ATP-dependent resolution of R-loops at the ColE1 replication origin by *Escherichia coli* RecG protein, a Holliday junction-specific helicase

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The RecG protein of *Escherichia coli* is a DNA helicase that promotes branch migration of the Holliday junctions. We found that overproduction of RecG protein drastically decreased copy numbers of ColE1-type plasmids, which require R-loop formation between the template DNA and a primer RNA transcript (RNA II) for the initiation of replication. RecG efficiently inhibited *in vitro* ColE1 DNA synthesis in a reconstituted system containing RNA polymerase, RNase HI and DNA polymerase I. RecG promoted dissociation of RNA II from the R-loop in a manner that required ATP hydrolysis. These results suggest that overproduced RecG inhibits the initiation of replication by prematurely resolving the R-loops formed at the replication origin region of these plasmids with its unique helicase activity. The possibility that RecG regulates the initiation of a unique mode of DNA replication, *oriC*-independent constitutive stable DNA replication, by its activity in resolving R-loops is discussed.

Keywords: RecG/recombination/R-loop/RNA–DNA helicase/stable DNA replication

Introduction

In *Escherichia coli*, the *recG* and *ruvA*, *B*, *C* genes are thought to play overlapping roles in homologous recombination and recombination repair, since single mutations in the *recG* or *ruv* genes were only mildly defective, whereas *recG ruv* double mutants were severely defective in these functions (Lloyd, 1991). Genetic evidence suggests that both *recG* and *ruv* are involved in the processing of Holliday recombination intermediates (Benson *et al.*, 1991; Lloyd, 1991). The biochemical properties of these gene products are consistent with their roles in Holliday junction processing. RuvA protein is a Holliday junction-specific binding protein that targets RuvB protein, which is a DNA helicase, to the junction by forming a complex, and the complex promotes migration of the junction by catalyzing strand exchange (Shiba *et al.*, 1991; Iwasaki *et al.*, 1992; Parsons *et al.*, 1992; Tsaneva *et al.*, 1992; Parsons and West, 1993). RecG protein also possesses junction-specific binding properties similar to those of RuvA and promotes migration of Holliday junction as does RuvAB (Lloyd and Sharples, 1993; Whitby

et al., 1994). However, RuvAB differs from RecG in possessing a 5' to 3' helicase activity with respect to single strand DNA while RecG shows a 3' to 5' DNA helicase activity (Tsaneva *et al.*, 1993; Whitby *et al.*, 1994).

In the RuvABC pathway, RuvC is a specific endonuclease that resolves Holliday junctions (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991). RecG, which functions in the alternative pathway, has been suggested to promote junction resolution by driving branch migration in the opposite direction from that promoted by RecA protein (Whitby *et al.*, 1993; Whitby and Lloyd, 1995). These authors proposed that RecG plays a role in preventing unproductive recombination by reversing the invasion of a 5' single-stranded DNA end into the homologous duplex (Whitby and Lloyd, 1995).

The *recG* and *ruv* genes play different roles in alternative mechanisms of stable DNA replication (SDR), which do not require the chromosomal replication origin (*oriC*), DnaA initiator protein or concomitant protein synthesis (Asai and Kogoma, 1994a). Mutations in the *ruv* or *recG* gene stimulate inducible SDR (iSDR), which is a part of SOS responses, suggesting that this mode of SDR involves D-loops or Holliday junctions made by RecA (Asai and Kogoma, 1994b). *recG* mutants, but not *ruv* mutants, stimulate constitutive SDR (cSDR), which was found originally in RNase HI-defective (*rnhA*) mutants, and *recG rnhA* double mutants are inviable. These results suggested that RecG, like RNase HI, plays an important role in the removal of R-loops. R-loops are thought to be required for the initiation of cSDR (Kogoma *et al.*, 1994; Hong *et al.*, 1995).

