Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6063–6067, July 1991
Biochemistry

Resolution of Holliday junctions *in vitro* requires the *Escherichia coli ruvC* gene product

(genetic recombination/post-replication repair/strand exchange/nuclease)

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Communicated by Martin Gellert, April 2, 1991

ABSTRACT **In previous studies, Holliday junctions generated during RecA-mediated strand-exchange reactions were resolved by fractionated *Escherichia coli* extracts. We now report the specific binding and cleavage of synthetic Holliday junctions (50 base pairs long) by a fraction purified by chromatography on DEAE-cellulose, phosphocellulose, and single-stranded DNA-cellulose. The cleavage reaction provided a sensitive assay with which to screen extracts prepared from recombination/repair-deficient mutants. Cells with mutations in *ruvC* lack the nuclease activity that cleaves synthetic Holliday junctions *in vitro*. This deficiency was restored by a multicopy plasmid carrying a *ruvC*$^+$ gene that overexpressed junction-resolving activity. The UV sensitivity and deficiency in recombinational repair of DNA exhibited by *ruv* mutants lead us to suggest that RuvC resolves Holliday junctions *in vivo*.**

Much of our current understanding of the enzymology of genetic recombination in *Escherichia coli* comes from studies of RecA and RecBCD. *In vitro*, RecA catalyzes homologous pairing and strand-exchange reactions between DNA molecules to form recombination intermediates, whereas RecBCD is a multifunctional enzyme with DNA helicase and single- and double-strand exonuclease activities (1). However, genetic studies indicate that the products of the *recF*, *recG*, *recJ*, *recN*, *recO*, *recQ*, *recR*, *ruvA*, *ruvB*, and *ruvC* genes are also required for normal levels of recombination (2). A number of these proteins have now been cloned, overexpressed, and purified; RecF binds single-stranded DNA (ssDNA) (3), RecJ is a ssDNA exonuclease (4), RecQ is a DNA helicase (5), and RuvB is an ATPase (6). Many proteins required for genetic recombination are also needed for the recombinational repair of DNA damage, and the *recA*, *recN*, *recQ*, *ruvA*, and *ruvB* genes are inducible and regulated by LexA protein (2, 7).

A vital step in the recombination of DNA is the resolution of recombination intermediates into mature heteroduplex products. To understand the biochemistry of the resolution process in *E. coli*, a system in which recombination intermediates could be made and resolved *in vitro* was developed (8). We utilized the homologous-pairing and strand-exchange properties of RecA to produce intermediates in which two duplex molecules were linked by a single crossover, or Holliday junction. These structures were used to detect an activity from fractionated *E. coli* extracts that resolved the intermediates into recombinant products. Resolution occurred by specific endonucleolytic cleavage at the site of the Holliday junction (9).

The presence of a nuclease that is specific for Holliday junctions is confirmed and extended by the present experiments. Using a more purified protein fraction than that reported (9), we show that the resolution activity binds to small synthetic Holliday junctions and cleaves them to produce nicked-duplex products. The development of this sensitive cleavage assay allowed us to screen a series of recombination/repair-deficient cells. We found that the specific nucleolytic activity was absent in strains carrying mutations in *ruvC*. A defect in Holliday junction resolution is consistent with the phenotypic properties exhibited by *ruv* mutants.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* N3312 (*recO1504*::Tn*5*), N3313 (*recR252*::Tn*10-9*), N3315 (*ruvA60*::Tn*10*), N3320 (*eda-51*::Tn*10 ruvC53*) were made by P1 transduction of strain NH5033 (*recB21 sbcB15 sbcC endA1*) (9) using RDK1541 (10), AM207 (11), N2057 (12), and CS85 (12) as donors, with selection for transposon antibiotic-resistance markers. The presence of the mutant allele was confirmed by sensitivity to UV irradiation and mitomycin C. N1373 is a *ruvC51* derivative of AB1157 (12).

