Characterization of the T4 Mre11/Rad50 complex and investigation of the Mre11 dimeric interface

Dustin William Albrecht

Iowa State University

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Characterization of the T4 Mre11/Rad50 complex and investigation of the Mre11
dimeric interface

by

Dustin William Albrecht

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fulfillment of the requirements for the degree of

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Scott Nelson, Major Professor
Michael Shogren-Knaak
Drena Dobbs

Iowa State University

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Chapter I: Introduction

General Introduction

Double-strand breaks (DSB) in DNA are one of the most harmful forms of DNA damage and may lead to genomic instability or cell death if they are not repaired efficiently (1). DSBs are formed through both internal and external causes. External causes include UV radiation, ionizing radiation (IR), and genotoxic chemicals including chemotherapeutic drugs. Internal causes of DSBs include reactive oxygen species, protein complexes that inhibit replication fork progression, and the deliberate formation during VDJ recombination in antibody development (2).

In eukaryotes there are three pathways for the repair of DSBs which have been described previously: homologous recombination (HR), non-homologous end-joining (NHEJ), and microhomology-mediated end-joining (MMEJ) (3). Mre11 and Rad50 (MR complex hereafter) are involved in all three of these pathways (3, 4). The pathway that is used is dependent on the nature of the broken ends and the phase of the cell cycle. DSBs that have a 3’ hydroxyl and a 5’ phosphate group are termed “clean” breaks and require no further processing before ligation. This kind of break is the only type that is suitable for repair via NHEJ. The Ku70/Ku80 complex that initiates NHEJ does not have a high affinity for breaks with more extensive damage or that have proteins bound. These types of breaks require processing (i.e. resection) and are thus candidates for HR or MMEJ (5). Repair through HR is prevalent during the S and G2 phases of the cell cycle due to the presence of replicated DNA and the availability of sister chromatids (5). NHEJ is active throughout the entire cell cycle but is the preferred mechanism during G1. MMEJ is also active throughout the whole cell cycle. A summary of this regulation is shown in Figure 1 (5).
The MR complex is heterotetrameric ($M_2/R_2$) and its role is best understood in the context of HR. Homologous recombination is a multi-step process that requires a number of proteins and is thought to be largely error-free (6). It is best understood in the *Saccharomyces cerevisiae* model system and the role of the MR complex has recently been explained in the literature (7-9). The MR complex works along with Xrs2 and Sae2 to perform the initial short range (50-100 nucleotides) resection of the 5’ end to create a 3’ overhang (7). After this initial processing, a more extensive resection (>500 nucleotides) is performed by Sgs1/Top3/Rmi1/Dna2 and RPA or Exo1 and RPA. Once resection is completed, Rad51 binds to the 3’ ssDNA tail, performs a search for homologous sequences, and promotes strand invasion into the homologous DNA. This creates a D-loop and formation of the D-loop allows the non-invading strand of the DSB to pair with the other strand of the homologous DNA. Both 3’ ends from the DSB are then used as primers for DNA synthesis, ultimately forming a pair of Holliday junctions (10, 11). After completion of DNA synthesis, the elongated strands are released by a DNA helicase or the Holliday junctions are resolved by a DNA nuclease and a ligase.

Homologous recombination in prokaryotes is performed following steps similar to those in eukaryotes (i.e. damage recognition, end processing, homology search/annealing, and DNA synthesis/strand dissocation) (12). Although there is are Mre11 and Rad50 homologs in prokaryotes they do not seem to be involved in DNA end resection. Prokaryotic resection is instead carried out by the RecBCD helicase/nuclease complex (13).

The role of the MR complex in NHEJ is less understood than in HR. NHEJ is a fairly simple process in which a heterodimer of Ku70 and Ku80 binds to the DNA ends with a high affinity and effectively blocks resection (14). The Ku heterodimer is also required for
recruitment of Dnl4 and the ligase-accessory protein Nej1 (15). The role of the MR complex in NHEJ is not completely defined although it has been implicated in bridging the ends of DNA and also the dissociation of the Ku heterodimer and Nej1 (15, 16).

The MR complex is also involved in MMEJ. MMEJ is an error-prone mechanism for repairing DSBs and utilizes small (5-25 base pair) sequences of microhomology (3). MMEJ begins in *S. cerevisiae* in much the same way as HR does, with the MR complex and Xrs2 performing 5’ resection at the site of the DSB. Once resected, Srs2 displaces Rad51 coating the ssDNA and regions of microhomology are annealed together (17). Before gap fill-in and ligation can occur, the 3’ flaps that are outside of the microhomologous region must be trimmed by the Rad1-Rad10 endonuclease, resulting in a deletion compared to the original sequence (17). The resulting gaps are then filled in via DNA synthesis with Pol4 and Polγ both implicated in this process. Finally, the ends are ligated together by either Cdc9 or Dnl4.

Another pathway which involves multiple rounds of annealing of microhomologies, DNA synthesis, and dissociation of DNA strands has been proposed and is demonstrated in Figure 2 (3).

The MR complex is present in eukaryotes, prokaryotes, archaea, and bacteriophage biological systems and performs DSB resection in all of these systems except prokaryotes. As previously discussed, the MR complex has an important role in the repair of DSBs. Therefore it is not surprising that diseases can be attributed to a dysfunctional MR complex phenotype. Mre11 deficiency has been linked to ataxia telangiectasia-like disorder (ATLD), IR sensitivity, and cerebellar dysfunction (18). Likewise, Rad50 has been linked to cancers and Nigmen breakage syndrome-like disorder (19, 20).
Rad50 is a member of structural maintenance of chromosomes (SMC) protein family. SMC proteins are a subset of the ATP Binding Cassette (ABC) protein superfamily which is highly conserved and abundant in all organisms (21, 22). ABC proteins form dimers and bind/hydrolyze ATP at the dimeric interface. There are six conserved motifs in the ABC protein family; the Walker A motif, Walker B motif, Q-loop, H-loop, D-loop, and Signature motif. The dimer is in a head-to-tail formation with the Walker A, Walker B, Q-loop, and H-loop motifs from one monomer and the D-loop and Signature motifs from the other monomer forming the ATPase active site (23). The structure of the ATPase active site of *Pyrococcus furiosus* (*Pfu*) has been solved in apo-, ATP-bound, and AMP-PNP-bound forms (24). The hydrolysis of ATP in ABC proteins is often coupled to an associated protein with Mre11 being the protein coupled to Rad50 (21, 25). In addition to the six motifs seen in ABC proteins, Rad50 has a long coiled-coil region that contains a conserved Cys-X-X-Cys motif. This motif dimerizes with another Rad50 monomer to create a zinc binding site (26). The function of the coiled-coil motif is not well defined but may play a role in tethering the DNA ends separated by the DSB (4).

Mre11 belongs to the Ser/Thr phosphatase protein family (27). The crystal structure of *Pfu* Mre11 has been solved and is shown to be able to bind dsDNA at its dimeric interface (28, 29). The nuclease activity of the MR complex is performed by Mre11. Mre11 exhibits a Mn\(^{2+}\)-dependent 3’ to 5’ exonuclease activity *in vitro* which differs from its apparent 5’ to 3’ exonuclease activity *in vivo* (30).

The T4 bacteriophage relies heavily on recombination for DNA replication due to the lack of transcription initiation after DNA replication has begun (31). A majority of the proteins involved in T4 phage HR have been characterized and T4 phage has been used as a
model system for studying nucleic acid proteins for more than two decades (32). The T4 phage homologs of Mre11 and Rad50 (gp47 and gp46, respectively) are required for in vivo HR and DSB repair and have not yet been extensively characterized. Our lab has been able to over-express and purify large quantities of T4 phage Mre11 and Rad50 while purification of the eukaryotic homologs has proven to be more difficult (30, 33). As discussed earlier, the structures of Pfu Mre11 and Rad50 proteins have been solved individually and, recently, structures of the MR complex have been solved for Pfu, Thermatoga maritima, and Methanococcus jannaschii (34-37). Although these studies have provided valuable structural information regarding Mre11-Rad50 interaction, nucleotide binding/hydrolysis, and DNA binding, the biochemical activity of the MR complex has not been sufficiently characterized. The lack of a complete understanding of the T4 phage Mre11 and Rad50, their homology to the eukaryotic proteins, and their high expression/simple purification make them an ideal system to study. The desire to gain a deeper understanding of the biochemical characteristics of the Mre11-Rad50 complex serves as the basis for this thesis project with an added focus on the significance of the Mre11 dimeric interface.

**Thesis Outline**

For my thesis project I examined the T4 Mre11 protein which is involved in the processing of DSBs. I performed initial characterization assays of the wild-type protein and also utilized site-directed mutagenesis to generate the L101D-Mre11 mutant. This mutant was designed to alter the dimeric interface of Mre11. The properties of this mutant have proved to be quite interesting and are considerable different than those of wild-type.

This thesis is setup to show the progress of my research project and contains a manuscript published in the Journal of Biological Chemistry and a second manuscript
submitted for publication to the same journal. Chapter II contains the first manuscript which discusses the initial biochemical characterization of T4 Mre11 and T4 Rad50. Chapter III contains the second manuscript which investigates the significance of the T4 Mre11 dimer interface and how altering the interface affects Mre11-Rad50 complex activity. Chapter IV will be a general conclusion discussing the accomplishments of my research project, future directions, and acknowledgements.

References


Figure 1. **Regulation of repair pathway choice.** The three modes of DSB repair are outlined. Chemically modified or damaged ends (stars) cannot be repaired by nonhomologous end joining (NHEJ) and require processing, whereas free ends can be processed by any pathway. Once initiated, NHEJ inhibits end processing via Ku and prevents microhomology-mediated end joining (MMEJ) or homologous recombination (HR). Conversely, MRX/N-Sae2/CtIP-dependent resection displaces Ku and inhibits NHEJ. Extensive resection results in Rad51 filament formation and inhibition of MMEJ. NHEJ is used primarily in the G1 phase of the cell cycle, and HR is used primarily in the S and G2 phases, following replication of the genome. MMEJ is active throughout the cell cycle. Negative interactions are shown by red lines, positive interactions by black arrows, and cell cycle transitions by grey arrows. (5).
Figure 2. **Model for MMEJ and alternative end-joining repair.** During the initial stages of MMEJ, Ku70–Ku80 (green) and Rad51 (red), which inhibit MMEJ, are prevented from binding or are removed. This enables 50–30 resection by the MRX complex, Sae2 and Exo1 (indicated by dark red partial circle) that reveals microhomologous sequences (blue boxes). These microhomologies transiently and dynamically anneal to each other. (i) In cases in which the annealing is stable, repair is completed by flap trimming, fill-in DNA synthesis and ligation, resulting in a deletion relative to the original sequence. Mismatch repair is not required for MMEJ, although it might have a supporting role. (ii) Alternatively, one or more translesion polymerases (yellow) can extend the annealed sequences (represented here by orange–blue boxes) using templated error-prone synthesis. Dissociation of the initial microhomologies and realignment at other microhomologous sequences, followed by flap trimming, fill-in DNA synthesis and ligation completes repair, resulting in a deletion plus insertion event. Many variations and iterations of (ii) can hypothetically occur, resulting in complex insertion–deletion junctions. (3).
Chapter II: Biochemical Characterization of Bacteriophage T4 Mre11-Rad50 Complex

Modified from a paper published in The Journal of Biological Chemistry

Timothy J. Herdendorf², Dustin W. Albrecht², Stephen J. Benkovic³⁴, and Scott W. Nelson²⁴

Abstract

The Mre11/Rad50 complex (MR) from bacteriophage T4 (gp46/47) is involved in the processing of DNA double-strand breaks. Here, we describe the activities of the T4 MR complex and its modulation by proteins involved in homologous recombination. T4 Mre11 is a Rad50- and Mn²⁺-dependent dsDNA exonuclease and ssDNA endonuclease. ATP hydrolysis is required for the removal of multiple nucleotides via dsDNA exonuclease activity but not for the removal of the first nucleotide or for ssDNA endonuclease activity, indicating ATP hydrolysis is only required for repetitive nucleotide removal. By itself, Rad50 is a relatively inefficient ATPase, but the presence of Mre11 and dsDNA increases ATP hydrolysis by 20-fold. The ATP hydrolysis reaction exhibits positive cooperativity with Hill coefficients ranging from 1.4 for Rad50 alone to 2.4 for the Rad50/Mre11/DNA complex. Kinetic assays suggest that approximately four nucleotides are removed per ATP hydrolyzed. Directionality assays indicate that the prevailing activity is a 3’to 5’ dsDNA exonuclease, which is incompatible with the proposed role of MR in the production of 3’

² Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011
³ Department of Chemistry, 414 Wartik Laboratory, The Pennsylvania State University, University Park, Pennsylvania, 16802
⁴ Author of correspondence
ssDNA ends. Interestingly, we found that in the presence of a recombination mediator protein (UvsY) and ssDNA binding protein (gp32), Mre11 is capable of using Mg\(^{2+}\) as a cofactor for its nuclease activity. Additionally, the Mg\(^{2+}\)-dependent nuclease activity, activated by UvsY and gp32, results in the formation of endonuclease reaction products. These results suggest that gp32 and UvsY may alter divalent cation preference and facilitate the formation of a 3’ ssDNA overhang, which is a necessary intermediate for recombination-mediated double-strand break repair.

