The phage T4 UvsW protein has been shown to play a crucial role in the switch from origin-dependent to recombination-dependent replication in T4 infections through the unwinding of origin R-loop initiation intermediates. UvsW also functions with UvSX and UvSY to repair damaged DNA through homologous recombination, and, based on genetic evidence, has been proposed to act as a Holliday junction branch migration enzyme. Here we report the purification and characterization of UvsW. Using oligonucleotide-based substrates, we confirm that UvsW unwinds branched DNA substrates, including X and Y structures, but shows little activity in unwinding linear duplex substrates with blunt or single-strand ends. Using a novel Holliday junction-containing substrate, we also demonstrate that UvsW promotes the branch migration of Holliday junctions efficiently through more than 1000 bp of DNA. The ATP hydrolysis-deficient mutant protein, UvsW-K141R, is unable to promote Holliday junction branch migration. However, both UvsW and UvsW-K141R are capable of stabilizing Holliday junctions against spontaneous branch migration when ATP is not present. Using two-dimensional agarose gel electrophoresis we also show that UvsW acts on T4-generated replication intermediates, including Holliday junction-containing X-shaped intermediates and replication fork-shaped intermediates. Taken together, these results strongly support a role for UvsW in the branch migration of Holliday junctions that form during T4 recombination, replication, and repair.

Homologous recombination plays essential, but seemingly paradoxical, roles in promoting genetic diversity through meiotic recombination and in maintaining genomic stability. The importance of recombination in genomic stability is ensured by its roles in the repair of DNA damage such as double strand breaks and in the restart of stalled replication forks (for review, see Ref. (1)). For all of these processes, the Holliday junction (HJ) is a common feature. HJs can be formed through an enzyme-mediated process that involves the invasion of a single-stranded portion of a DNA into a homologous sequence in another DNA. HJs may also be formed through regression of an inactive replication fork. The branch point of a HJ can migrate through strand exchange, as long as both participating duplex segments are homologous. Branch migration (BM) has been shown to be catalyzed by a number of enzymes including RecG (2) and RuvAB (3) from Escherichia coli, a subset of eukaryotic RecQ enzymes (4, 5), and Rad54 from human cells (6).

Bacteriophage T4 has been used as a model biological system since the early days of molecular biology and has been invaluable in advancing our understanding of many fundamental biological processes. The T4 genome encodes ~300 different proteins, including all essential enzymes at the replication fork, making it a relatively simple organism to study (7). Furthermore, T4 proteins show about as much sequence homology to eukaryotes as they do to prokaryotes (8). For example, T4 DNA polymerase (gp43) shows strong homology to the B family DNA polymerases of the archaean, eucaryal, and viral kingdoms (9); T4 thymidylate synthase and the replisome sliding clamp protein (gp45) show extensive conserved regions with the corresponding proteins from Bacteriadia and Eucarya; and a subunit of the DNA polymerase clamp loader, gp44, is homologous to eukaryotic replication factor C (7).

Replication proceeds through two distinct pathways in T4 infections (10). Origin-dependent replication initiates at specific replication origins in the early stages of infection by assembly of replication complexes onto persistent RNA-DNA hybrids (R-loops). Recombination-dependent replication (RDR) predominates later in infection, and involves the assembly of replication fork complexes onto D-loop recombination intermediates. The same enzymes involved in RDR are also involved in double-stranded DNA break repair, and thus the processes of recombination, replication and DNA repair are all tightly interconnected (11, 12).

The T4 UvsW gene was first identified through mutant phenotypes which included increased sensitivity to hydroxyurea, decreased recombination, increased sensitivity to UV, and decreased UV mutability (13, 14). Some UvsW mutants had dissimilar effects on replication and double-strand break repair, indicating that UvsW might have distinct multiple functions that could be uncoupled (14, 15). Subsequently, it was shown that the UvsW protein could unwind R-loops in an ATP-dependent manner in vitro. Furthermore, expression of UvsW at late times of infection represses origin-dependent replication presumably by unwinding the origin R-loop intermediate (16). In addition, UvsW protein was found to possess a branched-DNA specific helicase activity that was ATP-dependent and abolished by the K141R mutation in the Walker A motif of a...
UvsW-promoted Holliday Junction Migration

RecA-like domain of UvsW (15). Subsequent x-ray crystallography of the N-terminal region of UvsW-K141R confirmed that the protein fits the structural profile of a superfamily II DNA helicase and suggested unique structural domains for DNA binding (17). UvsW has also been shown to function as a 3′ → 5′ helicase and to promote single-stranded DNA annealing (18).

The UvsW protein can complement E. coli recG mnhA double mutants, most likely through unwinding of persistent R-loops in the bacterial chromosome (15). Accordingly, the E. coli RecG helicase shows very similar functional properties to UvsW, including R-loop unwinding, branched-DNA specific unwinding, and a weak 3′ → 5′ helicase activity, though there is only limited structural homology between the two (17, 19). RecG has been shown to promote BM of HJs in T4 in vitro, and to facilitate the regression of stalled replication forks (20, 21).

Double-strand break repair is only partially reduced in T4 mutants lacking gp49 (Endo VII; HJ cleaving endonuclease), but is completely lost in the uvsW/gp49 double mutant (15). This suggests that HJs can be processed by two different pathways in a T4 infection: Endo VII-catalyzed cleavage and a UvsW-promoted HJ resolving activity, most likely involving BM. To date, however, the enzyme(s) responsible for in vivo BM of HJs in T4 has not been identified, although the strand exchange enzyme UvsX and the helicase gp41 (in conjunction with its loader protein gp59) have been shown to promote 3-strand BM in vitro (22). The roles of UvsW protein in recombination, repair and mutagenesis, together with the similarities to RecG, suggest that UvsW could be a HJ BM enzyme. Here we confirm this prediction by showing that UvsW promotes the migration of a HJ through ~1000 bp of DNA, using a novel HJ-containing substrate that should be generally useful. We also show that UvsW can resolve HJs and replication fork intermediates composed of T4-modified DNA generated during T4 infection.