In order to define better the function of RecG and compare the properties of RecG with those of RuvAB, we constructed an overproduction system and purified RecG. In the course of this study, we observed that a recombinant plasmid derived from pUC19 that overproduced RecG protein showed a drastically reduced plasmid copy number. Since the initiation of DNA replication of ColE1-type plasmids requires a precursor RNA, called RNA II, which forms a stable RNA–DNA hybrid (R-loop) at the origin, we hypothesized that RecG itself resolves the R-loop and thereby inhibits the initiation of DNA replication. Here we present results that support this hypothesis.

Results

Overexpression of RecG protein drastically decreases plasmid copy number

To overproduce RecG protein, we first constructed pAF219 (Figure 1A). We noticed that plasmid DNA recovery from cells carrying this plasmid was very poor. To examine whether overexpression of *recG* was related to the decrease

Fig. 1. Decrease in the copy number of the ColE1-type plasmids by overproduction of RecG protein. (A) Schematic representation of the plasmid constructs that overproduce RecG and the control plasmids. The large arrows indicate the direction of the *recG* transcription and the small arrows indicate the direction of transcription initiated from the *lac* promoter (*plac*). (B) Effect of RecG overproduction on the plasmid copy numbers. Plasmid DNAs were prepared from 2 OD_{600 nm} units of *E. coli* AB1157 cells as the host. The samples were analyzed by 0.8% agarose gel electrophoresis, and DNA was stained with ethidium bromide. Lanes a–d, pUC derivative plasmids; lanes e–h, p15A derivative plasmids. (C) Effect of genetic backgrounds of the host strains on the plasmid copy number. Plasmid DNAs were prepared and analyzed as in (B). Lanes a and e, AB1157 (wild-type); lanes b and f, HRS2006 [Δ (*srfR-recA*)306::Tn10]; lanes c and g, HRS2004 (*rhA*399::cat); lanes d and h, HRS2000 (Δ *recG*100::Km^r).

in the plasmid copy number, we compared plasmid yields from AB1157 cells carrying plasmids with or without *recG* (Figure 1A and B, lanes a–d). The drastic decrease was observed only in pAF219 in which the *recG* coding

region lacking its cognate promoter is inserted downstream of the *lac* promoter of pUC19. The yield was normal with pAF218, which contained the same *recG* fragment as pAF219 but inserted in the opposite direction to the *lac* promoter, or with the plasmids which did not carry the *recG* gene (pUC18 and pUC19). The situation was essentially the same when we employed vectors which had the replicon derived from p15A, another ColE1-type plasmid (Figure 1A and B, lanes e–h).

We examined the effects of some mutations related to the *recG* functions on the plasmid copy number (Figure 1C). The copy numbers of pUC19 in various mutant strains including a Δ *recG* mutant were almost the same as that in the wild-type strain, although more plasmid multimers were observed in the *rhA* and *recG* strains. The copy numbers of pAF219 were much reduced regardless of the host strains examined. Since the copy number reduction did not require *recA* function, this phenomenon appeared independent of homologous recombination. We therefore considered the possibility that RecG might inhibit plasmid DNA replication directly.

RecG inhibits ColE1 DNA replication *in vitro*

To analyze the RecG effect on plasmid DNA synthesis *in vitro*, we purified RecG protein from the cells overproducing RecG by a T7 expression system (see Materials and methods), and the RecG peak fractions were analyzed by SDS-PAGE. The purity of the RecG protein in this preparation was >99% and it was DNase and RNase free, as assayed with several substrates including synthetic oligonucleotides and single- or double-stranded linear or circular DNAs (data not shown).

We studied the effect of RecG on the ColE1 plasmid replication *in vitro*, using the reconstitution system developed by Itoh and Tomizawa (1979). In this system, the precursor of primer RNA (called RNA II) synthesized by RNA polymerase forms a stable RNA–DNA hybrid (R-loop) at the replication origin (*ori*), and it is processed specifically by RNase HI to serve as a primer for deoxynucleotide polymerization by DNA polymerase I (Tomizawa *et al.*, 1977; Itoh and Tomizawa, 1979, 1980). We found that RecG inhibits incorporation of dTMP into the acid-insoluble fraction in a concentration-dependent manner (Figure 2).

