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Bacteriophage T4 UvsW protein is a helicase involved in recombination, repair and the regulation of DNA replication origins

Kelly Carles-Kinch[1], James W.George[2] and Kenneth N.Kreuzer[3]

Duke University Medical Center, Department of Microbiology and Program in Cell and Molecular Biology, Durham, NC 27710, USA

[1]Present address: Department of Basic Medical Sciences, Purdue University School of Veterinary Medicine, West Lafayette, IN 47907, USA

[2]Present address: Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, CA 94551-0808, USA

[3]Corresponding author

Bacteriophage T4 UvsW protein is involved in phage recombination, repair and the regulation of replication origins. Here, we provide evidence that UvsW functions as a helicase. First, expression of UvsW allows growth of an (otherwise inviable) *Escherichia coli recG rnhA* double mutant, consistent with UvsW being a functional analog of the RecG helicase. Second, UvsW contains helicase sequence motifs, and a substitution (K141R) in the Walker 'A' motif prevents growth of the *E.coli recG rnhA* double mutant. Third, UvsW, but not UvsW-K141R, inhibits replication from a T4 origin at which persistent RNA–DNA hybrids form and presumably trigger replication initiation. Fourth, mutations that inactivate UvsW and endonuclease VII (which cleaves DNA branches) synergistically block repair of double-strand breaks. These *in vivo* results are consistent with a model in which UvsW is a DNA helicase that catalyzes branch migration and dissociation of RNA–DNA hybrids. In support of this model, a partially purified GST/UvsW fusion protein, but not a GST/UvsW-K141R fusion, displays ssDNA-dependent ATPase activity and is able to unwind a branched DNA substrate.

Keywords: branch migration/helicase/recombination/repair/replication

Introduction

DNA replication, recombination and repair are tightly coupled in the bacteriophage T4 life cycle. At early times of infection, DNA replication initiates at distinct origins which coincide with recombination hotspots (reviewed in Kreuzer and Morrical, 1994; Mosig, 1994). When late gene expression begins, the origins are inactivated as replication initiation switches to a mechanism dependent on T4 recombination proteins (Luder and Mosig, 1982; Derr and Kreuzer, 1990). This recombination-dependent replication presumably occurs by conversion of recombination intermediates into replication forks (reviewed in Kreuzer and Morrical, 1994; Mosig, 1994). Furthermore, T4 recombination-dependent replication is closely related to recombinational repair (reviewed in Kreuzer and Drake, 1994). Not only does the repair of a double-strand break (dsb) utilize the same recombination proteins as late replication, but it can proceed via a mechanism involving extensive DNA replication (George and Kreuzer, 1996).

Phage T4 proteins that assist in strand invasion include gp46/47, UvsX, UvsY and gp32 (reviewed in Kreuzer and Morrical, 1994; Mosig, 1994). Gp46/47 is believed to constitute an exonuclease that generates single-stranded DNA (ssDNA) intermediates at duplex ends, while strand invasion is mediated by the RecA homolog UvsX, along with its accessory protein UvsY and the ssDNA binding protein gp32. The only T4 protein with a demonstrated role in the later stages of recombination is gp49 (endonuclease VII), which resolves Holliday junctions by means of a structure-specific endonuclease activity (Kemper and Brown, 1976; Mizuuchi *et al.*, 1982).

Recent studies of dsb repair in T4 suggest that gp49 is not the only branch-processing enzyme active during T4 infections (George and Kreuzer, 1996; Mueller *et al.*, 1996). In both studies, dsb repair was completely dependent on UvsX, UvsY, gp32 and gp46. However, the repair reactions were only partially reduced by knockout mutations in gene *49*, suggesting an alternate resolution pathway. Such a pathway could involve a branch migration enzyme which moves the junction to a free DNA end or which facilitates the action of a second junction-cleaving enzyme. The gene *41* helicase, together with its loading factor gp59, facilitates branch migration of a three-way junction *in vitro* (Salinas and Kodadek, 1995). However, gp59 did not target the helicase directly to the DNA branch, and no strong evidence implicates gp41 in branch migration *in vivo*. To date, no phage T4 protein has been shown to promote branch migration by interacting specifically with Holliday junctions.

In contrast, biochemical experiments indicate that *Escherichia coli* RuvAB and RecG proteins are both DNA helicases that facilitate branch migration in the bacterial system. In the case of RuvAB, RuvA binds to the junction and thereby targets the RuvB helicase (reviewed by West, 1996). RecG protein by itself recognizes DNA branches and has branch-specific helicase activity (Lloyd and Sharples, 1993a,b; Whitby *et al.*, 1994). Genetic analyses imply a functional overlap between RuvAB and RecG in the process of conjugal recombination (Lloyd, 1991).

Recent evidence strongly suggests that the helicase activity of RecG also represses the formation of persistent R-loops. *E.coli oriC*-independent replication, termed constitutive stable DNA replication (cSDR), occurs when RNase HI (product of *rnhA*) is mutationally inactivated (reviewed by Asai and Kogoma, 1994). This and other results imply that R-loops are normally removed in wild-type cells by RNase HI, but that they persist in *rnhA*-deficient cells and allow replication initiation. Hong *et al*.

(1995) found that *recG* mutants, like *rnhA* mutants, display cSDR and argued that the helicase activity of RecG inhibits R-loop formation. This argument is supported by the finding that an *rnhA recG* double mutant is lethal (Hong *et al.*, 1995), presumably because the accumulation of excess R-loops causes cell death (see Itaya and Crouch, 1991; Kogoma *et al.*, 1993). There is now direct evidence that RecG counters R-loop formation—purified RecG protein dissociates RNA from R-loops but not from simple RNA–DNA hybrids (Vincent *et al.*, 1996).

Mutations in the phage T4 *uvsW* gene cause multiple phenotypes, including increased sensitivity to hydroxyurea (HU) and to UV light, reduced recombination and reduced UV mutability (Hamlett and Berger, 1975; Conkling and Drake, 1984; Derr and Drake, 1990). UvsW has also been linked to the inactivation of origin replication at late times of infection, when the UvsW protein is normally expressed (Derr and Kreuzer, 1990). Thus, knockout mutations in *uvsW* suppress the arrested DNA synthesis caused by mutations (in genes *46*, *47*, *59*, *uvsX* or *uvsY*) that block recombination-dependent replication (Wu and Yeh, 1975; Cunningham and Berger, 1977; Yonesaki and Minagawa, 1987). The recent finding of R-loops at a T4 replication origin (Carles-Kinch and Kreuzer, 1997) is consistent with the possibility that UvsW inhibits origin initiation by countering R-loop formation, as discussed above for RecG. Furthermore, the recombination and repair phenotypes of *uvsW* mutants could be explained if the UvsW protein is involved in DNA branch migration. In this communication, we provide evidence that the physiological function of UvsW involves branch migration and the removal of RNA from R-loops. Furthermore, we show that UvsW is indeed a helicase that can act on DNA branches.

