Branch Migration of Holliday Junctions Promoted by the *Escherichia coli* RuvA and RuvB Proteins

I. COMPARISON OF RuvAB- AND RuvB-MEDIATED REACTIONS*

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The *Escherichia coli* RuvA and RuvB proteins mediate the branch migration of Holliday junctions in vitro. In the presence of stoichiometric amounts of RuvB (1 RuvB dimer/12 nucleotides), branch migration can occur without need for RuvA. However, RuvA is required when the RuvB concentration is reduced 4-fold or more. Under optimal conditions, we found the minimal protein requirement to be 1 RuvB dimer per 500–1100 nucleotides and 1 RuvA tetramer per 600–1200 nucleotides. To determine the roles of RuvA and RuvB in branch migration, we compared branch migration reactions mediated by RuvB only and by RuvA and RuvB. The time courses of the two reactions were similar, and both required ATP and Mg++. However, RuvB-mediated branch migration occurred at lower ATP concentrations (≥200 μM) and higher Mg++ concentrations (≥10 mM MgCl₂) than the reaction mediated by RuvA and RuvB (≥1 mM ATP, ≥5 mM MgCl₂). The Mg++ requirement for RuvB-mediated branch migration reflects the Mg++ requirement of RuvB for DNA binding (Müller, B., Tsaneva, I. R., and West, S. C. (1993) J. Biol. Chem. 268, 17185–17189) and can be overcome by addition of RuvA. These results indicate that RuvA protein facilitates the interaction of RuvB with DNA.

During genetic recombination and the recombinational repair of DNA damage in *Escherichia coli*, Holliday junctions made by RecA-mediated strand exchange are processed into recombinant products by three proteins encoded by the *ruv* gene locus (for reviews, see Refs. 1 and 2). The *ruvA* and *ruvB* genes form a DNA damage-inducible operon, regulated by the LexA repressor, and encode the 22-kDa RuvA and 37-kDa RuvB proteins (3–5). The *ruvC* gene, which encodes the 19-kDa RuvC protein, forms a separate operon with a gene of unknown function (*orf-26*) (6–8). Mutations in *ruvA*, *ruvB*, or *ruvC* give rise to mutants that show similar phenotypic properties. In an otherwise wild-type genetic background, *ruv* mutants are sensitive to DNA-damaging agents such as UV-light, ionizing irradiation, or mitomycin C (9), but are effectively recombination-proficient. However, in combination with *recBC sbcA, recBC sbcB(C), or recG* mutations, *ruv* mutants are recombination-defective (10–14).

The biochemical properties of the Ruv proteins have been studied in vitro, and their likely roles in recombination have been determined. The RuvC protein is a nuclease which resolves recombination intermediates by specific cleavage of the Holliday junction (15–17). The RuvA protein is a DNA binding protein that interacts with the RuvB ATPase (18, 19) to promote the branch migration of Holliday junctions (19–21). Branch migration has been demonstrated in vitro using (i) synthetic Holliday structures formed by annealing four oligonucleotides (21), and (ii) Holliday junctions made by RecA protein (20, 22). The reaction is dependent on ATP and Mg++ and occurs without a demonstrable polarity (20).

The interaction of RuvA with RuvB has been demonstrated by glycerol gradient centrifugation (23) and by the formation of specific RuvAB-Holliday junction complexes (24). Since RuvA protein binds specifically to Holliday junctions (21, 24), it is likely that one role of RuvA in branch migration is to direct the RuvB ATPase to the junction.

In this manuscript, we extend our studies of branch migration of Holliday junctions mediated by RuvA and RuvB. We confirm that stoichiometric amounts of RuvB can promote branch migration in the absence of RuvA (RuvB-mediated branch migration) and compare this reaction with that catalyzed by RuvA and RuvB (RuvAB-mediated branch migration). We find that the speed and efficiency of RuvB- and RuvAB-mediated branch migration reactions are similar. However, a systematic investigation of the ATP and Mg++ requirements shows that the requirements for branch migration are different and are related to the DNA binding properties of RuvA and RuvB (25). Although it is unlikely that the RuvB-mediated branch migration reaction occurs in vivo (since *ruvA* mutants are repair-defective), a comparison of the two in vitro reactions now allows us to define the individual roles played by the RuvA and RuvB proteins in branch migration and the recombinational repair of DNA.

