

Role of the *Escherichia coli* RecQ DNA helicase in SOS signaling and genome stabilization at stalled replication forks

Takashi Hishida,^{1,5,7} Yong-Woon Han,^{1,5,6} Tatsuya Shibata,¹ Yoshino Kubota,¹ Yoshizumi Ishino,³ Hiroshi Iwasaki,⁴ and Hideo Shinagawa^{1,2,8}

¹Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; ²CREST, Japan Science and Technology Agency, Osaka 565-0871, Japan; ³Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Fukuoka, 812-8581, Japan; ⁴Graduate School of Integrated Science, Yokohama City University, Yokohama, 230-0045 Japan

The RecQ protein family is a highly conserved group of DNA helicases that play roles in maintaining genomic stability. In this study, we present biochemical and genetic evidence that *Escherichia coli* RecQ processes stalled replication forks and participates in SOS signaling. Cells that carry *dnaE486*, a mutation in the DNA polymerase III α -catalytic subunit, induce an RecA-dependent SOS response and become highly filamented at the semirestrictive temperature (38°C). An *recQ* mutation suppresses the induction of SOS response and the filamentation in the *dnaE486* mutant at 38°C, causing appearance of a high proportion of anucleate cells. In vitro, RecQ binds and unwinds forked DNA substrates with a gap on the leading strand more efficiently than those with a gap on the lagging strand or Holliday junction DNA. RecQ unwinds the template duplex ahead of the fork, and then the lagging strand is unwound. Consequently, this process generates a single-stranded DNA (ssDNA) gap on the lagging strand adjacent to a replication fork. These results suggest that RecQ functions to generate an initiating signal that can recruit RecA for SOS induction and recombination at stalled replication forks, which are required for the cell cycle checkpoint and resumption of DNA replication.

[**Keywords:** RecQ DNA helicase; DNA replication; SOS induction; DNA homologous recombination]

Supplemental material is available at <http://www.genesdev.org>.

Received May 19, 2004; revised version accepted June 7, 2004.

Progression of replication forks is often blocked following an encounter with either DNA lesions on template strands or other impediments such as bound proteins, secondary structures, or topological stress. Under these circumstances, the efficient resumption of DNA synthesis is essential for the growth and survival of all organisms. The RecQ protein family is a group of highly conserved helicases that includes *Escherichia coli* RecQ, *Saccharomyces cerevisiae* Sgs1, *Schizosaccharomyces pombe* Rqh1, and five gene products in humans (Bachrati and Hickson 2003). Human RecQ homologs include BLM, WRN, and RECQL4, which are implicated in the human hereditary diseases Bloom's syndrome, Werner's syndrome, and Rothmund-Thomson syndrome, respectively (Ellis et al. 1995; Yu et al. 1996; Kitao et al. 1999).

The fact that human disorders associated with mutations in different RecQ helicases have distinct features implies that different human RecQ helicases function in distinct cellular processes. However, several lines of evidence indicate that some functions are conserved between the RecQ helicases of lower and higher organisms (Cobb et al. 2002; Bachrati and Hickson 2003). One conserved function among RecQ homologs involves the processing of replication forks when DNA replication has been arrested prior to completion (Murray et al. 1997; Courcelle and Hanawalt 1999; Doe et al. 2000; Frei and Gasser 2000). RecQ family members share a highly conserved helicase domain, but have little homology outside of this region (Bachrati and Hickson 2003). Several RecQ homologs possess a 3'-to-5' DNA helicase activity that is active on a variety of substrates, including G4 DNA and three- or four-strand DNA structures including D-loops, forked structures, and Holliday junctions (Harmon and Kowalczykowski 1998; Sun et al. 1998; Karow et al. 2000; Mohaghegh et al. 2001; Wu and Maizels 2001; Huber et al. 2002). It is likely, therefore, that the conserved RecQ helicase domain determines the enzyme's DNA substrate specificity and, in many in-

⁵These authors contributed equally to this work.

⁶Present address: The Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan.

Corresponding authors.

⁷E-MAIL hishida@biken.osaka-u.ac.jp; FAX 81-6-6879-8320

⁸E-MAIL shinagaw@biken.osaka-u.ac.jp; FAX 81-6-6879-8320.

Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1223804>.

stances, participates during the rescue of stalled replication forks.

E. coli RecQ belongs to the *recF* recombination pathway (Nakayama et al. 1985). The *recF* pathway proteins are required for the repair of gapped DNA and resumption of DNA synthesis following UV-induced DNA damage, and to stabilize the nascent strands at stalled forks (Horii and Clark 1973; Kolodner et al. 1985; Tseng et al. 1994; Courcelle et al. 1997). Recently, it was reported that the RecFOR complex loads RecA onto gapped DNA in vitro (Morimatsu and Kowalczykowski 2003). Although unlike *recF* mutations the *recQ* mutation does not render cells sensitive to UV irradiation, recent experiments have shown that RecQ together with RecF play an important role in the resumption of DNA synthesis following UV-induced DNA damage, by degrading the nascent lagging strand of the replication fork (Courcelle and Hanawalt 1999; Courcelle et al. 2003). RecQ also suppresses illegitimate recombination in cells with DNA damage (Hanada et al. 1997). These results indicate that RecQ plays important roles in the resumption of stalled DNA replication and prevention of chromosomal abnormalities.

In this study, we have analyzed the interaction of *E. coli* RecQ with synthetic DNA substrates resembling DNA replication forks. We demonstrate that RecQ binds leading-strand gapped fork DNA more efficiently than lagging-strand gapped fork DNA or Holliday junction DNA and is capable of converting the ssDNA gaps on the fork from the leading-strand DNA to the lagging-strand DNA by two-step reactions. We also show that RecQ at stalled replication forks acts to induce RecA-dependent SOS signaling. The results suggest that RecQ functions to generate an initiating signal that can recruit RecA for SOS induction and recombination in response to DNA replication interference, which are required for the cell cycle checkpoint and resumption of DNA replication. We propose here that prokaryotic and eukaryotic RecQ helicases play a role in coordinating a cell cycle checkpoint response with recombination and replication.

