Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5452–5456, June 1992
Genetics

Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions

(recombination/repair/SOS response/branch migration/strand exchange)

CAROL A. PARSONS*, IRINA TSANEVA*, ROBERT G. LLOYD†, AND STEPHEN C. WEST*‡

*Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, United Kingdom; and †Genetics Department, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, United Kingdom

Communicated by Philip C. Hanawalt, March 13, 1992 (received for review January 23, 1992)

ABSTRACT **The RuvA, RuvB, and RuvC proteins of *Escherichia coli* are required for the recombinational repair of ultraviolet light- or chemical-induced DNA damage. *In vitro*, RuvC protein interacts with Holliday junctions in DNA and promotes their resolution by endonucleolytic cleavage. In this paper, we investigate the interaction of RuvA and RuvB proteins with model Holliday junctions. Using band-shift assays, we show that RuvA binds synthetic Holliday structures to form specific protein–DNA complexes. Moreover, in the presence of ATP, the RuvA and RuvB proteins act in concert to promote dissociation of the synthetic Holliday structures. The dissociation reaction requires both RuvA and RuvB and a nucleotide cofactor (ATP or dATP) and is rapid (40% of DNA molecules dissociate within 1 min). The reaction does not occur when ATP is replaced by either ADP or the nonhydrolyzable analog of ATP, adenosine 5′-[γ-thio]triphosphate. We suggest that the RuvA and RuvB proteins play a specific role in the branch migration of Holliday junctions during postreplication repair of DNA damage in *E. coli*.**

Mutations in the *ruvA*, *ruvB*, or *ruvC* genes of *Escherichia coli* give rise to mutants that are phenotypically very similar. They are sensitive to DNA-damaging agents such as UV or ionizing irradiation and to chemical mutagens (1). Although *ruv* single mutants are recombination proficient, *recBC sbcA ruv*, *recBC sbcB ruv*, and *recG ruv* multiple mutants are defective in genetic recombination, indicating that the *ruv*-encoded proteins play a role in recombinational repair of damaged DNA (2–5).

The *ruv* locus on the *E. coli* chromosome consists of two operons: *orf26 ruvC* and *ruvA ruvB* (6–9). The latter operon is regulated by LexA repressor and the products of *ruvA* and *ruvB* are induced in response to DNA damage (6, 10, 11). Recent work has focused on the *in vitro* properties of the three Ruv proteins. RuvA protein has been purified and shown to bind DNA, and in the presence of DNA it stimulates the ATPase activity of RuvB (12, 13). Interestingly, the combined action of RuvA and RuvB proteins on a supercoiled plasmid containing a cruciform junction leads to reabsorption of the cruciform (13). The mechanism of reabsorption is unknown.

The RuvC protein was recently purified and shown to resolve recombination intermediates to produce duplex DNA products (14, 15). Resolution occurred by specific endonucleolytic cleavage at the site of the Holliday junction (14–16). In addition, RuvC also cleaves synthetic Holliday junctions and cruciform DNA structures that are extruded from supercoiled plasmids (14, 15, 17). The demonstration that RuvC encodes a Holliday junction resolvase is consistent with a role in the recombinational repair of DNA damage and led us to investigate the interaction of RuvA and RuvB proteins with Holliday junctions. We find that (*i*) RuvA protein recognizes and binds synthetic Holliday junctions, and (*ii*) RuvA and RuvB proteins promote ATP-dependent branch migration of the junction leading to dissociation of the synthetic Holliday structure.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Proteins and DNA. RuvA protein was purified from *E. coli* strain GTI265 (*ruvAB recA*) carrying the plasmids pGTI25 (*ruvA*$^+$) and pcI857 as described (18). RuvB was purified from *E. coli* FB800, a *ruvA60*::Tn*10* derivative of JM101, carrying the plasmid pGTI19 (*ruvB*$^+$) as described (18). Both proteins were estimated by SDS/PAGE to be >99% homogeneous. Concentrations are expressed in moles of monomeric protein.

