# RecA Protein of *Mycobacterium tuberculosis* Possesses pH-Dependent Homologous DNA Pairing and Strand Exchange Activities: Implications for Allele Exchange in Mycobacteria†

Moreshwar B. Vaze and K. Muniyappa*

*Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India*

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ABSTRACT: To gain insights into inefficient allele exchange in mycobacteria, we compared homologous pairing and strand exchange reactions promoted by RecA protein of *Mycobacterium tuberculosis* to those of *Escherichia coli* RecA protein. The extent of single-stranded binding protein (SSB)-stimulated formation of joint molecules by MtRecA was similar to that of EcRecA over a wide range of pH values. In contrast, strand exchange promoted by MtRecA was inhibited around neutral pH due to the formation of DNA networks. At higher pH, MtRecA was able to overcome this constraint and, consequently, displayed optimal strand exchange activity. Order of addition experiments suggested that SSB, when added after MtRecA, was vital for strand exchange. Significantly, with shorter duplex DNA, MtRecA promoted efficient strand exchange without network formation in a pH-independent fashion. Increase in the length of duplex DNA led to incomplete strand exchange with concomitant rise in the formation of intermediates and networks in a pH-dependent manner. Treatment of purified networks with S1 nuclease liberated linear duplex DNA and products, consistent with a model in which the networks are formed by the invasion of hybrid DNA by the displaced linear single-stranded DNA. Titration of strand exchange reactions with ATP or salt distinguished a condition under which the formation of networks was blocked, but strand exchange was not significantly affected. We discuss how these results relate to inefficient allele exchange in mycobacteria.

In recent times, tuberculosis has been one of the most intensively researched infectious diseases consequent to increased incidence of tuberculosis in individuals afflicted with HIV and the emergence of multidrug-resistant strains. It is believed that gene replacement would provide insights into the understanding of the role(s) of virulence determinants as well mechanisms of pathogenesis (reviewed in ref *1*). Consequently, there has been an increasing interest in the development of tools that would permit molecular genetic manipulation of mycobacteria (*1*). Although allele replacement has been achieved in *Mycobacterium smegmatis* (*2*−*4*) and *Mycobacterium intracellulare* at a low frequency (*5*), it has been technically limited in *Mycobacterium tuberculosis* (*3*, *5*, *6*) and *Mycobacterium bovis* BCG (*7*−*10*), perhaps due to their ability to promote random integration by nonhomologous recombination (reviewed in ref *11*). Furthermore, the genetic mechanisms that promote allele exchange in these organisms are not fully understood.

Genetic and biochemical analyses have shown that *Escherichia coli* RecA is essential for homologous genetic recombination, induced mutagenesis, and for the postreplication repair of damaged DNA (reviewed in refs *12*−*14*). *In vitro*, RecA protein (EcRecA)[1] or its analogues promote homologous pairing and strand exchange involving either three- or four-stranded DNA substrates. The staging of all the reactions promoted by EcRecA, or its analogues, requires its binding to both ssDNA and ATP (or ATP$\gamma$S) complexed with $Mg^{2+}$. There are at least three clearly separable phases in the overall pathway of RecA-mediated homologous recombination in a three-strand exchange reaction. The first phase, *presynapsis*, involves the cooperative polymerization of EcRecA on ssDNA, or duplex DNA along the minor grooves, to form a right-handed helical nucleoprotein filament with a pitch of 95 Å and an axial rise/nucleotide of 5.1 Å (*15*−*20*). The second phase is the *synaptic* phase which includes the search for homology on naked duplex DNA by the nucleoprotein filament followed by homologous alignment to produce either paranemic or plectonemic joint molecules with a short region of nascent heteroduplex DNA (*21*−*25*). The third phase, *strand exchange*, driven by ATP hydrolysis can either extend the length of heteroduplex DNA or cause disassociation of EcRecA in a polarized manner (5′ to 3′ with respect to ssDNA) (*26*−*28*). In *E. coli*, more

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* To whom correspondence may be addressed. Telephone: (91-80) 309-2235. Fax: (91-80) 334-1683/1814. E-mail: kmbc@biochem.iisc.ernet.in.

[1] Abbreviations: ATP$\gamma$S, adenosine 5′-*O*-(3′-thiophosphate); BSA, bovine serum albumin; DTT, dithiothreitol; EcRecA, *Escherichia coli* RecA protein; EDTA, ethylenediaminetetraacetic acid; form I, negatively superhelical DNA as isolated from *E. coli*; form II, circular duplex DNA with one interruption in either strand; form III, linear duplex DNA; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; kb, kilobase pair; Kglu, potassium glutamate; MES, 2-(*N*-morpholino)-ethane sulfonic acid; MOPS, 3-(*N*-morpholino)propane sulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MtRecA, RecA protein of *Mycobacterium tuberculosis*; ssDNA, circular single-stranded DNA; SSB, *E. coli* single-stranded binding protein; SDS, sodium dodecyl sulfate.

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than 20 gene products have been implicated to play a role in the overall pathway of homologous recombination promoted by RecA protein (reviewed in ref *13*). For instance, single-stranded binding protein (SSB) facilitates the formation of RecA−ssDNA nucleoprotein filament by melting the secondary structure, stabilizes the RecA−ssDNA nucleoprotein filament, and inhibits the transfer of RecA protein among nucleoprotein filaments (reviewed in refs *13* and *14*). In addition, SSB stimulates strand exchange by sequestering the 5′ end of the displaced single strand and thereby inhibits invasion of newly formed heteroduplex DNA. Although much of our current understanding of the mechanistic aspects of homologous recombination is gained from the study of the EcRecA paradigm, all organisms including humans possess RecA homologues (reviewed in ref *12*). Among these the Rad51 protein of yeast and its homologue in humans are of particular interest in light of conservation of structure of the nucleoprotein filament and mechanism of pairing and strand exchange with that of the prototypic EcRecA (*29*−*33*). Significantly, mice homozygous for a null mutation in *Rad51* gene produce embryos which die at an early stage of development, suggesting that it is necessary for somatic growth and survival as well (*34*).

The *rec*A gene of *M. tuberculosis* contains in-frame insertion of an intervening sequence (*35*, *36*). After synthesis of a RecA precursor, an internal domain, termed the intein, is excised from the precursor and the two flanking domains, called exteins, are ligated together to generate RecA protein (MtRecA). Previously, we reported purification and characterization of both the precursor and spliced form of RecA proteins of *M. tuberculosis* (*37*). We also demonstrated that the extent of SSB-stimulated formation of joint molecules by MtRecA between linear duplex and ssDNA was similar to that of EcRecA. Why, then, has allelic exchange been difficult to achieve in slow-growing mycobacteria? One way to address this question is to examine the overall pathway of homologous recombination promoted by MtRecA for its efficiency and limitations. Here, we describe homologous pairing and strand exchange reactions promoted by MtRecA and a model to illustrate why homologous recombination is inefficient in extent in *M. tuberculosis*. We believe that these results are potentially useful for developing strategies for allele exchange in mycobacteria.

## EXPERIMENTAL PROCEDURES

*Enzymes, Proteins, and DNA.* RecA protein of *E. coli* (*38*) and *M. tuberculosis* (*37*) and SSB (*39*) were purified, and their concentrations were determined as described. Phosphocreatine, creatine phosphokinase, MES, PIPES, HEPES, Tris, glycine, boric acid, DTT, MOPS, proteinase K, and tricine were obtained from SIGMA Chemical Company, St. Louis, MO. Restriction endonucleases were purchased from Amersham and used as suggested by the manufacturer. S1 nuclease was from United States Biochemicals, Cleveland, OH. ATP was from Amersham Pharmacia Biotech, England. Negatively supercoiled duplex (form I) and circular single-stranded DNA (ssDNA) from bacteriophage M13 were prepared as described (*40*). Fragments of linear duplex DNA were generated from complete digestion of M13 form I DNA by appropriate restriction endonucleases. Samples were electrophoresed in agarose gels, and DNA was isolated as described (*41*). DNA fragments were extracted from the gel with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated by ethanol. The concentration of DNA in stock solutions was determined at $A_{260\ nm}$ and expressed in terms of total nucleotides.