Results

RecQ helicase preferentially binds forked DNA with a gap on the leading strand

This study examines the interaction between *E. coli* RecQ helicase and synthetic forked DNA substrates that resemble DNA replication intermediates. DNA binding and DNA helicase activities were measured using ³²P-labeled DNA substrates. The substrates included ssDNA and double-stranded DNA (dsDNA) regions of different lengths, or included a Holliday junction structure. DNA substrates were incubated with RecQ at different concentrations, and DNA–RecQ complexes were measured using a gel mobility shift assay. As shown in Figure 1, we observed that two distinct RecQ–DNA complexes were formed with these DNA substrates. Slower migrating complexes were only formed at extremely high concen-

tration and were observed in all DNA substrates used in this study (Figs. 1, 2), indicating the formation of a structure-independent RecQ–DNA complex. These results suggest that RecQ nonspecifically binds DNA at higher protein concentrations and these RecQ–DNA complexes are not physiologically relevant. Faster migrating complexes were observed at lower RecQ concentrations with 3'-overhang, 3'-flap, or leading-strand gapped fork (LeGF) DNA (Fig. 1C,E,G). In complexes involving 3'-overhang DNA, faster migrating species are smeared on the gel, indicating the unstable complexes. Because these complexes were not observed with duplex DNA with 5'-overhang or with blunt ends, junctions between ssDNA and the 5' terminus of the duplex might be important for the formation of the faster migrating complexes. In addition, they are observed only when the DNA substrate has a 3'-ssDNA region longer than 20 nucleotides (nt) and the binding efficiency increases with increasing the length of the 3'-overhang from 20 to 50 nt (Fig. 2). Thus, RecQ bound 3'-overhang DNA more efficiently than 5'-overhang or blunt-end dsDNA of comparable length, which is consistent with previous studies (Harmon and Kowalczykowski 1998). Moreover, RecQ formed more stable complexes with LeGF and 3'-flap DNA than 3'-overhang DNA, the former mimicking the replication fork structures with ssDNA on the leading-strand DNA (Fig. 1). Especially, the preferential and stable binding to the LeGF DNA substrate (K_d = 13 nM) is not solely due to the presence of ssDNA because it has only an 8-nt ssDNA gap on the leading strand. On the other hand, RecQ bound poorly to lagging-strand gapped fork (LaGF; K_d = 129 nM) and the DNA gap (K_d = 301 nM), both of which have 8-nt DNA gaps also (Fig. 1K). These results demonstrate that RecQ preferentially binds forked DNA with a gap on the leading strand.

Structure-specific unwinding by RecQ helicase

The DNA substrate specificity of RecQ helicase activity was also examined using synthetic forked DNA structures (Fig. 3). At low concentrations of RecQ protein, blunt-ended dsDNA and Holliday junction (HJ) DNA were unwound with very low efficiency (Fig. 3A,I). RecQ unwound DNA substrates with a 3'-overhang significantly more efficiently than DNA substrates with a 5'-overhang (Fig. 3B,C), indicating that the polarity of the RecQ helicase is 3' to 5', as previously reported (Umezumi et al. 1990). This specificity reflects a preferential affinity of RecQ for 3'-overhang DNA (Fig. 1). Moreover, the kinetics of RecQ helicase activity indicates that it unwinds 3'-flap or LeGF DNA substrates more efficiently than 5'-flap, LaGF, DNA gap, or fork structure without any gap (Fig. 3D–I). When RecQ was added to a reaction containing 3'-flap or LeGF DNA substrate containing 5'-end-labeled nascent lagging strands, a band corresponding to 3'-overhang DNA appeared rapidly (<1 min) and the amount of 3'-overhang DNA substrate decreased as the lagging-strand DNA levels increased (Fig. 3E,G,K). When LeGF DNA substrates containing 5'-end-labeled

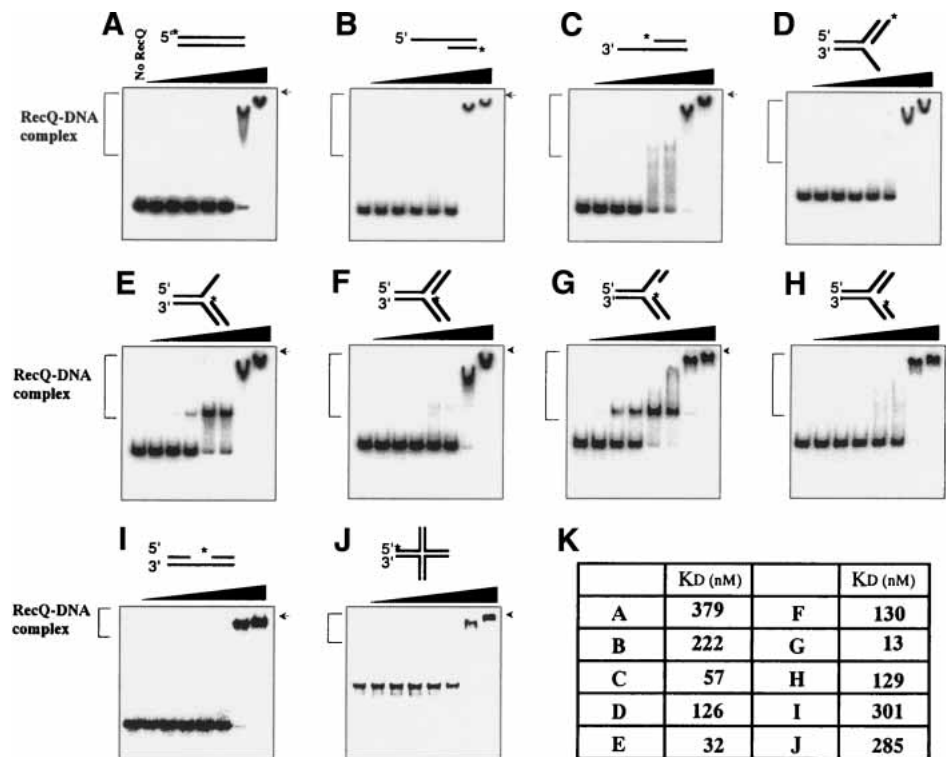


Figure 1. Gel mobility shift analysis of RecQ binding to forked DNA structures. Blunt-end (A), 5'-overhang (B), 3'-overhang (C), 5'-flap (D), 3'-flap (E), forked DNA with no gap (F), LeGF DNA (G), LaGF DNA (H), gapped DNA (I), or HJ DNA (J; 10 nM) was used in binding reactions. RecQ was added at various concentrations (1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1000 nM) in the absence of ATP. Protein–DNA complexes were analyzed by 6% nondenaturing PAGE. (*) 32 P-labeling. The position of the well on the gel is indicated by arrows. DNA-binding reactions were performed as described in Materials and Methods. (K) Summary of dissociation constants of RecQ with various forms of DNA substrates.

nascent leading strands were used in this reaction, production of the duplex with 5'-overhang DNA was observed (Supplementary Fig. S1). These results indicate that RecQ efficiently unwinds 3'-flap and LeGF DNA substrates, which is consistent with its binding affinity (Fig. 1) and 3'-to-5' helicase activity. Moreover, Figure 3E and G demonstrates that RecQ unwinds 3'-flap and LeGF DNA substrates by two distinct steps; RecQ first unwinds the parental strands, translocating 3' to 5' ahead of the fork, and subsequently unwinds and releases the nascent lagging strand.

Suppression of thermosensitivity of a *dnaE486* mutant by *recQ* mutation

If RecQ is involved in processing stalled DNA replication forks, it is predicted that RecQ will interact genetically with the DNA replication machinery. In *E. coli*, chromosome replication is performed by DNA polymerase III holoenzyme (Pol III), DNA helicase DnaB, and a DnaG primase. Pol III contains at least 10 different subunits (Kelman and O'Donnell 1995), in which the α -subunit is encoded by the *dnaE* gene and is responsible for the polymerizing activity of Pol III (Welch and McHenry 1982; Maki et al. 1985). *dnaE486* is a mutator allele (S885P; Wechsler and Gross 1971; Konrad 1978; Vande-

wiele et al. 2002), which causes temperature sensitivity for growth. We studied the effect of *recQ* mutation on the growth of *dnaE486* strains at different temperatures.

