J. Biochem. 131, 121–129 (2002)

Characterization of RecA424 and RecA670 Proteins from *Deinococcus radiodurans*[1]

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Received September 14, 2001; accepted November 6, 2001

RecA protein is considered to be the most important participant in the radiation resistance of *Deinococcus radiodurans*. However, it is still unclear how RecA contributes to the resistance. In this study, we identified a new *recA* mutation (*recA424*) in the DNA-repair deficient mutant strain KI696, the phenotype of which is remarkably different from mutant strain rec30 carrying *recA670*. The properties of the gene products from the *recA* mutants were compared. *recA424* could not complement the deficiency in *Escherichia coli* RecA, as found for *recA670*. *In vitro*, neither RecA424 nor RecA670 could promote DNA strand exchange under conditions in which wild-type RecA promoted the reaction, indicating that both RecA424 and Rec670 are defective in recombination activity. RecA424 promoted the autocleavage reaction of LexA *in vitro*, whereas RecA670 did not. The intracellular LexA level in KI696 was decreased following γ-irradiation. However, the LexA level in strain rec30 was constant irrespective of irradiation. These results indicate that RecA424 retains co-protease activity, whereas RecA670 does not. While strain rec30 is extremely radiation sensitive, strain KI696 is only slightly sensitive. Together, these observations suggest that the co-protease activity rather than the recombination activity of RecA contributes to radiation resistance in *D. radiodurans*.

Key words: co-protease activity, *Deinococcus radiodurans* RecA, DNA repair, DNA strand exchange, γ-ray resistance.

The RecA protein of *Escherichia coli* is a multifunctional protein with roles in the induction of the SOS response to DNA damage, SOS mutagenesis, homologous genetic recombination, and recombinational DNA repair (*1–3*). RecA is considered to promote the central steps of recombination *in vivo*: homologous pairing and strand exchange to establish a crossover, and branch migration. RecA promotes a DNA strand reaction *in vitro* that mimics the central steps of recombination *in vivo* (*1*). In the response to DNA damage, RecA is activated by the presence of single-stranded DNA, and mediates as a co-protease the proteolytic cleavage of LexA, UmuD and phage repressors. Cleavage of LexA results in derepression of the SOS regulon, a set of more than 30 genes required for DNA repair, mutagenesis and inhibition of cell division (*2, 4*).

Deinococcus radiodurans is characterized by its extraordinary resistance to the lethal and mutagenic effects of ionizing and UV radiation and to many other DNA damaging agents. This resistance is considered to be due to its capacity to repair DNA damage (*5–7*). The most noteworthy characteristic is a remarkable capacity for repairing DNA double-strand breaks (*8, 9*). At 3 kGy of γ-irradiation, the *D. radiodurans* genome sustains over 100 such breaks. However, they are perfectly repaired during post-irradiation incubation (*5*). The *D. radiodurans recA* gene was first isolated by Gutman *et al.* (*10*) through analysis of a mutant strain, rec30 (*11*), that shows reduced natural transformation efficiency, marked sensitivity to DNA damage, and slow growth. The *recA* gene was sequenced, and its product found to be 56% identical to *E. coli* RecA. Despite this similarity, *D. radiodurans recA* could not complement the deficiency of *E. coli* RecA. Instead, the expression of *D. radiodurans* RecA in *E. coli* resulted in cellular death, even at low levels (*10*). In *D. radiodurans*, the RecA protein is not detectably expressed in undamaged cells but is induced following γ-irradiation (*12*). From these results, it has been suggested that RecA is as toxic to *D. radiodurans* as it is to *E. coli* and is only tolerated during DNA repair. On the other hand, mutation in strain rec30 has been supposed to relate to its own regulation, since no protein, mRNA, or fragments of either were detected under any circumstances (*12*). Daly and Minton (*13*) suggested interchromosomal recombination repair as one mechanism by which *D. radiodurans* can reconstitute its chromosomes from many hun-

[1] This work was performed as part of an Atomic Energy Crossover Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and supported by a Grant-in-Aid for Scientific Research from MEXT.

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Abbreviations: ATPγS, adenosine-5′-*O*-[γ-thio]triphosphate; dsDNA, double-stranded DNA, LB, Luria-Bertani; MMC, mitomycin-C; PVDF, polyvinylidene fluoride; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA.

dreds of DNA fragments. Comparison of the wild-type strain and strain rec30 indicated that interchromosomal recombination could proceed in a *recA*-dependent manner. However, the significance of interchromosomal recombination in resistance to DNA damage is unresolved.

Recently, we discovered that *recA* in strain rec30 carries a missense mutation (*recA670*) (*14*). In *E. coli*, the RecA611 protein has been shown to have an amino acid substitution at a similar position to RecA670, and to be deficient in promoting both DNA strand exchange and the induction of SOS response (*15–17*). Consequently, it was suggested that the *D. radiodurans* RecA670 mutation causes functional defects that resemble those observed in *E. coli* RecA611. We succeeded in expressing *recA670* in *E. coli* (*14*). However, the mutant gene could not complement the deficiency of *E. coli* RecA. Under the same conditions, we also examined the expression and complementation of wild-type *recA* gene in *E. coli*. Contrary to the previous study, the wild-type gene was expressed without lethality and fully complemented the deficiency of *E. coli* RecA. Our *recA* sequence was perfectly consistent with that found in the *D. radiodurans* genome project (*18*). On the other hand, there are four amino acid differences between our sequence and the previously published sequence (*10*). Therefore, it was thought that the plasmid clone used in the previous study produced a mutant RecA protein that is lethal to *E. coli* cells.

