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The DNA Binding Preference of RAD52 and RAD59 Proteins

*IMPLICATIONS FOR RAD52 AND RAD59 PROTEIN FUNCTION IN HOMOLOGOUS RECOMBINATION**

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We examined the double-stranded DNA (dsDNA) binding preference of the *Saccharomyces cerevisiae* Rad52 protein and its homologue, the Rad59 protein. In nuclease protection assays both proteins protected an internal sequence and the dsDNA ends equally well. Similarly, using electrophoretic mobility shift assays, we found the affinity of both Rad52 and Rad59 proteins for DNA ends to be comparable with their affinity for internal sequences. The protein-DNA complexes were also directly visualized using atomic force microscopy. Both proteins formed discrete complexes, which were primarily found (90–94%) at internal dsDNA sites. We also measured the DNA end binding behavior of human Rad52 protein and found a slight preference for dsDNA ends. Thus, these proteins have no strong preference for dsDNA ends over internal sites, which is inconsistent with their function at a step of dsDNA break repair that precedes DNA processing. Therefore, we conclude that *S. cerevisiae* Rad52 and Rad59 proteins and their eukaryotic counterparts function by binding to single-stranded DNA formed as intermediates of recombination rather than by binding to the unprocessed DNA double-strand break.

The repair of DNA double-strand breaks (DSBs)[3] in cells is crucial for genomic integrity and viability. DSBs can be generated during the process of DNA replication on damaged templates or directly by exogenous DNA-damaging agents. A single unrepaired DSB can result in cell death (1). In eukaryotic cells, there are two major pathways to repair DSBs, homologous recombination and non-homologous end-joining (NHEJ). NHEJ repairs DSBs by ligating broken DNA ends, but this process is inherently error-prone (2). Homologous recombination, on the other hand, restores DSBs without the loss of genetic integrity by using a homologous sequence as template, such as a sister chromatid or homologous chromosome. In *Saccharomyces cerevisiae*, homologous recombination is the predominant mechanism for DSB repair but, when defective, the radiation sensitivity of NHEJ mutants is evident (3).

In *S. cerevisiae*, homologous recombination is mediated by proteins of the *RAD52* epistasis group, including *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, *XRS2*, and *RFA1*. A homologue of *RAD54*, *RDH54/TID1*, also plays a role in a subset of recombination-dependent DSB repair pathways (3, 4). Mutation of any of these genes confers radiation sensitivity. In particular, loss of *RAD52* function leads to the most severe recombination phenotype because the Rad52 protein is required for all recombination-dependent events (Ref. 5; for review, see Refs. 3 and 4). The Rad52 protein is conserved in most eukaryotic organisms ranging from yeast to human (6–8). *In vivo* it accumulates at DNA damage sites, forms discrete nuclear foci, and co-localizes with the DNA strand exchange protein, Rad51 (9–13). *In vitro* both yeast and human Rad52 proteins bind single-stranded DNA (ssDNA) and dsDNA, stimulate annealing of complementary ssDNA (14–17), and facilitate Rad51 protein-ssDNA filament formation in the presence of the eukaryotic ssDNA-binding protein, replication protein A, in a species-specific fashion (18–21). This level of functional conservation is consistent with the importance of the Rad52 protein in DNA recombination.

The Rad59 protein was identified in *S. cerevisiae* (22) and *Kluyveromyces lactis* (23) as a Rad52 protein homologue. It shares sequence similarity with the conserved N-terminal domain of Rad52 protein but lacks the C-terminal Rad51-interacting domain. Genetically, *RAD59* is dispensable in *RAD51*-dependent homologous recombination pathways but plays an important role in the *RAD51*-independent repair pathways (22), such as single-strand annealing (SSA) (24, 25), break-induced replication (BIR) (26, 27), and type II survival in telomerase-deficient cells (28, 29). *In vivo* Rad59 interacts with Rad52 protein (30), suggesting direct participation in a subset of *RAD52*-dependent homologous recombination events. The Rad59 protein possesses biochemical activities that are also found in other *RAD52* homologues, including the ability to bind ssDNA and dsDNA and to anneal complementary ssDNA (30–32).

Although both the homologous recombination and NHEJ pathways can repair DSBs, each functions differently in cells. NHEJ function is enhanced in haploid cells and in the G_1 phase of the cell cycle (33), whereas recombination functions in the

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[3] The abbreviations used are: DSB, double-strand breaks; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; hRad52, human Rad52; NHEJ, non-homologous end-joining; SSA, single-strand annealing; AFM, atomic force microscope; ExoIII, exonuclease III; MOPS, 4-morpholinepropanesulfonic acid.

S-G_2 phase (11, 34) or in diploid cells (35). It appears that pathway choice is determined by availability of a homologous sequence. However, it is unclear how and by what molecular mechanism pathway choice is regulated.

Human Rad52 (hRad52) protein was reported to localize preferentially at dsDNA ends and to protect dsDNA ends from nuclease degradation (36). These observations led to the hypothesis that Rad52 protein serves as a "molecular switch" or a "gatekeeper" to channel DSBs into the homologous recombination repair pathway rather than into the NHEJ pathway (36, 37). Soon afterward, a Rad52 homologue from the fission yeast *Schizosaccharomyces pombe*, the Rad22 protein, was reported to have the same ability to protect dsDNA ends from nuclease digestion and to localize to sites of DSBs *in vivo* (38). However, a subsequent report used atomic force microscopy (AFM) (39) to show that hRad52 has no preference for dsDNA ends; rather, it was shown to preferentially bind to ssDNA regions in dsDNA, regions even as small as 2 or 4 nucleotides. Because the functions of Rad52 protein are conserved among different organisms, we tested whether the *S. cerevisiae* Rad52 protein bound preferentially to dsDNA ends or to ssDNA. Answering this question could help address the issue of whether Rad52 regulates the channeling of DSB repair into different repair pathways in budding yeast. As an alternative, we also entertained the idea that Rad59 protein in *S. cerevisiae* might instead have assumed the role of gatekeeper, so we examined the dsDNA binding preference of Rad59 protein as well. We found no significant differences in dsDNA end binding *versus* internal binding for the yeast Rad52 and Rad59 proteins. Furthermore, we confirmed that the hRad52 had only a weak preference for dsDNA ends (39). Therefore, for these reasons and because these proteins bind ssDNA preferentially over dsDNA, we conclude that Rad52 and Rad59 proteins act only at later stages of recombination, after the DSBs are processed into ssDNA.