EXPERIMENTAL PROCEDURES

Enzymes—Dda helicase was a generous gift of Dr. Stephen W. White (St. Jude Children’s Research Hospital, Memphis, TN). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). RNase (DNase-free) was obtained from Roche Applied Sciences (Indianapolis, IN) and PreScission protease was obtained from GE Healthcare (Buckinghamshire, UK). Reverse gyrase was purified from Archaeoglobus fulgidus according to a published procedure (23).

Expression Plasmid Construction—The plasmid pKCK47 carries the UvsW gene (GenBank™ accession AF158101.6; Tulane T4-like genome data base) as a GST fusion in a pGEX-3X expression vector (Amersham Biosciences) (15). Repeated DNA sequencing of this construct as well as a PCR product of T4 genomic DNA consistently showed an AG → GA sequence inversion at T4 map position 114045 (update 3/28/2003). Thus, the data base sequence was incorrect, and the codon 457 should read GAC (aspartate) rather than AGC (serine). The plasmid pKCK48 carries a point mutation that causes amino acid substitution K141R in UvsW. To produce the in-frame expression constructs described below, both plasmids were modified by insertion of a cytosine two bases upstream of the UvsW start codon using a QuikChange™ site-directed mutagenesis kit (Stratagene). The plasmids were then digested with BamHI, and the resulting UvsW- and UvsW-K141R-containing fragments were ligated to BamHI-digested plasmid pGEX-6p-1 (Amersham Biosciences) producing the UvsW-containing plasmid pMRW47–6p, and the K141R-containing plasmid, pMRW48–6p. These constructs generated glutathione S-transferase (GST)-UvsW fusions under control of the IPTG-inducible Ptac promoter. The linker region of pGEX-6p-1 encodes a unique amino acid sequence that is cleaved by Prescision protease, allowing the selective cleavage of GST from the fusion products. The residual peptide GLPLGST remains attached to the N-terminal end of the purified proteins following cleavage.

A version of pGEX-6p-1 that had its ColE1-based origin of replication replaced with an R6K γ replication origin was constructed as follows. Plasmid pGEX-6p-1 was first digested with AlwNI and PflMI, producing two fragments, the smaller of which contained only the ColE1-based replication origin and flanking, non-coding, sequences. To obtain an R6K γ replication origin sequence with PflMI- and AlwNI-ligatable ends, the R6K γ-containing plasmid pGPS4 (New England Biolabs) was used as a template for PCR amplification using primers flanking the origin sequence (position 369 through 678) but designed to produce PflMI- and AlwNI-digestable ends. The purified PCR product was then digested with PflMI and AlwNI, repurified, and ligated to the purified, large AlwNI/PfMI pGEX-6p-1 digestion fragment (minus the ColE1 replication origin). The resulting plasmid (pMRW3) was transformed into a π-protein-expressing strain of E. coli, BW23322 (Δ(argF-lac), ΔuidA4::pir-116, rpoS396, rph-1, hsdR514, creC510, robA1), selected with ampicillin and confirmed by DNA sequence analysis.

To produce a UvsW-expressing version of pMRW3, both pMRW3 and pMRW47–6p were digested with Bsal and EcoNI. The gel-purified fragment containing the R6K γ replication origin from pMRW3 was then ligated to the UvsW-containing fragment of pMRW47–6p using T4 DNA ligase. The resulting plasmid (pMRW7) was obtained after transformation into BW23322 and confirmed by sequence analysis.

UvsW Protein Purification—E. coli strain BW23322 containing the pMRW7 plasmid was grown at 37 °C in 1 liter of Luria broth (LB) containing carbenicillin (100 μg/ml) with vigorous shaking to an A600 of ~1.0, at which point IPTG was added to 1 mM. After 1 h, cells were harvested by centrifugation at 4,000 × g (4 °C) and resuspended in 8 volumes of PBSX (10 mM Na2HPO4, 2 mM KH2HPO4, 3 mM KCl, 500 mM NaCl, pH 7.3) containing one Complete™ (Mini) Protease Inhibitor Mixture Tissue culture pellet (Roche Applied Science). Resuspended cells were frozen in dry ice/ethanol and either stored at −80 °C or used immediately as follows. After thawing, the cells were treated with Triton X-100 (0.2%) and lysozyme (1 mg/ml), refrozen and thawed, and then sonicated. Cell debris was removed by centrifugation at 45,000 × g for 20 min, followed by filtration through a 0.22-μm filter. ATP (2 mM) and Mg2+ (4 mM) were added to the homogenate which was incubated at 37 °C for 5 min. Glutathione-Sepharose 4B resin was then added and gently mixed for 1.5 h at 4 °C. This suspension was placed in a column, washed three times with PBSX, and then treated with RNase (5 μg/ml) by mixing one bed volume of an RNase-TE buffer (10 mM Tris-
UvsW-promoted Holliday Junction Migration

**Oligonucleotide Substrates—**DNA unwinding substrates (Table 1) were made by annealing the following oligonucleotides in the appropriate combinations, generally as described (24, 25). Ao (5'-AGCTGCGCAATTTCGCTGCTAAAGGATAGTGCAATTTCTTATT-3'), Bo (5'-CAAAGTATGAGCTTCTCGAGCTTGCGCTAGAAGGACGGT-3'), C0 (5'-TCTTTGGCCCAATAGCCAGGTTTACCCCGCCGACGTTCGAAAGCTTACCTT-3'), Do (5'-AAATAGAAGAATTTGCACCCTACCTTGGGTGACC-TCATATTGCCGCAAG-3'), E0 (5'-AAAAGACCAGGATCTCTGCTAGAGCTTTTACTTGT-3'), F0 (5'-AAGATAGATGAGCTTATTTCGTCTT-3'), G0 (5'-TTACCGAAGGCTCCGCTGTAGGCTT-3'), H0 (5'-AAATAGAAGAATTTGCACCCTACCTTGGGTGACC-TCATATTGCCGCAAG-3').

