

# The DNA Binding Properties of *Saccharomyces cerevisiae* Rad51 Protein\*

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***Saccharomyces cerevisiae* Rad51 protein is the paradigm for eukaryotic ATP-dependent DNA strand exchange proteins. To explain some of the unique characteristics of DNA strand exchange promoted by Rad51 protein, when compared with its prokaryotic homologue the *Escherichia coli* RecA protein, we analyzed the DNA binding properties of the Rad51 protein. Rad51 protein binds both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in an ATP- and Mg<sup>2+</sup>-dependent manner, over a wide range of pH, with an apparent binding stoichiometry of approximately 1 protein monomer per 4 (±1) nucleotides or base pairs, respectively. Only dATP and adenosine 5'-γ-(thiotriphosphate) (ATPγS) can substitute for ATP, but binding in the presence of ATPγS requires more than a 5-fold stoichiometric excess of protein. Without nucleotide cofactor, Rad51 protein binds both ssDNA and dsDNA but only at pH values lower than 6.8; in this case, the apparent binding stoichiometry covers the range of 1 protein monomer per 6–9 nucleotides or base pairs. Therefore, Rad51 protein displays two distinct modes of DNA binding. These binding modes are not inter-convertible; however, their initial selection is governed by ATP binding. On the basis of these DNA binding properties, we conclude that the main reason for the low efficiency of the DNA strand exchange promoted by Rad51 protein *in vitro* is its enhanced dsDNA-binding ability, which inhibits both the presynaptic and synaptic phases of the DNA strand exchange reaction as follows: during presynapsis, Rad51 protein interacts with and stabilizes secondary structures in ssDNA thereby inhibiting formation of a contiguous nucleoprotein filament; during synapsis, Rad51 protein inactivates the homologous dsDNA partner by directly binding to it.**

Homologous recombination is a ubiquitous biological process. Elucidation of this process has come mostly from detailed analysis of *Escherichia coli* RecA protein, a key enzyme in both homologous genetic recombination and recombinational repair of damaged DNA in the eubacteria. RecA-like proteins have been found in a variety of eukaryotes, from yeast to human. The first identified and the most extensively studied eukaryotic RecA protein-analog is the Rad51 protein of *Saccharomyces cerevisiae*, which is establishing the paradigm for RecA protein-like functions in the eukaryotes. The *RAD51* gene of *S. cerevisiae* is required for mitotic and meiotic recombination (1) and

for the repair of double-strand DNA breaks caused by ionizing irradiation (2). Biochemical studies of Rad51 protein demonstrated that it shares similar properties to RecA protein, including ssDNA<sup>1</sup>-dependent ATPase activity, homologous pairing, and DNA strand exchange activity (3–5), and the formation of helical filaments on both double-stranded DNA (dsDNA) and single-stranded DNA (4, 6) (for review, see Ref. 7).

*In vitro*, the most extensively investigated DNA pairing reaction is the three-strand exchange reaction, in which the pairing of circular ssDNA and homologous dsDNA yields nicked circular dsDNA and linear ssDNA. During the formation of a presynaptic complex in the first phase of the reaction, the RecA protein polymerizes on ssDNA in the 5' → 3' direction to form a right-handed helical structure in which the DNA is extended to 1.5 times its original length (8). Pairing with homologous duplex DNA in the second phase of the reaction results in the rapid uptake of the dsDNA into homologous register and the associated exchange of DNA strands. Then, as a final step of the reaction, unidirectional branch migration completes the exchange of DNA strands (9).

All DNA strand exchange proteins follow this general scheme for DNA strand exchange; however, the eukaryotic proteins (Rad51 protein for *S. cerevisiae* and human) display some unique characteristics (3–5, 10, 11). The DNA strand exchange reaction is very sensitive to Rad51 protein concentration, and it occurs only in a narrow range of protein concentration, which corresponds to a stoichiometry of 3–4 nucleotides of ssDNA per Rad51 protein monomer; an excess of Rad51 protein severely inhibits DNA strand exchange (4). Another important distinction is that, in contrast to the RecA protein-promoted reaction, Rad51 protein-promoted DNA strand exchange is almost totally dependent on the presence of a ssDNA-binding protein, either eukaryotic replication protein-A (RPA) or prokaryotic ssDNA-binding protein (SSB) (4, 5). Furthermore, in the presence of ATPγS, a non-hydrolyzable ATP analogue, the reaction requires a 6-fold excess of Rad51 protein above the aforementioned stoichiometric amount (3, 12).

In this paper, we describe the DNA binding properties of Rad51 protein from *S. cerevisiae*. These properties offer insight into the specific requirements for the DNA strand exchange reaction that were described above.

## EXPERIMENTAL PROCEDURES

**Reagents**—All chemicals were reagent grade; solutions were made using Barnstead NANOpure water. ATP, ADP, CTP, UTP, GTP, dATP,

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<sup>1</sup> The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DAPI, 4',6-diamidino-2-phenylindole; ATPγS, adenosine 5'-γ-(thiotriphosphate); etheno M13 DNA, modified M13 ssDNA containing 1,N<sup>6</sup>-ethenoadenosine and 3,N<sup>4</sup>-ethenocytidine residues; RFI, relative fluorescence increase; EtBr, ethidium bromide; bp, base pair; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; RPA, replication protein-A; SSB protein, single-stranded DNA-binding protein; MES, 4-morpholineethanesulfonic acid.

TTP, dCTP, and dGTP were purchased from Sigma. ATP $\gamma$ S and AMP-PNP were purchased from Boehringer Mannheim. Dyes were obtained from Molecular Probes, and their concentrations were determined using the following extinction coefficients: 4',6-diamidino-2-phenylindole (DAPI),  $33 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 345 nm and ethidium bromide (EtBr),  $5.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 546 nm (13).

**DNA**—Both ssDNA and dsDNA from M13 mp7 phage (viral (+) strand and replicative form I) were purified as described (14). M13 dsDNA was linearized by digestion with *Eco*RI. Etheno M13 DNA was prepared as described (15).

**Construction of the Rad51 Protein-overproducing Plasmid**—For overproduction of the Rad51 protein in *E. coli*, the *RAD51* gene was cloned under the control of the T7 promoter in the pET3a vector to form the plasmid pEZ5139. The *Nde*-*Bam*HI fragment that contains the *RAD51* gene was isolated from plasmid YEP13/Rad51-23 (16). Because the *RAD51* gene contains several sites for *Nde*I restriction endonuclease, the desired *Nde*-*Bam*HI fragment (1470 bp) was reconstituted from two independently purified fragments, *Nde*I-*Bsu*36I (230 bp) and *Bsu*36I-*Bam*HI (1240 bp). Both fragments were ligated with the *Nde*I-*Bam*HI-digested vector, pET3a (17). After transformation of *E. coli* strain DK1( $\Delta$ recA), the authenticity of recombinant plasmid was confirmed by sequencing the entire *RAD51* gene. Attempts to clone *RAD51* gene under T7 promoter using any other expression vectors were unsuccessful, presumably due to both the high uninduced level of expression and the toxicity of Rad51 protein to *E. coli*.<sup>2</sup>

