitutive RA observed in a *recD recJ* double mutant. Adaptive mutagenesis is another interesting phenomenon where creation of adaptive mutations also depends on recombination functions. It has been suggested that iSDR is also involved in adaptive mutation (Kogoma 1997). Thus, recombination-dependent generation of unmethylated dsDNA is a potentially dangerous event: it can either cause DNA cleavage by restriction–modification enzymes or induce spontaneous mutations.

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