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DNA Binding, Coprotease, and Strand Exchange Activities of Mycobacterial RecA Proteins: Implications for Functional Diversity among RecA Nucleoprotein Filaments[†]

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ABSTRACT: One of the fundamental questions concerning homologous recombination is how RecA or its homologues recognize several DNA sequences with high affinity and catalyze all the diverse biological activities. In this study, we show that the extent of single-stranded DNA binding and strand exchange (SE) promoted by mycobacterial RecA proteins with DNA substrates having various degrees of GC content was comparable with that observed for *Escherichia coli* RecA. However, the rate and extent of SE promoted by these recombinases showed a strong negative correlation with increasing amounts of sequence divergence embedded at random across the length of the donor strand. Conversely, a positive correlation was seen between SE efficiency and the degree of sequence divergence in the recipient duplex DNA. The extent of heteroduplex formation was not significantly affected when both the pairing partners contained various degrees of sequence divergence, although there was a moderate decrease in the case of mycobacterial RecA proteins with substrates containing larger amounts of sequence divergence. Whereas a high GC content had no discernible effect on *E. coli* RecA coprotease activity, a negative correlation was apparent between mycobacterial RecA proteins and GC content. We further show clear differences in the extent of SE promoted by *E. coli* and mycobacterial RecA proteins in the presence of a wide range of ATP:ADP ratios. Taken together, our findings disclose the existence of functional diversity among *E. coli* and mycobacterial RecA nucleoprotein filaments, and the milieu of sequence divergence (i.e., in the donor or recipient) exerts differential effects on heteroduplex formation, which has implications for the emergence of new genetic variants.

Homologous recombination, because of its fundamental roles in the maintenance of genome stability and evolution, is an essential cellular function common to all organisms. The prototype *Escherichia coli* RecA, a multifunctional protein, plays central roles in homologous recombination (HR),[1] DNA repair, and SOS response (*1*−*4*). The RecA family of proteins is ubiquitous and well-conserved among bacterial species (*2*, *3*). Many archaeal species possess two RecA homologues (RadA and RadB), and eukarya possess multiple homologues of RecA, including, Rad51, Rad51B, Rad51C, Rad51D, DMC1, XRCC2, and XRCC3 (*2*, *5*, *6*). RecA and its homologues function as polymers, consisting of hundreds of monomers that cooperatively polymerize on single-stranded DNA to form a helical nucleoprotein filament (*7*−*9*). The fundamental mechanism underlying HR, i.e., DNA strand exchange, is one of the most fascinating examples of molecular recognition and exchange between two biological polymers under physiologically relevant solution conditions.

The process of HR is kinetically divided into three phases: presynapsis, pairing and strand exchange, and resolution (*2*−*4*). In the presence of ATP, RecA polymerizes cooperatively on single-stranded DNA to generate a presynaptic nucleoprotein filament. During synapsis, the RecA nucleoprotein filament is aligned with homologous sequences in the duplex DNA to form joint molecules. The initial contacts are between nonhomologous regions of duplex DNA, resulting in the formation of large networks of DNA and RecA protein that are believed to contribute to the search for homology as evidenced by limited unwinding of duplex DNA (*2*). However, the molecular mechanism of homology search by the RecA nucleoprotein filament and subsequent homologous pairing are poorly understood. In the next phase, RecA catalyzes strand exchange between DNA molecules, resulting in the generation of extended regions of heteroduplex DNA. Finally, the recombination intermediates are processed and resolved by the RuvABC complex (*4*, *5*).

Homologous recombination, as opposed to site-specific recombination, is believed to be independent of DNA sequence. In particular, *E. coli* RecA and its homologues from bacteria, yeast, and humans are characterized by their ability to bind single-stranded DNA apparently in a sequence-independent fashion but pair with duplex DNA in a homology-dependent manner (*1*−*3*). However, EcRecA and yeast Rad51 proteins exhibit higher binding affinity and enhanced pairing activity with GT-rich nucleotide sequences (*10*−*13*). Consistent with this, GT-rich sequences have been found to be significantly

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[1]Abbreviations: ATPγS, adenosine 5′-*O*-(thiotriphosphate); dsDNA, double-stranded DNA; DTT, dithiothreitol; EcRecA, *Escherichia coli* RecA; EDTA, ethylenediaminetetraacetic acid; HR, homologous recombination; MMR, mismatch repair; MsRecA, *Mycobacterium smegmatis* RecA; MtRecA, *Mycobacterium tuberculosis* RecA; MtLexA, *M. tuberculosis* LexA; ODN, oligonucleotide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SE, strand exchange; ssDNA, single-stranded DNA.

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higher at recombination hot spots (*14*). Several other specific DNA sequences implicated in HR include the *E. coli* hot spot Chi (χ, 5′-GCTGGTGG-3′) (*15*, *16*), satellite repeat arrays in human and mammalian genomes (*17*−*19*), GAA·TTC (*20*) and CTG·CAG repeat sequences (*21*), and subtelomeric and telomeric DNA from yeast and human cells (*22*−*24*). The complete sequencing of numerous bacterial genomes suggests that HR might play an active role in the evolution of pathogenic microorganisms (*25*). Often, the DNA substrates derived from either M13 (41% GC) or φX174 (46% GC) phage chromosomes, below the average GC content (51%) of the *E. coli* chromosome, have been used to elucidate the molecular mechanism of HR *in vitro*. In view of this, it is important to understand how RecA functions in the context of native DNA, as well as the relationship between DNA binding affinities and its diverse biological activities. These parameters may include the native DNA substrates representing their genome sequences that are likely to affect the efficiency and fidelity of HR in bacteria as well as in other organisms (*4*, *5*, *26*).

One of the poorly understood aspects of molecular mechanisms of HR is how RecA or its homologues recognize several DNA sequences with high affinity and catalyze all the diverse biological activities. The complete sequencing of the genomes of various species of mycobacteria has disclosed that the GC content varies between 50 and 70%. The *Mycobacterium tuberculosis* H37Rv chromosome possesses high (66%) GC content (*27*). In addition, a group of genes belonging to the PE or PPE family of proteins contain very high (>80%) GC content (*27*). Consequently, correlation between binding of RecA to DNA and the diverse biological activities of the resulting nucleoprotein filament is a subject of considerable interest. To this end, we have investigated whether DNA substrates containing various GC contents affect the ability of *M. tuberculosis* and *Mycobacterium smegmatis* RecA proteins to bind ssDNA, catalyze coprotease activity, and promote DNA strand exchange (SE), in comparison to *E. coli* RecA. We observed that the extents of ssDNA binding and strand exchange promoted by *E. coli* and mycobacterial RecA proteins having various degrees of GC content were comparable, but they were distinct in their coprotease activity as well as sensitivity to inhibition by ADP. Moreover, the degree and context of sequence divergence (i.e., in the donor or recipient) have different effects on the extent of homeologous recombination, which has implications for the emergence of genetic variants.

EXPERIMENTAL PROCEDURES

Biochemicals, Enzymes, and DNA. All the fine chemicals were of analytical grade. Buffers were prepared using deionized water. T4 polynucleotide kinase was purchased from New England Biolabs. Radiolabeled ATP was purchased from BRIT (Mumbai, India). ATPγS was purchased from Merck, whereas dATP was procured from Amersham Biosciences. Phosphocreatine and creatine phosphokinase were purchased from Sigma. *M. tuberculosis* RecA (*28*), *M. smegmatis* RecA (*29*), *E. coli* RecA (*30*), and *M. tuberculosis* LexA (*31*) were purified to >98% homogeneity as described previously. The concentration of each protein was determined by the dye binding method (*32*). The protein preparations were free of contamination by any exonuclease or endonuclease activity.

Oligonucleotides used in this study were purchased from Sigma-Genosys (Singapore), and their sequences are listed in Table S1 of the Supporting Information. The ODNs were labeled at the 5′ end by using [γ-^{32}P]ATP and T4 polynucleotide kinase. ^{32}P-labeled duplex DNA (83 bp) was prepared by annealing labeled ODNs with an equimolar ratio of complementary unlabeled ODNs (*33*, *34*) at 95 °C for 5 min followed by gradual cooling to 24 °C .The annealing mixture was electrophoresed on a 10% polyacrylamide gel in 44.5 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA at 150 V for 7 h. The bands corresponding to the annealed substrates were excised from the gel and eluted into TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA]. The concentrations of unlabeled and labeled DNA were expressed in moles of nucleotide residues per liter.