*Buffers.* The following buffers were used in this study: MES-KOH ($pK_a = 6.1$) at pH 6; PIPES-KOH ($pK_a = 6.8$) at pH 6.5; HEPES-KOH ($pK_a = 7.5$) at pH 7; Tris-HCl ($pK_a = 8.1$) or HEPES-KOH ($pK_a = 7.5$) or MOPS-KOH ($pK_a = 7.2$) at pH 7.5; HEPES-KOH or tricine-KOH or Tris-HCl at pH 8; tricine-KOH ($pK_a = 8.1$) or borate-KOH ($pK_a = 9.2$) or Tris-HCl at pH 8.5; borate-KOH or glycine-KOH ($pK_a = 9.8$) or Tris-HCl at pH 9; glycine-KOH at pH 9.5 or pH 10 or 10.5. The final pH value of each buffer was determined at 37 °C. In all the experiments, pH was varied but total ionic strength of the buffer was maintained constant. The effect(s) of nucleotide cofactors and salt on the strand exchange reaction were carried out in Tris-HCl buffer at pH 7.5.

*Nitrocellulose Filter Binding Assay for Joint Molecules.* The formation of joint molecules was assayed as described (*42*). Reaction mixtures (25 $\mu$L) contained 33 mM buffer of different pH values, 1 mM DTT, 1.5 mM ATP, 5 mM phosphocreatine, 12 mM $MgCl_2$, 10 units of phosphocreatine kinase/mL, 0.1 mg/mL BSA, and 10 $\mu$M M13 or $\phi$X174 ssDNA. EcRecA or MtRecA (5 $\mu$M) was added to the reaction mixture and incubated at 37 °C for 5 min. For reactions involving SSB (0.75 $\mu$M), reaction mixtures were prepared as above, but incubation was extended for an additional 5 min. In all of the experiments, the reaction was started by the addition of 10 $\mu$M form III [$^3$H] DNA. After 10 min at 37 °C, reactions were stopped by the addition of 4 mL of a solution containing 1.5 M NaCl and 0.15 M sodium citrate (pH 7). Samples were filtered immediately over nitrocellulose filters (Sartorius, 0.45 $\mu$m), washed with 6 mL of the above solution, and dried under a heat lamp. The bound radioactivity was quantitated by liquid scintillation.

*Strand Exchange Assay.* Assays were performed as described (*43*). Reaction mixtures (40 $\mu$L) were prepared as described above, except that unlabeled form III DNA was used. After incubation at 37 °C for 60 min, the reactions were terminated by the addition of SDS to 0.1% and proteinase K to 0.2 mg/mL. Following incubation at 37 °C for 15 min, samples were loaded onto 0.8% agarose gel. Electrophoresis was carried out at 2 V/cm for 11 h in 89 mM Tris/borate buffer (pH 8.3). DNA was transferred onto a Hybond N+ membrane, visualized by Southern hybridization using $^{32}$P-labeled M13 ssDNA and autoradiography. The autoradiograms were quantitated using LKB ultroscan XL laser densitometer. In strand exchange reaction involving linear duplex DNA of varying length, reaction mixtures contained 10 $\mu$M duplex DNA and ssDNA at the indicated concentrations (see the legend to Figure 6).

*Analysis of Products of Strand Exchange with S1 Nuclease.* Three-strand exchange reactions were carried out as described above. The deproteinized DNA was precipitated with ethanol and collected by centrifugation. The dried pellets were suspended in water. Samples were incubated with 5 units/mL S1 nuclease at 37 °C for 10 min as recommended by the manufacturer. Reactions were terminated by the addition of EDTA to 25 mM and transferred immediately to 4 °C. The pH of the sample was adjusted to 7.5, incubated

with proteinase K (0.2 mg/mL) and SDS (0.2%) at 37 °C for 15 min. Samples were electrophoresed on an agarose gel, and DNA was visualized by Southern hybridization as described above.

*DNA Aggregation Assay.* Reaction mixtures were prepared as described in the assay for the formation of joint molecules with the following modifications. Briefly, reaction mixtures (40 $\mu$L) contained 5 $\mu$M M13 ssDNA, 2.5 $\mu$M of EcRecA or MtRecA, 0.3 $\mu$M SSB, 2.5 $\mu$M homologous (M13 form III [$^3$H]DNA) or heterologous (pUC18 form III $^{32}$P-labeled DNA) substrate. After incubation at 37 °C for 5 min, a 10 $\mu$L aliquot was taken for the determination of total radioactivity, and 30 $\mu$L was centrifuged at 27200*g* for 8 min at 24 °C. Radioactivity in the supernatant, and the pellet was quantitated by liquid scintillation (*43*). Typical recoveries of form III [$^3$H]DNA in the pellet and supernatant were 93% and above.

*Light Scattering.* Light scatter at 90° was monitored in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 $\mu$M EcRecA or MtRecA, diluted from a stock solution of 88 $\mu$M. Samples were incubated at 22 °C for 2 min. The excitation and emission wavelengths were both set at 410 nm (*44*). The scatter measurements were initiated with the addition of $MgCl_2$ or equivalent volume of water. ATP was added at the end of 15 min incubation as indicated in Figure 5.

## RESULTS

*Experimental Rationale.* To gain insights into the molecular basis of inefficient allele exchange in slow-growing mycobacteria, a number of experiments were carried out to determine the optimal reaction conditions for homologous pairing and strand exchange promoted by MtRecA. These include different temperatures, changes in buffer composition and pH values, metal ions, and alternate nucleotide cofactors. We found that the pH of the assay buffer evoked a significant effect on MtRecA, becoming efficient in strand exchange reaction at higher pH. Considering the relevance of strand exchange to allelic exchange, it is important to elucidate the mechanistic aspects of strand exchange. Since both MtRecA and EcRecA proteins were expressed in *E. coli* and purified by the same method, it is possible that the observed differences are physiologically relevant.

*MtRecA Displays a Sharp pH-Induced Transition in the Formation of Joint Molecules: Effect of SSB.* The formation of joint molecules between linear duplex and ssDNA promoted by EcRecA is greatly stimulated by SSB (*45*−*47*). In addition, maintenance of active nucleoprotein filaments requires the continuous presence of SSB in reaction mixtures (*48*, *49*). Using the same combination of substrates, we assayed the extent of formation of joint molecules promoted by EcRecA and MtRecA over the range of pH values from 6 through 10.5. The reaction mixtures contained stoichiometric amounts of the specified RecA protein and ssDNA, and SSB where mentioned, to facilitate complete covering of ssDNA (*45*−*47*). Samples for the homologous pairing reaction were prepared as follows: (a) M13 ssDNA was first incubated with MtRecA or EcRecA for 5 min at 37 °C, followed with SSB for an additional 5 min; (b) ssDNA was incubated with EcRecA or MtRecA at 37 °C for 5 min; (c) ssDNA was incubated with SSB for 5 min, which was

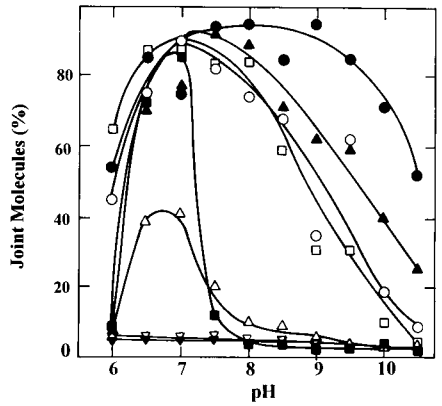


FIGURE 1: pH-Dependent formation of joint molecules by MtRecA and EcRecA and stimulation by SSB. The formation of joint molecules was assayed as described in the Experimental Procedures. The reaction mixtures contained (△) MtRecA incubated with ssDNA; (■) ssDNA incubated with SSB first, then with MtRecA; (▲) ssDNA incubated with MtRecA first, followed by SSB; (○) ssDNA incubated with EcRecA; (□) ssDNA incubated with SSB first, then with EcRecA protein; (●) ssDNA incubated with EcRecA first, followed by SSB. Control reactions were carried out with $\phi$X174 ssDNA with MtRecA (▼) and EcRecA (▽).