Results

Expression of UvsW rescues growth of an E.coli recG rnhA double mutant

The T4-encoded UvsW protein is able to inactivate phage replication origins, potentially by removing R-loops from the origin region (see Introduction). This possibility has led us to ask whether expression of UvsW could suppress the lethality of an *E.coli rnhA recG* double mutant, in which the accumulation of R-loops is thought to cause cell death (Hong *et al.*, 1995). A transduction experiment was performed with a P1 lysate from a donor strain carrying *recG-258::kan* and recipient strain KCK100 (*rnhA-339::cat*) or its *rnhA*$^+$ parent NapIV. Although the *recG* mutation was successfully introduced into the parental strain, we obtained essentially no kanamycin-resistant colonies from strain KCK100 unless it harbored a plasmid expressing either RNase HI (pPH310), RecG (pGS772), or UvsW (pKCK44) (Table I). The failure to obtain transductants in the plasmid-free KCK100 strain did not result from a general defect in P1 transduction, because a control mutation (*rfaG::*Tn*10-kan*) could be readily transduced into each strain. These results support the argument that UvsW decreases the number of R-loops on chromosomal DNA, perhaps as a functional analog of the RecG protein.

The amino acid sequence of RecG contains motifs generally conserved in DNA and RNA helicases (Gorbalenya *et al.*, 1989; Lloyd and Sharples, 1993b).

Table I. Expression of UvsW suppresses the lethality of *E.coli rnhA recG* double mutant

Recipient genotype	Protein provided by host plasmid	*recG* transductants[a]	*rfaG* transductants[a]
rnhA$^+$	None	940	570
rnhA::cat	None	0.5	450
rnhA::cat	RNase HI	655	420
rnhA::cat	RecG	265	129
rnhA::cat	UvsW	175	66
rnhA::cat	UvsW-K141R	0.5	335

[a]Transductants per 10^8 p.f.u.

Formation of *E.coli rhnA recG* or *rnhA rfaG* double mutants was assayed by means of phage P1-mediated transduction, as described by Miller (1992). Transductants were selected on the basis of the kanamycin-resistance element within the *recG* or *rfaG* gene of the donor strain (AQ8353 and JGF0, respectively). The *rfaG* donor served as a control for the efficiency of P1 transduction. Each transduction frequency is given as the number of kanamycin-resistant colonies per 10^8 plaque-forming units (p.f.u.) of phage P1, and is the average of two determinations. The reduced transduction efficiency of the cells expressing RecG or UvsW could result from interference of these proteins (at high levels) with transductional recombination.

UvsW also contains at least five of the seven defined helicase motifs, including the Walker 'A' consensus nucleotide-binding motif (motif I in Figure 1) common to many ATP-binding proteins and ATPases (Walker *et al.*, 1982). In other helicases, mutagenesis of the conserved lysine within this motif generally results in loss of ATPase and helicase activities (Sung *et al.*, 1988; Zavitz and Marians, 1992; George *et al.*, 1994). To determine whether the putative Walker 'A' motif is important for UvsW function, we mutated Lys141 to arginine (UvsW-K141R; indicated by an asterisk in Figure 1). We then asked whether UvsW-K141R could support growth of the *E.coli rnhA recG* double mutant. The P1 lysates carrying *recG-258::kan* and *rfaG::kan* were used in a transduction of KCK100 harboring a UvsW-K141R-expressing plasmid (pKCK46). No significant number of kanamycin-resistant colonies were obtained with the *recG::kan* donor, but the positive control *rfaG::kan* donor produced ample transductants (Table I). Therefore, the putative Walker 'A' motif of UvsW is important in suppressing lethality of the *rnhA recG* double mutant, consistent with the proposal that UvsW is a helicase.

The UvsW-K141R protein does not provide UvsW function to a T4 infection

We next tested the ability of the UvsW-K141R protein to complement a T4 *uvsW*Δ strain for a characteristic *uvsW* mutant phenotype, hypersensitivity to HU (Hamlett and Berger, 1975). Lawns of *E.coli* with one of three UvsW expression plasmids were grown on square Petri plates containing a concentration gradient of HU, and then equal numbers of either *uvsW*-proficient (K10) or deficient (K10-*uvsW*Δ) phage were spotted six times across the gradient (Figure 2). When the bacterial lawn contained a negative control plasmid in which most of the *uvsW* gene is inverted (pHEK11), the T4 *uvsW*Δ strain showed clear hypersensitivity to HU. When wild-type UvsW was expressed from the plasmid (pHEK4), the HU hypersensitivity of the T4 *uvsW*Δ strain was complemented and both T4 strains showed comparable growth. In the presence of

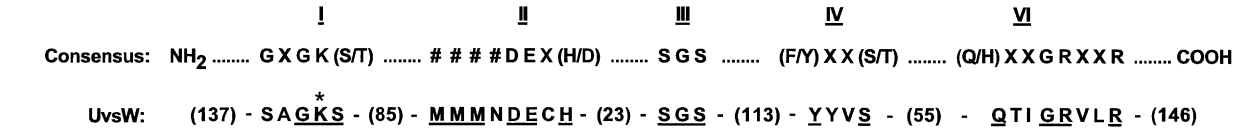

Fig. 1. Helicase motifs in the T4 UvsW protein. Five of the seven consensus motifs found in RNA and DNA helicases are shown (Gorbalenya *et al.*, 1989), with hydrophobic amino acids indicated by '#' and non-conserved amino acids by 'X'. The Walker 'A' motif corresponds to motif I. The remaining two helicase motifs (not shown) are very degenerate. Regions of UvsW that match the helicase motifs are listed below, with acceptable matches to specified residues underlined (note that unspecified 'X' residues are not underlined).

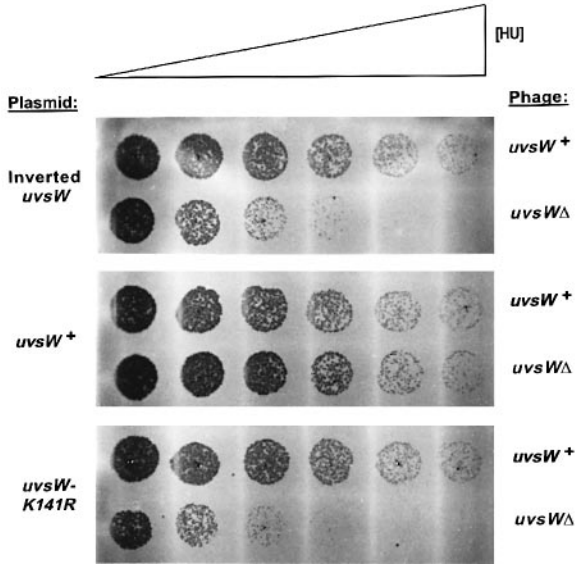

Fig. 2. Growth of T4 in cells expressing UvsW or UvsW-K141R on HU gradient plates. Lawns of *E.coli* strain MCS1 containing one of three plasmids (see below) were prepared on square Petri dishes with a gradient of HU up to 750 μg/ml (direction of concentration gradient indicated at top). Aliquots of ~300 plaque-forming units of T4 *uvsW*$^+$ (strain K10) or T4 *uvsW*Δ (strain K10-*uvsW*Δ) were spotted six times across the gradient. The top panel shows growth when the cells contained a negative control plasmid (pHEK11) with most of the 3′-end of the UvsW reading frame inverted in order to disrupt UvsW expression. In the middle and bottom panels, the cells harbored plasmids with an intact UvsW reading frame (pHEK4) or an identical insert that carries the *K141R* mutation (pKCK40), respectively.

the plasmid expressing UvsW-K141R (pKCK40), the T4 *uvsW*Δ strain was not complemented for HU hypersensitivity. We conclude that the K141R mutation blocks the normal function of the UvsW protein during a T4 infection.