Four oligonucleotides (49–51 nucleotides long) were annealed to produce synthetic Holliday junction DNA. The sequences of oligonucleotides 1, 2, 3, and 4 (15), annealing conditions, and purification have been described (19). The junction was labeled at the 5′ terminus of oligonucleotide 4. Linear duplex DNA was produced by annealing 5′ ^{32}P-labeled oligonucleotide 1 with its complement (oligonucleotide 5) (15). The specific activities of each DNA preparation varied from 0.5×10^6 to 1.7×10^7 cpm of ^{32}P per nmol. All DNA concentrations are expressed in moles of nucleotide residues. They were measured with DNA dipsticks (Invitrogen, San Diego) and are approximate because of their low concentrations.

To produce the markers for Fig. 3, 45 pmol of 5′ ^{32}P-end-labeled oligonucleotide 4 was annealed with excess (*i*) oligonucleotide 1, (*ii*) oligonucleotide 3, or (*iii*) oligonucleotides 1 and 3. DNA was annealed as described (19), and unincorporated label was removed by passage through a Sephadex G50 column.

Band-Shift Assay. Unless stated otherwise, reaction mixtures (20 μl) contained ≈0.6 μM [^{32}P]DNA in 50 mM Tris·HCl, pH 8.0/5 mM EDTA/1 mM dithiothreitol/bovine serum albumin (100 μg/ml). RuvA and RuvB proteins were added as indicated, and the reaction mixtures were incubated for 15 min on ice or at 37°C. Five microliters of gel loading buffer [40 mM Tris·HCl, pH 7.5/4 mM EDTA/25% (vol/vol) glycerol/bovine serum albumin (400 μg/ml)] was added immediately before loading onto low ionic strength 4% polyacrylamide gels. Electrophoresis was carried out at 4°C for 2.5 h at 160 V with continuous circulation of the buffer (6.7 mM Tris·HCl, pH 8.1/3.3 mM sodium acetate/2 mM EDTA). Gels were transferred onto Whatman 3MM paper, dried, and autoradiographed.

Junction Dissociation Assay. Reaction mixtures (20 μl) contained ≈0.6 μM DNA in a buffer containing 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 100 μM ATP, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml.

‡To whom reprint requests should be addressed.

Unless stated otherwise, incubation with RuvA and/or RuvB was for 15 min at 37°C. Reactions were stopped and deproteinized by addition of 2 μl of stop buffer (100 mM Tris·HCl, pH 7.5/5% SDS/250 mM EDTA/20 mg of proteinase K per ml) followed by incubation for 10 min at 37°C. DNA products were analyzed by electrophoresis through 10% polyacrylamide gels using a Tris borate buffer system followed by autoradiography. The percentage of DNA substrate dissociated was determined by using an LKB laser densitometer.

RESULTS

RuvA Protein Binds Synthetic Holliday Junctions. To determine whether purified RuvA protein binds specifically to Holliday junctions in DNA, we used small synthetic X junctions made by annealing four oligonucleotides (49–51 bases long). The same DNA structure was used previously in studies of RuvC protein (14, 15). It contains a central core of homologous DNA sequences [12 base pairs (bp)] flanked by heterologous sequences (18–20 bp). The junctions were ^{32}P-labeled at the 5′ terminus of oligonucleotide 4. When increasing amounts of purified RuvA were incubated with synthetic junction DNA, two defined protein–DNA complexes (designated I and II) were observed after electrophoresis through low ionic strength polyacrylamide gels (Fig. 1, lanes a–e). These were formed in a concentration-dependent manner. Under identical reaction conditions, the binding to linear duplex DNA was not observed with the band-shift assay (lanes f–j). Complexes formed between RuvA and the junction were stable and were not blocked by competition with exogenous poly(dIdC)·poly(dIdC) or calf thymus DNA (1000-fold excess) (data not shown). The homologous core of the junction was not required for binding since a second synthetic junction containing fully heterologous arm sequences (19) was also bound by RuvA (data not shown).