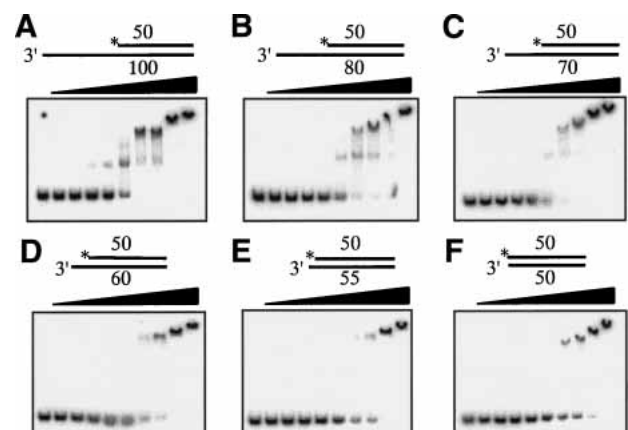


Figure 2. DNA-binding properties of RecQ depend on ssDNA overhang lengths. Substrates (10 nM) had 3'-overhang lengths of 50 nt (A), 30 nt (B), 20 nt (C), 10 nt (D), 5 nt (E), or 0 nt (F). RecQ concentrations (left to right lanes) were 1 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, and 1000 nM. (*) 32 P-labeling. Experiments are performed as described in Materials and Methods.

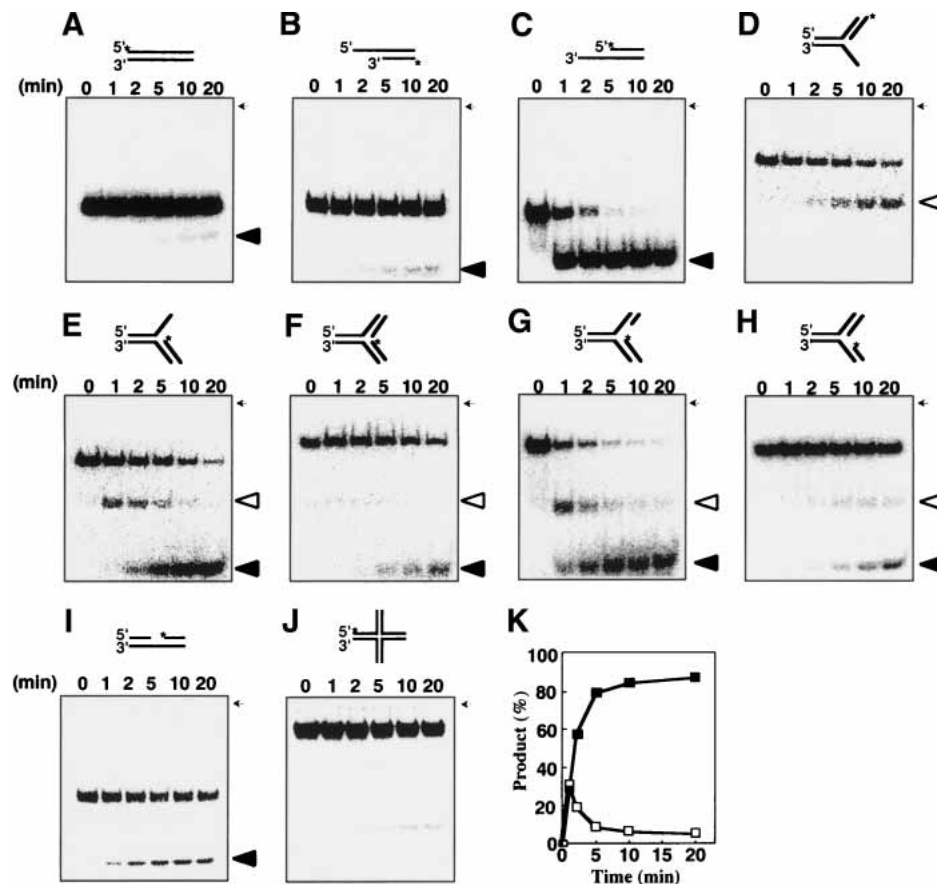


Figure 3. Time course analysis of RecQ-catalyzed DNA unwinding activity. (A–J) RecQ was incubated at 26°C with blunt-ended (A), 5'-overhang (B), 3'-overhang (C), 5'-flap (D), 3'-flap (E), forked DNA with no gap (F), LeGF DNA (G), LaGF DNA (H), duplex DNA with a gap (I), or HJ DNA (J). Reactions containing 10 nM RecQ, 10 nM DNA, and 1 mM ATP were carried out at 26°C and initiated by the addition of ATP. Aliquots were taken at 1, 2, 5, 10, and 20 min. Deproteinized reaction products except the HJ DNA (9%) were analyzed by 12% nondenaturing PAGE. Open triangles indicate 3'- or 5'-overhang DNA, and closed triangles indicate ³²P-labeled ssDNA. (K) The reaction products from G were quantified using a PhosphorImager (Fuji BAS1500) and plotted. Open and filled symbols represent 3'-overhang and 30-mer nascent lagging-strand DNA, respectively. (*) ³²P-labeling. The position of the well on the gel is indicated by arrows.

As shown in Figure 4A and Table 1, wild-type and $\Delta recQ$ cells grew normally at 30°C and 38°C, but *dnaE486* cells were severely defective in growth at 38°C. In contrast, we observed that a $\Delta recQ::cam$ mutation suppressed the growth defect of the *dnaE486* mutant at 38°C (Fig. 4A; Table 1). $\Delta recQ$ mutation could not suppress the growth defect of the mutant at 42°C (Fig. 4A; Table 1), at which temperature the DNA synthesis activity of Pol III is completely inactivated (Wechsler and Gross 1971). Similar results were also obtained when an *recQ61::Tn3* mutation was used (data not shown). This genetic interaction was further confirmed by examining the effect of $\Delta recQ$ mutation on growth rate and cell viability of the *dnaE486* mutant at 38°C and 42°C. Cells grown to early logarithmic phase at 30°C were shifted to 38°C or 42°C and plated on LB medium, and then the plates were shifted back to 30°C to allow surviving cells to form colonies (Fig. 4B,C). When incubated at the restrictive temperature (42°C), the plating efficiency of the *dnaE486* mutant was rapidly decreased, indicating the loss of vi-

ability during prolonged incubation at 42°C (Fig. 4B). The $\Delta recQ$ mutation failed to suppress the lethality of the *dnaE486* strain at 42°C. These results suggest that $\Delta recQ$ mutation does not relieve the loss of viability suffered by the DNA Pol III inactivation. In contrast, when incubated at the semirestrictive temperature (38°C), the proportions of *dnaE486* cells that could go on to form colonies hardly changed even after culturing for 5 h at 38°C (Fig. 4C), indicating that the *dnaE486* cells cease to grow at 38°C, but do not lose viability. Moreover, the absence of RecQ protein evidently suppressed the growth defect of the *dnaE486* mutant. These results suggest that RecQ plays a role in stalling the growth of the DNA Pol III mutant cells at 38°C.

