

Catalysis of ATP-Dependent Homologous DNA Pairing and Strand Exchange by Yeast RAD51 Protein

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The *RAD51* gene of *Saccharomyces cerevisiae* is required for genetic recombination and DNA double-strand break repair. Here it is demonstrated that RAD51 protein pairs circular viral single-stranded DNA from ϕ X 174 or M13 with its respective homologous linear double-stranded form. The product of synapsis between these DNA partners is further processed by RAD51 to yield nicked circular duplex DNA, which indicates that RAD51 can catalyze strand exchange. The pairing and strand exchange reaction requires adenosine triphosphate, a result consistent with the presence of a DNA-dependent adenosine triphosphatase activity in RAD51 protein. Thus, RAD51 is a eukaryotic recombination protein that can catalyze the strand exchange reaction.

Saccharomyces cerevisiae genes of the RAD52 epistasis group, namely, *RAD50*, *RAD51*, *RAD52*, *RAD53*, *RAD54*, *RAD55*, *RAD56*, and *RAD57*, are required for DNA double-strand break repair and genetic recombination (1). Mutants of the RAD52 group genes often exhibit meiotic inviability, which is likely to be the result of the requirement for genetic recombination in order to ensure the proper segregation of homologous chromosomes in meiosis I (2). Among these genes, *RAD51* is of particular interest because of the structural similarity to the recombination enzyme RecA from *Escherichia coli* (3–6). In the presence of adenosine triphosphate (ATP), RecA protein polymerizes on DNA to form highly ordered filaments and catalyzes the pairing and strand exchange between homologous DNA molecules (7). When expressed in *E. coli* and purified, RAD51 protein possesses DNA-binding activity that is ATP-dependent (3). RAD51 protein forms nucleoprotein filaments on double-stranded DNA (8). Despite these similarities to RecA, previous studies have found no DNA pairing and strand exchange activities associated with RAD51 protein (3, 8). Here it is demonstrated that RAD51 catalyzes homologous DNA pairing and strand exchange dependent on ATP.

For expression of the RAD51 protein in *S. cerevisiae*, the *RAD51* gene was placed under the control of the yeast alcohol dehydrogenase 1 (*ADC1*) promoter in the 2 μ multicopy vector pSCW231 to form plasmid pR51.1 (2 μ , *ADC1*-RAD51) (9). Increased production of RAD51 in yeast strain LP2749-9B harboring pR51.1 was verified by immunoblot analysis of yeast extracts with the use of affinity-purified polyclonal antibodies raised against a segment of RAD51 expressed in *E. coli* (10). A

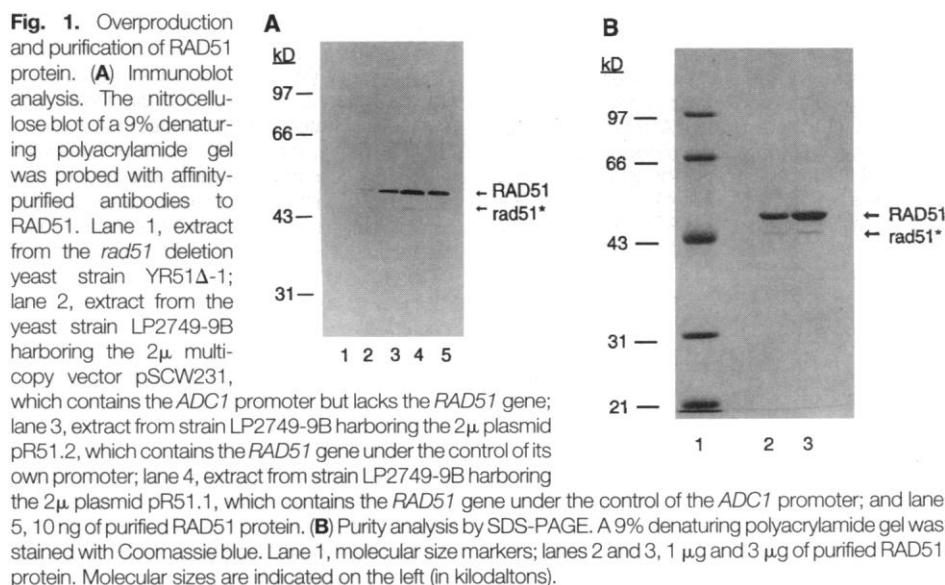
minor proteolytic form of RAD51, referred to as rad51* (Fig. 1A), was detected in yeast extracts by immunoblotting and persisted through protein purification (Fig. 1, A and B). The RAD51 protein was purified (11) to near homogeneity (Fig. 1B) and its identity was established by immunoblotting (Fig. 1A) and by sequencing peptides from a cyanogen bromide digest of the protein (12).