In this paper, we report the identification of a new *recA* mutation (*recA424*) in the DNA repair-deficient mutant strain KI696 (*19*), the phenotype of which is remarkably different from strain rec30 carrying *recA670*. Strain KI696 is sensitive to mitomycin-C (MMC) and UV, but only slightly sensitive to γ-irradiation, proficient in natural transformation, and shows a normal growth rate (*19*). To gain insight into the nature of deinococcal RecA, the properties of RecA424 were compared with those of the wild-type protein as well as RecA670.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions—*D. radiodurans* KD8301 (Ade^- $DNase^-$ Sm^R) (*20*) was used as the wild-type stain throughout this study. Strain rec30 was kindly provided by Dr. B.E.B. Moseley. *E. coli* JM107 and JM109 were purchased from Takara Shuzo (Kyoto). *E. coli* BLR(DE3) and pET3a were purchased from Novagen. *D. radiodurans* strains were grown in TGY broth containing 0.5% tryptone–peptone, 0.1% glucose, and 0.3% yeast extract, or on TGY agar at 30°C. Transformation of *D. radiodurans* was performed as described (*19*). The transformants were selected on TGY agar supplemented with 0.1 μg of MMC (Kyowa Hakko Kogyo, Tokyo) per ml. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. Ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml) were added as required.

Construction of Expression Plasmids—To isolate the *recA* coding region, PCR was carried out using pJTM89 (*14*) with the specific oligonucleotides 5′-GCGCCCCAGGAGCACATATGAGCAAGGACGCC-3′ and 5′-CACGCAGCAAGAGGATCCTTACGCTTCGGCGGC-3′ containing restriction sites (*Nde*I and *Bam*HI, underlined in the sequences). The PCR products were then digested with *Nde*I and *Bam*HI to adapt the termini for the in-frame insertion of *recA* into the *Nde*I–*Bam*HI sites of the pET3a vector. The resultant expression plasmid was designated pET3recAwt. The *recA424* and *recA670* mutations were introduced into pET3recAwt using a QuikChange Site-directed Mutagenesis Kit (Stratagene), and the resultant plasmids were designated pET3recA424 and pET3recA670. The DNA sequences of the plasmids were checked to confirm the lack of introduction of errors by PCR. DNA sequencing was done using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Other DNA manipulations were performed as described previously (*14, 20, 21*).

Protein Purification—*E. coli* BLR(DE3) carrying pET3recAwt were cultivated in LB broth containing ampicillin and tetracycline. The use of BLR(DE3) carrying Δ(*srl-recA*)*306*::Tn*10* prevents the copurification of *D. radiodurans* RecA with *E. coli* RecA. When the optical density at 600 nm reached approximately 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM, and growth was continued for an additional 3 h, after which the cells were harvested by centrifugation. Overproduction of mutant RecA proteins was carried out in the same manner using pET3recA424 and pET3recA670. The cell pellet was resuspended in cold buffer R (20 mM Tris-HCl [pH 7.5], 10% glycerol, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and stored at –80°C. All subsequent steps were carried out at 4°C unless otherwise indicated.

Cells were allowed to thaw with occasional stirring, and lysed by sonication for 10 min on ice. After the cell debris was removed by centrifugation at 10,000 ×*g* for 30 min, ammonium sulfate was added slowly to the supernatant to 50% saturation. The suspension was stirred for 1 h and then centrifuged for 30 min. The pellets were resuspended in 30 ml of buffer P1 (20 mM sodium phosphate [pH 6.4], 10% glycerol, and 0.1 mM EDTA). The suspension was dialyzed for 18 h against buffer P1. The dialyzed sample was then applied to a HiTrap SP HP column (1.6 × 10 cm, Amersham Pharmacia Biotech) equilibrated with buffer P1. The column was washed with 4 column volumes of buffer P1, and then eluted with 5 volumes of a linear NaCl gradient (0 to 1 M). Fractions containing RecA were pooled. The majority of RecA eluted at approximately 300 mM NaCl. The pooled fractions were concentrated and desalted using a Centriplus-30 unit (Millipore) with buffer P1.

For the purification of the wild-type RecA protein, the fraction was applied to a DNA-Cellulose (denatured) column (1.6 × 10 cm, Amersham Pharmacia Biotech) equilibrated with buffer P2 (20 mM sodium phosphate [pH 7.0], 10% glycerol, and 0.1 mM EDTA). The column was then washed in succession with 3 column volumes of buffer P2, 2 volumes of buffer P2 containing 50 mM NaCl, 1 volume of buffer P2 containing 50 mM NaCl and 1 mM ATP, and finally 1 volume of the same buffer without ATP. RecA was eluted with buffer P2 containing ATP. The fractions containing RecA were pooled, concentrated and desalted with Centriplus-30. This fraction was then loaded onto a HiTrap Heparin HP column (1.6 × 5 cm, Amersham Pharmacia Biotech) equilibrated with buffer P3 (10 mM sodium phosphate [pH 6.8], 10% glycerol, and 0.1 mM EDTA). The column was washed with 4 column volumes of buffer P3. Following elution with 6 volumes of a linear NaCl gradient (0 to 1 M), fractions containing RecA were pooled. The majority of RecA eluted at approximately 400 mM NaCl. To remove ATP bound to RecA, ammonium sulfate (0.25 mg/

ml) was added to the pooled fractions. After the suspension was stirred for 1 h, the supernatant containing ATP was removed by centrifugation, and the pellet was resuspended in buffer P4 (20 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol). The fraction containing highly purified RecA was concentrated and desalted using Centriplus-30 with buffer P4, and stored at –80°C.

For the purification of RecA424 and RecA670, the pooled RecA-containing fraction from the HiTrap SP HP column was loaded onto a Resource S column (0.5 × 5 cm, Amersham Pharmacia Biotech) equilibrated with buffer P1. The column was washed with 10 column volumes of buffer P1, eluted with 25 volumes of a linear NaCl gradient (0 to 0.5 M), and the fractions containing RecA were pooled. The majority of RecA424 and RecA670 eluted at approximately 300 and 100 mM NaCl, respectively. The pooled fractions were concentrated and desalted with Centriplus-30, and the fractions were then loaded onto a Mono S column (0.5 × 5 cm, Amersham Pharmacia Biotech) equilibrated with buffer P1. The column was washed with 10 column volumes of buffer P1, eluted with 20 volumes of a linear NaCl gradient (0 to 0.4 M), and the fractions containing RecA were pooled. The majority of RecA424 and RecA670 eluted at approximately 400 and 200 mM NaCl, respectively. The pooled fractions were concentrated and desalted using Centriplus-30 with buffer P4, and stored at –80°C.