The morphology of the *dnaE486* and *dnaE486* $\Delta recQ$ cells was analyzed at 6 h after the shift to 38°C (Fig. 5): ~80% of *dnaE486* cells were highly filamented, and the filamentation was much reduced in the *dnaE486* $\Delta recQ$ cells. In addition, the ability of $\Delta recQ$ mutation to suppress filamentation in the *dnaE486* mutant was associ-

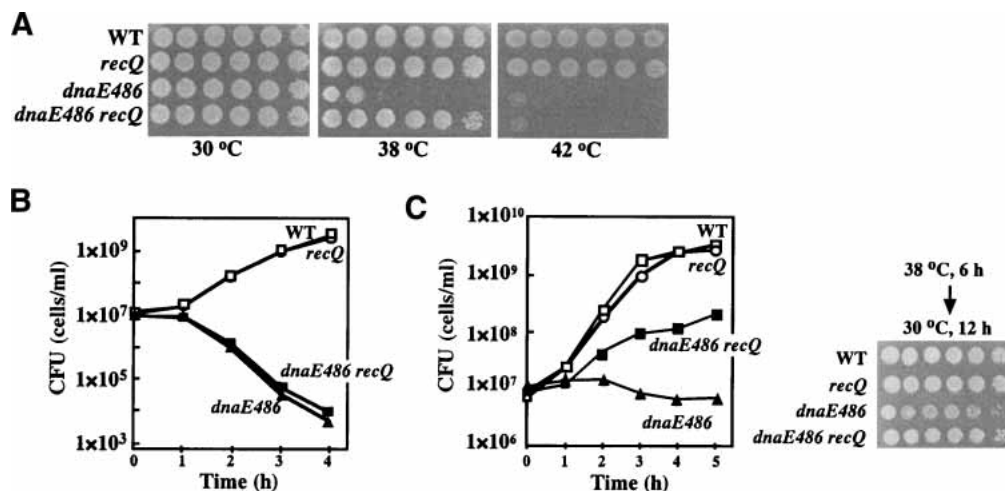


Figure 4. Genetic interaction of RecQ with DNA Pol III. (A) Cells were grown at 30°C in LB medium. Tenfold serial dilutions of cultures (1×10^8 cells/mL) of the indicated mutants were spotted onto LB plates. The plates were incubated at the indicated temperatures. (B) Cells were grown at 30°C to an O.D.₆₀₀ of 0.2, and the cultures were shifted to 40°C (time 0). Samples were taken at the indicated times, and colony-forming units were counted by plating them on LB plates, which were incubated at 30°C for 24 h. (C) Cells grown at 30°C to an O.D.₆₀₀ of 0.2 were shifted to 38°C (time 0). (Left) Cells were plated on LB plates following appropriate dilution and incubated for 24 h at 30°C to count colony-forming units. Cells growing at 30°C were spotted as described in A. (Right) The plates were incubated for 6 h at 38°C and then transferred to 30°C.

ated with appearance of a high proportion of anucleate cells (Fig. 5B). These results are consistent with the interpretation that $\Delta recQ$ mutation allows the replication fork to proceed in the Pol III mutant at 38°C, but that resumption in this situation leads to the loss of genome integrity. Thus, RecQ might interfere with DNA replication fork progression in the Pol III mutant, which may contribute to the faithful recovery of stalled replication forks and thereby the prevention of chromosome loss caused by aberrant DNA replication.

The effect of *recQ* mutation on SOS induction in response to defective DNA replication

If RecQ helicase generates ssDNA at a stalled replication fork, the ssDNA would recruit RecA and activate an SOS response (Harmon and Kowalczykowski 1998; Humayun 1998), which involves induction of DNA repair genes, inhibition of cell division, and subsequent filamentation. We suggest that the SOS response may be responsible for the cell filamentation observed in the *dnaE486* strain at 38°C, and that RecQ plays a role in this process, presumably by increasing the quantity of ssDNA present at stalled replication forks. The RecQ-dependence of the

dnaE486-induced SOS response was confirmed by measuring the expression of a UmuC–LacZ fusion protein as a colorimetric indicator of SOS response in *dnaE486* and *dnaE486* $\Delta recQ$ cells at 38°C. Figure 6A shows that β -galactosidase activity is induced in *dnaE486* cells at 6 h after the temperature shift, but it is induced to a significantly lower level in *dnaE486* $\Delta recQ$ cells. These results suggest that RecQ functions to generate the signal that activates the SOS response when DNA replication is interfered with, which subsequently blocks cell division and induces repair of DNA damage.

The role of RecQ in checkpoint signaling was also examined for a UV-induced SOS response. SOS induction was monitored by measuring degradation of LexA, the repressor for the SOS regulon. Figure 6B shows that in wild-type cells, UV-induced LexA degradation was dependent on RecA and was slightly delayed in an *recQ61::Tn3* mutant relative to wild-type cells, which was confirmed by quantitative analysis of the amount of LexA protein (Fig. 6C). Because UV-induced DNA lesions are primarily removed from the genome by nucleotide excision repair proteins, SOS signaling was also examined in a *uvrA6* mutant, in which DNA replication is frequently blocked and recovery is severely inhibited

Table 1. *recQ* mutation partially suppresses the temperature sensitivity caused by *dnaE486* mutation

Relevant genotype	30°C ^a	38°C ^a	42°C ^a
wt	1	1.0 ± 0.2	1.15 ± 0.25
<i>recQ</i>	1	0.97 ± 0.28	1.16 ± 0.2
<i>dnaE486</i>	1	$1.4 \times 10^{-3} \pm 0.3 \times 10^{-3}$	$3.4 \times 10^{-6} \pm 0.2 \times 10^{-6}$
<i>dnaE486 recQ</i>	1	0.93 ± 0.14	$3.9 \times 10^{-6} \pm 1.1 \times 10^{-6}$

^aCells were grown overnight at 30°C, plated on LB plates following appropriate dilutions, and then incubated for 30 h at 30°C, 38°C, or 42°C. Ratios of colony-forming units (CFUs) at 38°C or 42°C relative to CFUs at 30°C are shown.