^{32}P-labeled duplex DNA substrates used for strand exchange assays were made as follows: 50% GC duplex (labeled ODN2 and unlabeled ODN3), 60% GC duplex (labeled ODN4 and unlabeled ODN5), and 70% GC duplex (labeled ODN6 and unlabeled ODN7). Mismatch oligonucleotides (ODN8−12) having varying extents of mismatches were designed to be complementary to ODN2. The mismatches are randomly embedded in both single- and double-stranded DNA. For mismatch assays, radiolabeled duplex substrates were synthesized as follows: perfect duplex or 0% mismatch duplex (labeled ODN3 and unlabeled ODN2), 3% mismatch duplex (labeled ODN8 and unlabeled ODN2), 6% mismatch duplex (labeled ODN9 and unlabeled ODN2), 9% mismatch duplex (labeled ODN10 and unlabeled ODN2), 12% mismatch duplex (labeled ODN11 and unlabeled ODN2), and 15% mismatch duplex (labeled ODN12 and unlabeled ODN2).

Electrophoretic Mobility Shift Assay. The assay was performed as described previously (*28*, *29*). Reaction mixtures (20 μL) contained 20 mM Tris-HCl (pH 7.5 for EcRecA and MtRecA and pH 7.0 for MsRecA), 1.4 mM DTT, 0.1 mM ATPγS, 12 mM $MgCl_2$, 3 μM ^{32}P-labeled 83-mer ssDNA(+) with a varying percent GC content (ODN1, -2, -4, and -6), and increasing concentrations of EcRecA, MtRecA, or MsRecA. Reaction mixtures were incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 2.5 μL of 10× gel loading solution [50% glycerol, 0.42% (w/v) bromophenol blue, and 0.42% (w/v) xylene cyanol]. Samples were electrophoresed on a 6% native polyacrylamide gel in 0.5× TAE [20 mM Tris-acetate buffer (pH 7.4) containing 0.5 mM EDTA] buffer at 150 V for 3 h (*35*). The gels were dried and bands visualized using a Fuji FLA-9000 phosphorimager. The bands were quantified in UVI-Tech gel documentation station using UVI-BandMap version 97.04 and plotted using Graphpad Prism (version 5.0).

DNA Strand Exchange Assay. The assay was performed as described previously (*30*, *39*). Reaction mixtures (10 μL) containing 20 mM Tris-HCl (pH 7.5 for EcRecA and MtRecA and pH 7.0 for MsRecA), 3 mM dATP, 8 mM $MgCl_2$, 5 μM unlabeled ssDNA, and 2.5 μM RecA (from the indicated source) were incubated in the presence of the ATP regeneration system (5 mM phosphocreatine and 10 units/mL creatine phosphokinase) unless stated otherwise at 37 °C for 5 min. Reaction was initiated by the addition of 1 μM ^{32}P-labeled duplex DNA, and incubation at 37 °C was continued for an additional 10 min, unless otherwise stated. Reaction was terminated by the addition of 2.5 μL of 5× stop solution (5% SDS and 100 mM EDTA) followed by the addition of 1.4 μL of 10× gel loading solution [50% glycerol, 0.42% (w/v) bromophenol blue, and 0.42% (w/v) xylene cyanol]. Samples were loaded onto a 10% polyacrylamide gel and electrophoresed in 44.5 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA at 150 V for 7 h. The bands were visualized with a Fuji FLA-9000 phosphorimager and quantified in UVI-Tech

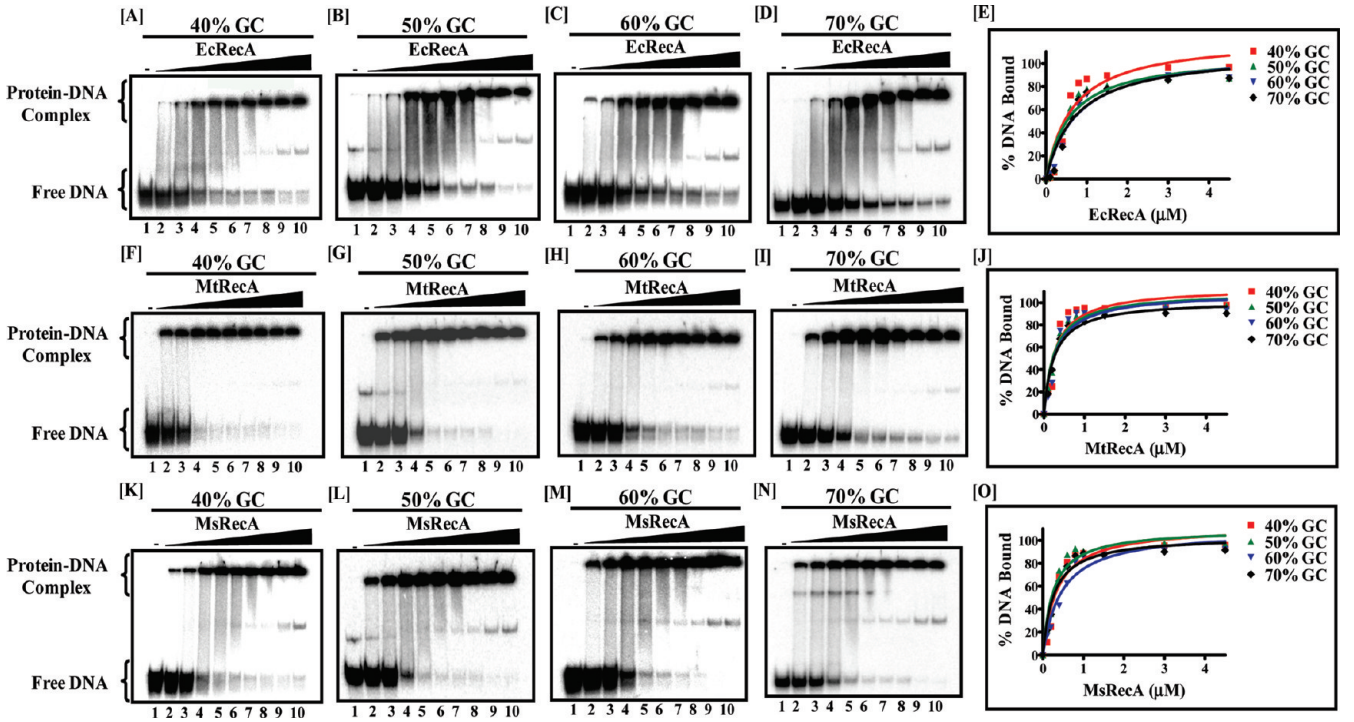

FIGURE 1: RecA proteins bind ssDNA substrates having various percent GC contents to comparable extents. Binding reactions were performed as described in Experimental Procedures. ^{32}P-labeled 83-mer ssDNA (3 μM) having various degrees of GC content [40% (A, F, and K), 50% (B, G, and L), 60% (C, H, and M), and 70% (D, I, and N)] were incubated in the absence (lane 1, A−D, F−I, and K−N) or presence of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 3, and 4.5 μM EcRecA [lanes 2−10, respectively (A−D)], MtRecA [lanes 2−10, respectively (F−I)], or MsRecA [lanes 2−10, respectively (K−N)]. Filled triangles on top of gel images represent increasing concentrations of RecA protein from the indicated source. The positions of free DNA and the RecA−DNA complex are shown on the left-hand side of the gel images. Graphic representation of the extent of binding as a function of increasing concentrations of RecA protein is shown in panels E, J, and O. The data points represent the mean of three independent experiments.

gel documentation station using UVI-BandMap version 97.04 and plotted using Graphpad Prism (version 5.0).

