Role of the Nuclease Activity of Saccharomyces cerevisiae Mre11 in Repair of DNA Double-Strand Breaks in Mitotic Cells

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ABSTRACT

The Rad50:Mre11:Xrs2 (RMX) complex functions in repair of DNA double-strand breaks (DSBs) by recombination and nonhomologous end-joining (NHEJ) and is also required for telomere stability. The Mre11 subunit exhibits nuclease activities in vitro, but the role of these activities in repair in mitotic cells has not been established. In this study we have performed a comparative study of three mutants (mre11-D16A, D56N, and -H125N) previously shown to have reduced nuclease activities in vitro. In ends-in and ends-out chromosome recombination assays using defined plasmid and oligonucleotide DNA substrates, mre11-D16A cells were as deficient as mre11 null strains, but defects were small in mre11-D56N and -H125N mutants. mre11-D16A cells, but not the other mutants, also displayed strong sensitivity to ionizing radiation, with residual resistance largely dependent on the presence of the partially redundant nuclease Exo1. mre11-D16A mutants were also most sensitive to the S-phase-dependent clastogens hydroxyurea and methyl methanesulfonate but, as previously observed for D56N and H125N mutants, were not defective in NHEJ. Importantly, the affinity of purified Mre11-D16A protein for Rad50 and Xrs2 was indistinguishable from wild type and the mutant protein formed complexes with equivalent stoichiometry. Although the role of the nuclease activity has been questioned in previous studies, the comparative data presented here suggest that the nuclease function of Mre11 is required for RMX-mediated recombinational repair and telomere stabilization in mitotic cells.

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UKARYOTIC organisms repair broken chromosomes by at least two distinct DNA repair pathways, homologous recombination and nonhomologous end-joining (NHEJ). The conserved Saccharomyces cerevisiae Rad50, Mre11, and Xrs2 proteins (referred to as RMX) play a unique role in that they function in both recombination and NHEJ repair. Yeast cells containing inactivated RMX genes are defective in NHEJ assays (e.g., homology-independent plasmid recircularization, sensitivity to in vivo expression of EcoRI endonuclease, deletion formation within dicentric plasmids, etc.) and also exhibit reduced efficiency of DSB-induced homologous recombination (Lewis and Resnick 2000; Sung et al. 2000; Symington 2002). RMX mutants also greatly increased frequencies of spontaneous chromosome rearrangements, shortened telomeres, defects in S-phase checkpoint responses to DNA damage, hypersensitivity to clastogenic chemicals and ionizing radiation and reduced recombination in meiosis (Chen and Kolodner 1999; Kouprina et al. 1999; Lewis and Resnick 2000; Gренон et al. 2001; Usui et al. 2001; Chang et al. 2002; D’Amours and Jackson 2002; Myung and Kolodner 2002).

Several of the metabolic defects described for yeast RMX mutants are also observed in mammalian cells upon inactivation of the corresponding gene orthologs. For example, mutations within the human genes hMRE11 and hNBS1 (hNBS1 is the apparent human equivalent of yeast XRS2) cause the human disorders Nijmegen breakage syndrome and ataxia telangiectasia-like disorder, respectively (Stewart et al. 1999). Cells derived from individuals with these disorders display multiple DNA damage response defects, including hypersensitivity to ionizing radiation and defective checkpoint responses. In addition, individuals with these disorders have an increased incidence of cancer (Petrini 1999; D’Amours and Jackson 2002). Further evidence suggesting a role for inactivation of the complex in cancer development has been obtained from directed sequencing of hMRE11 genes from random (Fukuda et al. 2001) and mismatch repair-deficient tumor cells (Giannini et al. 2002).

The Mre11 subunit of RMX has manganese-dependent 3’- to 5’ dsDNA exonuclease and ssDNA endonuclease activities that are active on a number of linear and circular DNA structures, including the tops of hairpin structures formed by inverted repeat sequences in...
forms the DNA-binding portion of the complex. Larger the N- and C-terminal Walker A and B motifs in proxim- by homologous recombination and stabilization of telo-
subunit forms a folded, antiparallel structure that places functions of the enzyme
in endonuclease and exonuclease activities, but whichligase IV complex, suggest an important role in RMX-
duplex are consistent with a role for its nuclease function(s) in
Other than this subunit and Lif1, a component of the DNA ligase IV complex, suggest an important role in RMX-
mediated repair by NHEJ (CHEN et al. 2001). Studies of the equivalent protein in higher eukaryotes (NB51) indicate that some activities of the complex, e.g. duplex DNA unwinding, are also dependent upon the presence of this subunit (PAUL and GELLERT 1999).

Structural studies of archaebacterial, yeast, and human Rad50 and Mre11 suggest that these proteins combine to form multimers whose unit structure consists of two molecules of each polypeptide (ANDERSON et al. 2001; CHEN et al. 2001; DE JAGER et al. 2001; HOPFNER et al. 2002). According to recent models, each Rad50 subunit forms a folded, antiparallel structure that places the N- and C-terminal Walker A and B motifs in proximity with each other. Association of two Mre11 molecules with the joined ends of two folded Rad50 subunits then forms the DNA-binding portion of the complex. Larger multimeric structures that can potentially form bridges between broken DNA ends or between adjacent sister chromatids have also been suggested, possibly resulting from Zn²⁺-mediated joining of Rad50 molecules at a “hinge” or “hook” region (DE JAGER et al. 2001; HOPFNER et al. 2002).