Inhibition of T4 origin-dependent replication by the UvsW protein

Phage T4 initiates DNA replication by both an origin-dependent and a recombination-dependent mode; recombination-deficient mutants can only replicate via the origin-dependent mode (see Introduction). When wild-type UvsW protein was inappropriately expressed at early times of infection, the genomic replication of a recombination-deficient (*uvsY*Δ) phage was severely depressed, suggesting that UvsW is a repressor of origin activity (Derr and Kreuzer, 1990). To test and explore this model further, we performed similar replication experiments with a T4 origin-containing plasmid in the presence of UvsW protein expressed at early times. *E.coli* BL21(DE3) cells with the origin plasmid and a second (compatible) plasmid expressing UvsW from a T7 promoter (pKCK41) were treated with increasing amounts of IPTG to induce expression of T7 RNA polymerase and thus UvsW protein. Induced cells were infected with a T4 *uvsY*Δ strain, and DNA was prepared and analyzed.

As expected, induction of increasing amounts of wild-type UvsW protein markedly inhibited replication of both the phage DNA and the origin-containing plasmid (Figure 3A and B, lanes 1–5), and a control infection with an expression plasmid containing an inverted *uvsW* gene had very little effect on DNA replication (Figure 3A and B, lanes 6–10). These results support the model that UvsW is an inhibitor of T4 origin-dependent replication. To determine whether the putative Walker 'A' motif of UvsW is required for origin repression, a comparable expression plasmid carrying the K141R mutation was also tested. Induction of the UvsW-K141R protein caused very little reduction in phage or plasmid DNA replication (Figure 3A and B, lanes 11–15). Therefore, the putative Walker 'A' motif of UvsW is crucial for the inhibition of replication origins by UvsW, consistent with the possibility that UvsW inhibits origin replication via helicase activity.

Functional overlap of UvsW and endonuclease VII (gp49)

As described in the Introduction, dsb repair in T4 is only partially reduced by a mutation in gene *49*, suggesting a second pathway for DNA branch processing (George and Kreuzer, 1996; Mueller *et al.*, 1996). Given the suggestion that UvsW is a branch migration enzyme like RecG, we asked whether UvsW participates in this second pathway for DNA branch processing.

A convenient system for analyzing dsb repair in T4 utilizes a plasmid containing an inverted repeat, with one copy of the repeat (the top segment in Figure 4A) containing a recognition site for the T4-encoded endonuclease I-*Tev*I (George and Kreuzer, 1996). Cleavage at the I-*Tev*I site triggers a repair event that uses the homologous segment (the bottom segment in Figure 4A) as template. Because the bottom segment does not contain an I-*Tev*I recognition site, the repair products are free of the I-*Tev*I site and therefore not subject to further DNA cleavage. The exchange of flanking DNA during the dsb repair event is revealed by the size of *Pac*I restriction fragments (see Figure 4A; see legend to Figure 4 for a more complete description of the repair products; also see George and Kreuzer, 1996).

A remarkable feature of dsb repair with this inverted repeat plasmid is that all detectable repair products are replicated in their entirety by the T4 replication machinery. This and other results led to the formulation of the extensive chromosomal replication (ECR) model for dsb repair (George and Kreuzer, 1996). The replicated nature of the repair products is easily assessed in the T4 system, because T4 incorporates hydroxymethylcytosine residues

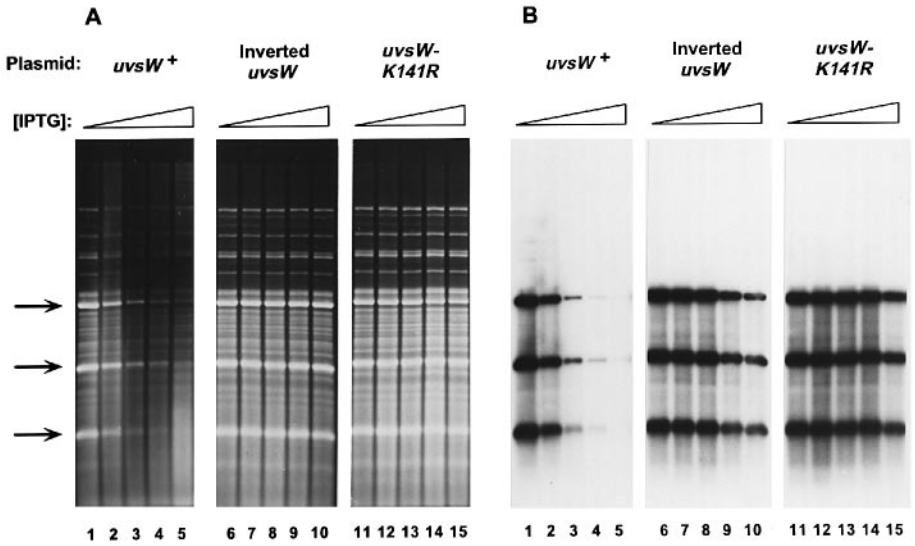

Fig. 3. Effect of inappropriate UvsW expression on T4 origin-dependent replication. *E.coli* BL21(DE3) cells with the appropriate plasmids (see below) were treated with IPTG at the following concentration (μM): 0 (lanes 1, 6, and 11), 50 (lanes 2, 7, and 12), 75 (lanes 3, 8, and 13), 100 (lanes 4, 9, and 14) or 200 (lanes 5, 10, and 15). Cells harbored plasmid pGN003 containing the T4 origin *ori*(*uvsY*) and either plasmid pKCK41 (encoding $UvsW^{+}$; lanes 1–5), pKCK42 (UvsW with a large fragment inverted to disrupt protein expression, lanes 6–10) or pKCK43 (UvsW-K141R, lanes 11–15). After a 19-min incubation, cells were infected with K10-*uvsY*Δ and incubated for 1 h at 37°C. Total DNA samples were isolated and digested with *Ase*I and *Hae*III. This combination of restriction enzymes allows visualization of plasmid DNA only if it has been replicated during T4 infection. T4 replication results in DNA in which every deoxycytosine residue is modified. *Ase*I cleaves such modified DNA, and pGN003 contains three *Ase*I sites. In contrast, *Hae*III cleaves only the unmodified (non-replicated) plasmid DNA, converting it into small fragments that migrate off the gel. T4 genomic DNA is cleaved by *Ase*I into a series of heterogenously sized fragments. The cleaved DNA fragments were separated on a 1% agarose gel and visualized by ethidium bromide staining (**A**) or Southern blot hybridization using a plasmid probe (**B**). Replicated plasmid fragments are indicated by arrows.

during DNA replication. These modified cytosines block the action of most restriction enzymes, including *Hae*III. Because the plasmid has numerous *Hae*III sites, any unreplicated plasmid DNA is cleaved into small fragments that migrate off the gel when *Hae*III is present in the digest. Replicated repair products are resistant to *Hae*III but can be cleaved by *Pac*I, which is one of the few restriction enzymes that cleaves T4-modified DNA.