EXPERIMENTAL PROCEDURES

Proteins—Exonuclease III (ExoIII) was purchased from Promega. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Yeast Rad52 protein was purified as described (18), except that the Superose-12 column was replaced with a Sephacryl-300 column. Rad59 protein was overexpressed in BLR(DE3) pLysS cells and purified as described (32). Human Rad52 protein was a generous gift from Dr. P. Sung of Yale University. In reactions where a protein was omitted, an equal amount of corresponding protein storage buffer was added instead.

DNA Substrates—All DNA concentrations are expressed in nucleotides. The complementary oligonucleotides PB77 and PB78, 100 nucleotides in length, were purchased, purified, and annealed as described (40). Concentrations of PB77 and PB78 were determined using nucleotide extinction coefficients of 8891 and 9737 M^{-1} cm^{-1} at 260 nm, respectively. PB77 and PB78 were first annealed to each other, and the resulting PB77-78 dsDNA was labeled by T4 polynucleotide kinase at the 5′-end on both strands. Unincorporated [γ-^{32}P]ATP was removed using a MicroSpin G25 desalting column (GE Healthcare). Plasmid pBR322 DNA was purified by conventional alkaline lysis followed by equilibrium ultracentrifugation in a CsCl-ethidium bromide gradient. Purified pBR322 was linearized with the indicated restriction endonucleases and purified by phenol extraction followed by ethanol precipitation. In all cases agarose gel electrophoresis was used to verify that the DNA was completely digested by the restriction endonuclease (data not shown). DNA concentration was determined using an extinction coefficient of 6500 M^{-1} cm^{-1} at 260 nm.

Nuclease Protection Assay—The ability of Rad52 and Rad59 proteins to protect dsDNA from degradation by nucleases was assayed in nuclease protection buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol) with the concentration of magnesium acetate indicated. Duplex DNA (PB77-78 that was 5′-end-labeled at both ends, 10 μM) was preincubated with the indicated amount of protein at 37 °C for 10 min before the addition of nuclease to start DNA degradation. ExoIII and HaeIII were titrated to give ~80% degradation of the free DNA substrate for the time specified. After incubation at 37 °C for 15 min, the reactions were deproteinized with 0.7% SDS and 1 mg/ml proteinase K (Roche Applied Science) and subjected to native polyacrylamide gel (8%) electrophoresis in 1× TBE buffer (89 mM Tris borate, pH 8.3, 2 mM EDTA). DNA substrate with the electrophoretic mobility of the remaining intact dsDNA was quantified using a Storm 820 system (GE Healthcare). The average band intensity for the dsDNA from control reactions lacking nuclease in each gel (Figs. 1–3, *panels A* and *B*, *lanes 1* and *10*) was defined as 100% protection. A box was drawn around the position of the intact dsDNA in the same gel (*lanes 1* and *10*); boxes of the same size and electrophoretic position were used in the sample lanes to determine the amount of intact dsDNA remaining. The band intensity in nuclease-only control (*lane 2*) was defined as 0% protection. The degree of dsDNA protection was determined using the formula dsDNA protected (%) = (band intensity − 0% control)/(100% control − 0% control) ×100%.

Electrophoretic Mobility Shift Assay—The reactions contained 10 μM 5′-end-labeled dsDNA (100 bp, PB77-78) and various amounts of unlabeled competitor dsDNA in electrophoretic mobility shift buffer (30 mM K^+-MOPS, pH 7.3, 1 mM dithiothreitol). Rad52 (1 μM), Rad59 (2.2 μM), or hRad52 (1.2 μM) protein was added to the dsDNA, and the reactions were incubated at 37 °C for 5 min to allow protein-DNA complex formation. The concentration of NaCl contributed by the protein storage buffer was 40, 50, and 25 mM in the Rad52-, Rad59-, and hRad52-containing reactions, respectively. Under these conditions, in the absence of unlabeled competitor DNA, the mobility of more than 90% of the DNA substrate was shifted. The reactions were analyzed by electrophoresis using 8% polyacrylamide gels in 1× TBE buffer and quantified using a Storm 820 system.

AFM—EcoRV- or PstI-linearized pBR322 DNA (10 μM) was incubated with various amount of yeast Rad52 protein (0.4–350 nM) in binding buffer (30 mM K-MOPS, pH 7.3, 3 mM magnesium acetate, 20 mM NaCl) in a total reaction volume of 20 μl at 37 °C for 5 min. The sample was applied onto freshly cleaved mica; after 5 min the surface was washed with 1 ml of distilled water and dried with compressed nitrogen gas. Protein-DNA complexes were examined using a Nanoscope IIIa AFM (Digital Instruments) operated in tapping mode. Images were captured at scan sizes of 0.6–1.7 μm and processed by first-order flatten-