RecA Coprotease Assay. The assay was performed as previously described (*36*). Reaction mixtures contained 20 mM Tris-HCl (pH 7.5 for EcRecA and MtRecA and pH 7.0 for MsRecA), 8 mM $MgCl_2$, 1 mM DTT, 3 mM dATP, 5 μM ssDNA (ODN2 or ODN6), and 2.5 μM RecA from the specified source. After incubation for 5 min at 30 °C, the reaction was initiated by the addition of 5 μM *M. tuberculosis* LexA and the mixture further incubated at 30 °C for 2.5, 5, 10, 15, 20, 30, 45, and 60 min. Reaction was terminated by boiling in SDS-Laemmli sample buffer. Samples were then subjected to 12.5% SDS−PAGE and then stained with silver nitrate. The bands were quantified in UVI-Tech gel documentation station using UVI-BandMap version 97.04 and plotted using Graphpad Prism (version 5.0).

RESULTS

Experimental Design. The relationship between the DNA sequence and the efficiency as well as fidelity of SE promoted by the RecA family of proteins is poorly understood. A number of studies have used ODN substrates to delineate the mechanistic aspects of HR promoted by EcRecA and its homologues (*37*−*39*). Accordingly, we chose to use ODN substrates whose base composition can be readily modified to suit the needs of the individual experiments. The probability of the formation of DNA secondary structure in ODNs was minimized as described previously (*40*).

Binding of RecA Proteins to ssDNA Containing Increasing GC Content. To investigate the effect of GC content on the DNA binding and SE efficiency of mycobacterial RecA proteins, in comparison to the prototype EcRecA, we prepared four types of 83-mer ODNs with increasing GC content (40, 50, 60, and 70%). Here we mention that previous studies have shown that >48-mer ODNs are sufficient to serve as substrates for the formation of active nucleoprotein filaments of the RecA−ssDNA complex as well as strand exchange (*41*). Reaction mixtures contained 20 mM Tris-HCl, 0.1 mM ATPγS, 12 mM $MgCl_2$, 3 μM ^{32}P-labeled 83-mer ssDNA having 40, 50, 60, or 70% GC content, and increasing concentrations of EcRecA, MtRecA, or MsRecA. After incubation at 37 °C for 10 min, samples were subjected to electrophoresis on native polyacrylamide gels as described in Experimental Procedures. At low protein concentrations, we observed a less well-defined protein−DNA complex as evidenced by the smeared distribution of radioactivity between the most retarded bands and free DNA, indicating either the formation of multiple species that likely correspond to different stoichiometric ratios in the protein−DNA complexes or the dissociation of protein−DNA complexes upon electrophoresis (Figure 1, EcRecA, panels A−D; MtRecA, panels F−I; and MsRecA, panels K−N). However, the level of the RecA−ssDNA complexes increased with increasing protein concentration, in a saturable manner. The bands corresponding to free and protein-bound DNA were quantified and expressed as the amount of DNA bound versus increasing concentrations of RecA from the indicated source (Figure 1, EcRecA, panel E; MtRecA, panel J; and MsRecA, panel O). Quantified results suggest that the formation of the RecA nucleoprotein complex plateaus at a DNA:protein stoichiometric ratio of ~3 nucleotide residues per

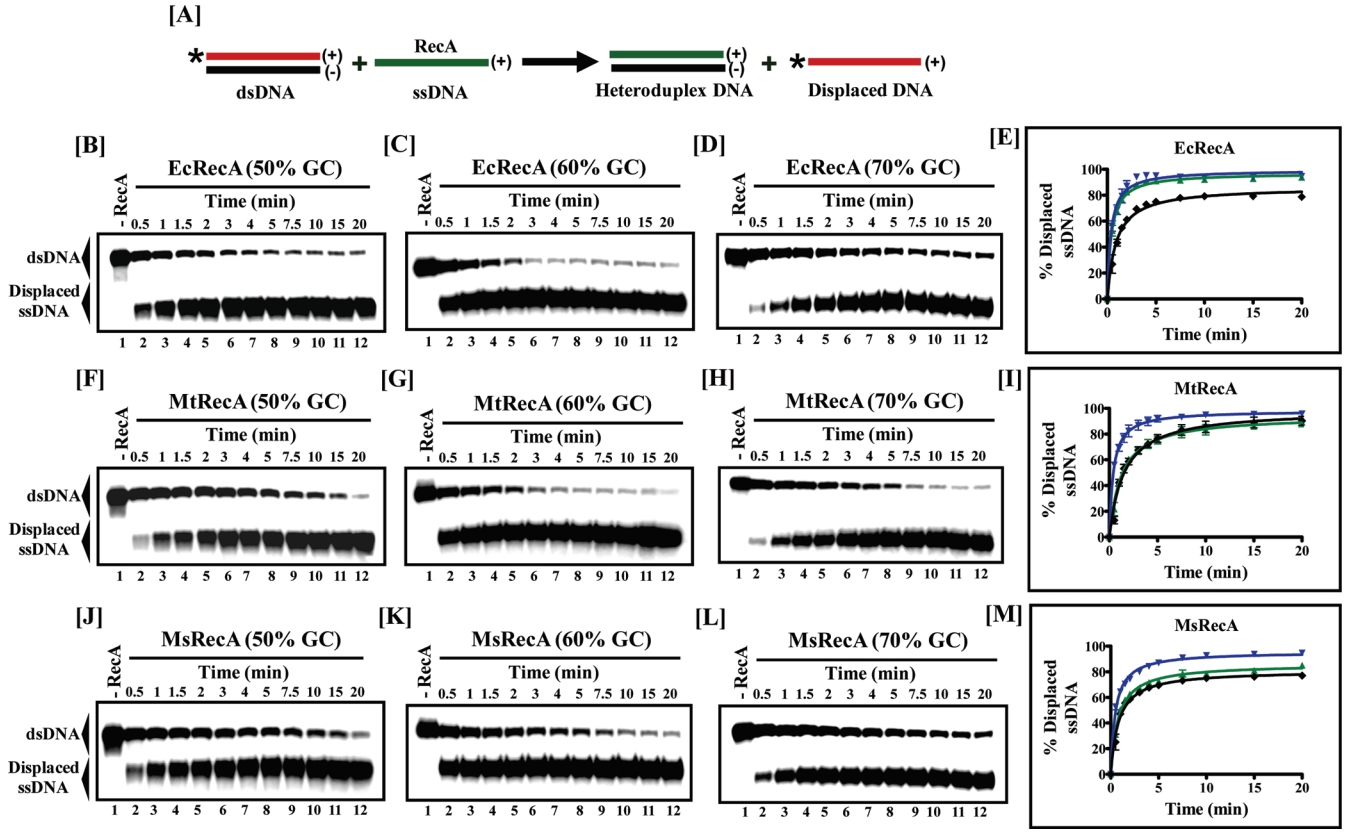

FIGURE 2: Effect of increasing GC content on strand exchange promoted by RecA proteins. (A) Schematic of the experimental design for strand exchange. An asterisk represents the labeled phosphate at the 5′ end. Reactions were performed as described in Experimental Procedures. Reactions were conducted using DNA substrates having 50 (B, F, and J), 60 (C, G, and K), and 70% (D, H, and L) GC content with EcRecA (B−D), MtRecA (F−H), and MsRecA (J−L), respectively. Lane 1 (B−D, F−H, and J−L) shows the control reaction performed in the absence of RecA. Lanes 2−12 (B−D, F−H, and J−L) show strand exchange reactions conducted for 0.5, 1, 1.5, 2, 3, 4, 5, 7.5, 10, 15, and 20 min, respectively. The positions of labeled dsDNA and displaced ssDNA are indicated on the left-hand side of the gel. Panels E, I, and M show the graphic representation of the extent of strand exchange as a function of reaction time: (◆) 70% GC content, (▲) 60% GC content, and (▼) 50% GC content. The data points represent the mean ± standard deviation of four independent experiments.

monomer and occurred with an efficiency of 90−100%. Considered together, these results are consistent with the notion that *E. coli* and mycobacterial RecA proteins share similar binding specificities for ssDNA containing various GC contents. We next examined the efficiency of binding of individual RecA proteins at different pH values to 83-mer ssDNA and 83 bp DNA duplexes containing increasing GC (40, 50, 60, and 70% GC) content. All three recombinases were able to bind single- and double-stranded DNA to similar extents across a wide pH range (pH 5−11) (data not shown).