The specific mechanism(s) by which the RMX nuclease complex mediates repair by recombination and NHEJ, activates checkpoints, inhibits chromosome rearrangements, and stabilizes telomeres is unknown. We and others established that some DSB repair phenotypes of RMX mutants can be suppressed by overexpression of the gene encoding Exo1, a 5′-to-3′ exonuclease (and also by telomerase RNA; LEWIS et al. 2002), suggesting that a critical function that has been lost in these mutants is DSB end-processing (CHAMANKHAK et al. 2000; SYMINGTON et al. 2000; TSUBOUCHI and OGAWA 2000; MOREAU et al. 2001; LEWIS et al. 2002). More specifically, these experiments revealed that the nuclease activity of Exo1 could partially substitute for RMX in recombinational repair, but not repair by the NHEJ pathway. The results with Exo1 are inconsistent with recent analyses of mutants with substitutions in the conserved phosphoesterase motifs of Mre11 (e.g., -D56N, -H125N, and -H125L/D126V), which suggested that nuclease activity is not required for several major functions of RMX in mitotic cells, including recombination, NHEJ, and telomere stabilization (BRESSAN et al. 1999; MOREAU et al. 1999; SYMINGTON et al. 2000; TSUKAMOTO et al. 2001; LOBACHEV et al. 2002).

While the Mre11 nuclease is clearly required for processing of special DNA structures, such as meiotic DSBs containing attached proteins or certain DNA secondary structures in mitotic cells (RATTRAY et al. 2001; LOBACHEV et al. 2002; SYMINGTON 2002), previous studies of known nuclease-defective alleles observed only minor effects on repair of DSBs induced by ionizing radiation, chemicals, or site-specific endonucleases during mitotic growth. We report here that cells expressing a mutant Mre11 protein (Mre11-D16A; motif I), which is deficient in endonuclease and exonuclease activities, but which retains the ability to bind DNA and to form multimers with Mre11, has multiple DNA metabolic defects that are consistent with a role for its nuclease function(s) in recombinational repair of DSBs and telomere stabilization, but not NHEJ in mitotic cells. Several phenotypes of mre11-D16A cells differ only in severity from cells expressing two other Mre11 variants shown to have reduced nuclease activities in vitro. Together with past observations of recombination-specific suppression by EXO1, these results suggest that catalytic activities established for Mre11 in vitro are in fact important for major functions of the enzyme in vivo such as repair of DSBs by homologous recombination and stabilization of telo-

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used for this work are shown in Table 1. rad50::hisG-URA3-hisG disruptions were generated using pNK83 (a generous gift from N. Kleckner) digested with EcoRI + BglII and exo1::URA3 disruptions were created using plasmid p244 cut with HindIII + KpnI (TRAN et al. 1999). Geneticin/G418 (BRL) and Hygromycin B (Boehringer Mannheim, Indianapolis) were added to plates for selection of resistant strains at concentrations of 200 and 300 μg/ml, respectively. 5-Fluoroorotic acid (5-FOA) used for se-

Plasmids used for expression studies were as follows: pRS314 (GEN/ARS, TRP1; SIKORSKI and HIETER 1989), pMre11-D16A (GEN/ARS, TRP1 mre11-D16A; this work), pSM258 (GEN/ARS, TRP1, MRE11; a kind gift from L. Symington), pSM904 (as pSM258, but mre11-H125N), pSM312 (as pSM258, but mre11-D56N), and pRS316Gal (LEWIS et al. 1998).

Site-specific mutagenesis of chromosomal and plasmid loci: A recently developed technique (delitto perfetto; STORICI et al. 2001) was employed to create substitutions in MRE11 at its natural locus on chromosome XIII (Figure 1A). A DNA frag-
ment containing selectable and counterselectable markers for G418 and URA3 flanked by MRE11 sequences was generated using primers MRE11.G and MRE11.U to amplify DNA in the cassette plasmid pCORE (Storici et al. 2001). Sequences of these and all other primers are available upon request. This fragment was used to insert the MRE11 gene on chromosome XIII between nucleotides (nt) 446 and A47 of the coding region. Cells were subsequently transformed with the 80-mer MRE11.a and MRE11.b and 5-FOA G418-sensitive cells were selected. Genomic MRE11 DNAs from three independent transformants were sequenced and found to contain a single mutation at codon 16 from GAT to GCT, changing the coding from aspartate to alanine. Using the same approach plasmid pSM258 (CEN/ARS, TRP1, MRE11) was modified in an MRE11-deleted strain background (YLKL-555) to create pMre11-D16A. All PCR reactions utilized Platinum PfX enzyme (GIBCO/Invitrogen).

Ends-in and ends-out chromosome recombination and NHEJ assays: Plasmid NHEJ assays were performed by LiAc transformation as previously described (Lewis et al. 2002) using uncut or BamHI-cut pRS314 with strains VLA6a (MRE11), YLKL503 (mre11Δ), and YLKL641 (mre11Δ-D16A). In these experiments the uncut pRS314 DNA serves as a control for variability in transformation efficiencies among different strains.

Ends-in recombination efficiencies of cells expressing mutant mre11 alleles were assessed using strain YLKL503 (mre11Δ) containing pRS314, pSM258, pSM304, pSM312, or pMre11-D16A. Cells were transformed with pKL37Y that had been cut inside URA3 with NotI. pKL37Y was created in the following way: A 1.2-kb HindIII URA3 gene fragment obtained from YEp24 was made blunt with T4 DNA polymerase and cloned into SalI/NotI-cut pRS309 that had also been made flush by extension of sticky ends with T4 DNA polymerase. The resulting plasmid, pKL37Y, is an integrating vector containing URA3 and HIS3. After digestion with NotI and transformation, Ura+ colonies formed by recombinational integration of the plasmid into the ura-3-5210 locus on chromosome V were scored. In this assay most transformants are Ura+. His+ integrants (see Figure 3B), with a small fraction (≈1%) of Ura+ His− cells presumed to arise by conversion of ura-3-52 on the chromosome. All transformation efficiencies (transformants per microgram of DNA) were normalized to those for uncut CEN/ARS plasmid DNA (pRS316Gal) transformed into the same competent cell preparations on the same day. Results presented are the mean ± SD of 3–5 experiments for each strain.