Plasmid pFB520, a derivative of pUC18 carrying a *Bam*HI–*Eco*RV fragment of the chromosomal *ruv* region, carries the *orf-33* and *ruvC* genes. It was derived from plasmid pFB512 (13, 14) by digestion with *Eco*RI and *Eco*RV. The 3′-OH recessed *Eco*RI end was filled-in using the Klenow fragment of DNA polymerase I and the plasmid was recircularized. pFB520 complements the UV sensitivity associated with *ruvC* mutant alleles.

Proteins. *E. coli* Holliday junction-resolving activity was prepared from strain NH5033. Cell growth, lysis, and purification by ammonium sulfate precipitation, DEAE-cellulose, and phosphocellulose chromatography (to produce fraction IV) were essentially as described (9). Fraction IV was dialyzed against R buffer [50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol] and loaded onto an 8-ml ssDNA-cellulose column (Sigma). The column was eluted with a 0–400 mM KCl gradient in R buffer and fractions were assayed for the resolution of strand-exchange intermediates made by RecA (9). Active fractions were divided and stored at −20°C (fraction V).

E. coli RecA was purified as described (15). Concentrations of RecA refer to moles of monomeric protein.

Preparation of Mutant Extracts. Cultures (1 liter) were grown with aeration at 37°C in Luria broth to an OD_{650} of 0.6 unit. Cells were collected by centrifugation, washed in 20 ml of ice-cold 100 mM Tris·HCl, pH 8.0/5% glycerol/2 mM EDTA, resuspended at 3 ml/g in the same buffer, and stored

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Abbreviation: ssDNA, single-stranded DNA.

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at −20°C. To the thawed cells, dithiothreitol was added to 1 mM, and 0.2 vol of lysozyme [5 mg/ml in 0.25 M Tris·HCl (pH 8.0)] was added. After 30 min on ice, Triton X-100 was added to 0.1% and incubation was continued for 10 min. Finally, 0.1 vol of 4% (wt/vol) sodium deoxycholate was added and lysates were centrifuged at 42,000 rpm for 60 min in a Beckman 60 Ti rotor at 4°C. Supernatants were collected and stored at −20°C.

Each extract (8 ml, routinely 30–50 mg of protein per ml) was applied directly to an 8-ml phosphocellulose column, equilibrated with R buffer, at a flow rate of 40 ml/h. The column was washed with the same buffer and 19 fractions were eluted with an 80-ml, 0–700 mM KCl gradient in R buffer. Each fraction was assayed for the cleavage of synthetic Holliday junction DNA. The same column was used repeatedly (after a high salt wash and reequilibration), and each run in a series was highly reproducible. Different columns were used for the experiments of Figs. 5 and 6.

DNA Substrates. Gapped circular duplex φX174 DNA and *Pst*I-linearized duplex φX174 DNA were prepared and purified as described (9). Linear duplex DNA was 3′-end-labeled using terminal transferase and [α-^{32}P]dideoxyadenosine 5′-triphosphate. Synthetic Holliday junction DNA was ^{32}P-end-labeled at the 5′ end and prepared by annealing oligonucleotides 1 (5′-GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCC-3′), 2 (5′-TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGTT-3′), 3 (5′-CAACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCGA-3′), and 4 (5′-ATCGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGT-3′) as described (16). Duplex DNA was made by annealing oligonucleotides 1 and 5 (5′-GGGTCAACGTGGGCAAAGATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-3′). Concentrations of synthetic X-junctions, approximate due to their low concentration, were measured using DNA DipSticks (Invitrogen, San Diego). All concentrations refer to moles of nucleotide residues.

Resolution of Strand-Exchange Intermediates. RecA-mediated strand-exchange reactions between gapped circular DNA and ^{32}P-labeled linear duplex DNA were performed as described (9). Reactions were stopped by addition of 0.1 vol of stop buffer [proteinase K (0.5 mg/ml)/5% (wt/vol) SDS/100 mM Tris·HCl, pH 7.5/250 mM EDTA] and incubated for 10 min at 37°C.

