A RecA Homologue in *Ustilago maydis* That Is Distinct and Evolutionarily Distant from Rad51 Actively Promotes DNA Pairing Reactions in the Absence of Auxiliary Factors†

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ABSTRACT: Two RecA homologues have been identified to date in *Ustilago maydis*. One is orthologous to Rad51 while the other, Rec2, is structurally quite divergent and evolutionarily distant. DNA repair and recombination proficiency in *U. maydis* requires both Rec2 and Rad51. Here we have examined biochemical activities of Rec2 protein purified after overexpression of the cloned gene. Rec2 requires DNA as a cofactor to hydrolyze ATP and depends on ATP to promote homologous pairing and DNA strand exchange. ATPγS was found to substitute for ATP in all pairing reactions examined. With superhelical DNA and a homologous single-stranded oligonucleotide as substrates, Rec2 actively promoted formation and dissociation of D-loops. When an RNA oligonucleotide was substituted it was found that R-loops could also be formed and utilized as primer/template for limited DNA synthesis. In DNA strand exchange reactions using oligonucleotides, we found that Rec2 exhibited a pairing bias that is opposite that of RecA. Single-stranded oligonucleotides were activated for DNA strand exchange when attached as tails protruding from a duplex sequence due to enhanced binding of Rec2. The results indicate that Rec2 is competent, and in certain ways even better than Rad51, in the ability to provide the fundamental DNA pairing activity necessary for recombinational repair. We propose that the emerging paradigm for homologous recombination featuring Rad51 as the essential catalytic component for strand exchange may not be universal in eukaryotes.

Repairing damaged DNA by homologous recombination is a universal mechanism for restoring integrity and maintaining stability of the genetic material. In the emerging view, a DNA duplex suffering a double-strand break is resected to reveal a protruding single-stranded stretch which is activated for strand invasion and information transfer from a homologous DNA sequence by the action of a homologous pairing protein (*2*). RecA protein of *Escherichia coli*, the prototype for this class of recombination proteins, loads onto single-stranded DNA to form a nucleoprotein filament which is the active principle in the search for DNA sequence homology (*3*, *4*). Pairing commences with the formation of a metastable complex of single-stranded and duplex DNA, followed by homology-dependent association between the single-stranded DNA and corresponding homologous duplex region unrestricted by topological barriers. In the absence of topological constraints, these paranemically associated complexes are processed further to heteroduplex joint molecules with Watson−Crick base pairing. The overall reaction has been studied using a variety of ingenious assays that monitor the dynamics of strand exchange as the invading single strand is transferred to the duplex with concomitant displacement of the corresponding resident strand.

Proteins structurally related to RecA including the Rad51 protein of *Saccharomyces cerevisiae* and the Rad51 and Dmc1 homologue of humans have been shown to promote homologous pairing and DNA strand exchange by mechanisms generally in accord with the RecA paradigm (*5*, *6*). However, detailed analyses have revealed intriguing mechanistic differences among all of these well-characterized proteins which likely could reflect important differences in their biological functions. The multiplicity of RecA homologues in eukaryotes and the accumulating evidence indicating an absence of functional redundancy also suggest diversification into different biological roles (*7*). In *S. cerevisiae*, the strand exchange reaction promoted by Rad51 alone is weak compared with RecA. However, it can be enhanced by addition of other proteins including the heterodimer of Rad55−Rad57, which are also structurally related to RecA, and by RPA, Rad52, and Rad54 (*8*−*15*). In light of these findings, a model that has emerged holds that Rad51 is a member of a multicomponent recombinational repair complex providing a strand pairing function whose activity is augmented and facilitated by the other components (*16*).

Ustilago maydis is an extremely radiation resistant fungus that is evolutionarily distant from *S. cerevisiae* and *S. pombe*. Two RecA homologues have been identified in *U. maydis*. One is orthologous to Rad51 (*17*) while the other, encoded by the *REC2* gene (*18*), is much more divergent, more than

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Table 1: Oligonucleotide Substrates Used in This Study

oligo-nucleotide	description	length (nt)	sequence (5′ → 3′)
RB1	5′ end homology substrate [no. 1 (*23*)]	63	ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCA-AAAATGACCTCTTATCAAAAGGA
RB2	3′ end homology substrate [no. 2 (*23*)]	63	TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCT-TAATTGCTGAATCTGGTGCTGT
RB3	[no. 45 (*23*)]	31	ACAGCACCAGATTCAGCAATTAAGCTCTAAG
RB4	complement of RB3 [no. 55 (*23*)]	31	CTTAGAGCTTAATTGCTGAATCTGGTGCTGA
RB5	[no. 5 (*23*)]	32	CCATCCGCAAAAATGACCTCTTATCAAAAGGA
RB6	[no. 6 (*23*)]	32	TCCTTTTGATAAGAGGTCATTTTTGCGGATGG
RB7	AT-rich strand exchange substrate [AT(−) (*21*)]	83	AAATGAACATAAAGTAAATAAGTATAAGGATAATACAAAAT-AAGTAAATGAATAAACATAGAAAATAAAGTAAAGGATATAAA
RB8	complement of RB7	83	TTTATATCCTTTACTTTATTTTCTATGTTTATTCATTTACTTA-TTTTGTATTATCCTTATACTTATTTACTTTATGTTCATTT
RB9	identical to M13 nt 182−267 [W16(−) (*21*)]	83	TTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATT-GCTGAATCTGGTGCTGTAGCTCAACATGTTTTAAATATGCAA
RB10	RNA identical to M13 nt 235−264	30	UUGAUAAGAGGUCAUUUUUGCGGAUGGCUU
RB3 + RB4	31 bp duplex substrate		
RB1 + RB6	5′ ssDNA tailed substrate		
RB2 + RB5	3′ ssDNA tailed substrate		
RB7 + RB8	AT rich duplex		

twice the size of Rad51, and without a structural equivalent in the databases. Both genes are expressed in mitotic cells. There is no redundancy in their functions as mutation in either results in loss of DNA repair and recombination proficiency. Genetic analysis indicates epistatic interaction between the two genes in certain recombination and repair functions, but some measure of independent activity in other functions (*17*). The phenotype of certain *rec2* deletion mutants created by gene disruption technology implies Rec2 protein is involved in chromosome segregation, mutagenesis, and meiosis in addition to recombination and repair. The N-terminal portion of the protein appears to constitute an interacting domain which can interfere in a dominant negative fashion with one or more other proteins to carry out these various functions (M. Kojic, C. Thompson, and W.K.H., unpublished material).

We are interested in understanding the role of Rec2 in governing chromosome dynamics and maintaining genomic stability and in learning about the relationship between the Rec2 and Rad51 proteins. Homologous pairing and DNA strand exchange activity associated with Rec2 protein was first discovered in protein preparations[1] purified by traditional means from cell-free extracts of *U. maydis* (*19*). Initial findings indicated that several of the pairing reactions catalyzed resembled those promoted by RecA protein, but with some notable differences including the apparent opposite polarity of DNA strand transfer (*20*). Unfortunately, the Rec2 protein contained in those preparations was found to be a proteolyzed fragment of the full length gene product raising concern about the extent to which the conclusions could generalized (*1*). Therefore, we have been interested in investigating the biochemical properties of the intact protein. Here we report a survey of findings on the properties and reactions promoted by Rec2 using full-length protein obtained after overexpression of the cloned gene in *E. coli*. These results offer new insights into the mechanism of recombinational repair promoted by Rec2 and suggest a pathway in mitotic cells for DNA pairing and strand invasion that could be independent of Rad51.

MATERIALS AND METHODS

Reagents. All chemicals were reagent grade and prepared with deionized water. Nucleotides were purchased from Sigma-Aldrich (St. Louis MO). Restriction enzymes, RecA protein, and T4 polynucleotide kinase were from New England Biolabs, Inc., (Beverly, MA). RNase A was from Worthington Biochemical Corp. (Lakewood, NJ), and stock solutions were held in boiling water for 10 min to inactivate any contaminating DNase. RNase H was from Gibco/BRL (Rockville, MD). Nickel-nitrilotriacetate conjugated with alkaline phosphatase (Ni-NTA AP conjugate) was obtained from Qiagen, Inc. (Valencia, CA). Oligodeoxyribonucleotides were synthesized and purified by HPLC at the Cornell University Bioresource Center (Ithaca, NY) and are described in Table 1. Sequences were based on those reported and utilized previously in studies on RecA, Rad51, and Dmc1 (*21*−*23*). Oligonucleotides were labeled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase. Duplex oligonucleotides were prepared by mixing labeled oligomers with a 2-fold molar excess of unlabeled complementary oligomer in solutions containing 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 M NaCl, heating to 95° for 5 min, then annealing at 65° for 45 min. Duplex oligomers were then purified by gel electrophoresis in 12% polyacrylamide gels containing 50 mM Tris, 50 mM boric acid, and 1 mM EDTA. DNA bands were localized by autoradiography, gel slices with the appropriate DNA were minced and overlaid with a solution containing 0.5 M ammonium acetate, pH 7.0, 10 mM EDTA, and 0.1% SDS,[2] and DNA was recovered after diffusion. DNA was then extracted with a solution of phenol and chloroform and recovered after precipitation from ethanol. Plasmid DNA was prepared after alkaline lysis of cells with SDS using a proprietary solid matrix binding system (Qiagen,

[1] The reader is advised that the proteolytic form of Rec2 purified originally from cell extracts of *U. maydis* was known as rec1 protein at the time before recombinant DNA technology enabled identification of the structural gene as *REC2* (*1*). W.K.H. regrets the confusion stemming from this terminology.