Ends-out gene conversion assays were performed using derivatives of the strain BY4742-TRP5-HPS (Table 1). This strain contains a selectable-counterselectable HgyB+ GALp:p53-V122A CORE cassette inserted into nucleotides 1002 and 1003 of the TRP5 gene in strain BY4742. This strain is used for quantitative analysis of oligonucleotide-mediated recombination events that result in perfect excision of the CORE cassette. The cassette used for these studies differs from the cassette previously described in Storici et al. (2001) in that hygromycin B resistance is selectable and resistance to the growth inhibitory effects of p53-V122A expression can be counterselected (Storici and Resnick 2003). p53-V122 is a variant of human p53 that is highly toxic to yeast cells when expressed from the GAL1 promoter (Storici and Resnick 2003). MRE11, mre11Δ, and mre11Δ-D16A cells (BY4742-TRP5-HPS, YLKL770, and YLKL771, respectively) were transformed with complementary 95-nt oligonucleotides TRP5.e and TRP5.f and frequencies of HgyB+ p53− cells quantitated as described previously for recombination-dependent delitto perfetto mutagenesis (Storici et al. 2001). The rad52Δ control cells used for Figure 4 were identical to the above strains except that an alternative cassette, URA3 + G418, was employed. BY4742-TRP5-CORE and YLKL769 were used for the latter assays.

Binding of Rad50 and Xrs2 to wild-type and mutant Mre11 and Mre11-D16A proteins: 6His-Mre11 and 6His-Mre11-D16A were purified from Escherichia coli strains tailored to express these proteins (Furuse et al. 1998). Nontagged Rad50, Mre11, and Xrs2 were overexpressed in yeast and purified to near homogeneity as described previously (Trujillo and Sung 2001; Trujillo et al. 2003). The concentrations of Rad50, Mre11, 6His-Mre11, 6His-Mre11-D16A, and Xrs2 were determined by densitometric scanning of 7.5% SDS-PAGE gels containing multiple loadings of the purified proteins against known amounts of bovine serum albumin run on the same gel (Trujillo et al. 2003).

Binding studies were conducted by incubating purified Rad50 (5 μg, 1.1 μM) or Xrs2 (2.3 μg, 0.8 μM) with and without purified Mre11 (3.5 μg, 1.5 μM) or Mre11-6His (3.5 μg, 1.5 μM) at 0°C in 30 μl of B buffer (20 mM K2HPO4, pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol) containing 150 mM KCl, 5 μg BSA, 10 mM imidazole, and 0.01% Igepal (Sigma). After 60 min of incubation, 10 μl of nickel-NTA-agarose beads (QIAGEN, Valencia, CA) were added and the reaction mixtures were left at 0°C for another 60 min, with gentle tapping every 2 min. The beads were washed twice with 30 μl of B buffer containing 20 mM imidazole before eluting the bound proteins from the nickel matrix with 30 μl of 200 mM imidazole in B buffer.

Cell survival assays: Survival after treatment with gamma radiation was monitored after exposure to a 137Cesium source emitting at a dose rate of 2.7 krad/min. Two or three independent log phase cultures containing YLKL503 (mre11Δ) cells with pRS314 or different MRE11 plasmids (see above) were irradiated and placed on ice and mean fractions of surviving cells were calculated after dilutions were spread onto synthetic media plates without tryptophan. Hydroxyurea survival assays were performed by dilution plating and fivefold dilutions of cells as described (Lewis et al. 2002). Strains used for the assays were YLKL503 containing pRS314 and MRE11 plasmids as above. Control strains were YLKL532 (Δrad51) and YLKL593 (Δyku70) containing pRS314. Cells were propagated on synthetic glucose plates minus tryptophan with increasing concentrations of hydroxyurea.

RESULTS

The mre11-D16A mutation greatly increases sensitivity to ionizing radiation: The endo- and exonuclease activities of Mre11 reside in conserved phosphodiesterase motifs located in the amino terminus of the protein (Figure 1B; Hopfer et al. 2001; D’amours and Jackson 2002). The aspartic acid residue in phosphoesterase domain I (D16) is associated with a manganese ion in the crystal structure of Pyrococcus furiosus Mre11 (the corresponding aspartic acid in P. furiosus Mre11 is the eighth residue of the protein; Hopfer et al. 2001). Conversion of this negatively charged residue to a neutral alanine produces a protein that has no detectable nuclease activities in vitro, but which retains the ability to bind to DNA and to other Mre11 molecules (Furuse et al. 1998). Our study was designed to assess the precise consequences of this and other substitutions known to produce proteins with no detectable nuclease activity on DSB repair capabilities in mitotic cells.

