DNA Structure-specific Nuclease Activities in the Saccharomyces cerevisiae Rad50-Mre11 Complex

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Saccharomyces cerevisiae RAD50 and MRE11 genes are required for the nucleolytic processing of DNA double-strand breaks. We have overexpressed Rad50 and Mre11 in yeast cells and purified them to near homogeneity. Consistent with the genetic data, we show that the purified Rad50 and Mre11 proteins form a stable complex. In the Rad50-Mre11 complex, the protein components exist in equimolar amounts. Mre11 has a 3′ to 5′ exonuclease activity that results in the release of mononucleotides. The addition of Rad50 does not significantly alter the exonuclease activity of Mre11. Using homopolymeric oligonucleotide-based substrates, we show that the exonuclease activity of Mre11 and Rad50-Mre11 is enhanced for substrates with duplex DNA ends. We have examined the endonuclease function of Mre11 on defined, radiolabeled hairpin structures that also contain 3′ and 5′ single-stranded DNA overhangs. Mre11 is capable of cleaving hairpins and the 3′ single-stranded DNA tail. These endonuclease activities of Mre11 are enhanced markedly by Rad50 but only in the presence of ATP. Based on these results, we speculate that the Mre11 nuclease complex may mediate the nucleolytic digestion of the 5′ strand at secondary structures formed upon DNA strand separation.

DSBs are induced by ionizing radiation and are also formed during initiating events in various modes of homologous recombination (1). Genetic studies in Saccharomyces cerevisiae have been instrumental in the discovery of genes required for recombination and DSB repair through homologous recombination. These genes, RAD50, MRE11, XRS2, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, and RDH54/TID1, are collectively referred to as the RAD52 epistasis group (2).

A number of studies in S. cerevisiae indicate that DSBs in homologous recombination events are processed in a nucleolytic fashion to generate an intermediate with overhanging 3′ ssDNA tails. The ssDNA tails are bound by Rad51 and other recombination factors, which function in concert to locates regions of homology on a corresponding DNA duplex (a homologous chromosome or sister chromatid) and form heteroduplex DNA joints (1, 2).

RAD50, MRE11, and XRS2 are involved in the nucleolytic processing of DSBs. Analysis of the Rad50 and Mre11 sequences reveals homology of these two proteins to Escherichia coli SbcC and SbcD, respectively (3), which combine to form a complex that exhibits both exon- and endonuclease activities, including the capacity to cleave hairpin structures (4). Mre11 from human and yeast possesses exo- and endonuclease activities (5–9). Interestingly, overexpression of Rad50 and Mre11 can allow DNA synthesis to efficiently progress through DNA sequences with a propensity to form secondary structures, suggesting that the Mre11 complex in yeast might also have the ability to cleave such DNA structures (10).

As evidenced by co-immunoprecipitation and two-hybrid experiments, Rad50, Mre11, and Xrs2 are associated in a complex (7, 11). Likewise, the human counterparts of these proteins, human Rad50, Mre11, and NBS1 (the Xrs2 equivalent) have also been purified from human cells as an endogenous complex (8). Remarkably, genetic evidence in S. cerevisiae implicates RAD50, MRE11, and XRS2 in other cellular functions including the formation of DSBs at meiotic recombination hotspots (1), non-homologous DNA end-joining, and the maintenance of telomere length (1, 12). More recently, Rad50 and Mre11 have been implicated in the adaptation to a Rad9/Rad17-mediated G2/M checkpoint after the introduction of an unreparable DSB (13).

The multifunctional nature of the Rad50, Mre11, and Xrs2 trio emphasizes the importance for the biochemical characterization of these proteins, singly and in combination, for activities germane for their biochemical functions. Here, we purify Rad50 and Mre11, reconstitute the Rad50-Mre11 complex, and describe the endo- and exonuclease activities of these factors. We speculate on how the DNA structure-specific endonuclease activity of Rad50-Mre11 could be utilized to create ssDNA tails that facilitate subsequent steps in recombination, including the formation of dual Holliday junctions.