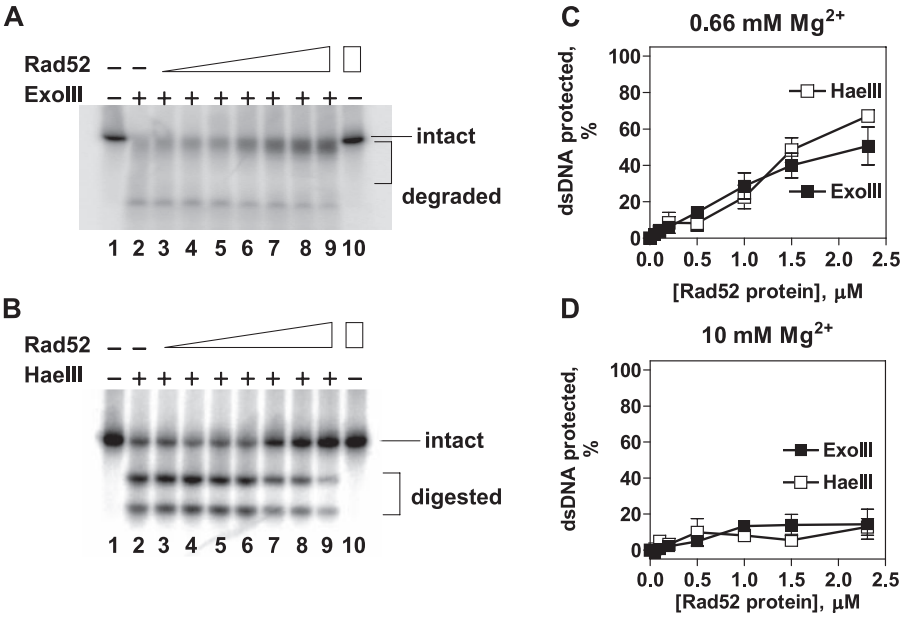

FIGURE 1. **Nuclease protection assays show that yeast Rad52 protein protects dsDNA ends and internal sites equally.** The 5′-end-labeled 100-bp dsDNA substrate (10 μM) was preincubated with increasing amounts of Rad52 protein at 0.66 mM magnesium acetate before the addition of ExoIII (*A*) or HaeIII (*B*). Rad52 protein concentrations were 0.05, 0.1, 0.2, 0.5, 1, 1.5, 2.31, and 2.31 μM in *lanes 3–10*, respectively. After 15 min the reactions were deproteinized and analyzed by gel electrophoresis. The results from reactions performed at 0.66 and 10 mM magnesium acetate (gel not shown) were quantified in *panels C* and *D*, respectively. Results are the average obtained from two independent experiments, and the *error bars* represent the variation.

ing to remove sample tilt. For Rad59 protein, magnesium acetate and protein concentrations were optimized to 5 mM and 100 nM, respectively, to facilitate discrete complex formation and to minimize aggregation.

RESULTS

Yeast Rad52 and Rad59 Proteins and hRad52 Protein Protect DNA Ends and Internal Sites Equally from Nucleolytic Degradation—We wanted to know whether the Rad52 and the Rad59 protein from budding yeast display a preference for binding to DNA ends as was reported for Rad52 homologues from human and fission yeast. Previously, this preference was demonstrated as an ability to protect DNA more effectively from exonucleolytic degradation than from endonucleolytic cleavage (36, 38). We, therefore, conducted such nuclease assays in the presence of Rad52 or Rad59 protein. The DNA substrate used in the nuclease assays was a blunt-ended, 5′-end labeled-100-bp DNA duplex (labeled on both strands) that contained one HaeIII site. This substrate was chosen to ensure that limited exonuclease degradation would be detected as a change in electrophoretic mobility. The DNA substrate was preincubated with various amounts of Rad52 or Rad59 protein and was then incubated with either ExoIII or HaeIII enzyme, each of which was present at an amount that degraded ~80% of the naked dsDNA substrate under the given reaction conditions. For both of the ExoIII or HaeIII protection assays, the percentage of label remaining at the position of the intact dsDNA was quantified because it is directly related to protection. We used two magnesium ion concentrations, 0.66 mM (low) and 10 mM (high), to permit comparison to previously published work (38). At these two Mg^{2+} concentrations, the activities of ExoIII and HaeIII were the same (data not shown); therefore, the same amount of each nuclease was used at the two different assay conditions.

We found that Rad52 protein protected dsDNA from degradation in a protein concentration-dependent manner in both the ExoIII- and HaeIII-containing reactions (Fig. 1, *A* and *B*). In the presence of ExoIII, the dsDNA migrated as a slightly faster and broader band due to removal of nucleotides by the exonuclease (Fig. 1*A*, *lane 2*). The addition of increasing amounts of Rad52 protein increased the fraction of dsDNA substrate that remained intact (Fig. 1*A*, *lanes 3–9*). Consistent with a previous report showing that DNA binding activity is sensitive to increased Mg^{2+} concentration (16), Rad52 protein protected the dsDNA better at 0.66 mM Mg^{2+} (Fig. 1*C*) than it did at 10 mM Mg^{2+} (Fig. 1*D*). Likewise, in the HaeIII nuclease protection assay, Rad52 protein prevented dsDNA cleavage (Fig. 1*B*), and it did so better at 0.66 mM Mg^{2+} (Fig. 1*C*) than it did at 10 mM Mg^{2+} (Fig. 1*D*). Most importantly, when the protection patterns for ExoIII and HaeIII were compared at the same magnesium ion concentrations (Fig. 1, *C* and *D*), protection was essentially the same, within experimental error. Thus, yeast Rad52 protein shows no preference for dsDNA end binding as measured by this nuclease protection assay.