**MATERIALS AND METHODS**

**Enzymes**—RecA protein of *E. coli* was purified using a modification of a previously published procedure (26). RuvA and RuvB proteins of *E. coli* were purified as described (21). Both proteins were greater than 99% pure, as judged by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. The concentrations of RuvA and RuvB were determined by the Bradford (34) and Lowry (35) methods (Bio-Rad and Sigma protein assay kits, respectively) using bovine serum albumin as a standard. In previous studies (20, 21, 22, 27), the protein concentrations were determined using ovalbumin as a standard, which led to an overestimate of the protein concentration. Unless stated otherwise, protein concentrations are defined in moles of protein monomers.

**DNA Substrates**—Gapped duplex DNA (gDNA)1 with a defined 162-nucleotide gap was prepared by annealing circular single-stranded ϕX174 (+) DNA with the denatured 5224-base pair PstI

1 The abbreviations used are: gDNA, circular ϕX174 duplex DNA with a single-stranded gap in the (+) strand between the PstI and AaII sites; ATPγS, adenosine 5’-O-(3-thiotriphosphate).
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Axolotl fragment of duplex ϕX174 DNA. DNA was prepared and annealed was performed essentially as described (28, 29). Homologous linear duplex DNA was produced by PstI cleavage of ϕX174 RF I DNA, followed by 3'-end-labeling using [α-32P]dATP and terminal transferase (Amersham). DNA concentrations were expressed in moles of phosphates per ml.

Preparation of Recombination Intermediates—Strand exchange reactions (100-150 µl) were performed in 20 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 2 mM ATP, 20 mM phosphocreatine, 6 units/ml phosphocreatine kinase, 2 mM dithiothreitol, and 100 µg/ml bovine serum albumin. gDNA (26 µM) and 32P-end-labeled linear duplex ϕX174 DNA (20 µM) were incubated in the reaction mixture for 2 min at 37 °C, and strand exchange was initiated by the addition of RecA protein (10 µM). Incubation was for 15 min at 37 °C. Reactions were stopped and deproteinized by addition of 1/4 volume of stop mixture (100 mM Tris-HCl, pH 7.5, 2.5% (v/v) SDS, 200 mM EDTA, and 10 mg/ml protease K) followed by incubation for 15 min at 37 °C. The mixture was then applied to a 3.5 M Sepharose CL-2B column equilibrated with 0.5 mM EDTA (pH 8.0), and the deproteinized DNA was collected in two drop fractions. The DNA concentration was determined by quantitation of 32P-labeled DNA.

Branch Migration of Recombination Intermediates—Unless otherwise indicated, branch migration reactions with recombination intermediates were performed in 20 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 2 mM ATP, 2 mM dithiothreitol, and 100 µg/ml bovine serum albumin at 37 °C. Reactions (20 µl) generally contained 26 nM RuvA and 16 nM RuvB, RuvA except that required branch migration) or 670 nM RuvB only (RuvB-mediated branch migration). The products of the reactions were analyzed by 0.8% agarose gel electrophoresis following deproteinization with SDS, EDTA, and protease K.

Agarose Gel Electrophoresis—Gels were run in TAE buffer (30) at room temperature at 6 V/cm with buffer recirculation. To visualize DNA by autoradiography, the gels were dried and exposed to Kodak XAR films. Autoradiographs were quantitated by densitometry using a Molecular Dynamics model 300 laser densitometer and ImageQuant software. The fraction of 32P-labeled linear DNA in recombinant intermediates was measured, and the percentage of recombination intermediates converted into branch migration products was determined.