DNA Strand Exchange Assay—The strand exchange reaction was assayed by assembling the following reaction mixture. First, 10 μM ϕX174 single-stranded DNA (ssDNA) (New England Biolabs) was preincubated in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM ATP or dATP, and 0.9 μM *E. coli* single-stranded DNA-binding protein (SSB) (Amersham Pharmacia Biotech) at 37°C for 10 min, after which RecA was added. The incubation was continued for an additional 10 min and the reaction was initiated by adding *Hinc*II-digested fragment of ϕX174 double-stranded DNA (dsDNA) (New England Biolabs) to the mixture at a final concentration of 20 μM. At selected times, the reaction was quenched by the addition of buffer consisting of 20 mM Tris-HCl (pH 7.5), 0.5% SDS, 40 mM EDTA, and 2 mg/ml of proteinase K (Qiagen). After incubation at 37°C for 30 min, the sample was subjected to 0.8% agarose gel electrophoresis. The substrate DNA and strand exchange products were visualized by staining with ethidium bromide (0.5 μg/ml).

Detection of Intracellular Levels of RecA and LexA—*D. radiodurans* cells were resuspended in 10 mM sodium phosphate buffer (pH 7.0), and γ-irradiated at a dose of 2 kGy. The cells were then incubated in fresh TGY broth at 30°C for 2 h with agitation, and then disrupted using a FastPrep Cell Disruptor FP120 (Savant Instruments, Holbrook, NY) with a FastPROTEIN BLUE Kit (Qbiogene, Carlsbad, CA). The cell debris was removed by centrifugation, and the supernatant was subjected to SDS-PAGE. The resolved proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), and incubated with *E. coli* RecA antiserum (diluted 1:500) or *D. radiodurans* LexA antiserum (1: 10,000), together with alkaline phosphatase–conjugated rabbit IgG antiserum (Applied Biosystems). The generation of the *E. coli* RecA antiserum and *D. radiodurans* LexA antiserum will be reported elsewhere. As a control, *D. radiodurans* GroEL (*22*) was detected using *E. coli* GroEL antiserum (1:4,000) (StressGen Biotechnologies, Victoria, British Columbia, Canada). Chemiluminescent signals on the PVDF membrane were visualized using a Lumi-Imager F1 Workstation (Roche Diagnostics).

LexA Cleavage Assay—The reaction mixture consisted of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 6 μM oligonucleotide (5′-GAGAACCATGGATGGACCAATAATAATGACTAGAG-3′; 35 mer), 1 mM adenosine-5′-*O*-[γ-thio]triphosphate (ATPγS), 4.2 μM RecA, and 0.4 μM *D. radiodurans* LexA. The purification of the LexA protein will be reported elsewhere. First, the mixture without LexA was combined in a microtube, and preincubated at 37°C for 10 min. Then, the reaction was initiated by the addition of LexA, and the reaction mixture was incubated at 37°C for 1 h. After the reaction was quenched, the reaction mixture was loaded onto a 15% SDS-polyacrylamide gel. The resolved proteins were transferred onto a PVDF membrane, incubated with *D. radiodurans* LexA antiserum, and the chemiluminescent signals were detected.

ssDNA-Dependent ATPase and dATPase Assay—ϕX174 ssDNA (10 μM) and RecA (12 μM) were incubated in a buffer of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 2 mM ATP or dATP at 37°C for 30 min. The sample was then loaded onto a Mono Q column (0.5 × 5 cm, Amersham Pharmacia Biotech) equilibrated with buffer P5 (20 mM sodium phosphate [pH 7.4] and 0.1 mM EDTA). The column was washed with 5 column volumes of buffer P5, and eluted with 10 volumes of a linear NaCl gradient (0 to 1 M). The ATP or dATP signal was monitored at 254 nm. The rate of ATP or dATP hydrolysis in μM/min was determined by the reduction in the integral value of the ATP or dATP peak area.

DNA-Binding Affinity—The DNA-binding reaction was assayed by assembling the following reaction mixture. ϕX174 ssDNA (10 μM) or *Stu*I-digested ϕX174 dsDNA (20 μM) was incubated in buffer comprising 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM ATPγS, and 1.5 μM RecA at 37°C for 10 min. The sample was then subjected to 0.8% agarose gel electrophoresis. The substrate DNA and DNA-RecA complex were visualized by staining with ethidium bromide.

Survival Measurements—Survival of *D. radiodurans* was assessed as described (*23*), and the viable colonies were enumerated after 3 days. Survival of *E. coli* was also evaluated as described (*14*), with viable colonies counted after 18 h.

N-terminal Amino Acid Sequencing—Amino acid sequence analysis was carried out by the Edman degradation method with a Shimazu protein sequence system model PSQ-1 (Shimazu, Kyoto).

RESULTS

Identification of the Mutation Site in Strain KI696—We found that plasmid pJTM89 containing the wild-type *recA* locus (*14*) could restore the MMC sensitivity of strain KI696 by natural transformation. To localize the mutation site in KI696, a deletion library constructed from pJTM89 was examined for transforming activity. As shown in Fig. 1, the mutation site in strain KI696 was located within a 441-bp region in the *recA* locus. To locate the precise position in the locus, a fragment containing this region was amplified from the genomic DNA of strain KI696, and the nucleotide sequence was compared to the wild-type sequence. As a re-

sult, the mutation was identified as a G:C to A:T transition at position 424 in the *recA* gene (Fig. 1). This allele is different from the *recA670* allele found in strain rec30 (*14*). We designated the allele *recA424*. Whereas the *recA670* mutation causes an amino acid substitution at position 224 (Gly to Ser) in RecA, the *recA424* mutation causes a substitution at position 142 (Glu to Lys).