RAD51 protein hydrolyzed ATP to adenosine diphosphate (ADP) in the presence of Mg^{2+} and single-stranded (ss) DNA (Table 1). Double-stranded (ds) DNA was much less effective in promoting ATP hydrolysis, and no hydrolysis was detected in the absence of DNA (Table 1). DNA-dependent ATP hydrolysis occurred slowly, with a k_{cat} of 0.7 min^{-1} when ss ϕ X DNA was used as a cofactor. In the Mono Q fractions from the last step of the purification, the adenosine triphosphatase (ATPase) activity coeluted with

RAD51 protein. RAD51 protein also hydrolyzed deoxyATP (dATP) with a similar efficiency and the same DNA cofactor requirement (13).

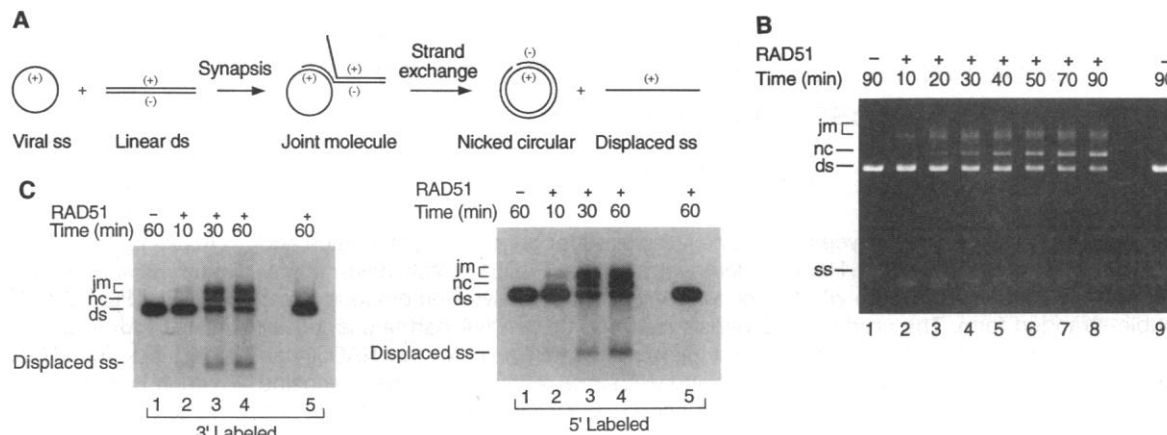
To test whether RAD51 protein catalyzes DNA pairing and strand exchange, we used as substrates circular viral ssDNA and linear duplex DNA that were used in studies with RecA protein (7). Different phases of the pairing and strand exchange reaction are shown (Fig. 2A). The ssDNA was preincubated with RAD51 protein and the *S. cerevisiae* single-strand DNA-binding protein replication protein A (RPA), which was purified as described (14), before the incorporation of dsDNA (15). RAD51 protein paired the circular ss ϕ X and linear ds ϕ X DNAs to form joint molecules, and an increasing amount of nicked circular dsDNA was produced as the reaction progressed (Fig. 2B). The formation of nicked circular dsDNA indicates that RAD51 protein can mediate complete strand exchange subsequent to pairing of the ss- and dsDNA molecules. The pairing and strand exchange activities coeluted chromatographically with RAD51 protein. This result and the high purity of RAD51 protein indicate that these activities are intrinsic to RAD51. RPA is an important accessory factor in pairing and strand exchange. In the presence of RPA, after 90 min of reaction, 26% and 36% of the input linear dsDNA had been converted by RAD51 to joint molecules and nicked circular dsDNA, respectively. Omission of RPA reduced the amount of joint molecules to 8% and that of nicked circular dsDNA to 1% of the input linear dsDNA after 90 min of reaction.