**EXPERIMENTAL PROCEDURES**

**Overexpression of Rad50 and Mre11 in Yeast**—The Rad50 and Mre11 genes were introduced into the vector pPM231 containing the galactose-inducible hybrid GAL-PGK promoter. The resulting plasmids, pR50.1 (2 μm, GAL-PGK-RAD50, LEU-2d) and pM11.1 (2 μm, GAL-PGK-MRE11, LEU-2d), were introduced into the protease-deficient strain BJ5464 (MATa ura3-52 trpl leu2Δ1 his3Δ12 pep4Δ HIS3 pro1Δ1 lip1Δ1 10R can1). Cultures were grown overnight to the stationary phase in synthetic medium lacking leucine and diluted 8-fold into leucine dropout synthetic media with 3% glycerol, 3% lactate, and 2% galactose. After 24 h of growth at 30 °C, cells were harvested by centrifugation.

**Purification of Rad50**—All the steps were carried out at 0 to 4 °C. Typically, cells (50 g) expressing Rad50 were resuspended in 75 ml of cell breakage buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 10 mM EDTA, 1.2 mM KCl, 1.0 mM diithotheitol, and 0.01% Nonidet P-40) with protease inhibitors (5.0 μg/ml each of aprotinin, leupeptin, chymostatin, pepstatin A, and 1 mM each phenylmethylsulfonyl fluoride and benzamidine) and lysed in a French press (SLM Aminco) at 20,000 p.s.i. The soluble fraction (Fraction I) was collected by ultracentrifugation.

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The abbreviations used are: DSB, double-strand break; BisTris, 2-(hydroxymethyl)iminodiacetic acid; Tris borate EDTA; PAGE, polyacrylamide gel electrophoresis; TBE, Tris borate EDTA.
(100,000 × g, 90 min) and treated with ammonium sulfate at 0.28 g/ml. The precipitate was collected by centrifugation (17,000 × g, 25 min), redissolved in 100 ml of K buffer (20 mM KH2PO4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Nonidet P-40, and 1.0 mM dithiothreitol) with protease inhibitors, and dialyzed against K buffer + 100 mM KCl. The dialyzed Fraction V was applied to a 20-ml Q-Sepharose Blue column, which was developed over a 200-ml gradient from 100 to 540 mM KCl, collecting 5.0-ml fractions. The peak fractions (about 350 mM KCl, Fraction III), as identified by Coomassie Blue staining of SDS-PAGE gels, were pooled and applied directly onto an 8.0-ml Affi-Gel Blue (Bio-Rad) column, which was developed with an 80-ml gradient from 100 to 1000 mM KCl, collecting 2.0-ml fractions. Peak elution occurred at about 1400 mM KCl, and these fractions were pooled (Fraction IV), dialyzed against K buffer + 100 mM KCl, and fractionated in a 6.0-ml macro-hydroxyapatite (Bio-Rad) column with a 50-ml gradient from 0 to 240 mM KH2PO4 in K buffer + 100 mM KCl, collecting 1.0-ml fractions. The peak fractions (~175 mM KH2PO4, Fraction V) were pooled, concentrated in a Centricron 30 concentrator to 0.7 ml, and subjected to gel filtration on a 35-ml Sepharose 6B column in K buffer + 100 mM KCl. The peak of Rad50 (eluted at 16–22 ml, Fraction VI) was further purified in a 1.0-ml Mono Q column using a 40-ml gradient from 100 to 370 mM KCl, collecting 1.0-ml fractions. Rad50 (eluted at about 170 mM KCl, Fraction VII) was concentrated in a Centricron 30 microcentrator to a final volume of 1.0 ml at 10 mg/ml. Rad50 was stored in small aliquots at −80°C. The Rad50 protein concentration was determined by densitometric scanning of a 7.5% SDS-PAGE gel containing multiple loadings of purified Rad50 against known amounts of bovine serum albumin run on the same gel.