[2] Abbreviations: ATPγS, adenosine 5′-*O*-(thiotriphosphate); bp, base pair; BSA, bovine serum albumin; dsDNA, double-stranded DNA; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; nt, nucleotide; NTA, nitrilotriacetate; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA.

Inc., Valencia CA) and purified further by velocity sedimentation through a sucrose gradient (5 to 20%) in 10 mM Tris-HCl, 1 mM EDTA, 0.25 M NaCl at 28 000 rpm for 20 h in an ultracentrifuge using the Sorvall AH629 swinging bucket rotor. M13 form I DNA was prepared in the same way from cells of *E. coli* strain K37 (HfrC) infected at a multiplicity of 20 and cultured for 3 h after addition of chloramphenicol to 60 μg/mL. DNA concentrations were determined spectrophotometrically using extinction coefficients $\epsilon^{260} = 6.8$ mM^{-1} cm^{-1} for duplex DNA and $\epsilon^{260} = 9.5$ mM^{-1} cm^{-1} for single-stranded DNA, respectively, and are expressed as moles of nucleotide unless otherwise indicated. Plasmid pT7-*ACT* (3.3 kbp) is a pT7-1 derivative (USB, Cleveland OH) containing a 538 bp fragment of the *S. cerevisiae* actin gene under control of a bacteriophage T7 promoter. Radiolabeled RNA was prepared by in vitro runoff transcription of the actin gene in pT7-*ACT* using T7 RNA polymerase and [α-^{32}P]GTP as described (*24*) after appropriate cleavage of the template DNA and was purified by gel filtration through Sepharose CL-6B. RNA concentrations were determined from the specific activity of the radiolabel. Plasmid pT7-*ACT* DNA and radiolabeled actin RNA were generously provided by Dr. Beate Schwer (Cornell University Weill Medical College). Oligoribonucleotide RB10 was synthesized by Oligos, Etc., (Wilsonville, OR). *E. coli* strain BL21(DE3) containing plasmid pJM126 expressing all three subunits of yeast replication protein A (RPA) was from Dr. Steven Brill (Rutgers University, Piscataway, NJ). RPA was purified as described (*25*).

Strains and Plasmids. *U. maydis* strains from the Cornell Medical College collection that were utilized included UCM5 (*leu1-1 ade1-1 a2b2*) and UCM174 (*rec2-1 leu1-1 ade1-1 a2b2*) in which *leu* and *ade* indicate mutation in biosynthetic genes for leucine and adenine, respectively, and *a* and *b* indicate mating type loci. Strains were transformed with pCM201 and pCM582 and tested for resistance to killing by methyl methanesulfonate (MMS) using media and procedures as described (*17*). For overproduction of the cloned Rec2 protein in *E. coli*, the *REC2* gene was cloned under the control of the T7 promoter in pET16b (Novagen, Inc., Madison WI). pCM538 is pET16b containing a 2.5 kbp DNA fragment with the *REC2* open reading frame engineered with an *Nde*I site at the initiation methionine fused in frame with a leader sequence encoding a decahistidine (His_{10}) stretch. pCM578 is pCR2.1 (Invitrogen, Carlsbad CA) containing the *His_{10}−REC2* open reading frame fusion downstream of a 0.5 kbp fragment (*pNAR*) from the promoter region of the *U. maydis* nitrate reductase gene (*26*). The *pNAR* fragment was amplified by PCR using primers designed to place an *Nco*I and an *Xba*I site at either end. After polymerase chain reaction it was inserted into plasmid pCR2.1. This plasmid was opened by cutting with *Nco*I and *Xho*I, and the *His_{10}−REC2* fusion was introduced as a 2.6 kbp *Nco*I−*Xho*I fragment to yield pCM578. The composite *pNAR His_{10}−REC2* element was moved as a 3.1 kbp *Eco*RI fragment into the *Eco*RI site of pBluescript II SK$^+$ (Stratagene, La Jolla CA) to yield pCM579. The element was reisolated from pCM579 as a *Apa*I−*Xba*I fragment and inserted into pCM201, a *U. maydis* autonomously replicating shuttle vector (*27*) to yield pCM582.

Purification of Rec2 Protein. All procedures were conducted at 0−4° and all centrifugations were at 15 000 rpm for 30 min unless otherwise indicated. Protein concentrations were determined as described (*28*). Rec2 was purified as the fusion protein with the N-terminal His_{10}-affinity tag from *E. coli* strain HMS174 carrying plasmids pCM538 and pLysS. Cells were cultured in LB medium containing 34 μg/mL chloramphenicol and 50 μg/mL carbenicillin at 37 °C. When the optical density reached 0.4 at λ_{600nm} isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM and cells were harvested by centrifugation after 2 h. The cell pellet was resuspended in 40 mL of a lysis buffer containing 25 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 0.1% Triton X-100, 10% glycerol. After addition of 0.5 mL of protease inhibitor cocktail solution P8849 (Sigma-Aldrich, St. Louis, MO), cells were ruptured by four bursts of sonication of 1 min duration with intermittent intervals of cooling on ice. Insoluble material was collected by centrifugation at 15 000 rpm for 30 min in a Sorvall SS34 rotor, resuspended in buffer B (25 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 10% glycerol) containing 2 M guanidine-HCl with the aid of a motorized Dounce homogenizer. The suspension was clarified by centrifugation, the supernatant discarded, and the insoluble pellet resuspended in buffer B containing 6 M guanidine-HCl. After another centrifugation to remove insoluble material, imidazole was added to the supernatant to a final concentration of 20 mM, and the solution was loaded onto a column (13 mL bed volume) of Ni-NTA (nickel-nitrilotriacetate agarose, Qiagen, Inc., Valencia CA) previously prepared by washing with 100 mL of the same buffer. After loading, the column was washed with 100 mL of the loading buffer, followed by a wash with buffer containing 100 mM imidazole. Fractions containing Rec2 protein were collected and pooled and then diluted to adjust the protein concentration to 50 μg/mL, the guanidine-HCl to 4 M and EDTA to 1 mM. The solution was dialyzed at 4° against buffer R (25 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1 mM EDTA, 2 mM DTT, and 0.05% Nonidet NP-40) containing 3.3 M guanidine-HCl. The guanidine-HCl concentration of the dialysis solution was decreased by 25% every 10 h by stepwise dilution with buffer R until the concentration reached 0.75 M. The protein solution was then dialyzed against buffer A (25 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, and 10% glycerol). This solution (Fraction I) was loaded onto a heparin-Sepharose column (5 mL bed volume, HiTrap Heparin, Pharmacia Biotech) which was then washed with 15 mL of buffer A. The protein was eluted with a 50 mL linear gradient (0 to 1 M) of NaCl. Fractions (1 mL) were collected and those containing the peak of ATPase eluting at ~0.5 M NaCl were pooled and dialyzed against buffer A (Fraction II). This fraction was loaded onto a MonoS 5/5 FPLC column (1.7 mL bed volume, Pharmacia Biotech) which was washed with 20 mL of buffer A containing 0.5 M NaCl followed by a 20 mL linear gradient (0.5 to 1.5 M) of NaCl. Fractions containing activity were pooled, dialyzed against buffer A (Fraction III), and stored at −80°. Generation of tryptic peptides, HPLC fractionation and protein sequencing were performed by the Protein/DNA Technology Center of the Rockefeller University (New York, NY). For western blot analysis protein samples were electrophoresed on a 10% polyacrylamide gel, and electrotransferred to PVDF membrane. Development was achieved by soaking the membrane in a 1000-fold dilution of alkaline-phosphatase conjugated Ni-NTA (Qiagen, Inc., Valencia, CA). Alterna-

tively blots were developed using antibodies raised to a 12 kDa fusion protein composed of a bacteriophage T7 leader sequence and a 96 amino acid sequence from the C-terminus of Rec2 (*20*).

ATPase. Reactions (50 μL) containing 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM [γ-^{32}P]ATP at 10^4 cpm/nmol, and 20 μM ϕX174 DNA were started by addition of enzyme and incubated at 37 °C. After 30 min, reactions were stopped by addition of 100 μL acetone, followed by addition of 10 μL of 10 mM KH_2PO_4, 100 μL 1% ammonium molybdate in 2 N H_2SO_4 and 700 μL of isobutanol:benzene (1:1) solution. The mixture was vortexed for 5 min and the phases separated by brief centrifugation in an Eppendorf microcentrifuge. Half of the organic phase was removed and radioactivity was determined in a scintillation counter after addition of 5 mL EcoLume fluor (ICN, Costa Mesa CA).