Gel-Retardation Assays. Reaction mixtures (20 μl) contained synthetic Holliday junction or linear duplex DNA (^{32}P-labeled at the 5′ terminus of oligonucleotide 1) in binding buffer (50 mM Tris·HCl, pH 8.0/5 mM EDTA/1 mM dithiothreitol) and various amounts of protein. After 15 min at 22°C, 5 μl of loading buffer [40 mM Tris·HCl, pH 7.5/4 mM EDTA/25% glycerol/bovine serum albumin (400 μg/ml)] was added and samples were electrophoresed through a low-ionic-strength 4% polyacrylamide gel (16).

Resolution of Synthetic Holliday Junctions. Reaction mixtures (20 μl) containing 5′-^{32}P-labeled junction DNA (≈0.6 μM) and Holliday junction resolution activity were incubated for 30–60 min at 37°C in cleavage buffer [50 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/5 mM potassium glutamate/1 mM dithiothreitol/bovine serum albumin (100 μg/ml)]. Samples were analyzed in one of two ways. (*i*) For native gels, reactions were stopped and deproteinized by incubation with 0.1 vol of stop buffer. DNA products were then electrophoresed at 4°C through native 10% polyacrylamide gels using a Tris/borate buffer system (16). (*ii*) For denaturing gels, reactions were stopped by addition of EDTA to 25 mM and the DNA was denatured and loaded onto 12% polyacrylamide gels containing 7 M urea (16). To assign the cleavage sites, 5′-^{32}P-labeled oligonucleotides were sequenced using the chemical method (17). A 1.5-base allowance was made to compensate for the nucleoside eliminated in the sequencing reaction. The cleavage sites were confirmed by comparison with fragments produced by restriction digestion.

RESULTS

Resolution of Holliday Junctions During RecA-Mediated Strand Exchange. In previous studies, we detected an activity that cleaves recombination intermediates when added to ongoing RecA-driven strand-exchange reactions (9). This activity has now been fractionated by ammonium sulfate precipitation and chromatography on DEAE-cellulose, phosphocellulose, and ssDNA-cellulose (fraction V).

The progress of RecA-mediated strand-exchange reactions between gapped duplex DNA and ^{32}P-end-labeled linear duplex DNA (8, 15) can be followed by agarose gel electrophoresis (Fig. 1*a*). At early time points (10–15 min), a large proportion of label is found as slow-migrating strand-exchange intermediates. At later times (20–30 min), nicked-circular duplex DNA is formed upon completion of strand exchange. Addition of fraction V to the reaction mixture results in the formation of linear duplex and nicked-circular DNA at early time points (Fig. 1*b*). In previous work, these products were identified as the "splice" and "patch" recombinant products resulting from Holliday junction resolution (8, 9).

Interaction with Synthetic Holliday Junctions. At this stage, two problems prevented our identification of the protein responsible for Holliday junction resolution: (*i*) fraction V was impure as determined by SDS/PAGE and (*ii*) attempts to detect activity (or lack of it) in small-scale cultures of mutant *E. coli* were unsuccessful due to the insensitivity of the resolution assay. We therefore sought other, more sensitive, assays that could be adapted to screen a series of mutant extracts.

During the preparation of fraction V, we observed that peak fractions were capable of binding and cleaving synthetic Holliday junctions. The synthetic Holliday junction was produced by annealing four oligonucleotides (49–51 bases long). Due to the sequences of the four single strands, the synthetic Holliday junction used in the following experiments contains a central core of homologous DNA sequences (12 base pairs long). When increasing amounts of fraction V were added to binding reaction mixtures containing synthetic Holliday junction DNA, protein–DNA complexes were formed, as observed by gel retardation (Fig. 2, lanes a–e). Under identical conditions, little or no binding was seen to a