RecG inhibits plasmid DNA replication with a pre-formed R-loop

To identify the step in plasmid replication that was inhibited by RecG, we prepared pNT35 DNA containing an R-loop at the *ori* region, by preferential transcription from the RNA II promoter using uridylyl (3' to 5') uridine (UpU) and rifampicin (see Materials and methods). The reaction products were analyzed by agarose gel electrophoresis (Figure 3). Two separate DNA bands were detected by ethidium bromide staining (lane b). The faster migrating band corresponded to that of pNT35 covalently closed circular DNA (cccDNA). More than 50% of DNA in the reaction mixture migrated as a band with a slower migration rate than the cccDNA. At the position corresponding to the slower band, we detected radiolabeled RNA (lane d). The faster RNA band should be free RNA, since the band position corresponded to that of the RNA released from the slower band by heat treatment (data not

Fig. 2. Inhibition of DNA replication of pNT35, a ColE1 derivative, by RecG *in vitro*. The *in vitro* plasmid DNA synthesis reaction was carried out using monomeric cccDNA of pNT35 as a template in the presence of various concentrations of RecG at 30°C for 60 min. DNA synthesis was measured by the incorporation of [α - 32 P]dTMP into the acid-insoluble fraction.

Fig. 3. Preparation of pNT35 containing the R-loop. pNT35 containing the R-loop was prepared and purified as described in Materials and methods. Products containing the R-loop were electrophoresed on 1% agarose gel, the gel was stained with ethidium bromide (lanes a–c) and the radioactive bands containing RNA were visualized by autoradiography (lanes d and e). The substrate and the reaction products are shown schematically on the right. Lane a, monomeric cccDNA of pNT35 (before transcription); lanes b and d, pNT35 containing the R-loop after transcription; lanes c and e, the reaction products after RNase HI treatment of pNT35 containing the R-loop. Note that [α - 32 P]UMP was incorporated into RNA by RNA polymerase-mediated transcription.

shown). When the transcription product was incubated with RNase HI at 37°C, the RNA migrating with the slower DNA band disappeared (lane e). This result indicates that the slower DNA band was plasmid DNA containing an RNA–DNA hybrid (R-loop).

We studied the effect of RecG on an *in vitro* replication system containing the plasmid with the R-loop, RNase HI, DNA polymerase I, ATP and dNTPs. In this system, RNA II constituting the R-loop is processed by RNase HI to become a functional primer (Itoh and Tomizawa, 1980). RecG inhibited DNA synthesis in this replication system in a concentration-dependent manner (Figure 4, closed circles). As a control experiment to assay the R-loop-independent DNA synthesis, we measured the dTMP incorporation by DNA polymerase I with pNT35 pre-treated with DNA ligase to reduce the nicks in the DNA template. Low but significant levels of DNA synthesis,

Fig. 4. Inhibition by RecG of *in vitro* DNA synthesis with the plasmid containing a pre-formed R-loop. DNA synthesis reactions were carried out using pNT35 containing the R-loop as a pre-primed template. In this system, the precursor RNA II in the R-loop is processed by RNase HI, and DNA synthesis is catalyzed by DNA polymerase I. RecG as indicated was added and the incorporation of [α - 32 P]dTMP into the acid-insoluble fraction was measured. ●, pNT35 containing the R-loop; ◇, cccDNA of pNT35 treated with RNA polymerase in the absence of NTPs; ■, monomeric cccDNA of pNT35; □, values of dTMP incorporation in the complete reaction system (●) subtracted from those of the control reactions (◇).

which were not affected by RecG, were observed (Figure 4, closed squares). As another control, we measured the dTMP incorporation using a plasmid template prepared in an RNA polymerase reaction that was devoid of NTPs. Slightly higher levels of DNA synthesis than in the former experiment, which were also unaffected by RecG addition, were observed (Figure 4, diamonds). The lower level of DNA synthesis might be caused by small amounts of the remaining nicks in the template DNA which served as the primer for DNA synthesis by DNA polymerase I, and the increase in the synthesis in the latter experiment might be due to the nicks introduced by the contaminating DNase in the RNA polymerase preparation. When we subtracted the dTMP incorporation in the system that lacks NTPs, which we considered due to the nick-translation type of DNA synthesis, from the incorporation in the complete system, a 50% inhibition of the DNA replication was attained by ~0.02 nM RecG (Figure 4, open squares).

