Role of RuvA in Branch Migration Reactions Catalyzed by the RuvA and RuvB Proteins of Escherichia coli*

(Received for publication, February 21, 1996, and in revised form, May 18, 1996)

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The RuvA and RuvB proteins of Escherichia coli promote ATP-dependent branch migration of Holliday junctions during homologous genetic recombination and DNA repair. In this process, RuvA acts as a specificity factor that targets RuvB, a hexameric ring motor protein, to the junction. Because elevated concentrations of RuvB can promote branch migration in the absence of RuvA, it has been suggested that RuvA acts as a molecular matchmaker. In the studies presented here, we compared the requirements for RuvAB- and RuvB-mediated branch migration reactions and found that reactions catalyzed by RuvB alone were highly sensitive to inhibition by NaCl, temperature, ADP, and ATPγS. Our observations indicate that the two reactions occur by distinct mechanisms and support the notion that RuvAB-mediated branch migration is physiologically more relevant than that catalyzed by RuvB. We also show that ongoing RuvAB-mediated branch migration reactions were blocked by the addition of polyclonal antibodies raised against RuvA. The role of RuvA is therefore unlikely to be restricted to RuvB targeting; instead, it is required continually during branch migration. Competition with excess synthetic Holliday junctions, sufficient to sequester released RuvA, failed to cause an immediate block and leads us to suggest that RuvAB promote branch migration by a processive mechanism.

Recent studies indicate that the DNA damage-inducible ruvA and ruvB gene products promote the formation of heteroduplex DNA during genetic recombination and postreplication repair (reviewed in Refs. 1 and 2). The product of ruvA, the 22-kDa RuvA protein, forms stable tetramers in solution (3), which interact directly with RuvB (4–6), a 37-kDa DNA-dependent ATPase (7). RuvA binds with high specificity to Holliday junctions (8, 9) and is thought to facilitate the loading of RuvB (10–12). In vitro studies indicate that RuvB protein forms hexameric ring structures in which DNA passes through the center of each ring (4, 13).

Using recombination intermediates made using RecA protein, it has been shown that stoichiometric concentrations of RuvB protein can promote branch migration in the absence of RuvA (14, 15). This observation led to the proposal that RuvA might function simply as a molecular matchmaker that plays no direct role in the catalysis of branch migration (16, 17). More recent studies argue against this viewpoint since RuvA: (i) changes the configuration of a Holliday junction to an open-square structure (18) that is energetically more favorable for branch migration (19, 20); (ii) is required for activation of the DNA helicase activity of RuvB (21, 22); and (iii) is required for branch migration through heterologous DNA sequences (23). Direct visualization of the RuvAB-Holliday junction complex by electron microscopy also supports the notion that RuvA is directly involved in the catalysis of branch migration (18). In these studies, RuvA was shown to bind the crossover point where it was flanked by two hexameric rings of RuvB. Confirmation of this tripartite structure was obtained by DNase I footprinting (11).

In the studies presented here, we have compared the requirements for RuvAB- and RuvB-mediated branch migration reactions. The observations indicate that the two reactions occur by distinct mechanisms and support the notion that RuvAB-mediated branch migration is physiologically more relevant than the reaction catalyzed by RuvB alone. Moreover, we provide evidence that RuvA plays a direct and continual role in the catalysis of branch migration.

MATERIALS AND METHODS

Enzymes and Reagents—RecA, RuvA, and RuvB proteins were purified as described previously except that the RuvB purification scheme contained an additional ammonium sulfate precipitation step prior to MonoQ fast protein liquid chromatography (3). Protein concentrations were determined using the Bio-Rad protein assay kit (24) using bovine serum albumin as standard and are expressed in moles of protein monomers. Where appropriate, proteins were diluted in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, 100 µg bovine serum albumin/ml, and 20% (v/v) glycerol. BSA and PstI were purchased from New England Biolabs, and terminal transferase was obtained from Amersham Corp.

