Characterization of DNA Strand Transfer Promoted by *Mycobacterium smegmatis* RecA Reveals Functional Diversity with *Mycobacterium tuberculosis* RecA†

N. Ganesh and K. Muniyappa*

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

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ABSTRACT: The RecA-like proteins constitute a group of DNA strand transfer proteins ubiquitous in eubacteria, eukarya, and archaea. However, the functional relationship among RecA proteins is poorly understood. For instance, *Mycobacterium tuberculosis* RecA is synthesized as a large precursor, which undergoes an unusual protein-splicing reaction to generate an active form. Whereas the precursor was inactive, the active form promoted DNA strand transfer less efficiently compared to EcRecA. Furthermore, gene disruption studies have indicated that the frequencies of allele exchange are relatively lower in *Mycobacterium tuberculosis* compared to *Mycobacterium smegmatis*. The mechanistic basis and the factors that contribute to differences in allele exchange remain to be understood. Here, we show that the extent of DNA strand transfer promoted by the *M. smegmatis* RecA in vitro differs significantly from that of *M. tuberculosis* RecA. Importantly, *M. smegmatis* RecA by itself was unable to promote strand transfer, but cognate or noncognate SSBs rendered it efficient even when added prior to RecA. In the presence of SSB, MsRecA or MtRecA catalyzed strand transfer between ssDNA and varying lengths of linear duplex DNA with distinctly different pH profiles. The factors that were able to suppress the formation of DNA networks greatly stimulated strand transfer reactions promoted by MsRecA or MtRecA. Although the rate and pH profiles of dATP hydrolysis catalyzed by MtRecA and MsRecA were similar, only MsRecA was able to couple dATP hydrolysis to DNA strand transfer. Together, these results provide insights into the functional diversity in DNA strand transfer promoted by RecA proteins of pathogenic and nonpathogenic species of mycobacteria.

Central to the mechanism of homologous recombination is the process of DNA strand exchange. A particularly convenient model to elucidate the molecular mechanism has been the three-strand exchange reaction between linear double-stranded and circular single-stranded DNA promoted by the ubiquitous family of RecA-like proteins. Detailed biochemical and structural studies have shown that the process of homologous recombination promoted by *Escherichia coli* RecA proceeds in kinetically distinguishable phases: The *presynaptic* polymerization of RecA protein on single-stranded DNA forming a helical nucleoprotein filament; *synapsis*, the homologous alignment of nucleoprotein filament comprised of RecA-ssDNA[1] with naked duplex DNA; and unidirectional *strand exchange*, which creates long stretches of heteroduplex DNA (reviewed in refs *1* and *2*). Furthermore, SSB stimulates the formation of RecA nucleoprotein filament, joint molecules, and strand exchange. The length of heteroduplex DNA generated by *E. coli* RecA in the presence of SSB extends over several kilobase pairs (*1*−*2*). While the mechanistic aspects of homologous recombination has been studied in considerable detail in *E. coli*, similar studies are required in other organisms to help define the general validity of the *E. coli* paradigm.

The members of the superfamily of RecA proteins are structurally conserved among eubacteria, eukarya, and archaea (*3*). However, the functional relationship among RecA proteins is poorly understood. In contrast to the much-studied *Escherichia coli* RecA, which follows the above scheme for effecting complete DNA strand exchange, *Mycobacterium tuberculosis* RecA displays some unique characteristic features (*4*−*7*). The genetic organization of the *M. tuberculosis recA* gene is different from that of the *Mycobacterium smegmatis* homologue by the presence of an intervening sequence in its *open reading frame*, yet the structural features of RecA proteins from the respective species are very similar (Figure 1). Phylogenetic relationships notwithstanding, a number of biologically significant questions remain. In particular, evidence from gene disruption studies in *M. tuberculosis* with small (*8*, *9*) and long (*10*) linear DNA fragments or suicidal vectors (*11*) has implicated that recombination machinery is inefficient, but also shows a high degree of illegitimate recombination. On the other hand,

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* Corresponding author: Tel: (+91.80) 394.2235 or 360.0278. Fax: (+91.80) 360 0814 or 0683. E-mail: kmbc@biochem.iisc.ernet.in.

[1] Abbreviations: bp, base pair; BSA, bovine serum albumin; DTT, dithiothreitol; EcRecA, *E. coli* RecA; EcSSB, *E. coli* SSB; EDTA, ethylenediaminetetraacetic acid; form I DNA, negatively supercoiled DNA; form II DNA, nicked circular double-stranded DNA, form III DNA, linear double-stranded DNA; HEPES, *N*-(2-hydroxyethyl)-piperzine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethane sulfonic acid; SSB, single-stranded binding protein; MsSSB, *M. smegmatis* SSB; MsRecA, *M. smegmatis* RecA; MtRecA, *M. tuberculosis* RecA, MtSSB, *M. tuberculosis* SSB.; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethane sulfonic acid); SDS, sodium dodecyl sulfate; ssDNA, M13 circular single-stranded DNA.

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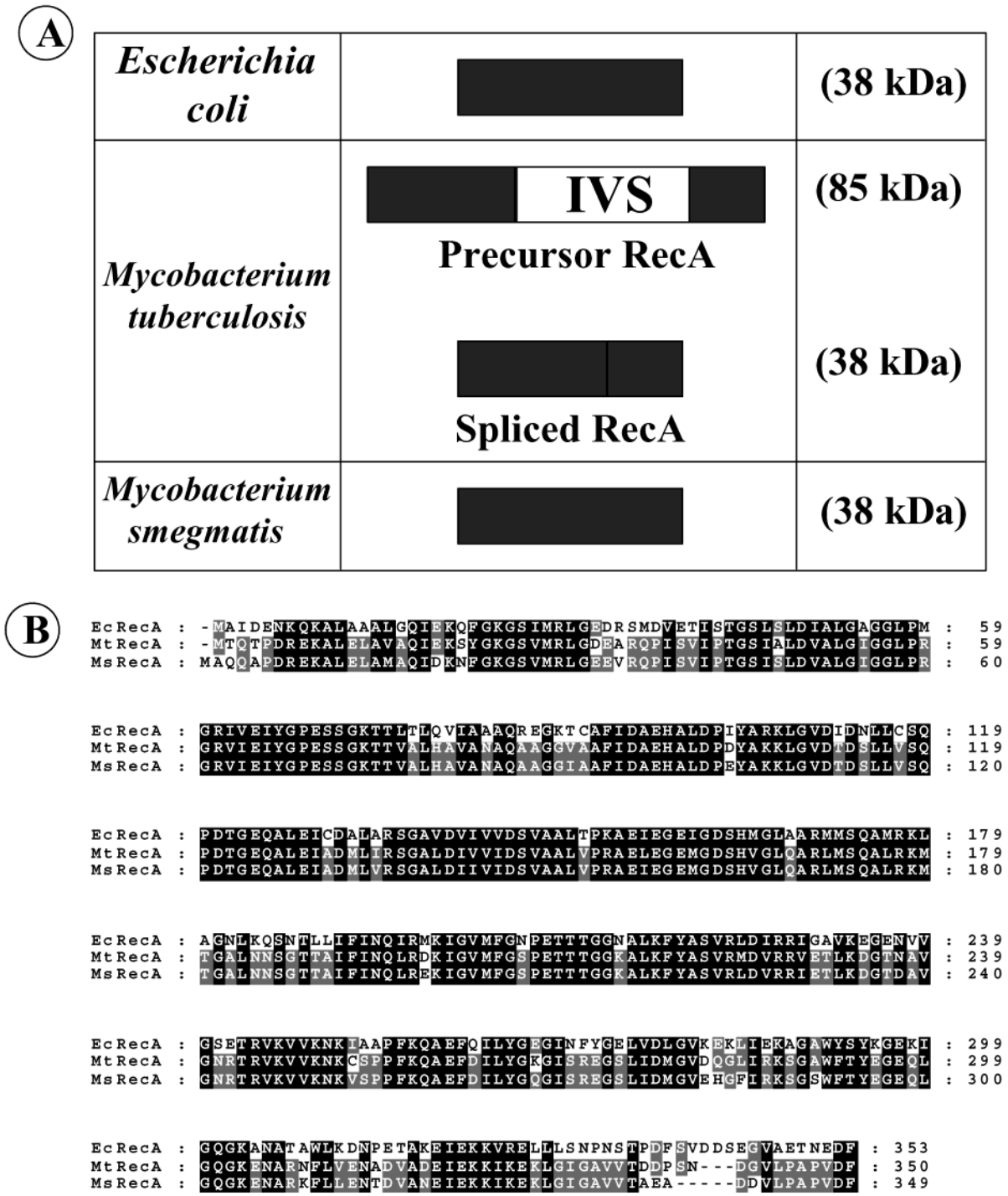