**Introduction**

Replication in T4\(^1\) phage is initiated through origin-dependent and -independent pathways (1). The origin-dependent pathway relies on the host cell RNA polymerase to synthesize an mRNA transcript, which is then processed and remains stably bound at the origin in what is known as an R-loop (2). The origin-independent pathway relies on homologous recombination (HR) within or between phage genome(s) (3). HR is essential to the lifecycle of T4 phage because the origin-dependent mode of initiation ends after only a few rounds of replication (4). A strong link between recombination and DNA repair was recognized early on using T4 phage as a model system (reviewed in (5)). In the late 1960’s it was reported that certain genes, when mutated, severely affected replication, recombination, and DNA repair (6). Indeed, the current models of DNA double-strand break (DSB) repair share many of the same steps as recombination-mediated initiation of replication (7, 8).

DSBs are one of the most lethal forms of DNA damage and unless repaired they may lead to gross chromosomal rearrangements, deletions, duplications, or cell death (9, 10).
DSBs are caused by exogenous agents such as ionizing and ultraviolet radiation and chemical mutagens or endogenous agents such as reactive oxygen species. In eukaryotic cells, DSBs are repaired through two pathways, non-homologous end joining (NHEJ) and HR; however, in T4 phage it appears that all DSBs are repaired through HR (4, 11).

The biochemical activities necessary for HR have been identified and in most cases, the proteins responsible for these activities are known (8, 12, 13). HR begins with the processing of a dsDNA end to produce a 3’ ssDNA overhang. In E. coli, this step is performed by the well-characterized helicase/nuclease complex, RecBCD (14). However, the genomes of eukaryotes, archaea, and T4 phage do not contain any obvious RecBCD orthologs and it appears that the Mre11/Rad50 (MR) complex, together with other proteins specific to each system perform the analogous function (15). Following end resection, the recombinase (RecA or RecA ortholog) binds to the ssDNA and forms a nucleoprotein filament that is capable of undergoing strand invasion and exchange with homologous DNA templates (16). This strand invasion event produces a D-loop structure that acts as a scaffold and primer for DNA synthesis (17). The HR pathway ends with either the resolution of the Holliday junction by a nuclease or the dissociation and strand annealing of the newly synthesized DNA strands (18).

In T4 phage, all of the proteins implicated in HR are well characterized except for Mre11 and Rad50 (gp47 and gp46, respectively) (19-24). In eukaryotic systems, the MR complex has long been associated with the repair of DSBs (25). In S. cerevisiae, null mutants of Mre11, Rad50, or Xrs2 (a third protein found in the S. cerevisiae MR complex) are unable to initiate and process meiotic DSBs and are sensitive to DNA damaging agents.
that produce DSBs such as ionizing radiation (IR) or methylmethane sulfonate (18). This led to a proposal that the MR complex is responsible for resection (5’ to 3’ dsDNA exonuclease activity) of DSBs (26). However, in vitro, the eukaryotic MR complex exhibits a 3’ to 5’ dsDNA exonuclease and a ssDNA endonuclease activity (27, 28). The products of these activities are not 3’ ssDNA overhangs and therefore the true function of the MR complex has been elusive.

Rad50 is a member of the Structural Maintenance of Chromosome (SMC) family of proteins, which are in turn members of the ATP Binding Cassette (ABC) super-family. All known ABC protein family members are dimeric and bind ATP at the interface between two monomers. It is thought that the energy of ATP hydrolysis is used to drive conformational changes within the ABC protein/domain, which are then propagated to other associated proteins or domains (29). Most ABC protein family members are membrane transporters, such as the CFTR transporter and p-glycoprotein, which cause cystic fibrosis and multi-drug resistance, respectively (30). In addition to transporters, the DNA repair enzymes MutS and RecF are ABC proteins. The structure of the nucleotide binding domain of Pyrococcus furiosus (Pfu) Rad50 has been solved in the apo and ATP-bound forms (31). The structures reveal significant conformational rearrangements that occur upon ATP binding and/or hydrolysis, which are thought to control the activity of Mre11 (32). Rad50 and related SMC proteins have an unusual architecture where the ABC protein motifs are split by a long coiled-coil region. In Rad50, the apex of the coiled-coil contains a conserved CXXC motif, which dimerizes with a second Rad50 monomer to create a zinc binding site similar to a zinc finger (33). The purpose of the coiled-coil motif is not entirely clear, but it is thought to be
involved in tethering the DSB to a homologous template or tethering the two ends of the DSB to each other (15). In eukaryotes the coiled-coil domain contains approximately 900 amino acids, whereas in T4 phage the coiled-coil contains around 200 residues. Presumably, the long coiled-coil region in eukaryotes is responsible for the expression difficulties encountered with the eukaryotic proteins. To date, only microgram quantities of eukaryotic Rad50 have been purified (27, 28, 34, 35).

Mre11 is a member of the ser/thr protein phosphatase family, which contains four conserved motifs and requires divalent cations for activity (36). Although the mutation of Mre11 has only a mild effect in S. cerevisiae (37), in human cells the nuclease activity of Mre11 is essential and its inactivation results in embryonic lethality and extreme sensitivity to IR (38). In humans, inherited Mre11 mutations cause ataxia telangiectasia-like disorder, the symptoms of which include hypersensitivity to IR and cerebellar degeneration (39). The Pfu Mre11 protein is dimeric and binds dsDNA near the dimeric interface (40). As mentioned above, in vitro assays reveal that Mre11 possesses a 3' to 5 dsDNA exonuclease activity, along with a ssDNA endonuclease activity (27).

Recently, several papers have clarified the role of the MR complex in the processing of DSBs. The Symington and Ira labs simultaneously demonstrated that DSBs are processed through a two-step mechanism involving the MR complex, Sae2, ExoI, Sgs1, and Dna2 (41, 42). In the initial step, the MRX complex, in conjunction with Sae2, remove approximately 100-200 bases from the 5' end of the DNA. In the second step, ExoI or Sgs1 with Dna2 catalyze rapid and processive removal of thousands of bases from the 5' end of the DNA.
The resulting 3’ ssDNA overhang can undergo Rad51-dependent HR or Rad52-dependent single-strand annealing.

In this report, we express, purify, and biochemically characterize the MR DNA repair complex from T4 phage. We find that the T4 MR complex shares all the same activities as its eukaryotic counterparts, but expression and purification of the Rad50 and Mre11 proteins is rapid and yields milligram quantities of purified proteins, which allows for the mechanistic examination of the complex. Our experiments indicate that the T4 MR complex is a DNA-activated ATPase, a Mn^{2+}-dependent ssDNA endonuclease and a Mn^{2+} and ATP-dependent 3’ to 5’ dsDNA exonuclease. ATP hydrolysis is positively cooperative with respect to ATP concentration and during the exonuclease reaction four nucleotides are removed per ATP hydrolyzed. While the observed 3’ to 5’ dsDNA exonuclease activity is incompatible with its proposed in vivo function, we find that the presence of two T4 recombination proteins, UvsY and gp32, alter the nuclease activity of the MR complex, which may promote the formation of a 3’ ssDNA recombinogenic end.

**Experimental Procedures**

*Cloning, Expression, and Purification of T4 Mre11 and wt-Rad50 and K42M-Rad50* - The open reading frames for gp46 and 47 were PCR amplified from bacteriophage T4 genomic DNA (Sigma-Aldrich) and cloned into the pTYB1 expression vector (NEB) using the NdeI and SapI restriction sites using the following primers: T4Rad50-NdeF: 5’-GGTGGTCATATGAAGATTTAATTAACTTAATAG; T4Rad50-SapR: 5’-GGTGGTTGCTTCCGCAATTAAACCATTACAGTAATCG; T4Mre11-NdeF: 5’-GGTGGTCATATGAAAATTTTAGGGTG; and T4Mre11-SapR 5’-
GGTGTTGTGCTTCCGATCATATTGTTGCCTCTACTACATATAG. The K42M-Rad50 mutant was generated using the QuickChange™ protocol (Stratagene) using the following mutagenic forward primer: K42M-Rad50F: 5’-

GGACGAAATGCGTGGTAGCTACTATGCTAGGAGCC-3’, where the mutant codon is highlighted in bold-face. The reverse mutagenic primer is the reverse-complement of the forward. The pTYB1 vector creates a fusion between the gene of interest and a self-cleavable Intein/Chitin binding domain purification tag. The gp46-pTYB1, K42M-gp46-pTYB1, and gp47-pTYB1 expression vectors were separately transformed into BL21(DE3) cells and plated on LB-agar plates containing 150 µg/ml ampicillin. A single colony from each plate was used to separately inoculate 50 ml flasks of LB-amp that was shaken for 16 hours at 37°C. 10 ml of starter culture was used to inoculate four, 1 liter flasks of LB-AMP per protein, which were shaken at 225 rpm at 37°C to an OD$_{600}$ of 0.8. The flasks were then cooled to 18°C and expression was induced by the addition of 0.2 mM IPTG. After 16 hours the cells were collected by centrifugation at 8000 g for 8 minutes and pellets were frozen at -20°C.

The purification scheme for T4 Rad50 (including K42M-Rad50) and Mre11 are identical through the chitin column elution step. A frozen cell pellet (approximately 15 g) was resuspended in 75 ml of ice-cold lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.0) and lysed by sonication. All purification steps were carried out at 4°C. The lysed cells were then centrifuged at 18000g for 60 minutes. The cell-free extract was loaded onto a column packed with 5 ml of chitin beads (New England Biolabs). The column was then washed with 100 ml of lysis buffer followed by an overnight wash with 1.5 L of wash
buffer (20 mM Tris-HCl, 1M NaCl, 1 mM EDTA, pH 8.0). Cleavage of the Intein-CBD tag was initiated by washing the column with 3 column volumes of cleavage buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 75 mM βME, pH 8.0) prior to stopping the buffer flow. The chitin column was incubated in cleavage buffer for 16 hours at 4°C, followed by elution of the tag-less protein. Mre11 was dialyzed twice against 1 L of storage buffer (20 mM Tris-HCl, 200 mM NaCl, 20% glycerol, pH 8.0), concentrated to 50-100 µM, dispensed into small aliquots, and frozen at -80°C. Rad50 was loaded directly onto a 20 ml cellulose phosphate (P11, Whatman) column, followed by a 10-column volume wash with P11 wash buffer (20 mM Tris-HCl, 200 mM NaCl, pH 8.0). T4 Rad50 was eluted from the P11 column with P11 elution buffer (20 mM Tris-HCl, 400 mM NaCl, 20% glycerol, pH 8.0), concentrated to 25-50 µM, dispensed into aliquots, and frozen at -80°C. The extinction coefficients for determining the concentration of T4 Mre11 and Rad50 are 69,130 cm⁻¹M⁻¹ and 33,140 cm⁻¹M⁻¹, respectively.

DNA Substrates - The M13 phage ssDNA was purified using PEG precipitation and phenol/chloroform extraction (43). The 50 bp dsDNA substrate was prepared by annealing equal amounts of two complementary oligonucleotides with the following sequences: ds50mer_top, 5’- CTC TTG GTG ATT ATG ATG GTT GCA ATA CAT TTA ATT TCA TTA TCA ATT AG-3’ and ds50mer_bottom, 5’- CTA ATT GAT AAT GAA ATT AAA TGT ATT GCA ACC ATC ATA ATC ACC AAG AG-3’. The 17 position 2-aminopurine (2-AP) containing substrate was of identical sequence to ds50mer_top except that the adenine at positions 17 (relative to the 3’ end of the DNA) was replaced with 2-AP. The DNA substrate with the 2-AP at the 1 position (3’ end) had an identical sequence to ds50mer_top.
except that the 3’ G was replaced with 2-AP. To prepare the 5’-labeled 50 bp DNA, the annealed duplex was diluted to a concentration of 5 μM and treated with polynucleotide kinase (NEB) and 10 μCi of [γ-32P] ATP in a volume of 20 μL (total ATP concentration of 0.165 μM) for 30 minutes. After 30 minutes cold ATP was added to a final concentration of 100 μM and allowed to react for 30 additional minutes. The reaction was then quenched at 65°C for 20 minutes, followed by removal of free nucleotides by passing the reaction through a G-25 Sephadex spin column. To prepare the 3’-labeled 50 bp DNA, the annealed duplex was diluted to a concentration of 10 μM and treated with 5 units of T4 DNA polymerase (Promega) in the presence of 1 mM dCTP, dTTP, and dATP in a final volume of 20 μL for 5 minutes. The reaction was then heated at 65°C for 20 minutes to inactivate the T4 polymerase. Following heat inactivation, 5 units of T4 exonuclease-deficient polymerase was added to the reaction, along with 20 μCi of [α-32P] dGTP, in a final volume of 38 μL. The reaction was allowed to proceed at 37°C for 30 minutes prior to the addition of 2 μL of 0.1 mM dGTP. The reaction was continued for 30 additional minutes, then heat inactivated for 20 minutes at 65°C. Finally, the reaction was passed through a G-25 Sephadex spin column to remove free nucleotides and precipitated protein. The uniformly labeled 1.68 kb dsDNA was prepared by performing a 100 μL PCR reaction in Taq polymerase buffer (Promega) using 5 units of Taq polymerase, 40 μCi of [α-32P] dGTP, 0.1 mM cold dNTP mix, 1 μM gp46-Nde-F, 1 μM gp46-Sap-R, and 100 ng of the gp46-pTYB1 vector. The labeled PCR product was purified using Qiagen’s PCR purification kit following the manufacturer’s protocol and quantitated using liquid scintillation counting.
Nuclease and ATP Hydrolysis Assays - The standard conditions for the nuclease reactions were 20 mM Tris-HCl, 50 mM KCl, 2 mM ATP, pH 7.6 and a divalent metal concentration indicated in the figure legends. The concentrations of ssDNA, dsDNA, Rad50, and Mre11 varied for each experiment and are given in the individual figure legends. The gp32 and UvsY proteins were purified by intein/chitin-based affinity chromatography as described (44). Reactions that were analyzed using 1% TAE-agarose gels (Figure 1) were quenched in 5 mM Tris-HCl, pH 7.5, 200 mM EDTA, 0.2% orange G, 0.015% bromophenol blue, 0.015% xylene cyanol FF, and 7.5% Ficoll 400. The agarose gels were stained with 10 μg/ml ethidium bromide for 30 minutes prior to visualization. Reactions that were analyzed using TBE-Urea-PAGE (15% polyacrylamide) (Figures. 2 and 7) were quenched with 50 mM EDTA, 95 % formamide, 0.015% xylene cyanol FF, and 0.015% bromophenol blue. The PAGE gels were ran for 2 hours at 35 mA, followed by overnight exposure to a phosphorimager plate and subsequent analysis using a Storm phosphorimager and ImageQuant software (GE Life Sciences). The reactions that were analyzed using PEI-TLC (Figures 4C, 4D, 5A, and 6) were quenched in an equal volume of 200 mM EDTA. The quenched samples were then spotted onto the PEI plates (1-2 μL volume) and developed in 300 mM KPi, pH 7.0. The TLC plate was then dried and exposed for 2 hours to the phosphorimager plate followed by analysis using the Storm phosphorimager and ImageQuant software.