To confirm that RecG does not inhibit the polymerization activity of DNA polymerase I directly, we assayed DNA synthesis using a defined template–primer, poly-(dA)·(dT)_{12–18}. In this reaction, the incorporation of dTMP was not affected by the addition of RecG (data not shown). These results suggest that RecG does not inhibit DNA elongation by DNA polymerase I.

RecG resolves the R-loop at ori in an ATP hydrolysis-dependent manner

The above results suggest that RecG inhibits DNA replication at the initiation step. Although it was conceivable that RecG might inhibit replication initiation by preventing processing of RNA II into the functional primer by RNase HI or by removing the RNA II from the R-loop by helicase activity, we thought the latter was the most likely possibility, for the following reasons. First, RecG has junction-specific DNA helicase activity (Whitby *et al.*, 1994) and possesses well-conserved motifs for RNA/DNA

Fig. 5. Resolution of the R-loop by RecG. pNT35 DNA containing the R-loop labeled with [α - 32 P]UTP was incubated with RecG (200 nM) in the presence of ATP at 37°C for 10 min (complete system). The reaction products were analyzed by electrophoresis on 1% agarose gel, and visualized by ethidium bromide staining (A) and autoradiography (B). Lane a, without RecG and ATP at 4°C; lane b, without RecG and ATP at 37°C; lane c, RNase HI (120 U) as a control; lane d, without RecG; lane e, complete system; lane f, ATP substituted by ATP γ S; lane g, ATP substituted by ADP; lane h, without ATP; lane i, monomeric cccDNA of pNT35 as a position marker.

helicases (Lloyd and Sharples, 1991; Kalman *et al.*, 1992). Second, the involvement of RecG in the resolution of R-loops by its helicase activity has been proposed from genetic studies on *cSDR* (Hong *et al.*, 1995). Third, the reduction of plasmid copy number was also observed in a *rnhA* mutant (Figure 1C, lane g). To test our hypothesis directly, we incubated the plasmid containing 32 P-labeled R-loop with RecG in the presence of $MgCl_2$ and ATP (Figure 5). The products of the reaction were analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide (Figure 5A) and autoradiography (Figure 5B). The bands containing the R-loop disappeared and the free RNA band increased during incubation with RecG (lane e). Omission of ATP (lane h) or substitution of ATP with the non-hydrolyzable analog of ATP, ATP γ S (lane f), or with ADP (lane g) abolished this effect, indicating dependence on the hydrolysis of ATP for the reaction. As shown previously, RNase HI diminished the R-loop band and increased the free RNA band (lane c).

To show that the removal of the RNA from the R-loop and the corresponding increase in the free RNA was not caused by the cleavage of the RNA in the RNA–DNA hybrid, we analyzed the sizes of the reaction products by 6% PAGE containing 8 M urea (Figure 6). A fast RNA band, which was observed in the reaction products by RNase HI (Figure 6A, lane d), was not detected in the RecG reaction products (Figure 6A, lane f). The largest RNA band was digested preferentially by RNase HI (Figure 6B, lane d), suggesting that it contained the RNA–DNA hybrid formed at the *ori* region. In fact, the size of the largest RNA corresponded to the expected size of RNA II (770 nucleotides) (Tomizawa and Masukata, 1987). These results support the conclusion that RecG resolves the R-loop by using its RNA–DNA helicase activity. Consistent with this notion, preliminary experiments with synthetic RNA–DNA hybrids suggest that RecG has a junction-specific RNA helicase activity.

Fig. 6. Products of the R-loop resolution. The reaction products described in Figure 5 were analyzed by electrophoresis on 6% PAGE containing 8 M urea at 1800 V for 45 min (A) and for 5 h (B). Lane a, size marker (pUC19 DNA digested by *Tth*HB8 I and end-labeled at the 5' end with [γ - 32 P]ATP); lane b, without RecG and ATP at 4°C; lane c, without RecG and ATP at 37°C; lane d, RNase HI (120 U) as a control; lane e, without RecG; lane f, complete system; lane g, ATP substituted by ATP γ S.