All oligonucleotides were purified by denaturing PAGE with gel extraction prior to annealing and, after annealing, by non-denaturing PAGE with electrophoresis and dialysis into 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and 50% glycerol. Protein concentration was determined (Bio-Rad protein assay), and samples were stored at −20 °C. The overall yield was ~600 μg.

The UvsW-K141R protein was prepared as follows. A 0.5-liter LB culture of BL21/pLysS containing pMRW48–6p was grown to an OD600 of ~1.1 and induced with IPTG at 0.5 mM. The protein purification was essentially the same as that used for UvsW, except that K141R was eluted from the column using HPB. The overall yield was 1.4 mg.

**Oligonucleotide-based Unwinding Assay—**Reactions were carried out in 20 μl of helicase reaction buffer (30 mM Tris-HCl (pH 7.5), 40 mM sodium acetate, 1 mM DTT, 5% glycerol, and 0.1 mg/ml bovine serum albumin) containing MgCl2 and ATP as indicated. Typically, substrates were added to yield concentrations of 0.5 nM (10 fmol per reaction), followed immediately by the addition of enzyme or control solution. Reactions were incubated at 37 °C for 15 min and then terminated by addition of a 2-μl stop buffer (100 mM Tris-HCl (pH 7.5), 50 mM EDTA, 2% SDS, 5 mg/ml proteinase K, 50% glycerol, 0.1% bromphenol blue, and 0.1% xylene cyanol) containing 50 nM of the unlabeled version of the substrate used for each particular reaction. The samples were incubated for 30 min at 30 °C and then separated on 7.5% polyacrylamide gels using 0.5 × TBE (44.5 mM Tris-HCl, 44.5 mM borate, 1 mM disodium EDTA) at 4 °C (typically at 12.5 V/cm). Gels were fixed in 10% acetic acid/methanol, dried briefly, exposed to a PhosphorImager screen overnight and visualized using PhosphorImager and ImageQuant software (Molecular Dynamics).

**DNA-trioxosalen Cross-linking—**DNA substrates (25–50 ng/μl) were treated with trioxosalen (final concentration 0.2 mM) and were exposed to a long-wave UV source for 20 min at room temperature.

**Double Holliday Junction Synthesis—**The Double Holliday Junction Substrate 2 (DHJS-2) was synthesized utilizing methods developed by Plank and Hsieh (26). Briefly, A/B and B/A large heterodimers were prepared by annealing and linking purified ssDNA, using *Archaeoglobus fulgidus* reverse gyrase as described (27). The reactions were then stopped by the addition of EDTA to 10 mM and SDS to 1%, and incubated at 80 °C for an additional 5 min. KCl was added to 500 mM, and the solution was cooled on ice for 15 min to precipitate the KDS and protein. The precipitate was removed by centrifugation at 20,000 × g for 20 min, and the cleared supernatant containing the DNA was loaded onto a Qiagen DEAE column. The column was washed with a solution containing 10 mM MOPS (pH 7.0), 1 mM NaCl, 4 mM urea, and 30% ethanol to remove any remaining ssDNA circles (28), and the large heterodimers were then eluted per kit instructions (Qiagen, Inc.). The two large heterodimers were then annealed and linked to each other using the same reaction conditions as above. This reaction was then stopped with the addition of EDTA to 10 mM and SDS to 1%, and the DNA was extracted with phenol/chloroform and precipitated with ethanol. The substrate was then dissolved in 10 mM Tris (pH 7.9), 0.1 mM EDTA and spectrophotometrically quantified.

**Holliday Junction Branch Migration Assay—**DHJS-2 was digested simultaneously with BamHI and AlwNI for 1 h at 30 °C in NEBuffer 2 (10 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 50 mM NaCl, 1 mM DTT). BM assays were carried out at 37 °C using the digested DHJS-2 in 20 μl of helicase reaction buffer (30 mM Tris-HCl, pH 7.5, 40 mM sodium acetate, 1 mM DTT, 5% glycerol, and 0.1 mg/ml bovine serum albumin) containing MgCl2 and ATP (or analogue) at the concentration indicated. Typically, DNA substrate concentrations were 7.5–15 ng/μl and substrate addition was followed immediately by the addition of enzyme or control solution. Reactions were terminated by addition of 2 μl of (10×) stop buffer (20% Ficoll, 1% SDS, 100 mM MgCl2, 10 μg/ml ethidium bromide, 0.1% bromphenol blue, and 0.1% xylene cyanol) and 2 μl of proteinase K (5 mg/ml). Following incubation at 30 °C for 30 min, the products were separated on a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were run overnight at 4 °C at 4 V/cm, destained, and the DNA was visualized using an Alphalager (Alpha Innotech Corp.).

**T4 Infection and DNA Purification—**Bacterial cells (*E. coli* CAG12135 derivative: *acrA::Tn10-kan, recA::Tn10-cam*, and *recD*) were grown with vigorous shaking at 37 °C to an A560 of 0.5 (~4 × 108 per ml), and infected with T4 strain K10-49m (amB262 [gene 38], amS29 [gene 51], nd28 [denA]), rIIPT8 (rII-
UvsW-promoted Holliday Junction Migration

denB deletion), amE727 (gene 49)] at a multiplicity of 6 PFU per cell. After 4 min at 37 °C without shaking, cells were incubated for an additional 28 min with shaking. Infected cells from 1 ml of culture were collected by centrifugation at 13,000 rpm for 2 min, and immediately resuspended in 300 µl of SDS lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 100 mM NaCl, 0.2% SDS). Proteinase K was added to 0.5 mg/ml, and the suspension was incubated at 65 °C for 2 h. Total nucleic acid was extracted sequentially with phenol/chloroform-isoamyl alcohol (24:1), and dialyzed overnight at 4 °C against TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA).