followed by either EcRecA or MtRecA at 37 °C for 5 min. Finally, in all the cases, the formation of joint molecules was initiated by the addition of linear M13 [$^3$H]DNA. Following incubation at 37 °C for 10 min, the extent of formation of joint molecules was determined by nitrocellulose filter binding assay. In reactions involving preincubation of ssDNA with EcRecA and then with SSB, the extent of formation of joint molecules was nearly constant over a wide range of pH values from 6.5 through 9.5 (Figure 1, filled circles). Likewise, in the absence of SSB or when ssDNA was preincubated with SSB, a similar pattern of behavior was observed in the formation of joint molecules by EcRecA at lower pH values. However, the extent of formation of joint molecules slightly decreased with increasing pH values (Figure 1, compare open circles and open squares versus filled circles). We will refer to this effect of pH on the formation of joint molecules as a pH transition. The pH-dependent transition in the formation of joint molecules by MtRecA displayed sharp changes: from the level of pairing nearly comparable to that by the EcRecA (Figure 1, filled triangles; incubation of ssDNA with MtRecA followed by SSB), to maximal activity in a narrow range at pH 7 (Figure 1, filled squares; incubation of ssDNA with SSB, followed by MtRecA), to sharply reduced level of activity with MtRecA at a narrow range of pH around 7 (Figure 1, open triangles). More importantly, the SSB-mediated half-maximal stimulation of the formation of joint molecules by MtRecA is associated with a shift of pH transition of about 2.5−3 units toward higher pH. Taken together, these results demonstrate that the formation of joint molecules by MtRecA is modified not only by the pH of the assay buffer but also by the presence or absence of SSB and the order by which it was added to the reaction solutions. Under these conditions, with heterologous combination of DNA substrates, RecA proteins failed to form joint molecules (Figure 1, open and filled inverted triangles).

*Strand Exchange Activity of MtRecA Is Sensitive to pH.* To assess whether DNA strand exchange function of MtRecA displays pH dependence, we have compared the three-strand

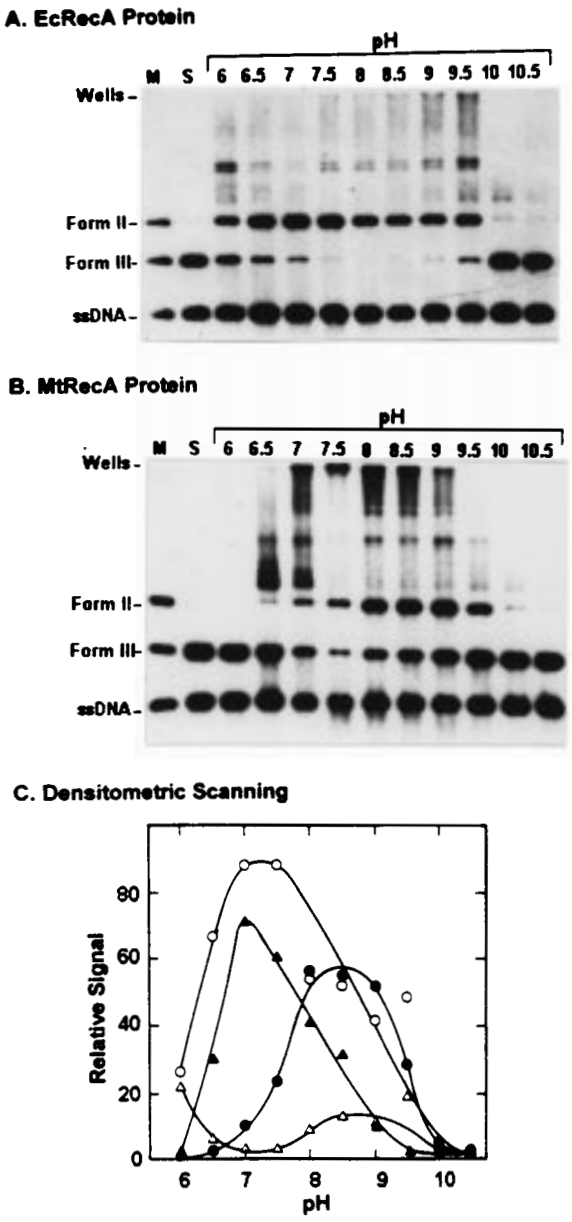


FIGURE 2: Comparison of pH dependence of strand exchange promoted by EcRecA and MtRecA in the presence of SSB. M13 ssDNA was incubated with EcRecA or MtRecA, then with SSB, in buffers of various pH. The reaction was initiated by the addition of M13 linear duplex DNA, and the formation of form II DNA was monitored as described in the Experimental Procedures: (A) EcRecA; (B) MtRecA; (C) quantitation of form II DNA and intermediates. The autoradiograms from panel A and B were scanned in a laser densitometer. The bands positioned between form II DNA and the wells are designated as networks and intermediates. Formation of form II DNA was by EcRecA (○) and MtRecA (●), respectively. The networks and intermediates were generated by EcRecA (△) and MtRecA (▲), respectively. M, markers; S, substrates.

exchange reaction promoted by MtRecA to that of EcRecA. The deproteinized products of the strand exchange reaction were separated by agarose gel electrophoresis, reinforced by Southern hybridization and visualized by autoradiography. Under these conditions, EcRecA catalyzed the formation of a maximal amount of form II DNA around neutral pH (Figure 2A). However, the rise of pH from 8 through 10.5 resulted in a progressive decline in strand exchange, and a small amount of the substrate DNA was converted into intermediates. In contrast, MtRecA-promoted strand exchange was less around neutral pH, although a weak band corresponding to form II DNA was evident (Figure 2B). The low yields of form II DNA coincided with the incorporation of most of form III DNA into intermediates that were trapped at various stages of strand exchange and some that failed to enter the gel. Significantly, the amount of homology-dependent DNA networks peaked around neutral pH. In contrast, MtRecA promoted complete strand exchange with a burst of form II DNA in the pH range 8 through 9 which was accompanied by a decline in the accumulation of intermediates. These results suggest that homology-dependent networks arise due to multiple synaptic events between linear duplex molecules and ssDNA. Treatment of reaction mixtures with proteinase K in the presence of SDS, or Pronase E, followed by extraction with phenol/chloroform/isoamyl alcohol solution failed to alter the extent of accumulation of DNA networks (data not shown). The simplest interpretation of these results is that DNA networks are stabilized by intermolecular base pairing as opposed to protein-dependent coaggregates generated by EcRecA in the absence of SSB (*50*). The patterns in Figure 2A,B were reinforced by quantitation by laser densitometer and extrapolated against the pH values of reaction mixtures. Figure 2C shows the extent of accumulation of homology-dependent DNA networks and form II DNA at different pH values. Both the formation of joint molecules (Figure 1) and strand exchange promoted by EcRecA were optimal around neutral pH (Figure 2C). In contrast, although efficient pairing was displayed by MtRecA at neutral pH (Figure 1), complete strand exchange occurred in alkaline buffers with a shift of optima by ~1.5 to 2 pH units. Above pH 10, strand exchange activities of both MtRecA and EcRecA were diminished to background levels. These results are consistent with the observations reported for EcRecA (*51*, *52*); however, reactions for MtRecA show a complex behavior (this study).