To examine the steady-state exonuclease activity of the MR complex, we employed a real-time continuous assay based on the enhancement of fluorescence when the nucleotide analog, 2-AP is excised from DNA (45). The assay was carried out at 30°C using excitation
and emission wavelengths of 310 and 375 nm, respectively, on a Cary Eclipse (Varian) spectrofluorometer. The reaction buffer contained 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl₂, 0.3 mM MnCl₂, 0.1 µM MR complex, and 1.3 µM 2-AP labeled dsDNA. The reactions were monitored for approximately 5-10 minutes and velocities were determined using at least 3 minutes of the time course.

To determine the steady-state ATP hydrolysis activity of Rad50 and the MR complex, we employed the standard coupled fluorometric ATP hydrolysis assay (46) carried out at 30°C using excitation and emission wavelengths of 340 and 460 nm, respectively, on either a SLM-Aminco (SLM Instruments, Inc.) or a Cary Eclipse spectrofluorometer (Varian). The reaction buffer contained 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl₂, 50 µM NADH, 150 µM phosphoenolpyruvate, 6.67 Units/ml of pyruvate kinase and 10 Units/ml of lactate dehydrogenase. In reactions where Mre11 was included, its concentration was always in slight excess over the Rad50 concentration. In reactions containing DNA, its concentration was held in excess over that of Rad50 or the MR complex and well above the $K_{activation}$ for DNA (Herdendorf and Nelson, unpublished). Steady-state rate constants were determined using Sigmaplot 10.0/Enzyme Kinetics Module 1.3 (Systat Software, Inc.).

**Results**

*Expression and Purification of T4 Rad50 and Mre11* - Over-expression of eukaryotic Mre11 and Rad50 requires the use of either *S. cerevisiae* or insect cells and yields only microgram amounts of purified protein (27, 28, 34). Moreover, eukaryotic Rad50 is difficult to over-express separately and requires co-expression of Mre11 to form significant amounts of soluble protein (47). We have achieved over-expression and purification of T4 Rad50 by
constructing a C-terminal fusion with the Intein/CBD domain provided by the pTYB1 vector. The CBD domain facilitates capture by chitin beads and the intein domain allows for reductant-mediated self cleavage (48). Following self-cleavage, the protein that elutes from the chitin beads contains a strong absorbance at 260 nm, indicating the presence of bound nucleotides or nucleic acids. Dialysis of the protein did not reduce the 260 nm absorbance, therefore we applied the eluted protein to phosphocellulose resin. The material that strongly absorbed at 260 nm was not retained by the column but nearly 100% of the protein was efficiently bound. Following a buffer wash, the NaCl concentration was increased from 200 to 400 mM, which eluted the Rad50 protein. This protein had a 280/260 nm ratio of 1.98, indicating that there is little to no nucleotide or nucleic acid contamination in our preparation of Rad50. Additionally, no appreciable enhancement of DAPI dye fluorescence was observed upon addition of protease-K digested Rad50, indicating the absence of contaminating DNA (data not shown). The overall yield for T4 Rad50 was ~3 mg protein per liter of LB (Fig. 1A, lane 3). The Rad50 Walker A motif mutant, K42M-Rad50, behaved essentially identical to the wild-type protein.

T4 Mre11 was also C-terminally fused to the intein/CBD domain provided by the pTYB1 vector. Mre11 was expressed in an identical fashion as Rad50 but was approximately 10-fold more abundant in the cell-free extracts as compared to Rad50. Additionally, no appreciable 280/260 nm absorbance ratio indicated that no nucleotides or nucleic acids were present in the protein that eluted from the chitin column. Therefore, only a single-column was necessary to achieve the desired level of purity. The overall yield for T4 Mre11 was ~25 mg protein per liter of LB (Fig. 1A, lane 2).
Requirements for Mre11 Nuclease Activity - To determine the requirements for the predicted Mre11 nuclease activity, we performed agarose gel electrophoresis-based nuclease assays. The metal, protein, and ATP dependence was tested using circular ssDNA (M13mp18) and linear dsDNA (Fig. 1B). The ssDNA nuclease reaction required the presence of Mre11, MnCl$_2$, and Rad50. Under these conditions, replacement of MnCl$_2$ with MgCl$_2$ or ZnCl$_2$ resulted in no observable nuclease activity (data not shown). The dsDNA nuclease reaction required the same components; however, ATP was also necessary for nucleolytic degradation of the dsDNA. In addition to defining the components required for nuclease activity, these results strongly argue against the presence of a contaminating nuclease in our preparations of either Mre11 or Rad50 (since both proteins are required). We next tested the endo/exo activity using linear and circular ssDNA or dsDNA (Fig. 1C). These reactions indicate that the T4 MR complex contains a ssDNA endonuclease activity (i.e., both linear and circular ssDNA are degraded) and a dsDNA exonuclease activity (i.e., only linear dsDNA is degraded). We also confirmed that the nuclease activity resides in the Mre11 subunit (Fig. 1D). This assignment is predicted based on sequence analysis; however, the nuclease subunit of the T4 MR complex had been previously assigned to Rad50 rather than Mre11 (49). We separately assayed the ability of Mre11 and Rad50 to degrade dsDNA at elevated concentrations of protein in the presence of MnCl$_2$ and ATP (Fig. 1D). Although the activity is >100-fold less than the MR complex, as expected, the nuclease activity resides within the Mre11 subunit.

Polarity of the dsDNA Exonuclease Activity of the MR complex - To determine the nuclease polarity of the MR complex, we compared the product pattern resulting from nuclease
activity using either 5’ or 3’ labeled 50 bp dsDNA (Fig. 2). The presence of intermediate
length products in reactions using the 5’ labeled DNA substrate and their absence in reactions
with the 3’ labeled DNA substrate is consistent with a 3’ to 5’ exonuclease.

*Steady-state ATP Hydrolysis Activity of Rad50 and the MR complex* – Rad50 is part of the
ABC protein super-family and it is known that Rad50 catalyzes the hydrolysis of ATP (50).
Additionally, the data shown in figure 1 indicates that ATP is necessary for the dsDNA
exonuclease activity of the MR complex. For these reasons, we examined the Mg-ATP
hydrolysis activity of the MR under conditions where the nuclease activity is negligible (i.e.,
in the absence of Mn^{2+}). We determined the steady-state Mg-ATP hydrolysis rate constants
for Rad50 and the MR complex with and without DNA (Table II). Since these reactions are
performed without Mn^{2+}, DNA is not consumed and can be considered an activator rather
than a substrate. We found that by itself, Rad50 is a relatively weak ATPase and the rate of
ATP hydrolysis is sigmoidally dependent on the concentration of ATP. The data fit best to
the Hill equation:

\[
v = \frac{V_{max} [S]^n}{K_m^n + [S]^n}
\]  

(eq. 1)

where \(v\) is velocity, \(V_{max}\) is the maximal velocity at saturating substrate concentration, \(K_m\) is
the Michaelis constant (substrate concentration where \(v = 0.5 \cdot V_{max}\)), and \(n\) is the Hill
coefficient. An F-test was employed to justify the use of the Hill equation over the standard
Michaelis-Menten equation. The data fitting yielded a \(k_{cat}\) of 0.15 sec^{-1}, a \(K_m\) of 16 µM, and
a Hill coefficient of 1.4. Addition of Mre11 results in minor changes in the kinetic constants
with 1.5- and 2.6-fold increases in the \(k_{cat}\) (0.226 sec^{-1}) and \(K_m\) (41.5 µM), respectively, and
no significant change in the Hill coefficient (1.5). Addition of dsDNA to Rad50 alone caused a 3.4-fold increase in $K_m$ (54 µM) and very minor decreases in $k_{cat}$ (0.10 sec$^{-1}$) and Hill coefficient (1.2). Addition of DNA to the MR complex caused a 14-fold increase in the $k_{cat}$ relative to the MR complex alone (3.2 sec$^{-1}$), an increase in Hill coefficient (2.4), and no significant change in the $K_m$ (49.2 µM). The large activation of ATPase activity is specific to linear dsDNA as circular plasmid DNA or a 50 base ssDNA oligonucleotide had no effect (data not shown). Mutation of the predicted Walker A lysine to methionine (K42M-Rad50) reduced the observed ATPase activity ~200-fold compared to Rad50 alone and ~4000-fold compared to the MR/DNA complex. This large decrease in activity is expected given the role the Walker A lysine plays in stabilizing negative charge that forms during the transition state for ATP hydrolysis. The residual activity that is observed represents the upper-limit for the level of ATPase contamination in our preparation of Rad50 (less than 0.025%).

Steady-state Exonuclease Activity of the MR Complex - To continuously monitor the exonuclease activity of the MR complex, we employed the fluorescent nucleotide analog, 2-AP, which has low fluorescence when incorporated into DNA and high fluorescence as a free nucleotide. We performed the assay under steady-state conditions ([DNA]>[enzyme]) with the 2-AP probe located at either the 1 or 17 position relative to the 3’ end of a 50 bp DNA substrate. As seen in Fig. 3A, when the 2-AP probe is located at the 1 position, the steady-state exonuclease activity of both the wild-type and K42M-Rad50 MR complex is essentially unaffected by the presence of either ATP or the non-hydrolyzable ATP analog, AMP-PNP ($k_{cat}$’s ranging between 2.3 and 3.2 min$^{-1}$). When the 2-AP probe is located at the 17 position (Fig. 3B), the steady-state exonuclease rate of both wild-type and the K42M-Rad50 MR
complex is much reduced compared to the 1 position substrate with \( k_{\text{cat}} \)'s of 0.03 min\(^{-1}\) and 0.015 min\(^{-1}\), respectively. Addition of ATP to the wild-type MR complex increases the exonuclease rate (0.24 min\(^{-1}\)) but decreases the rate of the K42M-Rad50 MR complex (0.008 min\(^{-1}\)). The addition of AMP-PNP decreases the rate of both the wild-type and K42M-Rad50 MR complex with \( k_{\text{cat}} \)'s of 0.001 min\(^{-1}\) and 0.006 min\(^{-1}\), respectively.

**Metal Dependence of ATP Hydrolysis and Exonuclease Activities** – Even though the nuclease activity strongly prefers MnCl\(_2\) over MgCl\(_2\), the ATP hydrolysis activity of the MR/DNA complex prefers MgCl\(_2\) (Fig. 4A). Under steady-state conditions, the apparent \( k_{\text{cat}} \) for ATP hydrolysis in the presence of 5 mM MgCl\(_2\) or 5 mM MnCl\(_2\), is 2.8 ± 0.3 and 0.23 ± 0.03 sec\(^{-1}\), respectively. Since the nuclease activity requires MnCl\(_2\), yet under physiological conditions the concentrations of Mg\(^{2+}\) far exceeds that of Mn\(^{2+}\) (51), we measured the rate of ATP hydrolysis at fixed MgCl\(_2\) (5 mM) while varying the concentration of MnCl\(_2\) from 0.1 to 5 mM (Fig. 4B). The ATP hydrolysis rate is inhibited by MnCl\(_2\) with an IC\(_{50}\) of 1.3 ± 0.1 mM. We next explored the metal dependence of the nuclease reaction in more detail. We employed the uniformly \([\alpha\text{-}^{32}\text{P}]\) dGTP labeled 1.6 kb PCR product as the exonuclease substrate and again varied the concentration of MnCl\(_2\) in the presence of 5 mM MgCl\(_2\) (Fig. 4C). The nuclease activity sharply increased between MnCl\(_2\) concentrations of 0 mM and 0.1 mM, followed by a slow decrease in nuclease activity between 0.4 mM and 5 mM. This data can be fit using the following uncompetitive substrate inhibition equation:

\[
\nu = \frac{v_{\text{max}} [A]}{K_a + [A] \left(1 + \frac{[A]}{K_I}\right)}
\]

(eq. 2)
where $v$ is velocity, $V_{max}$ is the maximal velocity at saturating activator concentration, $K_a$ is the Michaelis constant for the activator (concentration of A where $v = 0.5 \cdot V_{max}$), and $K_i$ is the inhibition constant. The data fitting yielded a $K_a$-$\text{Mn}^{2+}$ of 0.011 ± 0.002 mM and $K_i$-$\text{Mn}^{2+}$ of 4.1 ± 0.8 mM. Finally, we compared the MR complex’s nuclease activity with 5 mM MnCl$_2$ alone or with 5 mM MgCl$_2$ and 0.3 mM MnCl$_2$ in combination (Fig. 4D). The results of this assay indicate that the nuclease activity is essentially identical under both of these conditions.