RuvA and RuvB Promote Dissociation of the Junction. We next investigated the effect of RuvB protein and ATP on the

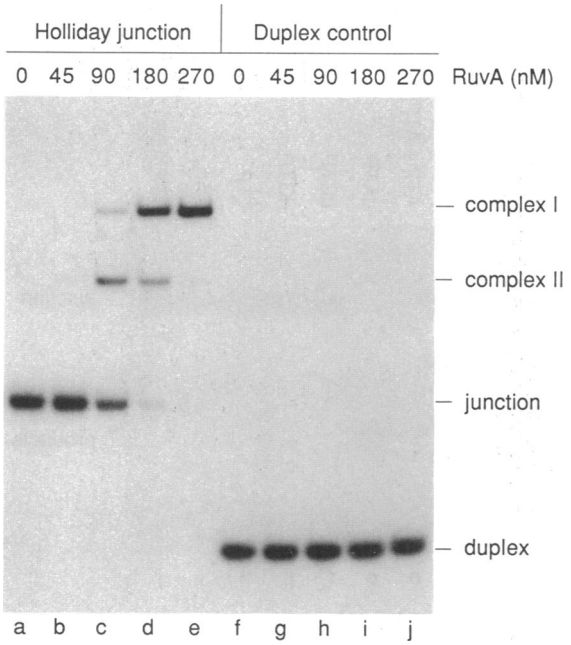

FIG. 1. Binding of synthetic Holliday junctions by purified RuvA protein, as detected by a band-shift assay. The indicated amounts of RuvA were incubated for 15 min on ice with ^{32}P-labeled synthetic Holliday junction (2.2×10^5 cpm) (lanes a–e) or linear duplex DNA (lanes f–j), as described. Complexes were separated on a low ionic strength polyacrylamide gel and radiolabeled DNA was detected by autoradiography. The two major RuvA–DNA complexes are designated I and II.

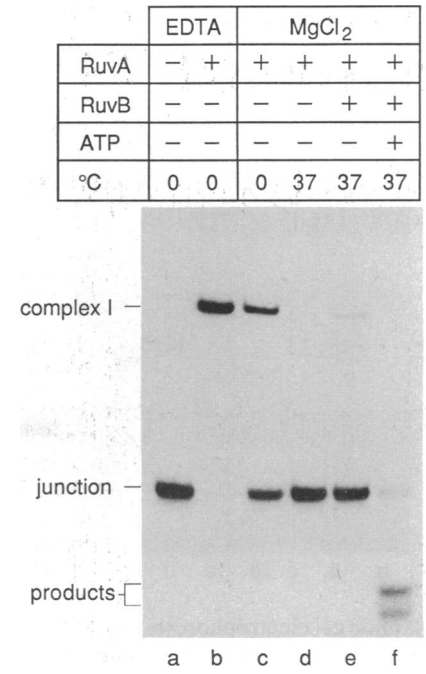

FIG. 2. Holliday junction binding by RuvA: Effect of $MgCl_2$, ATP, and RuvB. Reaction mixtures containing synthetic Holliday junction DNA (1.24×10^4 cpm) and RuvA protein (0.27 μM) were incubated in 50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/bovine serum albumin (100 μg/ml) supplemented with either 5 mM EDTA or 10 mM $MgCl_2$ as indicated. ATP (0.1 mM) and RuvB (1.08 μM) were also added as indicated and reaction mixtures were incubated for 15 min at 0°C or 37°C. DNA products were separated on a low ionic strength polyacrylamide gel and radiolabeled DNA was detected by autoradiography. The major RuvA–DNA complex is indicated.

RuvA–junction complex. In the previous experiment, binding was performed on ice in a buffer containing EDTA. When EDTA (Fig. 2, lane b) was replaced with 10 mM Mg^{2+}, a reduction in binding by RuvA was observed by this gel assay (lane c). If reaction mixtures were incubated at 37°C, we were unable to detect the formation of stable RuvA–junction complexes (lane d). No significant restoration of binding, or any additional bands, were observed when RuvB was added to a similar reaction mixture (lane e). However, when RuvA and RuvB were incubated with the junction in the presence of ATP and Mg^{2+} (lane f), we observed a complete loss of the junction DNA. Instead, two new, faster moving, ^{32}P-labeled bands were observed by gel electrophoresis.