Effects of Increasing GC Content on Strand Exchange Promoted by RecA Proteins. We next sought to determine the effects of increasing GC content on the kinetics of SE promoted by mycobacterial RecA proteins, in comparison to the effect of EcRecA (*38*, *42*). Reaction mixtures, in an ATP regeneration system, contained 20 mM Tris-HCl, 3 mM dATP, 8 mM $MgCl_2$, 5 μM ssDNA having 50, 60, or 70% GC content, and 2.5 μM RecA, from the indicated source. After incubation for 5 min at 37 °C, the reaction was initiated by the addition of 1 μM ^{32}P-labeled duplex DNA. Aliquots were removed at specified time intervals and processed as described in Experimental Procedures. We chose to perform the assays in the presence of dATP, instead of ATP, because dATP has been shown to confer stability to the RecA nucleoprotein filaments (*43*). The results show that EcRecA-promoted SE, between double- and single-stranded DNA with increasing GC content, was complete within 2 min (Figure 2B−E).

Using the same assay, we compared the effects of increasing GC content on SE promoted by mycobacterial RecA proteins. Like that promoted by EcRecA, the SE promoted by mycobacterial RecA proteins led to complete displacement of the labeled ssDNA from ^{32}P-labeled linear duplex DNA (Figure 2F−M). The bands were quantified from individual gels and plotted as a function of time. Quantified results suggest that, with substrates containing 50 and 60% GC content, the rate of SE promoted by EcRecA appeared to be synchronized over a time span of 0.5−2 min and then plateaued with an efficiency of >90% (Figure 2E). The difference in the rate and yields of SE is surprisingly small between substrates containing 50−60% GC content, indicating that this range is below the threshold value for inhibiting SE. On the other hand, the rate of SE was relatively slow and the yield was smaller with substrates having 70% GC content. By comparison, the kinetic behavior of mycobacterial RecA proteins with all the substrates was similar. The extent of SE promoted by mycobacterial RecA proteins with substrates was comparable with that observed for *E. coli* RecA. The parallel behavior between *E. coli* and mycobacterial RecA proteins suggests that a common mechanism may be responsible with respect to the effect of GC content. On the other hand, it is possible that the differences between *E. coli* and mycobacterial RecA−ssDNA filaments with high GC contents cannot be measured by this assay (see below). Assays performed in the presence or absence of an ATP regeneration system exclude the

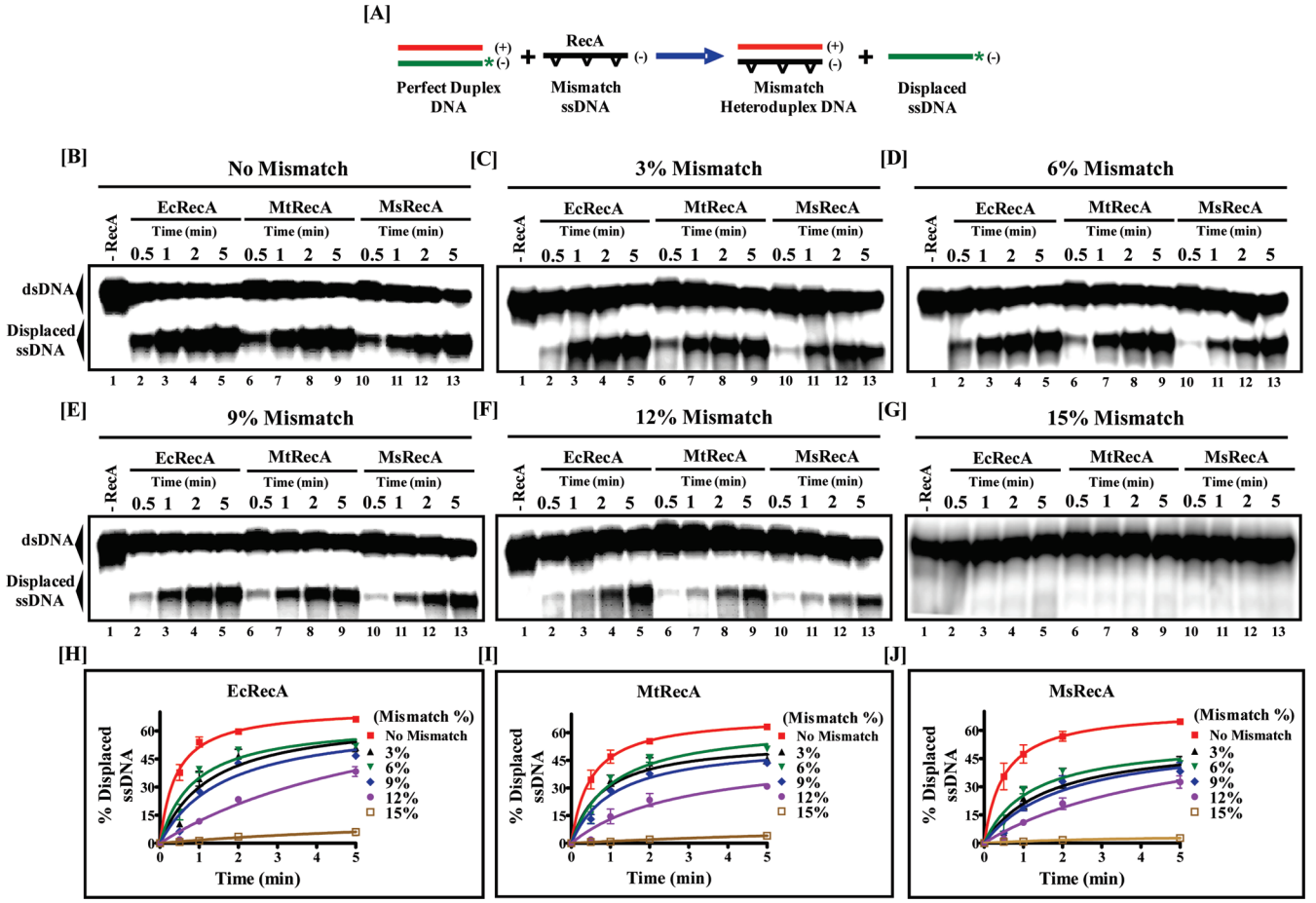

FIGURE 3: Effect of increasing sequence divergence in the donor on strand exchange promoted by RecA proteins. (A) Schematic depicting the experimental design. The triangular "bumps" on ssDNA and mismatch heteroduplex DNA indicate mismatched bases. An asterisk represents the labeled phosphate at the 5′ end. Reactions were performed with ssDNA containing 0, 3, 6, 9, 12, and 15% mismatched bases (B−G, respectively) with increasing concentrations of EcRecA, MtRecA, and MsRecA proteins as described in Experimental Procedures. Lane 1 (B−G) shows the control reaction performed in the absence of RecA. Lanes 2−5, 6−9, and 10−13 (B−G) shows strand exchange reactions conducted for 0.5, 1, 2, and 5 min with EcRecA, MtRecA, and MsRecA, respectively. The positions of labeled dsDNA and displaced ssDNA are indicated on the left-hand side of the gel images. Panels H−J show the graphic representation of the extent of strand exchange promoted by EcRecA, MtRecA, and MsRecA proteins, respectively, as a function of reaction time. The data points represent the mean ± standard deviation of three independent experiments.

possibility of instability of RecA nucleoprotein filaments consequent to ATP hydrolysis (Figure S1 of the Supporting Information).

Effect of DNA Sequence Divergence on Strand Exchange Promoted by RecA Proteins. A number of studies in bacteria, yeast, and humans have shown that the components of the mismatch repair (MMR) system play a crucial role in recombination between moderately divergent (homeologous) sequences (*44*). Genetic crosses between different bacterial species suggest that increasing amounts of base mismatches directly influence the frequency of HR (*45*, *46*). Furthermore, MMR enzymes have been shown to control the fidelity of HR *in vitro* (*47*). Because *M. tuberculosis* is devoid of recognized MMR components, we wished to examine the effect of DNA sequence divergence on SE promoted by mycobacterial RecA proteins (*48*, *49*). In these assays, we used single- and double-stranded DNA (83-mer ssDNA and 83 bp duplex DNA, respectively) in which the percent of base mismatches, ranging from 3 to 15%, was randomly embedded across the length of the donor strand or recipient duplex.