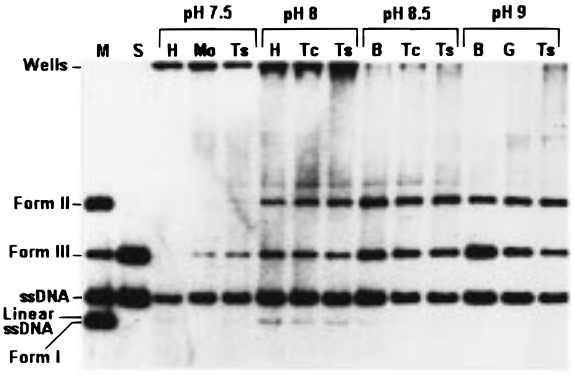


FIGURE 3: Strand exchange activity of MtRecA in buffers with varying pH and ions. Reactions were carried out as described in the legend to Figure 2 with the following buffers: (H), HEPES-KOH, (Mo) MOPS-KOH, and (Ts) Tris-HCl at pH 7.5; (H) HEPES-KOH, (Tc) tricine-KOH, and (Ts) Tris-HCl at pH 8; (B) borate-KOH, (Tc) tricine-KOH, and (Ts) Tris-HCl at pH 8.5; (B) borate-KOH, (G) glycine-KOH, and (Ts) Tris-HCl at pH 9. M, markers; S, substrates.

As the strand exchange activity was assayed at pH values from 7.5 through 9 in Tris-HCl buffers, it is possible that Tris or chloride ions are inhibitory for MtRecA to display its optimal activity. To corroborate pH dependence, experiments were carried out under conditions identical to those described above with different buffers in which the pH was

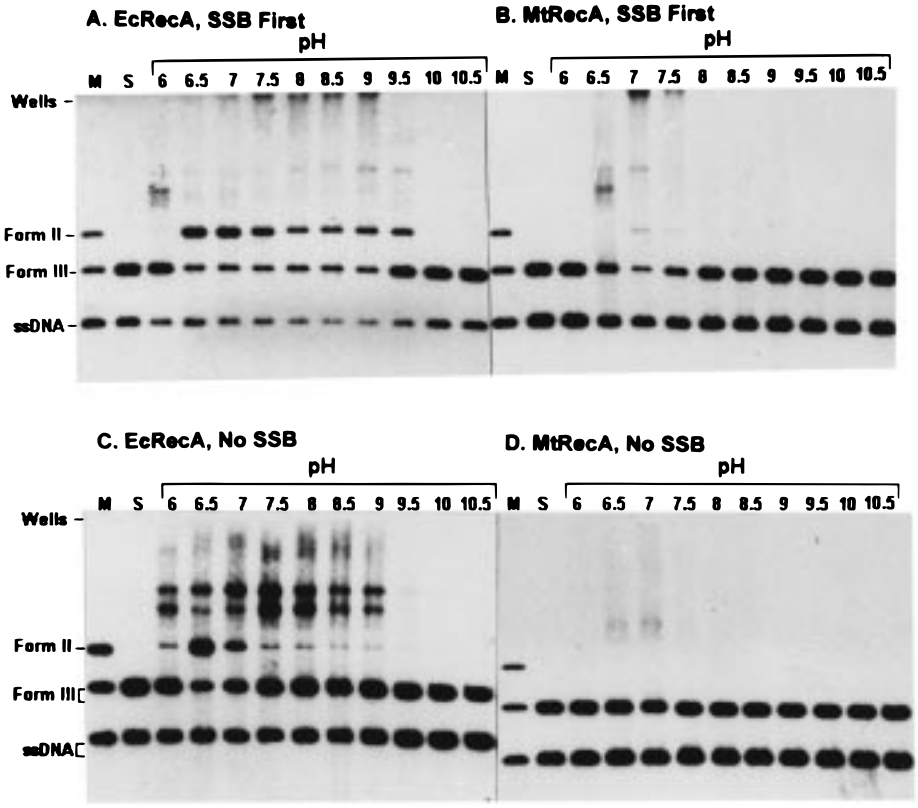


FIGURE 4: Comparison of strand exchange promoted by MtRecA and EcRecA and the effect of order of addition of reaction components. ssDNA was incubated with SSB first, and then with EcRecA or MtRecA or RecA proteins in the absence of SSB, as indicated. Reactions were initiated by the addition of form III DNA. The formation of DNA Form II and intermediates were assayed as described in the Experimental Procedures. M, markers; S, substrates.

maintained but the ions were altered. Figure 3 shows that the extent of accumulation of networks and form II DNA was similar at various pH values in different buffers, suggesting that the strand exchange activity of MtRecA is dependent on pH and independent of ionic composition of the buffer.

*SSB is Vital for the Strand Exchange Activity of MtRecA.* The efficiency of the three-strand exchange reaction is affected by the order in which ssDNA is incubated with SSB and EcRecA: reactions are most effective if SSB is incubated with ssDNA after EcRecA (*53*−*55*). However, if the order is reversed, or added concurrently, SSB improves the yield of form II DNA, in part, by inhibiting the invasion of the newly formed heteroduplex by the displaced linear ssDNA (*50*, *56*, *57*). Similarly, *recA441* displays enhanced recombination in vivo (*58*, *59*), and accordingly, its gene product competes with SSB for binding to ssDNA in vitro to generate homology-dependent DNA networks (*60*). A surprising feature of the reaction promoted by MtRecA is the formation of homology-dependent DNA networks that were not blocked by SSB. Moreover, unlike EcRecA, MtRecA does not effectively compete with SSB for binding to ssDNA (data not shown). These findings stimulated us to ask whether the sequence of incubations is significant to the MtRecA-promoted DNA strand exchange. For this purpose, we compared the strand exchange reactions promoted by MtRecA to those of EcRecA in the presence or absence of SSB at different pH values. Figure 4A shows that preincubation of ssDNA with SSB led to substantial strand exchange around neutral pH. However, the extent of formation of form II DNA slightly declined when compared to the reactions where ssDNA was first incubated with EcRecA (Figure 4A). In comparison, EcRecA produced a significant amount of hybrid DNA at pH 6.5 and then gradually declined over the pH range from 7.5 through 10 (Figure 4C). We note that, under these conditions, EcRecA produced steady-state levels of homology-dependent DNA networks. In contrast, in the SSB-first reactions, MtRecA generated small amounts of DNA networks and form II DNA only around neutral pH (Figure 4B). Significantly, in the absence of SSB, MtRecA failed to promote the formation of both intermediates as well as hybrid DNA (Figure 4D). These results suggest that SSB is vital for strand exchange activity of MtRecA.

*MtRecA Is Defective in $Mg^{2+}$-Induced Aggregation.* We reasoned that the basis for extensive generation of homology-dependent DNA networks by MtRecA is perhaps due to its ability to promote stable filament−filament interactions. It has been established that cations in the assay buffer exert a significant effect on the aggregation of wild-type EcRecA and its mutant derivatives, especially recA441 mutant protein (*44*). Increased concentrations of cations and polyamines induce precipitation of EcRecA in reaction mixtures which is alleviated by the presence of physiological concentrations of ATP or its analogues. *In vitro* conditions that are optimal for strand exchange typically contain 12 mM $MgCl_2$; therefore, we wondered whether homology-dependent formation of DNA networks is correlated with enhanced aggregation of MtRecA. To test this notion, MtRecA was incubated in assay buffers containing 12 mM $MgCl_2$. For reference purposes, EcRecA was used as internal control. In both we assayed the extent of aggregation by light scattering (*44*, *47*). Figure 5 shows that the kinetics of $Mg^{2+}$-induced aggregation

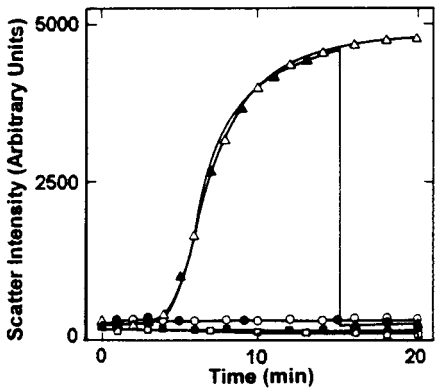


FIGURE 5: Effect of $Mg^{2+}$ on the aggregation behavior of EcRecA and MtRecA. Reactions were carried out as described in the Experimental Procedures. Proteins were incubated as follows: (□) EcRecA, no addition; (■) MtRecA, no addition; (△) EcRecA in the presence of 12 mM $MgCl_2$; (○) MtRecA in the presence of 12 mM $MgCl_2$; (▲) EcRecA, incubated with 12 mM $MgCl_2$. After 15 min, 0.5 mM ATP was added to the same sample, and the decrease in light scatter was recorded: (●) MtRecA incubated with 12 mM $MgCl_2$. After 15 min, 0.5 mM ATP was added, and recording was continued.

of EcRecA and the ATP-induced dissolution of aggregates were very rapid. In contrast, MtRecA failed to display the characteristic aggregation−disaggregation behavior associated with the prototypic EcRecA (reviewed in ref *13*).