Since neither RuvA nor RuvB proteins possess any nuclease activity on duplex DNA (data not shown), we wished to determine the nature of these DNA species. To do this, the products were electrophoresed through a 10% neutral polyacrylamide gel (Fig. 3, lane b) alongside a number of marker DNA species produced by annealing various combinations of oligonucleotides used to make the synthetic Holliday junction (lanes f–j). The upper product band was found to comigrate with one marker (^{32}P-labeled oligonucleotide 4 annealed with oligonucleotide 1; lane f) and the lower product band comigrated with a second marker (^{32}P-labeled oligonucleotide 4 annealed with oligonucleotide 3; lane g). As described in *Discussion*, these DNA forms are the products expected from dissociation of the Holliday structure by branch migration. They were only observed in complete reactions (lane b) and were not produced when RuvB (lane c), RuvA (lane d), or ATP (lane e) was omitted.

ATP Requirement. To determine the optimal amount of ATP for dissociation of the junction, reactions were set up over a range of ATP concentrations and the products were assayed

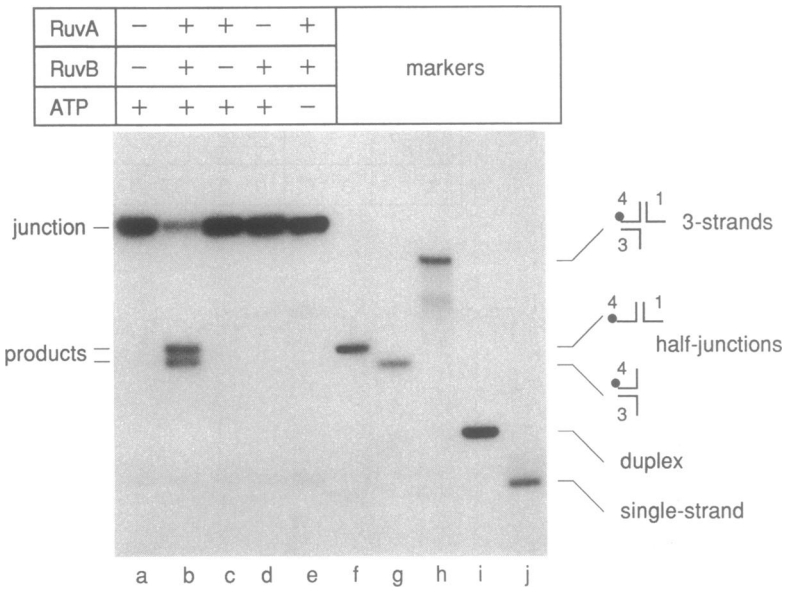

FIG. 3. Dissociation of synthetic Holliday junction by RuvA and RuvB proteins. Reaction mixtures containing synthetic Holliday junction DNA (1.6×10^4 cpm) were incubated for 15 min at 37°C as described. Lanes a–e, RuvA (1.58 μM), RuvB (0.95 μM), and ATP were absent or present as indicated. Reactions were stopped and products were analyzed on a 10% polyacrylamide gel as described. Lanes f–h, indicated combinations of oligonucleotides were annealed to provide markers. In each case, oligonucleotide 4 was 5′ ^{32}P-labeled. Lane i, linear duplex DNA; lane j, oligonucleotide 4. ^{32}P end labels are indicated by solid circles.

by polyacrylamide gel electrophoresis (Fig. 4*A*). To quantitate the data, the autoradiograph was scanned by using a laser densitometer and the results are shown in Fig. 4*C*. We found that concentrations of ATP > 100 μM supported junction dissociation with an optimum at ≈0.5 mM ATP. To investigate the speed of dissociation, a large-scale reaction was set up and samples were removed at various times after addition of RuvA and RuvB. We found that RuvAB-mediated dissociation was rapid, with 40% of the junctions being converted into products within 1 min (Fig. 4 *B* and *D*).