DNA Substrates—Closed circular duplex DNA were prepared from the plasmids pDEA-7Z f(+) and pDEA2 (25) by standard techniques. The plasmid pDEA2 (3.5-kilobase pairs) contains a 643-bp fragment of PACYC184 ligated to the 2.9-kilobase pair SphI-NsiI fragment of pDEA-7Z f(+). Circular (+) single-stranded DNA was prepared from pDEA-7Z f(+) using the helper phage M13K07 (Pharmacia). Phage were precipitated by the addition of polyethylene glycol/NaCl and purified by CsCl gradient centrifugation. Single-stranded DNA was isolated using phenol/sodium tetraborate. To prepare gapped duplex DNA, covalently closed circular duplex pDEA-7Z f(+) (3.0 kilobase pairs) was digested with BsaI and PstI to generate fragments of 2825 and 175 bp. The 2825-bp fragment was purified through a 5–20% neutral sucrose gradient and annealed to single-stranded DNA by heating to 95°C, followed by slow cooling to room temperature. The resultant gapped duplex DNA, containing a defined 175-nucleotide single-stranded gap, was isolated from excess circular single-stranded DNA by preparative agarose gel electrophoresis. Linear duplex DNA was prepared by PstI digestion of closed circular duplex pDEA2 DNA and 32P end-labeled using terminal transferase and [γ-32P]ATP (Amersham). Synthetic Holliday junctions, approximately 60 nucleotides in length, were produced by annealing four unlabeled oligonucleotides as described (10). All DNA concentrations are expressed in moles of nucleotide residues.

Preparation of Recombination Intermediates—RecA-mediated strand exchange reactions (200 µl) were performed in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, 100 µg bovine serum albumin/ml, and 20% (v/v) glycerol.
15 mM MgCl₂, 2 mM ATP, 2 mM dithiothreitol, and 100 µg of bovine serum albumin/ml. Gapped circular duplex DNA (15 µM) was preincubated with RecA protein (5.8 µM) for 5 min at 37 °C, and then the reaction was initiated by the addition of 32P-labeled and end-labeled linear duplex DNA (10 µM), followed by incubation for a further 30 min. Reactions were stopped by the addition of EDTA and SDS to 40 mM and 0.5% (w/v), respectively, and the DNA was deproteinized by addition of proteinase K to 2 mg/ml, followed by incubation for 10 min at 37 °C. The recombination intermediates (α-structures) were isolated on a 3.5-ml Sepharose CL-2B column, equilibrated with 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM dithiothreitol, and 100 µg bovine serum albumin/ml and could be stored at 4 °C for 2–3 days. DNA concentrations were determined by quantification of the 32P label.

Branch Migration Assay—Unless stated otherwise, reaction mixtures (100 µl) contained 3 µM 32P-labeled α-structures in 20 mM Tris-HCl, pH 8.0, 1 mM ATP, 1.5 mM dithiothreitol, and 100 µg of bovine serum albumin/ml. MgCl₂ was present at either 10 mM (RuvAB reactions) or 15 mM (RuvB reactions). Reactions were initiated by the addition of RuvB protein (1.35 µM), or a mixture of RuvA (22 nM) and RuvB (134 nM), and were incubated at 37 °C. At various times, samples (10 µl) were removed, and the reactions were stopped by the addition of one-tenth volume of 10 × stop buffer (100 mM Tris-HCl, pH 7.5, 5% (w/v) SDS, 0.25 mM EDTA, and 20 mg/ml proteinase K), followed by incubation at 37 °C for 10 min. Gel loading buffer (3 µl of 50% (v/v) glycerol, 50 mM EDTA, and 0.01% (w/v) bromophenol blue) was then added, and samples were analyzed by agarose gel electrophoresis. The gels were dried, and the 32P-labeled DNA was analyzed using a phosphorimager. Branch migration was quantified by calculating the percentage of 32P-labeled α-structures converted to 32P-labeled linear products.

Agarose Gel Electrophoresis—DNA products were analyzed by electrophoresis through 1.2% (w/v) agarose gels using TAE buffer (40 mM Tris-acetate, pH 8.5, and 1 mM EDTA). Ethidium bromide (0.5 µg/ml) was included in the gel and running buffer to minimize dissociation of the recombination intermediates during electrophoresis. Gels were run at 6 V/cm for 5 h with buffer recirculation.

ATPase Assay—Reactions were carried out essentially as described for branch migration but also contained 50 µCi (α-32P]ATP/ml. During incubation at 37 °C, aliquots (2 µl) were taken at the indicated times, and reactions were stopped by the addition of EDTA to 40 mM. Samples (1 µl) were spotted onto CEL 300 PEUV PhastGel (Pharmacia) thin layer chromatography plates, which were developed in 1 M formic acid/0.5 M LiCl. The percentage of (α-32P]ATP hydrolyzed to (α-32P]ADP was quantified using a Molecular Dynamics Model 425E PhosphorImager running ImageQuant software.