DNA was prepared from uninfected plasmid-containing cells and from cells infected with various T4 strains, digested with *Pac*I (Figure 4B, odd-numbered lanes) or *Pac*I plus *Hae*III (even-numbered lanes), and analyzed by Southern hybridization. The *Pac*I digestion of plasmid DNA from uninfected cells produced the two expected fragments, and, as expected, the addition of *Hae*III destroyed these two fragments (Figure 4B, lanes 1 and 2, respectively). Upon T4 infection, a massive amount of T4-replicated DNA was generated, as demonstrated by the large amount of *Hae*III-resistant material (Figure 4B, compare lanes 3 and 4). The replicated plasmid DNA consists entirely of products of a dsb repair event (see George and Kreuzer, 1996), and approximately half of the products have undergone exchange for the flanking DNA (Figure 4B, lane 4). As observed previously, a gene *49* mutation reduced the total amount of product without disturbing the ratio of exchange to non-exchange DNA, and also resulted in the appearance of slowly migrating forms that are probably branched DNA (Figure 4B, lane 6; also see George and Kreuzer, 1996). Turning to the possible role of UvsW, a *uvsW* deletion mutation also reduced the total amount of repaired product without disturbing the ratio of exchange to non-exchange DNA (Figure 4B, lane 8). Most importantly, when the gene *49* and *uvsW* mutations were combined, the repair reaction was reduced to levels much lower than those of either single mutant (Figure 4B, lane 10). We conclude that the *in vivo* functions of UvsW and gp49 overlap, arguing that UvsW participates in the resolution of branched DNA structures. Further experiments are necessary to deduce the precise structure of the slowly migrating DNA in the *49* mutant infection and to understand why this DNA form is not generated in the *uvsW* mutant infection.

ssDNA-dependent ATPase activity of GST/UvsW fusion protein

To begin a biochemical analysis of the UvsW protein, we fused the UvsW or UvsW-K141R coding sequence to the 3′ end of glutathione *S*-transferase (GST) under control of the *tac* promoter. The GST/UvsW fusion construct had the expected biological activity, as determined by complementation of the HU hypersensitivity of a T4 *uvsW*Δ phage (data not shown). The two fusion proteins were then partially purified using the affinity tag, as described in Materials and methods. The major band in each protein pool appeared to be the full-length fusion protein, and each pool also had a very similar profile of smaller, minor contaminant bands (data not shown). A Western blot analysis revealed that most or all of these contaminating proteins react with the anti-GST antibody (data not shown), indicating that they consist of degradation products of the full-length GST/UvsW fusions.

Results with the UvsW-K141R mutant described above indicate that the putative Walker ‘A’ motif of UvsW is important for the activity of the protein, strongly suggesting that UvsW catalyzes ATP hydrolysis. Indeed, the protein fraction containing the GST/UvsW fusion was

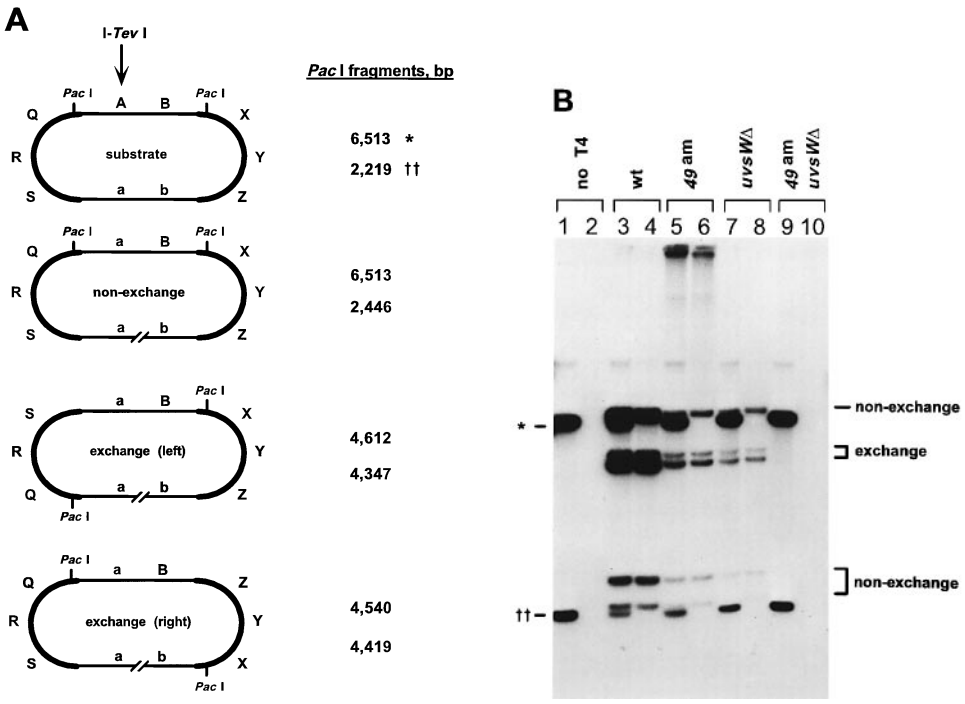

Fig. 4. Dsb repair is abolished in a gene *49 uvsW* double mutant. (**A**) Illustrations of the pJG6 plasmid substrate and three of the possible products of dsb repair. The two nearly identical homologous segments of pJG6 (coordinates 3–2064 and 4300–6397; coordinates are clockwise and relative to the *Eco*RI site at the border of the QRS segment and the AB segment; also see George and Kreuzer, 1996) are shown as thin parallel lines. The upper segment has a 56-bp DNA fragment containing the I-*Tev*I cleavage site (A), while the bottom segment has an unrelated 238-bp fragment (a) at the same location. The upper segment has a 248-bp fragment (B) not present in the bottom segment (site of missing fragment is labeled b). Within each segment, the two heterologies are separated by 506 bp of homology and are flanked to the left by 1079 bp and to the right by 231 bp of homologous DNA. The non-homologous DNA on the left and right sides of the plasmid are indicated by QRS (coordinates 6398–8732) and XYZ (coordinates 2065–4299), respectively. The cleavage sites for restriction enzyme *Pac*I are shown, along with the predicted sizes of the restriction fragments. The 2219-bp (††) and 6513-bp (*) *Pac*I fragments contain the recipient (i.e. cleaved by I-*Tev*I) and donor DNA, respectively. The three products shown have no exchange, an exchange on the left flank (i.e. flipping segment QRS) or an exchange on the right flank (i.e. flipping segment XYZ). The products shown have each undergone a dsb repair event that converts marker A into a (product 1 in the nomenclature of George and Kreuzer, 1996). A second set of products, which undergo conversion of both A and B into a and b (product 2 forms), have the following predicted sizes: 6513 and 2198 bp (non-exchange); 4364 and 4347 bp (exchange left); and 4419 and 4292 bp (exchange right). The double broken line in the lower segment of the three products reflects the fact that the products of dsb repair are long plasmid concatemers rather than monomeric circles (see George and Kreuzer, 1996). (**B**) Total DNA was isolated from uninfected cells (lanes 1 and 2) or from cells 30 min after infection by the indicated T4 phage mutant (lanes 3–10). Odd-numbered lanes contain *Pac*I-digested DNA and even-numbered lanes contain *Pac*I–*Hae*III double digests. Note that the migration of T4-replicated DNA is slightly retarded by the glucosyl residues on the hydroxymethylcytosine residues. The donor and recipient fragments are indicated by * and ††, respectively, and the positions of exchange and non-exchange products are indicated on the right. Internal size markers are provided by the *Pac*I digest of pJG6 DNA from uninfected cells (6513 and 2219 bp). The results in this figure are representative of four independent experiments. In this experiment, the amount of replicated repair products for the *49*, *uvsW* and *49 uvsW* mutants were 23%, 11% and <1% of the wild-type levels, respectively. In the other experiments, the level of product in the double mutant was as high as 5% of the wild-type level but was always much lower than the level in either single mutant infection.

found to contain a potent ATPase activity, whereas the GST/UvsW-K141R protein fraction displayed only ~2% of the activity of the wild-type fraction (Figure 5). The ATPase activity of both protein fractions was only detected in the presence of M13 ssDNA (Figure 5). We conclude that UvsW is a ssDNA-dependent ATPase and that its activity is greatly diminished by mutation of the invariant lysine within the Walker 'A' motif.