Incubation of RAD51 protein and RPA with a partial duplex consisting of a 32 P-labeled 41-nucleotide fragment annealed to circular viral M13 DNA (16) did not result in displacement of the annealed fragment, indi-



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Fig. 2. RAD51 catalyzes DNA pairing and strand exchange. **(A)** The DNA substrates and expected products of the pairing and strand exchange reaction. **(B)** Time-dependent formation of recombination products. Viral ss ϕ X 174 DNA and linear ds ϕ X 174 DNA (lanes 1 to 9) were incubated with RPA (lanes 1 to 8) and RAD51 protein (lanes 2 to 8) for the indicated times (15). **(C)** The ends of the linear dsDNA remain intact during pairing and strand exchange. Linear ds ϕ X DNA labeled with 32 P at the 3' end (left panel) or the 5' end (right panel) was used in the strand exchange reaction. The labeled DNA was incubated with viral ss ϕ X DNA for 60 min in the absence of RAD51 protein (lane 1) and with RAD51 protein for 10 min (lane 2), 30 min (lane 3), and 60 min (lane 4). Lane 5 is the same as lane 4, except that viral ss ϕ X DNA was omitted from the reaction mixture. The symbols in (B) and (C) are as follows: ss, viral circular single-stranded DNA; ds, linear double-stranded DNA; jm, joint molecules



cating the absence of a DNA helicase activity in these proteins. To demonstrate that the ends of the linear ds substrate remain intact in the RAD51-catalyzed pairing and strand exchange reaction, we used 3' and 5' 32 P end-labeled linear dsDNAs. Both the 3' and 5' ends of the input linear duplex DNA retain the 32 P label during pairing and strand exchange, thereby excluding the possibility that exonucleolytic resection of the linear dsDNA might have occurred during the reaction (Fig. 2C). In addition, these experiments also revealed the time-dependent displacement of the 32 P labeled (+) DNA strand from the linear dsDNA in the strand exchange reaction (Fig. 2C).

The RAD51-catalyzed pairing and

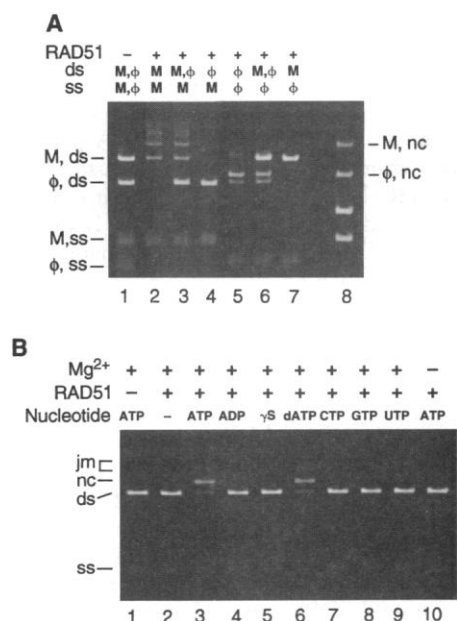
strand exchange reaction requires homology, because formation of joint molecules and nicked circular duplex DNA occurs only between homologous DNAs (Fig. 3A). As shown in Fig. 3B, pairing and strand exchange is dependent on ATP, which cannot be substituted by ADP, cytidine triphosphate, guanosine triphosphate, and uridine triphosphate. Whereas dATP is as effective as ATP in the reaction, the non-hydrolyzable ATP analog ATP γ S is inactive (Fig. 3B). Thus, ATP or dATP is required for the DNA pairing and strand exchange reaction. The pairing and strand

exchange reaction also has an absolute dependence on Mg^{2+} (Fig. 3B). Our results indicate that RAD51 mediates extensive pairing and strand exchange with a dependence on ATP. Thus, RAD51 represents a recombination protein from a eukaryotic organism that exhibits strong functional similarity to RecA (3, 8). Because RAD51 has been conserved during eukaryotic evolution (17), the RAD51 counterparts from higher eukaryotes are likely to catalyze ATP-dependent homologous pairing and strand exchange and thus have an indispensable role in genetic re-

Table 1. RAD51 protein is a DNA-dependent ATPase. Reaction mixtures (12.5 μ l) containing 1 mM [2,5,8- 3 H]ATP, 2.5 μ g of RAD51 protein (58 pmol), 6 mM $MgCl_2$, and 100 ng of viral ssDNA or linear dsDNA (300 pmol of nucleotides) were assembled in buffer R (15). Incubation was at 36°C for 70 min, and the amount of ATP hydrolysis was determined by thin layer chromatography with the use of PEI cellulose sheets. The amount of DNA used gave the optimal rate of ATP hydrolysis. The rate of ssDNA-dependent ATP hydrolysis by RAD51 is \approx 40-fold less than by RecA (7).