To test the effect(s) of DNA sequence divergence in the context of donor ssDNA, we have examined the kinetics of SE promoted by *E. coli* and mycobacterial RecA proteins between normal duplex and ssDNA containing increasing amounts of sequence divergence. Reaction mixtures contained 20 mM Tris-HCl, 8 mM $MgCl_2$, 3 mM dATP, 5 μM ssDNA (containing either no base mismatch or increasing amounts of base mismatches, ranging from 3 to 15%), and 2.5 μM RecA from the indicated source. SE was initiated by the addition of 1 μM ^{32}P-labeled duplex DNA, and aliquots were removed at specified time intervals and processed as described in Experimental Procedures. Panels B−G of Figure 3 show that the extent of heteroduplex formation by *E. coli* and mycobacterial RecA proteins was affected by the presence of increasing amounts of base mismatches in ssDNA. Quantification revealed that the rate as well as the extent of SE decreased rapidly as the percent of mismatched bases increased (Figure 3H−J). Compared to the homologous control, in the case of donor ssDNA having 12% mismatched bases, the extent of heteroduplex DNA formation decreased to ~50%, whereas the reaction was completely abolished with ssDNA having 15% mismatched bases. Reactions performed in the presence of ATPγS excluded the possibility of instability of RecA nucleoprotein filaments having various degrees of sequence divergence (Figure S2 of the Supporting Information).

To test the effect of sequence divergence in the context of the recipient duplex, we assayed the kinetics of SE promoted by

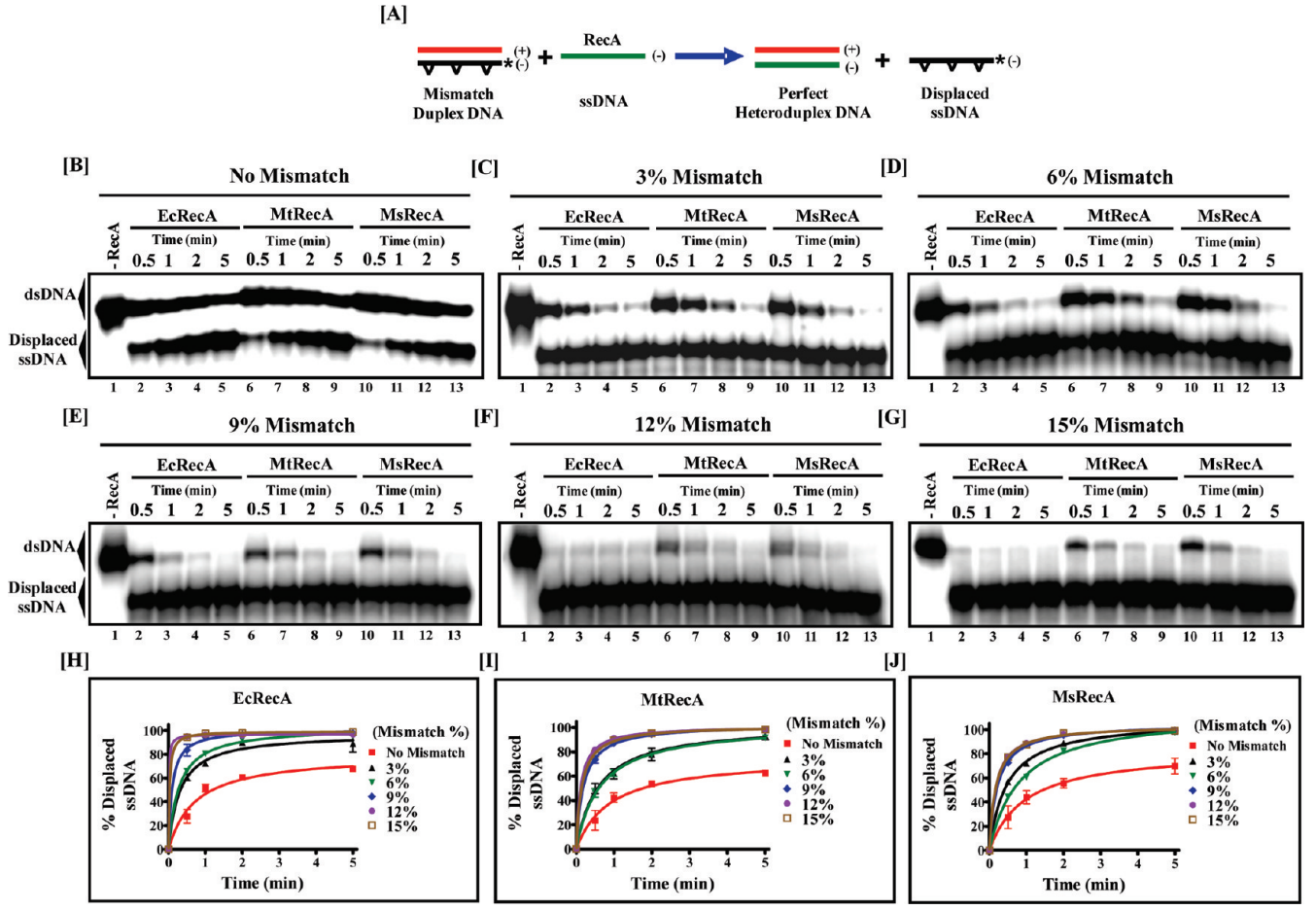

FIGURE 4: Effect of various degrees of sequence divergence in the recipient DNA on strand exchange promoted by RecA proteins. (A) Schematic depicting the experimental design. The triangular bumps on mismatch duplex DNA and displaced ssDNA indicate mismatched bases. An asterisk represents the labeled phosphate at the 5′ end. Reactions were performed as described in Experimental Procedures. Strand transfer reactions were initiated by the addition of ^{32}P-labeled linear duplex DNA having 0, 3, 6, 9, 12, and 15% mismatched bases (B−G, respectively) with RecA from the indicated source. Lane 1 (B−G) shows the control reaction performed in the absence of RecA. Lanes 2−5, 6−9, and 10−13 (B−G) represent reactions conducted for 0.5, 1, 2, and 5 min with EcRecA, MtRecA, and MsRecA, respectively. The positions of labeled dsDNA and displaced ssDNA are indicated on the left-hand side of the gel. Panels H−J show the graphic representation of the extent of strand exchange promoted by EcRecA, MtRecA, and MsRecA proteins, respectively, as a function of reaction time. The data points represent the mean ± standard deviation of three independent experiments.

E. coli and mycobacterial RecA proteins. The assays were performed as described above, except that the noncomplementary strand in the recipient duplex contained increasing amounts of base mismatches (ranging from 3 to 15%). Visual inspection of the gel electrophoresis images suggests that the extent of SE promoted by all three recombinases increased with increasing sequence divergence in the recipient duplex DNA (Figure 4B−G). Additionally, quantification revealed that the rate as well as the extent of heteroduplex formation was directly proportional to the percent base mismatches in the recipient duplex DNA (i.e., higher the percentage of mismatches, greater the SE efficiency) (Figure 4H−J). Using a similar pair of DNA substrates, we compared the effects of increasing amounts of sequence divergence in the context of both single- and double-stranded DNA on SE promoted by *E. coli* and mycobacterial RecA proteins (Figure 5B−G). The results suggest that the extent of SE promoted by all three recombinases was virtually similar, although to a lesser extent in the case of mycobacterial RecA proteins with substrates having 15% sequence divergence (Figure 5H−J).