Purification of Mre11—All the steps were carried out at 0–4°C. Typically, extract (Fraction I) was prepared from yeast cells (100 g) expressing Mre11 and subjected to ammonium sulfate precipitation at 0.28 g/ml. The resulting precipitate was dissolved in 130 ml of K buffer with protease inhibitors and dialyzed against K buffer + 100 mM KCl. The dialyze (Fraction II) was applied to a 25-ml Q-Sepharose column developed with a 250-ml gradient of 100–650 mM KCl, collecting 5.0-ml fractions. The peak fractions of Mre11 (~300 mM KCl, Fraction III), as identified by Coomassie Blue staining of SDS-PAGE gels, were combined, bound to a 6.0-ml macro-hydroxyapatite column, and eluted with a gradient of 0–270 mM KH2PO4 in K buffer + 100 mM KCl. The Mre11 peak fractions from the macro-hydroxyapatite step (~140 mM KH2PO4) were pooled (Fraction IV), concentrated using a Centricron 30 concentrator to ~4.0 ml, and subjected to gel filtration on a 200-ml Sephacryl 300 column in K buffer + 100 mM KCl. Mre11-containing fractions (eluted at 90–120 ml, Fraction V) were loaded onto a 1.0-ml Mono S column, which was developed with a 40-ml KCl gradient from 0 to 630 mM KCl, with Mre11 eluting at about 300 mM KCl. The peak fractions (Fraction VI) were concentrated in a Centricron 30 microcentrator to a final volume of 2.0 ml at 10 mg/ml. Purified Mre11 was stored at −80°C in small aliquots. The Mre11 protein concentration was determined by densitometric scanning of a 7.5% SDS-PAGE gel containing multiple loadings of purified Mre11 against known amounts of bovine serum albumin run on the same gel.

Reconstitution of the Rad50-Mre11 Complex—Fraction V Rad50 (15 mg) was mixed with Fraction V Mre11 (15 mg) in 4.0 ml and incubated on ice for 5 h to allow for complex formation. The Rad50-Mre11 complex was separated from free Mre11 by gel filtration on a 200-ml Sephacryl 6B column equilibrated in K buffer + 100 mM KCl. Fractions were collected over a 200-ml volume, with the Rad50-Mre11 complex eluting at ~80–100 ml. The Rad50-Mre11 complex was further purified on a 1.0-ml Mono S column with a 40-ml gradient (100–630 mM KCl), with the complex eluting at ~200 mM KCl. The peak fractions were pooled and concentrated to a final volume of 1.0 ml at 10 mg/ml.

DNA Substrates—Oligonucleotides were from Life Technologies, Inc. TP8 (9), a 74-mer, had the sequence 5’-GACCTGGCACACGTAGGACAGCATGGGATCTGGCCTGTGTTACACAGTGCTACAGACTGGAACAA-3’ (TP8), also a 74-mer, was identical to TP8, except for the absence of the 3’ overhang with the exonuclease-deficient Klenow polymerase and [α-32P]dATP. The labeling oligos were held at 70°C for 10 min and then allowed to cool slowly to room temperature to anneal. The annealed product was further purified on a 5% native polyacrylamide gel in 4°C TBE recovered by electrophoresis. The 5’-end-labeled duplex substrates were made by hybridizing oligo TP8 (10 mg) radiolabeled at the 5’ end with T4 polynucleotide kinase and [γ-32P]ATP to oligo TP9 (15 mg) held at 70°C in 30 mM Tris HCl, pH 7.0, with 200 mM KCl for 10 min, which was allowed to slowly cool to room temperature. Substrate concentrations were determined by titteration on a 10% native polyacrylamide gel in TBE against known amounts of DNA standards and by scintillation counting. The 3’-end-labeled duplex substrates were generated by the hybridization of oligo TP8E to TP9 and subsequent filling-in of the resulting single-base overhang with the exonuclease-deficient Klenow polymerase and [α-32P]dATP. The labeling reaction was halted by phenol/chloroform extraction followed by gel filtration in Biospin P-6 columns (Bio-Rad) to remove the unincorporated isotope.