Unwinding activity of GST/UvsW fusion protein

Branch migration enzymes such as *E.coli* RuvAB and RecG have a characteristic unwinding activity that acts on small branched duplex DNA but not on similarly sized linear duplexes (Lloyd and Sharples, 1993b; Whitby *et al.*, 1994). We used this assay to ask whether UvsW can unwind branched DNA.

Based on previous analyses of RuvAB and RecG (Parsons *et al.*, 1990; Lloyd and Sharples, 1993b; Whitby *et al.*, 1994), an oligonucleotide substrate consisting of a duplex Y junction and another consisting of a duplex linear with a sequence identical to two arms (left and right) of the Y substrate were prepared (see diagrams at top of Figure 6). In each case, only one strand of the substrate was labeled to simplify analysis of the products. As expected, heat denaturation of each substrate released the labeled single strand, which was unable to reanneal during the course of a mock reaction (Figure 6, compare lanes 1–2 and lanes 11–12). Incubation of the duplex Y substrate with increasing amounts of the GST/UvsW protein resulted in a strong unwinding reaction, with both partial duplex Y and single-strand products being generated (Figure 6, lanes 3–5). The GST/UvsW protein was also able to unwind partial duplex Y molecules that contain only two strands but are otherwise identical to the duplex Y substrate (data not shown). Unwinding activity on the duplex Y substrate was strictly dependent on both ATP and magnesium (Figure 6, compare lane 5 with lanes 9 and 10). The duplex linear oligonucleotide was not

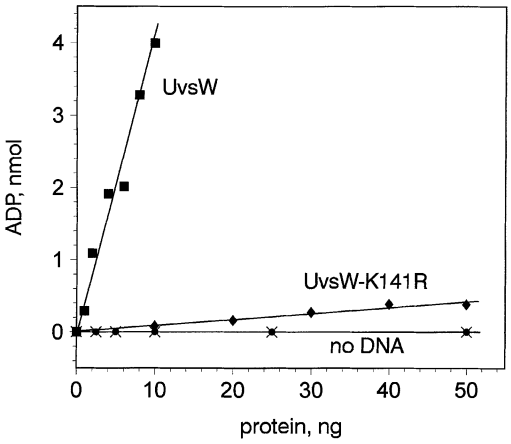

Fig. 5. ATPase activity of GST/UvsW and GST/UvsW-K141R fusion proteins. Reaction mixtures (20 µl) contained the indicated amount of the protein fraction containing either GST/UvsW (■,×), or GST/UvsW-K141R (●, ◆). Reactions contained either single-stranded M13 DNA (0.2 µg;■, ◆) or no DNA (×,●). Each data point represents the average of two determinations.

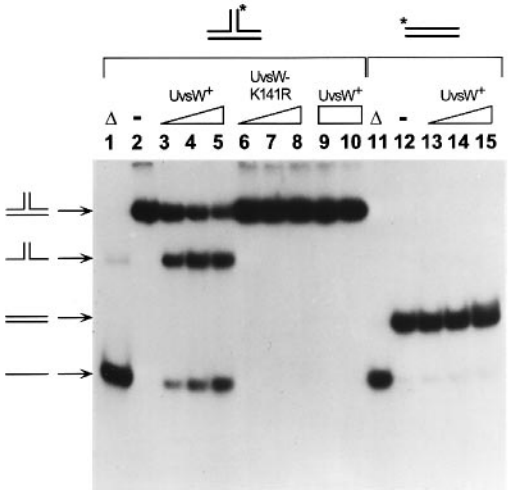

Fig. 6. Unwinding activity of the GST/UvsW fusion protein. Unwinding reactions contained a duplex Y branch substrate (lanes 1–10) or a linear duplex substrate (lanes 11–15). Lanes 1 and 11 contain DNA from controls in which the substrate was heat denatured and then incubated in a mock reaction. Lanes 2 and 12 contain DNA from controls with no added enzyme. Titrations of increasing amounts (50, 200 and 800 ng) of the wild-type GST/UvsW fusion protein were assayed in lanes 3–5 and 13–15. The same increasing amounts of the mutant GST/UvsW-K141R fusion protein were assayed in lanes 6–8. The wild-type GST/UvsW protein (800 ng) was tested in the absence of ATP or Mg^{2+} in lanes 9 and 10, respectively. All products were separated by electrophoresis through a non-denaturing polyacrylamide gel and visualized by autoradiography. The positions of migration of the two substrates and the two possible products are shown on the left.

unwound by the GST/UvsW protein (Figure 6, lanes 13–15), arguing that UvsW recognizes DNA branches. Importantly, the GST/UvsW-K141R protein displayed no detectable unwinding activity on the duplex Y substrate (Figure 6, lanes 6–8). This result provides strong evidence that the branched DNA unwinding activity is intrinsic to UvsW, and further validates the importance of the Walker 'A' motif for UvsW activity.

Discussion

We have explored the function of the T4 UvsW protein, and based on our results, we conclude that UvsW is a functional analog of the *E.coli* RecG protein. First, expression of wild-type UvsW can suppress the growth defect of an *E.coli recG rnhA* mutant. The lack of suppression with the UvsW-K141R protein is consistent with the model that UvsW replaces RecG (rather than RNase HI) because RecG is a helicase. Second, the GST/UvsW fusion protein displayed ssDNA-dependent ATPase activity, which was dramatically reduced by the K141R mutation. Third, the GST/UvsW protein, but not the GST/UvsW-K141R protein, effectively unwound a branched duplex DNA substrate but was unable to unwind a linear duplex of related sequence. Branched DNA unwinding was detected previously with RecG using the same duplex Y substrate (Lloyd and Sharples, 1993b; Whitby *et al.*, 1994).

RecG has also been shown to mediate branch migration of a Holliday junction during *in vitro* reactions (Whitby *et al.*, 1993). Based on the parallels between branch migration and unwinding activities, Whitby *et al.* (1994) argued that the helicase activity of RecG drives branch migration by directing localized unwinding of the Holliday junctions. Although UvsW has not been directly assayed for branch migration activity, it now seems highly likely that branch migration is a key function of UvsW during T4 infections. The branched DNA unwinding activity of the GST/UvsW fusion protein seems particularly indicative of a branch migration enzyme. We have also demonstrated a physiological relationship between UvsW and endonuclease VII (gp49), a nuclease that cleaves branched DNA during T4 infections (Kemper and Brown, 1976; Mizuuchi *et al.*, 1982; Flemming *et al.*, 1993). Single mutations in either *uvsW* or gene *49* modestly reduced the replication-coupled dsb repair reaction, whereas the double *uvsW 49* mutant was severely deficient (Figure 4; see also George and Kreuzer, 1996; Mueller *et al.*, 1996). Interestingly, preliminary results also indicate that a *uvsW*Δ *49*^{ts} double mutant phage is inviable at 30°C, a temperature that is normally permissive for the *49* mutant (J.George, unpublished data).