FIGURE 1: (A) Structural organization of RecA of *E. coli*, *M. tuberculosis* and *M. smegmatis*. IVS, intervening sequence. (B) Sequence alignment of EcRecA, MtRecA, and MsRecA. Protein sequence deduced from the nucleotide sequences were aligned using the Clustal W algorithm and displayed by GeneDoc Ver 2.6. Identical amino acids are shown in black, and similar residues are highlighted in gray.

several independent lines of evidence suggest that allele exchange in the fast-growing *M. smegmatis* appears to be relatively much higher (*12*−*16*). Interestingly, comparison of recombination frequencies in *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis*, using counter selection or specialized transduction approaches, has suggested that homologous recombination machinery in the three species is equally efficient (*17*, *18*). To reconcile these diverse observations, we reasoned that it is necessary to compare the mechanistic aspects of DNA strand exchange promoted by RecA from *M. tuberculosis* to its homologue from *M. smegmatis*.

It is generally accepted that the fast growing *M. smegmatis* (generation time of 2 h) could be an instructive model for studying basic cellular processes of relevance to slow-growing *M. tuberculosis* (generation time of 12 h). Here, we have studied the mechanistic aspects of DNA strand exchange, a central step in homologous recombination, promoted by MsRecA in comparison with MtRecA under a variety of solution conditions. Our findings suggest striking mechanistic differences in strand exchange promoted by RecA proteins of mycobacteria. Two prominent differences are noteworthy: First, in contrast with MtRecA, strand

exchange promoted by MsRecA occurs within a very narrow range of pH, and insensitive to the order of addition of SSB. Second, MsRecA was able to couple dATP hydrolysis to strand exchange at higher pHs, but not MtRecA. Together, these results provide insights into the mechanism of homologous recombination in pathogenic and nonpathogenic species of mycobacteria.

MATERIALS AND METHODS

Reagents, Proteins, and DNA. All chemicals were of reagent grade. Buffers were prepared using deionized water. Phage T4 polynucleotide kinase was obtained from New England Biolabs, USA. Phosphocreatine, creatine phosphokinase, MES, PIPES, HEPES, Tris, DTT, and proteinase K was purchased from Sigma-Aldrich, St. Louis, MO. Restriction endonucleases, ATP, and dATP were obtained from Amersham Biosciences, Hong Kong. *Escherichia coli* SSB (*19*), *E. coli* and *M. tuberculosis* RecA (*4*), SSB from *M. tuberculosis* and *M. smegmatis* (*6*) and *M. smegmatis* RecA[2] were purified and their concentrations were determined as described (*20*). Negatively supercoiled and circular single-stranded wild-type M13 DNA was prepared as described (*21*). The concentrations are expressed in moles of nucleotide residues. Aliquots were stored in 10 mM Tris-HCl buffer containing 0.1 mM EDTA (pH 7.5) at 4 °C.

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−HCl ($pK_a = 7.5$) at pH 7; Tris-HCl ($pK_a = 8.1$) at pH 7.5, 8, 8.5, or 9; glycine-KOH ($pK_a = 9.8$) at pH 9.5, 10, or 10.5. In all the experiments, pH was varied but total ionic strength of the buffer was maintained constant.

ATPase Assay. ATP hydrolysis catalyzed by RecA was measured as described (*22*). Reaction mixtures (20 μL) contained 33 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1.4 mM DTT, 0.1 mg/mL BSA, 0.8 μM of specified RecA, in the presence (3 μM) or absence of M13 ssDNA and varying concentrations of [α-^{32}P]dATP. Samples were incubated at 37 °C for 30 min. The reaction was stopped by the addition of EDTA to a final concentration of 25 mM. Aliquots (5 μL) were spotted on polyethyleneimine cellulose sheet and developed in a solution containing 0.5 M LiCl and 1 M formic acid. The amount of ATP hydrolyzed was quantified by scanning the autoradiogram in UVI−Tech gel documentation station equipped with UVI−BandMap software Ver 99. The reaction mixtures were mixed as mentioned above, except that the buffer type and pH were varied. The rate of ATP hydrolysis was calculated from the slope of the curve.

Three-Strand Exchange Assay. Assays were performed as described (*23*). Briefly, reaction mixture (40 μL) contained 33 mM buffer of different pHs, 1.5 mM ATP, 12 mM $MgCl_2$, 5 mM phosphocreatine, 10 U/mL phosphocreatine kinase, 1 mM DTT, 0.1 mg/mL BSA, 10 μM M13 ssDNA, and 5 μM of MsRecA or MtRecA. Reaction mixtures were incubated at 37 °C for 5 min before the addition of 0.66 μM of indicated SSB, and the incubation was continued for an additional 5 min. Strand exchange reaction was then initiated by the addition of 10 μM linear duplex M13 DNA (form I DNA cut with *Hinc* II). Unless otherwise stated, all reaction mixtures were incubated for 60 min at 37 °C. Samples were deproteinized by incubation with SDS (0.1%) and proteinase K (0.2 mg/mL) at 37 °C for 15 min and electrophoresed through 0.8% agarose gel in 89 mM Tris/borate buffer (pH 8.3) at 2 V/cm for 11 h. DNA substrates and products of the reaction were transferred onto a Hybond N^+ membrane, and visualized by Southern hybridization using ^{32}P-labeled M13 ssDNA and autoradiography (*24*). The bands were quantified in UVI−Tech gel documentation station using UVI−BandMap software ver. 99 and plotted using Graphpad Prism Ver.2.0.