D-Loop Formation. Reactions (30 μL) containing 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 100 μg of bovine serum albumin, 3 μM ^{32}P-labeled oligonucleotide, and 1 μM Rec2 protein were preincubated at 37 °C. After 10 min, $MgCl_2$ was increased to 13 mM and 50 μM M13 form I DNA was added so that the ratio of oligonucleotide to form I DNA (as molecules) was 10:1. Reactions were quenched and deproteinized by incubation for 10 min following addition of SDS to 0.5% and proteinase K to 100 μg/mL. DNA was analyzed by electrophoresis on 0.8% agarose gels in 40 mM Tris-acetate, pH 7.8, 1 mM EDTA. Gels were stained in ethidium bromide, dried onto DE81 paper, and radioactivity was detected using a Molecular Dynamics Storm 480 PhosphorImager.

R-Loop Formation and Extension. Reactions (30 μL) containing 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 100 μg of bovine serum albumin, 0.16 μM ^{32}P-labeled actin RNA, and 0.16 μM Rec2 protein were preincubated at 37 °C. After 10 min $MgCl_2$ was increased to 10 mM and 3.3 μM pT7-*ACT* form I DNA was added so that the molar ratio of oligonucleotide to form I DNA (as molecules) was 0.6 to 1. In control reactions, 30 ng of RNase A or 7.5 units of RNase H was added at the start of the reaction. Linear pT7-ACT DNA was generated by digestion with *Hin*dIII. At appropriate times reactions were deproteinized by the addition of proteinase K to 100 μg/mL and SDS to 0.5% and incubated at 37 °C for 10 min. Reactions were analyzed as above by electrophoresis in 0.8% agarose gels. To measure primer extension of R-loops, reactions were run as above but with 3 μM 30-mer oligoribonucleotide RB10 and 50 μM M13 form I DNA. *E. coli* DNA polymerase I Klenow fragment (1 unit) and 40 μM deoxynucleoside triphosphates containing 1.5 μCi [α-^{32}P]dATP were added at the start of the reaction.

DNA Strand Exchange. Reactions (30 μL) containing 3 μM single-stranded oligonucleotide in 30 mM Tris-acetate, pH 8.0, 1 mM magnesium acetate, 2 mM DTT, 100 μg/mL bovine serum albumin, 1.3 mM ATP, 20 mM phosphocreatine, 10 units/mL phosphocreatine kinase, and 1 μM Rec2 protein were preincubated at 37 °C. After 10 min, magnesium acetate was increased to 13 mM and duplex oligonucleotide was added such that the ^{32}P-labeled strand was 3 μM. Thus, the molar ratio of single-stranded DNA to duplex DNA (as molecules) was 1:1. When the effect of RPA was investigated, it was added to a concentration of 0.125 μM, for an RPA heterotrimer to single-stranded DNA nucleotide molar ratio of 1:24. After further incubation reactions were quenched and deproteinized by incubation for 10 min following addition of SDS to 0.5% and proteinase K to 100 μg/mL. Reaction products were examined after electrophoresis on 12% polyacrylamide gels containing 50 mM Tris, 50 mM boric acid, and 1 mM EDTA. Gels were dried onto Whatman 3MM paper and radioactivity was determined using a Molecular Dynamics Storm 480 PhosphorImager. Quantitation of captured phosphorimages was determined using ImageQuant software (Molecular Dynamics, Version 1.1).

DNA Binding. Reactions (20 μL) containing 30 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/mL bovine serum albumin, 1.0 mM ATPγS, and Rec2 protein were started by addition of 3 μM ^{32}P-labeled oligonucleotide. At appropriate times reaction mixtures were passed through a membrane filter disk (Gelman Metracel GN-6) prepared by scalding in boiling water for 10 min and then soaking for several hours in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (*29*). Disks were washed twice with 2 mL aliquots of 30 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, dried, and radioactivity then determined by scintillation counting after addition of 2 mL of Ecolume. DNA retained on filters in the absence of protein was less than 0.5%.

RESULTS

Activity of the His$_{10}$-Affinity Tagged Rec2 Protein in Vivo. We adopted affinity tagging as a general strategy to expedite purification of recombinant Rec2 protein. Of various tags available, we considered a leader peptide with a polyhistidine tract to be a good choice since such as tag would constitute a relatively minor modification in terms of mass and could enable purification on an immobilized metal affinity matrix. Therefore, we focused our efforts on modifiying Rec2 with a N-terminal leader containing a decahistidine sequence (His_{10}). As a necessary preliminary step to ascertain whether the modified protein would be biologically active, we designed an expression system for determining whether the recombinant protein could restore DNA repair proficiency to the *rec2-1* mutant.

We constructed a plasmid that allowed us to to express the gene fusion encoding the affinity-tagged Rec2 protein from a dedicated promoter. The vector contained a biosynthetic marker for selection and a fragment from the nitrate reductase gene with promoter activity (*pNAR*) controlling expression of the *REC2* gene fusion. As shown in Figure 1 resistance to killing by methylmethanesulfonate (MMS) was completely restored when the *rec2-1* mutant was transformed with the plasmid vector containing the modified *REC2* gene. In addition, there was no sensitization to MMS in wild-type cells transformed with the gene. These results indicated that the His_{10}-affinity tagged Rec2 protein is biologically active in DNA repair functions and furthermore displays no dominant negative interference when introduced into cells expressing endogenous Rec2 protein normally.

Purification of Rec2 Protein. Having established that the affinity-tagged Rec2 protein was biologically active, we inferred that its biochemical and enzymatic properties would be a valid representation of the native activity. Therefore, we overexpressed the gene in *E. coli* and commenced efforts

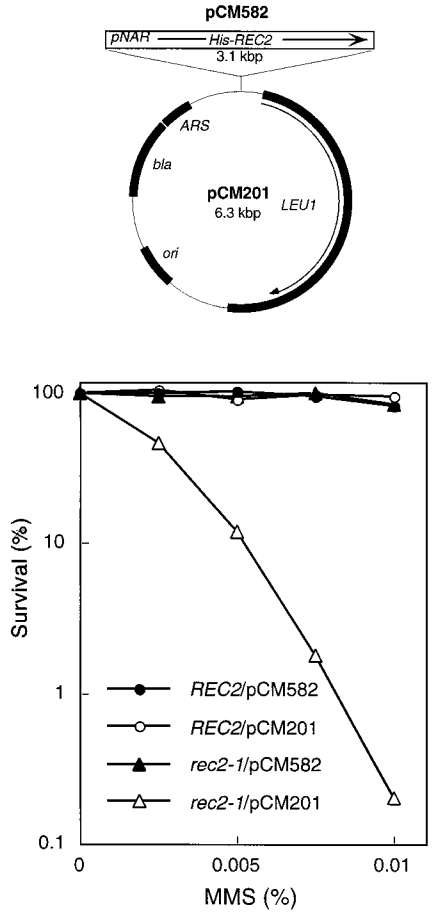

FIGURE 1: His10-Rec2 is proficient in DNA repair activity in vivo. Cell cultures of *U. maydis leu1* strains UCM5 (*REC2 leu1-1*) and UCM174 (*rec2-1 leu1-1*)were transformed to Leu^+ with the shuttle vector pCM201 or pCM582 containing the *His10-REC2* fusion under control of the *pNAR* promoter. Cultures were grown to a density of $\approx 5 \times 10^7$ cells/mL in selective medium, diluted appropriately and plated on solid nitrate minimal medium with the indicated levels of MMS. Survival was determined from the number of colonies counted after incubation 5 days at 32°.

to purify the protein. As was observed previously in a preliminary investigation (*1*), overexpression of the gene in *E. coli* led to production of protein that was sequestered in an insoluble form in inclusion bodies. After an extensive search to find conditions for producing the protein in a soluble form, we were unable to find any medium, temperature, osmotic additive or solvent condition that provided substantial improvement in solubility over what had been observed initially. Therefore, we concentrated efforts on optimizing conditions for refolding the protein into an active form after solubilization of inclusion bodies in 6 M guanidine hydrochloride. We examined a number of parameters including protein concentration, reducing conditions, solvent composition and regimen of denaturant removal. By trial and error, we found it best to purify the protein by affinity chromatography in the denatured state, then to refold it to an active form. Therefore, after isolation and judicious washing of the insoluble Rec2-containing inclusion bodies, we purified Rec2 protein solubilized in guanidine hydrochloride by chromatography on nickel-nitrilotriacetate agarose, an immobilized metal affinity matrix charged with Ni^{2+}. As can be seen by the SDS−gel analysis (Figure 2A) the protein was obtained in an essentially homogeneous form after elution from the column.