Effect of GC Content on RecA Coprotease Activity. To further address the effect of GC content, we used the RecA-mediated LexA cleavage assay because of its sensitivity and robustness (*2*, *3*). Reaction mixtures contained 20 mM Tris-HCl (pH 7.5 for EcRecA and MtRecA and pH 7.0 for MsRecA), 8 mM $MgCl_2$, 3 mM dATP, 5 μM ssDNA (ODN2 or ODN6), and 2.5 μM RecA from the specified source. After incubation at 30 °C for 5 min, the reaction was initiated by the addition of 5 μM *M. tuberculosis* LexA and the mixture further incubated at 30 °C for 2.5, 5, 10, 15, 20, 30, 45, and 60 min. The samples were resolved by SDS−PAGE (*36*). As shown in panels A and B of Figure 6, in the presence of ssDNA having either 50 or 70% GC content, EcRecA was able to cleave >80% of MtLexA within 10 min. By contrast, the cleavage promoted by mycobacterial RecA proteins was found to be dependent on the GC content. With ssDNA having 50% GC, the coprotease activity of mycobacterial RecA proteins was comparable to that of EcRecA (Figure 6C−F). However, with ssDNA having 70% GC, the half-maximal rate of LexA cleavage was 2-fold slower than that for ssDNA with 50% GC (Figure 6C−F). Taken together, these results suggest that the coprotease assay was able to uncover functional diversity among between *E. coli* and mycobacterial RecA proteins bound to ssDNA having various degrees of GC content.

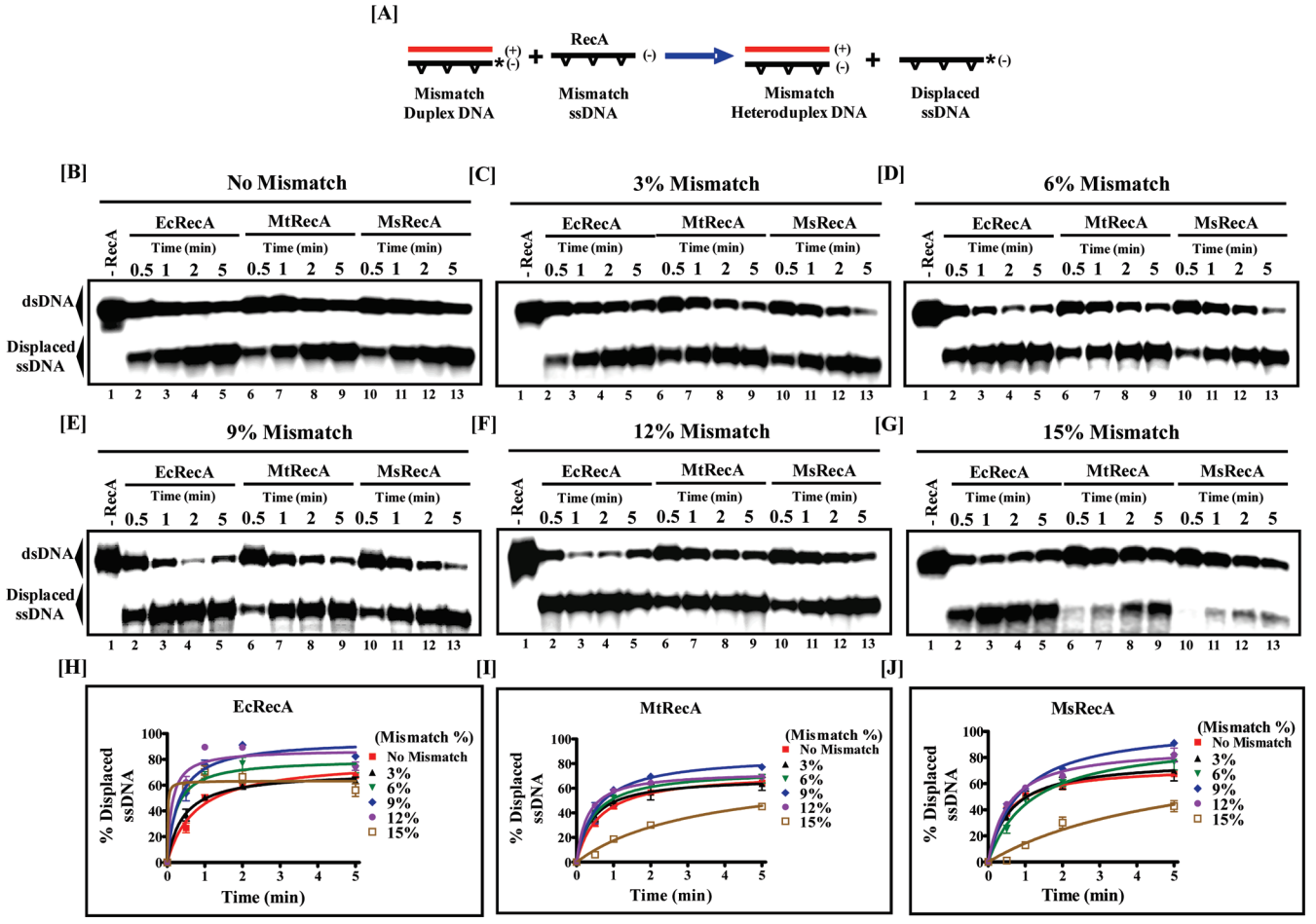

FIGURE 5: Effect of various degrees of sequence divergence in both the donor and recipient on strand exchange promoted by RecA proteins. (A) Schematic depicting the experimental design. The triangular bumps on mismatch duplex DNA and mismatch ssDNA indicate mismatched bases. An asterisk represents the labeled phosphate at the 5′ end. Reactions were performed as described in Experimental Procedures with ssDNA containing increasing amounts (3−15%) of sequence divergence. Strand transfer reactions were initiated by the addition of ^{32}P-labeled linear duplex DNA having 0, 3, 6, 9, 12, and 15% mismatched bases (B−G, respectively) with RecA from the indicated source. Lane 1 (B−G) shows the control reaction performed in the absence of RecA. Lanes 2−5, 6−9, and 10−13 (B−G) represent reactions conducted for 0.5, 1, 2, and 5 min with EcRecA, MtRecA, and MsRecA, respectively. The positions of labeled dsDNA and displaced ssDNA are indicated on the left-hand side of the gel. Panels H−J show the graphic representation of the extent of strand exchange promoted by EcRecA, MtRecA, and MsRecA proteins, respectively, as a function of reaction time. The data points represent the mean ± standard deviation of three independent experiments.

Effect of Nucleotide Cofactors on Strand Exchange Promoted by RecA Proteins. *E. coli* RecA forms an active nucleoprotein filament with ssDNA in the presence of ATP (*2*, *3*). However, the precise role of ATP hydrolysis in strand exchange promoted by RecA and its homologues remains elusive, because the SE reaction can also be conducted in the presence of ATPγS (ref *3* and references cited therein). Previous studies have shown that the ratio of ATP concentration to ADP concentration strongly influences the extent of SE promoted by EcRecA (*50*, *51*). To assess the effect of varying the ratios of ATP to ADP on SE promoted by mycobacterial RecA proteins, we performed assays in which the total concentration of the nucleotide cofactor remained constant (3 mM) but the percentage of ADP in the pool was varied from 0 to 60%. In the absence of ADP, RecA proteins catalyzed an efficient SE, consistent with the results described above (Figure 7A−D). Increasing the concentration of ADP in the reaction mixture tended to abolish the overall DNA SE reaction. The patterns observed for SE promoted by mycobacterial RecA proteins, as deduced by the percentage of displaced ssDNA, were similar to those seen with EcRecA (*50*−*52*). SE was abolished when the starting nucleotide pool contained ~50% ADP. These results are in accord with previous observations on the relative effects of the ADP:ATP ratio on SE promoted by EcRecA (*50*). By comparison, the difference in the half-maximal inhibition was ~10% between EcRecA and mycobacterial RecA proteins. Similar results were obtained when dATP and dADP were used in place of ATP and ADP as the nucleotide cofactors (data not shown).