TP8, TP7, and A74 were labeled at the 3’ end with terminal deoxynucleotidyltransferase and [α-32P]dATP. The labeled oligos were held at 70°C for 10 min and then allowed to cool slowly to room temperature to anneal. The annealed product was further purified on a 5% native polyacrylamide gel in 4°C TBE recovered by electrophoresis. The 5’-end-labeled duplex substrates were made by hybridizing oligo TP8 (10 mg) radiolabeled at the 5’ end with T4 polynucleotide kinase and [γ-32P]ATP to oligo TP9 (15 mg) held at 70°C in 30 mM Tris HCl, pH 7.0, with 200 mM KCl for 10 min, which was allowed to slowly cool to room temperature. Substrate concentrations were determined by titteration on a 10% native polyacrylamide gel in TBE against known amounts of DNA standards and by scintillation counting. The 3’-end-labeled duplex substrates were generated by the hybridization of oligo TP8E to TP9 and subsequent filling-in of the resulting single-base overhang with the exonuclease-deficient Klenow polymerase and [α-32P]dATP. The labeling reaction was halted by phenol/chloroform extraction followed by gel filtration in Biospin P-6 columns (Bio-Rad) to remove the unincorporated isotope.

Nuclease Assays—Various DNA substrates were mixed with Mre11 or Rad50-Mre11 complex (amounts indicated in figure legends) in reaction buffer (30 mM BisTris-HCl, pH 7.0, with 50 mM KCl, 2.5 mM MnCl2, 50 μg/ml bovine serum albumin, and 1.0 mM dithiothreitol). ATP or ATPγS (1.0 mM) were also added when stated. After incubation at 37°C or markers was purchased from Life Technologies, Inc.

Radial-labeling of Nuclease Substrates—DNA hairpin substrates were prepared by labeling HP2 and HP30 at the 3’ end with terminal deoxynucleotidyltransferase and [α-32P]dATP. The labeled oligos were held at 70°C for 10 min and then allowed to cool slowly to room temperature to anneal. The annealed product was further purified on a 5% native polyacrylamide gel in 4°C TBE recovered by electrophoresis. The 5’-end-labeled duplex substrates were made by hybridizing oligo TP8 (10 mg) radiolabeled at the 5’ end with T4 polynucleotide kinase and [γ-32P]ATP to oligo TP9 (15 mg) held at 70°C in 30 mM Tris HCl, pH 7.0, with 200 mM KCl for 10 min, which was allowed to slowly cool to room temperature. Substrate concentrations were determined by titteration on a 10% native polyacrylamide gel in TBE against known amounts of DNA standards and by scintillation counting. The 3’-end-labeled duplex substrates were generated by the hybridization of oligo TP8E to TP9 and subsequent filling-in of the resulting single-base overhang with the exonuclease-deficient Klenow polymerase and [α-32P]dATP. The labeling reaction was halted by phenol/chloroform extraction followed by gel filtration in Biospin P-6 columns (Bio-Rad) to remove the unincorporated isotope.

TP8, TP7, and A74 were labeled at the 3’ end with terminal deoxynucleotidyltransferase and [α-32P]dATP or [α-32P]dATP where indicated. TP8A+ indicates that the TP8 substrate (74-mer) was labeled at the 3’ end with an A, and TP8T+ indicates 3’-labeling with a T. For these reactions, a molar excess of oligonucleotide to isotope was used to minimize the possibility that multiple bases were added to a single oligonucleotide. The T-A hybrid was obtained by hybridizing TT74 to A74.
RESULTS

Rad50, Mre11, and Rad50-Mre11 Complex—SDS-PAGE analysis of extracts from yeast cells harboring the Rad50 expression plasmid (pR50.1) and the empty expression vector (pPM231) revealed the Rad50 protein band clearly. The observed size of the overexpressed Rad50 protein (150 kDa) (Fig. 1A, left panel) was in agreement with the predicted molecular mass for this protein (14). By immunoprecipitation, we have verified that Rad50 in wild type extract has the same gel size as the overexpressed protein. Rad50 was purified (Fig. 1A, middle panel) to near homogeneity (Fig. 1A, right panel).

Likewise, comparison of extracts from yeast cells harboring the Mre11 expression plasmid, pM11.1, versus pPM231 showed the overexpression of Mre11 (Fig. 1B, left panel). The apparent gel size of ~94 kDa for Mre11 was larger than the predicted size of 78 kDa for this protein (11). By immunoprecipitation, we have verified that the overexpressed Mre11 had the same gel size as Mre11 from wild type extract. Mre11 was also purified (Fig. 1B, middle panel) to near homogeneity (Fig. 1B, right panel).