**Rad50 ATP Hydrolysis and Utilization During the Exonuclease Reaction**- A TLC-based assay was employed to simultaneously monitor the ATP hydrolysis and exonuclease activity of Rad50 and Mre11, respectively, using MgCl$_2$ and MnCl$_2$ concentrations of 5 and 0.3 mM, respectively (Fig. 5A). To visualize both DNA and ATP, we used a uniformly [$\alpha$-$^{32}$P] dGTP labeled 1.6 kb PCR product as the DNA substrate and ATP that was labeled at the $\gamma$ phosphate with $^{32}$P. The concentration of ATP was held at 0.2 mM, which is approximately 4-fold above the $K_m$-ATP for the MR complex (Table II). The TLC assay enables visualization of both ATP/ADP and DNA/dGMP, making the determination of ATP utilization highly accurate. The MR complex was held in excess over the DNA substrate so that the rate of exonuclease activity reflects the rate of nucleotide excision and translocation and not productive DNA binding. As seen in Fig. 5B, the rate of exonuclease and ATPase activity is 0.1664 μM/sec$^{-1}$ and 0.0408 μM/sec$^{-1}$, respectively. This suggests that approximately four bases are removed for every ATP hydrolyzed. The concentration of dsDNA ends in this assay is 0.0217 μM, which represents the upper limit for the concentration of DNA-bound MR complex. Assuming a fully saturated dsDNA substrate
during the exonuclease reaction, the $k_{\text{cat}}$ for ATP hydrolysis is then 1.9 sec\(^{-1}\) and the maximum rate of nucleotide removal is 7.67 sec\(^{-1}\).

**The Effect of T4 UvsY and gp32 Proteins on the Exonuclease Activity of the MR Complex** – Consistent with MR complexes from human, *P. furiosus*, and *S. cerevisiae*, Mn\(^{2+}\) is the preferred metal for the exonuclease reaction in the T4 system. However, because physiological concentrations of Mg\(^{2+}\) far exceed those of Mn\(^{2+}\) (51), we further explored the ability of Mg\(^{2+}\) to support exonuclease activity. To quantify the exonuclease activity of the MR complex with Mg\(^{2+}\) and Mn\(^{2+}\) we employed the uniformly [$\alpha^{32}$P] dGTP labeled 1.6 kb PCR product and the TLC assay (Fig. 6). Protein was held in excess over the dsDNA substrate, therefore the loss of product can be fitted to a single-exponential decay with rate constants of 0.105 ± 0.001 min\(^{-1}\) and 0.009 ± 0.0005 min\(^{-1}\) for Mn\(^{2+}\) and Mg\(^{2+}\), respectively (an 11-fold difference between Mn\(^{2+}\) and Mg\(^{2+}\)). We then tested the effect of UvsY and gp32 on the exonuclease rates in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\). UvsY and gp32 were chosen as possible candidates for the modulation of MR complex activity because these two proteins bind to the product of the MR reaction (i.e., ssDNA) and participate in the subsequent step of DSB repair (strand invasion). We found that the presence of UvsY and gp32 increased the Mg\(^{2+}\)-dependent reaction by nearly 5-fold (rate constant of 0.0410 ± 0.0037 min\(^{-1}\)) while having no effect on the rate of the Mn\(^{2+}\)-dependent reaction (rate constant of 0.1031 ± 0.0104 min\(^{-1}\)). Separately, UvsY and gp32 had no measurable effect on the Mg\(^{2+}\)-dependent nuclease activity (data not shown). Several control experiments were performed to rule out the possibility of nuclease contamination in our preparations of UvsY and gp32 (Fig. 6). The Mg\(^{2+}\)-dependent exonuclease reaction required Rad50, Mre11, UvsY,
gp32, and ATP. Omission of Rad50, Mre11, or ATP resulted in a complete loss of the exonuclease activity, strongly arguing against the introduction of a contaminating nuclease present in the preparations of UvsY or gp32. We also examined the effect of UvsY and gp32 on the steady-state rate of ATP hydrolysis by the MR complex in the presence of a DNA substrate (Table II). Separately, UvsY and gp32 did not significantly alter the rate, whereas the combination reduced the rate by 1.9 fold.

The polarity of the UvsY/gp32-enhanced, Mg$^{2+}$-dependent exonuclease reaction, was examined under steady-state conditions using 3’ labeled 50 bp dsDNA. This substrate was chosen since the appearance of any DNA products of intermediate size represents a deviation from the strict 3’ to 5’ exonuclease reaction that was observed in Fig. 2. Under steady-state conditions (which are required when short DNA substrates are used), the Mg$^{2+}$-dependent reaction in the absence of UvsY/gp32 is nearly undetectable (i.e., the UvsY/gp32 enhancement of the steady-state Mg$^{2+}$-dependent reaction appears greater than the 5-fold observed in Fig. 6). Therefore, we compared the Mn$^{2+}$-dependent reaction to the Mg$^{2+}$-dependent reaction, both in the presence of UvsY/gp32. As shown in Fig. 7, in contrast to the reaction in the presence of Mn$^{2+}$, the Mg$^{2+}$-dependent reaction results in products with a length ranging between 15 and 25 bases, suggesting that an additional form of nuclease activity has occurred. Quantitation of this reaction reveals that approximately 32% of the products were between 15 and 25 bases with the remaining 68% as dGMP.

**Discussion**

Bacteriophage T4 is a well established model system for investigating the biochemistry of proteins involved in DNA replication, recombination, and repair (3, 52, 53).
Studies using the T4 phage model system led to the first proposed model for recombination-dependent DNA replication (RDR) (54). The RDR model begins with the formation of a single-stranded 3’ DNA end that subsequently undergoes strand invasion and pairing with a homologous dsDNA template. The single-stranded 3’ DNA can be formed as a result of incomplete replication of the lagging strand or through the action of a nuclease. Over the past three decades since the RDR model was proposed, all of the enzymes and proteins that are required to carry out RDR in T4 have been isolated and extensively characterized biochemically, with the notable exception of the nuclease complex thought to be responsible for the creation of the 3’ ssDNA overhangs [Rad50 (gp46) and Mre11 (gp47)]. Here we have described, for the first time, the expression, purification, and biochemical characterization of the Rad50 and Mre11 proteins from bacteriophage T4.

On a qualitative level it appears that the various activities of the MR complex are well conserved between all three kingdoms of life. The T4 MR complex is an ATPase, a ssDNA endonuclease, and a 3’ to 5’ dsDNA exonuclease. ATP hydrolysis is positively cooperative and is activated by the presence of Mre11 and DNA. We have found that during repetitive dsDNA exonuclease activity, approximately 4 nucleotides are removed for every ATP that is hydrolyzed and the maximum exonuclease rate is 7.7 bases/sec. ATP hydrolysis increases the rate of multiple nucleotide excisions but does not alter the rate of the first nucleotide excision. Interestingly, we have found that two T4 phage proteins that are necessary for the subsequent steps of RDR/DSB repair activate the Mg^{2+}-dependent nuclease reaction, which produces an altered product profile as compared to the Mn^{2+}-dependent exonuclease reaction.
To our knowledge, this is the first instance that the steady-state ATP hydrolysis rate constants have been reported for a Rad50 homolog in the presence and absence of Mre11 and a DNA substrate. We found that by itself, Rad50 is a relatively inefficient ATPase with a $k_{\text{cat}}$ of 0.15 sec$^{-1}$. The reaction is mildly positively cooperative with a Hill coefficient of 1.4, indicating Rad50 is at least a dimer, consistent with the structural analysis of $Pfu$ Rad50 and other ABC proteins (31, 32). Separately, the addition of DNA or Mre11 to Rad50 resulted in very minor perturbations in the kinetic parameters of Rad50. In contrast, the addition of both Mre11 and DNA resulted in a 20-fold increase in $k_{\text{cat}}$ and an increase in the Hill coefficient to 2.4. These reactions are performed with Mg$^{2+}$ as the only divalent cation so that there is essentially no nuclease activity under these conditions. This indicates that ATP hydrolysis can be uncoupled from nuclease activity and suggests that Mre11 and dsDNA act as allosteric activators of Rad50. The increase in the Hill coefficient from 1.4 to 2.4 suggests that in the presence of Mre11 and dsDNA, there are more than two ATP sites in communication with each other. It is not clear how this might occur, but it is tempting to speculate that binding of dsDNA promotes hetero-octamer (Mre11$_2$/Rad50$_2$)$_2$ formation through zinc-hook mediated tethering of Rad50’s coiled-coil domains (15). Hetero-octamers of the $Pfu$ MR complex have been previously observed in electron microscopy (33) and atomic force microscopy experiments (55) have revealed that DNA binding results in a straightening of Rad50’s coiled-coils, which prevents an intracomplex tether (hetero-tetramer) and promotes an intercomplex tether (hetero-octamer).

The exact function of Rad50’s ATP hydrolysis activity has been unclear (56). Based on structural changes between the ATP-free and bound forms of $Pfu$ Rad50, it has been
proposed that ATP hydrolysis induces a conformational change that may promote product release (dNMP) or drive MR complex translocation (31). Our data are inconsistent with the product release model, since the rate of excision of the first nucleotide at the 3’ end of the DNA is not affected by ATP. Additionally, the stoichiometry of ATP hydrolysis is one ATP per four nucleotides removed. If ATP hydrolysis was directly linked to product release, then a 1:1 correspondence between ATP hydrolysis and nucleotide removal would be expected. The data are more consistent with ATP being involved in the translocation of the MR complex following nucleotide excision and release. Since ATP and AMP-PNP do not affect the rate of the first nucleotide excision for either the wild-type or K42M-Rad50 MR complexes, it is unlikely that the MR complex must unwind or open the DNA duplex prior to nucleotide excision. In contrast, ATP hydrolysis increased the exonuclease rate when the probe is moved 17 bases away from the 3’ end, indicating that ATP hydrolysis is necessary for multiple, processive nucleotide excisions. This suggests that the energy of ATP hydrolysis is likely driving translocation of the MR complex. Consistent with this, when the probe is in the 17 position, AMP-PNP potently inhibits the exonuclease activity of the wild-type enzyme and both ATP and AMP-PNP inhibit the K42M-Rad50 MR complex. The observed inhibition is likely due to the stalling of the MR complex following nucleotide excision and product release (i.e., the MR complex is unable to translocate or dissociate from the DNA in the ATP-bound form). In the absence of ATP, the MR complex likely dissociates from the DNA substrate and rebinds at the new ss/dsDNA junction (non-processively). If this interpretation is correct, then the steady-state exonuclease rate constant in the presence of AMP-PNP is equal to the rate of dissociation of the MR complex from DNA. The 1:4 ATP/base stoichiometry suggests that the Mre11 is able to remove four
nucleotides before it is required to translocate to the new ss/dsDNA junction. This would require “scrunching” of the DNA, similar to that which has been proposed for the NS3 helicase (57).

The data shown in Figure 2 are consistent with a 3’ to 5’ dsDNA exonuclease. This activity is at odds with the proposed physiological function of the MR complex, a paradox that has been long recognized (15, 58, 59). It is conceivable that the observed 3’ to 5’ exonuclease activity is the relevant physiological activity and the MR complex does not directly participate in the production of a 3’ ssDNA overhangs. However, this possibility seems unlikely for the following reasons: 1) it is well established that in eukaryotes the nuclease activity of the MR complex is required for the removal of Spo11/Rec12, which is covalently linked to the 5’ end of the DSB during meiosis (60); 2) the nuclease activity of Mre11 is required for the removal of topoisomerase-5’ DNA covalent adducts that are stabilized by anti-cancer etoposide derivatives (10); 3) the nuclease activity of Mre11 is required for repair of IR-induced DSBs (38); and 4) in T4 phage, Mre11 and Rad50 are necessary for the initiation of recombination dependent replication and recombination-dependent DSB repair, which begins with the 5’ to 3’ resection of a DNA end (7, 61). Even with the above data, the precise role of the MR complex in DSB repair is still an open question and the polarity paradox remains. Therefore, we tested several T4 recombination proteins for a possible change in the nuclease mechanism of the T4 MR complex. In the presence of MnCl$_2$, we did not observe any significant differences in the rate or product profile of MR nuclease assays in the presence of UvsX, gp32, and UvsY, either alone or in combination. However, UvsY and gp32 were found to enhance the ability of the T4 MR
complex to utilize MgCl$_2$ as a cofactor for the nuclease activity. We examined the product profile of the MgCl$_2$-dependent reaction in the presence of UvsY and gp32 and in addition to the dGMP that is produced by 3’ to 5’ exonuclease activity, we also observed a distribution of products in the size range of 15 to 25 bases. These products could be the result of a 5’ to 3’ exonuclease or an endonuclease activity. We favor an endonuclease mechanism due to the absence of products between 50 and 25 bases, which would be produced if 5’ to 3’ exonuclease activity had occurred. Experiments are currently underway to determine the exact nature of the alteration in nuclease mechanism. A metal-dependent change nuclease mechanism has also been observed in the MR complex from the thermophilic organism *P. furiosus* when assayed at elevated (physiologically relevant) temperatures (59). In the case of the *Pfu* MR complex, the MgCl$_2$-dependent reaction produces a 3’ ssDNA overhang through endonucleolytic cleavage of sites close to the 5’ end of the dsDNA, similar to what we are proposing here for the T4 MR complex.