Two-dimensional Gel Analysis of T4 Replication Intermediates—Purified DNA from T4 infections was treated with the restriction enzyme PacI in 1× restriction buffer (50 mM NaCl, 10 mM Tris-HCl (pH 7.8), 3.5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml bovine serum albumin) at 37 °C overnight. The restriction digestion reaction was then supplemented to give the following concentrations: 1 mM ATP, 20 mM Tris-HCl, pH 7.8, and 68 nM purified UvsW (unless otherwise indicated). Reactions were incubated at 37 °C for the indicated time, followed by sequential DNA extraction with phenol/chloroform-isoamyl alcohol (24:1).

Two-dimensional agarose gel electrophoresis was carried out according to the procedure of Friedman and Brewer (29). Briefly, the first-dimension gel was a 0.4% agarose gel run in 0.5× TBE buffer for 30 h at 0.75 V/cm at room temperature. The desired gel lane was sliced from the first-dimension gel and cast across the top of the second-dimension 1% agarose gel, which was run in 0.5× TBE buffer containing 0.3 µg/ml ethidium bromide for 16 h at 4.5 V/cm at 4 °C. For Southern hybridization, agarose gels were transferred to a Nytran Supercharge nylon transfer membrane (Schleicher & Schuell BioScience, Inc) by the downward sponge method (30). The probe for the Southern blots consisted of a PCR fragment containing T4 origin ori(34) (T4 map positions 149.172–152.033 kb) labeled with [α-32P]dATP by using the Random Primed DNA Labeling kit (Roche Applied Sciences).

RESULTS

Cloning and Expression of UvsW—UvsW and UvsW-K141R had been cloned and expressed as GST fusion proteins previously by this laboratory (15). The purified GST-UvsW wild-type fusion protein possesses a branched-DNA specific and ATP-dependent helicase activity, and the lysine to arginine substitution (in the Walker A motif) abolished both ATPase and helicase activities.

We sought untagged versions of the proteins for further biochemical characterization. To this end we cloned UvsW and the UvsW-K141R mutant into a GST fusion expression vector containing a PreScission protease (Amersham Biosciences) recognition site in the GST linker region. PreScission protease also has a GST fusion tag, allowing it to be used on-column to cleave glutathione-bound fusion proteins containing the recognition site. The cleaved protein product is then eluted without the need for glutathione, leaving the protease bound to the column. This strategy worked well for generating substantial amounts of the highly purified UvsW-K141R mutant protein (Fig. 1, lane 1). Attempts to obtain the wild-type UvsW protein using this system, however, were hampered by plasmid instability and low copy number. Because UvsW is known to unwind R-loops at replication initiation sites, we reasoned that replacing the R-loop-dependent ColE1-based plasmid origin with an R6K γ-based replication origin might improve UvsW productivity. The R6K γ origin uses the π initiator protein with no R-loop intermediate (31). The plasmid was found to be stable in strain BW23322, which encodes a mutated version of π (pir-116) that results in an increased plasmid copy number (32).

Initially, we found that UvsW was highly contaminated with RNA and a co-purifying protein which was identified by mass spectral analysis to be the chaperonin GroEL. Both contaminants were effectively eliminated by including an RNase treatment and an incubation with ATP/Mg²⁺ during the purification process (33). Using these techniques and additional modifications of the standard extraction procedure, we were eventually able to obtain ~600 µg of highly purified, soluble UvsW protein from 1 liter of culture (Fig. 1, lane 2).

Unwinding of Oligonucleotide Substrates—The GST-UvsW fusion protein was previously shown to unwind a blunt-ended, fully duplex, branched-DNA Y substrate in the presence of ATP (15). We began by analyzing the unwinding activity in more detail using the oligonucleotide-based substrates shown in Fig. 2. As in previous studies (15), UvsW was able to unwind a duplex DNA Y substrate, but not a blunt-ended linear DNA substrate (Fig. 2). We did not detect significant unwinding of double-stranded substrates with either 5′ or 3′ single-stranded overhangs (the latter contrary to an earlier report (18)), or double-stranded DNA with a flayed single-stranded end (Fig. 2). In contrast, UvsW efficiently unwound the static X-junction into flayed duplexes (Fig. 2B). At an enzyme/substrate ratio of 2:1,
UvsW unwound essentially 100% of the static X-junctions in less than 1 min (data not shown). As expected, the 5′ → 3′ helicase Dda was able to unwind the 5′ single-strand overhang substrate and the flayed substrate, but was essentially inactive on the other substrates.

**UvsW Catalyzes Branch Migration of Holliday Junctions**—The above results suggest that UvsW may catalyze DNA BM.

We directly tested BM activity using a novel substrate and recently developed method. An *in vitro* synthesized substrate, DHJS-2, consists of two double-stranded DNA circles combined to form two HJs (Fig. 3) (26). The two DNA circles are largely homologous except for the ~300-bp duplex regions between the two HJs (red and blue) and two ~450-base single-stranded bubble segments (green; identical, non-complementary sequence). BM of the HJs in intact DHJS-2 is prevented by topological constraints. However, digestion of DHJS-2 with restriction enzymes AlwNI and BamHI generates two fragments, both of which contain a HJ that can migrate in one direction only (through the two homologous arms) (Fig. 4A). The smaller fragment (III in Fig. 4) can branch migrate through duplexes of unequal length, 1090 and 1032 bp. The larger fragment from DHJS-2 digestion (II in Fig. 4) can branch migrate through ~350 bp before encountering a non-complementary single-stranded bubble region where, we believe, BM terminates as an irreversible hemi-catenane.