To determine the impact of the D16A substitution on DNA repair in mitotic cells, the MRE11 locus on
chromosome XIII of strain VL6α was altered by the delitto perfetto method of oligonucleotide-mediated, site-specific mutagenesis (Storici et al. 2001; Storici and Resnick 2003) as shown in Figure 1A. Initially, a two-gene “CORE” cassette was integrated into MRE11 by PCR fragment-mediated gene targeting. One of the CORE genes provides for selection by resistance to G418 and the other for counterselection against URA3 after subsequent transformation with oligonucleotides. After transformation of cassette-containing cells with long, complementary oligonucleotides containing one or more sequence changes, transformants containing perfectly excised cassettes were identified by 5-FOA counterselection of Ura−cells and confirmation of loss of the selectable marker (G418r) along with sequencing of the resulting DNA locus (see MATERIALS and METHODS). A plasmid-borne version of MRE11 on pSM258 was similarly converted to mre11-D16A after propagation in an MRE11-deleted strain background, producing the plasmid pMre11-D16A.

mre11 null cells are hypersensitive to killing by many physical and chemical agents that induce DSBs, including ionizing radiation. For example, haploid mre11 mutants are fully as sensitive to ionizing radiation as strongly recombination-defective rad51, rad52, and rad54 strains (Saeki et al. 1980; Lewis and Resnick 2000; Bennett et al. 2001). Survival of logarithmically growing cells containing mre11-D16A was found to be reduced at all doses tested, although cells were not as sensitive as mre11Δ strains (Figure 2A). In contrast, the widely studied phosphoesterase motif II and III mutants mre11-D56N and -H125N displayed near-wild-type resistance up to 20 krad, corresponding to ~10−15 DSBs per haploid genome (Resnick and Martin 1976). This result is consistent with a recent report demonstrating that the latter two mutants have a weak radiation sensitivity that becomes apparent at relatively high doses (30–70 krad; Moreau et al. 2001).

Past experiments have established that the 5′-to-3′ exonuclease encoded by EXO1 can partially substitute for the RMX complex in recombinational repair of DSBs (Chamankhah et al. 2000; Tsutouchi and Ogawa 2000; Moreau et al. 2001; Lewis et al. 2002). Haploid exo1 mutants are not sensitive to radiation, but exo1 rmx double mutants exhibit slightly more gamma sensitivity than rmx single mutants and reduced repair proficiency in plasmid DSB repair assays (Symington et al. 2000; Lewis et al. 2002). To assess the possibility that the residual radiation resistance of nuclease-defective mre11-D16A cells is due to basal level expression of Exo1, double-mutant strains were constructed and tested for radiation sensitivity. exo1 mre11-D16A double mutants exhibited ~10-fold more killing at 20 krad than mre11-D16A cells did (Figure 2B). This suggests that a large fraction of radiation-induced DSBs in mre11-D16A cells are processed by the 5′-to-3′ exonuclease activity of Exo1. However, killing did not reach the level of exo1 mre11Δ double mutants, which were slightly more sensitive than mre11 single mutants.

Radiation-induced DSBs are repaired primarily by homologous recombinational mechanisms and current models propose that RMX initiates recombination by processing DSB ends to generate 3′ single-strand overhangs (Sung et al. 2000; Symington 2002). The gamma

### TABLE 1

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>MATα ura3-52 his3Δ200 trp1Δ63 his2-801 ade2-101 met14</td>
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</table>

**TABLE 1**: Yeast strains used in this study
sensitivity of the mre11-D16A mutants is in agreement with this model and may also be an indication that Mre11-D16A protein has reduced nuclease activity relative to the Mre11-D56N and Mre11-H125N enzymes (see Discussion).

**mre11-D16A cells are unable to repair a site-specific DSB by homologous recombination, but are proficient in NHEJ repair:** To address the consequences of the MRE11 mutations on repair by the two pathways we utilized separate assays that each relied on repair of a defined DSB structure created in a plasmid (shown schematically in Figure 3, A and B). For each assay a single, cohesive-ended DSB with 5' overhangs that were four bases long served as substrate for repair (see Materials and Methods). Cells lacking Rad50, Mre11, or Xrs2 have reduced ability to recircularize linear plasmids in vivo after cell transformation if the DSB is in a region that lacks homology with chromosomal DNA. This reduction in recombination-independent repair by NHEJ, typically ~10- to 100-fold, is not observed in mutants deficient only in the recombination pathway (e.g., rad51 or rad52). NHEJ repair events were scored as transformant cells that had recircularized the broken plasmid under conditions where repair by homologous recombination was not possible. Repair of the DSB by NHEJ was reduced 20-fold in mre11Δ strains (Figure 4A). Similar to a previous report for mre11-D56N and mre11-H125N mutants (Moreau et al. 1999), mre11-D16A strains exhibited approximately wild-type levels of NHEJ repair. This observation reinforces the idea that the nuclease functions of the complex are not required for RMX-mediated NHEJ repair. The proficiency at NHEJ also implies that each of the mutants is able to form productive RMX complexes in vivo.

RMX mutants exhibit reduced frequencies of ends-in (Cromie and Leach 2000; Symington 2002) DSB-induced plasmid:chromosome recombination. For assessment of DSB repair by recombination, cells were
transformed with a linearized integrating plasmid that could undergo recombination with a homologous chromosomal locus (shown schematically in Figure 3B). Cells were transformed with a HIS3 URA3 DNA fragment (cut within URA3 using NcoI) that could recombine with the chromosomal ura3-52 locus. Nearly all Ura+ recombinants arose from integration of the entire cut plasmid into the chromosome by ends-in recombination to produce Ura+ His+ cells, but a small fraction (typically ∼0.5–1% in wild-type cells) of DSBs were repaired by gene conversion of the chromosomal locus to produce Ura+ His− colonies (see below). For all experiments, transformation efficiencies (recombinants formed per microgram of DNA) were normalized to those for uncut CEN/ARS plasmids transformed into the same competent cell preparations on the same day.