ATPase Assay—Assays were carried out at 37 °C in 20 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 50 µM ATP, 5 µCi/ml [α-32P]ATP (3000 Ci/mmol), 2 mM dithiothreitol, and 100 µg/ml bovine serum albumin. Reactions (90 µl) were prewarmed for 2 min and started by addition of either RuvB (670 nM) or RuvA (26 nM) and RuvB (16 nM), Aliquots (9 µl) were stopped prior to the addition of protein or at the indicated times after protein addition by addition of SDS, EDTA, and proteinase K (to 0.5%, 40 mM, and 2 mg/ml, respectively). Samples were incubated for 10 min at 37 °C, and 1-µl aliquots were applied to a 5% polyacrylamide gel (15 cm) cast on a sequencing gel apparatus plate (18). Chromatograms were developed in 1 M formic acid, 0.5 M LiCl. The plates were dried and exposed to x-ray film, and the spots corresponding to ATP and ADP were excised. The amount of radioactivity in each spot was determined by scintillation counting using Aquasol scintillation fluid (Du Pont-New England Nuclear).

Preparation of Antibodies—Antibodies were raised against homogeneous RuvA and RuvB proteins (27, 28). Preimmune sera were taken prior to immunization. Ruv proteins (200 µg in Freund’s adjuvant) were injected subcutaneously 2-week intervals. Rabbit IgGs were isolated from serum taken after the third and fifth injection using protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) as described (31). The antibodies were then precipitated by the addition of 0.5 volume of saturated ammonium sulfate, collected by centrifugation (30 min, 3000 × g), resuspended in phosphate-buffered saline (171 mM NaCl, 3.3 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2), and dialyzed against 3 changes of 600 ml of phosphate-buffered saline (31). If necessary, antibodies were concentrated by centrifugation (2000 × g) using Centricon-30 microcentrifugators (Amicon). The antibody concentrations were determined assuming an A₂₈₀ of 1.35 for a concentration of 1 mg/ml.

Inactivation of RuvA or RuvB Using Polyclonal Antibodies—For recombination intermediates containing RuvA and RuvB, RuvA (0.5 pmol) or RuvB (0.3 pmol) was incubated in the presence or absence of antibodies in 31 mM Tris-HCl (pH 7.5), 3.1 mM ATP, 23 mM MgCl₂, 3.1 mM dithiothreitol, and 150 µg/ml bovine serum albumin (in 13 µl). During this preincubation, the RuvA antibodies (or its preimmune) were at 8 µg/ml, and RuvB antibodies (or preimmune) were at 4.6 µg/ml. Following a 10-min incubation, RuvB (0.3 pmol) or RuvA (0.5 pmol) was added. For reactions containing RuvB alone, RuvB protein (13.4 pmol) was incubated for 10 min in the absence or the presence of antibodies in 27 mM Tris-HCl (pH 7.5), 2.7 mM ATP, 20 mM MgCl₂, 2.7 mM dithiothreitol, and 133 µg/ml bovine serum albumin (in 15 µl). The antibody concentrations were as follows: RuvA antibodies (or preimmune), 0.17 mg/ml, and RuvB antibodies (or preimmune), 4 mg/ml. To all reactions, recombination intermediates were added to 2 µM (20 µl final volume), and incubation continued for 20 min. Reactions were stopped, and the DNA products were analyzed by agarose gel electrophoresis as described above.

RESULTS

In previous studies, we used recombination intermediates made by RecA to show that the RuvA and RuvB proteins catalyze the branch migration of Holliday junctions (20, 22). Two reactions were observed: (i) a RuvB-mediated reaction which required stoichiometric amounts of RuvB protein but was independent of RuvA protein, and (ii) a RuvA/RuvB-mediated reaction which required much lower concentrations of RuvB protein.

To understand the RuvAB- and RuvB-mediated branch migration reactions further, we used the same branch migration assay. In the following experiments, RuvB-mediated reactions will be carried out using 670 nM RuvB, whereas the RuvAB-mediated reactions will be performed at 26 nM RuvA and 16 nM RuvB.