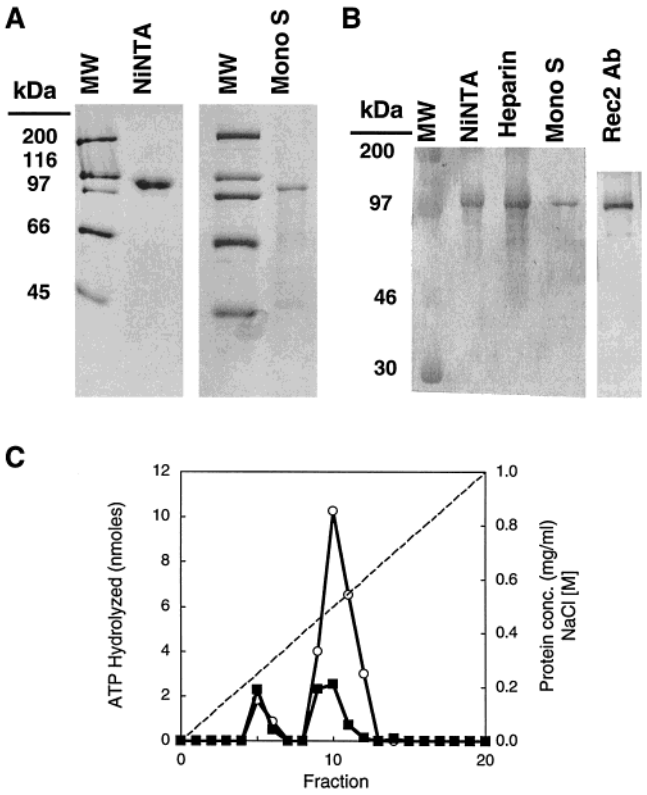

FIGURE 2: Analysis of recombinant Rec2 protein. (A) Samples of fractions containing Rec2 protein after Ni-NTA chromatography and MonoS FPLC were examined by SDS−gel electrophoresis in 10% polyacrylamide gels and visualized by staining with Coomassie blue. MW, molecular weight size standards. (B) Western blot hybridization was performed after electrophoresis in a 10% polyacrylamide SDS−gel. Samples included pooled fractions of Rec2 taken after chromatography on Ni-NTA (600 ng), heparin-Sepharose (800 ng), and MonoS (250 ng). PVDF membrane was probed using Ni-NTA AP conjugate for detection of polyhistidine tracts (left panel) or else using antibodies to the C-terminal Rec2 fusion protein (right panel). (C) Heparin-Sepharose chromatography was performed as described in Materials and Methods. Fractions (1 mL each) were monitored for DNA dependent ATPase activity (○) by assaying aliquots of 5 μL for 1 h under standard conditions and were also monitored for protein concentration (■). NaCl concentration represented by the dashed line was determined by conductivity using a Radiometer CDM80 instrument.

Fractions containing protein eluted from the column appearing >95% homogeneous were pooled. The protein concentration was then adjusted by dilution to no more than 50 μg/mL and a refolding regimen was initiated by ordered stepwise removal of guanidine hydrochloride. After completion of the procedure, activity was monitored by assaying for DNA-dependent ATPase activity. In successful preparations activity was detectable at this point. The solubilized protein was then chromatographed on a column of heparin-Sepharose. This served to separate active from inactive forms of the protein. No activity was found in the flow through which contained the bulk of the protein and which was composed presumably of incorrectly folded Rec2 conformers and aggregates. Only a small fraction of the total Rec2 protein loaded was retained by the column, but this appeared to be the enzymatically active form. Although the proportions were variable two peaks of DNA-dependent ATPase activity were usually observed; a small peak was usually followed by a larger peak (Figure 2C). The first peak was discarded as it exhibited some exonuclease contamination, but the

second peak of activity was processed further by chromatography on a MonoS column. By salt gradient elution from the column using the FPLC, a fraction was obtained that was free of contaminating phosphatase, exonuclease, and endonuclease activities and was used for all the studies reported below. Unfortunately, we were unable to remove a trace of protease activity as was evident by the appearance of Rec2 proteolytic degradation products that accumulated with time (Figure 2A). To minimize degradation, it is necessary to perform chromatography and dialysis steps as quickly as possible and to store Rec2 protein at −80°. In general, from three liters of cell culture, the final yield of Rec2 after MonoS chromatography is 0.5−1 mg of protein.

Protein Characterization. As noted previously (*1*) the Mr of Rec2 as determined by SDS−gel electrophoresis is approximately 110 kDa, which is anomalously high compared with the mass of 84 kDa predicted from the amino acid sequence. The basis of this disparity remains unknown. Antibodies raised against a 12 kDa fusion protein composed of a bacteriophage T7 gene 10 leader peptide and a 96 amino acid residue sequence from the carboxy-terminus of Rec2 cross-reacted with the 110 kDa protein indicating identity (Figure 2B). Edman degradation was performed on the purified protein from the MonoS fraction to establish identity, but it was found that the N-terminus was blocked. Therefore, tryptic peptides were prepared from approximately 100 pmol of protein and purified after fractionation by reversed-phase HPLC. Amino acid sequence determination performed on a well separated peptide yielded the sequence GNNTFQSRTFVROPIHASTK which corresponds precisely to the residues 284−303 within the Rec2 sequence. The basis for the failure of the Edman reaction on full-length protein is not known. However, the protein retained the polyhistidine leader sequence as evident by signal observed in Western blot analysis using nickel nitrilotriacetate conjugated to alkaline phosphatase as a probe (Figure 2B).

DNA-Dependent ATPase. Hydrolysis of ATP by Rec2 protein was dependent on a divalent cation and DNA as cofactors. Optimal activity was achieved with Mg^{2+} at 10 mM. When 10 mM Ca^{2+}, Zn^{2+}, or Mn^{2+} was substituted for Mg^{2+} there was 29%, 5%, or 1%, activity, respectively. No activity (<0.1%) was detectable in the absence of added divalent cation. ATP was hydrolyzed at nearly identical rates when single-stranded DNA or superhelical DNA was used as cofactor, but by comparison there was no ATP hydrolysis detectable in the absence of a DNA cofactor (Figure 3A). With linear duplex DNA as cofactor there was no detectable ATPase early in the reaction, but at a later point in the course of reaction hydrolysis of ATP became significant. This delay is reminiscent of the pronounced lag phase in ATP hydrolysis exhibited by RecA with duplex DNA as a cofactor. At neutral pH, RecA binds slowly to duplex DNA, but the nucleoprotein filament that is formed is quite similar in structure to that formed with single-stranded DNA. Thus, the large differential in initial rates of RecA-catalyzed ATP hydrolysis on single- versus double-stranded DNA has been attributed to a manifestation of a kinetic barrier in binding to duplex DNA (*30*, *31*). It is possible that a similar kinetic barrier in binding of Rec2 to duplex DNA is responsible for the lower initial rate of ATP hydrolysis.

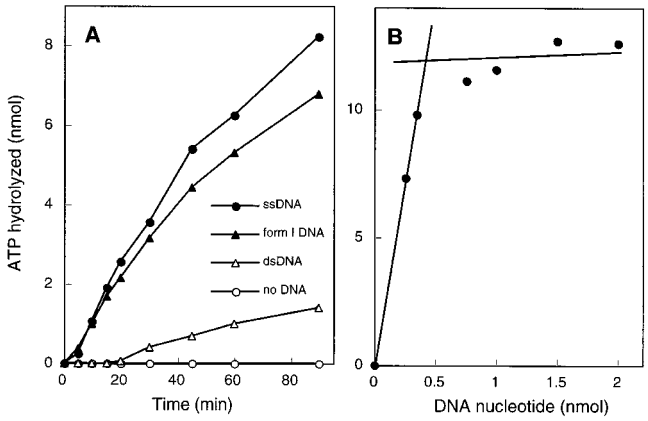

FIGURE 3: Rec2 is a DNA-dependent ATPase. (A) Time course of ATP hydrolysis. Reactions were performed as described in Materials and Methods with 2.3 μM Rec2 protein and either 10 μM M13 single-stranded DNA (●), 20 μM M13 form I DNA (▲), 20 μM M13 linear duplex form III DNA (△), or no DNA addition (○). (B) ATPase activity was determined after titration of DNA. Reactions contained 0.18 nmoles Rec2 protein and the indicated amounts of M13 single-stranded DNA and were performed under standard conditions for 1 h. The intersecting lines indicate the inflection point where the rate plateaued.

Titration of single-stranded DNA in reactions with a fixed level of Rec2 protein indicated that the system became saturated for ATP hydrolysis at a molar ratio of 2.5 mol of nucleotide/mole of Rec2 (Figure 3B). This calculation is based on the assumption that 100% of the Rec2 protein molecules present are active, which of course may be far from accurate given that the protein was prepared by refolding from the denatured state. With this proviso *k*cat was determined to be 1.1 min^{-1}. Oligonucleotides were poor cofactors for the ATPase activity and decreased in efficacy according to size. The rate of ATP hydrolysis with a 79-mer, 50-mer, 35-mer, or 20-mer at 20 μM nucleotide as cofactor was 21%, 8%, 5%, or 2.5%, respectively, of the rate with M13 single-stranded DNA.