The efficiency of ends-in recombinational repair was reduced ∼20-fold in mre11Δ strains (Figure 4B). Interestingly, mre11-D16A cells were as defective in recombinational repair of the plasmid DSBs as mre11Δ strains. In contrast, recombination was much higher in strains expressing the mre11-D56N and -H125N mutants (25 and 33% of wild-type levels, respectively). Over 99% of transformant colonies from wild-type cells contained integrated plasmids and were phenotypically Ura+ His+, with the remainder being Ura+ His− gene convertants. The corresponding numbers for mre11Δ and mre11-D16A cells were 99 and 96%, suggesting that crossover and noncrossover frequencies were not greatly affected.

We also determined if the severe recombination defect observed in the mre11-D16A cells was restricted to the types of ends-in plasmid:chromosome targeting events analyzed in Figure 4B. The chromosome mutagenesis procedure employed to create mre11-D16A involved replacement of a selectable-counterselectable cassette with homologous DNA contained within an oligonucleotide. This process requires a functional RAD52 gene (Storici et al. 2001) and involves the alternative, “ends-out” form of DSB-induced recombination (Cromie and Leach 2000; Symington 2002). The scheme used for the assays is depicted in Figure 3C. Briefly, wild-type and mutant cells containing a HygB′ GALp::p53-V122 cassette integrated into TRP5 were transformed with 95-mer DNA composed of upstream and downstream TRP5 sequences as described (Storici et al. 2001). Correct recombinational repair events resulted in cells that were HygB′ p53− and TRP5+. As shown in Figure 4C, no recombinants were observed when rad52 cells were assayed. Recombination frequencies in mre11Δ and mre11-D16A strains (recorded as integration events per 0.5 nmol of oligonucleotide DNA) were decreased to 2.1 and 1.3% of wild-type levels, respectively. Thus, mre11-D16A mutants are approximately as deficient as mre11 null cells in both classes of recombination events.

The nuclease mutants are differentially sensitive to the S-phase clastogens HU and MMS: Exposure of cells to high levels of the ribonucleotide reductase inhibitor HU leads to replication inhibition and formation of DSBs in chromosomal DNA (Merrill and Holm 1999; D’Amours and Jackson 2001). In contrast, low levels of HU produce few DSBs, but do result in activation of the S-phase checkpoint and killing of cells that are deficient in this checkpoint response. Early checkpoint activation events such as phosphorylation of Rad53 are inhibited and survival of RMX mutants is reduced after exposure to low levels of HU (D’Amours and Jackson 2001). Like HU, the DNA-methylating agent MMS induces DSBs during replication and is lethal to mutants defective in DSB repair and the S-phase checkpoint (Lewis and Resnick 2000; Usui et al. 2001; Chang et al. 2002).

We examined sensitivities of several repair-deficient mutant strains to a range of HU and MMS concentrations (Figure 5). Growth inhibition was apparent in mre11Δ strains at concentrations of HU as low as 5.0
mm. These cells were moderately more sensitive than Rec − rad51 cells and much more sensitive than NHEJ-deficient yku70 cells. Cells expressing the phosphoesterase mutants Mre11-D16A, -D56N, and -H125N required much higher doses of HU to detect loss of viability than did mre11 null cells. The mre11-D16A strains exhibited killing at a lower dose (40 mM) than that of either of the other nuclease mutants. A similar general pattern of survival was observed when cells were exposed to MMS (Figure 5B). Relative sensitivities could again be ordered as mre11Δ > rad51Δ > mre11-D16A > mre11-D56N or mre11-H125N (most sensitive to least sensitive). The greater killing of mre11-D16A cells compared to the other two mutants is qualitatively consistent with the radiation survival curves (Figure 2A).

Purified Mre11-D16A protein binds efficiently to Rad50 and Xrs2: Mre11 interacts with Rad50 and Xrs2 to form a trimeric complex (Sung et al. 2000; Symington 2002). To ask whether Mre11-D16A protein retains the ability to bind Rad50 and Xrs2, purified six-histidine-tagged Mre11-D16A was mixed with purified Rad50 or Xrs2, and the complexes formed between the protein pairs were isolated using nickel-NTA-agarose beads, which have high affinity for the histidine tag on Mre11-D16A. We included as positive control six-histidine-tagged wild-type Mre11 protein. As shown in Figure 6, A and B, while Rad50 and Xrs2 have no affinity for the nickel-NTA-agarose beads, a substantial portion of these two proteins became associated with the beads when tagged Mre11-D16A was present, indicating complex formation. Importantly, the histidine-tagged Mre11-D16A protein has the same affinity for Rad50 and Xrs2 as histidine-tagged wild-type Mre11 (Figure 6, A and B, lanes 4 and 8). Consistent with the affinity pulldown results, Mre11-D16A forms a trimeric complex with Rad50 and Xrs2 that has a component stoichiometry indistinguishable from that assembled with wild-type Mre11 (Chen et al. 2001; data not shown). These results, in conjunction with the previous work of Furuse et al. (1998), demonstrate that Mre11-D16A protein is proficient at both DNA binding and RMX complex formation.

**DISCUSSION**

The RMX complex is required for successful completion of several specific DNA metabolic processes in mitotic cells. These functions include repair by recombination and end-joining, telomere length maintenance, DNA replication-associated cell cycle checkpoints, inhi-
Figure 4.—DSB repair efficiencies of haploid cells expressing altered Mre11 proteins with reduced nuclease activity. Efficiencies of repair in wild-type cells by (A) end-joining, (B) ends-in recombination, and (C) ends-out recombination. Transformation efficiencies (repair events per microgram of DNA) in A and B were normalized to those for uncut CEN/ARS plasmids transformed into the same competent cell preparations on the same day. Numbers in parentheses indicate means derived from 3–5 assays for each strain. No recombinants were detected for rad52Δ strains in C. Error bars indicate standard deviations.