To prepare recombination intermediates, gDNA was incubated with homologous 32P-end-labeled linear duplex DNA in the presence of RecA protein, and the resulting DNA species were deproteinized and purified by gel filtration. The recombination intermediates (which form a diffuse band on a gel; Fig. 1A, lane a) look like a-structures in the electron microscope and are stable for several hours at 37 °C in the absence of added protein (22). When incubated with 26 nM RuvA and 16 nM RuvB, we observed a loss of label at the position of intermediates, indicating their dissociation with time (Fig. 1A, lanes b-g). This loss of label coincided with an increase in the amount of label at the position of circular and linear duplex DNA, as expected from branch migration and as described previously (20). At this RuvB concentration, the branch migration reaction shows an absolute requirement for RuvA (20). However, at much higher RuvB concentrations, the need for RuvA is overcome, and a similar time course was observed in the presence of 670 nM RuvB alone (Fig. 1A, lanes j-o). The two time courses, which appeared identical by visual analysis (Fig. 1A), were quantitated using a laser densitometer, and the amounts of 32P-labeled intermediates were determined at each time point. Fig. 1B shows the mean values calculated from five independent experiments and indicates that the rates of dissociation are similar within the limits of experimental variation.

Since RuvB protein was purified from an overexpression vector carried by a bacterial strain from which the ruvA gene was inactivated by transposon insertion (27), it is unlikely that trace amounts of contaminating RuvA protein could be contributing to the RuvB-mediated branch migration reaction. However, to unequivocally rule out this possibility, we tested the effect of polyclonal antibodies raised against RuvA and RuvB on branch migration reactions catalyzed by RuvAB (Fig. 2, lanes b-g) or RuvB alone (lanes h-l). We observed that branch migration reactions catalyzed by RuvAB (lanes b-g) were blocked by preincubation with antibodies raised against either RuvA (lane d) or RuvB (lane f). Similar reactions to which preimmune serum was added gave rise to branch migration products (lanes e and g), as did controls without antibody addition (lanes b and c). However, in RuvB-mediated branch migration reactions (lanes h-l), only the incubation with RuvB antibodies (lane k) prevented branch migration, since the RuvA antibodies had no effect on this
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A

FIG. 1. Time courses of RuvAB- and RuvB-mediated branch migration. A, analysis by agarose gel electrophoresis. Lanes a and i, recombination intermediates (final concentration 3.7 μM, 55% intermediates) were mixed with reaction buffer (in 40 μl) and stopped immediately as described under “Materials and Methods.” Lanes b–g, recombination intermediates were incubated for 2 min, supplemented with RuvA and RuvB, and further incubated (in 100 μl). Lanes j–o, as for lanes b–g, but RuvB only was added. Aliquots (15 μl) of the reaction mixtures were stopped at the times indicated (in minutes) and analyzed by 0.8% agarose gel electrophoresis followed by autoradiography. Lane h, XDNA cut with HindIII and 3' end-labeled using the Klenow fragment of DNA polymerase I and [γ-32P]ATP. B, quantitation of time courses. Five independent experiments comparing branch migration mediated by RuvB (670 nM) or RuvA and RuvB (26 nM and 16 nM, respectively) were performed. Reactions were stopped and analyzed as described in A, and the percentage of intermediates converted into products was determined as described under “Materials and Methods.” The mean values for each time point were determined and are shown with the lowest and highest observed values. In the five experiments, the DNA concentration was between 3.6 and 4.2 μg (32%, 50%, 55%, 64%, and 69% intermediates).

| 1st incubation | - | B | A | aA | B | pa | aB | pa | B |
|----------------|---|---|---|----|---|----|----|----|---|
| 2nd incubation | - | A | B | B | A | - | - | - | - |