*Length of Target Duplex Differentially Affects the Formation of DNA Networks and Hybrid DNA.* Genetic studies have revealed that allele exchange in mycobacteria is inefficient due to its propensity to promote illegitimate recombination (reviewed in ref *11*). In addition, the frequency of homologous recombination in *M. tuberculosis* is ~10 times less than that of *M. smegmatis*, varies from locus to locus, and appears to be dependent on the length of homology embedded in the targeting vector (*2*−*7*). To gain insights into the molecular basis of length dependency, experiments identical to those described in Figure 2 were carried out with a series of linear duplex DNA substrates. These were generated by cleavage of M13 form I DNA with appropriate restriction endonucleases. The sizes of the DNA substrates were from 1.08 through 6.4 kb, which was homologous to M13 ssDNA. We compared the products of strand exchange promoted by MtRecA to those of EcRecA using each of the substrates. Figure 6 shows the spectrum of products with each of these substrates. Both MtRecA and EcRecA promoted strand exchange with comparable efficiency from pH 6 through 10 with shorter duplex substrate (1. 08 kb). The only product was the hybrid DNA (gapped duplex) (Figure 6, panel A). Further lengthening of duplex DNA from 2.2 through 6.4 kb, had very little effect on strand exchange promoted by EcRecA (Figure 6, panels B, C, and D). However, the increase in the length of target duplex distinguished the spectrum of homology-dependent DNA networks generated by MtRecA. The intermediates formed by MtRecA around neutral pH with 2.2 and 4.18 kb duplex DNA migrated as a set of discrete bands as opposed to diffuse bands observed with the 6.4 kb substrate. With these substrates, both MtRecA and EcRecA formed approximately equivalent amounts of hybrid DNA (gapped duplex) over the pH range from 7 through 9.5. However, with 6.4 kb substrate, form II DNA peaked around neutral pH in case of EcRecA and at pH 9 with MtRecA. These results indicate that MtRecA promotes efficient formation of hybrid DNA with shorter duplex DNA, but with longer substrates the efficiency declines as a function of length.

*Dissolution of DNA Networks by S1 Nuclease.* The requirement for a long duplex substrate to generate homology-dependent DNA networks by MtRecA implies that joint molecules are converted to DNA networks, presumably by invasion of newly formed heteroduplex DNA by the displaced linear ssDNA. S1 nuclease has been extensively used to detect regions of single-stranded DNA in duplex molecules. Consequently, concentrations of S1 nuclease and time of incubation were chosen that would allow degradation of ssDNA and dissolution of DNA networks, but leave duplex DNA intact (data not shown). Aliquots were removed from strand exchange reactions by MtRecA and extracted with phenol/chloroform/isoamyl alcohol solution. DNA was precipitated with ethanol and collected by centrifugtaion. Purified DNA networks were incubated with S1 nuclease as described in the Experimental Procedures. Samples from the strand exchange reaction, as well as from S1 nuclease digestion, were loaded onto agarose gels for electrophoresis. The DNA was reinforced by Southern hybridization and visualized by autoradiography. Figure 7 shows that incubation with S1 nuclease resulted in the dissolution of DNA networks liberating original linear duplex DNA molecules. However, in addition to the linear duplex DNA, reactions carried out around neutral pH released discrete intermediates. On the basis of these results we conclude that MtRecA generates networks by multiple synaptic events involving both single- as well as double-stranded DNA.

*ATP Stimulates the Formation of Heteroduplex DNA by MtRecA.* Previously, we showed that MtRecA has low affinity for ATP with a $K_m$ value of 179 $\mu$M and a $K_{cat}$ of ~10 $min^{-1}$ (*37*). We examined more closely the effect of increased ATP concentrations on the generation of DNA networks, intermediates, and hybrid DNA by MtRecA at neutral pH. Reactions were performed as described in Figure 2, except that ATP was added to the reaction mixtures at the indicated concentrations. As ATP was increased from 3 to 10 mM, the formation of heteroduplex DNA was greatly enhanced with concomitant decline in the extent of DNA networks (Figure 8A). However, under conditions of ATP concentration equal to the $Mg^{2+}$, there was optimal formation of heteroduplex DNA with concomitant reduction in networks to background levels. Comparable results were also achieved with dATP, but required as much as twice the concentration to manifest the pattern seen with rATP. Although other explanations are possible, these results suggest that the differences may reflect on the reduced affinity of MtRecA for dATP. Quantitative estimation of products reinforces the differences in the extent of formation of heteroduplex DNA with dATP versus that of rATP (Figure 8B). It is possible that the effect of ATP is via its ability to dissolve coaggregates formed during the reaction and drive the intermediates toward hybrid DNA synthesis. However, under standard conditions, addition of ATP in the range of 5−10 mM abolished coaggregate formation, thereby inhibited joint molecules promoted by EcRecA (*61*). Thus, we measured the extent of coaggregates promoted by both EcRecA and MtRecA between linear duplex and ssDNA. We assembled nucleoprotein filaments comprised of EcRecA−ssDNA or MtRecA−ssDNA under standard conditions,