The mechanism behind the increased ability to use Mg$^{2+}$ as a cofactor is not clear but likely involves physical interaction(s) between the MR complex and UvsY/gp32. Based on our observation that 5 mM MgCl$_2$ does not inhibit the nuclease activity of the complex, even when the concentration of MnCl$_2$ is 50-fold lower (0.1 mM), it appears that the affinity of Mg$^{2+}$ for the Mre11 active site is low compared to Mn$^{2+}$. This is consistent with the fact that Mre11 crystals grown in the presence of MgCl$_2$ only have one of the two required divalent cation sites occupied (62). If this is correct and Mre11 normally has weak affinity for MgCl$_2$, then it is plausible to assume that UvsY and gp32 act in an allosteric manner to increase the affinity of Mg$^{2+}$ for the active site of Mre11. This hypothesis requires that gp32 and/or UvsY
directly interact with the MR complex, which has been previously observed in T4 phage lysate (63). Additionally, in vivo experiments have strongly suggested a physical interaction between gp32 and the MR complex (64). To enhance the affinity of Mg$^{2+}$ for Mre11, the interaction of gp32 and/or UvsY with the MR complex would have to reorient the active site in some manner. The nature of this putative active site perturbation is unclear and may require crystallographic analysis of the T4 MR complex.

The mechanism behind the Mg$^{2+}$-dependent change in product profile is also not clear and will require further investigation. One hypothesis is that the ssDNA endonuclease activity of the MR complex is enhanced in the presence of Mg$^{2+}$ and UvsY/gp32. This requires at least transient melting of the DNA duplex, which is an activity that has been previously observed in gp32 (65, 66). If gp32 and/or UvsY were to also protect the 3’ but not the 5’ strand of the DNA from endonucleolytic cleavage, then this would result in the production of a 3’ ssDNA overhang that is pre-bound with UvsY and gp32 and primed for the next step of recombination, filament formation by UvsX. It is clear from the data shown in Figure 7 that even the presence of UvsY and gp32, there is still a high level of 3’ to 5’ dsDNA exonuclease activity (nearly 70% of the products are produced as a result of exonuclease activity). This may indicate that additional proteins are required for the efficient production of a 3’ ssDNA overhang. Based on recent results from S. cerevisiae and P. furiosus, which demonstrated the involvement of helicases in MR-dependent DSB resection (42, 59), a possible candidate is UvsW helicase. UvsW helicase is known to be necessary for DSB repair (67, 68) and is a functional analog of Sgs1, which is the S. cerevisiae helicase involved in DSB resection. We are currently in the process of testing the above hypothesis.
With the biochemical characterization of the bacteriophage T4 MR complex reported here, the entire complement of T4 phage proteins involved in recombination and DSB repair have been purified and assayed in vitro. The bacteriophage T4 system provides milligram quantities of purified Mre11 and Rad50, which is not currently possible in the eukaryotic systems. Given the apparent conservation in DNA repair mechanisms between all kingdoms of life, it is expected that the T4 phage model system will provide valuable mechanistic insights into what has been termed the “keystone complex” of DNA repair (69).

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Footnotes

1 The abbreviations used are: T4 phage, bacteriophage T4; DSB, double-strand break; HR, homologous recombination; gp46, T4 gene product 46; gp47, T4 gene product 47; gp32, T4 gene product 32; UvsY, UV sensitivity protein Y; MR, Mre11/Rad50; SMC, structural maintenance of chromosomes; ABC, ATP binding cassette; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Pfu, Pyrococcus furiosus

References

Table I

**Steady-State Kinetic Constants for Bacteriophage T4 Rad50 ATP Hydrolysis**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>Hill (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad50$^a$</td>
<td>0.146 ± 0.003</td>
<td>16.0 ± 0.8</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Rad50/DNA$^b$</td>
<td>0.100 ± 0.003</td>
<td>54.1 ± 0.6</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Mre11/Rad50$^c$</td>
<td>0.226 ± 0.006</td>
<td>41.5 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Mre11/Rad50/DNA$^d$</td>
<td>3.2 ± 0.1</td>
<td>49.2 ± 1.6</td>
<td>2.4 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$Concentrations were as follows: 0.5 µM Rad50.

$^b$Concentrations were as follows: 0.5 µM Rad50, 2 µM DNA.

$^c$Concentrations were as follows: 0.5 µM Rad50, 0.63 µM Mre11.

$^d$Concentrations were as follows: 0.08 and 0.3 µM Rad50, 0.13 and 0.5 µM Mre11, 0.33 and 1 µM DNA.
### Table II

**The Effect of UvsY and gp32 (T4 SSB) on ATPase Activity of the Mre11/Rad50 Protein Complex**

<table>
<thead>
<tr>
<th>Protein Complex</th>
<th>$k_{cat}$ ($s^{-1}$) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mre11/Rad50/DNA</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>Mre11/Rad50/DNA/UvsY</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Mre11/Rad50/DNA/gp32</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>Mre11/Rad50/DNA/UvsY/gp32</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

$^a$Concentrations were as follows: Mre11, 83 nM; Rad50, 67 nM; DNA, 1 µM; UvsY, 550 nM; and gp32, 524 nM.

$^b$Apparent $k_{cat}$ (rate measured at saturating concentrations of ATP and DNA). The values reported are the average of three individual measurements. Errors represent the standard deviation.
Figure 1.  

A) SDS-PAGE analysis of purified bacteriophage T4 Mre11 and Rad50. Approximately 20 µg of protein was separated using 12% SDS-PAGE and stained with Coomassie blue R-250. Lanes 1-3 are molecular weight markers, Mre11, and Rad50, respectively.

B) The requirement for nuclease activity using circular ssDNA (0.4 µg) and linear dsDNA (0.4 µg) as substrates. The concentrations of MnCl$_2$ (Mn), Mre11 (M), Rad50 (R), and ATP were 5 mM, 0.3 µM, 0.3 µM, and 2 mM, respectively. The reaction was performed at 37°C for 30 minutes. The products of the reaction were analyzed using TAE-agarose gel electrophoresis and stained with ethidium bromide.

C) Nuclease reactions using circular and linearized forms of ss and dsDNA (0.4 µg each) using MnCl$_2$, Rad50 (R), Mre11 (M), and ATP concentrations of 5 mM, 0.3 µM, 0.3 µM, and 2 mM, respectively.

D) Nuclease assay carried out for 60 minutes using dsDNA (0.4 µg), 5 mM MnCl$_2$, 2 mM ATP, and the indicated concentrations of either Mre11 or Rad50.
Figure 2. Polarity of T4 Mre11/Rad50 dsDNA exonuclease activity. A) Analysis of nuclease reaction products using 16% UREA-PAGE. The reaction was carried out at 37°C and the time points for each lane are 0, 0.5, 1, 2, 4, and 8 minutes. The concentrations of dsDNA, Mre11, Rad50, MnCl₂, and ATP were 0.5 µM, 0.1 µM, 0.1 µM, 5 mM, and 2 mM, respectively. The dGMP band was identified by comparison to a reaction analyzed using PEI-TLC.
Figure 3. **Steady-state exonuclease activity of wild-type and mutant MR complexes.**  

**A)** Exonuclease activity using the 50 bp dsDNA substrate with the 2-AP probe located at the 3’ end of the DNA. The concentrations of DNA, Mre11, Rad50 (wild-type and K42M), ATP, and AMP-PNP were 1.3 µM, 0.105 µM, 0.1 µM, 0.25 mM, and 0.25 mM, respectively. The black and grey bars represent the wild-type and K42M-Rad50 MR complexes, respectively. 

**B)** Exonuclease activity using the 50 bp dsDNA substrate with the 2-AP probe located at position 17 relative to the 3’ end of the DNA. The concentrations of DNA, Mre11, Rad50 (wild-type and K42M), ATP, and AMP-PNP were 1.3 µM, 0.42 µM, 0.4 µM, 0.25 mM and 0.25 mM, respectively. The black and grey bars represent the wild-type and K42M-Rad50 MR complexes, respectively. The numbers above the low-activity conditions represent $k_{cat}$ (min$^{-1}$). The error bars for both A and B represent the standard deviation of at least three assays.
Figure 4. The dependence of Mg$^{2+}$/Mn$^{2+}$ cation on ATP hydrolysis and dsDNA exonuclease activity.  

A) The rates of ATP hydrolysis in the presence of 5 mM MgCl$_2$ or MnCl$_2$. The rates were determined using the coupled fluorometric assay as described in “Materials and Methods” using Rad50, Mre11, and DNA concentrations of 50 nM, 67 nM, and 260 nM, respectively.  

B) The effect of increasing MnCl$_2$ concentration on the rate of ATP hydrolysis in the presence of 5 mM MgCl$_2$. The concentrations of Rad50, Mre11, and dsDNA are identical to panel A.  

C) Quantification of a PEI-TLC exonuclease assay using the uniformly labeled 1.68 kb dsDNA using a fixed concentration of MgCl$_2$ (5 mM) and increasing concentrations of MnCl$_2$. The concentrations of Rad50, Mre11, and ATP were 0.5 µM, 0.5 µM, and 2 mM, respectively. Each reaction was carried out for 10 minutes using a dsDNA concentration of 0.024 µM.  

D) Time course of exonuclease activity at 5 mM MnCl$_2$ (closed diamonds) or 0.3 mM MnCl$_2$ and 5 mM MgCl$_2$ (closed squares) using the uniformly labeled 1.68 kb dsDNA (6 nM) and identical protein and ATP concentrations as panel C.
Figure 5. Kinetics of the Mre11/Rad50 dsDNA exonuclease reaction. A) An exonuclease reaction time course analyzed via PEI-TLC. The substrates (DNA and ATP) and products (ADP and dGMP) can be simultaneously monitored. The reactions were performed at 37°C containing Rad50 (0.5 µM), Mre11 (0.5 µM), ATP (2 mM), MgCl₂ (5 mM), MnCl₂ (0.3 mM), and 1.68 kb DNA (.024 µM). B) Quantification of the PEI-TLC plate shown in A). The closed diamonds represent the exonuclease reaction product (dGMP) and the closed squares represent the ATP hydrolysis product (ADP).
Figure 6. **The effect of UvsY and gp32 on the MgCl$_2$-dependent exonuclease reaction.** Quantification of a PEI-TLC exonuclease assay using the uniformly labeled 1.68 kb dsDNA. Reactions contained Rad50 (0.5 μM), Mre11 (0.5 μM), UvsY (1 μM, hexamer), gp32 (1 μM), ATP (2 mM), and either MgCl$_2$ or MnCl$_2$ (5 mM). The conditions are: MnCl$_2$, Mre11, Rad50, and ATP (closed squares); MgCl$_2$, Mre11, Rad50, and ATP (closed diamonds); MnCl$_2$, Mre11, Rad50, UvsY, gp32, and ATP (closed circles); MgCl$_2$, Mre11, Rad50, UvsY, gp32, and ATP (closed triangles); MgCl$_2$, UvsY, gp32, and ATP (open squares); MgCl$_2$, Mre11, Rad50, UvsY, gp32, and ATP omission (open circles).
Figure 7. **Comparison of nuclease products in reactions containing UvsY and gp32 with MgCl$_2$ and MnCl$_2$.** *A*) Analysis of nuclease reaction products using 16% UREA-PAGE. The time points for both reactions are 0, 0.5, 1, 2, 4, and 8 minutes. The concentrations of the Mre11, Rad50, and ATP for the MgCl$_2$ (5 mM) containing reaction were 0.1 µM, 0.1 µM, and 2 mM, respectively. The concentrations of the Mre11, Rad50, and ATP for the MnCl$_2$ (5 mM) containing reaction were 0.025 µM, 0.025 µM, and 2 mM, respectively. The concentrations of UvsY, gp32, and the $3'$-$32$P labeled 50 bp dsDNA for both reactions were 1 µM, 1 µM, and 0.5 µM, respectively. The inset highlights the portion of the gel corresponding to a mobility between 25 and 15 bases. *B*) The size distribution of the products contained in lane 4 of the Mg$^{2+}$-dependent reaction (black) and lane 6 of the Mn$^{2+}$-dependent reaction (light grey). These lanes are indicated by asterisks at the top of the gel. The nearly overlapping traces at the 50 base positions indicate that the reaction has proceeded to the same extent.
Chapter III: Disruption of Bacteriophage T4 Mre11 Dimer Interface Reveals Two-State Mechanism for Exonuclease Activity

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Dustin W. Albrecht\(^2\), Timothy J. Herdendorf\(^3\), and Scott W. Nelson\(^{23}\)

Abstract

The Mre11/Rad50 (MR) complex is a central player in DNA repair and is implicated in the processing of DNA ends caused by double strand breaks. Recent crystal structures of the MR complex suggest that several conformational rearrangements occur during its ATP hydrolysis cycle. A comparison of the Mre11 dimer interface from recent x-ray crystal structures suggests that the interface is dynamic in nature and may adopt several different arrangements. In order to probe the functional significance of the Mre11 dimer interface, we have generated and characterized a dimer disruption Mre11 mutant (L101D-Mre11). Although L101D-Mre11 binds to Rad50 and dsDNA with affinity comparable to the wild-type enzyme, it does not activate the ATP hydrolysis activity of Rad50, suggesting that the allosteric communication between Mre11 and Rad50 has been interrupted. Additionally, the dsDNA exonuclease activity of the L101D-MR complex has been reduced by 10-fold under conditions where processive exonuclease activity is required. However, we unexpectedly found that under steady state conditions, the nuclease activity of the L101D-MR complex is 5-fold greater than that of the wild-type complex. Based on steady state and single-turnover

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1 This research has been submitted to The Journal of Biological Chemistry for publication.
2 Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011
3 Author of correspondence
nuclease assays, we have assigned the rate-determining step of the steady state nuclease reaction to be the productive assembly of the complex at the dsDNA end. Together, our data suggest that the Mre11 dimer interface adopts at least two different states during the exonuclease reaction.