When partially purified Rad50 and Mre11 were mixed on ice and applied to a Sepharose 6B gel filtration column, the Mre11 profile was shifted to co-elute with Rad50 as a higher molecular weight species (not shown), suggesting that Rad50 and Mre11 formed a stable complex. Interestingly, whereas Rad50 alone did not bind to a Mono S column, it was efficiently retained after incubation with Mre11, which binds to Mono S, thus providing additional evidence that these proteins form a stable complex. For biochemical studies, the Rad50-Mre11 complex was assembled and purified to near homogeneity as described under “Experimental Procedures” and found to consist of equimolar amounts of the two proteins (Fig. 1C, right panel). Neither the addition of ATP nor Mn$^{2+}$ had any detectable effect on complex stability (not shown).

Mre11 Exonuclease Activity Has 3’ to 5’ Polarity and Has a Preference for Duplex DNA Ends—To examine the exonuclease activity of Mre11 and the Rad50-Mre11 complex, the 5’-labeled duplex (5’-Duplex) and the 3’-labeled duplex (3’-Duplex) substrates were used. Aside from the location of the 32P label, the 5’-Duplex and the 3’-Duplex shared the same DNA sequence. To follow the kinetics more accurately, the experiment was carried out at 4 °C. Digestion of the 5’-Duplex by either Mre11 or the Rad50-Mre11 complex as a function of time generated a variety of radiolabeled products of decreasing size (Fig. 2A). By contrast, digestion of the 3’-Duplex generated a single major product that migrated at the gel front (Fig. 2B). These results confirmed that the exonuclease activity of Mre11 is 3’ to 5’ in polarity. Likewise, the Rad50-Mre11 complex exhibited a 3’ to 5’ exonuclease activity. In contrast to human Rad50, which stimulated the exonuclease activity of Mre11 and Rad50-Mre11 on the 3’ Duplex was monitored in the presence of Mg$^{2+}$ or Mn$^{2+}$, as shown. Reactions were carried out as described in Fig. 2 for 2 min and were analyzed by thin layer chromatography. GMP and the reaction products generated by exonuclease III were run as standards. GMP$, guanosine monophosphate.

4 °C where indicated, the reaction was halted by 1/10 volume of 3% SDS and deproteinized with protease K (0.5 mg/ml) at 37 °C for 10 min. For oligonucleotide-based nuclease assays, one-third volume of 90% formamide with 0.05% bromphenol blue was added to each sample, which was boiled for 2 min before loading 15% denaturing polyacrylamide gel at 55 °C. After electrophoresis, the gels were fixed in 10% acetic acid, 10% methanol, dried, and subjected to autoradiography and phosphorimage analysis.

Thin Layer Chromatography—Portions of the exonuclease reactions were loaded onto polyethyleneimine plates (J. T. Baker Inc.), which were developed in 1.0 M formic acid and 0.3 M LiCl. Reaction products were visualized by autoradiography and quantitated by phosphorimage analysis.

The exonuclease activity of Mre11 and Rad50-Mre11 on the 3’ Duplex was monitored in the presence of Mg$^{2+}$ or Mn$^{2+}$, as shown. Reactions were carried out as described in Fig. 2 for 2 min and were analyzed by thin layer chromatography. GMP and the reaction products generated by exonuclease III were run as standards. GMP$, guanosine monophosphate.

![FIG. 2. Mre11 and the Rad50-Mre11 complex have 3’ to 5’ exonuclease activity. A, the 5’-labeled duplex substrate (5’ Duplex, 2.0 pmol) was incubated at 4 °C with 15.0 pmol of Mre11 or Rad50-Mre11 (R/M) in 50 μl. At the indicated times, 8.0-μl aliquots were removed, deproteinized, and run on a 15% denaturing polyacrylamide gel at 55 °C. B, nuclease reactions were performed on the 3’ Duplex using the same conditions in A.](image-url)