Purification of Polyclonal Antibodies—Rabbit polyclonal antibodies raised against RuvA protein were prepared as described (26) and purified using protein-A-Sepharose (27). The concentration was determined assuming an A280 of 1.35 for a concentration of 1 mg/ml.

RESULTS

Branch Migration Assay—To compare ATP-dependent branch migration reactions catalyzed by either RuvAB or RuvB, recombination intermediates were prepared using RecA protein. As shown in Fig. 1A, the DNA substrates used to make recombination intermediates, gapped circular pDEA-7Z f(+), and 3′-32P end-labeled Pst I-linearized pDEA2 DNA share 1536 bp of homology through which RecA drives strand exchange up to heterologous sequences that act as a block (643 bp in length). The recombination intermediates can then be deproteinized and isolated by filtration through a Sepharose CL-2B column. Addition of RuvAB to the deproteinized 32P-labeled recombination intermediates (α-structures) results in branch migration away from the heterologous block (i.e. reverse branch migration), leading to the formation of gapped circular and 32P-labeled linear duplex products (Fig. 1A). Because the region of heterology limits branch migration to one direction only, a single set of reaction products will be generated, and these can be quantified by determining the percentage of 32P-labeled linear DNA released. This system, therefore, has an advantage over methods described previously for measuring branch migration (8, 14, 15).

When 32P-labeled α-structures were incubated with increasing concentrations of RuvB protein in the presence or absence of RuvA, the release of 32P-labeled linear duplex DNA was visualized by agarose gel electrophoresis (Fig. 1B). The gel was quantified by phosphorimager analysis (Fig. 1C) and showed that 10- to 20-fold less RuvB was required for branch migration in the presence of RuvA than in its absence. These findings are consistent with previous studies performed using α-structures without a heterologous block (15).

Comparison of RuvAB- and RuvB-mediated Branch Migration Reactions—The magnesium requirements for the RuvAB- and RuvB-mediated reactions were determined and are shown in Fig. 2. In the presence of 22 mM RuvaA and 134 mM RuvB, branch migration occurred optimally at ≈6 mM Mg²⁺ (Fig. 2A). Branch migration catalyzed by 1.35 µM RuvB (in the absence of RuvA) required ≈12 mM Mg²⁺, as reported previously (15). However, at suboptimal concentrations of protein (5 mM RuvA + 66 mM RuvB, or 0.6 µM RuvB), more defined Mg²⁺ optima were observed (Fig. 2B). The optimum for RuvB-mediated branch migration was 6–9 mM MgCl₂, whereas the reaction catalyzed by RuvB alone was most efficient at concentrations ≈15 mM. These optima reflect the ability of RuvAB and RuvB
to bind DNA at the different concentrations of MgCl$_2$ (26). The effects of temperature on the RuvAB- or RuvB-mediated branch migration reactions were next investigated. Fig. 3A shows time courses of branch migration by RuvAB at 37, 21, and 0°C. At the lower temperatures, significant amounts of branch migration were observed, and remarkably, almost 20% of the recombination intermediates dissociated during 30 min incubation at 0°C. At this temperature, we were unable to detect ATP hydrolysis by RuvAB, indicating that the energy requirement for this reaction is low (data not shown). Dissociation of $\alpha$-structures was not observed in the absence of RuvAB, indicating that the result was not due to spontaneous branch migration (data not shown). In contrast, RuvB-mediated branch migration was more sensitive to low temperatures. Negligible linear product was detected when RuvB was incubated with the recombination intermediates at 0°C (Fig. 3B), in contrast to the reaction catalyzed by RuvAB.

The RuvAB- and RuvB-mediated reactions also displayed different sensitivities to the presence of monovalent ions (Fig. 4). We found that inclusion of 50–100 mM NaCl resulted in a stimulation of RuvAB-mediated branch migration and that concentrations greater than 300 mM NaCl were required to effect 50% inhibition. By contrast, the RuvB-dependent reaction was highly salt-sensitive, with 50% inhibition observed at 75 mM NaCl. The different effects of NaCl on RuvAB- and RuvB-catalyzed branch migration may indicate that these reactions occur by different mechanisms.