Restoration of DNA Repair Ability in the Transformant RT6961—Plasmid pKS5 (*14*), which consists of approximately half of the insert DNA of pJTM89 including the wild-type *recA* gene, could also restore the MMC sensitivity of strain KI696 by natural transformation (Fig. 1). To check whether the radiation resistance is completely restored in the transformant (RT6961), the survival rate of RT6961 after γ-irradiation was compared to that of strains KD8301 (carrying the wild-type *recA*) and KI696 (carrying *recA424*). While KI696 showed slight sensitivity to γ-irradiation, RT6961 was as resistant as KD8301 (Fig. 2), indicating that the *recA424* mutation is solely responsible for the sensitivity of KI696 to radiation.

To confirm that cells grown on MMC-supplemented TGY agar are not natural revertants, the following experiment was performed. First, a silent mutation was introduced at position 426 in the *recA* gene in plasmid pKS5. Second, MMC-resistant colonies were obtained by the transformation of strain KI696 with the mutated plasmid DNA. Then, genomic DNA was isolated from randomly chosen colonies, and the nucleotide sequence of the mutated region was determined by direct sequencing of the PCR products. In all colonies tested, the silent mutation was found to be at position 426 in the *recA* gene. Such colonies were as resistant to γ-irradiation as strains KD8301 and RT6961 (data not shown).

Under the same irradiation conditions, the survival rate of strain rec30 carrying the *recA670* mutation was examined (Fig. 2). Obviously, strain rec30 was much more sensitive to γ-irradiation than strain KI696, consistent with the results of previous studies (*10, 11, 24*). It has been shown that survival in strain rec30r (the previously isolated rec30 transformant with pBC2 DNA containing the wild-type *recA* gene) exposed to DNA-damaging agents is equivalent to that in the wild-type strain (*10*). We isolated a similar rec30 transformant (RT1) by transformation with pKS5 DNA. The transformant was as resistant to γ-irradiation as strain KD8301 (Fig. 2), indicating that the *recA670* mutation is solely responsible for the radiation sensitivity of strain rec30. From these observations, we concluded that the distinct phenotypes of strains KI696 and rec30 for γ-irradiation are due to different mutations in the *recA* gene.

Complementation of E. coli recA1 by recA424—Plasmid pKS5 carrying the wild-type *recA* gene has been shown to complement *E. coli* RecA deficiency (*14*). Whether the *recA424* gene complements the deficiency was tested. For this purpose, we introduced the *recA424* mutation into pKS5, thereby generating pKS424. Then, *E. coli* strain JM109 carrying *recA1* was transformed with the mutated plasmid. Survival measurements indicated that *recA424* could not complement the *E. coli* RecA deficiency (Fig. 3), suggesting that the RecA424 protein is defective in recombination activity, as is the RecA670 protein (*14*).

Purification of D. radiodurans RecA Protein—To examine the recombination and co-protease activities of *D. radiodurans* RecA *in vitro*, we purified the wild-type and two mutant proteins as described in Materials and Methods. The purified RecA proteins migrated in SDS–polyacrylamide gels with apparent molecular masses of 40 to 42 kDa (Fig. 4). This is close to the molecular mass (38,144 Da) calculated from the DNA sequence data (*13, 18*). Amino acid sequencing of these purified proteins revealed that the N-terminal sequence was Ser-Lys-Asp-Ala-Thr-Lys-Glu-Ile-Ser-Ala-Pro-Thr in all cases. This is completely consistent with the sequence from the second to thirteenth residues of

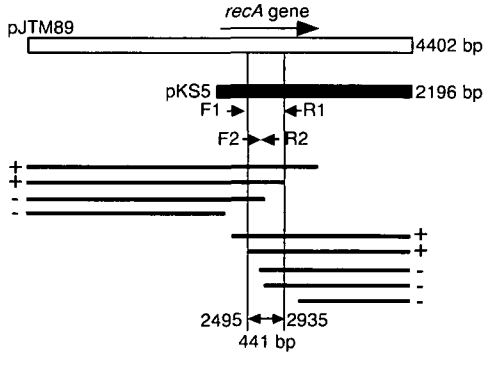

Fig. 1. **Localization of the mutation site in KI696.** The open and filled bars represent insert DNA of pJTM89 carrying the 4.4-kb *Apa*LI–*Sph*I fragment and pKS5 carrying the 2.2-kb *Xho*I–*Pst*I fragment, respectively. The arrow above the open bar indicates the position and orientation of the *recA* gene. Lines below the filled bar represent deletion plasmids derived from pJTM89. The transforming ability of these deletion plasmids in strain KI696 is indicated as positive (+) or negative (–) on the left or right side. The arrow at the bottom indicates the 441-bp region in which the mutation should be located. Numbers indicate the nucleotide positions corresponding to both ends of the region. Primers used to identify the precise mutation position are indicated by small arrows adjacent to their annealing sites. F1, 5′-GCAGCAAGGCCATCGAAACAGC-3′; R1, 5′-GGCGACCTTGTTCTTCACGGTC-3′; F2, 5′-ATCGTCGCGCAGGCCCAGAAAG-3′; R2, 5′-GGAGAGAATCGCCGTCAGCTTGC-3′.