Fig. 7. RecG concentration dependency of the R-loop resolution. The reactions were done as in Figure 5 with various concentrations of RecG as indicated at the top. Lanes a–j, 2 mM ATP; lanes k and l, no ATP.

Quantitative correspondence between R-loop resolution and the inhibition of plasmid replication by RecG

We next measured the release of RNA II from the R-loop as a function of the RecG concentration (Figure 7). To release 50% of the RNA from the R-loop, ~0.1 nM RecG was required. This concentration nearly corresponded to that required for a 50% inhibition of the plasmid DNA replication (Figure 4). These results strongly suggest that

efficient removal of the RNA II from the R-loop by the RecG helicase prevents the subsequent processes required for the initiation of the DNA replication. Since RecG at 0.1 nM could resolve ~1.5 nM of R-loop under these conditions, the reaction should be catalytic. RuvAB protein complex could resolve the R-loops but could do so only at an extremely high concentration, 1000-fold higher than the required RecG concentration (data not shown). Together with the *in vivo* result showing that overproduction of RuvAB proteins does not decrease the copy number of ColE1-type plasmids (our unpublished data), this activity of RuvAB would not be expected to have any physiological significance.

Discussion

We have observed that the *recG* gene carried by a pUC-derived plasmid reduced the plasmid copy number drastically when it was placed downstream of the *lac* promoter in the correct orientation but not when it was in the opposite direction (Figure 1B). This phenomenon was also observed with *recG* recombinant plasmids derived from the p15A plasmid. Since these plasmids have initiation mechanisms very similar to that of the ColE1 plasmid, we employed a well characterized ColE1-based *in vitro* system for the analysis of the underlying mechanism of this phenomenon. The initiation of ColE1-type plasmid replication requires synthesis of RNA II which hybridizes with the template DNA around the *ori* region to form an R-loop. The hybridized RNA II is processed to serve as a primer for the initiation of DNA synthesis by DNA polymerase I (Itoh and Tomizawa, 1980). We have shown that RecG promotes removal of the RNA from an R-loop and that this reaction requires ATP hydrolysis (Figure 5). Since the concentration of RecG that resolved 50% of the R-loop was similar to that required for 50% inhibition of DNA replication *in vitro* under similar experimental conditions (Figures 4 and 7), we think that the premature dissociation of the RNA from the R-loop is the likely cause of the replication inhibition *in vitro*. RecG did not inhibit the DNA elongation step by DNA polymerase I. Taken together, these results suggest that RecG overproduced from the recombinant plasmids inhibits replication initiation of the ColE1-type plasmids *in vivo* by resolving R-loops prematurely. Since R-loops are stable, as they contain an RNA–DNA hybrid region of ~220 bp (Masukata and Tomizawa, 1984), and the RecG resolves the R-loop catalytically (Figure 7), the present results suggest that RecG possesses an ATP-dependent RNA helicase activity which unwinds the RNA–DNA hybrid. However, RecG exhibits little activity in unwinding a short RNA hybridized to longer single-stranded DNA (our unpublished data).

RecG has a junction-specific DNA helicase activity that efficiently unwinds synthetic Holliday junctions (four-way junction), Y-junctions (three-way junction) and three-stranded junctions, but it shows extremely low helicase activity when assayed with partial duplex DNA substrates (Lloyd and Sharples, 1993; Whitby *et al.*, 1994; Whitby and Lloyd, 1995). The RNA helicase activity of RecG described here also seems to share similar junction specificity. However, this point should be tested rigorously by constructing various forms of junctions containing RNA–