Addition and omission	Percent conversion of ATP to ADP	k_{cat} (min^{-1})
- DNA	0	-
+ ϕ X ssDNA	22.8	0.7
+ ϕ X ssDNA, - Mg^{2+}	0	-
+ ϕ X dsDNA	3.3	0.1
+ M13 ssDNA	20.4	0.6
+ M13 ssDNA, - Mg^{2+}	0	-
+ M13 dsDNA	1.6	0.05

Fig. 3. RAD51-catalyzed pairing and strand exchange reaction is homology dependent and requires ATP. **(A)** Requirement for homology. Various combinations of M13 mp18 (abbreviated as M) and ϕ X 174 (abbreviated as ϕ) viral ssDNA and linear dsDNA were incubated with RAD51 protein for 90 min (15). Lane 8 contained a mixture of M13 nicked circular (M, nc) and ϕ X nicked circular (ϕ , nc) and supercoiled M13 and ϕ X DNAs (not marked) incubated in buffer without RAD51 protein. For clarity, the positions of joint molecules formed between the M13 substrates (lanes 2 and 3) and between the ϕ X substrates (lanes 5 and 6) are not marked. In lane 3, the M13 linear dsDNA but not the ϕ X linear dsDNA reacted with M13 viral ssDNA to yield recombination products. In lane 6, the linear ds ϕ X DNA but not the linear M13 DNA reacted with viral ss ϕ X DNA to yield recombinants. Conversion of joint molecules to nicked circular dsDNA occurred more efficiently with the ϕ X substrate than with the larger M13 substrate. **(B)** Nucleotide requirement. Reaction mixtures containing ϕ X substrate DNAs and one of various nucleotide cofactors (2.5 mM each) were assembled and incubated without the ATP-regenerating system for 90 min (15). Mg^{2+} was omitted from the reaction in lane 10. Symbols: γ S, ATP γ S; ss, viral circular single-stranded DNA; ds, linear double-stranded DNA; jm, joint molecules; and nc, nicked circular double-stranded DNA.



combination. In addition, our findings raise the possibility that the proteins encoded by the *S. cerevisiae* DNA repair gene *RAD51* (5, 18) and the meiosis-specific gene *DMC1* (19), both structurally related to *RAD51* protein, may also possess some or all of the *RAD51* enzymatic activities described herein.