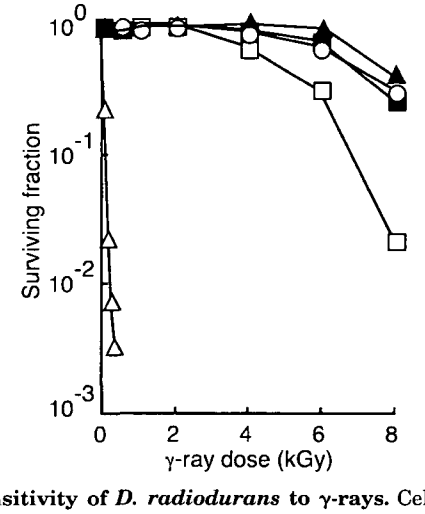

Fig. 2. **Sensitivity of *D. radiodurans* to γ-rays.** Cells grown to early stationary phase were resuspended in 10 mM sodium phosphate buffer (pH 7), challenged with ^{60}Co irradiation, spread on TGY agar, and incubated at 30°C. Symbols: open circles, KD8301 (wild-type *recA*); open squares, KI696 (*recA424*); filled squares, RT6961 (MMC-resistant transformant derived from strain KI696); open triangles, rec30 (*recA670*); filled triangles, RT1 (MMC-resistant transformant derived from strain rec30).

the primary structure predicted from the DNA sequence data. Thus, the purified proteins were confirmed to be *D. radiodurans* RecA. The wild-type RecA, RecA424, and RecA670 were purified to greater than 90% purity, with a respective yield of approximately 0.7, 0.3, and 0.2 mg from 1 liter of culture.

DNA Strand Exchange—To check whether the purified *D. radiodurans* RecA proteins retain recombination activity *in vitro*, a DNA strand exchange assay was performed. In the presence of ATP at pH 7.5, the wild-type RecA promoted an efficient strand exchange reaction between homologous linear dsDNA and circular ssDNA substrates to yield complete strand exchange products (gapped circular heteroduplex DNA) (Fig. 5A, lanes 3 and 5). As the addition of SSB to the reaction slightly enhanced the yield of the products (Fig. 5A, lane 5), we conducted all subsequent experiments in the presence of SSB. When either ssDNA, ATP or RecA was omitted from the reaction, no strand exchange product was observed (Fig. 5A, lanes 1, 2, and 4). At a constant reaction time, the yield of the products was dependent upon the concentration of the RecA

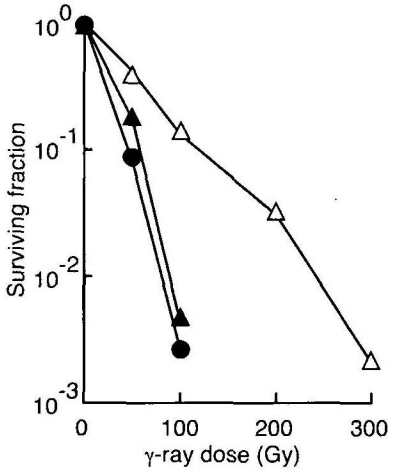

Fig. 3. **γ-ray sensitivity of *E. coli* strain JM109 (*recA1*) expressing the *recA424* mutant gene (filled circles).** The sensitivities of *E. coli* strains JM107 (JM109 but *recA*$^{+}$) (open triangles) and JM109 (filled triangles), both of which carry pUC18 are given for reference.

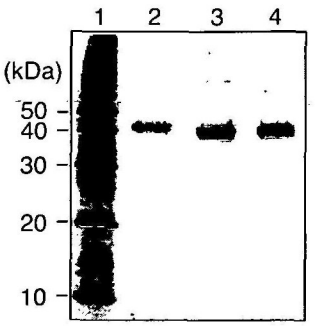

Fig. 4. **12.5% SDS-polyacrylamide gel electrophoregram of purified *D. radiodurans* wild-type and mutant RecA.** The purified RecA proteins were prepared as described in "MATERIALS AND METHODS." Each sample contained 2 μg of protein. Lane 1, 10-kDa protein ladder (Invitrogen); lane 2, wild-type RecA; lane 3, RecA424; lane 4, RecA670.