FIG. 2. Effect of antibodies on RuvAB- and RuvB-mediated branch migration. The reaction components and the order of addition are indicated, and full details are presented under “Materials and Methods.” RuvA or RuvB protein was incubated for 10 min with either RuvA antibodies, RuvB antibodies, or antibodies isolated from preimmune sera. Recombination intermediates, and, where indicated, RuvA or RuvB protein, were then added and incubation was continued for 20 min. The products of the reactions were deproteinized and analyzed by agarose gel electrophoresis. Lane a, recombination intermediates (2 μM) were incubated in the absence of Ruv proteins. Lanes b–g, reactions contained 26 nM RuvA and 16 nM RuvB. Lanes h–l, reactions contained 670 nM RuvB protein. The following nomenclature is used in the figure heading: RuvA antibodies (aA), preimmune antibodies (pa), RuvB antibodies (aB), preimmune antibodies (pa).

reaction (lane i). These results confirm that the branch migration reaction which occurs in the presence of stoichiometric amounts of RuvB is independent of RuvA.

To determine the effect of the RuvA and RuvB concentrations on branch migration, reactions containing different amounts of RuvA and RuvB protein were performed (Fig. 3). As shown above, reactions carried out with 670 nM RuvB (corresponding to a ratio of 1 RuvB monomer per 6 nucleotides) showed no requirement for RuvA. When the RuvB concentration was reduced to 160 or 16 nM, RuvA protein (7 nM) was required for branch migration. A plateau was reached at 14 nM RuvA, and, at very high concentrations (1.7 μM), we
observed that RuvA protein inhibited branch migration in the presence of 160 and 670 nM RuvB. We presume that the excess RuvA protein inhibits the reaction by competing with the RuvAB protein complex for DNA.

In the experiment shown in Fig. 3, efficient branch migration at a low RuvB concentration (16 nM) required ≥14 nM RuvA (at a DNA concentration of 4.2 μM, expressed in nucleotides). Other experiments performed at 8 nM RuvB required 27 nM RuvA (data not shown). These results indicate that RuvAB-mediated branch migration requires approximately 1 RuvB dimer per 500–1100 nucleotides and 1 RuvA tetramer per 600–1200 nucleotides. These estimates are based on observations which show that RuvA and RuvB are tetrameric and dimeric, respectively (23, 27).

The addition of RuvA and RuvB to RecA-mediated in vitro recombination reactions facilitates branch migration past UV-lesions (20). To determine whether RuvA was specifically required for this reaction, we tested the effect of UV-irradiation on the branch migration reaction catalyzed by RuvB or by RuvAB. We found that both reactions were slowed down to a similar extent, and the inhibitory effect was proportional to the dose of UV irradiation (data not shown). Thus, branch migration past UV-lesions does not specifically require the presence of RuvA protein.

To determine whether RuvAB- and RuvB-mediated branch migration reactions have different cofactor requirements, we varied the ATP and Mg2+ concentrations. Both RuvAB- and RuvB-mediated branch migration reactions were dependent upon the presence of ATP (Fig. 4). However, the ATP requirements were different, with the RuvB-mediated reaction requiring less ATP (≥0.2 mM) than the RuvAB-mediated reaction (≥1 mM).

Both reactions were dependent on the presence of Mg2+ (Fig. 5A), but again, the optimal Mg2+ concentrations were found to differ. RuvAB-mediated branch migration occurred with low efficiency at 5 mM MgCl2 and was maximally efficient at 10 mM MgCl2. In contrast, higher MgCl2 concentrations were required for RuvB-mediated branch migration. In this case, no reaction was observed at 5 mM MgCl2, and the optimal concentration was found to be ≥15 mM MgCl2. At low MgCl2, the inability of RuvB to promote branch migration (Fig. 5B, lane c) was overcome by addition of RuvA (lane b).

The effects of NaCl and pH on RuvAB- and RuvB-mediated branch migration were also investigated (at 15 mM MgCl2 and 2 mM ATP). Both reactions were inhibited in a similar way by NaCl, with 50% inhibition at 200 mM (data not shown). Branch migration occurred efficiently between pH 5.5 and pH 10.5, the highest pH tested (data not shown).