Uptake of Single-Stranded Oligonucleotides by Superhelical DNA. In studies with RecA protein on the interaction between superhelical form I DNA and homologous single-stranded DNA fragments, it was found that D-loops were formed initially but then were dissociated in reactions that were coupled with hydrolysis of ATP (*32*). We examined the activity of the Rec2 protein in D-loop formation using an agarose gel assay to monitor the course of reaction (Figure 4A). A short ^{32}P-labeled single-stranded synthetic oligonucleotide incubated in a reaction with Rec2 protein formed a complex with M13 form I DNA that was dependent on DNA sequence homology and ATP or ATPγS (Figure 4C). The complex was assayed by the appearance of a radiolabeled band in agarose gels with a mobility equivalent to that of form I DNA and was quantitated by measuring the intensity of the imaged bands. RecA protein promoted formation of an apparently identical complex with a yield of 87% compared to a yield of 82% for Rec2 (Figure 4C, lanes 2 and 3). No complex was formed if the single-stranded oligonucleotide was heterologous, if ADP was used in place of ATP, or if the duplex DNA was precut to the linear form III with a restriction endonuclease. Complexes were stable after deproteinization so long as the duplex DNA remained intact. However, once the DNA was cleaved with a restriction endonuclease, the complexes dissociated. All of these results

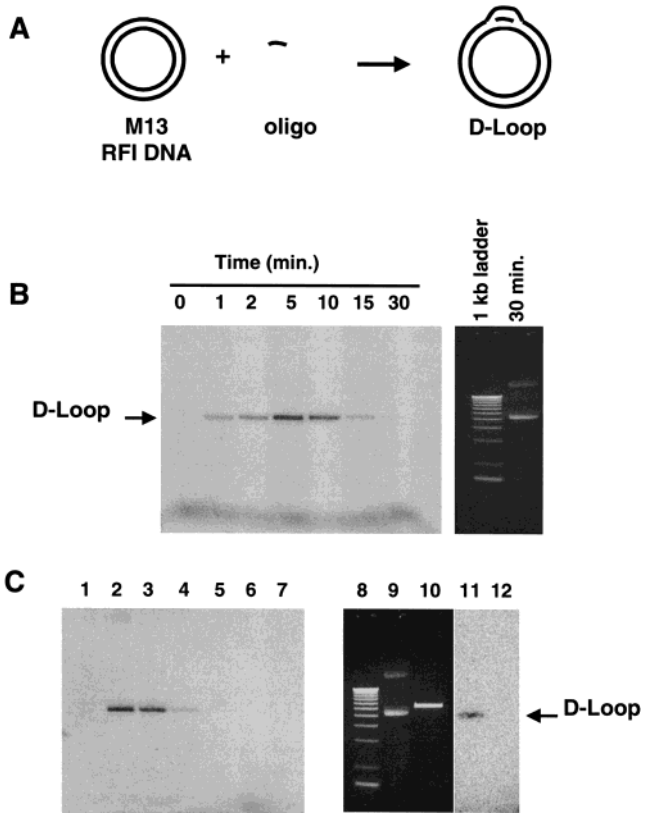

FIGURE 4: D-loop formation and dissociation promoted by Rec2. (A) Reaction scheme. Reactions were performed under conditions outlined in Materials and Methods. (B) Time course of D-loop formation with Rec2 protein using ^{32}P-labeled 83-mer oligonucleotide RB9 and M13 RFI DNA. The panel on the right indicates the state of the form I DNA after a 30 min reaction. The arrow indicates that the mobility of the complexes corresponds to form I DNA. (C) Reaction requirements for D-loop formation. Reactions with Rec2 were run for 5 min. (Left panel) Lane 1, complete reaction, no Rec2; lane 2, RecA protein control, 1 min; lane 3, complete reaction with Rec2 protein; lane 4, Rec2 with ATPγS substituted for ATP; lane 5, Rec2 without nucleotide cofactor; lane 6, Rec2 with RB7 (nonhomologous) substituted for RB9 (homologous); lane 7, Rec2 with M13 form III DNA substituted for form I DNA. Right panel. Ethidium bromide stained gel with lane 9, complete D-loop reaction; lane 10, complete reaction, but subsequently digested with *Bam*HI (100 units, 10 min); lanes 11 and 12, corresponding phosphorimager scan. Lane 8, size standards.

indicate that Rec2 catalyzes formation of D-loops which are maintained as long as the closed circular DNA remains unnicked (*33*). In addition, the results indicated that Rec2 actively dissociates D-loops over time (Figure 4B). We base this conclusion on the finding that the covalently closed circular DNA remained largely intact over the course of the reaction. Thus, the dissociation was not an artifactual consequence of spontaneous branch migration following destabilization of the structure by endonucleolytic cleavage of the DNA. Dissociation of D-loops appears to be an active process promoted by Rec2 and is a manifestation of an intrinsic ability to catalyze branch migration.

We tested whether Rec2 could promote formation of similar joint molecules with an RNA oligonucleotide. We took two approaches to investigate this question. First, we tested whether RNA prepared by runoff transcription performed in vitro could be taken up by a recipient superhelical molecule. For this experiment, a cloned 0.5 kbp DNA fragment placed under control of a bacteriophage T7

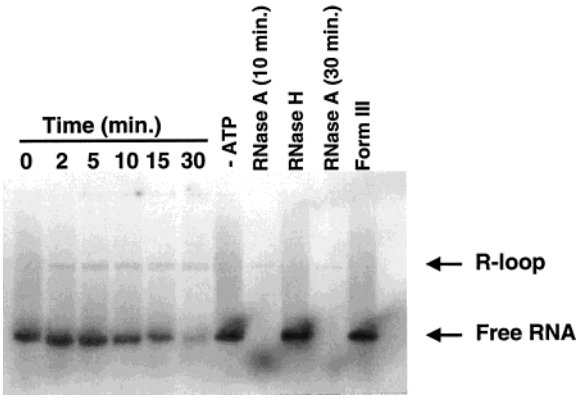

FIGURE 5: Rec2 promotes the formation of R-loops. Reactions were performed using ^{32}P-labeled actin RNA, obtained after in vitro runoff transcription of pT7-*ACT* DNA and homologous form I pT7-ACT DNA as described in Materials and Methods. Time course of R-loop formation with Rec2 protein. R-loop formation activity after 10 min is also depicted in reactions with no ATP, with addition of RNaseA or RNase H at the start of reactions, or with linear duplex form III DNA instead of form I DNA.

promoter was transcribed using T7 RNA polymerase and ^{32}P -labeled ribonucleoside triphosphates to generate radiolabeled RNA transcripts homologous to the 0.5 kbp DNA sequence. The ^{32}P -labeled RNA was then added to Rec2 pairing reactions with superhelical plasmid DNA containing the cloned DNA fragment and joint molecule formation was assayed by the agarose gel procedure as above. Formation of a complex was detected by the appearance of a radioactive band with a mobility similar to that of form I plasmid DNA containing the cloned insert (Figure 5). No complex was generated in the absence of ATP, when linear, rather than superhelical plasmid DNA was the duplex substrate in the pairing reactions, or when plasmid DNA with no homology to the transcribed RNA was used. The complex survived treatment with RNaseA, but not RNaseH. We interpret these results to mean that Rec2 can promote the uptake of RNA oligonucleotides into superhelical DNA to form a structure likely similar to that of a D-loop.

RNA−DNA hybrids are intrinsically more stable than their DNA−DNA counterparts. Given the higher stability we wondered if R-loops formed by Rec2 could serve as primer/template to initiate DNA synthesis. We tested whether R-loops formed in a reaction similar to that above, but with an invading 30-residue RNA oligonucleotide, could prime DNA synthesis in a coupled reaction with Klenow DNA polymerase. In this case reactions contained form I plasmid DNA, homologous 30-mer oligoribonucleotide, ATP, Rec2 protein, deoxyribonucleoside triphosphates and Klenow DNA polymerase. The course of reaction was monitored by the incorporation of [α-^{32}P]deoxyribonucleoside triphosphate into product made visible after polyacrylamide gel electrophoresis of the reaction products as above. In the complete reaction we observed a unique band with a mobility approximately that of superhelical DNA which was dependent on addition of Rec2 and oligoribonucleotide primer (Figure 6A). The unique band did not appear when reaction mixtures were prepared with RNase A.or RNase H and is thus likely to represent an R-loop extension product. This product remained undiminished over a time period in which D-loops as control were dissociated by Rec2 (Figure 6B). These results indicate that Rec2 could act in concert with a DNA polymerase to