RESULTS

Experimental Strategy. In principle, strand transfer promoted by the concerted action of MsRecA and MsSSB or MtRecA and MtSSB (hereafter referred to as cognate) were compared in a variety of solution conditions including various types and concentrations of salt, $MgCl_2$, nucleotide cofactor, and varying pHs in the presence of ATP regeneration system. Unless otherwise stated, reaction mixtures contained 1.5 mM ATP, 12 mM Mg^{2+}, and ssDNA was incubated first with RecA, then SSB, and finally form III DNA was added to initiate strand transfer reaction.

Strand Transfer Promoted by MtRecA and MsRecA Display Distinctly Different pH Profiles: SSB Is a Required Accessory Factor. To gain insights into the mechanistic aspects of DNA strand transfer promoted by MsRecA or MtRecA in vivo, we used an in vitro assay involving linear duplex and circular single-stranded DNA. DNA pairing and exchange results in the generation of joint molecule intermediates, form II DNA and displaced linear single-stranded DNA. In this assay, DNA pairing and exchange are dependent on SSB for extensive (several kilobase pair) strand transfer in the presence of ATP hydrolysis. Building on these observations, we compared MsRecA-promoted DNA strand transfer in the presence or absence of its cognate SSB. After halting the reaction by deproteinization, the reaction mixtures were separated on an agarose gel, and analyzed by Southern hybridization and autoradiography as described under Materials and Methods. In the absence of SSB, MsRecA was unable to promote strand transfer over the range of pH from 6 to 10.5 (Figure 2A). On the other hand, MsRecA in conjunction with its cognate SSB was able to execute DNA strand transfer with sharp pH optima. At pH 7, MsRecA with MsSSB generated significant amounts of form II DNA, and two groups of products corresponding to joint molecule intermediates and DNA networks, the latter failed to enter the gel (Figure 2B). Under similar conditions, MsRecA and MsSSB produced trace amounts of joint molecules and equivalent amount of form II DNA at pH 6.5 and pH 7.5, respectively.

In parallel experiments, we assayed MtRecA-promoted DNA strand transfer in the presence of its cognate SSB. As reported previously for MtRecA and EcSSB (*4*, *5*), MtRecA in the presence of MtSSB generated trace amounts of joint molecules, form II DNA, and large amounts of DNA networks in the pH range of 6.5−7.5. At higher pHs, we observed a gradual increase in the formation of form II DNA with a concomitant decrease in the amounts of DNA

[2] Ganesh, N., and Muniyappa, K. (2003) *Proteins*: *Struct., Funct, Genet.*, in press.

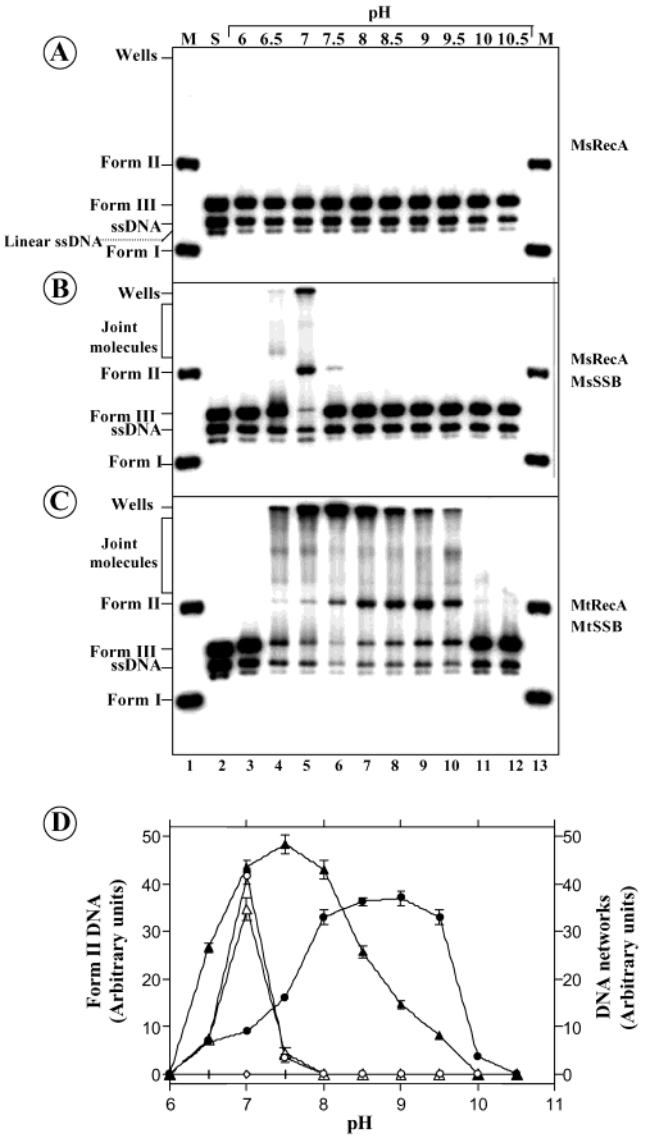

FIGURE 2: Strand transfer promoted by the concerted action of MsRecA and MtRecA display distinct pH profiles. Reactions were performed as described under Materials and Methods. (A) Reactions performed with MsRecA; (B) MsRecA and MsSSB; (C) MtRecA and MtSSB; (D) Quantification of form II DNA and DNA networks. The extent of formation of form II DNA and DNA networks (arbitrary units) is plotted as a function of pH. Open and closed circles correspond to form II DNA generated by MsRecA and MtRecA, respectively. Open and closed triangles represent DNA networks formed by MsRecA and MtRecA, respectively. Open diamonds represent form II DNA generated by MsRecA alone (panel A). The graph shows the mean with SD of three independent experiments. The position of substrates and reaction products are indicated on the left.

networks (Figure 2C). A quantitative analysis of band intensities of form II DNA and networks generated by MsRecA displayed a symmetrical profile around neutral pH. In contrast to MsRecA, MtRecA showed a broad peak of DNA networks in the pH range from 6.5 through 9, and a similar broad peak of form II DNA from pH 8 to 9.5 (Figure 2D). Control experiments indicated that MsRecA or MtRecA failed to promote strand transfer with heterologous DNA substrates (data not shown). Despite considerable structural and phylogenetic relatedness between MsRecA and MtRecA, our results indicate that their behavior is dissimilar in regard to DNA strand transfer.