Above, we showed that ATP was required for RuvAB- and RuvB-mediated branch migration. Analysis of the RuvB ATPase has shown that ADP is an effective competitor for ATP binding (18). In addition, ADP cannot replace ATP for RuvAB-mediated branch migration (20). To test whether ADP inhibits ATP-dependent branch migration, a series of reactions were performed in the presence of 2 mM ATP and various amounts of ADP. Both RuvAB- and RuvB-mediated reactions were found to be sensitive to the presence of ADP. However, the two reactions were inhibited to a different extent (Fig. 6). For example, the RuvB-mediated branch migration reaction was severely inhibited by 2 mM ADP (ADP:ATP = 1:1), whereas 6 mM ADP was required to inhibit the RuvAB-mediated reaction to the same extent (ADP:ATP = 3:1).

We found previously that ATPγS, a nonhydrolyzable analog of ATP, could not replace ATP in the RuvAB-mediated branch migration reaction (20), indicating that ATP hydrolysis was required. To test whether ATP hydrolysis was also required for RuvB-mediated branch migration, the two reactions were performed in the presence of 2 mM ATP and various amounts of ATPγS. As expected, we found that both the RuvAB- and RuvB-mediated branch migration reactions...
were sensitive to the presence of ATPγS (Fig. 7). RuvAB-mediated branch migration occurred in the presence of ATPγS, up to a concentration of 3 mM (ATPγS:ATP = 3:2). In this reaction, a complete block was observed at 5 mM ATPγS (ATPγS:ATP = 5:2). By comparison, we observed that RuvB-mediated branch migration was significantly more sensitive and was severely inhibited in the presence of 0.5 mM ATPγS and blocked by 1 mM ATPγS (ATPγS:ATP = 1:2).

The competition experiments with ADP and ATPγS confirm that ATP binding and hydrolysis are required for branch migration and show that the RuvB-mediated branch migration reaction is more sensitive to competitors than the RuvAB-mediated reaction. Interestingly, when we measured the amounts of ATP hydrolyzed during the RuvAB and RuvB reactions, we found that the RuvAB reaction was more energy efficient than the reaction catalyzed by RuvB.

The data presented in Fig. 8 show time courses of ATP hydrolysis in RuvAB- and RuvB-mediated branch migration reactions. In the RuvB-mediated reaction, significant amounts of ATP were hydrolyzed. However, in this experiment, we have been unable to correlate ATP hydrolysis with branch migration since the time course shows that ATP hydrolysis continues after 10 min, the time at which the majority of intermediates had been dissociated (Figs. 1 and 8). In contrast, the level of ATP hydrolysis in the RuvAB-mediated reaction hardly exceeded the background.

**DISCUSSION**

In this study, we have used deproteinized recombination intermediates to investigate the branch migration of Holliday junctions by the E. coli RuvA and RuvB proteins. We reported previously that RuvB protein is involved in two different types of *in vitro* branch migration reactions. In the presence of stoichiometric amounts of RuvB, branch migration occurred in the absence of RuvA, while RuvA was required for branch migration in the presence of lower amounts of RuvB (20). In this paper, we confirm that branch migration reactions can be mediated *in vitro* by RuvB alone and have compared the time courses and cofactor requirements of the RuvAB- and RuvB-mediated branch migration reactions. Although we observe a number of similarities, we also find conditions in which the two reactions can be distinguished from each other.

**FIG. 8.** ATP hydrolysis in the presence of recombination intermediates. Recombination intermediates (final concentration 3.8 μM, 69% intermediates) were incubated as described under "Materials and Methods" (90 μl containing 90 nmol of ATP). The reaction was started by the addition of RuvB (670 nM) or RuvA and RuvB (26 nM and 16 nM, respectively) and the amount of ATP hydrolyzed at the indicated times was determined as described under "Materials and Methods." The background amount of ADP (1 nmol) present prior to the addition of Ruv proteins has been subtracted.