**Proteins**—RecA protein was purified from *E. coli* JC12772 (18) using a preparative protocol based on spermidine acetate precipitation (19). SSB protein was purified as described (20), and RPA was purified as described (5, 21). *S. cerevisiae* Rad51 protein was purified from *E. coli* strain ENZ21 (a  $\Delta$ recA derivative of BL21(DE3)), carrying both plasmids pEZ5139 and pLysS. The cells (80 g) from a 10-liter culture that was induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside for 4 h were resuspended in 200 ml of 50 mM Tris-HCl (pH 7.5), 600 mM KCl, 5 mM EDTA, 10% sucrose, 10 mM  $\beta$ -mercaptoethanol, 5  $\mu$ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 0.1 mM benzimidazole. Cells were lysed by two cycles of freeze-thawing and by passage through a French pressure cell (Aminco Inc.). The insoluble material was removed by centrifugation at 40,000 rpm for 2 h at 4 °C. The proteins were precipitated from the supernatant with ammonium sulfate (0.24 g/ml) and resuspended in 120 ml of P buffer (20 mM potassium phosphate (pH 7.5), 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT), containing 50 mM KCl, 5  $\mu$ g/ml AEBSF, 0.1 mM benzimidazole. This solution was loaded at a flow rate of 120 ml/h onto an 80-ml Cybacron Blue-agarose column, equilibrated with P buffer. The column was washed with P buffer + 100 mM KCl until the  $A_{280}$  approached zero, and then 200 ml of a 0.2–1 M KCl gradient was applied. Fractions containing Rad51 protein were combined and dialyzed against P buffer + 100 mM KCl, 5  $\mu$ g/ml AEBSF, and 0.1 mM benzimidazole, and then loaded onto an 80-ml Q-Sepharose column, equilibrated with P buffer + 100 mM KCl. Proteins were fractionated with a 600-ml KCl gradient (200–800 mM KCl); Rad51 protein eluted at about 400 mM KCl. Pooled fractions were dialyzed against H buffer (5 mM potassium phosphate (pH 6.5), 0.5 mM DTT, 10% glycerol, 40 mM KCl) containing 5  $\mu$ g/ml AEBSF and 0.1 mM benzimidazole, and then loaded onto a 50-ml Bio-Gel hydroxyapatite column that was equilibrated with H buffer. Bound proteins were eluted with a 300-ml linear gradient of 20–200 mM potassium phosphate (pH 6.5) at a flow rate of 60 ml/h. Rad51 protein eluted at about 50 mM potassium phosphate. These fractions were pooled and diluted to bring the phosphate concentration to about 25 mM and then loaded onto a MonoQ (HR 10/10) column. A linear gradient (160 ml) of 200–600 mM KCl in P buffer was used to elute Rad51 protein (at about 400 mM KCl). Fractions containing purified Rad51 protein were dialyzed against storage buffer (20 mM Tris-HCl (pH 7.5), 1 mM DTT, 40% glycerol, 0.5 mM EDTA, 1  $\mu$ g/ml AEBSF, 0.05 mM benzimidazole), aliquoted, and stored at –80 °C. The final yield of Rad51 protein was about 15 mg from 10 liters of culture. The concentration of Rad51 protein was calculated using an extinction coefficient (determined from amino acid composition) of  $1.29 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm. The purity of Rad51 protein was >99% based on SDS-polyacrylamide gel electrophoresis.

Rad51 protein was also purified from *S. cerevisiae* strain LP2749-B, harboring pR51.3 (kindly supplied by Dr. P. Sung) (3) using the same protocol described above for the isolation of Rad51 protein from *E. coli*. Rad51 protein was eluted from MonoQ column in two separate peaks: 70% of the protein eluted at about 400 mM KCl (analogously to Rad51

protein from *E. coli*) and 30% of the protein eluted at about 500 mM KCl. We believe, but have not investigated the possibility further, that the protein in the second peak could have been post-translationally modified (e.g. perhaps phosphorylated). The proteins from both peaks displayed similar DNA binding and DNA strand exchange activities. The yield of Rad51 protein purified from the yeast cells was about 3 mg from 80 g cells.

**Etheno M13 DNA Binding Assay**—Etheno M13 DNA fluorescence was measured by exciting at 300 nm and monitoring at 405 nm using an SLM-Aminco 8000 fluorescence spectrophotometer (15). Protein titrations of etheno M13 DNA and salt titrations of the protein-etheno M13 DNA complexes were performed by adding aliquots of protein or concentrated salt (NaCl) solutions, respectively, to samples in reaction buffer (30 mM Tris acetate (pH 7.5) or Na-MES (pH 6.2), 0.1 mM DTT, 10 mM magnesium acetate). The concentration of etheno M13 DNA was 3  $\mu$ M nucleotides (or as indicated), and the concentration of nucleotide cofactors was 2 mM, unless otherwise indicated. An ATP-regenerating system (10 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate) was added to reactions with RecA protein but was omitted for Rad51 protein. Reactions were carried out at 37 °C with continuous stirring. The relative fluorescence increase (RFI) of etheno M13 DNA due to protein binding was measured as the ratio of the fluorescence increase upon the protein binding to the fluorescence of the free etheno M13 DNA and free protein. For salt titration experiments, the percentage of remaining protein-ssDNA complexes was determined by dividing the fluorescence of the protein-DNA complex, before salt titration, by the fluorescence of this complex after the salt titration (with corrections for dilution).

**dsDNA Binding Assay**—The fluorescence of dsDNA-DAPI or dsDNA-EtBr complexes was measured by exciting at 359 nm and monitoring at 461 nm for DAPI, and 510 and 595 nm for EtBr, respectively (22).<sup>3</sup> The binding of Rad51 protein or RecA protein to the dsDNA-DAPI (or dsDNA-EtBr) complexes causes the displacement of dye from the dsDNA, resulting in a fluorescence decrease. Reactions were performed in buffer containing either 30 mM Tris acetate (pH 7.5) or 30 mM Na-MES (pH 6.2) and 0.1 mM DTT; magnesium acetate and ATP concentrations were as indicated. The concentration of DAPI was 200 nM and that of EtBr was 2  $\mu$ M. The concentration of M13 dsDNA was 6  $\mu$ M (nucleotides). The experiments were conducted at 37 °C with continuous stirring. The extent of Rad51 (RecA) protein binding to dsDNA was determined from the ratio of the observed fluorescence signal upon the addition of the protein, relative to the initial fluorescence of the DAPI-dsDNA complex.

**Agarose Gel Mobility Shift Assay**—M13 DNA was mixed with varying concentrations of Rad51 protein in the indicated buffers in a total volume of 20  $\mu$ l and incubated at 37 °C for 30 min. Depending on the experiment, magnesium acetate and ATP either were present in the buffer or were added subsequently. When added separately, magnesium acetate or ATP were added, and incubation was continued for an additional 30 min. Sample loading buffer (3  $\mu$ l of 40 mM Tris acetate (pH 7.5), 50% glycerol, and a trace of bromophenol blue) was added to each sample, and the complexes were separated by electrophoresis through 0.8% agarose gel in TAE buffer (40 mM Tris acetate, 2 mM EDTA (pH 8.0)) at 120 V for 2 h and were visualized by EtBr staining.

## RESULTS

**Binding of Rad51 Protein to Etheno M13 ssDNA**—In the presence of ATP and Mg<sup>2+</sup>, the binding of Rad51 protein to etheno M13 DNA results in an increase in the fluorescence of this modified M13 DNA (2). This increase reaches a maximum when the stoichiometric ratio between Rad51 protein and ssDNA corresponds to 1 monomer of Rad51 protein to approximately 4 ( $\pm$ 1) nucleotides of ssDNA (Fig. 1). The relative fluorescence increase (RFI) value for Rad51 protein is 3.6–3.8, a value that is slightly lower than that for RecA protein (4.2), with this preparation of etheno M13 DNA, and under the same experimental conditions.

In the absence of ATP at pH 7.5, Rad51 protein does not show any significant increase in etheno M13 DNA fluorescence; this is unlike the behavior of RecA protein, which produces an RFI value in the absence of ATP of about 2 (not shown). The slight apparent increase in fluorescence upon addition of Rad51 pro-

<sup>2</sup> E. M. Zaitseva, E. N. Zaitsev, and S. C. Kowalczykowski, unpublished observations.

<sup>3</sup> E. N. Zaitsev and S. C. Kowalczykowski, submitted for publication.

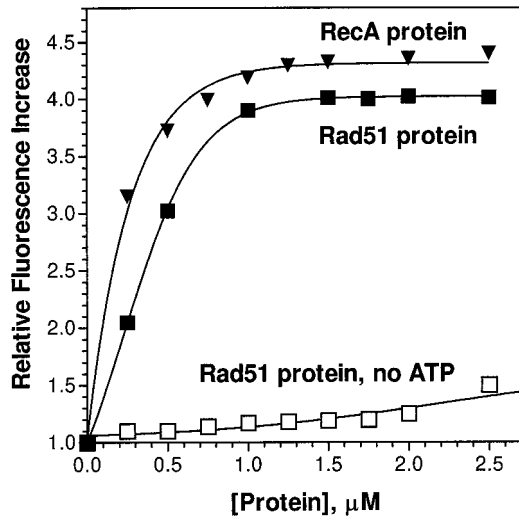


FIG. 1. **Binding of Rad51 and RecA proteins to etheno M13 DNA.** Reactions were performed in 30 mM Tris acetate buffer (pH 7.5), 10 mM magnesium acetate, 2 mM ATP at 37 °C, containing 3  $\mu$ M (nucleotides) of etheno M13 DNA. Titrations in the presence of ATP and magnesium ion with RecA protein ( $\blacktriangledown$ ) and Rad51 protein ( $\blacksquare$ ). The titration with Rad51 protein in the absence of ATP ( $\square$ ).

tein in the absence of ATP shown in Fig. 1 is probably due to light scattering from aggregation of Rad51 protein.