ATP Hydrolysis during Branch Migration—Previous studies have shown that protein-mediated branch migration is dependent upon ATP hydrolysis (14, 28). To determine whether ATP hydrolysis is directly coupled to the exchange of base pairs, RuvAB- or RuvB-mediated branch migration reactions were supplemented with [$\alpha$-32P]ATP, and the amount of ATP hydrolyzed was measured. In both reactions, the rate of ATP hydrolysis was approximately linear over a 30-min time course. The turnover number observed in the presence of RuvAB was calculated to be approximately 10.45 mol ATP/min/RuvB monomer. A similar rate of ATP hydrolysis was observed in control reactions containing linear duplex DNA (Fig. 5A). These results, combined with the observation that branch migration goes to completion within 5 min (Fig. 5B), indicate that the majority of ATP hydrolysis occurs in an uncoupled manner. Similar results were obtained in reactions containing RuvB alone ($K_{cat} = 7.4$ mol ATP/min/RuvB monomer), and again we were unable to detect ATP hydrolysis that was directly coupled to branch migration. No increase in ATP hydrolysis was observed with DNA substrates that were damaged by ultraviolet irradiation (data not shown).

Effect of ATPγS on RuvAB-mediated Branch Migration—To further investigate the relationship between ATP hydrolysis and branch migration, the sensitivity of the RuvAB- and RuvB-promoted reactions to inhibition by a nonhydrolyzable analog...
of ATP, ATPγS, was studied. Reactions carried out in the presence of RuvB alone (at 1 mM ATP) were completely inhibited by 1.5 mM ATPγS (Fig. 6A). In contrast, branch migration by RuvAB showed no inhibition at this concentration, and approximately 6 mM ATPγS were required to effect 50% inhibition.

At low concentrations of ATPγS (0.5 and 0.25 mM, respectively), the RuvAB- and RuvB-mediated branch migration reactions were stimulated rather than inhibited (Fig. 6A). Time courses of RuvAB-mediated branch migration carried out in the presence or absence of ATPγS showed an increased rate in the presence of the nonhydrolyzable ATP analog (Fig. 6B). The reason for this stimulation is unknown, but it may result from the stabilization of RuvAB complexes on DNA.

To determine whether the addition of excess ATPγS could block branch migration after initiation of the reaction in the presence of ATP, RuvAB reactions carried out in the presence of 0.25 mM ATP were supplemented with a 40-fold excess of ATPγS (Fig. 6C). The addition of ATPγS 1 or 2 min after the start of branch migration led to an immediate inhibition of linear product formation. A similar block to branch migration was observed when a 40-fold excess of ATPγS was added at the start of the reaction. In contrast, the addition of a 10-fold excess of ATPγS (i.e., at 10 mM ATPγS/1 mM ATP) failed to block branch migration completely and, in this case, the reaction occurred for 2–3 min before being fully inhibited (data not shown).

Effect of ADP—To determine the sensitivity of RuvAB or RuvB to ADP, reactions were set up containing 1 mM ATP and varying concentrations of ADP (Fig. 7A). We observed that RuvAB-mediated branch migration was unaffected by the presence of up to 2 mM ADP and required 10 mM ADP for complete inhibition. In contrast, the RuvB reaction was highly sensitive to the presence of ADP. RuvB-catalyzed branch migration was blocked completely by 4 mM ADP and 50% inhibition was observed at 1 mM ADP (Fig. 7A).

To determine whether ongoing branch migration reactions were blocked by the addition of ADP, a series of reactions containing 0.25 mM ATP were set up, and ADP (10 mM) was added prior to RuvB addition (0') or at 1 or 2 min after initiation of the reaction (Fig. 7B). We found that the addition of ADP either before or after the initiation of branch migration resulted in a block to the formation of linear product. These results confirm the need for a hydrolyzable nucleotide cofactor and indicate that inhibition by excess ADP occurs upon release of ADP-Pi, as nucleotide cofactors are bound ready for a second round of hydrolysis. Similar experiments were carried out in the presence of 1 mM ATP/10 mM ADP, and in this case, we observed a short delay of 1–2 min before the reaction stopped (data not shown). It is possible that hexameric rings of RuvB that have one subunit with ATP bound may be capable of limited branch migration.