The time courses of branch migration and the final yield of RuvAB- and RuvB-mediated products were very similar (Fig. 1). We determined the protein requirements for the two reactions and found that RuvB-mediated branch migration occurred at a ratio of 1 RuvB dimer/12 nucleotides of DNA. Interestingly, at this RuvB to nucleotide ratio, duplex DNA is maximally protected by RuvB (in the presence of ATPγS) from attack by DNase I and phosphodiesterase I (23). The need for high RuvB concentrations is likely to be due to the low affinity for DNA shown by RuvB protein. Alternatively, it is possible that RuvB-mediated branch migration requires the formation of a continuous nucleoprotein filament, as has been observed with RecA protein (32).

We determined the amounts of RuvA and RuvB required for RuvAB-mediated branch migration and found that the minimal protein requirement was 1 RuvB dimer per 500-1100 nucleotides and 1 RuvA tetramer per 600-1200 nucleotides.

Previously, we demonstrated that RuvA protein interacts with synthetic Holliday junctions to form specific protein-DNA complexes (21). More recently, we have used bandshift assays to detect the formation of a RuvA-RuvB-Holliday junction complex (24). In the experiments described here, we show that the requirement for RuvB is substantially reduced by the presence of RuvA. Presumably, the RuvA protein specifically targets RuvB to the region of the junction. This hypothesis is supported by recent observations which show that RuvA and RuvB interact directly in solution to form a RuvAB protein complex (23). The specific interaction of RuvA with the Holliday junction might also explain the observation that branch migration reactions are inhibited by excess RuvA (1 RuvA tetramer/10 nucleotides) (Fig. 3). Most likely, inhibition was due to competition between RuvA and RuvAB for the Holliday junction.

In addition to their protein requirements, branch migration reactions mediated by RuvAB or RuvB can be distinguished by their cofactor requirements. For example, RuvAB-mediated branch migration needed higher concentrations of ATP than the reaction performed by RuvB only (Fig. 4). Moreover, the addition of ADP (Fig. 6) or ATPγS (Fig. 7) to reactions...
containing ATP was found to inhibit branch migration by RuvAB or RuvB to different extents. Reflecting its low requirement for ATP, RuvB-mediated branch migration was more sensitive to the competitive effects of ADP and ATPγS. We cannot rule out the possibility that the difference in nucleotide cofactor requirements between the RuvAB- and RuvB-mediated reactions was caused by the change in RuvB concentration. However, we feel this is unlikely since RuvA directly stimulates the RuvB ATPase in the presence of DNA (19).

RuvAB- and RuvB-mediated branch migration reactions also had different Mg²⁺ requirements (Fig. 5), with the RuvB-mediated reaction requiring concentrations of MgCl₂ ≥ 10 mM. The need for ≥10 mM MgCl₂ correlates with the Mg²⁺ dependence of duplex DNA binding by RuvB, as observed by gel analyses and stimulation of the RuvB ATPase by DNA (25). In these experiments, we observed a direct interaction between RuvB and duplex DNA in the presence of ATP at MgCl₂ concentrations above 10 mM, indicating that Mg²⁺ affects the binding of RuvB to DNA.

The presence of RuvA protein allows branch migration at lower Mg²⁺ concentrations. In experiments that analyzed the interaction of RuvA and RuvB with DNA (25), we found that RuvA facilitates the interaction of RuvB with duplex DNA at low Mg²⁺ concentrations, leading to the formation of a complex consisting of RuvA, RuvB, and duplex DNA. From these results, we conclude that RuvA protein is able to facilitate the interaction of RuvB with DNA and more specifically with Holliday junctions.

In conclusion, our experiments confirm that RuvB-mediated branch migration can occur in the absence of RuvA, provided stoichiometric amounts of RuvB are present (20). This supports the notion that RuvB, which is an ATPase and contains structural motifs characteristic of DNA helicases (33), is the motor of branch migration. In the presence of lower concentrations of RuvB, RuvA is required for branch migration, and this reaction is likely to be more physiologically relevant than the RuvB-mediated reaction. Our study of RuvAB-mediated branch migration indicates that RuvA facilitates the interaction of RuvB with DNA, by the formation of a RuvA-RuvB-DNA complex, and directs RuvB to the Holliday junction (21, 24, 25). The formation of a RuvA-Holliday junction complex leads to movement of the joint by ATP-driven branch migration. The mechanism of this reaction is presently unknown, but is likely to involve strand separation followed by reannealing since RuvAB protein exhibits DNA helicase activity in vitro (33).