We discovered that preincubation of Rad51 protein in Tris acetate buffer at pH values higher than 7.0, containing 10 mM magnesium acetate but without ATP, inactivates the protein with regard to DNA binding (Fig. 2). The subsequent addition of ATP to such inactivated protein failed to restore binding activity to Rad51 protein. However, the prior presence of ATP under these conditions prevents the inactivation of Rad51 protein; the fluorescence increase after addition of etheno M13 DNA is the same as in the protein titration experiment (see Fig. 1).

**Sensitivity of Rad51 Protein-Etheno M13 DNA Complexes to Dissociation by NaCl**—The sensitivity of protein-DNA complexes to disruption by NaCl is a relative measure of the affinity of a protein for ssDNA (24). After formation of the Rad51 protein-etheno M13 DNA complex, its stability to dissociation by an increasing concentration of NaCl was examined. A decrease in fluorescence is observed upon the addition of NaCl until the complete dissociation of the protein-DNA complex occurs (Fig. 3). The salt titration midpoint for dissociation of the Rad51 protein in the presence of ATP and 10 mM magnesium acetate is about 550 mM NaCl; for comparison, the salt titration midpoint for RecA protein under identical conditions is about 700 mM NaCl (25) (data not shown).

**Dependence of Rad51 Protein-Etheno M13 DNA Complex Formation on NTP Cofactors**—The effects of different nucleotide cofactors on the binding of Rad51 protein to etheno M13 DNA was investigated (Table I). In addition to ATP, for which the RFI value for the complex was 3.6–3.8 (Fig. 1 and Table I), only dATP and ATP $\gamma$ S support binding. In the presence of dATP, the RFI value is 2.6–2.8, but the apparent binding stoichiometry is the same as that obtained with ATP (data not shown). No change in the fluorescence of etheno M13 DNA upon addition of Rad51 protein was detected for any of the other NTPs (Table I), implying that either Rad51 protein does not bind ssDNA under these conditions or if binding does occur but there is no extension of the etheno M13 DNA to produce a fluorescence change. Etheno M13 DNA complex formation by Rad51 protein was sensitive to the ratio of ATP to ADP; in the presence of a mixture containing 1 mM ADP and 1 mM ATP, the RFI decreased by 25%, in the presence of 3 mM ADP and 1 mM

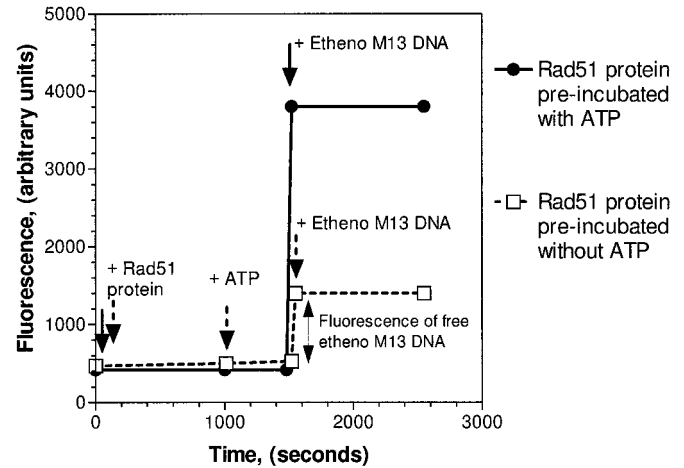


FIG. 2. **ATP prevents a magnesium ion-dependent irreversible inactivation of Rad51 protein.** Rad51 protein (1  $\mu$ M) was incubated in 30 mM Tris acetate buffer (pH 7.5) and 10 mM magnesium acetate, either with 1 mM ATP ( $\bullet$ ) or without ATP ( $\square$ ), for 15 min at 37 °C; ATP (1 mM) was then added to the mixture that did not have ATP. Components were added at the times indicated on the graph. The fluorescence was measured in arbitrary units. The concentration of etheno M13 DNA was 3  $\mu$ M (nucleotides).

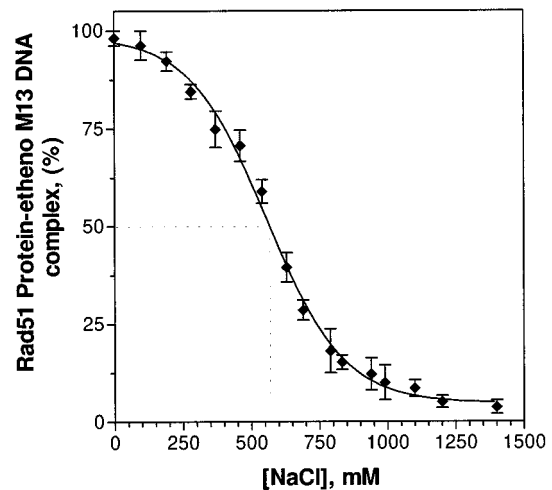


FIG. 3. **Salt titration of Rad51 protein-etheno M13 DNA complexes assembled in the presence of ATP.** After the Rad51 protein-etheno M13 DNA complex was formed, the change in fluorescence upon the addition of indicated amount of NaCl was monitored. The initial concentrations of etheno M13 DNA and Rad51 protein were 3 and 1.5  $\mu$ M, respectively. Reactions were performed in 30 mM Tris acetate buffer (pH 7.5) in the presence of 2 mM ATP and 10 mM magnesium acetate.

ATP, the RFI decreased by 80% (Table I).

Unlike RecA protein (15), ATP $\gamma$ S barely supported Rad51 protein binding to etheno M13 DNA, as indicated by the slight increase in fluorescence of etheno M13 DNA when a stoichiometric amount of Rad51 protein was present (RFI value of 1.3). However, ATP $\gamma$ S apparently binds to Rad51 protein and prevents ATP binding, since upon subsequent addition of ATP to the Rad51 protein/etheno M13 mixture that had been preincubated with ATP $\gamma$ S, no change in fluorescence was detected. However, addition of ATP $\gamma$ S to a preformed ATP-Rad51 protein-etheno M13 DNA complex resulted in a decrease in fluorescence (data not shown), suggesting that ATP $\gamma$ S could bind after ATP turnover. Increasing the Rad51 protein concentration above the stoichiometric ratio produced a substantial fluorescence signal in the presence of ATP $\gamma$ S; we observed RFI values of 1.3, 1.9, 3.0, and 3.3 at Rad51 protein concentrations that are 1-, 2-, 3-, and 5-fold higher than the stoichiometric

TABLE I  
Binding of Rad51 protein to etheno M13 DNA depends on nucleotide cofactor

The change in fluorescence was measured in 30 mM Tris acetate buffer (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, and indicated cofactor (1 mM) after the addition of 1  $\mu$ M Rad51 protein to 3  $\mu$ M etheno M13 DNA at 37  $^{\circ}$ C.

Nucleotide cofactor	Relative fluorescence increase
ATP	3.6–3.8
UTP	1
CTP	1
GTP	1
UTP	1
dATP	2.6–2.8
TTP	1
dCTP	1
dGTP	1
ATP $\gamma$ S <sup>a</sup>	1.3 (1.9, 3, 3.3)
AMP-PNP	1
ADP/ATP, 33%	3.4
ADP/ATP, 50%	2.7
ADP/ATP, 60%	2.2
ADP/ATP, 67%	1.9
ADP/ATP, 75%	1.4
ADP, 100%	1

<sup>a</sup> In the presence of ATP $\gamma$ S, the observed RFI depends on the Rad51 protein concentration (reported in parentheses are values for 2-, 3-, and 5-fold excesses above the stoichiometric concentration, as described in the text).

ratio, respectively.

**pH Dependence of Rad51 Protein Binding to ssDNA**—In the presence of ATP and Mg<sup>2+</sup>, no significant changes in the binding activity of Rad51 protein to etheno M13 DNA were observed upon changing the pH values over the range of 6.0–8.5 (Fig. 4). However, in the absence of ATP, an increase in etheno M13 DNA fluorescence upon Rad51 protein addition was detected only at pH values below 6.8. Furthermore, at these acidic pH values, the increase in etheno M13 DNA fluorescence upon the binding of Rad51 protein occurred in the absence of both nucleotide cofactor and magnesium ion. Without ATP and magnesium acetate, the maximum increase in etheno M13 DNA fluorescence is observed at pH 6.2, and no increase is detected at any pH values above 7.0. At a pH lower than 5.0, Rad51 protein seems to inactivate; an initial increase in fluorescence is seen immediately after Rad51 protein addition to the cuvette, but it gradually decreases to the fluorescence of free etheno M13 DNA (data not shown). The chemical nature of buffers did not affect the binding activity of Rad51 protein, because buffers with overlapping pH values (sodium citrate (pH 5.0–6.2), Na-MES (pH 5.8–7.2), and Tris acetate (pH 6.8–8.5)) showed no difference in RFI.