DISCUSSION

In this study, we show that the extent of ssDNA binding and SE promoted by mycobacterial RecA proteins with DNA substrates having various degrees of GC content was comparable with that observed for *E. coli* RecA. However, the rate and extent of SE promoted by these recombinases were proportional to the density of base mismatches in the donor ssDNA. Conversely, the rate and extent of SE were greatly enhanced with various degrees of sequence divergence in the recipient DNA. On the other hand, when mismatched bases were present in both the donor and recipient, strand exchange was not significantly affected, although SE promoted by mycobacterial RecA proteins was affected only

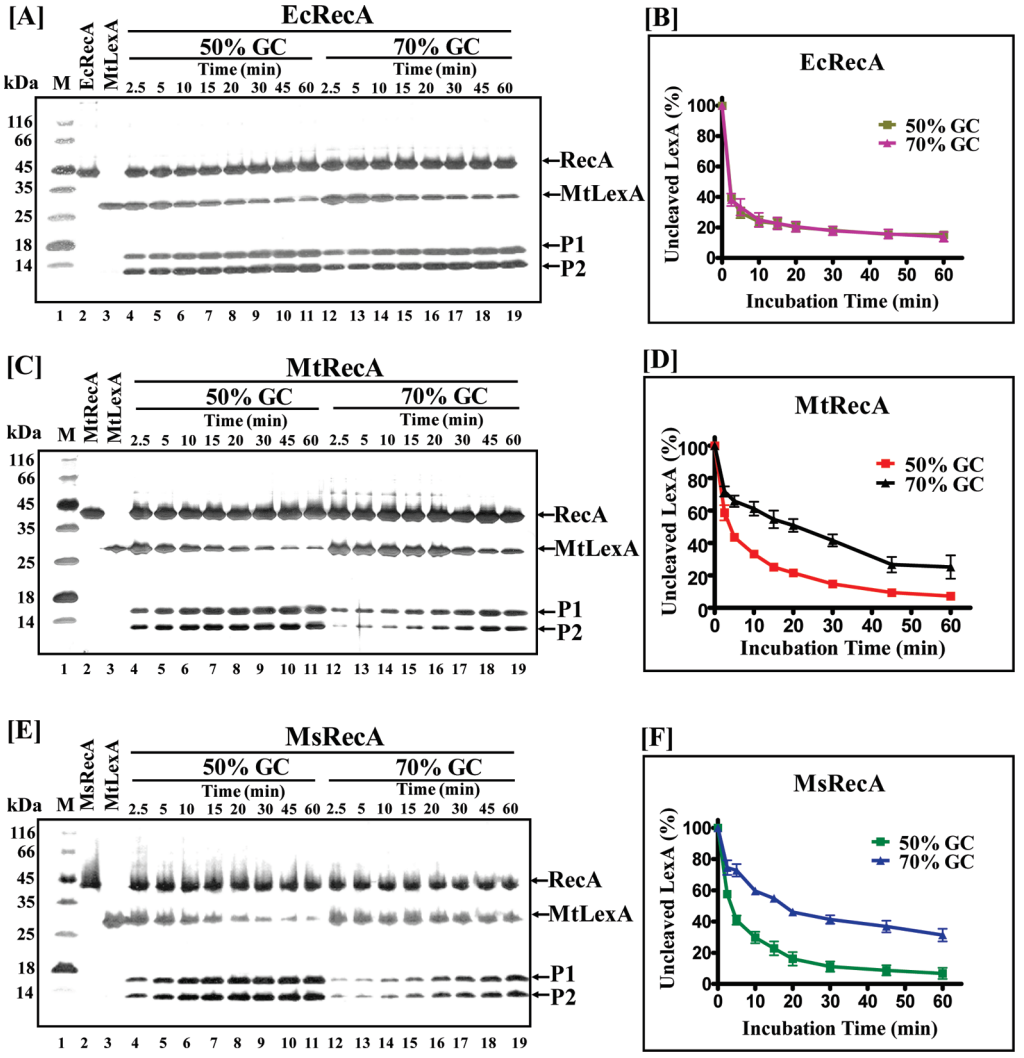

FIGURE 6: Effect of GC content on RecA coprotease activity. Reactions were performed using ssDNA containing 50 and 70% GC content with EcRecA, MtRecA, and MsRecA proteins (A, C, and E, respectively) as described in Experimental Procedures. Lane 1 (A, C, and E) shows SDS−PAGE standard molecular mass markers. Lane 2 (A, C, and E) shows RecA from the indicated source and lane 3 MtLexA; lanes 4−11 (A, C, and E) and lanes 12−19 (A, C, and E) show complete reaction mixtures incubated for 2.5, 5, 10, 15, 20, 30, 45, and 60 min, respectively, with ssDNA having either 50 or 70% GC content. The positions of RecA, LexA, and cleavage products (P1 and P2) are indicated on right-hand side of the gel images. Panels B, D, and F show the graphic representation of the amount of uncleaved MtLexA as a function of reaction time in the presence of EcRecA, MtRecA, and MsRecA, respectively. The data points represent the mean ± standard deviation of three independent experiments.

moderately at higher levels of sequence divergence. Additional analysis and comparison between *E. coli* and mycobacterial RecA proteins revealed important differences. When those data are considered together, this study extends our understanding of HR in mycobacteria and provides evidence of the possible existence of functional diversity among *E. coli* and mycobacterial RecA nucleoprotein filaments.

Several lines of evidence suggest that the formation of an active RecA nucleoprotein filament is modulated at least by two structural features of ssDNA (*2*, *3*). First, DNA secondary structure in general impedes the contiguous binding of RecA to ssDNA (*53*−*55*). Accordingly, RecA binds preferentially to poly(dT), because of its lack of secondary structure (*2*, *3*, *56*, *57*). Second, although the recombinases bind ssDNA primarily along the sugar phosphate backbone in a sequence-independent manner, several studies have shown that EcRecA and Rad51 proteins bind with high affinity to certain types of DNA sequences (*2*, *3*, *58*). An *in vitro* selection approach shows that EcRecA and yeast Rad51 proteins bind preferentially to GT-rich sequences (*10*−*13*). Using a variety of techniques, it has been shown that RecA and Rad51 proteins specifically bind di- or trinucleotide repeats such as CT-, GT-, and CA-repeating (*10*−*13*) and TTT-, CCC-, TCC-, and TAC-repeating sequences (*57*, *59*, *60*) in ssDNA. These and other findings have led to the inference that base-stacked purine-rich sequences hinder binding of RecA to ssDNA (*2*, *3*, *57*, *60*). A systematic assessment of the binding and coprotease activity of EcRecA using trinucleotide repeat sequences suggested a strong preference for the TGG-repeating sequence (*59*). These results are consistent with the crystal structure of the EcRecA nucleoprotein filament, which shows that the DNA in this complex has a triplet structure with large extensions occurring between triplets (*61*).

The biological effects of native DNA on the binding of RecA to ssDNA and activities of the resulting nucleoprotein filaments are poorly understood. So far, most studies on the mechanistic aspects of HR promoted by the RecA and Rad51 family of proteins have used plasmid- or phage-derived substrates. Alternatively, simple model substrates for the elucidation of various