RuvA Is Required Continually during Branch Migration—In the next experiment, we investigated whether ongoing branch
migration reactions could be blocked by sequestration of RuvA. To do this, RuvAB-mediated branch migration reactions were supplemented with an excess of synthetic Holliday junctions, to which RuvA binds efficiently (8). The amount of synthetic Holliday junction was predeterminded to be sufficient to bind all RuvA present in the reaction mixture and, indeed, caused a complete block if added prior to initiation of branch migration (Fig. 8). When the competitor junctions were added 1 min after the initiation of branch migration by RuvAB, we observed that branch migration occurred for a further 2–3 min before slowing (Fig. 8). The final yield of product was approximately 15% less than that observed in a control reaction, indicating that most molecules complete branch migration before protein dissociation. These results indicate (i) that RuvA is required continually during branch migration and (ii) that the RuvAB complex appears to turn over (i.e., RuvA dissociates from the DNA) only after a period of time ≥2–3 min.

In support of a continual and catalytic role for RuvA in the process of branch migration, we found that the addition of purified polyclonal antibodies raised against RuvA to ongoing RuvAB-mediated branch migration reactions led to an immediate block to linear product formation (Fig. 9). Preimmune sera, used as a control, had no effect on the yield of branch migration products (data not shown). These results argue against the proposal that RuvA acts as a molecular matchmaker that helps to load RuvB onto the DNA, yet plays no catalytic role in the branch migration process.

**DISCUSSION**

The principal conclusion of this work is that RuvA protein plays a direct catalytic role in the process of branch migration. Previous studies have shown that RuvA binds to Holliday junctions with a high specificity and directs the assembly of RuvAB-Holliday junction complexes (8–10, 12). Electron microscopic studies of complexes formed in the presence of ATP revealed tripartite structures in which RuvA was sandwiched between two hexameric rings of RuvB (18). This observation was supported by DNase I footprinting (11). However, difficulties in visualizing complexes formed with ATP, coupled with observations which show that under certain conditions stoichiometric amounts of RuvB can promote branch migration without the need for RuvA (14, 15), led us to carry out the present investigation. We found that branch migration reactions catalyzed by RuvB alone were highly sensitive to the inhibitory effects of NaCl, ADP, and ATPγS, whereas similar reactions promoted by RuvAB were relatively insensitive to these factors. These results indicate that the RuvAB-dependent reaction is the most relevant mechanism in vivo, consistent with genetic studies indicating that ruvA and ruvB mutants have similar phenotypic properties (29, 30). We suggest that the reaction driven by RuvB alone, which occurs only at elevated concentrations of MgCl₂ (>15 mM) and at stoichiometric concentrations of protein, is likely to be a consequence of nonspecific binding and translocation of hexameric rings of RuvB along DNA. Such a reaction is unlikely to be physiologically relevant since it lacks specificity and may occur by a mechanism that is similar to branch migration catalyzed by other nontargeted helicases, such as UvrD protein (31).

A direct role for RuvA in the catalysis of branch migration is also predicted by observations which indicate that RuvA (i)
promotes junction unfolding (18), (ii) stimulates the ATPase (6) and unwinding (21) activities of RuvB, and (iii) is required for branch migration through heterologous DNA sequences (23). In the experiments reported here, we extend these observations. The addition of antibodies raised against RuvA to ongoing RuvAB-mediated branch migration reactions caused an intermediate block, indicating the binding of antibodies to RuvA within an active branch migration complex. Moreover, competition by the addition of excess synthetic Holliday junctions was found to inhibit branch migration, but only after a delay of approximately 2–3 min. This lag may reflect the time at which the RuvA complex dissociates from the Holliday junction. Turnover such as this indicates that the RuvA complex may have a high affinity for DNA. It will, therefore, be of interest to further study the functional organization of the RuvB hexamer and its interplay with RuvA to gain new insight into the way in which hexameric ring motor proteins act on DNA.

Acknowledgments—We thank David Adams for the plasmids pDEA-TZ (f+) and pDEA2, Kevin Hiom for providing synthetic Holliday junction DNA, and Rajvee Shah for advice on substrate preparation. We thank David Adams and Angela Eggleston for suggestions and critical reading of the manuscript.

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Fig. 9. Rabbit polyclonal antibodies raised against RuvA block RuvAB-mediated branch migration. Branch migration reactions catalyzed by RuvAB (100 μl) were set up as described under "Materials and Methods." Purified polyclonal anti-RuvA antibodies (~1 μg) were added either before initiation (○) or 1 min after the initiation of branch migration by RuvAB (●). The control reactions (●) show branch migration by RuvAB in the absence of antibody. Branch migration was quantified as described in the Fig. 1 legend.