Titration of etheno M13 DNA with Rad51 protein at pH 7.5, pH 6.6, and pH 6.2 (Fig. 5) revealed that both the RFI value and the apparent binding stoichiometry are affected by pH and by the presence of both ATP and magnesium acetate. In the presence of ATP and magnesium ion, Rad51 protein binds to etheno M13 DNA over a wide range of pH (the RFI value is 3.8 over the pH range of 6.0–8.0 (Fig. 4)), with an apparent binding stoichiometry of 1 protein monomer per  $\approx$ 4–5 nucleotides of etheno M13 DNA (Fig. 5). However, in the absence of both nucleotide cofactor and magnesium ion, the RFI value increases with decreasing pH (the RFI values are 1.3 at pH 7.5, 2 at pH 6.6, and 3.2 at pH 6.2 (see Fig. 4)), and the apparent binding stoichiometry of Rad51 protein changes from no obvious plateau, to 6:1, to 9:1 nucleotides of ssDNA per protein monomer (Fig. 5).

The increase in RFI value with decreasing pH suggests either that the binding affinity of Rad51 protein to etheno M13 DNA is increasing or that the structure of the protein-DNA

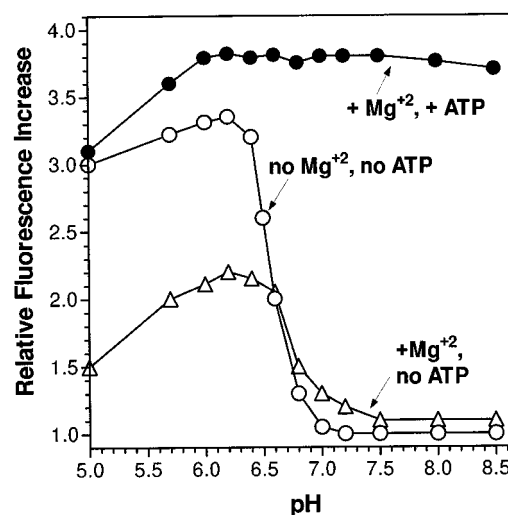


FIG. 4. Dependence of Rad51 protein-etheno M13 DNA complex formation on pH. The RFI values obtained upon addition of Rad51 protein (2  $\mu$ M) to etheno M13 DNA (3  $\mu$ M) were measured as described under "Experimental Procedures." Buffers containing 30 mM sodium citrate, Na-MES, or Tris acetate were employed for the pH intervals 5.0–6.0, 5.5–7.2, and 6.8–8.5, respectively. Reactions were performed in the presence of 2 mM ATP and 10 mM magnesium acetate ( $\bullet$ ); in the presence of 10 mM magnesium acetate but without ATP ( $\Delta$ ); and neither magnesium acetate nor ATP ( $\circ$ ).

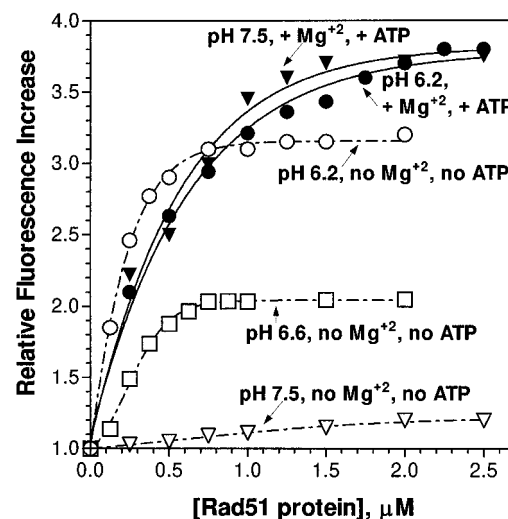


FIG. 5. Complexes with two distinct stoichiometries are formed between Rad51 protein and ssDNA. Etheno M13 DNA (3  $\mu$ M) was titrated with Rad51 protein in standard buffers at pH values of 7.5, 6.6, and 6.2, either in the presence or the absence of both ATP (2 mM) and magnesium acetate (10 mM). The filled symbols represent experiments in the presence of ATP and magnesium acetate, and the open symbols represent experiments without ATP or magnesium acetate: 30 mM Na-MES, (pH 6.2) ( $\circ$ ); 30 mM Na-MES (pH 6.2), 2 mM ATP, 10 mM magnesium acetate ( $\bullet$ ); 30 mM Tris acetate (pH 7.5) ( $\nabla$ ); 30 mM Tris acetate (pH 7.5), 2 mM ATP, 10 mM magnesium acetate ( $\blacktriangledown$ ); 30 mM Na-MES (pH 6.6) ( $\square$ ).

complex is changing. Since the salt titration midpoint is a relative measure of DNA binding affinity (15), the stability of Rad51 protein-etheno M13 DNA complexes, formed at different pH values, to disruption by NaCl was examined (Fig. 6). The salt titration midpoint values are 60 mM NaCl and 110 mM NaCl at pH 6.6 and pH 6.2, respectively, indicating that the interaction of Rad51 protein with ssDNA in the absence of ATP and magnesium acetate is stronger at reduced pH. When compared with the results that were presented in Fig. 3, it is evident that the stability of complexes formed in the presence of both ATP and magnesium acetate is higher than those

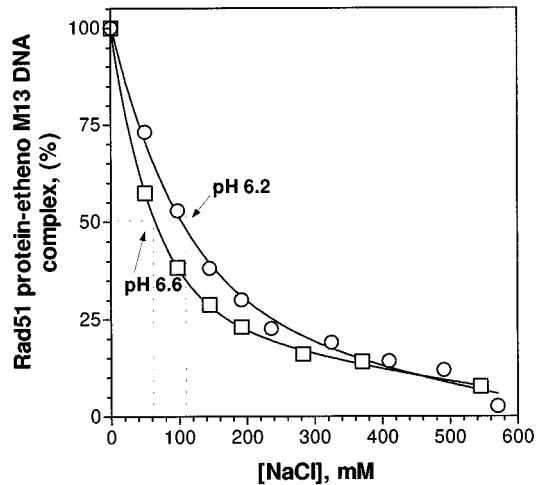


FIG. 6. Rad51 protein-etheno M13 DNA complexes assembled in the absence of nucleotide cofactor and magnesium ion have a greater stability at lower pH values. Salt titrations were performed with complexes that were formed in the absence of ATP and magnesium acetate in 30 mM Na-MES buffer at pH 6.2 (○) and pH 6.6 (□). Both reactions contained 3  $\mu$ M (nucleotides) of etheno M13 DNA and 2  $\mu$ M Rad51 protein.

formed in the absence of ATP cofactor and magnesium acetate (midpoints of 550 mM NaCl and 110 mM NaCl, respectively), which correspond to the behavior of RecA protein (15).

To verify these fluorescence measurements, Rad51 protein-native M13 ssDNA complexes were examined using a gel mobility shift assay (Fig. 7). The mobility of Rad51 protein-ssDNA complexes, formed with increasing concentrations of Rad51 protein in the absence of ATP and magnesium acetate at pH 6.2, was different than the mobility of free DNA; this change in mobility paralleled the fluorescence titration shown in Fig. 5. Also in agreement with the fluorescence data, only a slight change in mobility of M13 ssDNA was observed upon addition of Rad51 protein at pH 7.5 in the absence of ATP and magnesium acetate, showing that the absence of an increase in fluorescence of etheno M13 DNA was due to an absence of complex formation and not just to a failure to induce a change in fluorescence.