Figure 5.—Assessment of S-phase clastogen sensitivities of mre11-D16A, mre11-D56N, and mre11-H125N mutants. Haploid yeast cells were pronged to synthetic glucose plates lacking tryptophan and containing increasing concentrations of (A) hydroxyurea or (B) MMS.

proteins containing alterations within one or more conserved phosphoesterase motifs in the nuclease domain. Four mutant proteins described in the literature, Mre11-D16A (motif I, Furuse et al. 1998), Mre11-D56N and Mre11-H125N (motifs II and III, Moreau et al. 1999; Symington et al. 2000; D’Amours and Jackson 2001; Rattray et al. 2001; Tsukamoto et al. 2001; Lobachev et al. 2002), and Mre11-H213Y (motif IV, Tsukamoto and Ogawa 1998; Usui et al. 1998; Chamankhah and Xiao 1999; Lee et al. 2002), have been evaluated for proteins containing alterations within one or more conserved phosphoesterase motifs in the nuclease domain. Four mutant proteins described in the literature, Mre11-D16A (motif I, Furuse et al. 1998), Mre11-D56N and Mre11-H125N (motifs II and III, Moreau et al. 1999; Symington et al. 2000; D’Amours and Jackson 2001; Rattray et al. 2001; Tsukamoto et al. 2001; Lobachev et al. 2002), and Mre11-H213Y (motif IV, Tsukamoto and Ogawa 1998; Usui et al. 1998; Chamankhah and Xiao 1999; Lee et al. 2002), have been evaluated for both in vitro nuclease activities and multiple in vivo consequences. The D16, D56, and H213 residues are each associated with an Mn2+ ion in the crystal structure of Mre11, while the histidine at position 125 is thought to be involved in the phosphodiester hydrolysis reaction (Hoppner et al. 2001).

Characteristics of cells expressing each of the mutant proteins are summarized in Table 2. An additional less well-characterized mutant, mre11-H125L/D126V, was included in the table because of its similarity to the mre11-H125N allele, although nuclease activities of this protein have not been measured in vitro. One of the mutants listed in the table, mre11-H213Y, behaves essentially like a null mutation in most in vivo assays and is also defective in protein:protein interactions. Thus, this protein is deficient in nuclease activities and also in other functions of the enzyme.

Three of the mutant proteins depicted in Table 2 (D56N, H125N, and D16A) are particularly useful for
analysis of cellular requirements for the Mre11 nuclease activities. Each of these proteins has been reported to have no detectable nuclease activities in vitro, but the mutant proteins retain many Mre11 functions. For example, each of the proteins is proficient for DNA repair by NHEJ and the purified proteins are capable of RMX complex formation in vitro (Symington 2002; Figure 6; Table 2).

Several common DNA repair and chromosome stability defects are found in cells expressing the altered proteins. For example, all of the mutants are unable to complete meiotic DSB processing. In addition, each mutant is more sensitive than wild-type cells to ionizing radiation, MMS, and HU. mre11-D16A cells consistently demonstrated a stronger sensitivity to the clastogens than did the D56N and H125N mutants. In the two assays of recombinational repair of a defined DSB presented here, the D16A mutant behaved as a null while the D56N and H125N mutants displayed modest reductions. This result is qualitatively consistent with the relative radiation, MMS, and HU sensitivities. Another property of mre11-D16A strains is that telomeres are shortened in these mutants, unlike mre11-D56N or mre11-H125N cells (Furuse et al. 1998; Moreau et al. 1999). This property has previously been observed in RMX-deleted cells and in strains containing deletions of other NHEJ genes, including YKU70, YKU80, SIR2, SIR3, and SIR4 (Lewis and Resnick 2000). If this defect in telomere maintenance is due to a greater reduction in nuclease activity in the Mre11-D16A protein (discussed below), it would be supportive of models that postulate a role for RMX in processing of chromosome ends to generate single-stranded DNA overhangs (Diede and Gottschling 2001).

Of central importance is the question of why the D16A mutant has more severe defects in mitotic cells than the other phosphoesterase mutants do. The RMX complex has ssDNA endonuclease and 3’-to-5’ dsDNA exo-nuclease activities, as well as a weak DNA helicase activity. In addition, the Mre11 subunit forms specific associations with DNA, Rad50, Xrs2, and possibly other proteins (Sae2?) and may also be subject to post-translational modification in mitotic cells (D’Amours and Jackson 2002; Symington 2002). Although each of these activities and associations may vary in the three nuclease mutants, we favor the simplest explanation; i.e., the D16A protein is more defective than the other mutants in nuclease processing of DSBs in vivo.

Support for this proposal comes from several considerations. First, many phenotypic differences between the mutants are simply a matter of degree. For example, radiation, MMS, and HU sensitivities and plasmid/chromosome recombination are reduced in all of the mutants and mre11-D16A cells are simply more defective than the others.