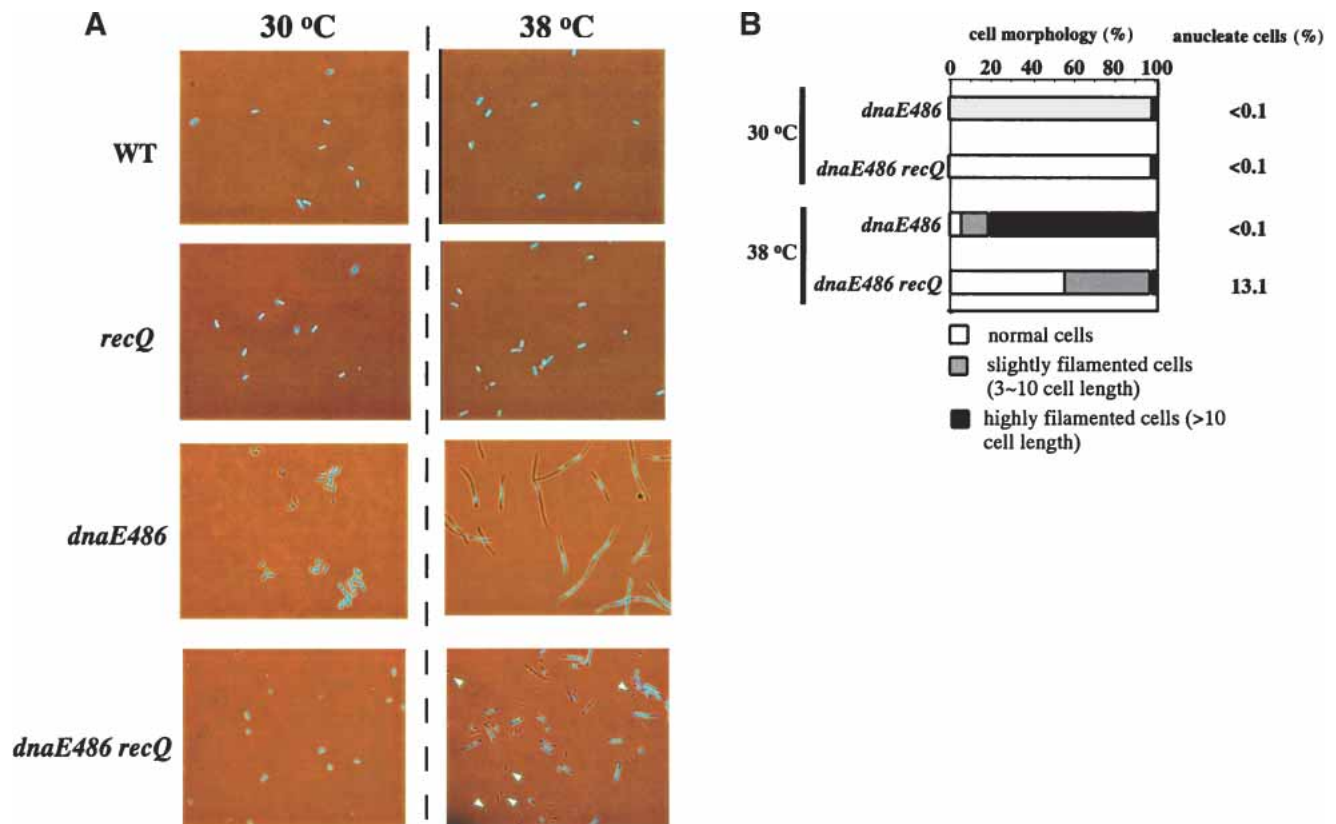


Figure 5. Morphological analysis of wild-type, $\Delta recQ$, *dnaE486*, and *dnaE486* $\Delta recQ$ mutant cells. (A) Cells were grown to early log phase at 30°C, and then grown for 6 h at 38°C. Cells were stained with DAPI (4',6'-diamidino-2-phenylindole) and observed under a phase contrast microscope. White arrowheads highlight some anucleate cells. (B) Distribution of cell sizes. Cells grown for 6 h at 38°C were stained with DAPI. The shapes and chromosomal DNA were monitored under a microscope. Proportions of normal cells are indicated by white bars. Slightly (gray bars) and highly (black bars) filamented cells are defined as cells larger than three and 10 cell lengths in size regardless of the number of nucleoids, respectively. At least 300 cells were examined.

(Courcelle et al. 1999, 2003). In log-phase cells exposed to 5 J/m² of UV, LexA degradation was significantly delayed in the *uvrA6 recQ61::Tn3* double mutant relative to the *uvrA6* single mutant (Fig. 6B,C). These results suggest that RecQ also plays a role in induction of SOS in cells with UV-induced DNA damage.

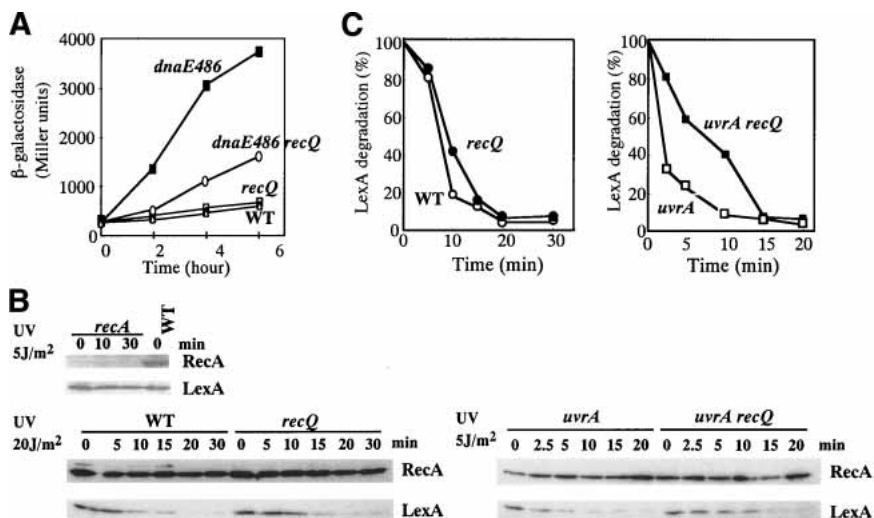
Discussion

This study characterizes the interaction between *E. coli* RecQ and forked DNA substrates resembling stalled DNA replication forks and proposes that the action of *E. coli* RecQ at such structures plays a role in replication restart and in cell cycle checkpoint activation in cells with DNA damage. The evidence for this is as follows: (1) RecQ preferentially binds forked DNA in vitro when the substrate has a gap on the leading strand (Fig. 1). (2) RecQ unwinds the parental strand of such fork moving with a 3'-to-5' polarity ahead of the fork and subsequently switches back to unwind the lagging strand of a forked substrate in vitro with a 3'-to-5' polarity releasing the nascent lagging strand (Fig. 3). (3) RecQ is required for a normal SOS signaling in response to DNA replica-

tion interference caused by *dnaE486* mutation or UV-induced cellular stress. These results suggest that RecQ generates ssDNA gap(s) on the lagging strand of a stalled replication fork; subsequent events (e.g., homologous recombination) promote recovery from DNA damage.