DHJS-2 can be made in substantial quantities and can therefore be used at concentrations sufficient for visualizing with ethidium bromide staining after agarose gel separation. Fig. 4B illustrates some of the properties of this substrate relevant to our BM assay. Untreated DHJS-2 migrates as two main bands, representing covalently closed and nicked forms (lane 1). Upon digestion with BamHI and AlwNI, the two single HJ-containing fragments II and III are produced (lane 4). When heated briefly to 65 °C, the HJs migrate spontaneously so that the larger fragment (II) becomes trapped as a hemi-catenane migrating more slowly, and fragment III resolves into the two expected 1032- and 1090-bp duplex fragments (lane 6). Digestion of the precursor plasmids pDHJS AS* and pDHJS BS* provides duplex markers that exactly co-migrate with the resolved III fragments (lanes 7 and 8), excluding the possibility that the resolved bands result from melting of the heterologous ends. As expected, inter-strand crosslinking of fragments II and III with trioxsalen (immediately following digestion) prevents BM upon heating of the fragments (compare lanes 5 and 6).

Spontaneous BM of the HJs in DHJS-2 can occur as soon as the topological constraints have been removed by restriction enzyme digestion. The rate of spontaneous migration is very sensitive to temperature, ionic strength, and Mg$^{2+}$ concentration (34). Under our conditions spontaneous BM was robust in the absence of Mg$^{2+}$, much slower at Mg$^{2+}$ levels as low as 2–3 mM, and undetectable at Mg$^{2+}$ concentrations of 10 mM or higher. Spontaneous BM during long periods of gel electrophoresis was prevented by running at 4 °C and including ethidium bromide (0.5 μg/ml in both gel and buffer).

When DHJS-2 fragments II and III were incubated for 15 min at 37 °C in the presence of 3.5 mM Mg$^{2+}$ and 1 mM ATP, there is no discernible resolution of fragment III into the two small products (Fig. 5, lane 1). However, limited spontaneous BM apparently occurred, resulting in some of fragments II and III migrating slightly slower in the gel (compare Fig. 5, lane 1 with Fig. 4, lane 4 and 5; also see below). Increasing concentrations of UvsW allowed complete resolution of fragment III to the two expected small fragments and fragment II to the slowly migrating hemi-catenane form (Fig. 5, lanes 2–7). The resolution products of fragment III can be detected as early as 1 min after start of incubation with UvsW, suggesting that UvsW can promote BM at rates of roughly 20 bp/sec. One caveat, however, is that the earliest resolution products could be derived from a...
UvsW-promoted Holliday Junction Migration

subpopulation of fragment II in which the HJ has already spontaneously migrated toward the resolving end of the fragment prior to UvsW action.

The BM activity of UvsW was largely inhibited when ATPγS was substituted for ATP (Fig. 5, compare lane 7 with lanes 8 and 9), indicating that ATP hydrolysis is required for this activity.

The low levels of BM seen may result from partial hydrolysis of ATPγS by UvsW (not tested). When ADP was substituted for ATP, UvsW-catalyzed BM did not occur (Fig. 5, lanes 10 and 11). Interestingly, the limited amount of spontaneous BM was also inhibited, as evidenced by the tighter banding patterns of fragment II and III (compare lanes 1 and 10). Even in the presence of ATP, the K141R mutant of UvsW was unable to stimulate BM, and also appears to stabilize the HJs against spontaneous BM (lanes 12–14). The well characterized T4 helicase Dda, in the presence of ATP, had no detectable effect on BM (compare lanes 15–17 with lane 1), even when the enzyme to substrate ratio was increased to 200:1.

The BM activity of UvsW increased disproportionately when the enzyme to substrate ratio was increased from 25:1 to 50:1 (Fig. 5, compare lanes 6 and 7). This effect is reproducible (and confirmed by band intensity quantification) and does not appear to be due to simple dilution effects on enzyme stability or activity. One possible explanation is that the single-stranded bubble region of fragment II acts as an inhibitor through non-productive binding of the enzyme; eventual saturation of the single-stranded DNA with increasing enzyme concentration would then explain the sudden increase in UvsW-dependent BM activity. In support of this explanation, we found that UvsW BM activity could be inhibited by adding single-stranded DNA (data not shown).

**UvsW Binding Can Inhibit Spontaneous Branch Migration**—The above experiments suggested that bound UvsW can stabilize HJs against spontaneous BM. We therefore examined the...
effects of UvsW, UvsW-K141R and Dda helicase on the HJ substrates under solution conditions that favor spontaneous BM (Mg$^{2+}$ reduced to 0.5 mM). In reactions without protein, spontaneous BM was indicated by the altered migration of fragments II and III on the gel and a gradual appearance of the two resolved fragment III bands (Fig. 6). Resolution of fragment III was nearly complete after 30 min at 37 °C, indicating an overall spontaneous BM rate of roughly 20 bp/min (Fig. 6, lanes 1–6). When UvsW or UvsW-K141R were included without added ATP (molar ratio of enzyme to HJ of 58), spontaneous BM was not detectable (Fig. 6, lanes 7–16). This result strongly suggests that specific binding of either enzyme to the HJ itself prevents BM. This binding apparently requires little or no Mg$^{2+}$, because UvsW-K141R still prevents BM under conditions in which all Mg$^{2+}$ (carried through from restriction digestion) has been theoretically chelated by EDTA (data not shown). Dda did not slow spontaneous BM at an enzyme to HJ ratio of 58:1 (lanes 17–21) or 290:1 (data not shown).