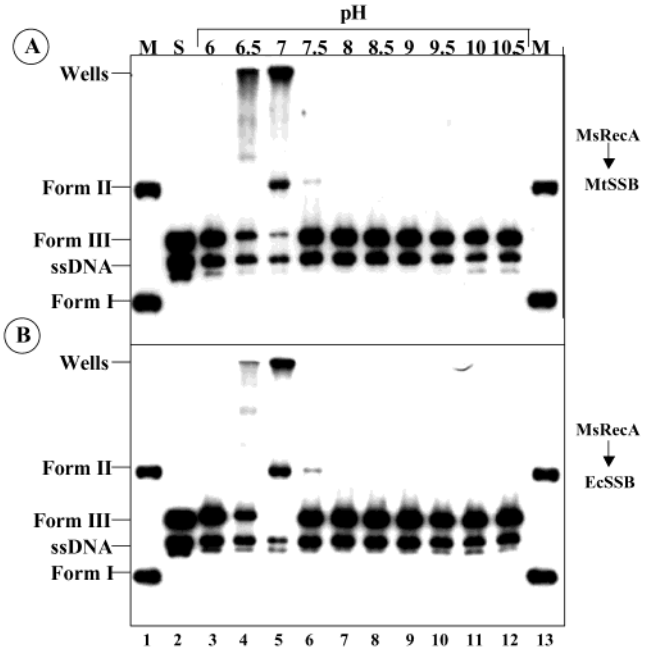

FIGURE 3: Heterologous SSBs stimulate strand transfer reaction promoted by MsRecA. Reactions were performed with MtSSB (A) or EcSSB (B) as described in Materials and Methods.

The above results suggest that MsRecA-promoted DNA strand transfer requires the participation of SSB for optimum activity. To understand the specificity of SSB, we tested the abilities of heterologous SSBs to stimulate MsRecA-promoted strand transfer. Interestingly, the levels of heteroduplex DNA generated by MsRecA in the presence of MtSSB (Figure 3A), EcSSB (Figure 3B) or MsSSB (Figure 2B) appeared to be quantitatively comparable. This result is interesting in the light of our previous studies, which showed specific interaction between MsSSB and MtSSB with their cognate RecAs, both in vivo and in vitro (*6*). However, heterologous SSBs are functionally interchangeable with cognate SSB in EcRecA-promoted strand transfer, indicating a high level of functional conservation of the gene products (*20*). The significance of specific interaction between MsSSB and MsRecA on strand transfer in vitro remains to be established. However, we cannot exclude the possibility that different experimental conditions might be needed to detect the consequences of MsRecA−MsSSB interaction on strand transfer. Alternatively, it is likely that specific interaction between MsRecA and its cognate SSB might be important particularly in the early stages of homologous recombination.

Effect of Order of Addition of SSB on Strand Exchange Promoted by MsRecA. Several lines of evidence suggest that the biochemical activities of eubacterial RecAs, and their eukaryotic homologues, are greatly influenced by the order in which RecA and SSB incubated with ssDNA. The inhibitory effect imposed by SSB on EcRecA-promoted DNA pairing and strand transfer is robust in the absence of ATP hydrolysis (*25*, *26*). The alleviation of inhibition involves partial displacement of EcSSB from ssDNA by EcRecA. Furthermore, although the mechanism is not understood, the combined activities of RecF, RecO, and RecR have been shown to replace SSB and confer stability to the RecA nucleoprotein filament (*1*−*3*). Accordingly, incubation of MtSSB with ssDNA prior to MtRecA inhibited stand transfer at higher pHs (compare Figure 4A with Figure

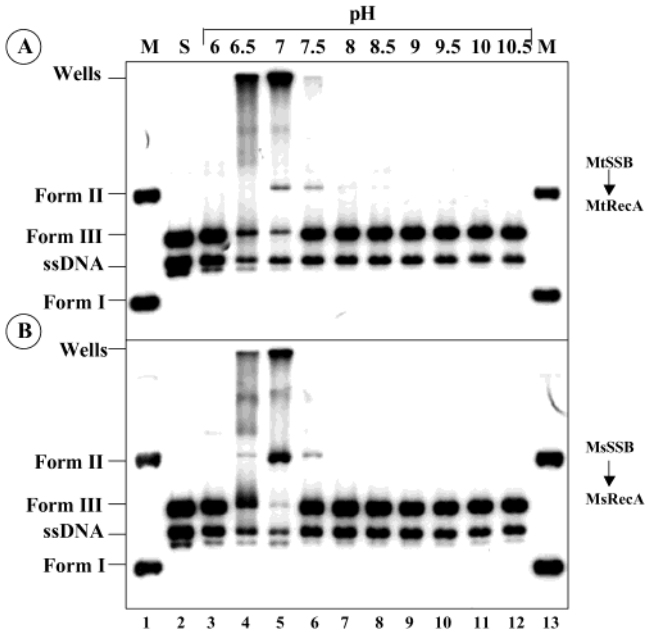

FIGURE 4: Incubation of ssDNA with SSB prior to RecA failed to inhibit MsRecA- promoted, but not MtRecA-promoted, strand transfer. Reactions were performed as described under Materials and Methods. The standard order of incubation was reversed by incubation of ssDNA with the specified SSB for 5 min, and then with its cognate RecA for 5 min. Reaction was initiated by the addition of form III DNA. (A) Incubation of ssDNA with MtSSB prior to MtRecA. (B) Incubation of ssDNA with MsSSB prior to MsRecA.

2C). In contrast, addition of MsSSB prior to MsRecA displayed robust DNA strand transfer (compare Figure 4B with Figure 2B). Corroborating this result, we observed that MsRecA-promoted DNA strand transfer was not affected by incubation of ssDNA with heterologous SSBs prior to MsRecA (data not shown). Hence, our finding that same experimental test yields opposite results suggests that the mechanism of strand transfer promoted by MsRecA and MtRecA in the presence of SSB is likely to be different.

Effect of Mg^{2+} on Strand Transfer Reaction Promoted by MsRecA or MtRecA. Previously, it was observed that 10−12 mM Mg^{2+} was necessary for EcRecA to display its optimal strand transfer activity (*1*, *2*). To test whether there is a causal relationship between recombination activities of mycobacterial RecAs and Mg^{2+}, we compared the consequences of increasing concentration of Mg^{2+} on MsRecA and MtRecA-promoted strand transfer. The assays were performed at their optimal pH values: pH 7 and 8.5 for MsRecA and MtRecA, respectively. After halting the reaction by deproteinization, we analyzed the reaction mixtures on an agarose gel and visualized by Southern hybridization and autoradiography. As shown in Figure 5, quantitative analysis indicated that increasing concentrations of Mg^{2+} led to a gradual increase in the formation of form II DNA by both MsRecA and MtRecA, reaching its maximum at 8 and 10 mM, respectively. However, the efficiency was slightly lower with MtRecA compared to MsRecA (Figure 5). Upon further increase in Mg^{2+} concentration, the extent of form II DNA decreased with concomitant increase in the production of DNA networks. The overall pattern is consistent with the observed effects of Mg^{2+} on strand exchange promoted by EcRecA and its variant RecA K72R (*27*).