![FIG. 3. The exonuclease activity of Mre11 and the Rad50-Mre11 (R/M) complex is dependent on Mn$^{2+}$. The exonuclease activity of Mre11 and Rad50-Mre11 on the 3’ Duplex was monitored in the presence of Mg$^{2+}$ or Mn$^{2+}$, as shown. Reactions were carried out as described in Fig. 2 for 2 min and were analyzed by thin layer chromatography. GMP and the reaction products generated by exonuclease III were run as standards. GMP$, guanosine monophosphate.](image-url)
quired Mn$^{2+}$ (Fig. 3), which could not be substituted by Mg$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, or Co$^{2+}$ (not shown). As expected, the exonuclease activity of Mre11 was unaffected by ATP or ATP$\gamma$S. Interestingly, whereas ATP was clearly not a requirement for the Rad50-Mre11 exonuclease function, ATP$\gamma$S caused partial inhibition (Fig. 4, A and B). For example, whereas $\approx 73\%$ of the labeled nucleotide was released from the duplex by Rad50-Mre11 with or without ATP in 2 min, only $\approx 36\%$ was liberated in the presence of ATP$\gamma$S (Fig. 4, A and B). These results indicated that the ATP$\gamma$S-bound form of Rad50-Mre11 is less active exonucleolytically. Other results indicated that Rad50 and the Rad50-Mre11 complex possessed only a low level of ATP$\gamma$S-bound activity with $k_{cat}$ values of 0.10 min$^{-1}$ and 0.12 min$^{-1}$ at 37 °C, respectively. The exonuclease activity of Mre11 and Rad50-Mre11 was maximal from pH 6.4 to 7.2 and was considerably less active above pH 7.6. As expected, Rad50 alone was devoid of any exonuclease activity (not shown).

We next examined whether Mre11 and Rad50-Mre11 also acted exonucleolytically on ssDNA. For this purpose, oligonucleotide TP8 was labeled at the 3’ end with either an A or T residue, as described under “Experimental Procedures.” Incubation of these substrates with Rad50-Mre11 (Fig. 5A) or Mre11 (not shown) resulted in the release of the $^32$P-labeled nucleotides as observed for the 3’-Duplex (Fig. 2B). However, we also wished to consider the possibility that hybridization of the ssDNA end in TP8 to short complementary sequences within the oligonucleotide substrate or between different TP8 molecules might transiently generate a duplex end for the Rad50-Mre11 exonuclease function to act upon. To examine this, we labeled the homopolymeric oligonucleotides T74 and A74, consisting of a run of 74 T or A residues, at the 3’ end with a labeled T or A residue, respectively. Such substrates should be largely devoid of secondary structure. As shown in Fig. 5B, Rad50-Mre11 had little ability to digest these homopolymers. Importantly, the hybrid generated by the radiolabeled T74T* substrate with the unlabeled A74 substrate (T*A Hybrid) was now digested by Rad50-Mre11 exonucleolytically (Fig. 5B), albeit less extensively than the 3’-Duplex (Fig. 2B). We attribute this difference to the fact that the TA Hybrid is
likely a population of annealed products, some of which have persistent ssDNA overhangs or have the radiolabeled end embedded in concatamers, thus preventing the labeled end from degradation. Similar results were obtained using Mre11 alone (data not shown) and with another unrelated enzyme, exonuclease III, which is a known dsDNA-specific 3' to 5' exonuclease (Fig. 5C). Taken together, the results demonstrated that the exonuclease activity of Mre11 and Rad50-Mre11 has a clear preference for duplex DNA ends and that digestion of the TP8 oligonucleotides as initially observed was likely enhanced by transient base-pairing events that generated duplex DNA ends.

**DNA Structure-specific Endonuclease Activity of Mre11 and Rad50-Mre11**—Both Mre11 (5–7) and Rad50-Mre11 (data not shown) were capable of digesting circular plasmid-length ssDNA to products of higher gel mobility, indicating the action of an endonuclease. The fact that neither Mre11 nor Rad50-Mre11 cleaved a homopolymeric oligonucleotide endonucleolytically (Fig. 5B) suggested that they might be acting on secondary structures within the ssDNA substrate. Therefore, it was of considerable interest to examine the endonucleolytic function on defined hairpin substrates that mimic structures expected in the ssDNA molecule. Difficulties in discerning endo-versus exonuclease activities on such substrates were overcome by the inclusion of a homopolymeric poly(dA) tail at the ends of the hairpins, guided by our observation that such homopolymers were relatively resistant to exonucleolytic cleavage (Fig. 5B). Interestingly, both Mre11 and Rad50-Mre11 were capable of making specific incisions resulting in the creation of two major endonucleolytic products, designated A and B. Product A was the result of an incision at the distal end of the hairpin relative to the 3'-labeled extremity. Importantly, as determined by analysis of reaction products on sequencing gels, the relative position of the incision remained the same regardless of whether a fully paired hairpin (HP2) or a hairpin with a 30-base homopolymeric loop (HP30) was used (Fig. 6). Product B resulted from another incision at the junction of the duplex with the 3' ssDNA poly(dA) overhang (Fig. 6). Owing to the higher temperature (37°C) and increased incubation times (10–40 min) employed for these experiments compared with the conditions (4°C and 0.5–2.0 min) used to examine the exonuclease activity of the homopolymeric substrates in Fig. 5B, a modest amount of mononucleotide (AMP*) was observed (Fig. 6).