The behavior of Rad59 protein was also evaluated in these assays. Similar to Rad52 protein, Rad59 protein protected the dsDNA from both exonuclease and endonuclease degradation in a protein concentration-dependent manner (Fig. 2, *A* and *B*). In agreement with the previously reported sensitivity of DNA binding to increased Mg^{2+} concentration (31), Rad59 protein protected the dsDNA to a greater extent at the lower Mg^{2+} concentration (Fig. 2*C*) than at the higher Mg^{2+} concentration (Fig. 2*D*). More importantly, Rad59 protein showed almost the same level of protection against ExoIII- and HaeIII-mediated degradation at the low Mg^{2+} concentration and, actually, better protection against HaeIII digestion at the high Mg^{2+} concentration at the highest protein concentration used. Thus, the results obtained from the nuclease assays do not demonstrate preferential DNA end binding for either Rad52 or Rad59 proteins.

Finally, we tested the DNA end binding preference of human Rad52 protein using the same nuclease protection assays. Like the yeast Rad52 and Rad59 proteins, hRad52 protein protected dsDNA in a protein concentration-dependent manner (Fig. 3, *A* and *B*). The protection from HaeIII degradation was better than from ExoIII degradation at the low Mg^{2+} concentration (Fig. 3*C*) and marginally better at the high Mg^{2+} concentration (Fig. 3*D*). Unlike the yeast Rad52 and Rad59 proteins, however, the

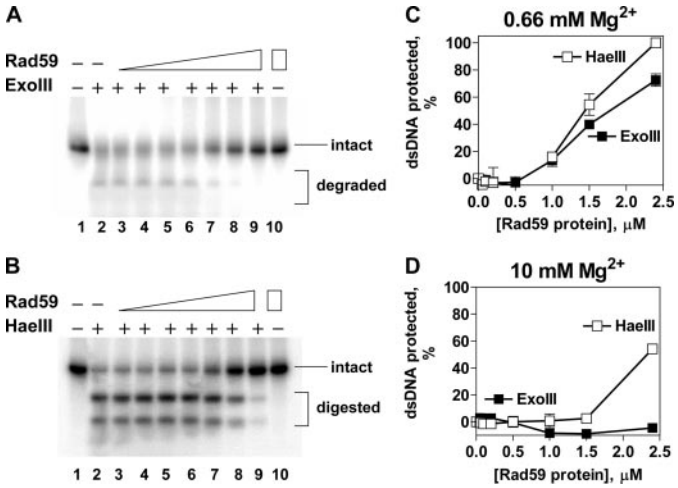

FIGURE 2. **Nuclease protection assays show that yeast Rad59 protein protects dsDNA ends and internal sites equally.** The 5′-end-labeled 100-bp dsDNA substrate (10 μM) was preincubated with increasing amounts of Rad59 protein at 0.66 mM magnesium acetate before the addition of ExoIII (*A*) or HaeIII (*B*). Rad59 protein concentrations were 0.05, 0.1, 0.2, 0.5, 1, 1.5, 2.4, and 2.4 μM in *lanes 3–10*, respectively. After 15 min, the reactions were deproteinized and analyzed by gel electrophoresis. The results from reactions performed at 0.66 and 10 mM magnesium acetate (gel not shown) were quantified in *panels C* and *D*, respectively. Results are the average obtained from two independent experiments, and the *error bars* represent the variation.

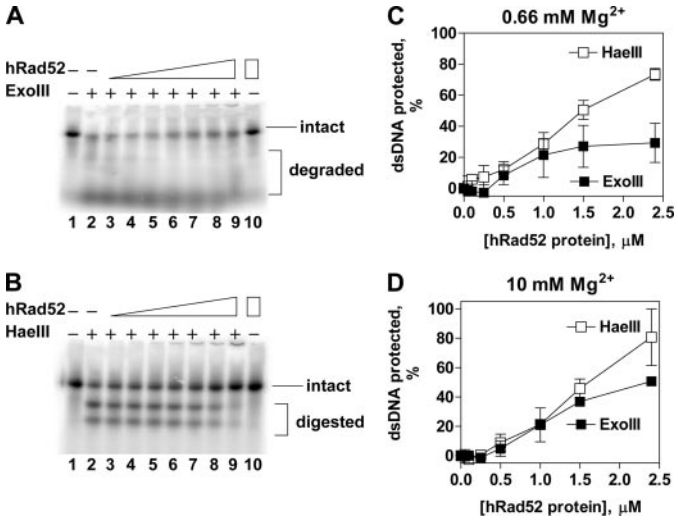

FIGURE 3. **Nuclease protection assays show that human Rad52 protein protects dsDNA ends and internal sites equally.** The 5′-end-labeled 100-bp dsDNA substrate (10 μM) was preincubated with increasing amounts of hRad52 protein at 0.66 mM magnesium acetate before the addition of ExoIII (*A*) or HaeIII (*B*). The hRad52 protein concentrations were 0.05, 0.1, 0.2, 0.5, 1, 1.5, 2.4, and 2.4 μM in *lanes 3–10*, respectively. After 15 min the reactions were deproteinized and analyzed by gel electrophoresis. The results from reactions performed at 0.66 and 10 mM magnesium acetate (gel not shown) were quantified in *panels C* and *D*, respectively. Results are the average obtained from two independent experiments, and the *error bars* represent the variation.

protection by hRad52 protein was only slightly reduced at the higher Mg^{2+} concentration (Fig. 3*D*), indicating the DNA binding activity of hRad52 protein is less sensitive to an increased concentration of Mg^{2+} than that of yeast Rad52 and Rad59 proteins.

Yeast Rad52 and Rad59 Proteins and Human Rad52 Protein Bind to DNA Ends with Minimal Preference Relative to Internal Sites—To confirm the results from the nuclease protection assays, we designed a direct competition experiment to examine the dsDNA binding specificity of Rad52 and Rad59 proteins. The same 5′-end-labeled 100-bp dsDNA substrate was incubated with a fixed concentration of protein so that ~90% of the dsDNA substrate had a resultant electrophoretic mobility that caused it to remain in the well of the gel. This protein-DNA complex was then titrated with increasing amounts of unlabeled, EcoRV- or HaeIII-digested pBR322 DNA. There is only one EcoRV site in pBR322 DNA, but there are 22 HaeIII sites; thus, the HaeIII-linearized pBR322 DNA yields 22 times more dsDNA ends than the EcoRV-linearized DNA. Therefore, if a protein has a high affinity for dsDNA ends, then the same concentration of HaeIII-linearized pBR322 DNA should be a 22-fold better competitor for protein binding than the EcoRV-linearized DNA.