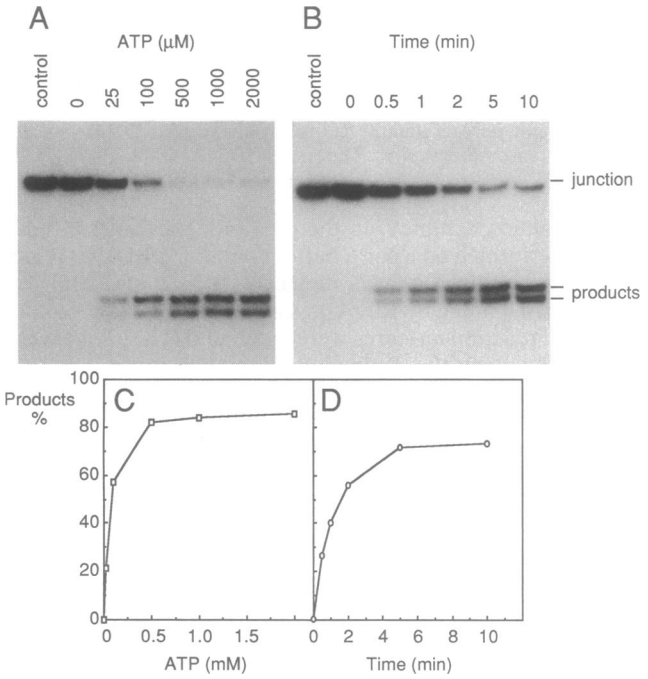

FIG. 4. RuvAB-mediated dissociation of the synthetic Holliday junction: ATP requirement and time course. (*A*) Reaction mixtures containing Holliday junctions (1.6×10^4 cpm), RuvA (1.58 μM), and RuvB (0.95 μM) were incubated for 15 min at 37°C at the indicated ATP concentrations. (*B*) Large scale reaction mixture (140 μl) containing 1.2×10^5 cpm of Holliday junction DNA was supplemented with RuvA (1.58 μM) and RuvB (0.95 μM), and at the times indicated 20-μl aliquots were removed for analysis. The first time point (designated 0 min) represents a sample stopped after 12 sec. The ATP concentration was 100 μM. All reactions were stopped and deproteinized, and the products were analyzed by electrophoresis through 10% polyacrylamide gels as described. (*C* and *D*) Autoradiograms of *A* and *B* were analyzed by laser densitometry and the amount of DNA product is expressed as percentage of total DNA. Backgrounds observed in the absence of ATP (4.3%), or at 0 min (1.6%), have been subtracted. Control lanes, DNA incubated without enzymes.

Although RuvAB-mediated dissociation of the junction occurred in the presence of ATP (Fig. 5, lane b) or dATP (lane e), it did not occur in the presence of ADP (lane i) or the nonhydrolyzable analog of ATP, adenosine 5′-[γ-thio]-triphosphate (lane c). In addition, we found that 2 mM ADP completely inhibited reactions occurring in the presence of 0.5 mM ATP (lane j). Other nucleotide cofactors, including dCTP and TTP, were able to support junction dissociation at a reduced level (as shown by overexposing autoradiograms similar to that of Fig. 5).

Stoichiometric Requirement for RuvA and RuvB. To determine the protein requirements for dissociation, reaction