**Effect of ATP on the Endonuclease Function of Rad50-Mre11**—We addressed the question of whether ATP was important for the structure-specific endonucleolytic function of Mre11 and Rad50-Mre11. Where indicated, MnCl₂ was omitted or substituted with MgCl₂, and ATP (1.0 mM) was omitted or replaced with ATP-S. Portions (5.0 μl) of each sample were removed after 20-min incubations at 37°C, deproteinized, and analyzed. A and B mark the sites of specific incisions. Bl, blank (no protein); –Me, no metal cofactor; γS, ATP-S.
enhanced, whereas that of Mre11 was unaffected (Fig. 7, lanes 3 and 9). For instance, whereas only 11% of HP2 was converted to products A and B by Rad50-Mre11 after 40 min in the absence of ATP (Fig. 7, lane 11), ~52% was converted into these products when ATP was present (Fig. 7, lane 9). Importantly, ATP-S did not stimulate the endonuclease activity of Rad50-Mre11 (Fig. 7, lanes 6 and 12), nor did ATP-P if any influence on the endonuclease function of Mre11. As expected, the structure-specific endonuclease activities had a specific requirement for Mn²⁺ as cofactor (Fig. 7).

**DISCUSSION**

*Exonuclease Activity of Mre11 and Rad50-Mre11—*Mre11 and Rad50-Mre11 exhibit dsDNA exonuclease activity with a 3’ to 5’ polarity that releases mononucleotide products. Although neither Mre11 nor Rad50-Mre11 are significantly stimulated by ATP for its exonuclease function, the activity of Rad50-Mre11 is inhibited by ATP-P or ATP-P-S. Interestingly, whereas hRad50 has been shown to stimulate the exonuclease activity of human Mre11 (9), we find that S. cerevisiae Rad50 does not appear to significantly affect the exonuclease activity of S. cerevisiae Mre11. We also demonstrate that Mre11 with or without Rad50 has a clear preference for duplex DNA ends. The apparent ability of Mre11 to digest ssDNA substrates as seen by Usui et al. (7) was likely enhanced by the formation of transient duplex ends within the oligonucleotide substrates.

*DNA Structure-specific Endonuclease Activity of Mre11 and Rad50-Mre11—*Based on our finding that the exonuclease activity of Mre11 was attenuated by homopolymeric ssDNA sequences, we designed hairpin structures with poly(dA) extensions to allow us to discern the endonuclease function. Using this approach, we were able to observe two distinct endonucleolytic activities within hairpin substrates. One of the endonucleolytic products was formed upon cleavage at the distal end of a hairpin loop relative to the labeled 3’ end. The second major endonucleolytic product was formed upon cleavage at the junction between the duplex DNA molecule and the 3’ ssDNA extensions. Although Mre11 was capable of making both incisions alone, Rad50 stimulated these activities markedly, but ATP was specifically required for the maximal expression of the endonucleolytic activities of only the Rad50-Mre11 complex. Similar DNA structure-specific endonuclease activities were first reported by Paull and Gellert (15) for the human Mre11 protein, but our current results point to two major differences concerning how these endonucleolytic activities in the yeast and human counterparts are regulated. Specifically, whereas the ability to incise a fully paired DNA hairpin and a 3’ ssDNA tail for hMre11 is largely dependent on both the human Rad50 and NBS1 proteins (15), S. cerevisiae Rad50 alone is sufficient to stimulate these activities in yeast Mre11, although it remains possible that Xrs2 may further enhance the endonucleolytic function of Rad50-Mre11. Second, although the incision of a 3’ ssDNA tail but not a DNA hairpin by the human Rad50-Mre11-NBS1 complex requires ATP, the yeast Rad50-Mre11 complex needs ATP for the maximal expression of both structure-specific activities.