A previous study found that RecQ unwound a diverse set of DNA substrates with equal efficiency, but bound these DNA substrates with different affinities (Harmon and Kowalczykowski 1998). We also found that RecQ bound and unwound all types of DNA substrates used in this study at a high concentration (100 nM), but it did so with different efficiencies at a low concentration (10 nM; Figs. 1, 3; data not shown). It is difficult to compare the previous study with the present study because the assay systems were different; in the previous study, helicase activity was quantified using fluorescent dye displacement, and in this study, the helicase activity was quantified by separating reaction substrate and product by polyacrylamide gel electrophoresis. However, it should be noted that the substrate specificity for RecQ unwinding is largely dependent on RecQ protein concentration. Indeed, it has also been shown that RecQ only partially unwinds dsDNA at low concentration (10 nM), and an

Figure 6. RecQ is required for proper SOS induction in response to DNA replication interference. (A) Expression of the *umuDC::lacZYA* fusion gene in the *dnaE486* strain grown at the semirestrictive temperature. Overnight cultures of wild-type cells, $\Delta recQ$, *dnaE486*, or *dnaE486* $\Delta recQ$ mutants were grown at 30°C, diluted into fresh LB medium, and incubated at 38°C. Aliquots were sampled at specific intervals and examined for β -galactosidase activity. Values are averages of the enzyme activities in Miller units of duplicate samples measured after the shift to 38°C. (B) Effect of *recQ* mutation on UV-induced LexA degradation. Measurements of LexA degradation were performed as described in Materials and Methods. The cells were UV-irradiated (time 0), and aliquots were sampled at intervals. Samples were analyzed by 15% SDS-PAGE, and RecA and LexA proteins were detected by immunoblotting with anti-RecA and anti-LexA antibodies. The reproducibility of the data was confirmed by four independent experiments. (C) The relative amounts of LexA protein shown in B were plotted after they were normalized to the values at time zero. (Open circles) Wild type; (filled circles) *recQ61::Tn3*; (open squares) *uvrA6*; (filled squares) *uvrA6 recQ61::Tn3*.



increase in the RecQ helicase concentration from 10 nM to 100 nM resulted in a higher rate of DNA unwinding (Harmon and Kowalczykowski 2001). An earlier *in vivo* study shows that the expression of the *recQ* gene is regulated by the SOS regulon, and overproduction of RecQ severely inhibits cell growth (Nakayama et al. 1985; Irino et al. 1986) and makes wild-type cells sensitive to UV (data not shown), suggesting that the nonspecific DNA binding and unwinding activity of RecQ observed at higher concentration may interfere with DNA repair and replication. These results support that RecQ has structure specificity for DNA binding and unwinding at physiological concentrations *in vivo* similar to the one shown *in vitro* in this study.

Enzymatic and biological functions of *E. coli* RecQ helicase at a stalled replication fork

This study shows that $\Delta recQ$ mutation suppresses the temperature-sensitive growth defect of *dnaE486* cells at 38°C (Fig. 4). However, the *dnaE486* $\Delta recQ$ double mutant accumulates anucleate cells and has a lower proportion of viable cells than wild-type or *dnaE486* cells (Fig. 4; data not shown). These results suggest that *dnaE486* cells that lack RecQ continue to replicate DNA in an unregulated manner at 38°C, so that DNA synthesis by the mutant DNA Pol III causes genomic instability and cell death. In contrast, *dnaE486* cells with active RecQ induce SOS response, restrict DNA replication, and become highly filamented. This study also shows that *recQ* mutation causes a slight delay in UV-induced SOS response, and nucleotide excision repair-deficient *uvrA recQ* cells have a significant delay in UV-induced SOS response (Fig. 6). Thus, RecQ is required for the efficient induction of SOS in response to replication block. It is possible that the role of RecQ is to generate ssDNA as

part of the SOS signaling process. This would activate RecA, promote cleavage of LexA, induce DNA repair, and inhibit cell division (Humayun 1998). This possibility is consistent with the results that the RecF pathway is necessary for the initiation of recombination repair of ssDNA gaps and that RecQ helicase and RecJ exonuclease cooperatively promote degradation of nascent DNA on the lagging strand at blocked replication forks, thereby allowing RecA to bind to exposed ssDNA (Harmon and Kowalczykowski 1998; Courcelle and Hanawalt 1999; Morimatsu and Kowalczykowski 2003). Although we have not examined the effects of *recJ* mutation, we observed that a $\Delta recA$ mutation also suppressed the temperature sensitivity and filamentation of the *dnaE486* mutant at 38°C (T. Hishida, T. Shibata, and H. Shinagawa, unpubl.), consistent with our conclusion that SOS induction is responsible for the growth arrest and filamentation in the *dnaE486* strain grown at 38°C.

Based on the evidence discussed above, we propose a model for the role of RecQ in replication restart and cell cycle signaling (Fig. 7). This model includes two distinct RecQ-dependent steps. In the first step, RecQ binds an ssDNA gap on the leading strand of a DNA replication fork and unwinds parental dsDNA in front of the fork. In the second step, RecQ switches to the lagging strand and unwinds nascent lagging-strand DNA. On both strands, RecQ moves in the 3'-to-5' direction. This model predicts that RecQ processes gapped forks into DNA structures that serve to activate RecA and induce the SOS response, which leads to a delay in cell growth, prevention of replication and chromosome segregation, and the expression of specific repair genes. It remains to be determined whether the same RecQ molecule tracks on both strands or the two reactions involves two RecQ molecules. However, this model gives an answer to the questions about how RecQ helicase resolves structures

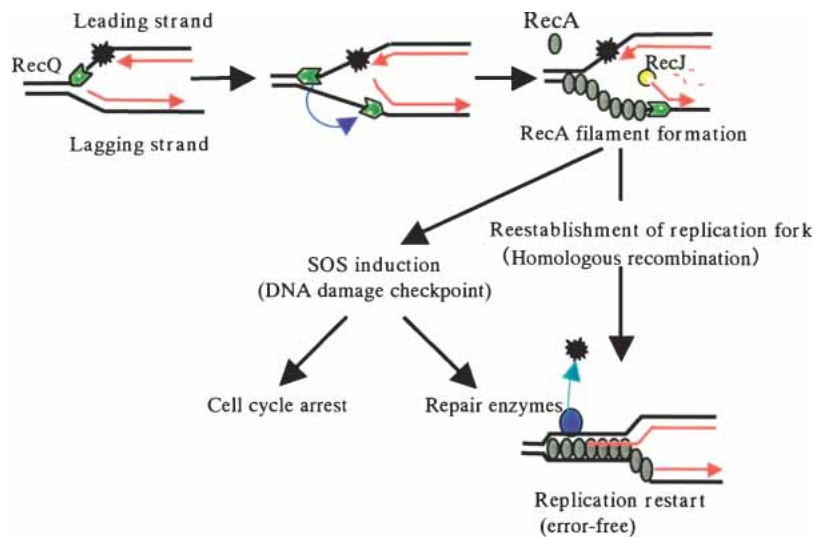


Figure 7. A model for the roles for RecQ in the rescue of a stalled replication fork. RecQ binds an ssDNA gap on the leading strand and unwinds the template dsDNA ahead of the fork, which might generate positive supercoiling and contribute to the elimination of structures that impede fork movement (e.g., G4 DNA structure). Subsequently, RecQ switches to the lagging strand and creates an ssDNA region by unwinding the lagging strand, which may be an initiating signal for RecA-dependent SOS induction and recombination repair. SOS induction in response to DNA replication interference leads to a delay in cell growth, prevention of replication and chromosome segregation, and the induction of specific repair genes. See text for further details. Red arrowheads indicate the 3'-OH groups of the leading and lagging strands.