**Introduction**

Double-strand breaks (DSB)\(^1\) are among the most harmful forms of DNA damage, and they may lead to deletions, duplications, or cell death if they go unrepaired (1). DSBs are common and can result from both internal and external causes. External causes include ultraviolet radiation, ionizing radiation (IR), and genotoxic chemicals. Internal causes include reactive oxygen species and protein complexes that interfere with replication fork progression. DSBs are also deliberately formed during the development of antibodies through VDJ recombination (2).

The three pathways for repairing DSBs in eukaryotic cells have been described in the literature: homologous recombination (HR), non-homologous end-joining, and microhomology-mediated end-joining (3). Mre11 and Rad50 form a heterotetrameric complex (Mre11\(_2/Rad50_2\), referred to as the MR complex hereafter) that is involved in all three of the above mentioned DSB repair pathways (3, 4). The role of the MR complex is best understood in HR, which involves several steps and requires multiple proteins (5). The MR complex is involved in the initial step of HR, which is the resection of the 5’ strand of the DSB to create 3’ single-stranded DNA. The role of the MR complex in this process has been recently elucidated using the *Saccharomyces cerevisiae* model system (6–8). Resection is a two-step process in eukaryotes with the MR complex being essential for the first step. In the first step of *S. cerevisiae* cells, the MR complex works along with Xrs2 and Sae2 to
remove 50-100 nucleotides (nts) from the 5’ end of the DSB (6). In the second step, the intermediates created by the MR complex are further resected (>500 nts) by Sgs1/Top3/RmiI and RPA or ExoI and RPA. Upon completion of DSB resection, Rad51 promotes strand invasion of the ssDNA into a region of homologous DNA and creates a D-loop that is used as a primer for DNA synthesis (9). Upon completion of DNA synthesis, either the extended strand is liberated from the template by a DNA helicase, or the Holliday junction is resolved by a DNA nuclease and ligase (10).

The MR complex is present in eukaryotes, archaea, prokaryotes, and some bacteriophage biological systems (4). While the MR complex homologs from eukaryotes, archaea, and bacteriophage participate in DSB resection, the prokaryotic MR homolog, the SbcCD complex, does not seem to be involved in DSB resection, instead prokaryotic resection is carried out by the RecBCD helicase/nuclease complex (11). The T4 bacteriophage homologs of the MR complex (gp46/gp47) are required for in vivo homologous recombination and DSB repair and T4 bacteriophage has been used as a model system for the investigation of nucleic acid enzymes for many years (12–14).

Rad50 belongs to the structural maintenance of chromosomes protein family, which is a subset of the ATP binding cassette (ABC) protein superfamily (15, 16). The ABC superfamily of proteins is broadly distributed and contains a well-conserved dimeric nucleotide binding domain (NBD) that binds and hydrolyzes ATP at its dimeric interface (17). The energy obtained from ATP hydrolysis is used by ABC proteins to promote a variety of biological functions (17). T4 Rad50 (gp46) is an inefficient ATPase ($k_{cat}$ of 0.15 s$^{-1}$), but becomes much more active ($k_{cat}$ of 3.2 s$^{-1}$) upon the addition of Mre11 and double-stranded DNA (dsDNA) (18). Although it does not affect the initial rate of nucleotide
removal, ATP hydrolysis increases the rate of repetitive nucleotide removal leading to the hypothesis that ATP hydrolysis is involved in MR complex translocation along the DNA substrate (18, 19).

Mre11 is a member of the Ser/Thr protein phosphatase superfamily, which requires divalent cations for their catalytic activity (20). In vitro assays have shown that T4 Mre11 (gp47) contains the nuclease subunit of the MR complex and has single-stranded DNA (ssDNA) endonuclease, dsDNA exonuclease, and a probable end-dependent dsDNA endonuclease activity (18). Knockout of Mre11 in S. cerevisiae causes only mild effects, but has a much more profound impact in humans (21, 22). Mre11 deficiency in humans can lead to ataxia telangiectasia-like disorder, which causes IR sensitivity and cerebellar dysfunction (23).

Several recent crystal structures have greatly clarified the architecture of the heterotetrameric MR complex. A combination of small angle x-ray scattering and x-ray crystallography using full-length Mre11 and Rad50 with the majority of its coiled-coil and all of the zinc-hook removed revealed that the M2R2 heterotetramer can exist in at least two different conformations, termed open and closed (24–28). In the open conformation, the nucleotide binding domains (NBDs) of Rad50 are far away from each other with Mre11 acting as a bridge holding the heterotetramer together (24). In the presence of ATP or an ATP analog, the Rad50 nucleotide binding domains come together and form the classic sandwich dimer that has been observed in many ABC proteins. A surprising feature of these new structures has been the relative position of Mre11 with respect to the coiled-coil domain of Rad50. Instead of being placed between the coiled-coils of Rad50 as it has been traditionally depicted in schematic models, Mre11 embraces Rad50 on the surface of Rad50.
opposing the coiled-coil domain (27, 28). As a consequence of this arrangement, the nuclease active sites of Mre11 are somewhat protected by Rad50 so that it appears difficult for anything larger than ssDNA to approach the Mre11 active site. Hopfner and colleagues have identified at least four separate Mre11-Rad50 interfaces that contribute to stabilizing the MR complex heterotetramer (27). The most extensive interface is formed through a C-terminal helix-loop-helix of Mre11 that interacts with the base of the coiled-coil domain of Rad50. Also interacting with the base of the coiled-coil of Rad50 is the linker domain that separates the HLH and Cap domains of Mre11. The remaining interfaces are formed through interactions between the Mre11 cap and phosphoesterase domains and the nucleotide binding domain of Rad50. Any or all of these domains may be involved in allosteric signal transmission between Mre11 and Rad50. Besides the four heterosubunit interfaces, there are three additional homosubunit interfaces, two from the Rad50 dimer and one from the Mre11 dimer. The most extensive homosubunit interface is that of the NBD of Rad50, which is typical of all ABC proteins and has been readily observed in many x-ray crystal structures (15). The second Rad50-Rad50 interface is the zinc-hook, which is found at the apex of the coiled-coil domain (29). This interface is defined by a shared Zn$^{2+}$ binding site that is formed through a tetrathiolate linkage where each coiled-coil provides two cysteine residues. While this type of binding site generally binds Zn$^{2+}$ with extremely high affinity and is therefore thought to be extremely stable, atomic force microscopy experiments suggest that the Rad50 zinc-hook domain is dynamic and able to respond to DNA binding by Rad50 (30). The Mre11-Mre11 homodimeric interface is relatively small, made up of a four-helix bundle with two helices being provided by each subunit (Fig. 1 and Fig 2A). The interaction between these helices is largely hydrophobic with the exception of two salt bridges. X-ray
crystal structures and protein cross-linking indicate that the Mre11 dimeric interface undergoes small conformation rearrangements upon ATP binding by Rad50 (24, 27).

To explore the significance of the Mre11 dimeric interface, we utilized site-directed mutagenesis on T4 Mre11. A hydrophobic residue (Leu\textsubscript{101}) located in the Mre11 dimeric interface was chosen and mutated. In an effort to determine the significance of the dimer interface, we investigated the effects of the mutation on the MR complex in terms of Rad50 and DNA affinity, activation of Rad50 ATP hydrolysis activity, and nuclease activity. Our assays show that disruption of the Mre11 dimeric interface has no effect on the affinity of Mre11 for Rad50. Also, the disrupted dimeric interface appears to have little effect on MR complex DNA binding and ssDNA endonuclease activity. However, the disruption has profound effects on the ATPase activity of Rad50 and the exonuclease activity of Mre11 in both steady state and pre-steady state assays.

**Experimental Procedures**

*Mutagenesis, Protein Expression, and Purification* – The L101D-Mre11 mutant was constructed using the Stratagene QuikChange site-directed mutagenesis protocol. The presence of the mutation and the integrity of the entire open reading frame were verified using DNA sequencing. Bacterial expression and purification of the L101D-Mre11, wild-type Mre11 (WT-Mre11) and Rad50 proteins were carried out as previously described (18)(31).

*Circular Dichroism (CD) Spectroscopy* – CD studies on WT- and L101D-Mre11 were performed at 22°C on a Jasco J710 CD spectrometer in a 1-cm cell using a protein concentration of approximately 0.25 mg/ml. The buffer used was 10 mM Tris-HCl, 200 mM NaCl, pH 7.6. Spectra were collected from 200 to 260 nm in increments of 0.2 nm. Each
spectrum was blank-corrected and L101D-Mre11 was normalized to the ellipticity of the WT protein at 210 nm to correct for differences in protein concentration.

ATP Hydrolysis Assays – Determinations of $K_m$, $k_{cat}$, and level of DNA activation were performed as previously described (18).

Competition by L101D-Mre11 with WT-Mre11 for Rad50– The relative affinity of the L101D-Mre11 mutant for Rad50 was determined using a coupled ATPase assay (32). All assays were done at 30° C. The reaction was performed in a buffer containing 50 mM Tris-HCl, 50 mM KCl, 5 mM MgCl$_2$, 0.1 mg/ml BSA (referred to hereafter as ‘standard buffer’) with 1 mM ATP, 1 µM DNA, 300 µM NADH, 2 mM phosphoenolpyruvate, 6 units/ml pyruvate kinase, and 10 units/ml lactate dehydrogenase at pH 7.6 in a volume of 125 µL. The sequence of the oligonucleotides used to create the DNA substrate were the following, ds50-F: 5’-

CTCTTGGTGTATTGATGGTGTGCAATACATTTAATTTCATTATCAATAAG-3’,

ds50-R: 5’-

CTTATTGATAATGAAATATGTATTGCAACCATCATCATACACCAAGAG-3’. The oligonucleotides were dissolved in sterile water, quantitated by measuring the absorbance at 260 nm mixed in equal molar ratios, and annealed by heating to 80° C for 5 minutes and slowly cooled to room temperature. In each assay, WT-Mre11 and Rad50 each had a concentration of 0.1 µM while the L101D-Mre11 concentration had values of 0, 0.05, 0.1, 0.2, 0.4, 0.7, and 1.0 µM in each respective assay. ATP hydrolysis was assayed spectrophotometrically on a Varian Cary 50 UV-Vis spectrophotometer by monitoring absorbance at 340 nm. All assays were started with addition of the preassembled WT-MR complex.
**Nuclease Assays**—The ssDNA endonuclease activity was monitored using single-strand circular M13 DNA as a substrate (1 µg per lane). The reactions were carried out in standard buffer with 0.3 mM MnCl₂. Mre11 and Rad50 concentrations were held at 0.3 µM. At the indicated time points, 10 µL of reaction was removed and quenched with EDTA. Each time point was then run out on a 1% TAE-agarose gel containing ethidium bromide and visualized using UV light.

The steady state product profile of the MR complex was visualized using denaturing polyacrylamide gel electrophoresis (PAGE). All assays were done at 30° C in standard buffer with 0.3 mM MnCl₂ in the presence of 1.3 µM hexachlorofluorescein (HEX) labeled DNA substrate. The HEX label was attached to the 5’ end of the ds50-F primer and a phosphorothioate linkage was introduced between the 1st and 2nd nucleotides of the ds50-R substrate (relative to the 3’ end). The phosphorothioate linkage prevented Mre11 from excising nucleotides from the unlabeled DNA strand. The Mre11 and Rad50 concentrations varied and are denoted in each individual figure legend. Reactions that used an ATP regeneration system contained 1 mM ATP, 7 mM phosphocreatine, and 2 units of creatine kinase. The reactions were started by addition of the DNA substrate (containing ATP and MnCl₂) to an equal volume of the protein solution. At times 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 minutes a 12 µL aliquot was removed and quenched with an equal volume of quench buffer containing 50% formamide and 100 mM EDTA. The zero time point was taken prior to the initiation of the reaction by sequentially adding an aliquot of the protein solution and an aliquot of the DNA solution to the quench buffer. Reaction products were resolved with 16% denaturing PAGE containing 7.5 M urea in 1X TBE buffer. Gels were run for 3 – 3.5 h at a constant power of 60 W. The gel was visualized on a
Typhoon Phosphorimager using a 532 nm laser and a 555 nm bandpass filter and analyzed using the ImageJ software (NIH).