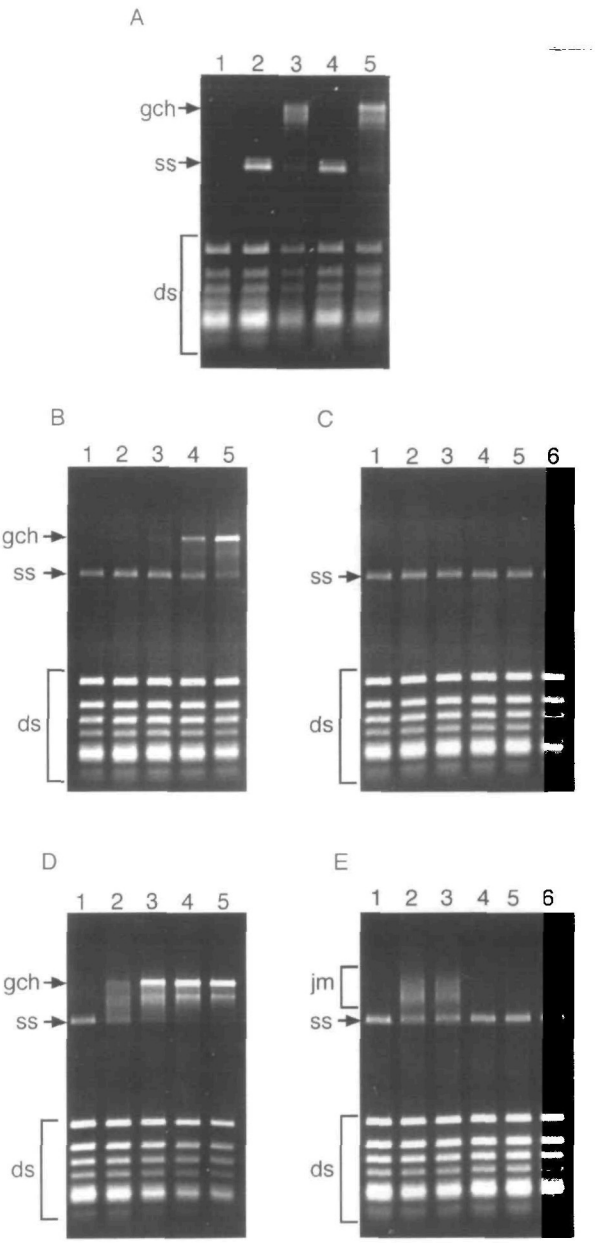

Fig. 5. **DNA strand exchange.** ϕX174 ssDNA (10 μM) and the *Hin*cII-digested fragment of ϕX174 dsDNA (20 μM) were incubated with *D. radiodurans* RecA in the buffer described in "MATERIALS AND METHODS." (A) Wild-type RecA. Lane 1, ssDNA was omitted; lane 2, ATP was omitted; lane 3, SSB was omitted; lane 4, RecA was omitted; lane 5, complete reaction. Reaction time was 30 min. (B) Wild-type RecA in the presence of ATP. Lanes 1 to 5, the concentration of RecA was varied at 0, 1.5, 3, 6, and 12 μM RecA, respectively (reaction time was 30 min). (C) RecA424 (lanes 1 to 3) and RecA670 (lanes 4 to 6) in the presence of ATP. The concentration of RecA was 12 μM. Lanes 1 and 4, 0 min reaction time; lanes 2 and 5, 15 min reaction time; lanes 3 and 6, 30 min reaction time. (D) Wild-type RecA in the presence of dATP. Lanes 1 to 5, the concentration of RecA was varied at 0, 1.5, 3, 6, and 12 μM RecA, respectively (reaction time was 30 min). (E) RecA424 (lanes 1 to 3) and RecA670 (lanes 4 to 6) in the presence of dATP. The concentration of RecA was 12 μM. Lanes 1 and 4, 0 min reaction time; lanes 2 and 5, 15 min reaction time; lanes 3 and 6, 30 min reaction time. The positions of dsDNA (ds), ssDNA (ss), the complete strand exchange product (gapped circular heteroduplex DNA, gch), and the strand exchange intermediate (joint molecule, jm) are indicated.

protein. We obtained a maximum yield with 12 μM RecA (Fig. 5B). Next, we examined whether the mutant RecA proteins promote the DNA strand exchange reaction. As shown in Fig. 5C, no DNA strand exchange was observed with RecA424 or RecA670 under conditions in which the wild-type RecA could promote the reaction. A further increase in the reaction time up to 1h and/or in the RecA concentration to 24 μM had no effect (data not shown). These results indicate that both RecA424 and RecA670 are defective in recombination activity *in vitro*. This is consistent with the experiment showing the failure of complementation of a *recA1 E. coli* strain by *recA424* (Fig. 3) and *recA670* (*14*).

We also found that wild-type RecA promoted the DNA strand exchange reaction more efficiently when dATP was used as the nucleotide cofactor instead of ATP. A substantial level of products was accumulated even at 3 μM RecA (Fig 5D, lane 3). In the presence of dATP, RecA424 (12 μM) could convert substrate DNA to strand exchange intermediates (joint molecules) to a certain extent at reaction times of 15 and 30 min (Fig. 5E, lanes 2 and 3). However, no complete strand exchange product was observed with RecA424. In the case of RecA670, neither a complete nor an intermediate strand exchange product was detected under the same conditions (Fig 5E. lanes 5 and 6). A further increase in the reaction time and/or RecA concentration did not improve the reaction (data not shown).

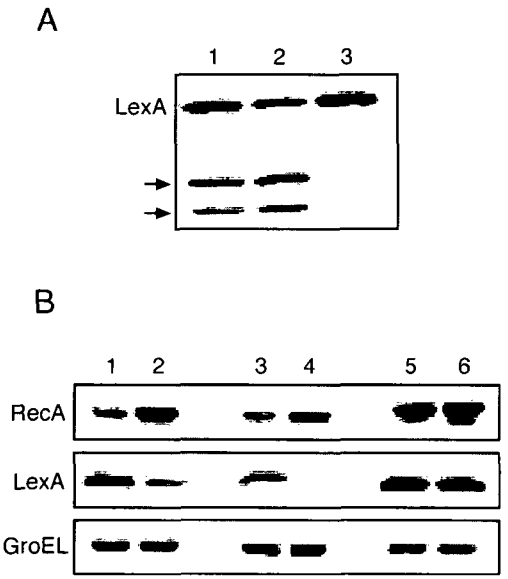

Fig. 6. **LexA cleavage activity.** (A) RecA-mediated LexA cleavage reaction *in vitro*. *D. radiodurans* LexA (0.4 μM) was incubated with *D. radiodurans* RecA (4.2 μM) for 1 h in the buffer described in "MATERIALS AND METHODS," and subjected to Western analysis with *D. radiodurans* LexA antiserum. The arrows on the left indicate the positions of breakdown products of LexA. Lane 1, wild-type RecA; lane 2, RecA424; lane3, RecA670. (B) Changes in intracellular RecA and LexA levels following irradiation. Early stationary phase cells were resuspended in 10 mM sodium phosphate buffer (pH 7) and divided into two fractions. One fraction was irradiated at a dose of 2 kGy, and the other fraction was not irradiated. The cells were then incubated in fresh TGY broth for 2 h. The protein extracts were subjected to Western analysis with *E. coli* RecA antiserum (upper), *D. radiodurans* LexA antiserum (middle) and *E. coli* GroEL antiserum (lower). Each sample contained 10 μg of protein. Lanes 1 and 2, strain KD8301 (wild-type *recA*); lanes 3 and 4, strain KI696 (*recA424*); lanes 5 and 6, strain rec30 (*recA670*). Odd and even numbered lanes contained non-irradiated and irradiated samples, respectively. The level of GroEL was constant irrespective of irradiation in all the strains tested.