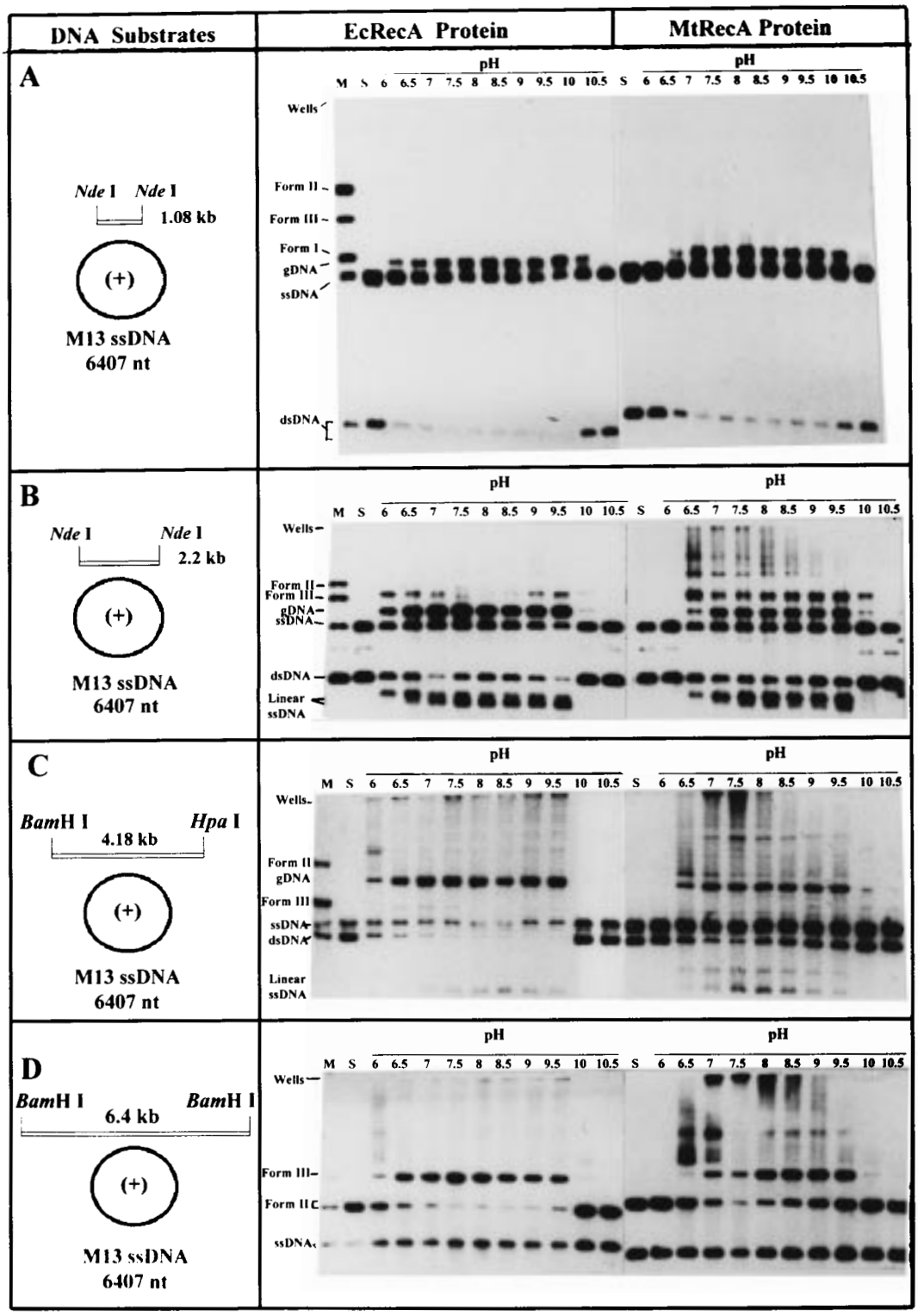


FIGURE 6: Effect of length of duplex DNA on strand exchange promoted by MtRecA and EcRecA. Duplex DNA of varying lengths was generated by cleavage of M13 form I DNA by appropriate restriction enzymes as shown in the left column. Nucleoprotein filaments composed of RecA−ssDNA−SSB were assembled in the presence of ATP or ATP regeneration system as described in the Experimental Procedures. Reaction mixtures contained 10 $\mu$M duplex DNA, and ssDNA was varied to give a stoichiometric ratio of 2 ssDNA per duplex DNA molecule. Panel A: reaction carried out with 1.08 kb duplex DNA. Samples were loaded onto 0.8% agarose gel and electrophoresed at 2 V/cm for 18 h at 4 °C. Panel B: Reactions were carried out with 2.2 kb duplex DNA. Samples were loaded onto 0.8% agarose gel and electrophoresed at 1.3 V/cm for 10 h. Panel C: Reactions carried out with 4.187 kb duplex DNA. Samples were loaded onto 1% agarose gel and electrophoresed at 3 V/cm for 7 h. Panel D: Reactions carried out with 6.407 kb duplex DNA. Samples were loaded onto 0.8% agarose gel and electrophoresed at 2 V/cm for 11 h. The concentrations of ssDNA used were 60, 29, 16, and 10 $\mu$M in panel A, B, C, and D, respectively. DNA was transferred to nylon membranes, visualized by Southern hybridization and autoradiography (*41*). gDNA: gapped circular duplex DNA. ds DNA: linear duplex DNA. M, markers; S, substrates.

added either linear duplex M13 [$^3$H]DNA or heterologous $^{32}$P-labeled pUC19 DNA with the indicated amounts of ATP. After incubation for 10 min, we assayed the amount of labeled DNA in the pellet as well as in the supernatant as

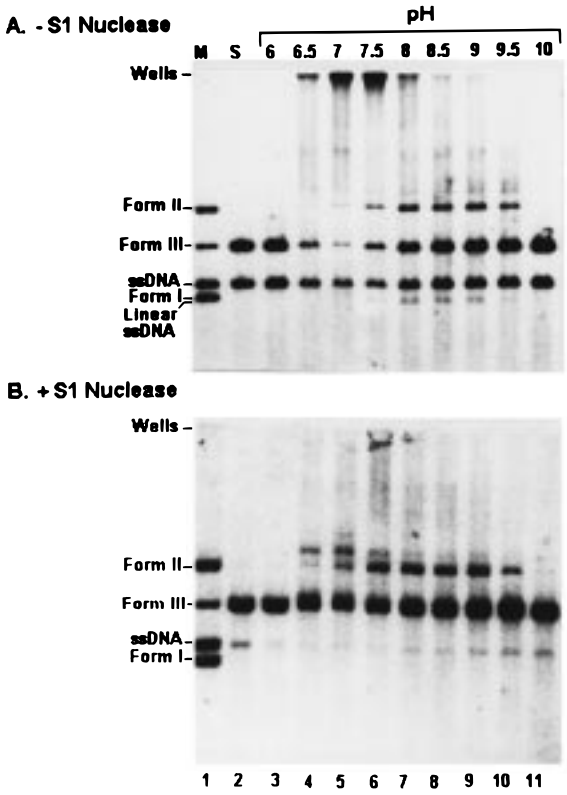


FIGURE 7: Single-stranded DNA connects DNA networks. Strand exchange reactions were carried out as described in the legend to Figure 2. Samples were deproteinized, precipitated with ethanol, and sedimented by centrifugation. Aliquots were incubated with S1 nuclease as described in the Experimental Procedures. Panel A: Strand exchange reaction promoted by MtRecA. Panel B: Samples of the strand exchange reaction after incubation with S1 nuclease. M, markers; S, substrates.

described in the Experimental Procedures. We observed that in the presence of 0−3 mM ATP, coaggregates peaked at 3 mM with virtually all of the input heterologous or homologous duplex DNA included in the pellet by both MtRecA and EcRecA (Figure 8C). However, coaggregates appeared to decrease somewhat at higher concentrations of ATP, but the yield was still significant at 10 mM. Due to the complexities involved in analyzing the DNA networks, no effort was made to reason a molecular basis for this phenomenon.

*Salt Stimulates Strand Exchange Promoted by MtRecA.* The effect of increasing concentrations of ATP on the formation of heteroduplex DNA is difficult to reconcile in view of the fact the intracellular levels of ATP are somewhat less. It is possible that the *in vivo* ionic environment could augment the requirement of ATP. It has been established that ionic strength of the assay buffer significantly modifies the activities of EcRecA. For instance, the products of homologous pairing and strand exchange reactions peaked at 20−50 mM KGlu and then decreased to background levels at higher concentrations (*62*). We compared the effect of KGlu on the strand exchange reaction promoted by MtRecA to those of EcRecA at neutral pH. Reactions were performed in the presence or absence of KGlu as described in Figure 2. Consistent with earlier results (*62*), KGlu stimulated strand exchange activity of EcRecA at 25−50 mM and then decreased to <10% at 350 mM (Figure 9A). In contrast, similar experiments carried out with MtRecA displayed two