**Effect of  $Mg^{2+}$  on the Formation of Rad51 Protein-ssDNA Complexes**—The requirement for  $Mg^{2+}$  in Rad51 protein-etheno M13 DNA complex formation shows a dependence on both the presence of ATP and the pH value (Fig. 8). In the presence of ATP, at neutral pH (pH 7.5), no binding was detected until the  $Mg^{2+}$  concentration reached 2 mM; however, at an acidic pH (pH 6.2), Rad51 protein bound to etheno M13 DNA in the absence of magnesium acetate to yield an RFI value of 3.2. The RFI displayed the curious trend of initially decreasing from the RFI of 3.2 at 0 mM  $Mg^{2+}$  to 2.6 at 4 mM  $Mg^{2+}$  but then increasing at higher  $Mg^{2+}$  concentrations and finally saturating at 7–10 mM magnesium acetate. Titrations of etheno M13 DNA with Rad51 protein in the presence of ATP (at pH 6.2) also revealed a change in the apparent binding stoichiometry that depends on  $Mg^{2+}$  concentration; the apparent binding stoichiometry changes from 6:1 (nucleotides of ssDNA per protein monomer) at 1–2 mM magnesium acetate to a stoichiometry of about 4:1, at 7–10 mM magnesium acetate (data not shown).

In the absence of ATP, Rad51 protein does not change the etheno M13 DNA fluorescence at pH 7.5, regardless of the magnesium ion concentration, but, at pH 6.2, the maximum RFI value is seen at 0 mM magnesium acetate (Fig. 8). Increasing the magnesium acetate concentration in the absence of ATP at pH 6.2 reduces the RFI (compare the RFI value of 3.2 at 0 mM and 1.8 at 10 mM magnesium acetate) and, by inference, the

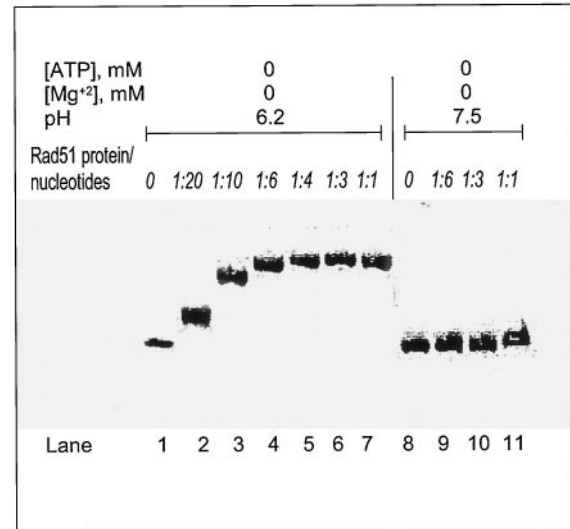


FIG. 7. The binding of Rad51 protein to M13 ssDNA detected by a gel mobility shift assay parallels the fluorescent assays. Rad51 protein was bound to DNA at the indicated ratio (given in protein monomer per nucleotides) in Tris acetate (pH 7.5) (lanes 2–7) or Na-MES (pH 6.2) (lanes 9–11) for 30 min at 37 °C, and the complexes were analyzed by electrophoresis through a native 0.8% agarose gel as described under “Experimental Procedures.” Lanes 1 and 8 contain free DNA. The concentration of M13 ssDNA was 30  $\mu$ M.

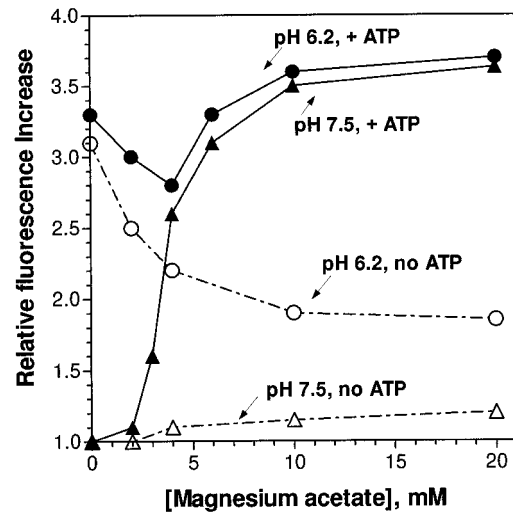


FIG. 8. Magnesium ion dependence of Rad51 protein binding to etheno M13 DNA. Each point represents the relative fluorescence increase value for etheno M13 DNA obtained upon addition of Rad51 protein (2  $\mu$ M) at different concentrations of magnesium acetate. Filled symbols represent experiments in the presence of 2 mM ATP; open symbols represent experiments without ATP: Na-MES (pH 6.2) (○), Na-MES (pH 6.2), ATP (●); Tris acetate (pH 7.5) (△); Tris acetate (pH 7.5), ATP (▲). Concentration of etheno M13 DNA was 3  $\mu$ M (nucleotides).

binding affinity of Rad51 protein to etheno M13 DNA.

Finally, a mobility shift assay (Fig. 9) shows that the migration of the Rad51 protein-ssDNA complexes formed at these experimental conditions is different. The Rad51 protein-ssDNA complexes formed in the presence of magnesium acetate and ATP (lanes 8–10) migrated faster than ones formed in the presence of ATP alone (lane 5) and in the absence of magnesium acetate and ATP (lane 1). In the presence of ATP, the Rad51 protein-ssDNA complexes formed at 4 mM magnesium acetate had a mobility faster than the complexes formed at 1 and 10 mM magnesium acetate (compare lane 9 to lanes 8 and 10). This complicated dependence of Rad51 protein-ssDNA

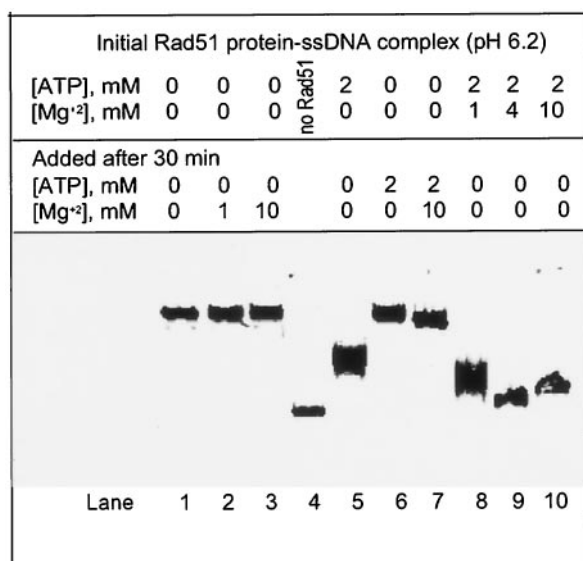


FIG. 9. The binding of Rad51 protein to ssDNA in the absence of both ATP and magnesium ion is not interconvertible. Complexes of Rad51 protein and M13 ssDNA were formed in Na-MES buffer (pH 6.2) at the indicated initial concentrations of ATP and magnesium acetate and were incubated for 30 min at 37 °C. Then, as indicated, ATP and magnesium acetate were added, and the incubation was continued for 30 min more. Sample loading buffer was added, and the reaction products were analyzed by gel electrophoresis as described under "Experimental Procedures." Lane 4 contains free DNA. The concentrations of Rad51 protein and M13 ssDNA were 15 and 30  $\mu\text{M}$  nucleotides, respectively.

complex mobility on magnesium ion concentration parallels the dependence of RFI (see Fig. 8) on  $\text{Mg}^{2+}$  concentration; the lowest RFI is seen at 4 mM magnesium acetate. This might indicate that a  $\text{Mg}^{2+}$ -dependent transition between two different modes of Rad51 protein binding to ssDNA occurs at about 4 mM magnesium acetate.

**Two Different Binding Modes of Rad51 Protein to ssDNA Are Not Interconvertible**—The previous binding data (Figs. 5–8) suggest that Rad51 protein exhibits two different modes of binding to ssDNA, which depend on ATP and  $\text{Mg}^{2+}$  concentration. These two complexes have different apparent binding stoichiometries and different mobilities through agarose gels. To test whether one binding mode can be converted to the other, the Rad51 protein-ssDNA complexes that form in the absence of ATP and  $\text{Mg}^{2+}$  were incubated with subsequently added ATP alone (Fig. 9, lane 6), magnesium acetate alone (lanes 2 and 3), or both ATP and magnesium acetate (lane 7). Fig. 9 shows that these complexes are not simply interconvertible; the mobility of the existing complexes does not change after addition of ATP and  $\text{Mg}^{2+}$ .

**Binding of Rad51 Protein to dsDNA**—To investigate the binding of Rad51 protein to dsDNA, we employed both a dye-displacement assay, and a gel mobility shift assay. The dyes used were DAPI and EtBr. DAPI interacts with dsDNA in the minor groove and EtBr intercalates between bases of dsDNA to produce dye-dsDNA complexes with increased fluorescence (26, 27). These fluorescent dye-dsDNA complexes were used previously to study RecA protein binding to dsDNA by monitoring the decrease in fluorescence upon protein binding (22).<sup>3</sup> The binding of Rad51 protein to dye-dsDNA complexes results in the displacement of the dye from dsDNA and, consequently, the fluorescent signal decreases (Fig. 10, A and B).