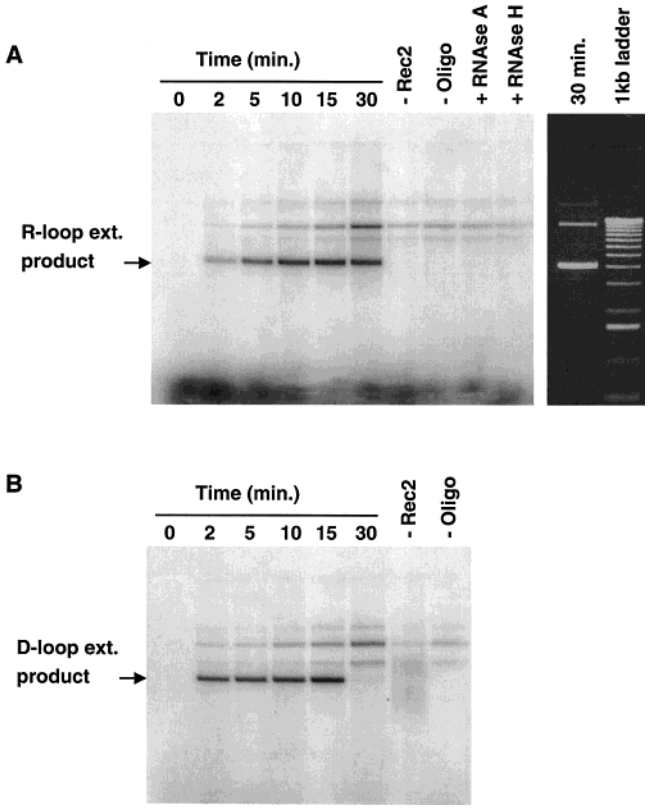

FIGURE 6: Primer extension of Rec2-promoted R-loops. Complete reactions containing Rec2, M13 form I DNA, oligonucleotide RB10, ATP, Klenow DNA polymerase, deoxynucleoside triphosphates, and [α-^{32}P]dATP were performed as described in Materials and Methods. (A) Time course of R-loop primer extension product formation after addition of M13 RFI DNA to start the reaction. Controls include reactions lacking Rec2 protein, oligonucleotide RB10, as well as reactions containing RNase A and RNase H which were run for 10 min. An ethidium bromide stained agarose gel of the 30 min reaction is also shown. (B) Primer extension of D-loops formed by Rec2. Reactions were identical to those in (A), but with DNA oligonucleotide RB9 in place of RB10. A time course of extension product formation is shown as well as reactions lacking Rec2 protein or oligonucleotide RB9.

initiate RNA-primed DNA synthesis at a site independent of an origin of replication when provided an RNA fragment homologous to a given DNA sequence.

DNA Strand Exchange. We measured DNA strand exchange using a paired set of oligonucleotide substrates consisting of a duplex prepared by annealing two complementary strands and a homologous single-stranded oligomer. The set contained an 83-mer duplex in which one of the two strands was labeled with ^{32}P, plus an unlabeled 83-mer single-stranded DNA which was identical in sequence to the labeled strand in the duplex. Strand exchange was monitored after electrophoresis in polyacrylamide gels and was evident when radiolabel associated with the faster migrating duplex was transferred to the position of the slower migrating single-stranded oligonucleotide (Figure 7). Rec2-catalyzed strand exchange was completely dependent on ATP, although ATPγS, but not ADP could substitute as a nucleotide cofactor. DNA sequence homology was also required. No product was formed if a heterologous single-stranded oligomer was substituted for the homologous single-stranded 83-mer. This result indicates that product formation was not merely an artifactual consequence of DNA melting due to helicase activity, but was a result of a concerted exchange

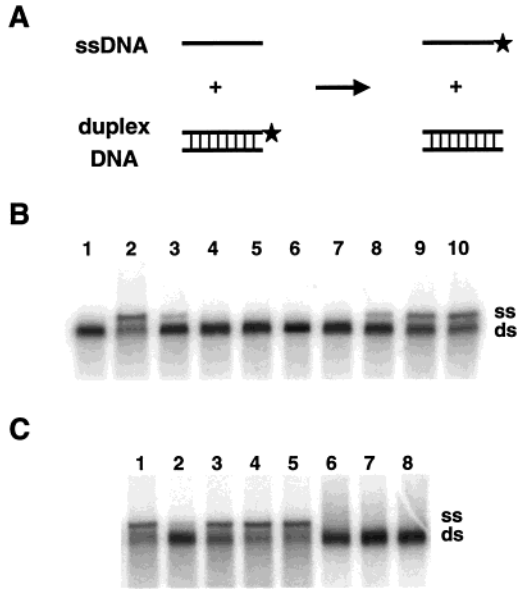

FIGURE 7: DNA strand exchange promoted by Rec2. (A) Scheme of the reaction. Reactions contained 3 μM each single-stranded oligonucleotide RB7 and duplex *RB7+RB8 [the asterisk (*) indicates the ^{32}P-labeled strand]. Strand exchange yields labeled single-stranded oligonucleotide *RB7 and unlabeled duplex RB7+RB8. (B) Time course with 1 μM Rec2 protein and reaction requirements. Lane 1, complete reaction, no Rec2 protein; lane 2, 1 μM RecA control, 10 min; lane 3, ATPγS substituted for ATP; lane 4, no nucleotide cofactor; lane 5, ADP substituted for ATP; lane 6−10, Rec2 time course, 0, 5, 10, 30, and 60 min respectively; (C) Rec2 protein titration and homology requirement. Lane 1, 1 μM RecA control, 1 μM 10 min; lane 2−5, 0.2 μM, 0.4 μM, 0.8 μM, and 1.6 μM Rec2 protein, respectively; lane 6, RecA with RB9 (nonhomologous) substituted for RB7 (homologous); lane 7, same as lane 6 but with Rec2; lane 8, no protein.

of the labeled strand in the duplex for the unlabeled homologous single-stranded partner.

Strand Exchange Enhanced by Duplex DNAs with Single-Stranded Tails. We investigated if there was an end preference for Rec2-mediated pairing. The determination was conducted using a single-stranded oligonucleotide homologous to the duplex DNA as in the assay for strand exchange described above, but additionally containing a heterologous sequence attached 5′ or 3′ end of the homologous sequence. This procedure was adapted from an established protocol developed in an analysis of preferential end pairing by human and yeast Rad51 proteins (*23*). As was reported for Rad51 protein, we observed that the Rec2-promoted reaction was faster and more efficient when the homologous region was at the 5′ end of the single-stranded oligonucleotide (Figure 8). The initial rates of exchange of oligonucleotides with 5′-end homology and 3′-end homology were 21 fmol oligonucleotide/minute and 11 fmol oligonucleotide/minute, respectively. RecA protein was also examined and in agreement with previously published work the preference was for the 3′ end of single-stranded DNA (data not shown). Thus, Rec2 displays a bias for DNA pairing at the 5′ end of single-stranded DNA that distinguishes it from its prokaryotic counterpart. This pairing bias is consistent with the bias noted in the original report using the native DNA pairing activity purified from *U. maydis* (*20*).

It was reported that Rad51 protein from both yeast and human promoted strand exchange faster and to a greater extent when duplex DNA with a single-stranded tail was used

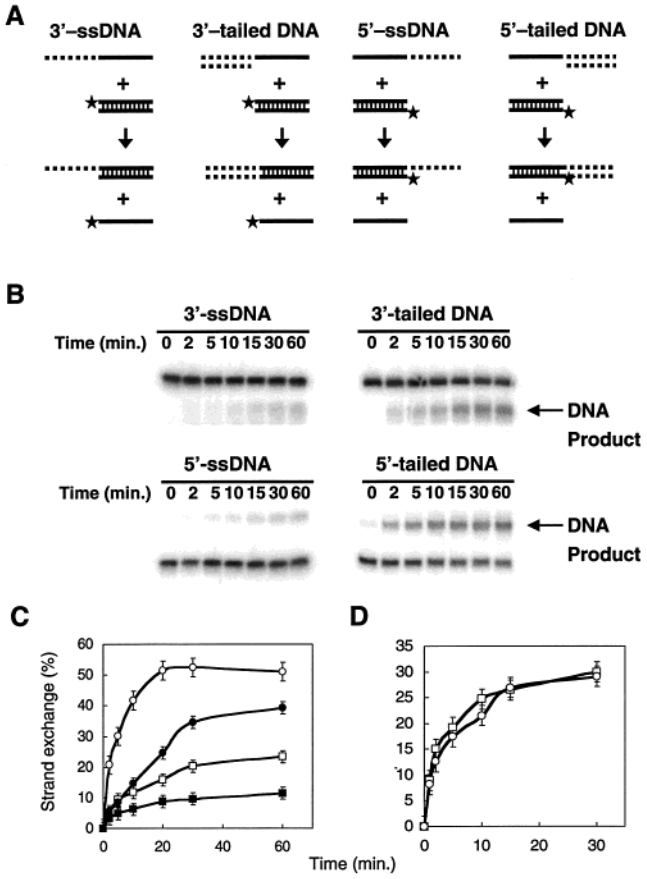

FIGURE 8: End-pairing bias and preference for tailed duplex DNA. (A) Reaction scheme. Reactions contained either single-stranded oligomers RB1 or RB2, or single-stranded tailed duplex RB1+RB6 or RB2+RB5. Strand exchange was initiated by the addition of ^{32}P-labeled duplex DNA RB3+*RB4 [the asterisk (*) indicates the ^{32}P-labeled strand] that was homologous to either the 3′ or 5′ end of the single-stranded DNA or the tailed duplex DNA. The solid line indicates homology to the duplex DNA while the dashed line indicates heterology. DNA strand exchange between substrates with 3′ homology results in displacement of ^{32}P-labeled RB4. Strand exchange between substrates with 5′ homology results in formation of ^{32}P-labeled heteroduplex. (B) Time course of Rec2 promoted strand exchange with each type of substrate. (C) Graphical representation of strand exchange after quantitation of phosphorimager scans. 5′-tailed DNA (○), 5′-ssDNA (□), 3′ tailed DNA (●), 3′-ssDNA (■). (D) Same as panel C except using RecA protein (gels are not shown for the sake of clarity).