Second, studies utilizing either overexpression or inactivation of EXO1 in RMX mutants also provide support. Overexpression of the 5’-to-3’ exo activity of Exo1 partially rescues repair of DSBs induced by radiation, MMS, EcoRI, and HO in RMX mutants, as well as the mitotic recombination defects of the mutants (Lee et al. 2002; Lewis et al. 2002; Symington 2002 and references within). This effect is likely due to enhanced processing of the broken DNA ends by Exo1 to create 3’ tailed substrates for the Rad51/Rad52 strand exchange complex. We note, however, that EXO1 overexpression does not rescue meiotic DSB repair (involving removal of DNA ends containing attached protein by Mre11 endonuclease activity), inverted repeat-stimulated recombination (thought to involve endonuclease cleavage of hairpin loops), or shortening of telomeres (which might also involve endo cleavage of T-loop structures; Diede and Gottschling 2001; Symington 2002; K. Lobachev and M. Resnick, unpublished results). These latter results clearly point to the importance of the endonuclease activity of Mre11 in vivo. The endo activity may also be important in resection of damage-induced DSB ends in mitotic cells, possibly in conjunction with the

**Figure 6.**—Analysis of binding of wild-type Mre11 and mutant Mre11-D16A protein to Rad50 and Xrs2. (A) Rad50 alone, Rad50 with nontagged Mre11, Rad50 with 6His-Mre11, or Rad50 with 6His-Mre11-D16A (6His-D16A) were incubated with nickel-NTA-agarose beads and washed twice with buffer before the bound proteins were eluted with 200 mM imidazole. The supernatant (S), first wash (W1), second wash (W2), and eluate (E) fractions were run in a 7.5% denaturing polyacrylamide gel followed by staining with Coomassie blue. (B) Xrs2 alone, Xrs2 with Mre11, Xrs2 with 6His-Mre11, and Xrs2 with 6His-Mre11-D16A (6His-D16A) were incubated with nickel-NTA-agarose and bound proteins were eluted as in A. Note that binding of Rad50 and Xrs2 to the nickel-NTA matrix is dependent on the histidine tag on the Mre11 protein (compare lanes 4 and 12 in both A and B).
### TABLE 2

Impact of Mre11 proteins with reduced in vitro nuclease activities on DNA repair and stability

<table>
<thead>
<tr>
<th>Allele</th>
<th>In vitro nuclease activities</th>
<th>Associations</th>
<th>Plasmid diploid recombination</th>
<th>Survival</th>
<th>DSB repair by recombination</th>
<th>Telomere stability</th>
<th>Meiotic recombination</th>
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<tr>
<td></td>
<td>Endo</td>
<td>Exo</td>
<td>DNA</td>
<td>Mre11</td>
<td>R/X</td>
<td>NHEJ</td>
<td>R/X</td>
</tr>
<tr>
<td><strong>MRE11</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>mre11Δ</strong></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>mre11-D16A</strong></td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>mre11-D56N</strong></td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>mre11-H125N</strong></td>
<td>---</td>
<td>---</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>mre11-H125L/D126V</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td><strong>mre11-H213Y</strong> (mre11-58)</td>
<td>---</td>
<td>---</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>ND</td>
</tr>
</tbody>
</table>

References are as follows: Mre11-D56N and Mre11-H125N (Moreau et al. 1999; Symington 2002; this work), Mre11-H125L/D126V (Bressan et al. 1998; Lee et al. 2002), Mre11-D16A (Furuse et al. 1998; this work), and Mre11-H213Y (Mre11-58; Tsubouchi and Ogawa 1998; Usui et al. 1998; Chamankhah and Xiao 1999; Lee et al. 2002). NA, not applicable; ND, no data; +, wild-type efficiency; +++, higher than wild type; −, slight deficiency; −−, moderate deficiency; −−−, strong deficiency.

* Binding of mutant Mre11 protein to Rad50/Xrs2.

* Mutants exhibit more killing than wild-type cells only at high doses of radiation (>30 krad) or high concentrations of MMS (this work; Moreau et al. 1999); survival of mre11-H125L/D126V mutants was also near wild type over the dose range 0–30 krad (Bressan et al. 1998).

* Different levels of MMS sensitivity were reported (Tsubouchi and Ogawa 1998; Usui et al. 1998; Lee et al. 2002).
weak helicase activity of the complex (Trujillo and Sung 2001; Symington 2002). The major point here is that increased levels of a nuclease (Exo1) rescues clastogen sensitivities and recombination defects of RMX mutants during mitotic growth, suggesting that nuclease processing is the function that is missing.

Analyses of the ionizing radiation sensitivities of mre11-D16A and mre11-H125 mutants with and without a functional EXO1 gene present also lend support to this premise. mre11-D16A strains were more sensitive than the other nuclease mutants and mre11-D16A exo1 double mutants exhibited a linear, dose-dependent reduction in survival that was greater than that of mre11-D16A single mutants (~10-fold difference at 20 krads). This indicates that much of the resistance in the D16A single mutants was due to basal levels of Exo1. The strong sensitivity of these cells and its dependence on Exo1 seem most consistent with the idea that very little or no nuclease activity is retained in the Mre11-D16A complex in vivo, although other factors may also be involved.

In contrast to results with D16A, radiation survival was high in mre11-H125N mutants and was not reduced further in mre11-H125N exo1 double mutants at doses up to 50 krads (Moreau et al. 2001; Figure 2), which corresponds to ~40 DSBs per G2 cell (Resnick and Martin 1976). If the Mre11-H125N protein is nuclease deficient, then this result would indicate that cells lacking both RMX and Exo1 nuclease activities are largely proficient at processing of radiation-induced DSBs for recombinational repair. Put another way, this would mean that the major enzymatic activities defined for Mre11 (and Exo1) in vitro are not essential for a major function of the complex in vivo (repair of chemically and physically induced DSBs). It seems more likely that survival is high in mre11-H125N cells lacking the "backup" Exo1 nuclease activity because the mutant RMX complex has residual nuclease activity in vivo.