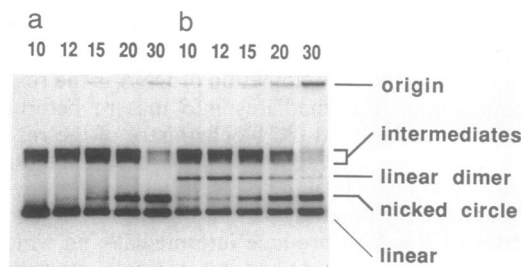


FIG. 1. Resolution of strand-exchange intermediates by fraction V. A 110-μl reaction mixture containing 5.8 μM gapped φX174 DNA and 2.6 μM RecA was incubated for 5 min at 37°C. ^{32}P-labeled linear duplex φX174 DNA (4.5 μM) was added to initiate strand exchange and incubation was continued. At the indicated times (in min after addition of the linear DNA), 10-μl samples were removed and processed in one of two ways. Reactions were stopped (*a*) or supplemented with 7.2 μg of fraction V (FV) and incubation was continued for 5 min (*b*). Reactions were then stopped as above. DNA products were analyzed by 0.7% agarose gel electrophoresis and autoradiography.

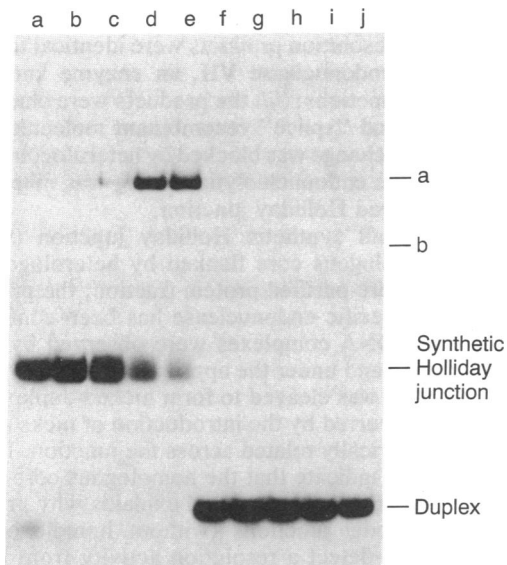


FIG. 2. Binding of synthetic Holliday junctions by fraction V. Fraction V was incubated with 0.12 μM 5'-^{32}P-labeled synthetic Holliday junction (lanes a–e) or linear duplex DNA (lanes f–j). Lanes: a and f, fraction V omitted; b and g, 0.3 μg of fraction V; c and h, 0.6 μg of fraction V; d and i, 1.2 μg of fraction V; e and j, 1.8 μg of fraction V. Complexes were separated on low-ionic-strength polyacrylamide gels and radiolabeled DNA was detected by autoradiography. The major complexes are designated a and b.

linear duplex control (lanes f–j). The observed binding to junction DNA was highly specific, was stable to KCl concentrations in excess of 0.3 M, and was not competed away by a 500-fold excess of competitor DNA [either poly(dI-dC)·poly(dI-dC) or calf thymus DNA] (data not shown).

In reaction mixtures containing Mg^{2+}, the synthetic junction used in the binding assay was cleaved by fraction V to form products (Fig. 3, lanes a–e) that comigrated with a linear duplex marker (lane f). To determine whether the junction had been resolved by the introduction of specific nicks, four junctions were prepared (5'-^{32}P-end-labeled in strand 1, 2, 3, or 4) and treated with fraction V, and the DNA products were analyzed on a denaturing gel (Fig. 4*A*). As reference markers, we used G+A and T+C sequencing ladders. The data in Fig. 4*A* show that fraction V resolved the synthetic Holliday

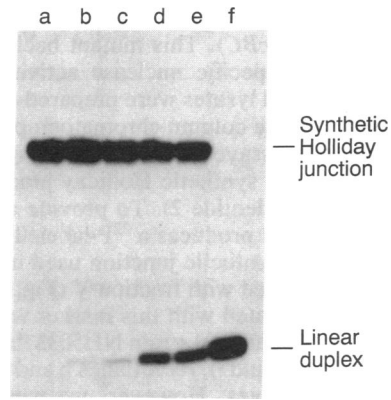
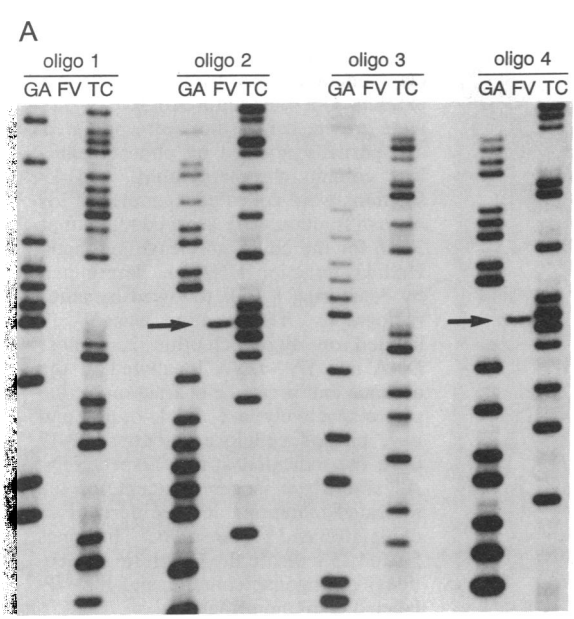