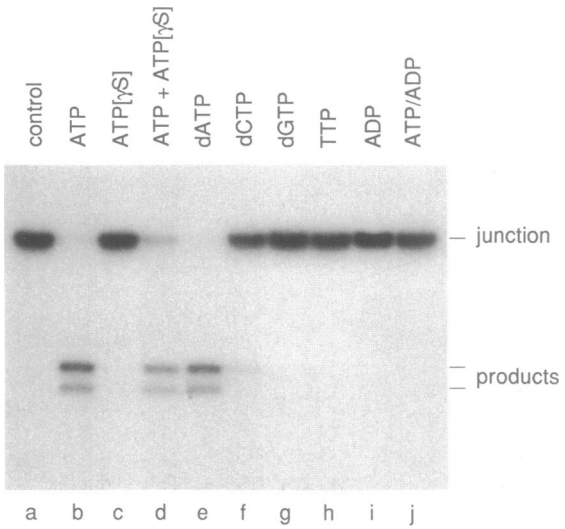

FIG. 5. Effect of nucleotide cofactors on Holliday junction dissociation by RuvA and RuvB. Lane a, control reaction, Holliday junction DNA (1.27×10^4 cpm) incubated without proteins. Lanes b–i, reaction mixtures contained DNA, RuvA (1.58 μM), and RuvB (0.95 μM) in reaction buffer from which ATP had been omitted. Each reaction mixture was supplemented with 0.5 mM ATP, adenosine 5′-[γ-thio]triphosphate (ATP[γS]), dATP, dCTP, dGTP, TTP, or ADP as indicated. Lane j, reaction mixture contained 0.5 mM ATP and 2 mM ADP. Products were analyzed by polyacrylamide gel electrophoresis as described in Fig. 4 legend.

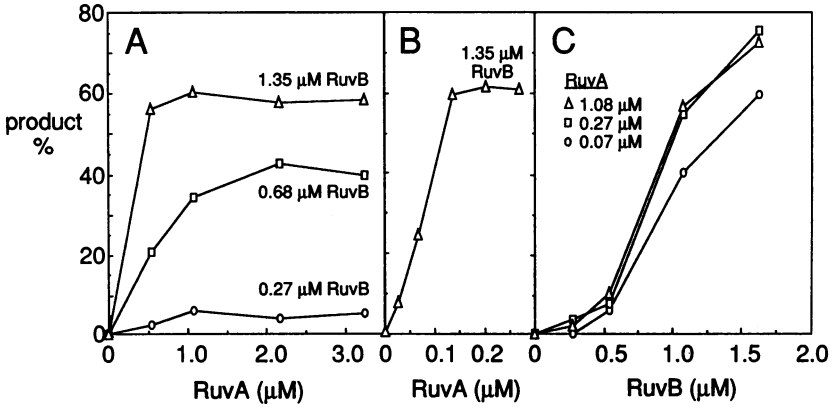

FIG. 6. Stoichiometric requirement for RuvA and RuvB for Holliday junction dissociation. (*A* and *B*) Increasing amounts of RuvA protein were added to reaction mixtures containing synthetic Holliday junction DNA (5×10^3 cpm) with 0.27, 0.68, or 1.35 μM RuvB. (*C*) Increasing amounts of RuvB protein were added to reaction mixtures containing synthetic Holliday junction DNA (5×10^3 cpm) and 0.07, 0.27, or 1.08 μM RuvA. All reaction mixtures were incubated for 15 min at 37°C. Reactions were stopped and deproteinized, and the products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The yield of products was quantitated by using a laser densitometer as described in Fig. 4 legend. Backgrounds (≈4%) observed in the absence of the enzyme that was varied have been subtracted. In these experiments, the DNA concentration was ≈0.6 μM, corresponding to a Holliday junction concentration of ≈3 nM (200 nucleotides per junction).

mixtures were set up in which the concentration of either RuvA or RuvB was kept constant as the other protein was varied (Fig. 6). When RuvB was present at a high level (1.35 μM), we observed a requirement for ≈0.15 μM RuvA (Fig. 6 *A* and *B*). At lower RuvB concentrations, more RuvA (≈1.5 μM) was required. This result may indicate that RuvB is directed to the junction via its interaction with RuvA.

The percentage of junctions dissociated during the course of the reaction was directly related to the concentration of RuvB (Fig. 6*C*). At high (1.08 μM), medial (0.27 μM), or low (0.07 μM) concentrations of RuvA, similar-shaped curves were obtained when RuvB was varied. These results, together with the requirement for ATP, indicate that RuvB (an ATPase) drives the dissociation reaction.