REFERENCES

1. West, S. C. (1992) Annu. Rev. Biochem. 61, 603-640
2. Tsaneva, I. R., Muller, B., and West, S. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6063-6067
3. Shiba, T., Iwaseki, H., Nakata, A., and Shinagawa, H. (1991) Mol. Gen. Genet. 185, 352-355
4. Benson, F. E., Illing, G. T., Sharples, G. J., and Lloyd, R. G. (1988) Nucleic Acids Res. 16, 5141-5150
5. Sharples, G. J., Benson, F. E., Illing, G. T., and Lloyd, R. G. (1990) Mol. Gen. Genet. 221, 219-226
6. Sharples, G. J., Lloyd, R. G. (1991) J. Bacteriol. 173, 771-7715
7. Takahagi, I., Iwaseki, H., Nakata, A., and Shinagawa, H. (1991) J. Bacteriol. 173, 5747-5753
8. Otsui, N., Iyehara, H., and Hideshima, Y. (1974) J. Bacteriol. 117, 337-344
9. Stacey, K. A., and Lloyd, R. G. (1976) Mol. Gen. Genet. 143, 223-232
10. Lloyd, R. G., Benson, F. E., and Shurvinton, C. E. (1984) Mol. Gen. Genet. 194, 303-309
11. Luini-DeLuca, C., Lovett, S. T., and Kolodner, R. D. (1989) Genetics 122, 269-278
12. Benson, P., Collier, S., and Lloyd, R. G. (1991) Mol. Gen. Genet. 225, 266-272
13. Lloyd, R. G. (1991) J. Bacteriol. 173, 5414-5418
14. Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G., and West, S. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5452-5456
15. Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G., and West, S. C. (1991) Nature 354, 506-510
16. Iwaseki, H., Takahagi, M., Shiba, T., Nakata, A., and Shinagawa, H. (1991) EMBO J. 10, 4381-4389
17. Iwaseki, H., Shiba, T., Makino, K., Nakata, A., and Shinagawa, H. (1989) J. Mol. Biol. 217, 605-620
18. Shibata, T., Iwaseki, H., Nakata, A., and Shinagawa, H. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 8445-8449
19. Tsaneva, I. R., Muller, B., and West, S. C. (1992) Cell 99, 1171-1180
20. Parsons, C. A., Tsaneva, I., Lloyd, R. G., and West, S. C. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5452-5456
21. Muller, B., Burdett, I., and West, S. C. (1992) EMBO J. 11, 2685-2693
22. Shibata, T., Iwaseki, H., Nakata, A., and Shinagawa, H. (1993) Mol. Gen. Genet. 239, 157-163
23. Morgan, H. K., Morgan, J. E., and West, S. C. (1993) J. Bacteriol. 175, 6059-6067
24. Parsons, C. A., and West, S. C. (1993) Mol. Cell. Biol. 13, 2171-2177
25. Cox, M. M., McEntee, K., and Lehman, I. R. (1981) J. Biol. Chem. 256, 4774-4778
26. Tsaneva, I. R., Illing, G. T., Lloyd, R. G., and West, S. C. (1992) Mol. Gen. Genet. 235, 1-10
27. West, S. C., Cassuto, E., and Howard-Flanders, P. (1982) Mol. Gen. Genet. 187, 209-217
28. Muller, B., Jones, C., Kemper, B., and West, S. C. (1990) Cell 60, 329-336
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Harlow, E., and Lane, D. P. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Staszak, I., and Epstein, E. H. (1988) in Genetic Recombination (Kucherlapati, R., and Smith, G. R., eds) pp. 265-308, American Society for Microbiology, Washington, D.C.
32. Tsaneva, I. R., Muller, B., and West, S. C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1316-1319
33. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275