Another question that must be addressed is the following: If the nuclease activity of mre11-D16A mutants is absent (or greatly reduced), why is radiation resistance not reduced to the level of mre11 null strains? We suggest that an important difference here is the presence or absence of the RMX complex bound to DSB ends. Structural studies have indicated that two Mre11 molecules bind to the proximal ends of two folded, fibrous Rad50 subunits to form the DNA-binding portion of the complex (Anderson et al. 2001; Chen et al. 2001; De Jager et al. 2001; Hoffner et al. 2002). The structures imply that RMX might potentially form a bridge between two DNA ends in a broken molecule or between adjacent sister chromatids in a replicated chromosome. The latter structure would be consistent with the observation that Rad50 is structurally similar to SMC proteins required for sister chromatid cohesion (Hoffner et al. 2000). It is possible that this "tethering" function of RMX is retained in the mutant Rad50/Mre11-D16A/Xrs2 complex, although nuclease activities are reduced. We infer that this tethering, combined with redundant nuclease activities, provides an explanation for the observation that the radiation sensitivity of mre11-D16A cells did not reach that of mre11Δ cells. After exposure to ionizing radiation, the tethering function would keep sister chromatids (or possibly broken DNA ends) in proximity and enhance the likelihood that a break is processed by Exo1 or another partially redundant nuclease and repaired by the dominant pathway of radiation repair in yeast, homologous recombination.

mre11-D16A mutants were not as radiation sensitive as mre11Δ cells, but they were as defective as null cells in the ends-in and ends-out recombination assays. It is possible that the impact of RMX DNA bridging is less in the plasmid/chromosome and oligonucleotide:chromosome DSB repair assays than in the radiation survival assays, since the latter are almost completely dependent on sister chromatid exchanges. DNA tethering by mutant RM*X complexes may also explain why spontaneous recombination rates of diploid cells are not elevated in the three mutants with reduced nuclease activities (Table 2). Unlike other RAD52 group mutants, diploid strains lacking RMX display increased spontaneous recombination between homologous chromosomes, possibly because of a reduced preference for interactions between sister chromatids (Symington 2002). The absence of high spontaneous recombination rates in the three nuclease mutants may be an indication that RMX complexes containing Mre11-D16A, Mre11-D56N, and Mre11-H125N are still capable of forming bridges between sister chromatids, and therefore the strong preference for sister-sister recombination has been retained.

mre11-D56N and mre11-H125N mutants have only slight reductions in mitotic DSB repair, but they show strong defects in assays of inverted repeat-stimulated recombination in mitotic cells and DSB processing in meiotic cells (this work; Rattray et al. 2001; Lobachev et al. 2002; Symington 2002). The latter two processes are likely to involve endonucleolytic cleavage of transiently formed hairpin structures and protein-bound DNA ends, respectively, and they cannot be rescued by overexpression of EXO1. It is possible that the mutant D56N and H125N complexes have a reduced level of endonuclease activity in vivo and that the type of end-processing required for these structures cannot be supplied by backup enzymes such as Exo1. If this is true, then the reduced levels of RMX endonuclease activity in the mutants might be limiting for these repair events, but not for others that can also be performed by redundant nucleases. Other possibilities, such as impacts on helicase activity or postendonucleolytic processing by the exonuclease cannot be ruled out, however.

Finally, we note that D16 of S. cerevisiae Mre11 is completely conserved among many related yeasts (H125 also), but D56 is changed to a valine in the yeast S. kluuyeri (Saccharomyces Genome Database; http://www.
yeastgenome.org/). The reduced evolutionary conservation of this aspartic acid, one of several residues found in association with Mn\(^{2+}\) ions in the \textit{P. furiosus} Mre11 crystal structure, suggests that its contributions to the phosphodiesterase reaction may be less critical than those of other residues such as D16.

In summary, cells expressing Mre11-D16A exhibit several dramatic mitotic DNA repair defects that are more severe than those seen in two widely studied phosphoesterase mutants with reduced \textit{in vitro} nuclease activities. The mutant protein exhibits normal RMX complex formation and DNA binding \textit{in vitro} and \textit{mre11-D16A} cells are proficient at NHEJ repair \textit{in vivo}. We suggest that the strong radiation sensitivity and recombination defects are due primarily to lack of nuclease processing by the mutant Rad50/Mre11-D16A/Xrs2 complex. This conclusion is contrary to those of previous mutant studies proposing a limited role for the Mre11 nuclease activity in mitotic cells (e.g., Bressan et al. 1999; Moreau et al. 1999; Syminton et al. 2000; Tsukamoto et al. 2001; Lobachev et al. 2002) and suggests the possibility that some mutants such as \textit{mre11-D56N} and -H125N may have residual nuclease activity \textit{in vivo}. We note that more subtle distinctions are also possible. For example, the endo- and exonuclease activities, whose precise roles in DNA processing \textit{in vivo} remain unclear, may be differentially affected in the mutants. It is likely that the residual radiation resistance in haploid \textit{mre11-D16A} mutants and the absence of high spontaneous recombination in \textit{mre11-D16A} diploids arise, at least in part, because the mutant complex retains the ability to tether sister chromatids and/or DSB ends. It is intriguing that telomeres are shortened in \textit{mre11-D16A} cells. This result might also be due to a greater loss of nuclease activities in this mutant and supports the idea that chromosome ends (possibly forming T-loop structures) may require processing by RMX to create substrates for DNA replication by telomerase (Diede and Gottschling 2001).

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**LITERATURE CITED**


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