DISCUSSION

Using a simple band-shift gel assay, we have shown that the *E. coli* RuvA protein interacts with small synthetic Holliday junctions to produce specific protein–DNA complexes (Fig. 1). The complexes were formed in a concentration-dependent manner and were not blocked by competition with excess nonspecific competitor DNA. Under identical binding conditions and RuvA concentrations, binding to a duplex control was not observed by this assay.

Shiba *et al.* (13) have shown that the ATPase activity of RuvB protein is stimulated by the presence of RuvA and DNA, suggesting direct interaction between RuvA and RuvB proteins. They also showed that a cruciform structure, present in a supercoiled plasmid, was reabsorbed after addition of RuvA, RuvB, and ATP. Although the mechanism of cruciform reabsorption is unknown, these results led Shiba *et al.* (13) to propose that the RuvA and RuvB proteins interact to facilitate disruption and re-formation of hydrogen bonds by a process similar to branch migration.

The results presented in Fig. 3 show that the combined action of RuvA and RuvB leads to dissociation of synthetic Holliday structures to form products with a fast mobility through polyacrylamide gels. To determine the nature of these dissociation products, they were run alongside a series of marker DNA species produced from the oligonucleotides used to make the junction (Fig. 3). We identified the products as ^{32}P-labeled strand 4 with either strand 1 or strand 3. As shown in Fig. 7, these products correspond to those expected from branch migration of the ^{32}P-labeled junction.

Other work from this laboratory confirms that RuvA and RuvB proteins promote branch migration. In work to be reported elsewhere (21), we used RecA protein to initiate strand-exchange reactions between gapped circular and linear duplex φX174 DNA. Intermediates of the reaction (α structures containing Holliday junctions) were isolated after deproteinization and were mixed with RuvA and RuvB in the presence of ATP. We observed that the recombination intermediates dissociated rapidly by branch migration (through 2000–3000 bp) to form either the starting substrates or heteroduplex DNA products.

From the experiments described here with synthetic junctions, we suggest specific individual roles for the RuvA and RuvB proteins in branch migration. First, binding of synthetic Holliday structures by RuvA (Fig. 1) indicates that it may provide specificity and target RuvB (the ATPase) to the junction point. Second, the requirement for ATP for branch migration (Figs. 4 and 5), together with our observation that junction dissociation is directly related to the concentration of RuvB protein (Fig. 6*C*), suggests that RuvB is the motor

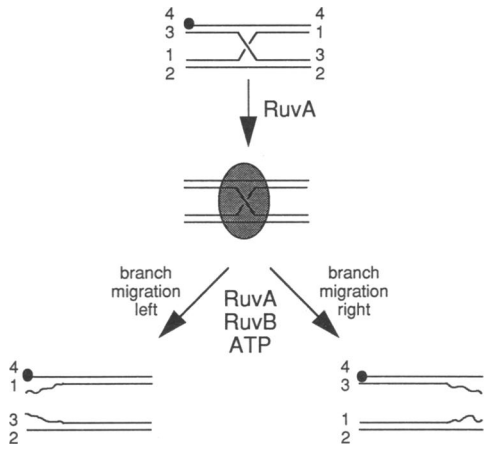

FIG. 7. Diagram indicating interaction and processing of synthetic Holliday junctions by RuvA and RuvB proteins. Synthetic Holliday junction (*Upper*) labeled at the 5′ terminus of oligonucleotide 4 (solid circle) is bound by RuvA protein. RuvA and RuvB proteins act in concert to promote branch migration in the presence of ATP, leading to dissociation of the junction. Branch migration to the left or right gives rise to pairs of products (as indicated; *Lower*). Because of the unique ^{32}P end label, only two of the four possible products will be labeled. ^{32}P end labels are indicated by solid circles.

that drives the branch migration reaction. However, while we have shown a requirement for ATP (and this cannot be fulfilled by either ADP or the nonhydrolyzable analog of ATP, adenosine 5'-[γ-thio]triphosphate), we have not as yet demonstrated that branch migration is coupled to the hydrolysis of ATP. Further work will be required to define the role of ATP and the detailed nature of the RuvA–RuvB association.