The binding of Rad51 protein to dsDNA is also magnesium ion-dependent. As can be seen in Fig. 11, in the presence of ATP, Rad51 protein binds dsDNA poorly at 1–3 mM magnesium acetate. An increase in the magnesium ion concentration be-

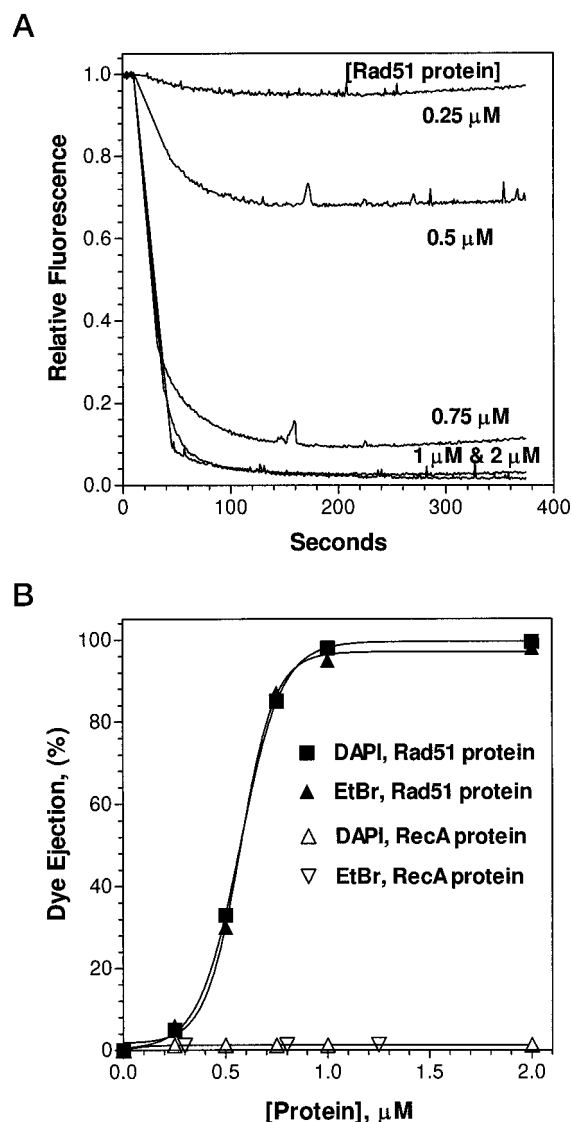
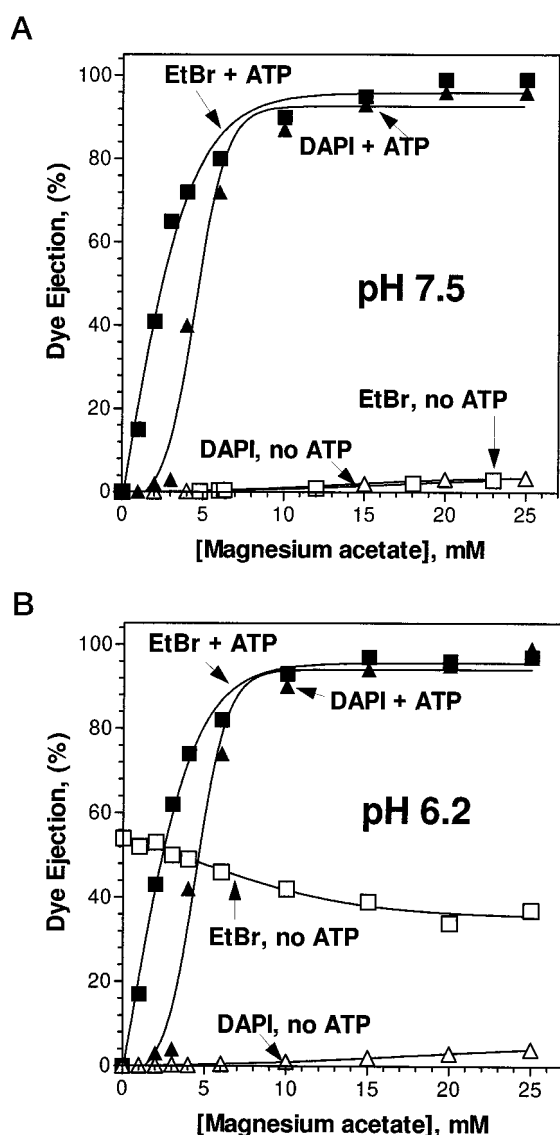


FIG. 10. Rad51 protein binding to dsDNA monitored by the dye displacement assay. Binding of Rad51 protein to dsDNA was measured by observing the displacement of a DNA-bound fluorescent dye upon protein-DNA complex formation. A, decrease in fluorescence of dsDNA-DAPI complexes upon addition of indicated concentrations of Rad51 protein. B, dependence of DAPI and EtBr displacement from dsDNA on the concentration of Rad51 and RecA proteins. Reactions contained 30 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 2 mM ATP, 200 nM DAPI (or 2  $\mu\text{M}$  EtBr), and 6  $\mu\text{M}$  (nucleotides) dsDNA. Filled symbols represent experiments with Rad51 protein, and open symbols represent experiments with RecA protein: DAPI displacement by Rad51 protein (■), EtBr displacement by Rad51 protein (▲), DAPI displacement by RecA protein (△), and EtBr displacement by RecA protein (▽).

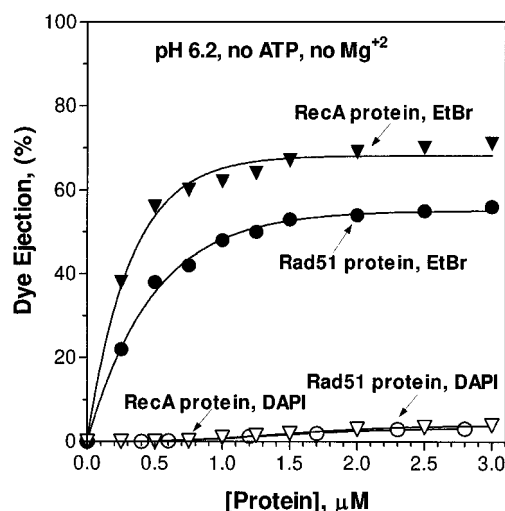
yond 4 mM magnesium acetate results in an increase in the apparent affinity of Rad51 protein for dsDNA, with apparent saturation occurring above 10 mM magnesium acetate. At these saturating concentrations of magnesium acetate, the extent of Rad51 protein binding is the same for both the DAPI and EtBr assays (Fig. 11, A and B), and the apparent binding stoichiometry is unchanged at approximately 1 monomer per 4.5 ( $\pm 0.5$ ) bp, whereas at the lower concentrations of magnesium acetate, the binding of Rad51 protein becomes progressively more sigmoid (Fig. 10B and other data not shown). The efficiency of EtBr displacement from dsDNA by Rad51 protein at low  $\text{Mg}^{2+}$  concentrations (1–3 mM magnesium acetate) is higher than the displacement of DAPI (Fig. 11, A and B); this may reflect differing affinities and modes of binding for each of these dyes



**FIG. 11. Dependence of Rad51 protein binding to dsDNA on magnesium ion concentration.** The extent of DAPI and EtBr displacement from dsDNA was determined upon the binding of Rad51 protein to dye-dsDNA complexes at different concentrations of magnesium acetate. *A*, reactions were performed in Tris acetate buffer (pH 7.5). *B*, reactions were performed in Na-MES buffer (pH 6.2). *Filled symbols* represent experiments in the presence of 2 mM ATP, and *open symbols* are in the absence of ATP: EtBr displacement ( $\square$ ,  $\blacksquare$ ) and DAPI displacement ( $\triangle$ ,  $\blacktriangle$ ). The concentration of dsDNA was 6  $\mu$ M nucleotides. The concentration of Rad51 protein was 2  $\mu$ M.

and is consistent with DAPI being a more effective competitor than EtBr of Rad51 protein-dsDNA complex formation. Furthermore, in the presence of ATP, the binding of Rad51 protein to dsDNA is equally effective at pH 6.2 and at pH 7.5 (Fig. 11, *A* and *B*).