FIG. 3. Resolution of synthetic Holliday junction by fraction V. Fraction V was incubated with synthetic junction DNA, and the DNA products were deproteinized and separated by electrophoresis through a native 10% polyacrylamide gel. Lanes: a, fraction V omitted; b, 0.3 μg of fraction V; c, 0.6 μg of fraction V; d, 1.2 μg of fraction V; e, 1.8 μg of fraction V; f, linear duplex marker. All DNA was ^{32}P-labeled at the 5' terminus of oligonucleotide 1.

junction by the introduction of single nicks into strands 2 and 4. Incision occurred at precisely the same site in each strand within the homologous core at the sequence 5'-GT↓CC-3' (Fig. 4*B*). Unpublished results indicate that the resolution reaction is dependent upon homology and DNA sequence since (*i*) a junction without homology (16) was not resolved by fraction V and (*ii*) a junction with a 26-base-pair homologous core (18) was cleaved in both orientations at a number of specific sites. We have not observed specific cleavage of linear duplex DNA (oligonucleotides 1 or 2 annealed with their complementary strands) or of supercoiled form I plasmid DNA by fraction V.

Resolution Activity Is Dependent on *ruvC*$^+$. The specific cutting of synthetic Holliday junction DNA provided a sensitive assay with which to assay mutant strains for the absence of resolution activity. Since gel-filtration experiments indicated that the active component of fraction V has a native molecular mass of ≈20 kDa (as measured by chromatography through Ultrogel AcA54 using R buffer/0.5 M KCl; data not shown), we limited our screen to recombination/repair mutants with deficiencies in genes that encode proteins of <40 kDa. The strains N3312 (*recO1504*::Tn*5*), N3313 (*recR252*::Tn*10-9*), N3315 (*ruvA60*::Tn*10*), N3320



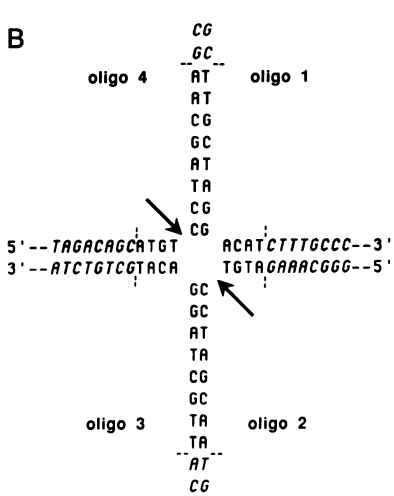


FIG. 4. Mapping of the cleavage sites. (*A*) Four synthetic junctions (5'-^{32}P-labeled on oligonucleotide 1, 2, 3, or 4, as indicated in the heading) were incubated for 60 min with fraction V (0.6 μg). Reaction products were analyzed on denaturing polyacrylamide gels, followed by autoradiography. G+A (lanes GA) and T+C (lanes TC) sequencing ladders flank the fraction V cleavage reaction products (lanes FV). Only part of each ladder is shown. (*B*) Central core of the junction in which the sites of cleavage are indicated with arrows. The junction point is arbitrarily positioned within the homologous core of the junction, and heterologous sequences are italicized.