DNA hybrids. RuvAB has little activity to resolve R-loops (data not shown), and overproduction of RuvAB or RuvABC did not affect plasmid copy number (our unpublished data). Although RecG and RuvB share the ATP binding motif A (GxGKT) and specialized motif B (DExH) for ATP binding, only RecG contains the well-conserved seven motifs shared by the RNA/DNA helicase superfamily II, which includes RNA helicases involved in mRNA splicing and transcription regulation (Gorbalenya *et al.*, 1989; Lloyd and Sharples, 1991; Kalman *et al.*, 1992).

cSDR is an alternative mechanism for the initiation of chromosomal replication observed in *rmhA* mutants devoid of RNase HI. It does not require the normal replication origin, *oriC*, the initiation protein DnaA and new protein synthesis for the initiation of new rounds of DNA replication (Asai and Kogoma, 1994a). However, it requires RNA synthesis for initiation, which differs from another alternative mechanism, SOS-regulated iSDR. Although both SDRs require RecA function, only iSDR requires RecBC(D) function. Based on these findings, it was proposed that RecBCD and RecA promote the formation of D-loops during iSDR by their recombination functions, and RecA promotes the formation of R-loops in cSDR by assimilating an RNA transcript into duplex DNA (Asai and Kogoma, 1994a). The D-loops or R-loops thus formed may provide sites for the PriA-mediated primosome assembly. Like ColE1 replication, the invading single strand 3' ends within D-loops or R-loops may serve as primers for the initiation of leading strand synthesis (Kogoma, 1996). RecG, like RNase HI, was proposed to function in removing RNA from the R-loop using its helicase activity (Hong *et al.*, 1995). *recG* mutants, in which the R-loops are expected to be more stable, also exhibit cSDR, and a combination of *rmhA* and *recG* mutations is lethal to the cell (Hong *et al.*, 1995). The latter phenomenon suggests that the persistence of R-loops is harmful to the cell. The present work provides biochemical evidence that RecG indeed resolves R-loops and, therefore, it gives credence to the hypothesis that RecG controls the initiation of DNA replication primed by R-loops at non-*oriC* sequences *in vivo*. Although the physiological significance of cSDR had been unclear, very recent work from Kogoma's laboratory demonstrated that a cSDR-like activity is activated in wild-type cells at the time of entry into the stationary phase (Hong *et al.*, 1996). Overproduction of RecG at the level that greatly reduced the copy number of the plasmids did not affect the growth of the host cell significantly. RecG might not affect the normal mode of chromosomal replication and, therefore, might not interact with the RNA–DNA hybrid formed at the *oriC* region and with the hybrids formed by primase at the replication fork. The structures of these hybrids may be different from those of the R-loops formed at the ColE1 *ori* region and the *oriK* regions in cSDR.

Plasmid copy numbers of pUC19 and pSTV29 were not affected significantly in a *recG* deletion mutant. This does not necessarily mean that *recG* when expressed at normal physiological levels does not play any role in resolving R-loops and in regulating initiation of the plasmid replication. It is more likely that other gene product(s) can substitute for RecG in its role in resolving R-loops in *recG* mutant cells. The R-loop could be

resolved by the endonuclease activity of RNase HI and by exonuclease activity of DNA polymerase I.