In the absence of ATP, the DAPI displacement assay shows that Rad51 protein does not eject DAPI from dsDNA, regardless of pH (Fig. 11, *A* and *B* and Fig. 12). However, displacement of EtBr from dsDNA is observed at pH 6.2 but not at pH 7.5 (Fig. 11*B* and Fig. 12). This is also consistent with the idea that EtBr is more readily displaced or actually facilitates Rad51 protein binding, as it does for RecA protein (28). The extent of EtBr displacement upon Rad51 protein binding is about 50%, and the apparent binding stoichiometry is different than in the presence of ATP (1 Rad51 protein monomer per 6 bp of DNA *versus* 1 per 4.5 bp, respectively). In the absence of

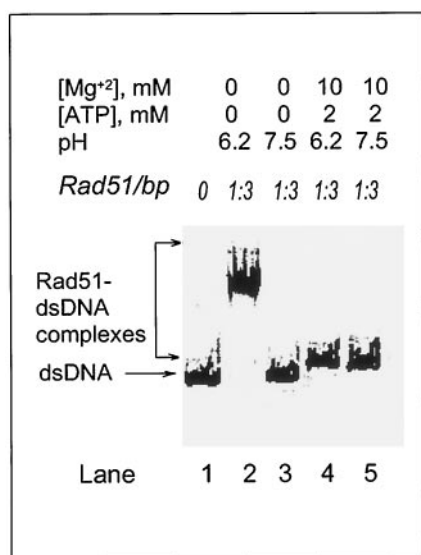


**FIG. 12. Binding of Rad51 protein and RecA protein to dsDNA in the absence of nucleotide cofactor and magnesium ion.** Titrations with Rad51 protein and RecA protein were performed using either dsDNA-DAPI or dsDNA-EtBr complexes in Na-MES buffer (pH 6.2) in the absence of magnesium acetate and ATP. The concentration of dsDNA was 6  $\mu$ M (nucleotides). *Filled symbols* represent EtBr displacement experiments, and *open symbols* represent DAPI displacement experiments: EtBr displacement by Rad51 protein ( $\bullet$ ), EtBr displacement by RecA protein ( $\blacktriangledown$ ), DAPI displacement by Rad51 protein ( $\circ$ ), and DAPI displacement by RecA protein ( $\triangledown$ ).

ATP, the maximum extent of EtBr displacement occurs in the absence of magnesium acetate and decreases with increasing magnesium ion concentration (Fig. 11*B*), presumably due to a reduction in binding affinity of Rad51 protein to dsDNA. Interestingly, in the absence of ATP and magnesium ions, RecA protein binds dsDNA ejecting EtBr but not DAPI, exhibiting a behavior similar to Rad51 protein (Fig. 12). Analogous results were obtained using the mobility shift assay (see Fig. 13); complexes of Rad51 protein and M13 dsDNA formed in the presence of ATP at pH 7.5 and at pH 6.2 exhibit the same mobility through agarose gels (Fig. 13, *lanes 4* and *5*). However, in the absence of ATP and magnesium acetate, the binding of Rad51 protein to dsDNA is pH-dependent. The formation of Rad51-dsDNA complexes in the absence of ATP and magnesium acetate occurred only at reduced pH and was not detected at pH 7.5 (Fig. 13, *lanes 2* and *3*). This difference suggests the existence of two different modes of Rad51 protein binding to dsDNA, and these modes are dependent on the presence or absence of ATP.

#### DISCUSSION

In this study, we examined the binding of Rad51 protein to ssDNA and dsDNA using both spectrofluorimetric and gel assays. The speed and convenience of the fluorimetric etheno M13 DNA binding assay permitted a rapid survey of binding behavior, and although indirect, the fluorimetric assay produced results that coincided with the more direct but tedious gel mobility shift assay. This correspondence suggests that, for Rad51 protein, an increased fluorescence signal in the etheno M13 DNA binding assay generally coincides with an increased amount of protein-ssDNA complex. Based on these two assays, we make several conclusions about the DNA binding properties of Rad51 protein. At pH 7.5, the protein binds to ssDNA in an ATP- and  $Mg^{2+}$ -dependent manner, with an apparent binding stoichiometry of about 1 Rad51 protein monomer per 4 nucleotides of DNA. However, in the absence of ATP, Rad51 protein shows a unique pH dependence of ssDNA binding. In contrast to RecA protein, Rad51 protein does not bind ssDNA in the absence of ATP at pH 7.5. Instead, it irreversibly loses DNA



**FIG. 13. Binding of Rad51 protein to dsDNA detected by a gel mobility shift assay parallels the EtBr displacement assay.** Rad51 protein was bound to M13 dsDNA at a stoichiometry of 1 protein monomer to 3 base pairs (6 nucleotides) of DNA (lanes 2–5), at the indicated conditions, for 15 min at 37 °C; complexes were analyzed as described in Fig. 7. Lane 1 contains free dsDNA. The concentration of dsDNA was 30  $\mu$ M (nucleotides). The concentration of Rad51 protein was 5  $\mu$ M.

binding activity when magnesium acetate is present, probably due to aggregation, since the subsequent addition of ATP to Rad51 protein does not restore DNA binding activity (Fig. 2). Strangely, this inactivation does not occur at pH 6.2; rather, in the absence of ATP, Rad51 protein binds to ssDNA at acidic pH values, saturating at an apparent binding stoichiometry of 1 protein monomer per 7–9 nucleotides of DNA. Thus, Rad51 protein exhibits two different ssDNA-binding modes, one ATP-dependent and the other ATP-independent, with the transition controlled by the bound nucleotide cofactor. Moreover, these ssDNA binding modes are not inter-convertible (Fig. 9), a behavior similar to that observed for RecA protein (29–31).

The stability of Rad51 protein-ssDNA complexes was examined using salt titration experiments. The salt titration midpoint for the dissociation of Rad51 protein- and RecA protein-etheno M13 DNA complexes was found to be 550 and 700 mM NaCl, respectively. Without knowledge of the salt concentration dependence of the affinity constant, it is not possible to compare the apparent affinities at these two different salt concentrations. However, the data do show that ATP induces a high affinity, high RFI (*i.e.* extended) Rad51 protein-ssDNA complex with characteristics similar to those of RecA protein (9).

The most notable difference between RecA and Rad51 proteins is in their dsDNA binding activities. To bind dsDNA, RecA protein must overcome a kinetic barrier that limits nucleation on dsDNA. Therefore, at neutral pH values, the binding of RecA protein to dsDNA is so slow that it is practically negligible (32). Reduced pH (pH 6.2) greatly facilitates the nucleation step and, consequently, the binding to dsDNA (33, 34). In contrast to RecA protein, Rad51 protein much more easily circumvents the slow nucleation step and binds to dsDNA in a pH-independent manner.

Interestingly, Rad51 protein binds dsDNA poorly at low Mg<sup>2+</sup> concentrations (1–3 mM), conditions that facilitate dsDNA binding for RecA protein. In the absence of ATP, both proteins bind to dsDNA only at reduced pH 6.2, with an apparent binding stoichiometry of approximately 1 protein monomer per 4.5 bp of DNA. Binding to dsDNA at this pH for both Rad51

and RecA proteins is accompanied by displacement of EtBr but not DAPI. In contrast, the ATP-dependent binding of RecA and Rad51 proteins results in the displacement of both EtBr and DAPI. The ATP-dependent binding is likely to occur through the minor groove of dsDNA, given that DAPI is a minor groove binder. Therefore, as for ssDNA, Rad51 protein displays two different modes of dsDNA binding that are modulated by ATP binding, and these two modes differ from each other in binding stoichiometry.

Although the intracellular pH of *S. cerevisiae* is in the range of 6.15–6.6 (35, 36), the ability of Rad51 protein to bind DNA in the absence of ATP at low pH conditions is unlikely to be physiologically significant. However, *in vitro*, this characteristic may be useful; for example, we successfully used a change in pH from 6.2 to pH 7.5 to elute Rad51 protein from ssDNA-cellulose as a useful purification step.<sup>2</sup> Interestingly, both Rad51 and RecA proteins show significant similarities in DNA binding properties at reduced pH conditions; conversely, at neutral pH, both the efficient binding of Rad51 protein to dsDNA in the presence of ATP and the inability to bind ssDNA in the absence of ATP differentiate Rad51 protein from RecA protein.