The results obtained with Rad52 protein are shown in Fig. 4*A*. The retention of labeled dsDNA in the well was inversely dependent on the concentration of the unlabeled competitor DNA. Quantification of the gel (Fig. 4*B*) shows that the amount of the EcoRV-digested pBR322 needed to liberate the labeled dsDNA due to competitive binding of the Rad52 protein is similar to the amount of the HaeIII-digested pBR322. This indicates that the EcoRV-digested pBR322 dsDNA, although possessing 22 times fewer dsDNA ends, competes for Rad52 protein binding as efficiently as the HaeIII-digested pBR322 DNA.

Identical experiments were performed using Rad59 protein. Only the quantification is shown here (Fig. 4*C*) because the gels were essentially the same as those shown for Rad52 protein. Just as for Rad52 protein, the HaeIII-digested pBR322 competed equally well for Rad59 protein binding compared with the EcoRV-linearized pBR322. These results are consistent with our nuclease protection studies (Figs. 1 and 2), and they show that neither Rad52 protein nor Rad59 protein has a strong preferential affinity for dsDNA ends.

When the human Rad52 protein was examined using this assay, the results were similar, although the HaeIII-linearized pBR322 DNA was found to compete for hRad52 protein binding slightly better than the EcoRV-linearized pBR322 DNA (Fig. 4*D*). The concentration of competitor DNA required to dissociate the hRad52 protein-dsDNA complex to free ~50% of the labeled dsDNA was about 20 and 40 μM for the HaeIII- and EcoRV-cut DNA, respectively. Although this difference suggests that hRad52 protein has a somewhat higher affinity for dsDNA ends than for internal sites, this 2-fold difference is much less than the 22-fold difference expected. We, therefore, estimate that the hRad52 protein has at most an ~100-fold greater affinity for dsDNA ends than internal dsDNA sites. However, the results from the nuclease protection experiments suggest little or no preferential binding.

Visualization of Rad52- and Rad59-DNA Complexes—We used AFM to directly visualize yeast Rad52- and Rad59-DNA complexes on linearized plasmid dsDNA. We observed that Rad52 protein often forms discrete bead-like complexes on plasmid dsDNA and that it binds to both dsDNA ends and internal sites (Fig. 5*A*). In AFM, tip convolution results in measured widths that are larger than the actual widths of DNA or protein-DNA complexes. The width of naked dsDNA was measured to be 11.7 ± 1.8 nm ($n = 19$), which is ~10 nm larger

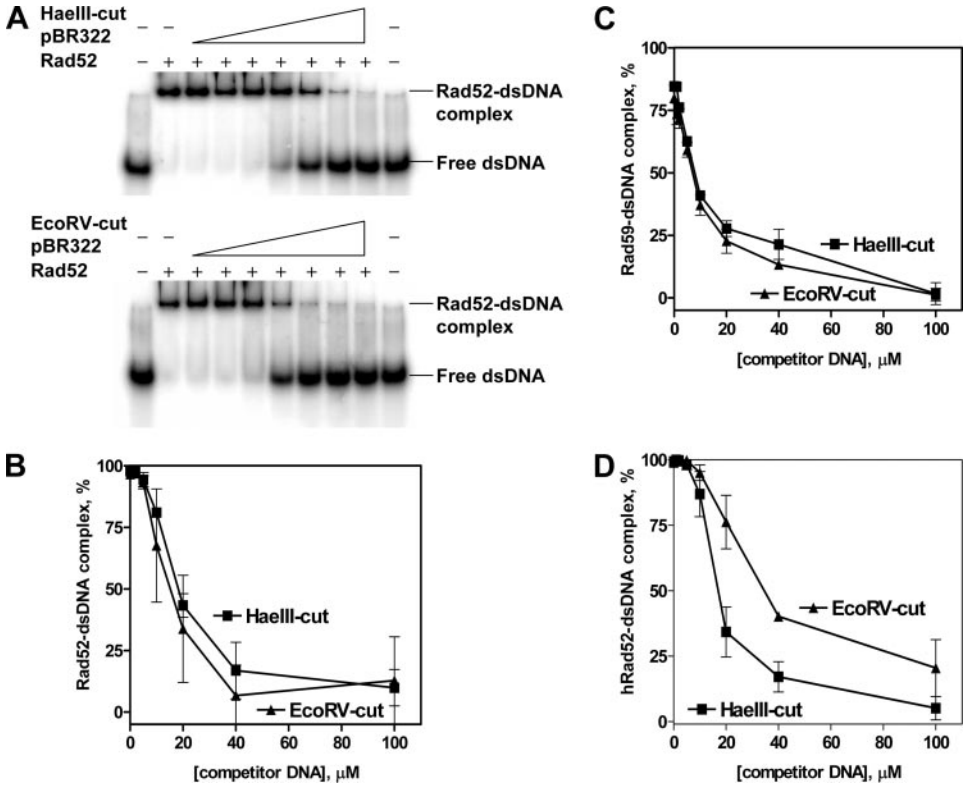

FIGURE 4. **Competitive DNA binding assays reveal no preferential affinity for dsDNA ends by yeast Rad52 and Rad59 proteins and by human Rad52 protein.** *A*, the 5′-end labeled 100-bp dsDNA substrate (10 μM) was incubated with a saturating amount of Rad52 protein and various amounts (0–100 μM) of unlabeled competitor DNA, EcoRV (*upper panel*)- or HaeIII (*lower panel*)-digested pBR322. The Rad52-dsDNA complex and the unbound dsDNA were then resolved by native gel electrophoresis. The fraction of labeled DNA that remained bound by Rad52 protein were quantified and plotted in *panel B*. The same competitive DNA binding assays performed using Rad59 and human Rad52 proteins (gels not shown) were quantified and plotted in *panels C* and *D*, respectively. Results are the average obtained from two independent experiments, and the *error bars* represent the variation.