Materials and methods

Bacterial strains, plasmids and growth conditions

Escherichia coli K-12 strains HRS2004 (*rnhA399::cat*), HRS2006 [Δ (*srlR-recA*)306::Tn10] and HRS2000 (Δ *recG*100::Km^r) were made by P1 phage-mediated transduction of the *rnhA399::cat* allele from KHG557 (a gift from T.Horiuchi, National Institute for Basic Biology, Okazaki, Japan), the Δ (*srlR-recA*)306::Tn10 allele from DM2571 (Ennis *et al.*, 1985) and the Δ *recG*100::Km^r allele from HRS1997 (H.Iwasaki, unpublished), respectively, into AB1157 (Bachman, 1972). BL21(DE3) was used as a host strain for overproduction of RecG using a T7 expression system (Studier *et al.*, 1990). The *recG* gene was subcloned from the clone 571 of Kohara's *E.coli* genome library (Kohara *et al.*, 1987) into pACH191, a pACYC174 derivative containing a *KpnI* restriction site (H.Iwasaki, unpublished), as a 7 kb *KpnI* fragment. pAF201 is a pT7-7 (Tabor, 1990) derivative that carries the 2.3kb *PstI*–*Clal* fragment containing the *recG* coding region and expresses the *recG* gene under the control of the T7 expression system. Plasmids pAF218, pAF219, pAF518 and pAF519 were obtained by cloning the coding region of the *recG* gene contained on a 2.0 kb *PstI*–*HindIII* fragment into the same restriction sites of pUC18, pUC19 (Yanisch-Perron *et al.*, 1985), pSTV28 and pSTV29 (Takara Biomedicals), respectively (Figure 1A). Plasmid pNT35, a smaller derivative of ColE1 plasmid (Masukata and Tomizawa, 1984), was used for the preparation of the R-loop and as a template for DNA synthesis *in vitro*. Bacteria were cultured in LB medium or grown on LB plates (Sambrook *et al.*, 1989) at 37°C and, when required, antibiotics, ampicillin, chloramphenicol and tetracycline were used at final concentrations of 50, 10 and 10 µg/ml, respectively.

Chemicals and enzymes

Poly(dA)-(dT)_{12–18}, *E.coli* DNA polymerase I, RNA polymerase, single strand DNA binding protein (SSB) and chromatography media were purchased from Pharmacia. UpU and *E.coli* RNase HI were from Sigma and Takara Biomedicals, respectively.

Purification procedure for RecG protein

RecG protein was purified from BL21(DE3) carrying pAF201. Cells were grown in LB with ampicillin to an OD_{600 nm} of 0.5, and induced by 1 mM isopropyl-β-D-thiogalactopyranoside and grown further for 6 h. The cells were collected by centrifugation and disrupted by sonication in a lysis buffer (20 mM Tris–HCl, pH 8.5, 10% glycerol, 7 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100, 400 mM NaCl). RecG protein in the purification steps was identified by SDS–PAGE. Clear supernatant was obtained by centrifugation at 40 000 g for 30 min. After nucleic acids in the supernatant were removed by the addition of polyethylenimine (0.5%), proteins containing RecG were precipitated with 70% saturation of ammonium sulfate. The pellet was resuspended in R-buffer (20 mM Tris–HCl, pH 7.5, 10% glycerol, 7 mM β-mercaptoethanol) and dialyzed against R-buffer + 500 mM NaCl. The dialyzed solution was diluted 5-fold, and applied to a DEAE–Sephacrose column equilibrated with R-buffer + 100 mM NaCl. The flow-through fraction was loaded directly onto an SP–Sephacrose column equilibrated with the same buffer, and eluted with a linear gradient of NaCl (0.1–1.0 M) in R-buffer. Fractions containing RecG, which were eluted at ~500 mM NaCl, were pooled. The pooled fraction was applied to a HiLoad 26/60 Superdex 200 gel filtration column equilibrated with R-buffer + 1 M NaCl. RecG fractions were diluted 10-fold and applied to a HiTrap heparin column equilibrated with R-buffer + 200 mM NaCl, and eluted with a linear gradient of NaCl (0.2–1.0 M) in R-buffer. The pooled RecG fraction was diluted with R-buffer (in this case pH 8.5), applied to a HiTrap Q column equilibrated with R-buffer (pH 8.5), and eluted with a linear gradient of NaCl (0–1.0 M) in R-buffer (pH 8.5). The RecG fraction was dialyzed against the storage buffer (10 mM Tris–HCl, pH 8.0, 0.05 mM EDTA, 3.5 mM β-mercaptoethanol, 500 mM NaCl and 50% glycerol) and stored at –20°C. Protein concentrations were measured by a modified Bradford method with a Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard.