*The Mre11 Endonucleolytic Function and DSB Processing—*Results from a number of genetic studies in S. cerevisiae indicate that during DSB processing, the DNA strands that contain the 5’ termini are resected preferentially (1). Given this observation, it is clear that the Mre11 exonuclease activity, which has a 3’ to 5’ directionality, cannot be solely responsible for the resection of the 5’ DNA strands. Like its human counterpart, yeast Mre11 also possesses the ability to incise DNA hairpin structures as well as 3’ ssDNA tails that border a duplex region. The highly conserved nature of these endonucleolytic activities suggests that they are germane for the processing of DNA DSBs. We speculate that these DNA structure-specific activities may be used in conjunction with a DNA helicase to process DNA double-strand breaks. The engagement of the DNA ends by the Rad50-Mre11-Xrs2 complex and recruitment of a DNA helicase would lead to catalytic unwinding of the two DNA strands, creating 3’ and 5’ single-stranded overhangs (Fig. 8A, Step I). We also surmise that this helicase may be activated by a ssDNA region generated by the 3’ to 5’ exonuclease activity of the complex or through the limited capacity of Rad50 to partially unwind duplex DNA (15). As postulated in Fig. 8A, the endonucleolytic function of the Rad50-Mre11-Xrs2 complex may recognize and incise secondary structures present in the DNA strands that harbor the 5’ termini of the DNA break (Fig. 8A, Step II). This hypothetical model (Fig. 8) resembles the mode of DSB processing in E. coli, during which the ends of DSBs are acted upon by the integrated helicase/nuclease action of the RecBCD protein complex (16).

The end result of such a mode of processing would in fact create not only a 3’ ssDNA tail but also a shorter 5’ ssDNA tail...
as well (Fig. 8A, Step III). Given that Rad51 can efficiently utilize both 3′ and 5′ ssDNA tails for DNA joint formation (17), the DNA intermediate depicted in Fig. 8A (III) is ideally suited for the formation of double Holliday junctions (Fig. 8B) known to exist during meiotic recombination (1).

At this time, we can only speculate as to the reason(s) why secondary structures within the 3′ strand are not also acted upon by the Rad50-Mre11-Xrs2 complex. Genetic and biochemical evidence clearly indicates that either the 3′ strand is protected or that the 5′ strand is preferentially excised. We suggest that the 3′ ssDNA generated as result of the helicase action might be subject to preferential binding by a variety of proteins that would, in turn, function to protect the 3′ ssDNA tail and/or melt the secondary structures that might otherwise be susceptible to the action of Rad50-Mre11-Xrs2. Among such protein factors might be RPA, Rad51, and other recombination factors that have been shown to localize to the site of a DSB (18–20). We wish to emphasize that the proposed model is suggestive and must be validated experimentally.

Functional Redundancy of the Mre11 Nuclease in Mitotic cells—Point mutations that render Mre11 nuclease deficient cause no apparent anomalies in DSB processing in mitotic cells. We would imagine that the most important role of the Rad50-Mre11-Xrs2 complex in end-processing in such instances is to recruit a DNA helicase to the DSB. Once a branched DNA structure is generated as a result of catalytic DNA strand separation by the helicase (Fig. 8A, Step II), the fact that nuclease-deficient mutants of Mre11 are still capable of DSB processing (21) would argue that the 5′ ssDNA strand in the branched structure is also eventually accessible to other nucleases, as suggested by the work of Symington et al. (21).

It has been shown that in the absence of Mre11, DNA ends can still be processed, perhaps by a dsDNA exonuclease (21). However, the low processivity of 5′ strand resection in such instances is evidenced by highly reduced conversion tract lengths (21). This observation strongly implicates the Rad50-Mre11-Xrs2-dependent pathway as the preferred mechanism for DSB processing in wild type cells.

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