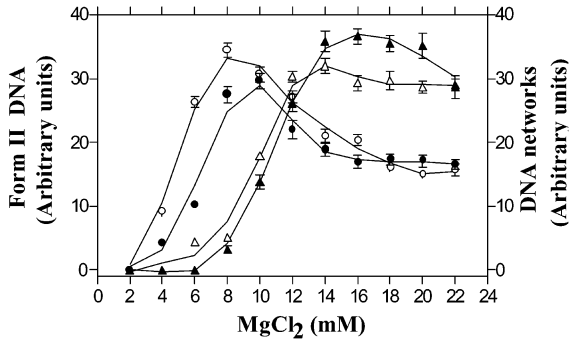

FIGURE 5: MsRecA and MtRecA-promoted strand transfer is affected by Mg^{2+} concentration. Reactions were performed as described under Materials and Methods at pH 7 and 8.5 for MsRecA and MtRecA, respectively. The extent of formation of form II DNA and DNA networks (arbitrary units) are plotted as a function of Mg^{2+} concentration. Open and closed circles correspond to form II DNA generated by MsRecA and MtRecA, respectively. Open and closed triangles represent DNA networks formed by MsRecA and MtRecA proteins, respectively. The graph shows the mean with SD of three independent experiments.

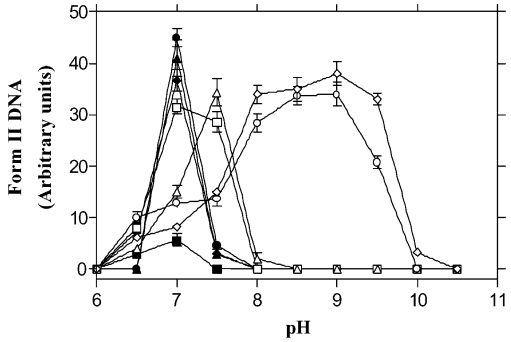

FIGURE 6: MtRecA-promoted, but not MsRecA-promoted, strand transfer is differentially affected by increasing Mg^{2+} concentrations as well as varying pH. Reactions were performed with 4 mM (squares), 6 mM (triangles), 8 mM (circles), or 12 mM (diamonds) Mg^{2+} as described under Materials and Methods. The formation of form II DNA (arbitrary units) at the above concentrations of Mg^{2+} is plotted as a function of pH. Open and closed symbols correspond to form II DNA generated by MtRecA and MsRecA, respectively. The graph shows the mean with SD of three independent experiments.

We extended our analysis to examine the effects of increasing concentrations of Mg^{2+} at varying pHs on DNA strand transfer. Under these conditions, the profiles of form II DNA generated by MtRecA and MsRecA showed striking differences (Figure 6). Quantitative analysis indicated that MtRecA was able to generate maximum amounts of form II DNA at pH 7 in the presence of 4 mM Mg^{2+}, while MsRecA produced barely detectable amounts of the same product (Figure 6, compare open versus closed squares). At 6 mM Mg^{2+}, MsRecA was able to produce optimal amounts of form II DNA at pH 7 (Figure 6, closed triangles). Similar results were obtained with MtRecA, except that the optimal activity occurred at pH 7.5 (Figure 6, open triangles). The results obtained in the presence of 8 or 12 mM Mg^{2+} appear to be significant. Specifically, the formation of maximum amounts of form II DNA generated by MtRecA occurred at alkaline pH, while that of MsRecA peaked at pH 7 (Figure 6, compare open versus closed circles and diamonds). Furthermore, the profile of formation of form II DNA was sharp in the case of MsRecA, while it was broad in the case of MtRecA. Two general explanations might account for the contrasting

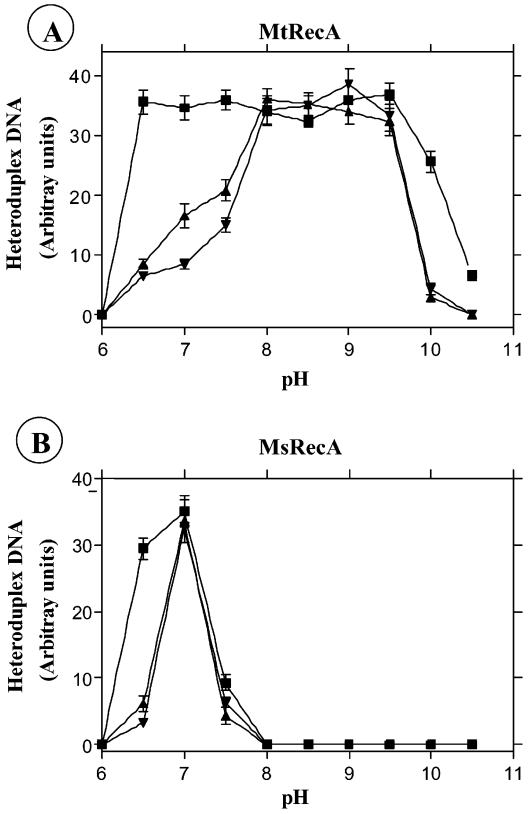

FIGURE 7: The length of duplex DNA affects MtRecA-promoted, but not MsRecA-promoted, strand transfer at varying pHs. Reactions were performed as described under Materials and Methods, except the indicated fragments of DNA were used: 2.2 kb (closed square); 4.2 kb (closed triangle); or 6.4 kb (closed inverted triangle). (The DNA fragments were generated by cleavage of Form I M13 DNA with appropriate restriction enzymes). The extent of heteroduplex DNA (arbitrary units) generated by MtRecA (A) or MsRecA (B) is plotted as a function of varying pH. The graph shows the mean with SD of three independent experiments.

outcomes of strand transfer in these experiments. (a) Nucleoprotein filaments of MtRecA-ssDNA might form differently as a function of Mg^{2+} concentration and varying pHs. (b) Higher pH might influence the ability of MtRecA to carry out any of the steps involved in the conversion of DNA substrates to products.

Effect of Target Length on Strand Transfer Reactions by MsRecA and MtRecA. To gain insights into the relationship between the target length and generation of heteroduplex DNA, we examined strand transfer with varying lengths of substrate DNA. Increasing length of linear DNA from 2.2 to 6.4 kb caused changes in the profiles of MsRecA or MtRecA-promoted strand transfer. With 2.2 kb substrate, MtRecA was able to generate maximum amounts of hDNA in the pH range from 6.5 to 9.5 (Figure 7A). With an increase in the length of linear duplex DNA, formation of form II DNA shifted to alkaline pHs, and the optimal reaction was observed in the pH range from 8 to 9.5. In contrast to MtRecA, MsRecA-promoted strand transfer was at its maximum at pH 7, regardless of the length of the linear duplex DNA (Figure 7B). The overall efficiency of product formation by both Ms and Mt RecA proteins was largely similar.