than expected (2 nm). Similarly, the diameter of the Rad52 protein complexes was measured to be about 28.1 ± 3.4 nm (n = 10), implying that its actual diameter is at most 18 nm. To verify that Rad52 protein did not prefer to bind dsDNA ends, we reduced the protein concentration until only a few Rad52 protein complexes were bound to each dsDNA molecule. On 40 clearly resolved individual EcoRV-linearized pBR322 molecules, 88 bound Rad52 proteins were discerned; 79 of these proteins (90%) were bound to internal DNA sites, whereas only 9 were located close to a DNA end (10%). Notably, even though some of the complexes visualized contained as many as five Rad52 protein bound to the DNA, we did not observe any dsDNA molecules with both ends occupied. Our results with yeast Rad52 protein are consistent with the prior AFM study of human Rad52 protein-dsDNA complexes, which showed that only 6–18% of hRad52 protein was bound at the end of blunt-ended dsDNA (39). In contrast, with PstI-linearized pBR322, which has 4-nucleotide 3′-overhangs on both ends, 17 of 55 Rad52 complexes observed (31%) were bound to ends. This result shows that the presence of an ssDNA tail as short as 4 nucleotide results in 3-fold more binding of Rad52 at the DNA ends and is the same as that reported for hRad52 protein (39).

We also examined complexes of Rad59 protein and EcoRV-linearized pBR322 dsDNA. We saw that Rad59 protein also formed discrete complexes (Fig. 5*B*). The size and shape of the Rad59 complexes are highly variable, suggesting the presence of different oligomeric states. For these molecules, the width of the naked dsDNA was measured to be 9.5 ± 0.8 nm (n = 15), which is ~7.5 nm larger than the actual width of DNA. The observed diameter of the Rad59 complexes ranged from 13 to 30 nm (n = 33). The average diameter of the smallest complexes was measured to be 14.8 ± 1.2 nm (n = 16) or at most 7 nm after correction, suggesting these complexes are Rad59 monomers. To determine whether Rad59 protein displays any preference for dsDNA ends, we decreased the Rad59 protein concentration to 100 nM (the resultant ratio was 1 protein/100 nucleotides). The 35 individual dsDNA molecules that could be clearly resolved contained 105 bound Rad59 proteins; 99 of the proteins (94%) were bound to internal sites, whereas only 6 were bound to a dsDNA end (6%). As for Rad52 protein, even though some of the DNA molecules visualized contained as many as 10 Rad59 protein bound to the DNA, none had both ends occupied by Rad59 protein. Thus, our observations using AFM confirm the biochemical data that neither Rad52 nor Rad59 protein have a preference for dsDNA ends.

DISCUSSION

In this report we showed that the *S. cerevisiae* Rad52, *S. cerevisiae* Rad59, and human Rad52 proteins do not have a significant preference for binding to dsDNA ends. Although our conclusions differ, our experimental results are not necessarily inconsistent with those previously reported for hRad52 and *S. pombe* Rad22 proteins (36, 38), where an apparent end binding preference had been inferred. In fact, many of the previous interpretations can be explained by our observations. First, the protein concentration required to achieve efficient protection from exonuclease digestion was extremely high compared with that of dsDNA ends (158 proteins per dsDNA end). A reasonable explanation for this requirement of excess protein over DNA ends is that most of the Rad52 protein is sequestered by binding to the internal DNA sites. Second, in the previous reports DNA end binding was measured either by the release of label from a uniformly labeled plasmid DNA using trichloroacetic acid precipitation (36) or by the change in mobility of plasmid DNA using agarose gel electrophoresis (38). ExoIII, the nuclease used in the exonuclease protection assays, is a distributive enzyme that hydrolyzes ~6 nucleotides before dissociating from a dsDNA end (41), leaving a ssDNA tail. Given that