ahead of the fork that impede fork movement (e.g., G4 DNA) and how RecQ degrades nascent DNA on the lagging strand in collaboration with RecJ nuclease. If RecQ acts *in vivo* in a manner similar to that suggested by our *in vitro* data, we predict that the unwinding activity of RecQ affects DNA topology and requires the coordinated activity of a DNA topoisomerase because there are no dsDNA ends. It has been shown that the RecQ family of helicases and topoisomerase III (Top III) functionally interact in many organisms (Gangloff et al. 1994; Harmon et al. 1999; Bennett et al. 2000; Wu et al. 2000). Indeed, recent biochemical evidence has revealed that BLM and Topo III α of human and RecQ helicase and Topo III of *E. coli* work together to comprise a potent and concerted single-strand DNA passage activity and changes topology of DNA *in vitro* (Harmon et al. 1999, 2003; Wu and Hickson 2003). Thus, RecQ and Topo III might act in a coordinated manner to process stalled replication forks, resolving topological hinderance and/or decatenating precatenanes that accumulate behind the replication fork during DNA replication. Future studies will be required to investigate the joint action of RecQ and Topo III at stalled forks.

Evidence shown here indicates that RecQ is required for the recovery of stalled replication forks. It should be noted that deficiency in RecQ does not cause reduced viability or UV sensitivity (data not shown). These results suggest that not all stalled replication forks are rescued by RecQ, and that stalled DNA replication forks can be rescued by several mechanisms. If RecQ is capable of converting the ssDNA gaps on the fork from the leading-strand DNA to lagging-strand DNA, RecQ may affect the polarity of RecA-catalyzed strand exchange. Indeed, RecA can promote regression of forks with a leading-strand gap but not with a lagging-strand gap *in vitro* (Robu et al. 2001). Deficiency in RecQ may therefore lead to accumulation of leading-strand gaps, which are substrates for RecA-dependent fork regression. In this case, RecQ may unwind a stalled replication fork to prevent it from becoming a Holliday junction by the re-

gression of a stalled fork with a leading-strand gap, rather than promoting resolution of the junction by reverse branch migration.

In this study, we aimed at determining the role of RecQ at stalled replication forks *in vivo* and *in vitro*, and found that RecQ preferentially binds and unwinds the LeGF structure. However, RecQ helicase action may not be solely confined to the LeGF DNA because RecQ also unwinds 3'-overhang DNA (Fig. 3). RecQ is a multifunctional enzyme that acts as an initiator of homologous recombination and a suppressor of illegitimate recombination (Ryder et al. 1994; Hanada et al. 1997; Harmon and Kowalczykowski 1998). Therefore, the above model represents the simplest explanation for the role of RecQ in the rescue of stalled replication forks. RecQ may be involved in other processes like resolution of entangled chromosomes or aberrant recombination events.

Conserved functions of RecQ family helicases at stalled replication forks

This study suggests that *E. coli* RecQ generates ssDNA in cells with a DNA replication defect as part of a cell signaling process that induces SOS response, activates a cell cycle checkpoint, and subsequently permits DNA replication to resume. The eukaryotic homologs of RecQ, including Sgs1, Rqh1, BLM, and WRN, may also perform similar functions. In the yeast *S. cerevisiae*, *sgs1* mutation is associated with a hyperrecombination phenotype and hypersensitivity to HU and MMS in S phase (Gangloff et al. 1994; Mullen et al. 2000). Sgs1 is required for HU-induced activation of Rad53 as a sensor protein, which acts in parallel with Rad24 (Frei and Gasser 2000). In human cells, WRN and RPA colocalize in discrete nuclear foci and interact with p53 when cell growth is arrested (Spillare et al. 1999; Constantinou et al. 2000; Sakamoto et al. 2001). BLM is part of the BASC complex (BRCA-1-associated genome surveillance complex), and BRCA1, BLM helicase, and the RAD50-MRE11-NBS1 complex colocalize to large nuclear foci that contain

PCNA when DNA synthesis is inhibited (Wang et al. 2000). Recent study shows that BLM interacts with RAD51 and transports p53 to stalled replication forks (Sengupta et al. 2003). Moreover, it has been reported that ssDNA coated by replication protein A (RPA, a eukaryotic ssDNA-binding protein) is a key structure for checkpoint activation in response to double-strand breaks and replication interference (Zou and Elledge 2003). These findings suggest that *E. coli* RecQ and eukaryotic RecQ homologs play similar roles in replication-associated homologous recombination and checkpoint activation: ssDNA produced by RecQ helicase activity may be the initiating signal for the checkpoint response in both bacteria and eukaryotes.

The work presented here has uncovered a role of the *E. coli* RecQ helicase in the integration of the checkpoint response with DNA recombination and DNA replication and provides important clues to understanding the complex interplay between DNA replication, recombination, and checkpoint functions, especially at stalled replication forks.

Materials and methods

Media and growth condition

Standard methods for *E. coli* genetics and recombinant DNA techniques were as described by Miller (1992) and Sambrook et al. (1989). Unless otherwise stated, cells were grown in Luria broth (LB) or M9 salts-glucose minimal medium (Miller 1992) supplemented with appropriate amino acids and thiamine. Ampicillin (50 µg/mL), tetracycline (10 µg/mL), and kanamycin (30 µg/mL) were used as needed.

Bacterial strains and plasmids

Bacterial strains are listed in Table 2. A $\Delta recQ::cat$ allele was constructed by replacing the SacII–ClaI fragment of the *recQ* coding region with the chloramphenicol-resistance gene, and it was introduced into an *recD* strain (D301) as described (Russell et al. 1989). The gene disruption was confirmed by PCR using appropriate primers. The isogenic derivatives of AB1157 strain were constructed by transduction with P1 phage (Miller 1992).

pLysS and pSQ211 were used for the overexpression of RecQ (Harmon and Kowalczykowski 1998). pSK1002 was used for SOS induction assays (Shinagawa et al. 1983).