ATP or Salt Facilitates Strand Transfer Reaction Promoted by MsRecA and MtRecA. To assess whether the solution parameters would influence MsRecA- or MtRecA-promoted

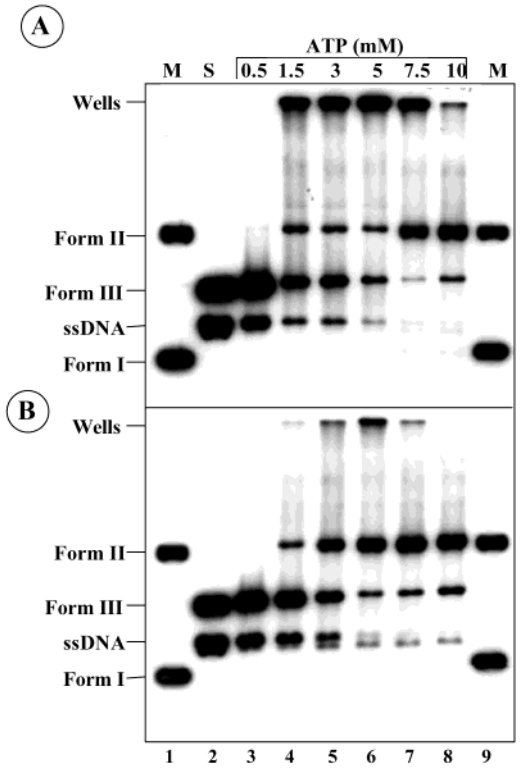

FIGURE 8: ATP facilitates MtRecA- and MsRecA-promoted strand transfer. Reactions were performed for MtRecA at pH 7.5 (A) and MsRecA at pH 7 (B) with ATP concentrations as indicated on top of each lane in the absence of ATP-regeneration system as described under Materials and Methods. The position of substrates and products are indicated on the left.

stand transfer, we performed assays with increasing concentrations of ATP. 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 5 to 10 mM, MtRecA-promoted DNA strand transfer was enhanced with concomitant decrease in the extent of DNA networks (Figure 8A). Under similar conditions, the effect of ATP was robust on MsRecA-promoted strand transfer, and required much less ATP compared to MtRecA (Figure 8B). Overall, we observed a 3-fold increase in the levels of form II DNA at 10 mM ATP with MsRecA or MtRecA. The simplest interpretation of these results is that ATP causes dissolution of DNA networks in a nonspecific manner, thereby driving the reaction toward the formation of form II DNA.

It has been demonstrated that physiological concentrations of salt in the assay buffer influences the activities of EcRecA and Rad51 protein (*28*, *29*). We therefore compared the effect of an in vivo solute, potassium glutamate, on MsRecA or MtRecA-promoted strand transfer. Interestingly, its effect on MsRecA or MtRecA-promoted strand transfer was strikingly different (Figure 9). In the absence of potassium glutamate, MtRecA displayed minimal strand transfer (Figure 9, open triangles). Addition of 150 mM potassium glutamate led to a significant increase in MtRecA-promoted strand transfer with concomitant decline in DNA networks. On the other hand, the effect of potassium glutamate on MsRecA-promoted strand transfer was relatively insignificant.

dATP Hydrolysis Catalyzed by MsRecA and MtRecA. In reactions promoted by EcRecA, ATP hydrolysis has two

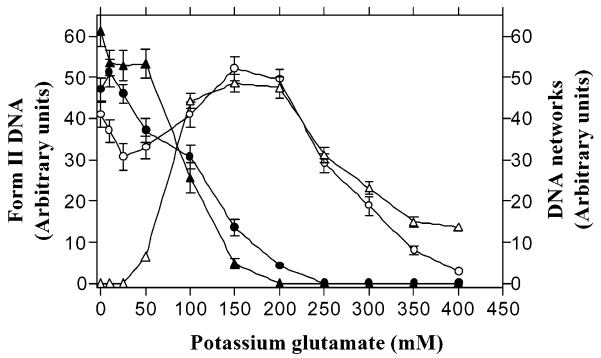

FIGURE 9: Potassium glutamate facilitates MtRecA- and MsRecA-promoted strand transfer. Reactions were performed for MsRecA at pH 7 and MtRecA at pH 7.5 with increasing concentrations of potassium glutamate as described under Materials and Methods. The extent of formation of form II DNA and DNA networks (arbitrary units) was plotted as a function of potassium glutamate concentration. Open circles and triangles correspond to form II DNA (arbitrary units) generated by MsRecA and MtRecA, respectively. Closed circles and triangles represent DNA networks produced by MsRecA and MtRecA, respectively. The graph shows the mean with SD of three independent experiments.

functions: it facilitates 5′ → 3′ disassembly of nucleoprotein filament and DNA strand transfer. EcRecA catalyzes hydrolysis of ATP as well dATP almost to a similar extent. Although the mechanistic basis is not understood, RecA proteins from Gram-positive bacteria such as *Bacillus subtilis*, *Deinococcus radiodurans*, and *Streptococcus pneumoniae* preferentially hydrolyze dATP over ATP (*30*−*32*). Since mycobacteria are Gram-positive, we reasoned that MsRecA and MtRecA might prefer dATP compared to ATP. Hence, we assayed ssDNA-dependent dATP hydrolysis catalyzed by MsRecA and MtRecA over a wide range of pH values from 6 to 10.5. Reaction mixtures contained MsRecA or MtRecA (0.8 μM), ssDNA (3 μM), and ^{32}P-labeled dATP (1 mM). These conditions ensured that there was sufficient ssDNA to bind all of RecA in the reaction mixture. In the absence of ssDNA, both MsRecA as well as MtRecA failed to promote dATP hydrolysis (Figure 10A). On the other hand, in reactions containing ssDNA the rate of dATP hydrolysis catalyzed by MsRecA or MtRecA increased up to pH 9, and then declined thereafter. Under these conditions, both the RecAs displayed a broad optimum of dATPase activity from pH 7 through 9.5.

To examine the kinetics of dATP hydrolysis catalyzed by MsRecA or MtRecA, we performed assays with increasing concentrations of dATP. As a control, we also measured the dATPase activity of *E. coli* RecA. In the absence of ssDNA, MsRecA or MtRecA displayed no measurable dATP hydrolysis (Figure 10B). However, in the presence of ssDNA, the rates of dATP hydrolysis catalyzed by MsRecA or MtRecA increased linearly with dATP concentration. However, the rates were significantly lower compared to EcRecA (Figure 10B). The $S_{0.5}$ values for dATP was 30 μM for EcRecA, whereas 75 and 90 μM for MtRecA and MsRecA, respectively. The apparent turnover rate $k_{cat(ssDNA)}$ for MsRecA and MtRecA was 13 and 11.8 min^{-1}, respectively. Under similar conditions, the $k_{cat(ssDNA)}$ for EcRecA was 23 min^{-1}, in agreement with the value reported previously (*4*). These data indicate that the $k_{cat(ssDNA)}$ values for MsRecA or MtRecA are ~2-fold lower compared with the values for EcRecA. Under similar conditions, MsRecA or MtRecA