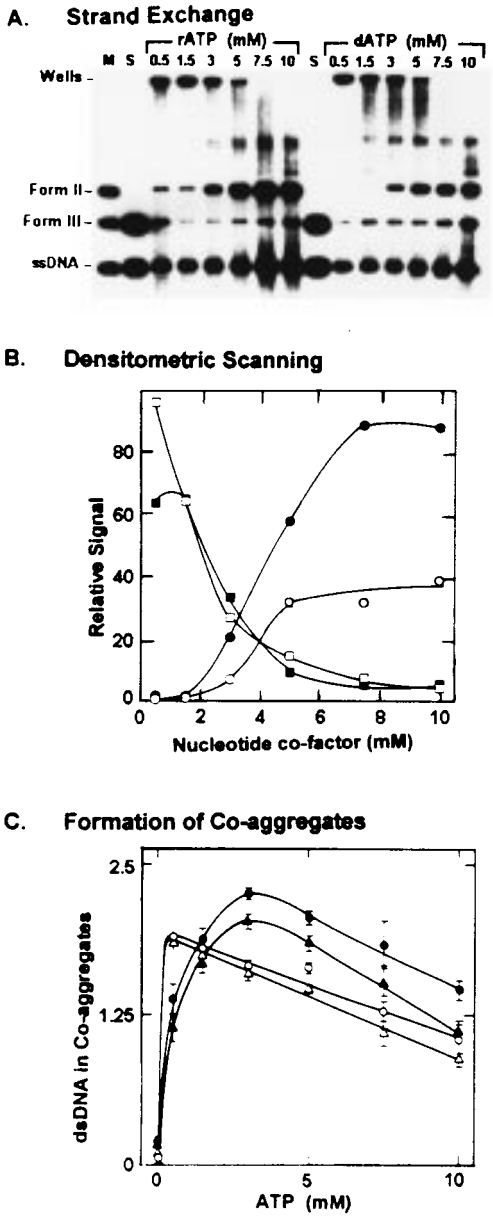


FIGURE 8: Effect of ATP on the strand exchange reaction promoted by MtRecA. Panel A: Reactions were carried out in Tris-HCl buffer at pH 7.5 in the presence of rATP or dATP at the concentrations indicated. Panel B: Quantitative estimation of DNA products. Autoradiogram from Figure 8A was scanned in a laser densitometer, and the relative signals were plotted versus nucleotide concentrations: the extent of formation of networks in the presence of rATP (■) or dATP (□); formation of form II DNA in the presence of rATP (●) or dATP (○). Panel C: Effect of increasing concentrations of ATP on the formation of coaggregates. The assay was performed in Tris-HCl buffer at pH 7.5 with the indicated concentrations of ATP as described in the Experimental Procedures: (○) EcRecA, homologous DNA; (△) EcRecA, heterologous DNA; (●) MtRecA, homologous DNA; (▲) MtRecA, heterologous DNA.

correlations. First, in the presence of 10−150 mM KGlu, DNA networks gradually dissolved with concomitant increase in the formation of hybrid DNA. Second, surprisingly MtRecA was more tolerant to KGlu: concentrations that completely abolished DNA networks generated steady-state amounts of heteroduplex DNA (Figure 9B). The generality of this phenomenon was established by titrations carried out with NaCl. The effect was fairly uniform except that the

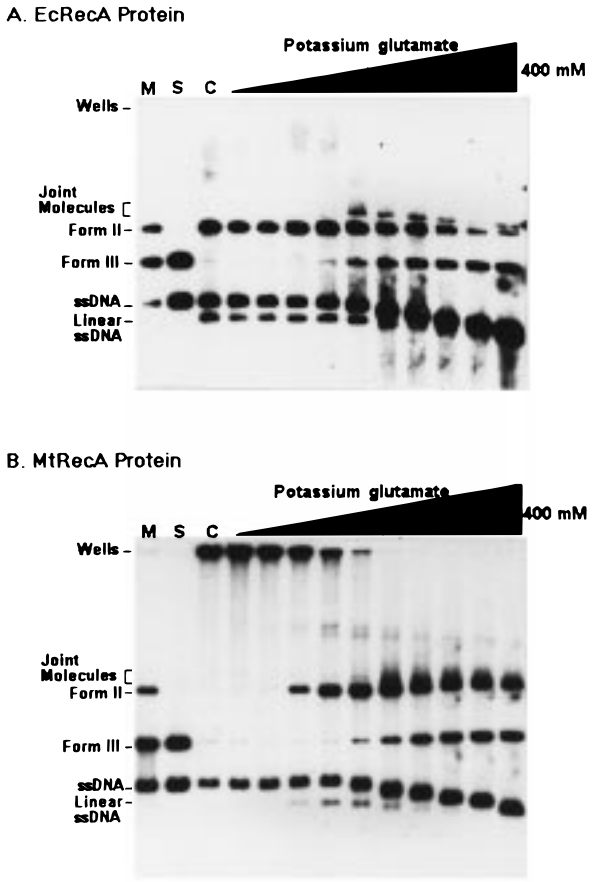


FIGURE 9: Comparison of the effect of potassium glutamate on the strand exchange reaction promoted by EcRecA or MtRecA. The reactions were carried out in Tris-HCl buffer at pH 7.5 in the presence of potassium glutamate at (left to right): 0, 10, 25, 50, 100, 150, 200, 250, 300, 350, and 400 mM. Samples were loaded onto 0.8% agarose gel and electrophoresed as described in the legend to Figure 2. ssDNA migrated faster at high concentrations of potassium glutamate; probably it attains a folded conformation. M, markers; S, substrates; C, control reaction.

profiles shifted to lower concentrations of NaCl (data not shown).

## DISCUSSION

Our results demonstrate that the formation of joint molecules promoted by the concerted action of MtRecA and SSB is maximal around neutral pH; however, optimal strand transfer manifests at higher pH. A second and striking observation is that shorter fragments of duplex DNA facilitate completion of strand exchange by suppressing the formation of DNA networks. Although the kinetics of homologous pairing and strand exchange promoted by MtRecA are comparable to that of EcRecA, notable differences in the mechanistic aspects and requirements do exist. Both MtRecA and EcRecA assemble on ssDNA to form nucleoprotein filaments, catalyze ATP hydrolysis, and promote strand exchange. However, unlike EcRecA, homologous pairing and strand exchange promoted by MtRecA is greatly modified by ionic milieu and SSB is vital for strand exchange. Furthermore, the pH dependence of pairing and strand exchange mirror differences in the invasion of duplex DNA by the displaced single-stranded DNA to generate networks.

To monitor homologous pairing and strand exchange, it was first necessary to determine how MtRecA interacts with ssDNA. Previous work showed that MtRecA assembles cooperatively on ssDNA to form a nucleoprotein filament in the presence of ATP or ATP$\gamma$S,. The extent of SSB-stimulated formation of joint molecules by MtRecA was similar to that of EcRecA (*37*). Why, then, is allelic exchange inefficient in *M. tuberculosis*? A priori, there is no obvious reason efficient homologous pairing does not lead to completion of strand exchange culminating in hybrid DNA. Intriguingly, however, the strand exchange activity of MtRecA is reduced significantly around neutral pH. As noted elsewhere for EcRecA, there exists a close relationship between the rate of ATP hydrolysis and extension of heteroduplex DNA (*63*). In this scenario, it remained possible that the slowing of strand exchange implies some defect in a coupled reaction such as ATPase activity needed to recycle MtRecA monomers, similar to that of EcRecA (*64*, *65*). Although the ATPase activity of MtRecA is quantitatively less compared to that of EcRecA, the profiles are invariant in the pH range of 7−9.5, conditions at which the latter displays pH differential in strand exchange (data not shown). Further, MtRecA and EcRecA hydrolyze ATP in the pH range of 7.5 through 9.5 with a monomer $k_{cat}$ of ~10 and 22 min$^{-1}$, respectively, comparable to those reported previously (*37*, *51*, *52*, *66*). In contrast to the situation observed for the wild-type, two mutant EcRecA proteins (G160N and H163A) which are inactive in assay conditions at pH 7.5 assemble nucleoprotein filaments and promote both ATPase and strand exchange reactions in the pH range 6.2−6.8 (*51*, *52*, *67*). Interestingly, the same mutant proteins were competent in DNA strand exchange reactions in the presence of dATP or ddATP over the pH range 6−8.2 (*66*). Addition of other NTPs such as ATP$\gamma$S and dATP had no effect on MtRecA at different pH values (data not shown).

A number of biochemical experiments have established that SSB helps EcRecA-promoted ATP hydrolysis, homologous pairing, and strand exchange reactions (reviewed in refs *12*−*14*). It has been proposed that the stimulative effects of SSB are due to its ability to remove secondary structure from ssDNA, which otherwise impedes the polymerization of RecA (*45*, *46*). Subsequently, SSB becomes integrated into the nucleoprotein filament in substantial amounts and confers stability (*48*, *49*). Given this general mechanism of stimulation, and in light of structural and functional similarity among RecA proteins from bacteria to humans, studies carried out with homologues often have included *E. coli* SSB as a common ingredient for optimal activity. Consistent with these findings, we showed earlier that the formation of joint molecules by MtRecA was stimulated by SSB, at a concentration less than the stoichiometric ratio, in standard assay conditions at pH 7.5 (*37*).