(*eda51*::Tn*10 ruvC53*) were made by P1 transduction into NH5033 (*endA recB sbcBC*). This mutant background was used to minimize nonspecific nuclease activity. One-liter cultures were grown and lysates were prepared and fractionated by phosphocellulose column chromatography. Samples of each fraction were assayed, using denaturing PAGE, for the ability to cleave the synthetic Holliday junction (^{32}P-5′-end-labeled on oligonucleotide 2). To provide a marker for specific cleavage (which produces a ^{32}P-labeled fragment 23 nucleotides long), the synthetic junction used in the experiment of Fig. 4 was treated with fraction V (Fig. 5, lane FV). A fragment that comigrated with this marker was produced by fractionation of the parental strain NH5033 (lanes c and d) and by its *recO* (lanes h and i), *recR* (lanes n and o), and *ruvA* (lanes s and t) derivatives. However, we were unable to detect specific cleavage activity in extracts from the *ruvC53* strain N3320 (Fig. 5, lanes u–y).

A Multicopy Plasmid Carrying the *ruvC* Gene Overexpresses Resolution Activity. To complement the defect in resolution activity found in the *ruvC* mutant, we used plasmid pFB520 that carries the *ruvC* gene from the *E. coli* chromosome (Fig. 6*A*). To prevent problems with plasmid instability in the *endA recB sbcBC* background, we used strains derived from wild-type strain AB1157 (i.e., *endA*$^+$ *recB*$^+$ *sbcB*$^+$ *sbcC*$^+$). Extracts were prepared from AB1157, N1373 (*ruvC51*), and N1373 pFB520, and phosphocellulose fractionation was carried out as above. Although the wild-type genetic background resulted in an increase in nonspecific nuclease activity, resolution-specific fragments were observed in the AB1157 extract (Fig. 6*B*, lanes c and d) that were absent in extracts from plasmid-free N1373 (*ruvC51*) (lanes g–l). However, extracts prepared from N1373 carrying pFB520 showed overexpression of the resolution activity (Fig. 6*B*, lanes m–r). Related plasmids carrying *ruvC*, but lacking *orf-33*, were also able to complement the defect in resolution observed with *ruvC* mutants. These results indicate that resolution depends upon a functional *ruvC*$^+$ gene and that multicopy plasmids carrying *ruvC*$^+$ produce elevated levels of resolution activity.

DISCUSSION

In previous work, we detected an activity from partially purified extracts of *E. coli* that resolved Holliday junctions made by RecA (9). Several lines of evidence suggested the presence of a Holliday junction-specific endonuclease: (*i*) the fraction was active upon intermediates generated by strand exchange; (*ii*) the resolution products were identical to those produced by T4 endonuclease VII, an enzyme known to resolve Holliday junctions; (*iii*) the products were characteristic of "patch" and "splice" recombinant molecules; and (*iv*) when strand exchange was blocked by heterologous DNA sequences, specific endonucleolytic cutting was mapped to the site of the stalled Holliday junction.

By using a small synthetic Holliday junction (with a 12-base-pair homologous core flanked by heterologous sequences) and a more-purified protein fraction, the presence of this junction-specific endonuclease has been confirmed. Specific protein–DNA complexes were observed by a gel-retardation assay, and under the appropriate reaction conditions, the junction was cleaved to form nicked-duplex products. Cleavage occurred by the introduction of nicks at sites that were symmetrically related across the junction. Preliminary experiments indicate that the homologous core of this junction is required for cleavage and explains why previous attempts with model junctions (without homologous sequences) failed to detect a resolution activity from *E. coli* (unpublished results).

More importantly, the cleavage of this model Holliday junction provided a sensitive assay with which to screen mutant cell extracts. The activity that resolves Holliday junctions *in vitro* was found to be absent from extracts prepared from cells carrying either *ruvC51* or *ruvC53* mutant alleles.