A variety of previous *in vivo* results are also consistent with UvsW playing a key role in branch migration. Mutations that inactivate the protein reduce phage recombination, repair and mutagenesis (Hamlett and Berger, 1975; Conkling and Drake, 1984; Derr and Drake, 1990), and each of these defects could reflect a perturbation in branch migration of recombination intermediates. Furthermore, sedimentation analyses indicated that *uvsW* mutations result in an unusual structure of the complex phage genomic DNA network that accumulates during infection (Hamlett and Berger, 1975; Wu and Yeh, 1978a,b). More specifically, *uvsW* mutants show a reduction and delay of DNA packaging into phage heads, which could be explained by an excess of unresolved branches (see Flemming *et al.*, 1993).

To summarize our current view of branch processing during T4 infection, endonuclease VII (gp49) clearly plays an important role in resolving branches prior to DNA packaging, and we now believe that UvsW plays a major role in branch migration. It is not yet clear if the two proteins generally act together, as do the RuvAB (branch migration) and RuvC (branch cleavage) proteins of *E.coli* (reviewed by West, 1996). An alternative possibility is that

UvsW and endonuclease VII participate in two different mechanisms of branch processing, perhaps with additional phage-encoded proteins involved in each. A strong precedent for this view is provided by studies of *E.coli*, which currently provides the best developed system for understanding branch processing. At least five *E.coli* proteins—RuvA, RuvB, RuvC, RecG and Rus—are involved in the processing of recombinational branches (reviewed by West, 1996). We currently have an incomplete understanding of the reasons for multiple branch processing enzymes in *E.coli*, and it is not clear whether multiple mechanisms will be the norm in other biological systems. Further work with the phage T4 system may help to resolve these issues.

In addition to its likely role in branch migration, we have strong (albeit indirect) evidence that UvsW acts as an RNA–DNA helicase. First, various repair phenotypes associated with mutational inactivation of *uvsW* closely resemble those of a T4 *rnh* (RNase H) mutant (Woodworth and Kreuzer, 1996). Second, expression of UvsW from a plasmid suppresses the lethality of an *E.coli recG rnhA* double mutant, in which an accumulation of R-loops is believed to cause cell death (Table I; see Introduction). Notably, UvsW-K141R, which is inactive in a DNA unwinding assay (Figure 6), is unable to support growth of the double mutant bacteria (Table I). Third, expression of UvsW inhibits replication from a phage origin at which R-loops form and presumably act as replication intermediates (Figure 3; Carles-Kinch and Kreuzer, 1997). Finally, the similarities between RecG and UvsW also support the prediction that the UvsW unwinding activity functions on RNA–DNA substrates, because RecG has recently been shown to dissociate RNA from R loops (Vincent *et al.*, 1996).

For both RecG and UvsW, the relationship between the putative RNA–DNA unwinding and the branched DNA unwinding activity is of mechanistic and biological importance. The RNA–DNA unwinding activity of RecG was detected on R loops but not on simple RNA–DNA hybrids (Vincent *et al.*, 1996), arguing that a branched form is required for this activity. The biological significance relates to the precise role of RecG in inhibiting cSDR in *E.coli* and of UvsW in inhibiting T4 origin-dependent replication. We believe that the putative RNA–DNA unwinding activity of UvsW is responsible for inactivating T4 replication origins at late times of infection, when UvsW protein is normally produced (see Introduction). Consistent with this model, the inappropriate production of the wild-type UvsW protein at early times effectively blocked origin-dependent replication (Figure 3). UvsW might act on established R-loops, removing the transcript from the DNA template via the unwinding activity. Alternatively, UvsW could inhibit formation of the R-loop by actively opposing the invasion of RNA into the DNA duplex, as has been proposed for the inhibiting role of RecG in cSDR (Hong *et al.*, 1995).

In summary, we have shown that the phage T4 UvsW protein is a helicase that acts on branched DNA and is likely to play a major role in branch migration of Holliday junctions during a T4 infection. UvsW also inhibits T4 origin-dependent replication, presumably by means of an RNA–DNA unwinding activity that removes R-loops from the origin. So far, the known biological and biochemical properties of UvsW strongly resemble those of *E.coli* RecG protein, although the two proteins do not share significant amino acid homology outside of the short motifs common to all DNA helicases.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, alkaline phosphatase, T4 polynucleotide kinase, proteinase K and PEI–cellulose were purchased from various commercial sources. Oligonucleotides were synthesized by the Duke University Botany Department Oligonucleotide Synthesis Facility. Radiolabeled nucleotides were obtained from Amersham Life Science and Dupont NEN and Nytran membranes from Schleicher and Schuell. Cells were grown in either L broth [NaCl (10 g/l), Bacto-Tryptone (10 g/l), and yeast extract (5 g/l)], or M9 media [K_2HPO_4 (7 g/l), KH_2PO_4 (3 g/l), NaCl (0.5 g/l), NH_4Cl (1 g/l), glucose (10 g/l), $MgSO_4$ (0.48 g/l), $CaCl_2$ (11 mg/l), tryptophan (13.3 mg/l), thiamine (0.67 mg/l), $FeCl_3 \cdot 6H_2O$ (27 μg/l) and $MnCl_2 \cdot 4H_2O$ (2 μg/l)] supplemented with casamino acids (2 g/l). Liquid media were supplemented with carbenicillin (200 μg/ml) or ampicillin (25 μg/ml) for selection of pBR322-derived plasmids or with chloramphenicol (10.2 μg/ml) for selection of pLysE or pGN003. L plates used in P1-mediated transduction assays contained the same ingredients as in L broth, as well as Bacto-Agar (13.5 g/l) and kanamycin (50 μg/ml). The EHA plates used to titer T4 phage consisted of Bacto-Tryptone (13 g/l), Bacto-Agar (10 g/l), NaCl (8 g/l), sodium citrate (2 g/l) and glucose (1.3 g/l).

Strains

Bacterial and phage strains are summarized in Table II. *E.coli* strain MIC1020 (*rnhA-339::cat*) was a generous gift from Dr Robert Crouch (National Institute of Health; Itaya and Crouch, 1991), and strain AQ8353 (*recG-258*::Tn*10-kan*) was kindly provided by Dr Tokio Kogoma (University of New Mexico; Hong *et al.*, 1995). The *E.coli rnhA*-deficient strain KCK100 was constructed by a P1-mediated transduction with a MIC1020 donor and NapIV recipient; the desired transductants were selected by chloramphenicol resistance. All T4 strains are derivatives of strain K10 (see Table II).