The pre-steady state exonuclease assays were performed as described for the steady state assays with some exceptions. The DNA substrate was identical, except that a biotin group was attached to the 3’ end of the ds50-R DNA strand. The reactions included a two-fold molar excess of streptavidin (tetramer) relative to DNA concentration in order to completely block the nuclease activity of Mre11 on the unlabeled DNA strand under high enzyme concentrations. The concentrations of DNA, Rad50, and Mre11 were 100 nM, 3 µM, and 3.15 µM, respectively. ATP concentrations used were 5 x the \( K_m \)-ATP for each protein and no ATP regeneration system was used. Time points for the assays were 0, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, and 120 seconds.

The uniformly labeled 1.96 kb dsDNA used in the exonuclease reaction shown in Fig. 2D was prepared by performing a 100 µL PCR reaction in Long Amp polymerase buffer using 5 units of Long Amp polymerase, 30 µCi of [\(-^{32}\)P] dGTP, 0.25 mM cold dNTP mix, 1 µM T7 promoter primer, 1 µM T7 terminator primer, and 50 ng of the gp46-pET28a vector. The labeled PCR product was purified using the Qiagen PCR purification protocol. Three more PCR reactions were setup with the same conditions, but lacking the [\(-^{32}\)P] dGTP. They were subject to the same PCR cycle and purification protocols and quantitated using their A\(_{260}\) values. The three values were averaged and that value was used for the [\(-^{32}\)P] dGTP labeled product. The exonuclease reaction was performed in the standard buffer with 0.3 mM MnCl\(_2\), 7mM phosphocreatine, and 2 units/20 µL of creatine kinase at pH 7.6. Assays were performed at 30°C and with 5 nM of uniformly [\(-^{32}\)P] dGTP labeled 1.96 kb dsDNA. The ATP concentration was kept at 5 x the \( K_m \)-ATP for each protein. Mre11 and
Rad50 were at concentrations of 1.25 µM and 1.0 µM, respectively. The assays were started by addition of the DNA substrate to the protein solution. The assays were quenched by adding 2 µL of the assay to an equal volume of 250 mM EDTA. Time points of 0, 1, 2, 4, 8, 16, and 32 minutes were taken for both the WT- and L101D-MR complex. The zero time point was taken prior to the initiation of the reaction by sequentially adding an aliquot of the protein solution and an aliquot of the DNA solution to the quench buffer. The reactions were analyzed by spotting 2 µL onto Baker-flex cellulose PEI-F TLC plates and developed in 300 mM KPi pH 7.0. The TLC plates were dried, then exposed for 2 hours to a PhosphorImager plate. After exposure, the plate was visualized on a Typhoon Phosphorimager and analyzed on the ImageJ software.

2-Aminopurine Assays – Nuclease activity was also probed spectrofluorometrically using DNA substrates with a fluorescent 2-aminopurine deoxyribonucleotide (2-AP) at either the 1st, 2nd, 5th, 9th, 12th, 17th, or 22nd position relative to the 3’ end of the substrate. The assays were performed in the standard buffer with 0.3 mM MnCl₂ and 1.3 µM DNA substrate. In assays containing ATP, the ATP concentration was 5 x the $K_m$-ATP for each protein. Steady state exonuclease assays were performed at 30°C, pH 7.6, in a volume of 300 µL. The concentration of the MR complex was held at 400 nM. An MR concentration of 100 nM was used in the assays probing the 1st and 2nd positions in the presence of ATP. An MR concentration of 50 nM was used in the L101D-MR complex assay probing the 1st position in the absence of ATP. Mre11 was always held in slight excess over Rad50 (1.05:1 ratio, Mre11:Rad50). The 2-AP was excited at a wavelength of 310 nm and the increase in fluorescence was monitored at 375 nm on a Varian Cary Eclipse spectrofluorometer. Pre-steady state assays were performed using a Bio-logic SFM-400 stopped flow apparatus held
at 30°C in the standard assay buffer with 0.3 mM MnCl$_2$. In assays containing WT-MR complex, the ATP, DNA, and protein concentrations were 1 mM, 200 nM, and 3 µM, respectively. In assays containing the L101D-MR complex, the ATP, DNA, and protein concentrations were 1 mM, 200 nM, and 2 µM, respectively. The nucleotide, DNA, and protein were held in separate syringes and simultaneously injected into the flow cell at time zero.

**DNA Binding Assays** – The DNA binding affinity of the MR complex was determined using a fluorescence polarization assay carried out with a Synergy 2 Multi-Mode microplate reader. All assays were done at ambient temperature in the standard buffer with 10 nM hexachlorofluorescein labeled DNA substrate. Mre11 and Rad50 concentrations used were 12 µM followed by a series of ten two-fold serial dilutions and a zero protein condition. In the MR complex assays, Mre11 was always held in slight excess over Rad50 (1.05:1 ratio, Mre11:Rad50). The ATP concentration was kept at 5 x the $K_m$-ATP for each protein. The reactions were started by the sequential addition of the protein and the DNA substrate (containing ATP, when applicable) to a well in a black 96-well microplate. The total volume for each individual well was 100 µL. Samples were excited at 520 nM and polarization data was collected at 560 nM. Data was analyzed and $K_d$ values were determined using SigmaPlot.

**Oligomeric Determination of the T4 Mre11/Rad50 Complex** – Oligomeric determination was carried out as previously described with the exception that 200 mM NaCl was used in the buffer instead of 50 mM NaCl (18).
Results

Rationale for the L101D mutation – The T4 homology model generated with the Swiss-model server (33) using the structure of the *Pyrococcus furiosus* Mre11 (PDBid 3DSC) indicated that Leu\textsuperscript{101} is the T4 phage amino acid corresponding to residue Leu\textsuperscript{97} from *P. furiosus*, which when mutated has been shown to disrupt dimerization (34). Although it is possible that the T4 homology model may not be entirely accurate, it is highly likely that the prediction for the core T4 Mre11 structure is correct. To date, the published Mre11 structures from a prokaryote (*Thermotoga maritima*), two eukaryotes (human and *Schizosaccharomyces pombe*) and two archaea (*P. furiosus* and *Methanococcus jannaschii*) all share a very similar core catalytic domain (28, 34–37). The dimeric interface of the prokaryotic and archaea Mre11 structures vary somewhat with respect to the orientation of the monomeric subunits, but the residues that make up the hydrophobic interface are highly conserved (37). The equivalent residue in *S. pombe* Mre11 (Ile\textsuperscript{148}) is part of the hydrophobic interface as well (35). However, the human Mre11 dimer interface is somewhat different, with the equivalent residue (Leu\textsuperscript{143}) being part of the interface helix, but not spanning the interface. Instead, it may be involved in an interaction with an adjacent helix in the same subunit (36). Despite these differences, we can conclude with some degree of confidence that Leu\textsuperscript{101} resides in or at least very near the T4 phage Mre11 dimeric interface. We expected that introduction of a negatively charged side chain into this interface would destabilize this region and therefore test the role of this interface in the overall functioning of the MR complex.

Steady state ATP hydrolysis Activity – The addition of WT-Mre11 and dsDNA to Rad50 results in a 22-fold increase in $k_{\text{cat}}$-ATP, a 3-fold increase in $K_m$-ATP, and an ATP
cooperativity increase (i.e., Hill coefficient) from 1.4 to 2.4 (18). These parameters were determined for the L101D-MR complex to determine the effect of disrupting the dimeric interface on the allosteric activation of Rad50 (Table 1). The $k_{\text{cat}}$-ATP of the L101D-MR-DNA complex was 10-fold lower than that of the WT-MR-DNA complex, whereas the $K_m$-ATP and ATP cooperativity was effectively unchanged. In the absence of dsDNA, the $k_{\text{cat}}$-ATP of the L101D-MR complex was nearly normal, indicating that the binding of L101D-Mre11 to Rad50 does not negatively affect the conformation of Rad50 in the absence of dsDNA. Consistent with this, the CD spectrum of L101D-Mre11 was essentially identical to that of the WT enzyme (supplemental Fig. S1).

DNA Binding – To determine the relative affinities of WT- and L101D-MR complexes for dsDNA and ssDNA, we employed a fluorescent polarization assay using either dsDNA or ssDNA with a single 5’ hexachlorofluorescein label. As seen in Table 2, Rad50 by itself binds dsDNA with relatively high affinity and is unaffected by the presence of ATP ($K_d$ values of 0.14 and 0.22 µM in the absence and presence of ATP, respectively). Neither WT- nor L101D-Mre11 displays measurable dsDNA binding at concentrations up to 12 µM. Surprisingly, when either WT- or L101D-Mre11 was added to Rad50 in the absence of ATP, the resulting MR complex had weaker affinity for both dsDNA and ssDNA as compared to Rad50 by itself ($K_d$ values of 0.69 and 0.62 µM for the WT- and L101D-MR complex, respectively). However, upon addition of ATP, tight binding is restored ($K_d$ values of 0.15 and 0.13 µM for the WT- and L101D-MR complex, respectively). The affinity of Rad50 for ssDNA is nearly identical to that of dsDNA ($K_d$ values of 0.16 and 0.26 µM in the absence and presence of ATP, respectively). Also, similar to what is observed with dsDNA, both the WT- and L101D-MR complex have lower affinity for ssDNA as compared to Rad50 alone.
(K\textsubscript{d} values of 0.62 and 0.96 µM for the WT- and L101D-MR complex, respectively).

However, unlike dsDNA binding, the inclusion of ATP does not greatly affect the affinity of the MR complex for ssDNA (K\textsubscript{d} values of 0.48 and 0.39 µM for the WT- and L101D-MR complex, respectively).

*Competition by L101D-Mre11 with WT-Mre11 for Rad50* – To compare the affinity of WT-Mre11 and L101D-Mre11 for Rad50 a spectrophotometric ATPase assay was utilized. In the assay, WT-Mre11 and Rad50 were both held at a concentration of 0.1 µM and DNA was saturating at 1 µM. The concentration of L101D-Mre11 was varied between 0 and 1.0 µM. The relatively low \(k\text{cat}-ATP\) of the L101D-MR complex compared to the WT-MR complex made this a suitable assay (i.e., formation of the L101D-MR complex will result in a lower activity compared to the WT-MR complex). The apparent \(k\text{cat}-ATP\) (i.e., a specific activity determination at saturating concentrations of ATP and DNA) was determined and plotted for each condition (Fig. 2B). As seen in Fig. 2B, L101D-Mre11 successfully out-competed WT-Mre11, with a calculated IC\textsubscript{50} of 0.11 ± 0.01 µM, which indicates equal affinity since the concentration of WT-Mre11 was 0.1 µM.

*Oligomeric Determination of the T4 Mre11/Rad50 Complex* – We previously determined that the T4 WT-MR complex is a heterotetramer (Mre11\textsubscript{2}/Rad50\textsubscript{2}) in solution using sedimentation equilibrium analytical ultracentrifugation (AUC) (31). Here, we again used AUC to determine the oligomeric state of WT-Mre11, L101D-Mre11, and the L101D-MR complex. Due to the instability of Rad50 alone during the prolonged AUC run (~96 hours), we are unfortunately unable to determine its oligomeric state in the absence of bound Mre11. The determined molecular weights are provided in Table 3. We found that both WT- and L101D-Mre11 protein are monomeric in the absence of Rad50 (41.4 and 40.9 kDa,
respectively, with a calculated MW for the monomer of 39.1 kDa). As suggested by the competition assay, L101D-Mre11 forms a stable complex with Rad50 and has the expected molecular weight for a stable Mre11<sub>2</sub>/Rad50<sub>2</sub> heterotetramer of 227.7 kDa (supplemental Fig. S2), with a calculated MW for the heterotetramer of 205.6 kDa. A model containing an equilibrium between MR complex tetramers and dimeric and/or monomeric subunits was not required to produce small residuals.

*Nuclease Activity* – The ssDNA endonuclease activity was probed using a circular M13 substrate. As shown in Fig. 2C, under these conditions neither WT-Mre11 nor L101D-Mre11 alone is effective as an ssDNA endonuclease. However, in the presence of Rad50, even in the absence of ATP, the endonuclease activity of both the WT- and L101D-MR complex is apparent. The Rad50-induced enhancement of endonuclease activity is another indication that the L101D mutation does not affect the ability of Mre11 to bind to Rad50. Additionally, the mutation does not appear to have a significant impact on the ssDNA endonuclease of the complex, consistent with results from the *P. furiosus* system (34).