**UvsW Unwinds Bona Fide T4 Recombination and Replication Intermediates**—T4 DNA is highly modified in vivo by hydroxymethylation and glucosylation of cytosine residues, and this modified DNA should also be a substrate for UvsW if it catalyzes BM in vivo. Various recombination and replication intermediates of T4 DNA can be separated and visualized as particular arcs by two-dimensional agarose gel electrophoresis and Southern hybridization with a DNA probe for the 6.2 kb ori(34)-containing PstI restriction fragment (Fig. 7). The X and Y arcs consist of HJ-containing intermediates and replication fork-shaped intermediates, respectively (29). During an infection by an EndoVII-deficient strain of T4, an unusually high level of X-shaped intermediates are generated as a consequence of the inability of this variant to resolve HJs generated from DNA replication and recombination. We treated such purified DNA in vitro with purified UvsW for increasing incubation times. In the presence of ATP, X structures decreased over time and were essentially gone by 30 min, but no resolution was detected when ATP was absent (Fig. 7). These results indicate that UvsW promotes ATP-dependent BM of HJs in T4-modified DNA to the ends of the restriction fragment. The Y arc, containing replication fork-like intermediates, also diminished over time in the presence of ATP and UvsW (Fig. 7). This result suggests that UvsW promotes replication fork regression of the Y structures to form HJ-containing intermediates, followed by subsequent resolution to linear DNA. Because the end-products of this UvsW-mediated reaction were not identified in this experiment, we cannot exclude the possibility that UvsW promotes resolution of fork structures through another mechanism (e.g. unwinding of the duplex parental segment).

**DISCUSSION**

Previous biochemical studies of the T4 protein UvsW have been hindered by difficulties in obtaining usable amounts of the
Our laboratory previously purified a GST-UvsW fusion protein (15) and more recently, a hexahistidine-tagged version of UvsW was successfully purified (18). Here we report a purification scheme based on GST affinity tagging and on-column affinity tag cleavage. This method allowed us to purify 600 μg of un-tagged, wild-type UvsW for biochemical studies. An important feature of the method involves substitution of the ColE1 origin of replication of the plasmid vector with an R6K-γ replication origin, so that plasmid replication occurs without an R-loop initiation intermediate.

We confirmed the branched-DNA specific helicase activity of UvsW using oligonucleotide based substrates. Under our conditions, UvsW showed no helicase activity on three non-branched DNA substrates, including duplex DNA oligomers with 3’ or 5’ single-stranded overhangs and a duplex with blunt ends. UvsW has been reported by Nelson and Benkovic (18) to unwind duplex DNA oligomers with a 3’ single-stranded overhang. An additional discrepancy is that Nelson and Benkovic found a strong stimulation of UvsW-promoted branched DNA unwinding by gp32, but we found that gp32 was not stimulatory for either UvsW-mediated oligonucleotide unwinding or BM activity (data not shown). The unwinding activities of UvsW reported by Nelson and Benkovic in the absence of gp32 were dramatically weaker than in our experiments, and the oligonucleotide substrates were different between the two studies. Additional experiments are necessary to resolve these discrepancies and determine whether UvsW is strictly dependent on a branch point for activity.

While UvsW showed no unwinding activity using a duplex substrate with flayed (non-complementary) single-stranded ends (Fig. 2B, lanes 3 and 4), it was capable of unwinding the Y shaped substrate constructed by annealing this flayed substrate with an oligonucleotide complementary to the flayed ends (Fig. 2B, lanes 8 and 9). The activity was both ATP-dependent and sensitive to Mg²⁺ concentration (data not shown). Unwinding activity with this substrate was incomplete under our standard reaction conditions and was unexpectedly inhibited by increasing the concentration of enzyme. This effect was reproducible and may be relevant to a proposed mechanism of unwinding/BM (see below). In comparison, unwinding of the HJ-like substrate was fast and very efficient. This substrate is best described as a static X-junction because, unlike a true HJ, the branch point cannot migrate due to non-homologous arms. Unwinding of this substrate produced two different detectable flayed products when the substrate was labeled on only one strand (Fig. 2B, lanes 13 and 14). This result indicates that the enzyme has no preference for how it orients on the branch point.
point. In addition, no single-stranded products were formed from the static-X junction, consistent with the lack of activity of UvsW on flayed or blunt-ended duplex substrates. We conclude that UvsW strongly prefers branched DNA with at least two duplex arms, with the HJ-like substrate being most highly preferred.

Based on genetic studies, UvsW was implicated in the generation or processing of recombination intermediates such as HJs (15). The branched-DNA specific unwinding activity and strong preference for the static-X structure described herein is consistent with UvsW acting as a HJ BM enzyme. We examined this possibility using a novel BM assay developed as a modification of a previously described method (26). The basis for this assay is a double HJ containing substrate, DHJS-2, synthesized in vitro. The two HJs are constrained from migrating by regions of heterology in one direction and by topological constraints in the other. Upon digestion with the appropriate restriction enzymes, two HJ-containing fragments are released and BM within these fragments can be readily detected by formation of resolution products. Using this assay, we demonstrated that UvsW efficiently promotes BM of HJs through at least 1000 bp under our conditions. This activity is very sensitive to [Mg$^{2+}$], with concentrations greater than 3.5 mM (when [ATP] = 1 mM) becoming progressively more inhibitory, and complete inhibition evident at about 10 mM Mg$^{2+}$ (data not shown). This enzymatic activity is dependent on ATP, cannot be supported by ADP, and is only weakly supported by ATPγS (Fig. 5).

The UvsW-K141R mutant contains a mutation in the Walker A motif and is totally inactive for BM (Fig. 5). This mutation inactivates one of two RecA-like domains found in UvsW, significantly inhibiting ATP hydrolysis (15). The loss of BM activity in this mutant is consistent with models in which two functional RecA domains are required for translocation of a monomeric helicase (35). The UvsW-K141R mutant appears to stabilize HJs against spontaneous BM in the presence of ATP arguing that K141R can bind HJs but cannot translocate (branch migrate). Similarly, in the absence of ATP, either wild-type UvsW or UvsW-K141R also strongly inhibit spontaneous BM. These results argue that specific binding of UvsW to HJs does not require ATP binding or hydrolysis, nor does it require [Mg$^{2+}$] higher than 0.5 mM.