Plasmid DNA preparation

For comparison of plasmid copy number, plasmid DNAs were purified from 2 OD_{600 nm} units of cells with QIAprep Spin Plasmid Miniprep kit (QIAGEN), and were suspended in 50 µl of TE buffer (10 mM Tris–

HCl, pH 8.0, 1 mM EDTA). Plasmid pNT35 DNA in monomeric ccc form was used as a template for *in vitro* DNA replication and for preparation of R-loops. The plasmid was amplified in DH5α (Hanahan, 1983), a *recA E.coli* host, and purified by the alkaline lysis method, followed by two cycles of equilibrium centrifugation in ethidium bromide–CsCl (Sambrook *et al.*, 1989). To seal the remaining nicks in the circular DNA, the preparation was treated with T4 DNA ligase and then deproteinized with phenol. This plasmid preparation was called monomeric cccDNA.

Preparation of plasmid DNA containing an R-loop

Monomeric cccDNA of pNT35 (2 µg) was incubated at 44°C for 10 min with 9.8 U of *E.coli* RNA polymerase in a reaction mixture (75 µl) containing 20 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.05% BSA, 40 µg/ml SSB, 2 mM UpU and 2 µM GTP, CTP and ATP. At the end of the incubation period, rifampicin (20 µg/ml) was added to inhibit the re-initiation of RNA synthesis, and incubated for another 5 min. To this, 25 µl of NTP mixture (1.2 mM of CTP, GTP and UTP containing [α -³²P]UTP, and 6 mM ATP) were added, and the incubation was continued for 10 min. When the R-loop was used as a pre-primed template for DNA synthesis, the reaction was carried out in the absence of the isotope-labeled UTP. The reaction was stopped by addition of 25 µl of stop buffer (1 µg/ml proteinase K, 20 mM EDTA, 0.5% SDS) and incubated at 37°C for 10 min. pNT35 containing the R-loop was purified by gel filtration through a MicroSpin S-400 HR column (Pharmacia).

R-loop resolution assay

Approximately 3 nM (in terms of plasmid molecules) of pNT35 DNA with R-loop and RecG as indicated were incubated in a reaction mixture (20 µl) containing 20 mM Tris–HCl, pH 8.0, 4 mM MgCl₂, 1 mM DTT, 0.05% BSA and 2 mM ATP for 10 min at 37°C. Reactions were stopped by the addition of 10 µl of stop buffer and incubated for a further 10 min. The reaction products were analyzed by electrophoresis on 1% agarose gels containing TAE buffer (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA) and by 6% PAGE containing 8 M urea with TBE buffer (45 mM Tris-borate, pH 8.0 and 1 mM EDTA). When the reaction products were analyzed by 6% PAGE containing 8 M urea, the samples were dried, resuspended in 30 µl of loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue), heated at 70°C for 3 min and loaded onto the gel.

In vitro DNA synthesis

Three kinds of assay of DNA synthesis were employed in this study. (i) When pNT35 monomeric cccDNA (7 nM) was the template DNA, the procedures of Itoh and Tomizawa (1979) were followed. RNA polymerase (0.3 U), RNase HI (0.06 U), DNA polymerase I (0.3 U) and RecG as indicated were added to start the reaction. (ii) When pNT35 with a pre-formed R-loop was used as the template, ~3 nM R-loop and RecG as indicated were first incubated in 9 µl of a DNA synthesis buffer (20 mM Tris–HCl, pH 8.0, 4 mM MgCl₂, 0.05% BSA and 1 mM DTT) on ice for 10 min. The reactions were initiated by the addition of 21 µl of a solution containing DNA polymerase I (0.3 U), RNase HI (0.06 U), 2 mM ATP, 5 µM of dTTP containing [α -³²P]dTTP and 50 µM of the other dNTPs in the buffer. The mixtures were incubated at 37°C for 5 min and the reactions were stopped by the addition of 2 µl of 0.5 M EDTA. (iii) When poly(dA)-(dT)_{12–18} was used as a template–primer, 0.003 OD_{260 nm} units of the template–primer and RecG as indicated were incubated in the DNA synthesis buffer containing 2 mM ATP, 1 mM [α -³²P]dTTP and DNA polymerase I (0.3 U). The reaction mixture was incubated at 37°C for 60 min and stopped by the addition of 2 µl of 0.5 M EDTA. The dTMP incorporated was measured by acid-insoluble radioactivity.

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