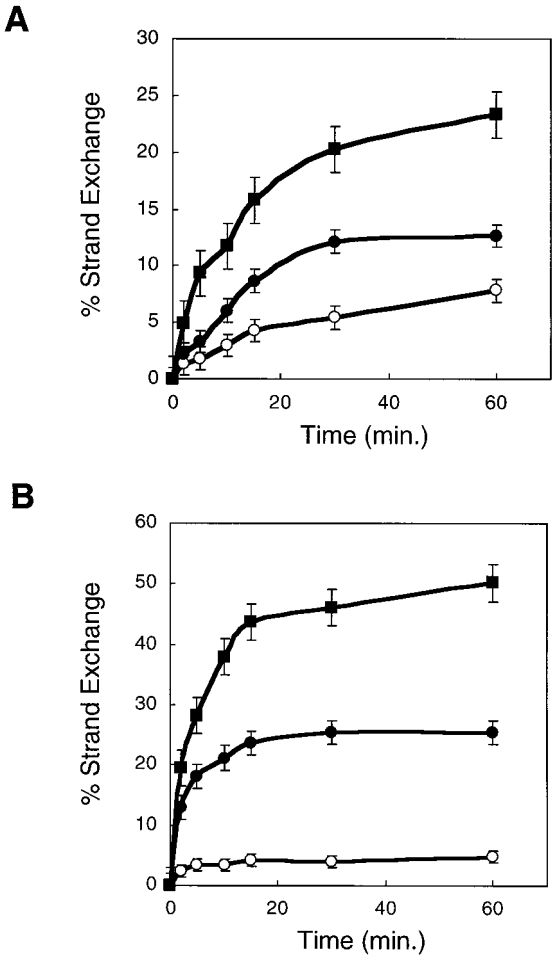

FIGURE 9: RPA inhibits strand exchange promoted by Rec2 protein. (A) Reactions contained single-stranded oligomer RB1 and ^{32}P labeled duplex RB 3+*RB4 [the asterisk (*) indicates the ^{32}P-labeled strand]. RPA was either prebound for 2 min before addition of Rec2 (○), or added immediately after Rec2 (●), Standard reaction without RPA (■). (B) Reactions contained tailed duplex DNA RB1+RB6 and ^{32}P-labeled duplex RB3+*RB4. RPA was either prebound (○) or added immediately after Rec2 (●). Standard reaction without RPA (■).

in reactions (*23*). The reported increase in reaction was greater when the single-stranded DNA tail was located at the 5′ end of a duplex rather than the 3′ end (Figure 8A). Stimulation by tailed DNA was not observed with RecA protein. Therefore, we investigated whether Rec2-catalyzed strand exchange activity was stimulated by tailed DNA. Using identical substrates as those described by the Kowalczykowski laboratory in their Rad51 analysis (*23*), we found that tailed duplexes were indeed preferred over single-stranded DNAs both in terms of the rate and extent of reaction promoted by Rec2 (Figure 8, panels B and C). In addition, the increase in reaction was greater when the single-stranded tail protruded from the 5′ end of a duplex rather than the 3′ end. Initial rates of strand exchange with 5′- and 3′-single-stranded tailed molecules were 65 and 34 fmol oligonucleotide/min, respectively. As a control, we used RecA protein and found that there was no preference for tailed duplexes over single-stranded DNA molecules (Figure 8D). In these regards, the preference of Rec2 for tailed duplexes parallels the findings with Rad51.

The single-strand DNA binding protein RPA has been reported to stimulate Rad51-promoted strand exchange strongly (*8*, *15*, *34*). However, RPA inhibits strand exchange when it binds to single-stranded DNA prior to Rad51. Rad51 will subsequently displace RPA in a process mediated by Rad52 (*11*, *13*, *35*) thereby enabling strand exchange to proceed, but after a lag corresponding to the time required for Rad51 to displace the RPA. Tailed DNA substrates were found to enable Rad51 to compete more effectively with RPA with the result that strand exchange was not inhibited (*23*).

We observed that RPA blocked Rec2-catalyzed strand exchange with tailed DNA when it was added to reactions prior to addition of Rec2 (Figure 9B). However, there was no indication over the course of reaction that Rec2 could overcome the inhibition. Furthermore, there was no indication that RPA was stimulatory. When RPA was added to reactions following addition of Rec2, there was still inhibition of strand exchange, although not to the extent observed when RPA was added first. Thus, it appears there is strong competition between the two proteins in DNA binding. RPA was less

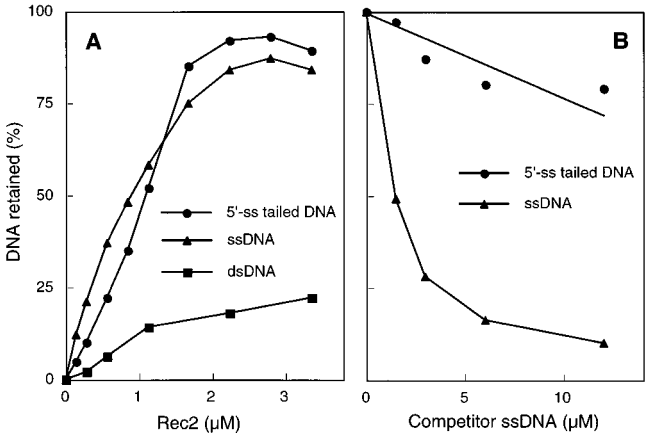

FIGURE 10: Rec2 preferentially binds to duplex DNA with a single-stranded tail. Complex formation was measured between DNA and Rec2 by retention to nitrocellulose filters as outlined in Materials and Methods. ^{32}P-labeled DNA substrates used were single-stranded oligonucleotide *RB9, duplex DNA RB3+*RB4 or tailed duplex DNA RB1+*RB6 [the asterisk (*) indicates the ^{32}P-labeled strand]. (A) DNA substrates were mixed with increasing amounts of Rec2 protein and complex formation was measured in the presence of ATPγS. (B) Competition experiments were performed using single-stranded oligonucleotide RB9 as the competitor. Single-stranded oligonucleotide *RB9 or tailed duplex DNA RB1+*RB6 was present in binding reactions at 3 μM (nucleotide) with 1 μM Rec2 plus the indicated concentrations of competitor oligonucleotide. After 10 min at 37 °C retention of ^{32}P-labeled DNA to nitrocellulose filters was measured.

inhibitory when single-stranded DNA, rather than tailed DNA was used in reactions (compare Figure 9, panels A and B). The reason for the different effect of RPA in the Rec2 system is not clear, but it is possible that it reflects a requirement for a protein−protein interaction that is absent in this system because the RPA utilized was from a heterologous source, i.e., from *S. cerevisiae*, or else a need for a mediator such as Rad52 to displace RPA.

Binding Preference of Rec2 To Tailed DNA Reflects the Enhanced Activity in Strand Exchange. We investigated binding of Rec2 to DNA to address the question of why tailed DNA was a preferred substrate. We assayed DNA binding using a membrane filter retention procedure that relies on trapping DNA bound in protein complexes but not free DNA. Under the conditions employed, which included the addition of ATP-γS as cofactor, complexes formed readily between Rec2 and tailed DNA or single-stranded DNA, but poorly with duplex DNA (Figure 10A). However, we could distinguish little discrimination in the formation of Rec2 complexes with tailed DNA versus single-stranded DNA. Therefore, we performed competition experiments to measure the stability of preformed Rec2−DNA complexes when challenged with single-stranded DNA. Complexes were formed by incubating Rec2 and radiolabeled tailed DNA or single-stranded DNA in the presence of ATP-γS. Unlabeled single-stranded DNA was then added in molar excess and the level of complexes was determined. The results demonstrate that the stability of complexes formed between Rec2 and tailed DNA was greater than that with single-stranded DNA (Figure 10B).