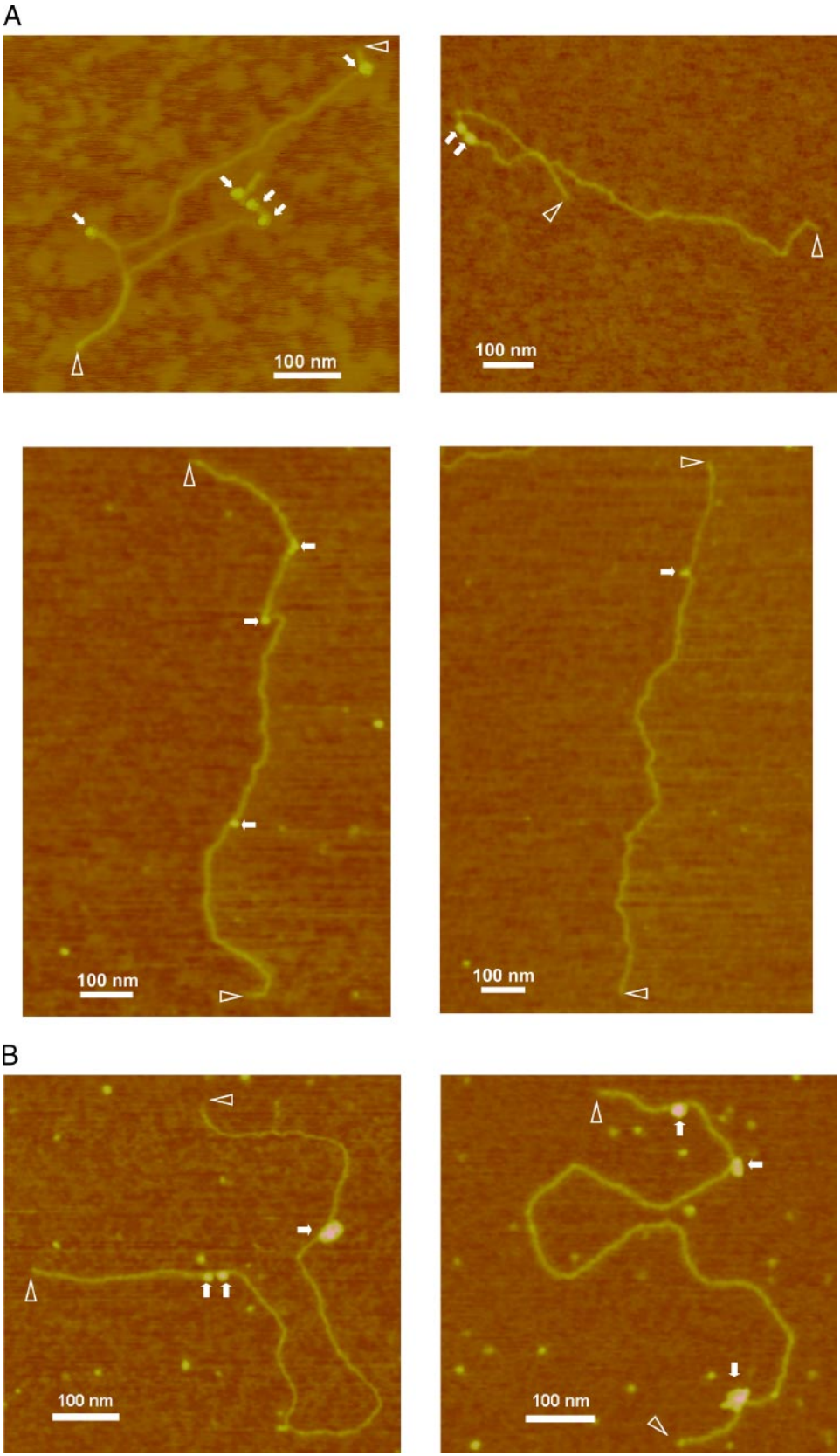

FIGURE 5. **Visualization of Rad52-dsDNA and Rad59-dsDNA complexes by atomic force microscopy.** Linearized pBR322 DNA complexed with yeast Rad52 or Rad59 protein was examined by tapping mode AFM at ambient room temperature and humidity as described under "Experimental Procedures." *A*, representative images of Rad52 protein-dsDNA complexes are shown. Rad52 protein is indicated with *closed arrows*, and the dsDNA end is indicated with *open triangles*. *B*, representative images of Rad59 protein-dsDNA complexes are shown. Rad59 protein is indicated with *closed arrows*, and the dsDNA end is indicated with *open triangles*. For both panels, maximum image height scale is 10 nm.

Rad52 (16), hRad52 (39), and Rad59 (31) proteins have a greater binding affinity for ssDNA than for dsDNA, the previous studies cannot eliminate the alternative explanation that Rad52 protein protected the remainder of the plasmid DNA substrate by binding to the ssDNA region that was generated by a limited extent of exonucleolytic degradation. Indeed, the limited degradation (evident in the slight smearing of the mobility) of the dsDNA in the ExoIII protection assay (Fig. 1*A*) indicates that Rad52 protein cannot efficiently protect the dsDNA substrate until the ends are processed into ssDNA tails. Furthermore, previous AFM results (39) with hRad52 and our findings with yeast Rad52 show that the presence of just a 4-nucleotide ssDNA tail results in a 2–3-fold increase in Rad52 binding to a DNA end. Thus, the previous findings and our interpretation of those findings are consistent with our results here showing that the affinity of Rad52 protein for internal DNA sequences is comparable with its affinity for dsDNA ends.

Moreover, in the work reporting the DNA end binding preference of the *S. pombe* Rad22 protein (38), the two nuclease protection assays were conducted in buffers recommended by the manufacturers, *i.e.* 0.66 mM Mg^{2+} for ExoIII and 10 mM Mg^{2+} with 50 mM NaCl for HaeIII.[4] Because the DNA binding affinity of Rad52 and Rad59 proteins is reduced by increasing concentrations of monovalent and divalent cations (14, 16, 31), the ability of these proteins to protect DNA in nuclease protein assays is concomitantly reduced (Figs. 1 and 2). If the DNA binding affinity of *S. pombe* Rad22 protein is similarly sensitive to Mg^{2+} and NaCl concentration, then it is likely that the Rad22 protein also would be less able to protect dsDNA from both endo- and exonuclease digestion at high Mg^{2+} and NaCl concentrations. Hence, the comparison of nuclease protection experiments that were performed at different reaction conditions could lead to an erroneous conclusion. If one were to compare our exonuclease protection results obtained for Rad52 protein at 0.66 mM Mg^{2+} (Fig. 1*C*, *ExoIII trace*), where strong protection is observed, to the endonuclease protection results at 10 mM Mg^{2+} (Fig. 1*D*, *HaeIII trace*), where weak protection is observed, then one would incorrectly conclude that Rad52 protein had a significant binding preference for dsDNA ends. It remains to be determined whether our simple explanation affords a straightforward explanation for the apparent inconsistency between our data with the *S. cerevisiae* proteins and the *S. pombe* homologue (38).