Experiments very similar to those described above were carried out to evaluate the impact of SSB on pH-dependent formation of joint molecules and strand exchange promoted by MtRecA or EcRecA. In particular, the effects of SSB on these processes as promoted by MtRecA contrasts with its effect on ATP hydrolysis. In the absence of SSB, or prior incubation of ssDNA with SSB, the formation of joint molecules by MtRecA occurred over a very narrow range of pH values. However, the magnitude of the pH differential in a MtRecA-promoted reaction can be altered by SSB. Joint molecules were stimulated significantly by SSB over a wide range of pH values. Furthermore, MtRecA displays maximal

strand exchange in the pH range 8−9, as opposed to pH 6.5−7.5 by EcRecA (compare Figure 2 versus Figure 4). This set of limitations with respect to the effect of SSB on ATPase and DNA strand exchange is similar to those of *E. coli* recA430 protein in standard assay conditions at pH 7.5 (*64*). These results suggest that the inability of MtRecA protein to displace SSB might foster inefficient homologous recombination.

In three-strand exchange reactions promoted by EcRecA, SSB or its functional homologues such as phage T4 gene 32 protein counteract a back-reaction that confers slowness and incompleteness of strand exchange (*50*). A notable feature of this reaction is that the 5′ end of the displaced strand invades the nascent hybrid DNA, or other joint molecules, to generate DNA networks that persist despite extensive deproteinization. SSB inhibits the reinvasion of the duplex DNA by preferentially binding to the 5′ end of the displaced linear strand (*55*, *57*). Intriguingly, in the presence of SSB under conditions optimal for EcRecA-mediated DNA strand exchange, MtRecA generated heterogeneous DNA networks. The intermediates are apparently trapped at various stages of strand exchange and shunted to unusual structures. The extent of formation of DNA networks, intermediates, and DNA products shows an excellent dependence on pH, ionic strength, and ATP, suggesting that the physical environment plays a critical role in strand exchange promoted by MtRecA. There are corresponding observations with phage T4 uvsX-promoted strand exchange *in vitro* (*68*). In contrast to MtRecA, altering the assay conditions such as the concentrations of uvsX protein or DNA, ionic strength or temperature did not significantly modify the outcome of the reaction (*68*). The mechanistic basis of the formation of DNA networks has not been reported.

The results presented here demonstrate that *E. coli* SSB is vital to homologous pairing and strand exchange reactions promoted by MtRecA *in vitro*. The ability of SSB to stimulate steps that are essential to recombination is a hallmark of the class of helix-destabilizing proteins (*12*, *13*). In support of lack of specificity, SSB has been shown to stimulate homologous pairing and strand exchange reactions promoted by phage T4 uvsX protein (*69*, *70*), RecA protein of *Bacillus subtilis* (*71*) and *Pseudomonas aeruginosa* (*72*), archaeal RadA protein (*73*), and yeast Rad51 protein (*74*−*76*). The observation that SSB stimulates the activities of MtRecA in vitro is also consistent with genetic data that show complementation of *E. coli recA*Δ cells for recombination and repair by *M. tuberculosis recA* (*35*, *36*).

In contrast to strand exchange as promoted by EcRecA, we have found that structural factors such as the length of the duplex DNA substrate greatly influences MtRecA-promoted formation of hybrid DNA, intermediates, and networks in a pH-dependent manner. Whereas RecA-mediated pairing between single- and double-stranded DNA have been described in detail, there are few observations that bear on the mechanism of strand transfer with shorter fragments of duplex and how it helps overcome secondary pairing interactions with additional DNA molecules. Significantly, our results reveal a striking correlation between the length of the duplex DNA substrates and the formation of DNA networks. One plausible explanation is that, consistent with the view that a rapid phase of strand exchange involves nascent hybrid DNA in the range 1−2 kb (*63*, *77*), shorter DNA substrates do not contain adequate lengths of ssDNA for invasion-generating DNA networks. To understand whether solutes alter strand exchange promoted by MtRecA, we sought a means to inhibit the formation of DNA networks.

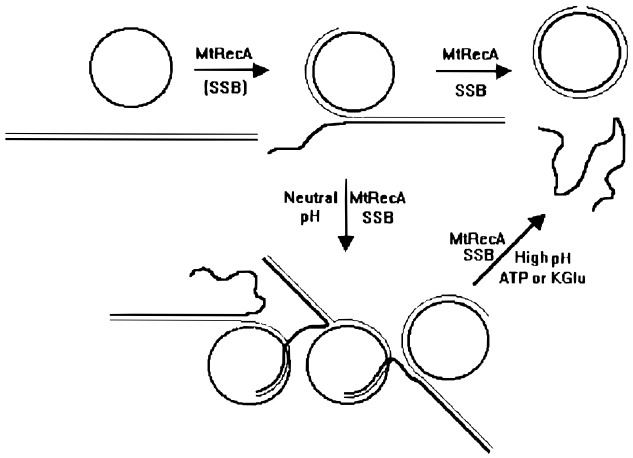


FIGURE 10: A generalized model for the formation of DNA networks produced by the invasion of the displaced single-stranded DNA by MtRecA. For details, see text.

The observations presented in this report can be rationalized in the context of a generic model (Figure 10). Although some features of this model are arbitrary, it is intended to integrate the observed mechanistic constraints in the pathway to the generation of DNA products during MtRecA-promoted reaction. We have illustrated a scenario in which the 5′ end of the displaced single-stranded DNA invades hybrid DNA. This essentially involves short stretches of ssDNA as deduced from the dissolution of networks by S1 nuclease. The formation of DNA networks might block strand exchange and resolution of branched recombination intermediates to DNA products. These observations suggest a strong correlation between the intrinsic capacity of MtRecA to halt strand exchange between long homologous DNA substrates *in vitro* to that of inefficient allelic exchange in *M. tuberculosis*.

What is the role of physical environment in the regulation of recombination? The pH dependence is unlikely to arise from structural differences at the ATP- and DNA-binding motifs in view of the fact that amino acid residues at these sites show a high degree of identity and homology between MtRecA and EcRecA proteins. Indeed, the calculated pI of these two proteins is about 4.9−5 (data not shown). Nevertheless, the subtle structural changes induced by ionic milieu is sufficient to cause distinct physiological changes in MtRecA. On the basis of these results, we speculate that high pH is required to trigger a conformational change in MtRecA for efficient strand exchange activity. An example is the observation that two mutant RecA proteins (G160N and H163A) that are ineffective at pH 7.5 are able to carry out DNA strand exchange at pH 6−6.7 (*66*). Alternatively, an increase in the concentration of ionic strength or ATP may enhance the efficiency of strand exchange at physiological pH. Support for this view can be found in the signal transduction pathways (*78*, *79*) and differences in binding specificity of DNA-binding proteins caused by the physical

[2] Peter Jenner, NIMR, London, personal communication.

environment or generated by interacting proteins (*80*, *81*). The intracellular pH of *M. tuberculosis*, to our knowledge, has not been reported in the literature. However, the pH of the cell-free lysate of *Mycobacterium microti* (closely related to *M. tuberculosis*) has been determined and appears to be in the range of ~6.85.[2] A long-standing question is how the biological specificities of macromolecules are influenced by the physical environment in a compartmentalized manner. It is possible that by engineering appropriate changes either in MtRecA or in the local environment, the efficiency of allele exchange may be enhanced in mycobacteria.

The results presented in this paper have important implications with regard to allele exchange events in *M. tuberculosis*. In an effort to generate attenuated mutants, a number of groups have attempted allele exchange in *M. tuberculosis*. The major obstacle is that illegitimate recombination events predominate those of homologous recombination. With shorter DNA substrates, two groups have observed an enhancement in the frequency of allele replacement in *M. bovis* and *M. smegmatis* (*7*−*9*). However, recent studies have shown a marginal increase in allele exchange with an increase in the size of target duplex DNA substrate (*6*). To our knowledge, the data presented in this report are not in conflict with the above findings. The differences observed may result from different experimental conditions, or locus being targeted, or the presence of a *cis*-acting putative recombination hot spot analogous to the $\chi$ site in the substrate. From these results, we suggest that multiple synaptic events may contribute to inefficient homologous recombination. Finally, these findings are relevant to our understanding of the mechanistic aspects of homologous pairing and strand exchange promoted by MtRecA and provide useful insights into inefficient allele exchange events in mycobacteria.

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