We have also shown that purified UvsW can resolve both T4-modified DNA X and Y structures generated during a T4 infection (Fig. 7). The X structures are presumably HJs generated through either RDR or recombinational repair, while the Y structures are simple replication fork intermediates. Under our in vitro conditions with no additional proteins, X structures presumably resolve into linear forms when the HJ migrates off the end of the restriction fragment. The diminishing intensity of the Y arc with UvsW incubation time parallels that of the X arc, implying that these structures are also being resolved by UvsW (Fig. 7B). One explanation is that the replication fork structures are resolved by UvsW into HJ X structures, which are then resolved into linear monomeric forms. Regression of stalled replication forks has been inferred to be one pathway through which stalled replication forks are processed in E. coli (1, 36).

The E. coli RecG protein has also been shown to catalyze BM and regression of stalled replication fork-like structures in vitro (20, 37). While UvsW and RecG share little structural homology, the remarkably similar functional properties of the two suggest that they catalyze BM through a similar mechanism. The crystal structure of RecG suggested a model of RecG-promoted HJ BM (37, 38). In this model, RecG binds the HJ in an orientation that directs translocation along one arm. Two homologous DNA side arms are directed through DNA binding channels in the enzyme that are only large enough for one strand of each arm, and as the enzyme translocates along the directional arm, it unwinds and subsequently anneals the second strand of each side arm to each other. An important component of the model is a protein wedge domain, which directs HJ binding and steers the second strands of the side arms to facilitate their annealing. As previously described by others (37, 38), this mechanism can explain HJ BM, regression of replication forks into HJs, and unwinding of branched oligonucleotides (see Fig. 8). While no structure similar to the RecG wedge domain has been reported for UvsW, the crystal structure of a UvsW-branched DNA complex has yet to be determined and a functionally related domain may ultimately be found. This model is based on only one functional UvsW (or RecG) unit binding each HJ, oriented with respect to one directional arm. The activity would presumably be more efficient if two functional enzymes bound per HJ, as long as they were on opposing arms. However, if two enzymes bind such that one is on a directional arm and the other on a side arm, translocation will be effectively blocked. This model might explain the results observed with the Y oligonucleotide substrate used in this study, where unwinding was inhibited at higher enzyme concentrations. The Y structure that we used might allow binding of more than one enzyme per junction, but since there are no truly opposing arms in this structure, such binding would be counterproductive for unwinding.

In our BM assay we observed essentially complete resolution of a HJ-containing structure (fragment III) by apparent unidirectional migration through ~1000 bp of DNA. If we assume that UvsW binds the HJs of our substrate randomly with respect to orientation on the HJ, there will be a 50% probability of moving the HJ in either direction. When bound in the productive orientation, complete resolution would occur if the enzyme is highly processive. If UvsW activity is more distributive, multiple cycles of productive and counterproductive BM would occur before BM happens to reach the DNA ends and release the product fragments. Clearly, the rate of UvsW-catalyzed BM would be affected by conditions that affect processivity. For these reasons, and the fact that the starting position of BM in our substrate may not be fixed, measuring the absolute rate of UvsW-promoted BM using our assay is problematic. Nevertheless, assuming the HJs in our substrate are relatively immobile prior to the assay and that the enzyme is highly processive, we calculated an approximate rate of UvsW-promoted BM of 20 bp/s (data not shown). For comparison, the rate of RecG- and RuvAB-promoted BM has been estimated to be 8–40 bp/s (39) and 10 bp/s (40), respectively, while the rate of RecA-driven strand exchange was estimated to be 3–10 bp/s (41, 42).

UvsW acts as a regulator of R-loop initiated replication and also plays roles in T4 replication, recombination and repair,
very likely through its ability to catalyze BM of HJs. UvsW could play any of several possible roles in these processes. Models for double-strand break repair and daughter-strand gap repair involve the formation of D-loop structures that could be stabilized by HJ formation and migration. RDR also requires the formation of a D-loop intermediate which, again, could be stabilized by UvsW extending the paired region by BM. UvsW might also stimulate 3-strand BM, a reaction that could promote conservative RDR (e.g. synthesis-dependent strand annealing). Finally, BM of recombinational HJs may be critical for branch resolution, as suggested in the Introduction.

Additional roles for UvsW BM activity can also be envisioned. The restart of stalled replication forks through pathways involving fork regression would likely require a BM enzyme (1). Fork regression can promote repair or bypass of DNA damage without recombination. An in vitro system that recapitulates a bypass pathway has been reconstituted using T4 proteins by Kadyrov and Drake (43). In this system, the strand exchange protein UvsX and the Dda helicase promoted strand switching by T4 polymerase. This partnership of UvsX and Dda can be rationalized through a mechanism in which Dda unwinds the leading strand and UvsX then allows strand invasion of the displaced leading strand into the complementary nascent lagging strand. While this pathway, in effect, allows fork regression, UvsW seems better suited for this role. UvsW acts on branched DNA including replication fork-like structures, and inactivation of UvsW causes hypersensitivity to DNA damage (not true for inactivation of Dda).

In an accompanying report (46) our colleagues present the structure of the UvsW protein determined by x-ray crystallography. Remarkably, the closest structural homolog of UvsW is the eukaryotic protein Rad54. Rad54 is a highly versatile protein that plays a central role in DNA recombination and repair processes (44). The precise mechanism of Rad54 function remains uncertain, but, like UvsW, Rad54 promotes HJ BM. Further characterization of UvsW and Rad54 will be required before the full extent of functional similarity is known, but it seems evident that a comparative analysis of the viral and eukaryotic recombination systems will be productive.

Many of the enzymes involved in T4 replication and recombination interact in ways that modify and regulate their activities (7, 45), and it seems probable that the activity of UvsW is regulated similarly. The little-studied protein UvsW.1 and the single-strand-binding protein gp32 have been reported to modulate UvsW activity in vitro (18) and other interacting proteins may also be found. UvsW acts in the same recombinational pathway as UvsX and UvsY, suggesting interactions with one or both of those proteins. The HJ cleaving enzyme endonuclease VII (gp49) could also act in concert with UvsW. Many fundamental questions still remain regarding the role of HJ BM in DNA replication, recombination and repair, and further studies of T4 UvsW protein may help to answer these questions.