One clue to the mechanism of branch migration catalyzed by RuvA and RuvB is provided by the present experiments. The synthetic junction used here contains a homologous core of 12 bp flanked by heterologous DNA sequences (18–20 bp long). These regions of heterology are needed to block spontaneous branch migration of the junction. However, dissociation of the junction by RuvA and RuvB (to form the products shown in Fig. 7) requires melting of these heterologous sequences. How this occurs is currently unknown, and it will be of interest to determine whether the RuvA and RuvB proteins promote branch migration via a specialized or targeted helicase activity.

The RuvA and RuvB proteins form part of the cellular SOS response and are induced after DNA damage (6, 11, 20). Mutants in *ruvA*, *ruvB*, or *ruvC* are UV sensitive and are recombination deficient in combination with *recB sbcA* (3, 4), *recB sbcB(C)* (2), or *recG* (5). The phenotypic properties of *ruvA* and *ruvB* mutants, combined with the *in vitro* properties of RuvA and RuvB proteins, indicate a primary role in branch migration during postreplication (recombinational) repair of DNA lesions. We visualize that RuvA and RuvB recognize Holliday junctions made by RecA and promote branch migration, leading to rapid and extensive formation of heteroduplex DNA. The Holliday junctions may then be resolved by the specific nuclease activity of RuvC protein (14).

1. Otsuji, N., Iyehara, H. & Hideshima, Y. (1974) *J. Bacteriol.* **117**, 337–344.
2. Lloyd, R. G., Benson, F. E. & Shurvinton, C. E. (1984) *Mol. Gen. Genet.* **194**, 303–309.
3. Lloyd, R. G., Buckman, C. & Benson, F. E. (1987) *J. Gen. Microbiol.* **133**, 2531–2538.
4. Luisi-DeLuca, C., Lovett, S. T. & Kolodner, R. D. (1989) *Genetics* **122**, 269–278.
5. Lloyd, R. G. (1991) *J. Bacteriol.* **173**, 5414–5418.
6. Shinagawa, H., Makino, K., Amemura, M., Kimura, S., Iwasaki, H. & Nakata, A. (1988) *J. Bacteriol.* **170**, 4322–4329.
7. Sharples, G. J., Benson, F. E., Illing, G. T. & Lloyd, R. G. (1990) *Mol. Gen. Genet.* **221**, 219–226.
8. Takahagi, M., Iwasaki, H., Nakata, A. & Shinagawa, H. (1991) *J. Bacteriol.* **173**, 5747–5753.
9. Sharples, G. J. & Lloyd, R. G. (1991) *J. Bacteriol.* **173**, 7711–7715.
10. Shurvinton, C. E., Lloyd, R. G., Benson, F. E. & Attfield, P. V. (1984) *Mol. Gen. Genet.* **194**, 322–329.
11. Benson, F. E., Illing, G. T., Sharples, G. J. & Lloyd, R. G. (1988) *Nucleic Acids Res.* **16**, 1541–1550.
12. Iwasaki, H., Shiba, T., Makino, K., Nakata, A. & Shinagawa, H. (1989) *J. Bacteriol.* **171**, 5276–5280.
13. Shiba, T., Iwasaki, H., Nakata, A. & Shinagawa, H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8445–8449.
14. Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991) *Nature (London)* **354**, 506–510.
15. Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6063–6067.
16. Connolly, B. & West, S. C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8476–8480.
17. Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A. & Shinagawa, H. (1991) *EMBO J.* **10**, 4381–4389.
18. Tsaneva, I. R., Illing, G. T., Lloyd, R. G. & West, S. C. (1992) *Mol. Gen. Genet.*, in press.
19. Parsons, C. A., Kemper, B. & West, S. C. (1990) *J. Biol. Chem.* **265**, 9285–9289.
20. Shurvinton, C. E. & Lloyd, R. G. (1982) *Mol. Gen. Genet.* **185**, 352–355.
21. Tsaneva, I. R., Müller, B. & West, S. C. (1992) *Cell*, in press.