Our results suggest that the hRad52 and the *S. pombe* Rad22 proteins protect dsDNA from exonuclease degradation by binding to the ssDNA tail of dsDNA or by saturating the entire dsDNA molecule rather than by preferential binding to the blunt dsDNA end. All of the Rad52 homologues that we examined protected dsDNA from endonuclease degradation as well as they protected the DNA from exonuclease degradation with no strong bias. In addition, when we substituted T7 gene 6 exonuclease (a 5′ to 3′ exonuclease) for ExoIII in our end-protection assays (using 5′-end-labeled dsDNA), comparable protection was observed for Rad52, hRad52, and Rad59 to that seen with ExoIII.[5]

In the electrophoretic mobility shift experiments we also observed that a dsDNA competitor with a low concentration of ends was as efficient a competitor for Rad52 protein binding as dsDNA with a high concentration of ends. Experiments using AFM to directly observe Rad52 protein-dsDNA or Rad59 protein-dsDNA complexes also revealed no strong preference for dsDNA ends by either protein. A similar conclusion was reached for hRad52 using AFM to show that the protein bound to ssDNA regardless of the presence of dsDNA ends or the dsDNA end structure (39); in competition experiments with DNA that had an internal ssDNA gap or an ssDNA tail as part of the dsDNA substrate, hRad52 protein bound exclusively to the ssDNA region. These findings are consistent with our conclusion that there is no preferential dsDNA end binding by Rad52 protein and its homologues. Rather, because they have a much greater affinity to ssDNA, these proteins are localized to DSB sites after the dsDNA end is processed into ssDNA.

It is our interpretation that, because Rad52 protein does not have a significant specificity for dsDNA ends, it cannot control the choice of repair pathway for DSBs, homologous recombination *versus* NHEJ. One of the most notable differences between the NHEJ and homologous recombination pathways is the level of DNA processing at the first step of repair. Although extensive nucleolytic degradation may occur before end joining (33, 42), typically only limited DNA processing is involved in NHEJ (33, 43). In contrast, DSB repair via homologous recombination requires extensive DNA processing to generate 3′-terminated ssDNA (44). If Rad52 protein indeed competed for dsDNA ends with the NHEJ pathway, then one would expect that in the absence of *RAD52* the rate of DSB processing would be greatly reduced. Instead, DSB processing is either unaffected (45) or slightly increased (46, 47) in *rad52* mutant cells. The increased DNA degradation seen in the *rad52* mutant is likely due to the inability of the homologous recombination pathway to repair DSBs at a later stage, presumably because Rad52 protein is not available to promote the assembly of Rad51 protein onto ssDNA tails. Furthermore, in a plasmid-rejoining experiment where yeast cells were directly transformed with linear dsDNA containing various lengths of an ssDNA overhang, the efficiency of dsDNA rejoining was either solely dependent on the *yKU70* and *yKU80* or on the *RAD52* (48); when the ssDNA overhang was less than eight nucleotides, plasmid re-ligation was solely dependent on the yeast Ku70-80 proteins but not Rad52; with longer ssDNA overhangs, plasmid re-ligation was largely dependent on *RAD52*, presumably via a SSA-type mechanism but not on y*KU70-80*. These results also imply that the NHEJ pathway is essentially not in competition with the homologous recombination pathways.

Cytological evidence also argues that Rad52 protein cannot be the first recombination factor to act on the DSB in homologous recombination pathways. During meiosis, in response to DSB formation, Rad52 protein forms nuclear foci that extensively co-localize with, but do not form earlier than replication protein A foci (12). This observation suggests that localization of Rad52 protein onto the DSB sites requires processing of the DSB into ssDNA. In contrast, in irradiated cells, ~50% Mre11 foci do not co-localize with replication protein A foci, and for-

[4] W. J. Kim, personal communication.
[5] Y. Wu and S. C. Kowalczykowski, unpublished observations.

mation of Mre11 foci precedes that of Rad52 foci (49), indicating *MRE11* acts upstream to *RAD52* before DSB resection.

Taken together, Rad52 protein is unlikely to be a gatekeeper in DSB repair. The steps at which Rad52 protein acts are after the DSB has been resected to form ssDNA. Rad52 protein is important in several critical post-processing aspects of homologous recombination, such as facilitating Rad51-ssDNA complex formation (18–21, 50) and the annealing of complementary ssDNA in the presence of replication protein A (14–17). For all of these essential biochemical activities, Rad52 protein acts on ssDNA rather than dsDNA. Likewise, Rad59 protein has no preferential end binding affinity, but rather, it binds to ssDNA with a greater affinity than to dsDNA (31). Rad59 protein interacts with Rad52 protein *in vivo* (30) and enhances the annealing function of Rad52 under suboptimal conditions *in vitro* (32). Hence, we believe that the substrate on which Rad59 protein acts is also ssDNA.

If Rad52 protein does not regulate the choice of DSB repair pathways, then which proteins(s) channels DSBs to the appropriate repair pathways? The *S. cerevisiae* Mre11·Rad50·Xrs2 (MRX) complex or the mammalian Mre11·Rad50·Nbs1 (MRN) complex is likely to be such a regulator. The MRX/MRN complex is important in both repair pathways as well as in intra-S phase checkpoint control (51–53). Mre11 localizes to DSB sites independently of DSB processing (49, 54). Deletion of *MRE11*, *RAD50*, and *XRS2* retards DSB processing (44, 45, 55) in unsynchronized cells and completely abolishes it in G_2-arrested cells (54). All of these phenotypes are expected for proteins that are important in the channeling function. In *S. pombe*, another pathway was also reported to act at the stage of DSB processing (56). This pathway is under the control of cellular Cdc2-cyclin B activity and specifically regulates homologous recombination events in G_2 independently of and in parallel with *RAD50*. Thus, it seems that there are multiple regulatory mechanisms to channel DSBs to different repair pathways and that the choice of DSB repair pathway must be made at a stage much earlier than the Rad52-dependent steps. The mechanism of this choice is yet remains to be elucidated.

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