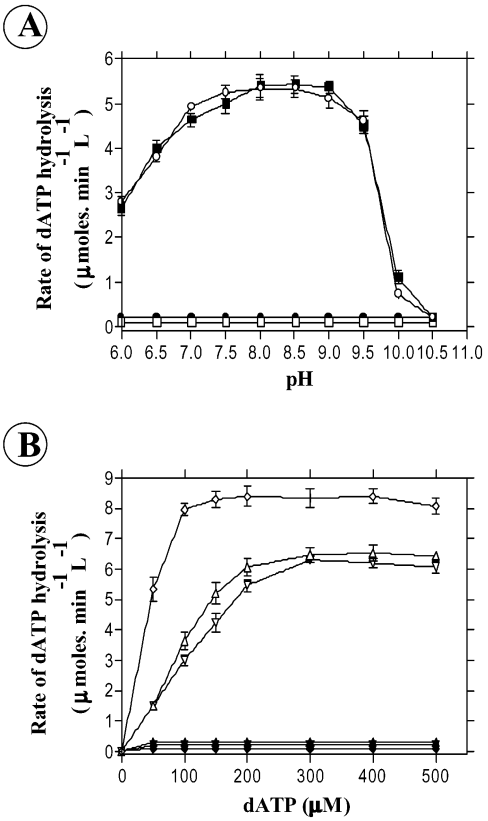

FIGURE 10: ssDNA- and pH-dependent dATP hydrolysis catalyzed by different RecA proteins. (A) Rate of dATP hydrolysis is plotted as a function of pH. The assay was performed with 1 mM dATP as described under Materials and Methods. The rate of hydrolysis catalyzed by MtRecA (closed squares) or MsRecA (open circles) in the presence ssDNA; and MtRecA (open squares) or MsRecA (closed circles) in the absence of ssDNA. (B) Rate of hydrolysis is plotted as a function of dATP concentration. The rate of hydrolysis catalyzed by EcRecA (open diamonds), MtRecA (open triangles) or MsRecA (open inverted triangles) in the presence of ssDNA; and EcRecA (closed diamonds), MtRecA (closed triangles), or MsRecA (closed inverted triangles) in the absence of ssDNA. The graphs show the mean with SD of three independent experiments.

catalyzed hydrolysis of ATP with similar kinetic constants (data not shown).

dATP Supports MsRecA, but not MtRecA-Promoted Strand Transfer. To explore whether dATP can support MsRecA- or MtRecA-promoted strand transfer, assays were performed with dATP as the nucleotide cofactor. The data in Figure 11A show that MtRecA-promoted strand transfer led to the formation of large amounts of DNA networks, minimal amount of joint molecule intermediates, and barely detectable amounts of form II DNA except at pH 9. Under identical conditions, MsRecA-promoted strand transfer resulted in the formation significant amounts of form II DNA, with maximum at pH 9 (Figure 11B). Intriguingly, MsRecA failed to generate form II DNA at neutral pH as in the presence of ATP (compare Figure 11B with Figure 2B). The basis for MsRecA-promoted strand transfer to alkaline pH in the presence of dATP remains to be investigated.

DISCUSSION

The results presented here have led to four principal conclusions. First, MsRecA by itself was unable to promote strand transfer across a wide range of pHs. Both cognate as well as noncognate SSBs stimulated MsRecA-promoted

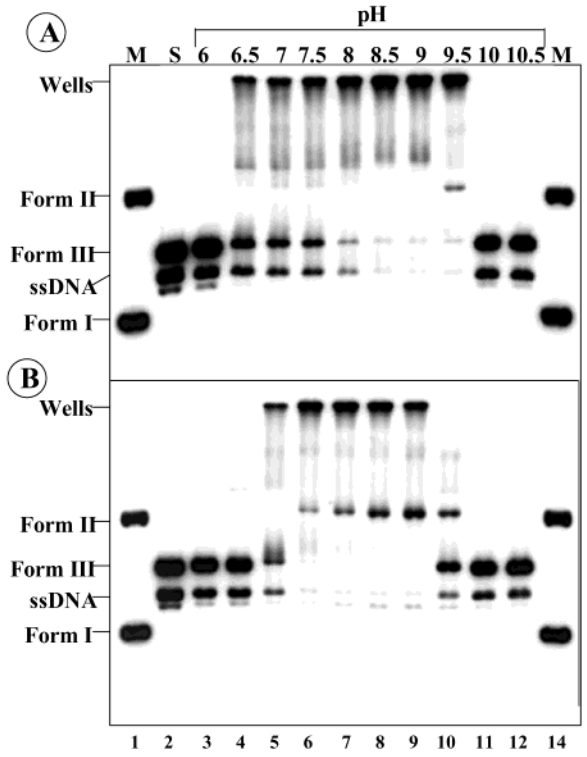

FIGURE 11: Effect of dATP on MtRecA- and MsRecA-promoted strand transfer at varying pHs. Reactions were performed as described under Materials and Methods, except dATP, instead of ATP, was used as the cofactor. (A) Reactions performed with MtRecA in the presence of MtSSB. (B) Reactions performed with MsRecA in the presence of MsSSB. The position of substrates and reaction products are indicated on the left.

strand transfer, even when added prior to RecA. This is in contrast to the inhibitory effect imposed by SSB on MtRecA-promoted strand transfer. Second, strand transfer promoted by MsRecA and MtRecA displayed distinctly different pH profiles. Third, both MsRecA and MtRecA were strongly stimulated by factors that suppressed the formation of DNA networks, which, in turn, stimulated strand exchange by ~3-fold. Fourth, MtRecA and MsRecA catalyzed dATP hydrolysis at similar rates with broad pH profiles. However, MsRecA was able to couple dATP hydrolysis mechanistically to strand transfer albeit at higher pHs, whereas MtRecA did not. We consider the implications of these results with respect to the mechanism of homologous recombination in mycobacteria.

Currently, limited information is available regarding the mechanistic aspects of DNA pairing and strand transfer promoted by RecA proteins of mycobacteria. A comparison of the structural features of MsRecA and MtRecA is instructive: pair wise comparison of amino acid sequence disclosed that MsRecA is 89% identical to MtRecA, and 60% to EcRecA (Figure 1B). As demonstrated in the case of EcRecA, the amino acid residues that are crucial for DNA-binding, ATP-binding and hydrolysis, and monomer−monomer interactions are entirely conserved among these RecAs. Moreover, the crystal structure of apo- and ATP-bound forms of MsRecA revealed a filamentous, three-domain structure with molecular surfaces rich in acidic amino acid residues similar to that of MtRecA (ref *33*; Datta et al.[3]). These findings indicate a high degree of structural conservation between MsRecA and MtRecA.