Plasmids

Plasmid pPH310, a generous gift from Dr Marc Drolet (University of Montreal), is a pBR322 derivative that encodes the *E.coli rnhA* gene under the control of the IPTG-inducible *tac* promoter (Drolet *et al.*, 1995). The plasmid pGS772, kindly provided by Dr Robert Lloyd (University of Nottingham), contains the coding region for the *E.coli recG* gene downstream of the T7 promoter within pT7-7 (Lloyd and Sharples, 1993a). The pLysE plasmid that expresses T7 lysozyme has been described elsewhere (Studier, 1991).

Plasmid pGN003 (G.Nosrati and K.Kreuzer, unpublished results) carries the T4 replication origin *ori*(*uvsY*) inserted between *Eco*RI and *Pst*I sites within the pSU19 multiple cloning site (Bartolome *et al.*, 1991). Plasmid pJG6, the test plasmid for dsb repair, was described by George and Kreuzer (1996).

The T4 *uvsW* gene was cloned downstream of the T7 promoter within pET11d (Novagen) to create the plasmid pHEK4 (H.Kreuzer and K.Kreuzer, unpublished results). First, the *Cla*I site within pET11d was destroyed by cleaving the plasmid with *Cla*I, extending the recessed 3′ ends with Klenow fragment, and religating the newly formed blunt ends. Next, a 5637-bp *Nco*I–*Bam*HI fragment of the vector was ligated to a double-stranded oligonucleotide containing a *Cla*I site, *Nco*I and *Bam*HI sticky ends and ~45 bp of the 5′ end of the *uvsW* gene (5′-CATGGATATTAAAGTACATTTTCACGACTTCAGTCATGTACGCATCGATG-3′/ 5′-GATCCATCGATGCGTACATGACTGAAGTCGTGAAAATGTACTTTAATATC-3′). The resulting plasmid, pHKW01, was linearized with *Cla*I and ligated to the 1903-bp *Cla*I fragment from plasmid pLDΔ60 which contains the remaining 3′ end of the *uvsW* gene (Derr and Kreuzer, 1990). Isolated plasmid DNA was sequenced to determine the orientation of the *Cla*I fragment (pHEK4, proper orientation to create an intact *uvsW* gene; pHEK11, inverted *Cla*I fragment).

Plasmid pHEK4 was used in the site-directed mutagenesis of a potential helicase motif encoded within *uvsW*. First, the *Eco*RI site within the vector sequence of the plasmid was destroyed by partially digesting the DNA with *Eco*RI, gel purifying linear plasmid, extending the 3′ recessed ends with Klenow fragment and religating the blunt ends. Next, plasmid DNA lacking the *Eco*RI site within the vector was digested with *Sal*I and *Eco*RI, and the resulting 7460-bp fragment was

Table II. Bacterial and phage T4 strains

Strain	Genotype	Source
E.coli AQ8353	*recG-258::*Tn*10-kan*	Hong *et al*. (1995)
E.coli BL21	F$^-$ *ompT hsdR*$_B^-$ *hsdM*$_B^-$	Studier *et al*. (1990)
E.coli BL21(DE3)	F$^-$ *ompT hsdR*$_B^-$ *hsdM*$_B^-$ λDE3	Studier *et al*. (1990)
E.coli JG99S	*recA1 relA spoT1 thi-1 deoB13 rpsL*	George and Kreuzer (1996)
E.coli JGF0	F$^-$ *thr-1 leu-6 lacY1 galK2 mtl-1 proA2 his-4 argE3 rpsL31 ara-14 xyl-5 tsx-33 supE44 rfaG::*Tn*10-kan*	J.George and K.Kreuzer (unpublished)
E.coli KCK100	*hsdM*$_K^+$ *hsdR*$_K^-$ *hsdS*$_K^+$ *rgl* (B1$^-$) *rnhA-339::cat*	P1.MIC1020×NapIV, chloramphenicol selection
E.coli MCS1	*supD araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL pro* (uncharacterized proline auxotrophy)	Kreuzer *et al*. (1988)
E.coli MIC1020	F$^-$ *thr-1 leu-6 lacY1 galK2 mtl-1 proA2 his-4 argE3 rpsL31 ara-14 xyl-5 tsx-33 supE44 rnhA-339::cat*	Itaya and Crouch (1991)
E.coli NapIV	*hsdM*$_K^+$ *hsdR*$_K^-$ *hsdS*$_K^+$ *rgl* (B1$^-$)	Nelson *et al*. (1982)
T4 K10	*amB262* (gene *38*) *amS29* (gene *51*) *nd28* (*denA*) *rIIPT8* (*denB-rII* deletion)	Selick *et al*. (1988)
T4 K10-*uvsY*Δ	As K10, *uvsY*Δ1	Kreuzer *et al*. (1988); Kreuzer and Kreuzer (1994)
T4 K10-*49*^{am}	As K10, *amE727* (gene *49*)	Kreuzer and Kreuzer (1994)
T4 K10-*uvsW*Δ	As K10, *uvsW*Δ1	Derr and Kreuzer (1990)
T4 K10-uvsWΔ *49*^{am}	As K10, *uvsW*Δ1 *amE727* (gene *49*)	K10-*49*^{am}×K10-*uvsW*Δ

purified. Two oligonucleotides were designed so that their 15-base 3′ ends annealed to one another (5′-CGATAGTCGACGAAGTCATCAGC-CATCTGAGTTGTCAGAGCAGTTGTTGGAACAATGATAAGAA-TTTTACCTTCATAATTCTCG-3′/5′-GTAGAATTCTTAATCTTC-CAACATCTGCAGGTAGATCTTTAATTCAAGCTTTGCTTGCGCG-ATATTATCTCGAGAATTATGAAGG-3′). The 3′ ends of the annealed oligonucleotides were extended by Klenow fragment, and the resulting double-stranded DNA, containing part of the *uvsW* coding sequence with the amino acid change K141R, was digested with *Sal*I plus *Eco*RI and ligated to the isolated vector fragment to create plasmid pKCK40.

Plasmids pKCK41 and pKCK42 were constructed by ligating the 7538-bp *Bgl*II–*Xba*I fragment from pHEK4 and pHEK11, respectively, to a double-stranded oligonucleotide flanked by *Bgl*II and *Xba*I sites (5′-GATCTTAAGAAATTAATACGACTTACTATAGGGGAATTGT-GAGCGGATAACAATTCCCCT-3′/5′-CTAGAGGGGAATTGTTAT-CCGCTCACAATTCCCCTATAGTAAGTCGTATTAATTTCTTAA-3′). This oligonucleotide contains a single point mutation (–7C to T) within the pET11D T7 promoter which substantially reduces promoter strength (Ikeda *et al*., 1992).

Plasmid pKCK43 was constructed by ligating the 5630-bp *Nco*I–*Bam*HI fragment of pKCK41, which includes the mutated T7 promoter, to the 1954-bp *Nco*I–*Bam*HI fragment of pKCK40, which contains the *uvsW-K141R* mutation.

Plasmid pKCK44 was constructed by ligating *Bam*HI-linearized pBR322 to the 2058-bp *Bam*HI–*Bgl*II fragment from pHEK4 so that the 5′ end of *uvsW* is proximal to the pBR322-*tet*(C) promoter. Plasmid pKCK46 was constructed by ligating the 4465-bp *Nco*I–*Bam*HI fragment from pKCK44, which contains the pBR322 vector, to a 1954-bp *Nco*I–*Bam*HI fragment from pKCK40, which includes the *uvsW-K141R* mutation.