LexA Cleavage—To check whether the purified RecA mutant proteins retain co-protease activity, we performed a RecA-mediated LexA cleavage assay. As shown in Fig. 6A, *D. radiodurans* LexA protein was cleaved when incubated with either the wild-type RecA or RecA424 to yield two breakdown products (lanes 1 and 2). *E. coli* RecA mediates the proteolytic cleavage of the Ala-Gly bond between positions 84 and 85 in *E. coli* LexA (*25*). These two amino acids at the cleavage site are also conserved in *D. radiodurans* LexA (Ala-83 and Gly-84) (*26*). The two breakdown products observed in Fig. 6A are thought to be the N-terminal and C-terminal fragments of LexA cleaved between Ala-83 and Gly-84. Under the same reaction conditions, RecA 670 could not promote the self-cleavage reaction (Fig. 6A, lane 3). These results indicate that RecA424 retains co-protease activity while RecA670 does not.

To confirm the co-protease activity of the mutant RecA proteins *in vivo*, we examined the intracellular level of LexA in strains KI696 and rec30. In strain KD8301, which carries the wild-type *recA*, the level of RecA was increased, while the level of LexA was decreased after γ-irradiation (Fig. 6B, lanes 1 and 2). In strain KI696 carrying *recA424*, RecA was induced after irradiation as in the case of KD8301. A decrease in the level of LexA was also observed in KI696 after irradiation (Fig. 6B, lanes 3 and 4). This result is consistent with that showing co-protease activity of RecA424 *in vitro* (Fig. 6A, lane 2). In contrast, no change was observed in the LexA level in strain rec30 after irradiation (Fig. 6B, lanes 5 and 6). It has been reported that RecA is not detected in rec30 under any circumstances (*12*). However, we did detect RecA in rec30. The reason for this difference is unclear. However, our results clearly indicate that RecA670 is defective in co-protease activity *in vitro*. Moreover, we found that the basal level of RecA was much higher in rec30 than in KD8301 and KI696. Since LexA is not involved in the induction of RecA in *D. radiodurans* (*26*), the increased basal level of RecA in unirradiated rec30 cells is not due to the lack of derepression of *recA*. This point must be clarified in future studies because it might provide a clue as to the still-unknown mechanism of RecA induction.

DISCUSSION

In the previous study, we showed that the highly radiation sensitive strain *D. radiodurans* rec30 carries the *recA670* mutation (*14*). It has also been shown that disruption of *D. radiodurans recA* remarkably affects the sensitivity of cells to γ-rays (*10*). Thus, RecA is believed to be the most important participant in the radiation resistance (*27*). In the present study, we identified the mutation in strain KI696 that prevents the repair of cross-links in DNA induced by MMC (*19*). The strain exhibits a slightly radiation-sensitive phenotype. Surprisingly, the mutation in strain KI696 is located in the *recA* gene (*recA424*). This indicates that the difference in phenotype between strains KI696 and rec30 is due to different mutations in the *recA* gene.

recA670 causes an amino acid substitution at residue 224 of the RecA protein (G224S) (*14*). We have speculated that

RecA670 is defective in both recombination and co-protease activity due to the similarity of the mutation site with *E. coli* RecA611 in which the amino acid substitution is located in α-helix G (part of the putative ssDNA-binding domain), thereby lowering the affinity for ssDNA (*16*). In this study, we show that RecA670 did not promote DNA strand exchange (Fig. 5), and was defective in co-protease activity *in vitro* and *in vivo* (Fig. 6). These results are consistent with our previous speculation. On the other hand, *recA424* causes an amino acid substitution at residue 142 of the RecA protein (E142K). This position corresponds to the aspartic acid at residue 130 (D130) in *E. coli* RecA. In *E. coli*, D130 is present in an amphipathic α-helix that is designated helix E (*28*). D130 is located on the hydrophilic face of helix E, and the side chain of D130 projects towards the hydrophilic surface of the RecA protein. Helix E, together with β-strand 3 in the same monomer, is predicted to be involved in interactions that form the subunit-subunit interface by packing against the α-helix A and β-strand 0 of another monomer (*29*). *recA424* could not complement *E. coli* RecA deficiency (Fig. 3). In addition, the *in vitro* assay indicated that RecA424 did not produce the complete DNA strand exchange products (Fig. 5). These results demonstrate that RecA424 is defective in recombination activity. Consequently, we suggest that the amino acid substitution in RecA424 causes a structural perturbation by changing the electric charge at the subunit–subunit interface.

In *E. coli*, several RecA mutants carrying mutations in the subunit interface altering the electric charge have been reported. RecA H97A and RecA K248A are defective in filament assembly, ATPase, DNA binding and DNA strand exchange (*30*). However, there is an apparent difference between these *E. coli* RecA mutants and RecA424. The activation of self-cleavage of LexA by RecA requires the formation of a stable ternary complex of RecA, ssDNA and nucleotide cofactor (*2*). Since RecA424 retains co-protease activity both *in vitro* and *in vivo* (Fig. 6), it seems to be proficient in filament assembly. The DNA strand exchange experiment indicated that RecA424 could convert substrate DNA to strand exchange intermediates (joint molecules) to a certain extent in the presence of dATP (Fig. 5E). This process might play a part in filament assembly in RecA424.

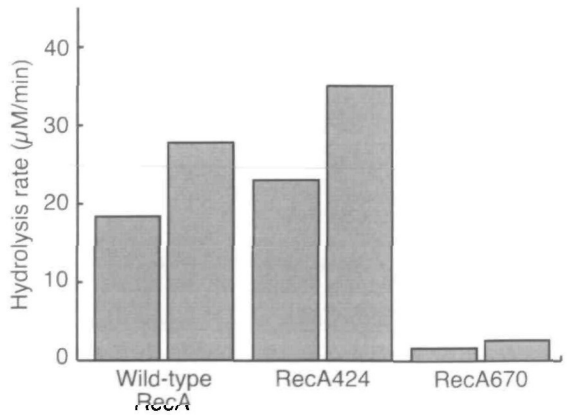

Fig. 7. **ssDNA-dependent ATPase and dATPase activity.** Reactions were carried out as described in "MATERIALS AND METHODS." Open and closed bars indicate the ATP and dATP hydrolysis rates, respectively. Plotted data are the means of at least three experiments.