An interesting question that arises from our studies is whether we can gain insights into the mechanism of homologous recombination in vivo from studying the functional characteristics of RecAs. The efficiency of three-strand transfer promoted by MsRecA is equivalent to MtRecA, implying that the pair of in vitro model substrates might be the physiological substrates for both the proteins in vivo. This is supported by the fact that transient single-stranded regions are generated in the genome as a consequence of normal DNA metabolism such as replication, transcription, and recombination (*2*, *34*). These regions could serve as substrates for the assembly of RecA nucleoprotein filament. Both MsRecA and MtRecA require cognate or noncognate SSBs to display maximum DNA strand transfer activity. The observation that MsRecA and MtRecA failed to promote efficient strand transfer in the absence of SSB reflects their inability to melt secondary structure in ssDNA (*35*−*38*). Moreover, it is possible that SSB might perform a postsynaptic role during which it sequesters the displaced linear single-strand when RecA nucleoprotein filament pairs with linear duplex DNA (*39*, *40*). However, the striking features that emerge from these studies is that, despite overall structural similarity, MsRecA and MtRecA differ functionally in critically important ways such as pH optimum for strand transfer, ability to couple dATP hydrolysis to strand transfer, and insensitivity to the order in which SSB is added to the reaction.

Under optimized conditions, the prototype EcRecA in conjunction with its SSB promotes extensive strand transfer using the energy released from ATP hydrolysis. Similarly, for complete strand transfer MsRecA and MtRecA require the presence of cognate or noncognate SSB. Unlike MsRecA, MtRecA failed to generate form II DNA even though the rate of dATP hydrolysis was similar to MsRecA across a wide range of pHs. Similarly, the ability of ScRad51 to promote strand exchange is modulated by RPA and Rad52 (*41*−*44*). It is possible that MtRecA and MsRecA function in a similar manner in vivo to accelerate-limited strand exchange. However, from the biochemical data available at present on strand transfer, it is difficult to infer why allele exchange is inefficient and random integration is efficient in *M. tuberculosis*, whereas it is relatively efficient in *M. smegmatis*.

The inhibition of MtRecA-promoted strand transfer at higher concentrations of Mg^{2+} might arise due to discontinuities in the nucleoprotein filament (reviewed in refs *1* and *2*). The observed effects of Mg^{2+} on MtRecA-promoted strand transfer as a function of pH are different from MsRecA. Intriguingly, the generation of form II DNA occurred at alkaline pHs with increasing concentrations of Mg^{2+}. Consistent with yeast Rad51 protein (*29*), nonproductive interaction between linear duplex and ssDNA affected the extent of strand exchange promoted by MsRecA. The data presented here establish that potassium glutamate or ATP facilitates strand exchange through inhibition of DNA network formation.

Normally, the optimal conditions for EcRecA-promoted DNA pairing and strand transfer involve 10−12 mM Mg^{2+}, 1−3 mM ATP, and an ATP-regeneration system (*1*−*3*). The

[3] Datta, S., Krishna, R, Ganesh, N., Chandra, N. R., Muniyappa, K., and Vijayan, M. (2003), submitted for publication.

Mg·ATP complex is the substrate in ATP hydrolysis catalyzed by RecA. Many lines of evidence indicate that the different phases of EcRecA-promoted homologous recombination show different dependence on Mg^{2+} concentration. The formation of RecA nucleoprotein filament is robust at 1−2 mM Mg^{2+}, whereas excess of that is needed for formation of pairing intermediate as well as strand transfer. To account for these differences, it has been proposed that excess Mg^{2+} facilitates the formation of discontinuous RecA nucleoprotein filament, which, in turn, enhances the formation of complete strand transfer product over time (*2*−*3*, *32*). How might then increasing concentrations of ATP, or Mg·ATP complex, convert DNA networks into form II DNA? One simple model would be that ATP functions directly to chelate Mg^{2+}, and thereby facilitates strand transfer. In this model, there will not be any free Mg^{2+} available for stabilization of the pairing intermediate and heteroduplex DNA. Hence, our finding that excess of ATP stimulates strand transfer implicate a model that is independent of its ability to chelate Mg^{2+} present in the assay buffer.

Both MsRecA and MtRecA were able to promote DNA pairing in the presence of either ATP or dATP, as evidenced by the accumulation of joint molecule intermediates, but the nucleotide cofactors had differential effect on formation of form II DNA. Importantly, the optimal pH for dATP-dependent strand exchange promoted by MsRecA was not optimal for MtRecA. With either of the nucleotide cofactors, SSB was extremely crucial for MsRecA promoted strand exchange. In contrast, MsRecA by itself was able to catalyze dATP hydrolysis similar to that of MtRecA, but 2-fold less compared to EcRecA. The basis for the differential effects displayed by MsRecA remains to be investigated. However, these findings are reminiscent of the results reported for *S. pneumoniae* SSB and its cognate RecA in the presence of dATP (*32*). Similarly, *D. radiodurans* RecA competes very well for ssDNA binding with EcSSB in the presence of dATP as monitored by ATPase assays. Addition of EcSSB prior to *D. radiodurans* RecA abolished strand exchange (*31*). These findings suggest that MsRecA and MtRecA although structurally quite similar are functionally distinct.

An important observation concerns the differential effects of pH, ATP, and Mg^{2+}on MsRecA- and MtRecA-promoted strand transfer. Previous studies have shown that the intracellular pH of *M. smegmatis* is in the narrow range of pH 6.1 to 7.2 (*45*), coincident with the optimum pH displayed MsRecA to effect maximal strand exchange. The intracellular pH of *M. tuberculosis* H37Rv has been reported to be in the range of 6.2 and 7.3 (*46*). Although the jury is still out on the question, it is possible that strand transfer is performed in a compartmentalized manner wherein the pH is most favorable. In considering the mechanistic basis for pH-dependent strand transfer, it is possible that MsRecA or MtRecA isomerize to a strand exchange conformational state at neutral and higher pHs, respectively. The precise structural nature of this isomerization is unclear. However, it has been shown that wild-type and variant forms of EcRecA display different pH-dependent conformational states (*47*−*49*). The stimulating effect of ATP on strand transfer at physiological pH is difficult to reconcile in light of the fact that the intracellular levels of ATP in eubacteria is in the range of 5 mM. Since stimulation was associated with the dissolution of DNA networks, reminiscent of the effect seen with salt, it is possible that the in vivo ionic environment might augment the requirement for higher concentrations of ATP.

It has been noted that dATP has a marked effect on the biochemical properties of EcRecA (*50*). Most importantly, dATP-dependent strand exchange reaction is facilitated under conditions that are not optimal for strand transfer reactions in the presence of ATP. We also note that substitution of dATP for ATP has been shown to stabilize RecA nucleoprotein filaments, but do not enhance D-loop formation (*1*−*2*). Our finding that both MsRecA and MtRecA hydrolyze dATP to a similar extent, but not strand transfer, highlights an important difference between RecAs of mycobacteria. The type of the nucleotide cofactor or its levels in these organisms is unknown, and consequently, the physiological significance of the effects of dATP remains to be established.

Understanding of homologous recombination in mycobacteria, and especially the basis for inefficient allele exchange in *M. tuberculosis*, is a dauntingly difficult task. The present results indicate that although MsRecA and MtRecA are phylogenetically conserved, they display striking differences in homologous strand transfer reactions. This study further suggests that a complex process such as homologous recombination in vivo may be deciphered by first understanding the biochemical properties of each component of the pathway, and subsequently integrating them to obtain a complete picture.

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