Acknowledgments—We thank Dr. Stephen W. White, Xiaoping He, and their colleagues at St. Jude Children’s Hospital for Dda protein, for mass spectral analyses of the purified proteins and for general advice and comments. We would also like to thank Dr. Nicole Rabaud for her assistance in preparing the manuscript.

REFERENCES
1. McGlynn, P., and Lloyd, R. G. (2002) Nat. Rev. Mol. Cell. Biol. 3, 859–870
2. Whitby, M. C., Vincent, S. D., and Lloyd, R. G. (1994) EMBO J. 13, 5220–5228
3. Tsaneva, I. R., Muller, B., and West, S. C. (1992) Cell 69, 1171–1180
4. Pank, J. L., Wu, J., and Hsieh, T. S. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11118–11123
5. Karow, J. K., Constantinoiu, A., Li, J. L., West, S. C., and Hickson, I. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6504–6508
6. Bagreev, D. V., Mazina, O. M., and Mazin, A. V. (2006) Nature 442, 590–593
7. Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kunisawa, T., and Ruger, W. (2003) Microbiol. Mol. Biol. Rev. 67, 86–156, Table of contents
8. Bernstein, H., and Bernstein, C. (1989) J. Bacteriol. 171, 2265–2270
9. Spencer, E. K., Rush, J., Fung, C., Reha-Krantz, L. J., Karam, J. D., and Konigsberg, W. H. (1988) J. Biol. Chem. 263, 7478–7486
10. Kreuzer, K. N. (2000) Trends Biochem. Sci. 25, 165–173
11. George, J. W., Stohr, B. A., Tomso, D. I., and Kreuzer, K. N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8290–8297
12. Kreuzer, K. N. (2005) Annu. Rev. Microbiol. 59, 43–67
13. Derr, L. K., and Kreuzer, K. N. (1990) J. Mol. Biol. 214, 643–656
14. Derr, L. K., and Drake, J. W. (1990) Mol. Gen. Genet. 227, 257–264
15. Carles-Kinch, K., George, J. W., and Kreuzer, K. N. (1997) EMBO J. 16, 4142–4151
16. Dudas, K. C., and Kreuzer, K. N. (2001) Mol. Cell. Biol. 21, 2706–2715
17. Sickmier, E. A., Kreuzer, K. N., and White, S. W. (2004) Structure 12, 583–592
18. Nelson, S. W., and Benkovic, S. J. (2007) J. Biol. Chem. 282, 407–416
19. McGlynn, P., and Lloyd, R. G. (1999) Nucleic Acids Res. 27, 3049–3056
20. McGlynn, P., and Lloyd, R. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8227–8234
21. Whitby, M. C., and Lloyd, R. G. (1998) J. Biol. Chem. 273, 19729–19739
22. Salinas, F., and Kodadek, T. (1995) Cell 82, 111–119
23. Rodriguez, A. C. (2002) J. Biol. Chem. 277, 29865–29873
24. Brosh, R. M., Jr., Opresko, P. L., and Bohr, V. A. (2006) Methods Enzymol. 409, 52–85
25. Constantinoiu, A., and West, S. C. (2004) Methods Mol. Biol. 262, 239–253
26. Plank, J. L., and Hsieh, T. S. (2006) J. Biol. Chem. 281, 17510–17516
UvsW-promoted Holliday Junction Migration

27. Plank, J. L., Chu, S. H., Pohlhaus, J. R., Wilson-Sali, T., and Hsieh, T. S. (2005) *J. Biol. Chem.* **280**, 3564–3573
28. Bregeon, D., and Doetsch, P. W. (2004) *BioTechniques* **37**, 760–762, 764, 766
29. Friedman, K. L., and Brewer, B. J. (1995) *Methods Enzymol.* **262**, 613–627
30. Ming, Y. Z., Di, X., Gomez-Sanchez, E. P., and Gomez-Sanchez, C. E. (1994) *BioTechniques* **16**, 58–59
31. Abhyankar, M. M., Reddy, J. M., Sharma, R., Bullesbach, E., and Bastia, D. (2004) *J. Biol. Chem.* **279**, 6711–6719
32. Metcalf, W. W., Jiang, W., and Wanner, B. L. (1994) *Gene (Amst.)* **138**, 1–7
33. Thain, A., Gaston, K., Jenkins, O., and Clarke, A. R. (1996) *Trends Genet.* **12**, 209–210
34. Panyutin, I. G., and Hsieh, P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2021–2025
35. Patel, S. S., and Donmez, I. (2006) *J. Biol. Chem.* **281**, 18265–18268
36. Marians, K. J. (2004) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **359**, 71–77
37. Briggs, G. S., Mahdi, A. A., Weller, G. R., Wen, Q., and Lloyd, R. G. (2004) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **359**, 49–59
38. Singleton, M. R., Scaife, S., and Wigley, D. B. (2001) *Cell* **107**, 79–89
39. Whitby, M. C., Ryder, L., and Lloyd, R. G. (1993) *Cell* **75**, 341–350
40. Grigoriev, M., and Hsieh, P. (1998) *Mol. Cell* **2**, 373–381
41. Cox, M. M., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3433–3437
42. West, S. C. (1992) *Annu. Rev. Biochem.* **61**, 603–640
43. Kadyrov, F. A., and Drake, J. W. (2004) *J. Biol. Chem.* **279**, 35735–35740
44. Heyer, W. D., Li, X., Rolfsmeier, M., and Zhang, X. P. (2006) *Nucleic Acids Res.* **34**, 4115–4125
45. Kodadek, T., and Alberts, B. M. (1987) *Nature* **326**, 312–314
46. Kerr, I. D., Sivakolundu, S., Li, Z., Buchsbaum, J. C., Knox, L. A., Kriwacki, R., and White, S. W. (2007) *J. Biol. Chem.* **282**, 34392–34400