This property of RecA424 is reminiscent of *E. coli* RecA K72R. It has been shown that *E. coli* RecA K72R promotes a limited DNA strand exchange reaction only if dATP is used as the nucleotide cofactor, forms filaments on DNA, and facilitates LexA cleavage at a reduced rate (*31, 32*). The mutation is at a conserved site in the P-loop for ATP binding (*33*). Tyrosine residue 264 of *E. coli* RecA constitutes part of the ATP-binding domain (*34*), and RecA protein with this residue replaced by phenylalanine or serine is defective in promoting the strand exchange reaction but proficient in promoting the LexA cleavage reaction *in vitro* (*35*). It has been proposed that helix E acts as a liaison connecting the subunit-subunit interface to the DNA and ATP binding sites (*29*). Therefore, we tentatively thought that the mutation in RecA424 modifies the ATP-binding affinity; thereby RecA424 shows similar properties to *E. coli* RecA K72R and the tyrosine-264 mutants. To check this possibility, we examined ssDNA-dependent ATP and dATP hydrolysis by *D. radiodurans* RecA. As shown in Fig. 7, however, RecA424 catalyzed the hydrolysis of ATP and dATP as did the wild-type RecA, whereas RecA670 lost almost all hydrolytic activity. We next examined the possibility that RecA424 modifies DNA-binding affinity. As shown in Fig. 8, RecA670 lacks ssDNA- and dsDNA-affinity. RecA424 showed a higher affinity for ssDNA and dsDNA than the wild-type RecA. Therefore, it was thought that the deficiency in DNA strand exchange activity of RecA424 is due to the modified DNA-binding affinity.

Daly and Minton (*13*) suggested *recA*-dependent intermolecular recombination repair as one mechanism by which *D. radiodurans* can reconstitute its chromosome from many hundreds of DNA fragments. To rationalize the importance of *recA*-dependent intermolecular recombination in efficient double-strand break repair in *D. radiodurans*, they further proposed a special form of redundancy wherein chromosomes exist in pairs, linked to each other by thousands of persistent Holliday junctions, and hypothesized that RecA plays a crucial role in the pre-aligned repair reactions by mediating strand invasion (*36, 37*). However, the optical mapping study provided no evidence of DNA molecules con-

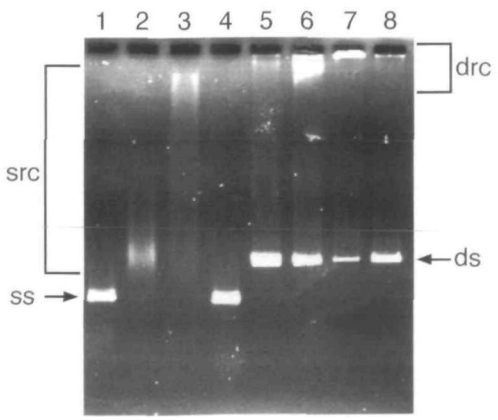

Fig. 8. **DNA-binding affinity.** ϕX174 ssDNA (10 μM; lanes 1 to 4) or *Stu*I-digested ϕX174 dsDNA (20 μM; lanes 5 to 8) was incubated with *D. radiodurans* RecA (1.5 μM) in the buffer described in "MATERIALS AND METHODS." Lanes 1 and 5, RecA was omitted; lanes 2 and 6, wild-type RecA; lanes 3 and 7, RecA424; lanes 4 and 8, RecA670. The positions of ssDNA (ss), dsDNA (ds), ssDNA-RecA complex (src), and dsDNA-RecA complex (drc) are indicated.

taining Holliday junctions (*38*). *D. radiodurans* wild-type RecA promoted the DNA strand exchange reaction in the presence of ATP at pH 7.5 (Fig. 5, A and B) and the reaction proceeded more efficiently when dATP was used as the nucleotide cofactor instead of ATP (Fig. 5D). The hydrolytic rate of dATP was higher than that of ATP (Fig. 7). In *E. coli*, the hydrolytic rate is also higher with dATP by about 20%, and the rate of DNA strand exchange is enhanced (*39, 40*). Thus, *D. radiodurans* RecA behaved in a similar way to *E. coli* RecA regarding DNA strand exchange and nucleotide cofactor hydrolysis. It has also been shown that *Bacillus subtilis* RecA catalyzes the hydrolysis of ATP and dATP, can use either ATP or dATP as a cofactor for the DNA strand exchange reaction, and the hydrolytic rate is higher with dATP (*41*). It seems that the nucleotide cofactor specificity of RecA is basically conserved among *E. coli, B. subtilis*, and *D. radiodurans*. *In vivo*, *D. radiodurans* wild-type *recA* fully complemented the deficiency of *E. coli* RecA (*14*). However, γ-ray resistance was not enhanced over that of *recA*$^+$ *E. coli*. Thus, there is no evidence at present that *D. radiodurans* RecA has a special property in recombination repair. The RecA, in turn, shares the functions of RecA from *E. coli* and *B. subtilis*, as expected from the high degree of amino acid sequence similarity among them.

The results of our experiments demonstrate that RecA424 from strain KI696 is defective in recombination activity. However, strain KI696 is only slightly sensitive to γ-irradiation whereas strain rec30 is extremely sensitive. How can the distinct phenotypes of these two mutant strains be explained? One possibility is that the recombination activity of RecA424 is still fully developed in *D. radiodurans* for some reason. However, this possibility seems unlikely because RecA424 cannot complement the *E. coli* RecA deficiency at all. Although we cannot exclude the existence of a still-unknown mechanism that potentiates the effectiveness of recombination function of RecA in *D. radiodurans*, such a mechanism has not been found yet. Another possibility is that the loss of the co-protease activity has a fatal influence on the DNA repair ability of *D. radiodurans*. We suggest that the co-protease activity rather than the recombination activity of RecA contributes to the extraordinary radiation resistance of *D. radiodurans*.

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