The *ruv* locus of *E. coli* is required for normal levels of genetic recombination, DNA repair and cell division. Cells carrying *ruv* mutations are sensitive to UV irradiation, ionizing irradiation, and mitomycin C (19). They are recombination-deficient in a *recBC sbcBC* or *recBC sbcA* background, although *ruv* single mutants show only slight recombination deficiency (20–22). The *ruv* locus is located at minute 41 on the *E. coli* linkage map and mutations in any one of three genes, designated *ruvA*, *ruvB*, and *ruvC*, confer the recombination/repair-defective phenotype (13, 14, 23). The *ruvA* and *ruvB* genes are part of the inducible SOS system of DNA repair and are regulated by LexA protein (13, 23, 24).

Genetic evidence indicates that the *ruv* gene products are involved in a late step in recombinational repair and it has been suggested that they are involved in the resolution of Holliday junctions (20, 25). The observation that the recombination-defective phenotype exhibited by *ruv* mutants is

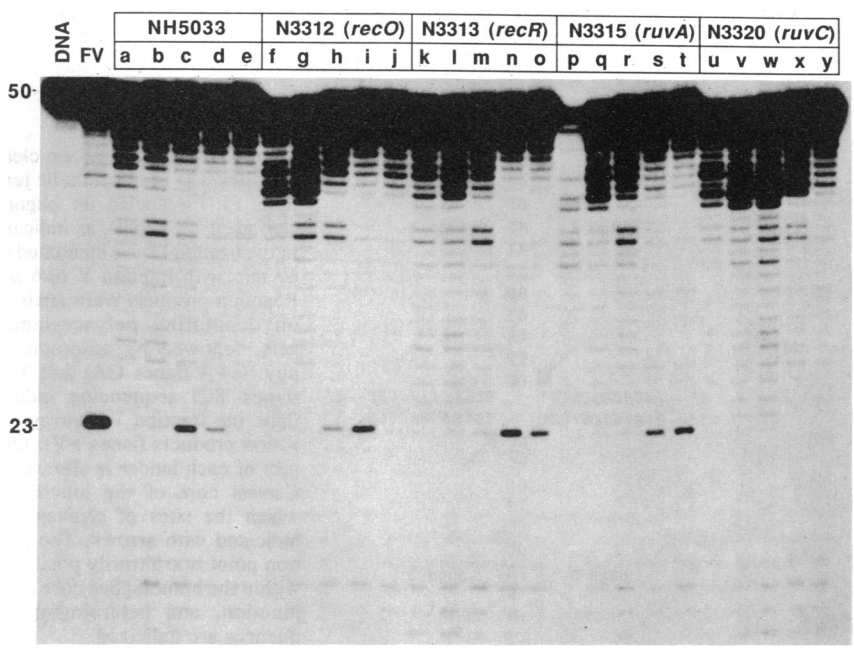


FIG. 5. Resolution of synthetic Holliday junction DNA by mutant *E. coli* extracts. One-liter cultures of five strains were grown, cell lysates were prepared and partially purified by phosphocellulose column chromatography, and 19 fractions were collected. Samples (2 μl) of each fraction were assayed (30 min at 37°C) for the ability to cleave synthetic Holliday junction DNA, as determined by denaturing PAGE followed by autoradiography. The junction was 5′-^{32}P-labeled on oligonucleotide 2. Lanes: DNA and FV, DNA incubated in the absence and presence of fraction V (0.6 μg), respectively; a–e, f–j, k–o, p–t, and u–y, phosphocellulose fractions 13–17 from the indicated strain, respectively. All strains are isogenic except for the indicated mutations and are derivatives of NH5033 *endA recB sbcBC*. The labels 50 and 23 indicate the length (in nucleotides) of oligonucleotide 2 and the ^{32}P-labeled resolution fragment.