The DNA binding properties of Rad51 protein explain some features of the DNA strand exchange mediated by this protein. The formation of an active and contiguous nucleoprotein filament on ssDNA is the first step of recombination. In contrast to the RecA protein-dependent reaction, DNA strand exchange mediated by Rad51 protein is nearly absolutely dependent on RPA (or SSB protein) function (3, 5), a protein whose role is to remove DNA secondary structure from ssDNA (37–39). In the absence of RPA, it is likely that Rad51 protein binds to and stabilizes dsDNA within the regions of ssDNA containing secondary structure. The Rad51 protein-dsDNA complexes formed within regions of secondary structure are probably stable since Rad51 protein displays both a high affinity for dsDNA and a low rate of redistribution. This conclusion is based on the fact that the fluorescence of either the etheno M13 DNA-Rad51 protein or the DAPI-dsDNA-Rad51 protein complexes does not change upon the subsequent addition of a 3-fold excess of competitor DNA (either dsDNA or ssDNA, respectively) during the time scale of a typical DNA strand exchange reaction.<sup>2</sup> In addition, a low rate of Rad51 protein monomer redistribution is expected due to its low ATPase activity; hence, the Rad51 protein-dsDNA complex formed on DNA secondary structure is more stable than the ssDNA complex because of a 2.5–10-fold lower rate of dsDNA-dependent ATP hydrolysis compared with the ssDNA-dependent rate (3, 5). Therefore, the secondary structure within ssDNA that is stabilized by Rad51 protein contributes to the low efficiency of DNA strand exchange promoted by Rad51 protein in the absence of a ssDNA-binding protein. The same considerations potentially explain the higher activity of  $\phi$ X174 ssDNA *versus* M13 ssDNA in DNA strand exchange promoted by Rad51 protein<sup>2</sup> (3, 40), since M13 ssDNA has a higher level of secondary structure compared with  $\phi$ X174 ssDNA.

DNA strand exchange mediated by Rad51 protein is very sensitive to an excess of protein (4). The binding of Rad51 protein to dsDNA inhibits DNA strand exchange by forming nucleoprotein filaments on both DNA partners in the reaction. Our DNA binding data explain why presynaptic complex formation is optimal at 3–4 mM Mg<sup>2+</sup> ion concentrations (4, 12); at these conditions, the inhibitory effect of the DNA secondary structure is minimized because the stability of DNA secondary structure is low and the binding of Rad51 protein to dsDNA is minimal (Fig. 8 and Fig. 11).

In a recent paper, the binding stoichiometry of Rad51 protein



to etheno M13 DNA was reported as 1 protein monomer per 5.5 to 7 nucleotides (41), values which, considering experimental error, may overlap with our own. In addition, there might be some systematic differences arising from the different Rad51 proteins used. In that work, Rad51 protein was purified from a baculovirus expression system, and this protein lacked 22 amino acid residues from the N terminus due to an alternative site of translation initiation (40).

In sum, we have demonstrated the existence of two different DNA binding modes for Rad51 protein; the mode utilized depends on a complex interplay of pH and of ATP and Mg<sup>2+</sup> concentration. The DNA binding properties of yeast Rad51 protein, as discussed above, explain some of the known differences in the requirements of, and the conditions for, DNA strand exchange promoted by Rad51 protein when compared with the RecA protein-mediated reaction. The limitations of DNA strand exchange mediated by Rad51 protein clearly suggest the requirements of auxiliary proteins needed to enhance the efficiency of this reaction. The studies reported here may provide a basis for understanding the mechanism of the stimulatory effects of Rad55/Rad57 proteins (42), Rad52 protein (43–45), and Rad54 protein (23) and, hence, represent a first step in obtaining the complete picture of yeast homologous recombination.

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## REFERENCES

- Petes, T. D., Malone, R. E., and Symington, L. S. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics* (Broach, J. R., Jones, E., and Pringle, J., eds) Vol. 1, pp. 407–521, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Shinohara, A., Ogawa, H., and Ogawa, T. (1992) *Cell* **69**, 457–470
- Sung, P. (1994) *Science* **265**, 1241–1243
- Sung, P., and Roberson, D. L. (1995) *Cell* **82**, 453–461
- Sugiyama, T., Zaitseva, E. M., and Kowalczykowski, S. C. (1997) *J. Biol. Chem.* **272**, 7940–7945
- Ogawa, T., Yu, X., Shinohara, A., and Egelman, E. H. (1993) *Science* **259**, 1896–1899
- Bianco, P. R., Tracy, R. B., and Kowalczykowski, S. C. (1998) *Frontiers Biosci.* **3**, D570–D603
- Egelman, E. H., and Stasiak, A. (1986) *J. Mol. Biol.* **191**, 677–697
- Kowalczykowski, S. C. (1991) *Annu. Rev. Biophys. Biophys. Chem.* **20**, 539–575
- Baumann, P., Benson, F. E., and West, S. C. (1996) *Cell* **87**, 757–766
- Gupta, R. C., Bazemore, L. R., Golub, E. I., and Radding, C. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 463–468
- Sung, P., and Stratton, S. A. (1996) *J. Biol. Chem.* **271**, 27983–27986
- Eggleston, A. K., Rahim, N. A., and Kowalczykowski, S. C. (1996) *Nucleic Acids Res.* **24**, 1179–1186
- Messing, J. (1983) *Methods Enzymol.* **101**, 20–78
- Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181**, 281–295
- Basile, G., Aker, M., and Mortimer, R. K. (1992) *Mol. Cell. Biol.* **12**, 3235–3246
- Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
- Uhlir, B. E., and Clark, A. J. (1981) *J. Bacteriol.* **148**, 386–390
- Griffith, J., and Shores, C. G. (1985) *Biochemistry* **24**, 158–162
- LeBowitz, J. (1985) *Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication. Ph.D. Thesis*, The Johns Hopkins University, Baltimore
- Alani, E., Thresher, R., Griffith, J. D., and Kolodner, R. D. (1992) *J. Mol. Biol.* **227**, 54–71
- Zaitsev, E. N., and Kowalczykowski, S. C. (1998) *Nucleic Acids Res.* **26**, 650–654
- Petukhova, G., Stratton, S., and Sung, P. (1998) *Nature* **393**, 91–94
- Kowalczykowski, S. C. (1990) in *Landolt-Bornstein: Numerical Data and Functional Relationships in Science and Technology (New Series) Group VII: Biophysics, Nucleic Acids* (Saenger, W., ed) Vol. 1, pp. 244–263, Springer-Verlag, Berlin
- Lauder, S. D., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **234**, 72–86
- Trotta, E., D'Ambrosio, E., Del Grosso, N., Ravagnan, G., Cirilli, M., and Paci, M. (1993) *J. Biol. Chem.* **268**, 3944–3951
- Wang, J. C. (1974) *J. Mol. Biol.* **89**, 783–801
- Thresher, R. J., and Griffith, J. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5056–5060
- Lee, J. W., and Cox, M. M. (1990) *Biochemistry* **29**, 7666–7676
- Lee, J. W., and Cox, M. M. (1990) *Biochemistry* **29**, 7677–7683
- Yu, X., and Egelman, E. H. (1992) *J. Mol. Biol.* **227**, 334–346
- Kowalczykowski, S. C., Clow, J., and Krupp, R. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3127–3131
- McEntee, K., Weinstock, G. M., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8835–8844
- Pugh, B. F., and Cox, M. M. (1987) *J. Biol. Chem.* **262**, 1326–1336
- de Bongioanni, L. C., and Ramos, E. H. (1988) *Rev. Argent. Microbiol.* **20**, 1–15
- Haworth, R. S., and Fliegel, L. (1993) *Mol. Cell. Biochem.* **124**, 131–140
- Muniyappa, K., Shaner, S. L., Tsang, S. S., and Radding, C. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2757–2761
- Kowalczykowski, S. C., Clow, J. C., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193**, 81–95
- Kowalczykowski, S. C., and Krupp, R. A. (1987) *J. Mol. Biol.* **193**, 97–113
- Namsaraev, E., and Berg, P. (1997) *Mol. Cell. Biol.* **17**, 5359–5368
- Namsaraev, E. A., and Berg, P. (1998) *J. Biol. Chem.* **273**, 6177–6182
- Sung, P. (1997) *Genes Dev.* **11**, 1111–1121
- Sung, P. (1997) *J. Biol. Chem.* **272**, 28194–28197
- New, J. H., Sugiyama, T., Zaitseva, E., and Kowalczykowski, S. C. (1998) *Nature* **391**, 407–410
- Shinohara, A., and Ogawa, T. (1998) *Nature* **391**, 404–407