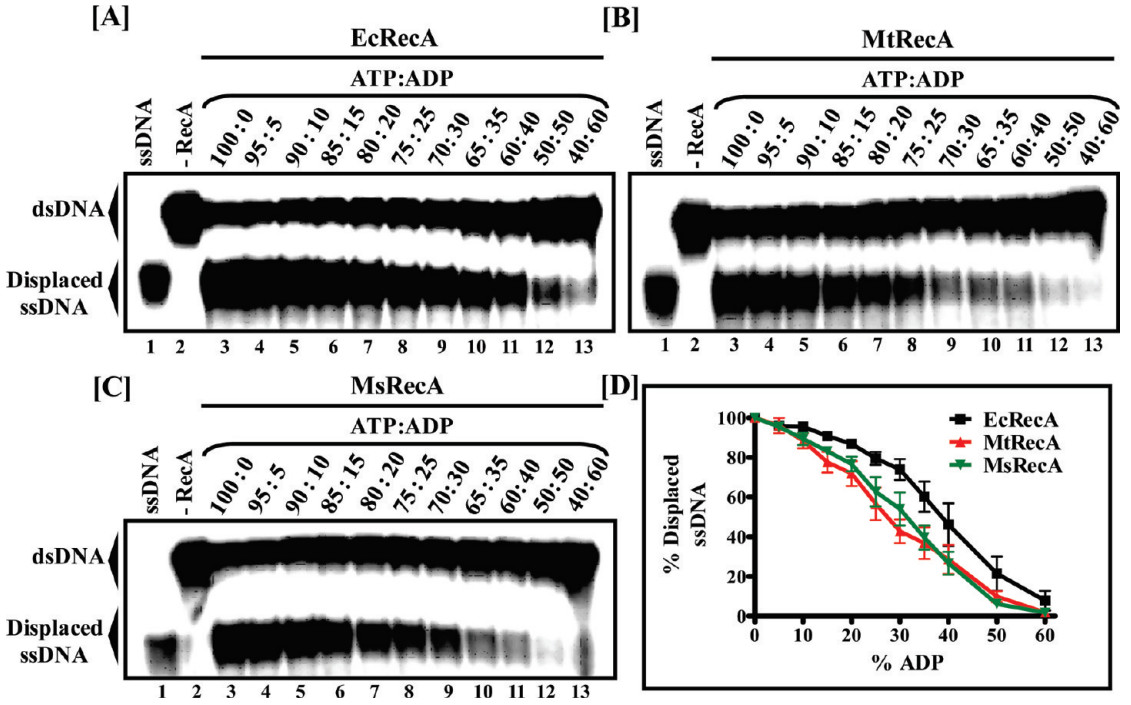

FIGURE 7: Effect of varying ratios of ATP to ADP on strand exchange promoted by RecA proteins. Reactions were performed as described in Experimental Procedures. Nucleoprotein filaments of EcRecA (A), MtRecA (B), and MsRecA (C) were formed with ssDNA having 50% GC content in the presence of the indicated ATP:ADP ratios: lane 1 (A−C), ssDNA marker; lane 2 (A−C), control reaction performed in the absence of RecA. The position of labeled dsDNA and displaced ssDNA is indicated on the left. Panel D shows a graphical representation of the extent of inhibition of strand exchange as a function of increasing ADP concentrations. The percent activity (shown as % displaced ssDNA) in the absence of ADP (lane 3, A−C) has been normalized to 100% reaction. The data points represent the mean ± standard deviation of three independent experiments.

stages of HR entail ODNs, mimicking the native DNA. In this study, to correlate the binding affinity with the biological activity of mycobacterial RecA proteins with their native DNA sequences, we used a series of ODNs containing various degrees of GC content (from 40 to 70%). Interestingly, we observed that the extent of ssDNA binding and SE promoted by mycobacterial RecA proteins with DNA substrates having various degrees of GC content was comparable with that observed for *E. coli* RecA.

The success of HR depends on the accurate search for homologous sequences by the RecA and Rad51 family of proteins (*2*−*6*, *26*). Numerous studies in a number of model organisms have shown that the rate of HR between divergent sequences is lower than that between identical sequences (*2*−*6*). Similarly, studies in prokaryotes and eukaryotes have demonstrated that MMR proteins play important roles in blocking HR between diverged homologous sequences. *In vitro* studies suggest that spontaneous branch migration through regions of heterologous DNA is inefficient (*62*). The effect of mismatches on strand exchange promoted by RecA *in vitro* has been studied previously (*63*−*70*). Strand exchange between DNA from bacteriophages fd and M13, whose sequences are diverged by 3%, is nearly normal (*63*). However, EcRecA-mediated strand exchange stalls in short regions or long regions of heterologously double-stranded DNA (*63*−*65*) and nucleotide mismatches (*66*, *70*). Previous studies have shown that EcRecA does not significantly discriminate between perfect and imperfect matches in the double-stranded DNA, until the fraction of mismatches approaches 10% (*64*, *71*).

The molecular mechanism of HR in mycobacteria is poorly understood. Because *M. tuberculosis* is devoid of recognized MMR components (*48*, *49*), we investigated the effect of mismatched bases on SE by mycobacterial RecA proteins. Our results with substrates containing various degrees of sequence divergence in the donor, recipient, or both are striking. The level of strand exchange progressively decreased with the increasing density of mismatched bases in the donor, whereas exchange was enhanced with the increasing density of mismatched bases in the recipient. Our data are consistent with previous findings for the low fidelity of EcRecA-promoted recognition of homology, SE, and stability of synaptic intermediates formed between ODNs having sequence divergence (*66*). We note that the MMR pathway averts genetic exchange between homeologous sequences in bacteria (*45*) and between diverged repetitive sequences in the *E. coli* chromosome and viruses (*46*, *72*). The precise mechanism by which *E. coli* and mycobacterial RecA proteins modulate homeologous recombination remains unclear. However, mismatched bases may interfere with HR between donor and recipient sequences in the following two ways. First, the mismatch may interfere with the homology search or invasion of the homeologous duplex by the nucleoprotein filament, resulting in a decrease in the level of formation of heteroduplex DNA. Second, the mismatch may alter the stability of the heteroduplex DNA. In the case of the donor, the inhibitory effect of the mismatch on SE may arise from the fact that the mismatches may interfere with the homology search and/or strand invasion of the homeologous duplex. Consistent with this notion, DNA duplexes bearing mismatched bases lead to its unwinding and, therefore, enhance the rate and extent of strand exchange (*62*). This view is in line with current thinking about heteroduplex DNA rejection in yeast (*26*). How is inhibition of SE between mismatched base pairs achieved *in vivo*? Consistent with our data, one study has shown that 10−12% of DNA sequence divergence restricts initiation of recombination in *M. tuberculosis* (*73*). On the basis of the foregoing, we are inclined to believe that the activities

of *E. coli* and mycobacterial RecA proteins are nearly indistinguishable from the perspective of SE and may have an inherent capacity to control the fidelity of HR even in the absence of an MMR pathway.

Emerging evidence suggests that the biochemical paradigms established with *E. coli* RecA are insufficient for fully understanding HR in other bacteria. A few examples have to be mentioned, however. First, *Deinococcus radiodurans* RecA protein binds preferentially to double-stranded DNA and promotes an inverse reaction of strand transfer (*74*, *75*). Second, *D. radiodurans* RecA (*74*, *75*) and mycobacterial RecA proteins (*29*, *30*) promote ATP- and dATP-dependent reactions and SE, respectively, with distinctly different pH profiles. On the other hand, hRad51 and hRad54 proteins work together to bypass mismatched base pairs during strand exchange (*76*). Functional diversity among *E. coli* and mycobacterial RecA nucleoproteins filaments was apparent in the presence of varying ratios of ATP and ADP (Figure 7). In these experiments, lower modulator ATP:ADP ratios exerted an inhibitory effect on SE promoted by mycobacterial RecA, compared to EcRecA. Furthermore, the mycobacterial SSB proteins, like the *E. coli* SSB, modulate the function of their cognate RecA but have very different effects (*29*, *30*, *35*). For example, mycobacterial SSB proteins physically interact with their cognate RecA in solution (*35*). The significance of this study with respect to RecA−DNA recognition is further emphasized, in particular, in the coprotease activity. The overall three-dimensional structures of *E. coli* and mycobacterial RecA proteins are very similar (*77*−*80*). This suggests that despite the observed structural redundancy between EcRecA and mycobacterial RecA proteins, these proteins are not functionally similar.

In summary, several details of this work suggest that specific DNA sequence in RecA nucleoprotein filaments is linked to processes of HR and SOS response and provides insights into the existence of some degree of functional diversity among RecA nucleoprotein filaments. Building on these findings, we posit structural diversity among (as well as within) RecA nucleoprotein filaments. Therefore, it would be interesting to determine the crystal structure of RecA bound to ssDNA having different base compositions. Finally, we describe the effect of homeologous sequences in the context of the donor, recipient, and donor and recipient on SE and speculate that the context-dependent sequence divergence may have important implications for the emergence of new genetic variants.

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SUPPORTING INFORMATION AVAILABLE

Sequences of oligonucleotides used in this study (Table S1), effect of the ATP regeneration system on strand exchange promoted by RecA proteins (Figure S1), and effect of sequence divergence on strand exchange promoted by RecA proteins in the presence of ATPγS (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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