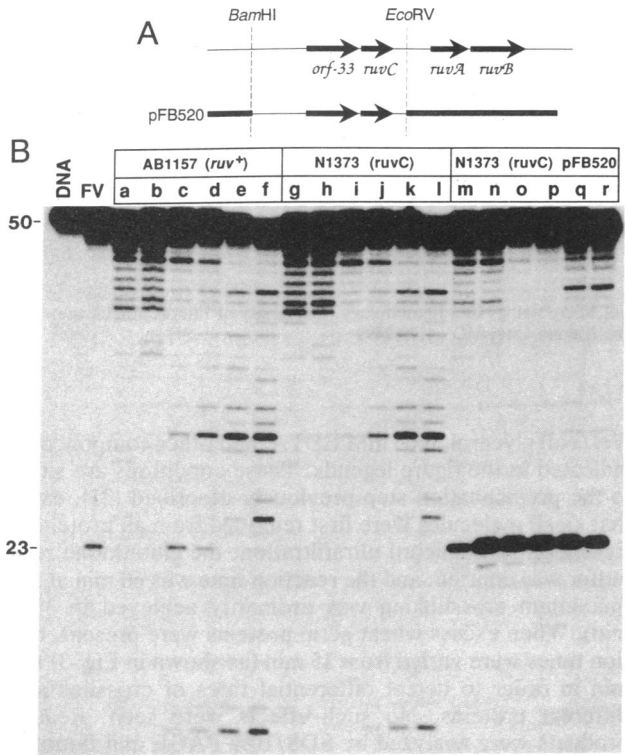


FIG. 6. Complementation of *ruvC* defect and overexpression of resolution activity by a multicopy plasmid carrying *ruvC*$^+$. (*A*) Map of the *ruv* region of the *E. coli* chromosome and construction of pFB520 plasmid. Vector sequences (solid bars) are not drawn to scale. (*B*) One-liter cultures of the indicated strains were grown, fractionated, and assayed for activity using a denaturing polyacrylamide gel as described in Fig. 5. In this experiment 17 fractions were collected. Lanes: DNA and FV, DNA incubated in the absence and presence of fraction V (0.3 μg), respectively; a–f, g–l, and m–r, phosphocellulose fractions 12–17 from the indicated strain, respectively.

seen only in *recBC sbcBC* or *recBC sbcA* genetic backgrounds may indicate two alternative pathways for resolution, one utilizing Ruv and the other RecBCD. A role for RecBCD in the resolution process has been suggested (26, 27), and it has been shown that RecBCD is capable of cleaving DNA at D-loops (28). However, *in vitro* efforts to demonstrate Holliday junction resolution by RecBCD have been largely inconclusive (29).

The experiments shown here demonstrate that *ruvC* mutants lack a nuclease that acts upon Holliday junctions. The most simple interpretation of these results is that *ruvC* encodes a Holliday-junction resolvase. This interpretation is reinforced by observations that extracts made from cells carrying a *ruvC*$^+$ multicopy plasmid show a large increase in specific resolution activity. The native molecular mass of the resolution activity is ≈20 kDa, a size consistent with that predicted from the DNA sequence of *ruvC* (unpublished results).

Whereas resolution of the synthetic Holliday junction needs *ruvC* gene product, as yet we find no requirement for *ruvA* or *ruvB*. Strain N3315, which carries a *ruvA*::Tn*10* insertion that exerts a polar effect on *ruvB* (14), contains resolution activity when assayed using the synthetic Holliday junction. However, RuvA and RuvB may be required for the recognition of the Holliday junction by RuvC *in vivo*. In recent studies, RuvB was purified and shown to hydrolyze ATP (6). The ATPase activity was enhanced by interaction with RuvA in the presence of ssDNA (30). It is possible that the RuvAB complex may move along DNA (using the energy derived from ATP hydrolysis) until a Holliday junction is encountered and may help target RuvC to the junction. A system of this type would be advantageous during post-replication repair of DNA when there are many Holliday junctions in need of resolution. This hypothesis has some similarities with the excision repair system of *E. coli*, where UvrA and UvrB target the site of the lesion in readiness for the UvrC nuclease (31).

In recent work, we have constructed a plasmid in which the *ruvC* gene was placed under control of the *lac* promoter. The overexpressed RuvC protein has a molecular mass of 20 kDa as detected by SDS/gel electrophoresis and copurifies with the resolution activity (unpublished results).

We thank Dr. Hideo Shinagawa for communication of results prior to publication. This work was supported by the Imperial Cancer Research Fund (S.C.W.) and by the Science and Engineering Research Council, the Medical Research Council, and the Wellcome Trust (R.G.L.).

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