Purification of recombinant RecQ protein

The *E. coli* BL21 (DE3) strain carrying pLysS and pSQ211 was grown at 37°C to an O.D.₆₀₀ of ~0.4 in Luria broth containing ampicillin. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was incubated for 18 h at 18°C. After the cells were harvested, the cells were resuspended in buffer A (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.1% Triton, 1 mM dithiothreitol [DTT], 10% Glycerol). They were disrupted by sonication and centrifuged at 40,000 × g for 30 min. Polyethyleneimine was added to a final concentration of 0.05%, and the mixture was stirred for 30 min and then centrifuged at 27,000 × g for 15 min. Ammonium sulfate was gradually added to the supernatant to achieve 40% saturation. The mixture was stirred for 30 min and then centrifuged at 27,000 × g for 15 min. The pellet was resuspended in RQ-buffer (20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1 mM DTT) containing 1 M ammonium sulfate, then centrifuged at 27,000 × g for 15 min, and the pellet was removed. The supernatant was loaded onto HiLoad 16/10 Phenyl Sepharose HP (Amersham Pharmacia), and the proteins were eluted with a 100-mL linear gradient from 1 M to 0 M ammonium sulfate in RQ buffer. The fractions containing RecQ protein were diluted twofold with RQ buffer and loaded onto a Hi-Trap Heparin column (Amersham Pharmacia). The proteins were eluted with a 100-mL linear gradient from 0 M to 1 M NaCl in RQ buffer. RecQ eluted at 300 mM NaCl. The RecQ protein peak fractions were applied to a Superdex 200 column (Amersham Pharmacia). The RecQ protein peak fractions were applied to a Hydroxyapatite column (Bio-Rad), and the proteins were eluted with a 100-mL linear gradient from 20 mM to 600 mM phosphate in phosphate buffer (phosphate at pH 6.8, 1 mM EDTA, 1 mM DTT). The RecQ protein peak fractions were applied to Hi-Trap Heparin to concentrate RecQ protein. The RecQ protein thus purified showed a single band on SDS-PAGE (Supplementary Fig. S2). Protein concentration was determined by the Bradford assay.

Synthetic DNA substrates

Oligonucleotides 1 (F3-d47), 2 (49N-2), 3 (d22), 4 (d20), and 5 (d28) were described previously (Komori et al. 2002). The se-

Table 2. *E. coli* strains

Strain	Relevant genotype	Reference or source
AB1157	<i>rec⁺, ruv⁺, gyr⁺, dna</i>	Bachmann 1972
E486	<i>dnaE486</i>	Wechsler and Gross 1971, NIG ^a
KD1996	<i>recQ61::Tn3, polA12</i>	NIG
OH1000	AB1157 but $\Delta recQ::Cam$	Lab stocks
TS1504	AB1157 but <i>recQ61::Tn3</i>	AB1157 × P1 KD1996
CAG12204	Hfr[PO3]btuB3192::Tn10kan	Singer et al. 1989
CAG18436	<i>zae-502::Tn10</i>	Singer et al. 1989, NIG
HRS2006	AB1157 but $\Delta(srl-recA)306::Tn10$	Lab stocks
TS1502	AB1157 but <i>dnaE486 zae-502::Tn10</i>	AB1157 × P1 TS1501
TS1507	TS1502 but $\Delta recQ::Cam$	TS1502 × P1 OH1000
AB1886	AB1157 but <i>uvrA6</i>	Brandsma et al. 1988, NIG
TS1520	AB1157 but <i>uvrA6, recQ61::Tn3</i>	AB1886 × P1 TS1504
TS1501	E486 but <i>zae-502::Tn10</i>	E486 × P1 CAG18436

^aNIG: NIG (National Institute of Genetics, Japan) collection.

quences of oligonucleotides 6(49R) and 7(d30-2) are 5'-AGC TATGACCATGATTACGAATTGCTTAATTCGTGCAGGC ATGGTAGCT-3' and 5'-CTGATACGAATTCGTGCAGGCA TGGTAGCT-3', respectively. The 5'-overhang, 3'-overhang, 5'-flap, 3'-flap, and blunt end DNAs were made from oligonucleotides 1 + 4, 2 + 3, 1 + 2 + 4, 1 + 2 + 3, and 2 + 6, respectively. Forked DNAs with no gap or with ssDNA on the leading or lagging strand were made from 1 + 2 + 5 + 7, 1 + 2 + 4 + 7, and 1 + 2 + 3 + 5, respectively. The Holliday junction was constructed as described previously (Hishida et al. 1999).

Helicase assay

Reaction mixtures (20 μ L) contained RecQ (10 nM), substrate DNA (10 nM), 1 mM ATP, 20 mM Tris-acetate (pH 8.0), 1 mM DTT, and 1 mM Mg-acetate. After incubation for the indicated time at 26°C, reactions were stopped with 5 μ L of stop buffer (50 mM Tris-HCl at pH 7.5, 100 mM EDTA, 1% SDS, and 7.5 mg/mL proteinase K) and incubated for 10 min. Reaction products were analyzed by electrophoresis on a 12% polyacrylamide gel (PAGE), except the Holliday junction (9% PAGE), and visualized using a Fuji BAS2000 image analyzer.

DNA-binding assay

Reaction mixtures contained 20 mM Tris-acetate (pH 8.0), 1 mM DTT, 10 nM substrate DNA, and the indicated concentration of RecQ protein. Reactions were incubated at 37°C for 10 min. Products were analyzed on 6% PAGE in TAE buffer and visualized using a Fuji BAS2000 image analyzer. Binding affinity was calculated as the reciprocal of the RecQ concentration at which half the available DNA was bound and half was unbound.

SOS induction

Induction of the SOS response was monitored by measuring synthesis of β -galactosidase (Miller 1992) in strains harboring a *umuDC::lacZYA* fusion gene on pSK1002 (Shinagawa et al. 1983). Enzyme units are given as a fraction of the O.D.₆₀₀ of the culture at the time of sampling. The detection of LexA degradation was carried out as described previously (Yasuda et al. 1998). Log-phase cultures were treated with chloramphenicol (100 μ g/mL) to inhibit the resynthesis of LexA and incubated for 10 min at 37°C. Then, the cells were UV-irradiated (time 0), and aliquots were sampled at intervals. Samples were analyzed by 15% SDS-PAGE, and RecA and LexA proteins were detected by immunoblotting with anti-RecA and anti-LexA antibodies. The *recQ61::Tn3* strain was used in this assay because the *Δ recQ::cam* strain is resistant to chloramphenicol.

Western blots

Cells (1×10^8 cells/mL) were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% SDS, and bacterial protease inhibitor cocktail (Sigma). After boiling, aliquots of the sample were applied to 15% SDS-PAGE gels. The RecA and LexA proteins were detected with rabbit antibodies raised against RecA and LexA, using the ECL Western blotting analysis system (Amersham Pharmacia).

Morphological analysis

The cells were grown in liquid LB to early logarithmic phase (1×10^7 cells/mL) at 30°C, and grown for 6 h at 38°C. Cells were harvested, fixed with methanol, and then stained with DAPI (4',6-diamino-2-phenylindole). Cell morphology was moni-

tored under a fluorescence microscope (Zeiss Axioplan2) as previously described (Ishioika et al. 1998).

Acknowledgments

We thank B. Michel, K. Umezu, J. Courcelle, J.M. Murray, and R.G. Lloyd for their critical reading of this manuscript. Y.-W.H. was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists. This work was supported by Grants in Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan to H.S.

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