Mazin et al. investigated the same question of why tailed DNA was the preferred substrate for Rad51-promoted DNA strand exchange (*23*). By examining the affinity for DNA using a gel mobility shift assay, they observed formation of complexes between Rad51 and tailed DNA under conditions in which no complexes were apparent using single-stranded DNA and in which complex formation with duplex DNA was dependent on length. They also showed in competition experiments with duplex DNA as competitor that the fraction of complexes composed of Rad51 bound to tailed DNA was greater than the fraction with Rad51 bound to double-stranded DNA. These findings led them to conclude that the Rad51 binding preference for tailed DNA was responsible for the enhanced strand exchange promoted by Rad51.

By comparison, it would appear that Rec2 and Rad51 have very different preferences in the formation of protein−DNA complexes with single-stranded and duplex DNA at least as measured by the two different assay systems. Nevertheless we reached the same conclusion as Mazin et al. (*23*) regarding tailed DNA: enhanced binding governs the preference by Rec2 in strand exchange.

DISCUSSION

U. maydis contains at least two RecA homologues that are expressed in mitotic cells. One is highly related in structure to Rad51 (*17*), while another, Rec2, the subject of this report, is much more divergent and apparently without a close relative among the known RecA homologues (*18*). The major conclusion to be drawn from this present study is that Rec2, purified after overexpression of the cloned gene and in the absence of any auxiliary proteins, is fully capable of promoting DNA pairing and strand exchange reactions that resemble those catalyzed by RecA and Rad51 proteins. This indicates that Rec2 has the innate ability to perform the homology recognition and strand invasion steps envisioned as central to the mechanism of double-strand-break repair and recombination. As such, it may be incorrect to accept as universal a generalization emerging from studies on the RecA homologues in *S. cerevisiae* that in mitotic cells Rad51 provides the essential catalytic activity for strand exchange while the more divergent homologues serve in an ancillary fashion to enhance Rad51 activity (*5*, *10*, *16*, *36*).

In *S. cerevisiae*, Rad55 and Rad57 have no reported strand pairing activity on their own, but serve as cofactors to stimulate the Rad51-driven reaction (*15*). Genetic analysis supports the notion that Rad55 and Rad57 are not essential for strand exchange since overexpression of *RAD51* can suppress the DNA repair deficiency of *rad55* or *rad57* mutants (*37*, *38*). A similar mechanism may be operational in *S. pombe* where genes orthologous to *RAD55* and *RAD57* are present and the DNA repair deficiency in an *rhp57* mutant can be partially suppressed by increasing the copy number of *rhp51*$^+$ encoding the Rad51 ortholog (*39*, *40*). But in *U. maydis*, the relationship between Rad51 and Rec2 appears to be different (*17*). Epistasis analysis indicates some independence in action of the two genes in repair of UV damage and in mitotic recombination suggesting there might be separate functional branches in the recombinational repair pathway. Sequence comparison reveals no strong similarity to suggest that Rec2 might be orthologous with Rad55 or Rad57. Furthermore, genetic analysis indicates that DNA repair proficiency is not restored to a *rec2* mutant upon introduction of a multicopy plasmid containing the *RAD51* gene. Thus, a paradigm for homologous pairing featuring a multicomponent interacting complex composed of *RAD52*

epistasis group gene products with Rad51 as the DNA pairing catalytic component might be widespread, but not universal.

As demonstrated in this study, Rec2 has certain enzymatic features in common with both RecA and Rad51. Like RecA, Rec2 is completely dependent on a DNA cofactor in hydrolysis of ATP, in contrast to Rad51 (*8*), but the rate at which Rec2 hydrolyzes ATP is much slower by comparison with RecA, and appears to be in the range of that observed for Rad51 (*8*, *41*, *42*). Our preparations of refolded protein are likely to contain only a fraction of active molecules, but nevertheless, the turnover number for Rec2 would still appear to be an order of magnitude lower than that of RecA. Pairing reactions promoted by Rec2 are completely dependent on an ATP cofactor, although hydrolysis is not necessary, as evidenced by the ability of ATPγS to substitute for ATP. Rec2 actively promotes the formation and the dissociation of D-loops with superhelical DNA and a homologous single-stranded oligonucleotide, in a manner resembling the reactions promoted by RecA, albeit with with slower kinetics. This activity stands in contrast to Rad51 which has almost negligible ability to form D-loops in the absence of Rad54 (*10*, *12*, *43*).

In confirmation of findings made some years ago using a proteolyzed form of Rec2 protein purified from *U. maydis* extracts (*20*) and in agreement with findings reported with Rad51 (*44*), the recombinant Rec2 protein prefers to pair duplex DNA and single-strands with the opposite polarity of that preferred by RecA. It is well established in studies on RecA and Rad51 that protruding single strand stretches at the ends of DNA duplexes, overall base composition, and secondary structure in single-stranded DNA have profound effects on the efficiency of strand pairing (*34*, *45*, *46*). We made no systematic analysis of the effects of these parameters in this current study, but gave due consideration in the design of substrates such that the base composition would be uniform throughout the length of the DNA to be tested. Base composition is a particularly important issue in experiments addressing polarity of strand exchange since a high GC content strongly inhibits pairing by RecA and Rad51 (*47*). The reaction to establish end preference that we performed utilized blunt-ended duplex DNA with 42% CG content. Efficient pairing was observed with both Rec2 and the RecA control, and the preference for Rec2 pairing at the 5′-end of single-stranded DNA was quite evident in comparison with the opposite preference exhibited by the RecA control run in parallel. Thus, Rec2 is like the yeast and human Rad51 proteins in the bias for pairing. The molecular basis for this difference in preference is unknown, but it is possible that it represents an opposite modality in the polymerization of protein monomers along the DNA backbone as has been suggested (*23*). What biological purpose might be served by such a mechanism is an open question, but one advantage offered could be a higher probability of assembly of Rec2 at the 5′-region of linear single-stranded DNA and conversely could free the 3′-end of an invading DNA strand from the constraint or confines of the nucleoprotein filament. Following strand exchange an available 3′-terminus could serve as a primer for further processing by DNA synthesis machinery as envisioned in synthesis-dependent strand annealing variants of the double-strand break repair model (e.g., ref *48*) and break-induced replication (*2*).

Accumulating genetic evidence in *E. coli* has suggested that one form of recombination-dependent replication known as constitutive stable DNA replication is achieved by RecA-facilitated joint molecule formation between an RNA transcript and the bacterial chromosome (*49*). Biochemical support for this idea comes from studies showing RecA can promote annealing of an RNA oligonucleotide with a free single-stranded DNA or with a preformed single-stranded loop in a DNA heteroduplex, (*50*) and furthermore, can perform an inverse strand exchange reaction between a DNA heteroduplex and a homologous single-stranded RNA (*51*, *52*). All of these pairing reactions performed with RNA are much less efficient than with DNA, presumably due to steric hindrance encountered during protein-promoted extension of the RNA phosphodiester backbone in preparing for intermolecular association (*53*). Thus, the question might be raised as to the universality of such a mechanism. Our demonstrations that Rec2 can promote uptake by superhelical DNA of RNA oligonucleotides to form a joint molecule raises the notion that the free energy of superhelix formation might be an important factor enabling the reaction. Furthermore, since the joint molecules formed in the Rec2-catalyzed reaction can serve as a primer/template for DNA synthesis, it would appear that there is support for extending the paradigm of an RNA-based pairing mechanism into the realm of eukaryotes. However, unlike the situation in prokaryotes, there is little genetic evidence available to suggest a role for RNA-mediated recombinational replication in eukaryotes. Still, one can imagine scenarios by which RNA-mediated recombination-dependent replication might reconcile many perplexing biological phenomena. For instance, it is possible that RNA transcripts or fragments derived perhaps from the mRNAs of highly expressed genes might be utilized to serve as primers for the localized DNA replication of the homologous genetic locus. This could lead to the amplification of that particular gene as seen with *dhfr* (dihydrofolate reductase) (*54*) and *mdr1*, (multiple drug resistance) (*55*) in mammals and might be the basis for generation of double minute chromosomes (*56*).

Progress in analyzing the mechanism of DNA pairing by Rec2 has been hampered by lack of success in overproducing a soluble form of recombinant Rec2. However, it is clear from the studies reported here that even activity reconstituted from denatured recombinant protein exhibits robust DNA pairing and strand exchange without addition of any auxiliary factors. Thus, in mitotic cells of *U. maydis* it seems likely that Rec2 can provide the fundamental enzymatic activity necessary for recombinational repair. It will be interesting to learn if Rad51 can perform similar functions in *U. maydis*, or if it has evolved to serve in an auxiliary fashion to enhance the activity of Rec2. Perhaps the presence in *U. maydis* of not one, but two separate, parallel pathways for double-strand break repair might account for the extreme radioresistance of this organism (*57*). We have overexpressed the *U. maydis* Rad51 protein in a soluble form and have started to investigate activity of the purified protein in DNA strand-pairing reactions. We look forward to experimentation that will provide some understanding of the relationship between the two proteins and that will perhaps offer deeper insight into their functions in recombinational repair.

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