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9. For overexpression of *RAD51* protein in *S. cerevisiae*, the *RAD51* gene from nucleotide position –22 relative to the first ATG translation initiation codon until 180 nucleotides downstream of the TAG termination codon was placed under the control of the yeast alcohol dehydrogenase I (*ADC1*) promoter in the multicopy vector pSCW231 (20) to yield plasmid pRS1.1. This plasmid was introduced into yeast strain LP2749-9B (*MATa, his3-Δ1, leu2-3, leu2-112, pep4-3, trp1, ura3-52*) (21).
10. A hybrid polypeptide comprising the COOH-terminal 225 amino acid residues of *RAD51* protein fused to the NH₂-terminal 15 residues of the *E. coli* transcriptional terminator ρ was expressed in *E. coli* and purified from inclusion bodies by preparative SDS-polyacrylamide gel electrophoresis (PAGE). Polyclonal antibodies against the ρ -*RAD51* fusion protein were raised in rabbits and purified with the use of a Sepharose column containing the ρ -*RAD51* antigen. Extract (fraction I) was prepared (20) from 100 g of strain LP2749-9B harboring pRS1.1. *RAD51* and ~20% of total protein in fraction I were precipitated by ammonium sulfate (0.22 g/ml), redissolved in 150 ml of buffer K [20 mM KH₂PO₄ (pH 7.5), 10% glycerol, 0.5 mM dithiothreitol (DTT), and 0.5 mM EDTA] to give conductivity equivalent to 140 mM KCl (fraction II). This was applied onto Q Sepharose (1.6 cm by 10 cm), which was developed with a 300 ml, 200 to 600 mM KCl gradient. The *RAD51* protein peak (fraction III, with conductivity of ~350 mM KCl) was dialyzed against K + 40 mM KCl and fractionated in Biogel-hydroxyapatite (HTP) (1 cm by 5 cm) with a 75 ml, 0 to 120 mM KH₂PO₄ (pH 7.5) gradient in 10% glycerol, 40 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA. *RAD51* protein elutes from HTP at ~50 mM KH₂PO₄, and the pool of which (fraction IV) was subjected to molecular sizing in Sephacryl S200 (1.6 cm by 35 cm) developed with K + 200 mM KCl. The S200 pool (fraction V) was chromatographed in Mono Q (HR5/5) with the use of a 40 ml, 200 to 500 mM KCl gradient in K. Purified *RAD51* (fraction VI; 2.5 mg) elutes from Mono Q at 350 mM KCl and was stored in this buffer (K + 350 mM KCl) at –70°C in 100- μ l portions.
11. Purified *RAD51* protein was subjected to NH₂-terminal sequencing, but no sequence information could be obtained most probably due to a blocked NH₂-terminus. To circumvent this, three high-performance liquid chromatography (HPLC)-purified peptides derived from a cyanogen bromide digest of *RAD51* protein were sequenced instead, and they yielded perfect matches to the following portions of *RAD51* protein: (i) residues 23 to 32, (ii) residues 94 to 100, and (iii) residues 284 to 289.
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14. Reaction (final volume, 12.5 μ l) contained buffer R [30 mM potassium MES (pH 6.5), bovine serum albumin (100 μ g/ml), 1 mM DTT, 2 mM ATP, and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine phosphokinase (1.4 U/ml)], 16 mM MgCl₂, 60 ng (180 pmol) of nucleotides of circular viral ssDNA in 1 μ l of TE [10 mM tris-HCl (pH 7.5), 0.2 mM EDTA], 50 ng (75 pmol) of base pairs of Pst I-linearized dsDNA in 1 μ l of TE, 1 μ g of RPA (8.5 pmol) in 1 μ l of storage buffer [25 mM tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 1 mM DTT, and 100 mM NaCl], and 2.5 μ g of *RAD51* protein (58 pmol) in 2.5 μ l of storage buffer [20 mM KH₂PO₄ (pH 7.5), 10% glycerol, 350 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA]. To assemble the reaction mixture, we incubated *RAD51* protein with viral ssDNA at 36°C for 10 min in the presence of 6 mM MgCl₂, followed by the addition of RPA and a 3-min incubation at 36°C, and finally linear dsDNA and 10 mM MgCl₂ in 1 μ l were added and the reaction proceeded at 36°C for varying times. Reaction mixtures were deproteinized by treatment with 0.5% SDS and proteinase K (1 mg/ml) at 36°C for 20 min and subjected to electrophoresis in 0.8% agarose gels in 30 mM tris acetate (pH 7.4), 0.5 mM EDTA. After staining with ethidium bromide, gels were photographed through a red filter with Polaroid type 55 films. Labeling of 3' end of Xho I-linearized ϕ X DNA was done with the Klenow polymerase and [α -³²P]thymidine triphosphate, and 5' end-labeling of Pst I-linearized ϕ X DNA was done with T4 polynucleotide kinase and [γ -³²P]ATP after removal of the preexisting 5' phosphate with calf intestinal alkaline phosphatase. In experiments that used radiolabeled DNA, gels were treated with ethidium bromide and then dried onto a sheet of Whatman 3MM paper. The dried gels were examined under ultraviolet light and the positions of various ethidium bromide-stained DNA species were marked with Glo-Bug (x-ray marking solution from Bel-Art Products) on the edge of the gels before exposure to x-ray films.
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Interaction of Dbf4, the Cdc7 Protein Kinase Regulatory Subunit, with Yeast Replication Origins in Vivo

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DNA replication in the budding yeast *Saccharomyces cerevisiae* initiates from origins of specific DNA sequences during S phase. A screen based on two- and one-hybrid approaches demonstrates that the product of the *DBF4* gene interacts with yeast replication origins in vivo. The Dbf4 protein interacts with and positively regulates the activity of the Cdc7 protein kinase, which is required for entry into S phase in the yeast mitotic cell cycle. The analysis described here suggests a model in which one function of Dbf4 may be to recruit the Cdc7 protein kinase to initiation complexes.

The autonomously replicating sequence ARS1 is a chromosomal origin of DNA replication in yeast (1) composed of three functional domains designated A, B, and C (2). Domains A and B of ARS1 constitute an efficient yeast replication origin (2, 3) that does not activate significant levels of transcription when placed upstream from the *lacZ* reporter gene (4) (Fig. 1). Proteins that interact with ARS1 either directly by sequence-specific DNA binding or indirectly by interaction with one or more protein

components of initiation complexes might activate transcription when fused to the Gal4 transcriptional activation domain (GAD) (Fig. 1A). This possibility has formed the basis of a genetic screen designed to identify gene products that might interact with an intact and functional replication origin.

A yeast strain containing an ARS1-*lacZ* reporter construct was tested for expression of β -galactosidase after transformation with a library of yeast complementary DNAs (cDNAs) fused to GAD. Five positive clones that expressed β -galactosidase in an ARS1-dependent manner were identified and contained overlapping sequences from the previously characterized *DBF4* gene (5) fused in-frame to GAD. One clone, C2 (Fig. 1B), was further characterized. In subsequent experiments, transcriptional activa-

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