To produce a GST/*uvsW* gene fusion, a *Bam*HI site was first cloned immediately upstream of *uvsW* by ligating the *Ava*I–*Nco*I 3297-bp fragment of pHEK4, which contains the *uvsW* gene, to a double-stranded oligonucleotide containing a *Bam*HI site flanked by *Ava*I and *Nco*I sticky ends (5′-CCGAGCCAGGATCCA-3′/5′-CATGTGGATCCTGGC-3′). The resulting plasmid, pKCK47A, was then digested with *Bam*HI and the 1965-bp fragment containing *uvsW* was gel purified and ligated to a *Bam*HI-linearized pGEX-3X vector (Pharmacia) containing the GST coding sequence downstream of the *E.coli tac* promoter. The 5′ end of *uvsW* is proximal to the GST coding sequence in plasmid pKCK47. To create a GST fusion with *uvsW*–K141R (plasmid pKCK48), the ~4400-bp *Pst*I fragment from pKCK47 (containing the 3′ end of *bla* and 5′ end of GST/*uvsW*) was gel purified and ligated to the 1708-bp gel-purified *Pst*I fragment from pKCK40 (containing the 5′ end of *bla* and the 3′ end of *uvsW* including the K141R mutation).

Gradient plates

Sensitivity of phage to HU was analyzed using gradient plates as described by Woodworth and Kreuzer (1996). EHA agar (25 ml) with HU (750 μg/ml) was poured into square Petri plates that had one edge resting on a pencil. Once the agar solidified, the plate was leveled and a second drug-free layer of EHA agar (25 ml) was added to each plate. *E.coli* lawns were produced by growing strain MCS1 with the indicated plasmid to a density of ~8×10^8 cells per ml, and then plating cells (0.5 ml) in 5 ml drug-free top agar. Aliquots of ~300 T4 plaque-forming units were spotted across the gradient (phage dilutions were titered before use to confirm their concentration).

Origin plasmid replication assays

E.coli strain BL21 (DE3) harboring the indicated plasmids was grown at 37°C in M9 media with vigorous shaking to a density of 4×10^8 cells per ml, and then treated with the indicated amount of IPTG. Cells were further incubated under the same conditions for 19 min (to a cell density of 5.6×10^8 per ml), at which time they were infected with T4 K10-*uvsY*Δ at a multiplicity of three plaque-forming units per cell. The infected cultures were incubated for 3 min without shaking to allow for phage adsorption, and then for an additional 1 h at 37°C with vigorous shaking. Total DNA was isolated as described previously (Kreuzer *et al*., 1988). Linearized pSU19 was labeled by the random-primed method (Boehringer-Mannheim kit) and used as probe for the Southern hybridization of replicated plasmid DNA.

Dsb repair assay

The dsb repair assay was described previously by George and Kreuzer (1996). Briefly, *E.coli* JG99S containing plasmid pJG6 were grown with vigorous shaking in L broth at 37°C to a density of 4×10^8 cells per ml and then infected with the indicated T4 strain at a multiplicity of three plaque-forming units per cell. After allowing phage adsorption for 3 min without shaking, cells were returned to vigorous shaking for 27 min. Total DNA was then isolated as described (Kreuzer *et al*., 1988; infected cells were lysed at 37°C instead of 65°C).

Purification of GST/UvsW fusion proteins

E.coli strain BL21 (without the λ lysogen DE3) harboring pLysE and either pKCK47 or pKCK48 was grown with vigorous shaking in 1 l of L broth at 37°C until the optical density (600 nm) reached 0.6. The cells were then treated with IPTG (1 mM), incubated with vigorous shaking at 37°C for another 30 min, collected by centrifugation and frozen at –80°C. The cells were later thawed and lysed by sonication. A 75% slurry of glutathione–Sepharose 4B (1.33 ml; Pharmacia) was washed with 10 ml ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and the resin was collected by centrifugation. The washed resin was added to the cell extract, incubated at 25°C for 30 min with slow stirring, and the protein-bound resin was then collected by centrifugation at 4°C for 10 min at 5000 r.p.m. in an SA600 rotor. The resin was resuspended in 10 ml ice-cold PBS, slowly loaded into a column at 4°C, and the column was washed with an additional 20 ml of ice-cold PBS. Protein was eluted from the column by incubating the resin for 10 min at room temperature with 10 mM reduced glutathione (in 50 mM Tris–HCl, pH 8). Eluted samples were pooled (~4 ml total) and dialyzed for 24 h into UvsW storage buffer (50 mM Tris–HCl,

pH 8, 50 mM NaCl, 1 mM Na_3EDTA, 5 mM β-mercaptoethanol and 50% glycerol).

ATPase assays

The release of [α-^{33}P]ADP from [α-^{33}P]ATP was measured by thin-layer chromatography on PEI–cellulose (Matson and Richardson, 1983). Reaction mixtures (20 µl) consisted of 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM NaCl, 2 mM dithiothreitol, 100 µg/ml BSA, 0.2 µg single-stranded M13 DNA (if appropriate), 12 nmol ATP, 0.2 µCi [α-^{33}P]ATP (2000 Ci/mmol) and protein as indicated. Reactions were incubated for 15 min at 37°C and stopped by the addition of Na_3EDTA (50 mM). The products were separated by thin-layer chromatography and quantitated by direct radioisotope counting using an AMBIS radioisotope imager.

Unwinding assays

Oligonucleotides 1 (5′-CAAAGTAAGAGCTTCTCGAGCTGCGCTA-GCAAGCCAGAATTCGGCAGCGT-3′, 2 (5′-GACGCTGCCGAATT-CTGGCTTGCTAAAGGATAGGTCGAATTTTCTCATTTT-3′), 3 (5′-AAAATGAGAAAATTCGACCTATCCTTGCGCAGCTCGAGAAGC-TCTTACTTTG-3′) and 4 (5′-CAAAGTAAGAGCTTCTCGAGCTGC-GCAAGGATAGGTCGAATTTTCTCATTTT-3′) were used to construct the duplex Y (oligonucleotides 1, 2 and 3) and duplex linear (oligonucleotides 3 and 4) substrates as described by Whitby *et al.* (1994). Briefly, one oligonucleotide of each substrate was labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP, and the specific activity was determined by measuring incorporated counts using DE81 filter paper (Sambrook *et al.*, 1989; the chemical quantity of oligonucleotide was measured prior to the labeling reaction by UV absorption). The labeled oligonucleotide was separated from unincorporated label by gel filtration through Sephadex G-50. The labeled oligonucleotide was then annealed with equal quantities of the appropriate unlabeled strands, and the duplex Y and duplex linear substrates were purified on a 10% native polyacrylamide gel.

The unwinding reactions (20 µl) contained 10 fmol oligonucleotide substrate, 20 mM Tris–HCl, pH 7.5, 5 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM ATP, BSA at 100 µg/ml, and the indicated amounts of GST/UvsW or GST/UvsW-K141R. After incubating for 15 min at 37°C, reactions were terminated by addition of 0.2% SDS, 17 mM EDTA, 13% glycerol and proteinase K (200 µg/ml). Reaction products were separated by electrophoresis through 10% polyacrylamide gels with TBE running buffer and visualized by autoradiography.

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