The exonuclease activity of the WT and L101D-MR complexes were first investigated using a uniformly P-32 labeled 1.96 kb linear blunt-ended DNA substrate. Nuclease activity was detected by separating the substrate and products using TLC, visualized by exposure to a Phosphorimager plate, and quantified using ImageJ (Fig 2D). The WT-MR complex exonuclease activity was fit to an equation with single-exponential and linear terms:

\[
y = a(1 - e^{-bx}) + (cx + d) \quad \text{(Eq. 1)}
\]
The single-exponential represents the dsDNA exonuclease activity and the much slower linear phase presumably represents a ssDNA nuclease reaction. The analysis provided a rate of 9.9 ± 1.2 nts removed per second. Because the slow linear phase was less apparent compared to the WT reaction (likely due to the slower exponential phase), the data for the L101D-MR complex were fit to a simple single-exponential equation:

\[ y = a(1 - e^{-bx}) \]  \hspace{1cm} (Eq. 2).

The determined rate constant for the L101D-MR complex was 1.1 ± 0.1 nts removed per second.

The steady state nuclease activity was then assayed using a denaturing PAGE gel to visualize the product profile of a 50 bp hexachlorofluorescein labeled DNA substrate (Fig. 3A). The ATP-dependent activity produces short DNA products (i.e., less than 10 nts) at the early time points, indicating that the WT-MR complex is reasonably processive. The L101D-MR complex does not appear to have the same level as processivity. It does, however, appear to be more efficient at initiating the nuclease reaction compared to the WT-MR complex, as judged by the more rapid loss of the 50 bp DNA substrate. We also monitored the steady state exonuclease activity of the WT- and L101D-MR complex using a real-time fluorescent assay using 2-AP as a reporter for nucleotide removal. Liberation of the 2-AP from the dsDNA substrate results in a large increase in fluorescence at 375 nm. We placed 2-AP probes at various positions along the DNA substrate and followed their removal at saturating concentrations of ATP. As seen in Fig 3B, the L101D-MR complex
outperforms the WT-MR complex at each 2-AP position by a range of 4 to 13-fold, consistent with the PAGE analysis.

In contrast to the ATP-dependent exonuclease reaction, in the absence of ATP, the L101D-MR complex has lower activity compared to the WT-MR complex. PAGE analysis of the nuclease reaction (Fig. 4A) indicates that while the L101D-MR complex appears to initiate the nuclease reaction normally (a single cut is seen at the first time point), it is very slow to make subsequent nucleotide excisions. The ATP-independent exonuclease reaction carried out by the WT-MR complex appears less processive than in the presence of ATP, but DNA products of less than 10 nts in length are still observed starting at approximately the 50 minute time point. The 2-AP fluorescence assay (Fig. 4B) is consistent with the PAGE analysis, when the 2-AP probe is at the 1st position the WT- and L101D-MR complexes have nearly identical exonuclease rates. However, when the 2-AP probe is moved away from the 3’ end, the exonuclease activity of the WT-MR complex exceeds that of the L101D-MR complex.

We next examined the pre-steady state exonuclease activity of the WT- and L101D-MR complex. The product profile of the ATP-dependent reaction was visualized using denaturing PAGE. In the pre-steady state reaction, the concentration of protein is held in a 30-fold excess over the concentration of DNA substrate. As shown in Fig. 5A, similar patterns are seen in the pre-steady state product profile as were seen in the steady state assay (Fig. 3A). The WT-MR complex produces products of the final length (distributed around 5 nts) even at the earliest time-point. Because no intermediate lengths of product DNA are observed, we tested the possibility that the products are the result of an endonuclease activity cutting approximately ~5 bp from the 5’ P-32 label instead of the expected 3’ to 5’
exonuclease activity. We performed a reaction under identical conditions but used a DNA substrate containing a phosphorothioate linkage 24 nts away from the 3’ end. As seen in Fig 5B, a build-up of products is seen at the exact position of the phosphorothioate linkage, indicating that the products produced in Fig. 5A are the result of exonuclease activity. The pre-steady state product profile of the L101D-MR complex reaction mirrors that of the steady state reaction. In contrast to the WT-MR complex, intermediate lengths of DNA are observed between the full-length DNA substrate and the final product. The smallest product in the L101D reaction does not appear until approximately the 30 second time point and a progressive decrease in product length is observed with increasing time.

We also employed the 2-AP probe to monitor the ATP-dependent pre-steady state exonuclease activity of the WT- and L101D-MR complexes in using stopped-flow fluorescence. The 2-AP probe was placed at either the 1st or 12th nucleotide relative to the 3’ end of the DNA substrate. As seen in Fig. 6, the results mirror that of the pre-steady state reactions analyzed using denaturing PAGE. The WT-MR complex is significantly slower than the L101D-MR complex at removing the 2-AP probe located at either the 1st or 12th position (Fig. 6A and 6B, blue traces); however, the WT-MR complex product curve using the 12th position DNA substrate displays no lag period (Fig 6A, red trace), whereas a prominent lag is observed in the L101D-MR complex reaction (Fig. 6B, red trace).

Discussion

It is thought that the Rad50 and Mre11 subunits of the MR complex are allosterically coupled to each other (24, 25, 27, 28, 38). X-ray crystal structures and protein-protein crosslinking experiments have demonstrated that ATP binding and/or hydrolysis by Rad50 affects the conformation of Mre11 (25, 27). Our data has shown that the communication
between Rad50 and Mre11 is bidirectional, with Mre11 affecting the catalytic activity of Rad50 (18). WT-Mre11, along with dsDNA, increases the ATP hydrolysis activity of T4 Rad50 by approximately 20-fold (18). A similar degree of activation (20-fold) is observed with nuclease-deficient Mre11 mutants, indicating that the activation is not directly related to the nuclease activity of Mre11. However, this allosteric activation by DNA may not be general to MR complexes from all species. The ATPase activity of the MR complex from *P. furiosus* is unaffected by addition of dsDNA (39), although it is unclear if the lack of activation is due to the absence of a zinc-hook in the mutant Rad50 protein that was used. We have found that in the T4 system, site-directed mutation of the zinc-hook completely eliminates the DNA activation of the ATPase activity. In contrast to WT-Mre11, L101D-Mre11 only activates Rad50 ATP hydrolysis two-fold in the presence of dsDNA. This decrease in activation is not due to a decrease in the stability of the complex because nuclease, Rad50 competition, and AUC experiments all indicate that the affinity of L101D-Mre11 for Rad50 is unaffected by the mutation. DNA binding experiments also indicate that the L101D-MR complex has normal affinity towards dsDNA.

The decrease in DNA activation of the L101D-MR complex suggests that when DNA is present, there is a tight coupling between the altered structure of the Mre11 dimer interface and the structure/activity of Rad50. On the other hand, the ATPase activity of the L101D-MR complex is nearly normal without DNA, suggesting that the tight coupling between Mre11 and Rad50 requires the formation of the MR-DNA complex. Alternatively, it is possible that the Mre11 dimer interface only forms in the presence of dsDNA so that the introduction of the L101D mutation has no effect on the conformation of Mre11 in the absence of DNA. However, the available crystal structures of the MR complex argue against
this possibility since they have all shown a dimeric Mre11, regardless of the presence or absence of a bound DNA substrate (25, 28, 34, 36, 37).

The ATP-independent nuclease activity of L101D-Mre11 on ssDNA (Fig. 2C) and dsDNA with the 2-AP probe at the 1st position (Fig. 4B) demonstrates that the intrinsic nuclease activity of L101D-Mre11 is unaffected. This indicates that the Mre11 active site is unperturbed, suggesting that the conformation of the L101D-Mre11 subunit itself is not greatly affected by the introduction of the negative charge into the dimer interface. However, the ATP-dependent exonuclease activity of the L101D-MR complex, when assayed using a 1.96 kb dsDNA substrate, is reduced by 9.2-fold (Fig. 2D). Since the intrinsic nuclease rate of L101D-Mre11 appears to be normal, the reduction in repetitive exonuclease activity is likely caused by a reduction in processivity or the translocation rate, which may be linked to the reduced ATP hydrolysis of the L101D-MR-DNA complex.

Based on the decrease in exonuclease activity of the L101D-MR complex when assayed with the 1.96 kb DNA substrate, we were surprised to observe elevated nuclease rates when assayed with the 50 bp dsDNA substrate. In the presence of ATP, the L101D-MR complex displays higher activity than the WT-MR complex when the 2-AP nuclease probe is placed at positions ranging from 1 to 22 (Fig. 3B). We have previously determined the rate-limiting step of the steady state nuclease reaction to be the productive assembly of the MR complex onto the DNA substrate (31, 38). Therefore, the higher activity of the L101D-MR complex suggests that its productive assembly rate is increased compared to the WT-MR complex. We are using the term “productive assembly” to represent an event that differs from simple DNA binding. Our DNA binding assays indicate that the L101D-MR complex has nearly identical affinity for duplex DNA as the WT-MR complex. Additionally, a
comparison of the ATP hydrolysis to the exonuclease rates suggests that the WT-MR complex likely undergoes several rounds of unproductive binding events prior to successfully removing the first nucleotide from the DNA substrate (the $k_{cat}$’s for ATP hydrolysis and 1$^{st}$ nucleotide removal are 3.2 and 0.15 s$^{-1}$, respectively).

The PAGE analysis of a 5’ labeled DNA substrate is consistent with a faster assembly rate and also reveals that the processivity of the L101D-MR is greatly reduced compared to the WT-MR complex (Fig. 3A). In the WT-MR complex reaction, very few intermediates are observed between the full-length DNA substrate and the final products that are less than 10 nts in length. This pattern is highly indicative of a slow rate-limiting assembly step, followed by a rapid (relative to the acquisition time) and processive exonuclease reaction. On the other hand, in the case of the L101D-MR complex, the pattern is more indicative of rapid assembly and a rate-limiting nucleotide excision or translocation step (Fig. 3B). Since the intrinsic nuclease activity of the L101D-MR complex is unaffected, it suggests that the defect lies in the translocation step that follows nucleotide excision. Also, the L101D-MR complex does not excise as many nucleotides from the DNA substrate as the WT enzyme (i.e., the smallest product for WT is approximately 3 nts, whereas for L101D it is approximately 6 nts). The cause of this difference is not clear, but it is possible that the size of the DNA remaining is simply related to the melting temperature of the DNA strand and the speed at which the MR complex moves along the DNA substrate (i.e., the WT complex excises more nucleotides prior to DNA strand melting due to its higher processivity and speed of translocation). This explanation is consistent with the production of a range of products rather than a single discreet band.
The more rapid assembly of L101D-MR complex as compared to the WT complex is difficult to explain within the context of an Mre11 dimer interface that is stable and unchanging throughout the nuclease reaction cycle. The x-ray crystal structures of the truncated MR complex in the absence and presence of ATP suggest minor changes in the Mre11 dimer interface and biochemical experiments suggest a dynamic interface as well (25, 27). Protein cross-linking experiments performed in the Hopfner laboratory indicate that binding of ATP to the MR complex alters the conformation of the Mre11 dimer interface leading to a decrease in cross-linking efficiency (27). Inclusion of ADP rather than ATP increases cross-linking efficiency, indicating that ATP and ADP stabilize different forms of the Mre11 dimer interface. It is unclear whether the L101D mutation is stabilizing the apo, ADP, or ATP form of the Mre11 dimer interface, or if it produces an alternative conformation that the WT-MR complex does not normally adopt during its nuclease cycle. It is tempting to speculate, however, that because L101D-MR complex has a more rapid productive assembly rate and a normal intrinsic nuclease rate, the mutation stabilizes the native conformation of the MR complex that is most competent for initiation of nuclease activity.

The pre-steady state kinetic data is also consistent with a slow rate-limiting assembly of the WT-MR complex, followed by a rapid and processive exonuclease reaction. In the reaction shown in Fig. 5A the ratio of protein to DNA is 30:1 and the reaction rate has reached a protein-independent phase, such that increasing the concentration of protein has no effect on the apparent rate of the reaction. Even at these extremely high protein concentrations, the product pattern is similar to that seen in the steady state nuclease reaction shown in Fig. 3A. The absence of observable intermediates in the pre-steady state WT
reaction indicates that the rate-limiting step remains productive assembly, even under high-protein conditions.

The shape of the product formation curve in the pre-steady state stopped-flow experiments (Fig. 6A and 6B) also provides information regarding the rate-limiting steps of the nuclease reaction. In the situation where productive assembly is fast compared to the translocation/nuclease rate, a distinct lag phase should be seen as the probe is moved away from the 1st position, analogous to what is often observed in all-or-none helicase unwinding assays (40). This is precisely what is observed in the L101D-MR complex reaction, consistent with the conclusions from the steady state and pre-steady state PAGE analysis. On the other hand, the product formation curve of the WT-MR complex shows no apparent lag as the probe is moved away from the 3’ end of the DNA. Again, as the PAGE analysis suggested, this is consistent with a rate-limiting productive assembly step, followed by rapid exonuclease activity and translocation.

Based on the data presented here, we propose a two-state working model for the Mre11 dimer interface (Fig. 7). The first state (the ‘nuclease initiation state’) forms during the initiation of nuclease activity and is promoted by the L101D mutation. The second state (the ‘translocation state’) forms during the processive exonuclease reaction, likely an ATP hydrolysis-driven translocation step, and is hindered by the L101D mutation. The exact nature of the Mre11 dimeric interface in these two states is unknown. Given the extreme nature of the Leu$^{101}$ to Asp mutation, it is tempting to speculate that in the nuclease initiation state, the Mre11 dimeric interface is altered in such a way as to remove the leucine residue from the hydrophobic pocket that is observed in the x-ray crystal structures. We propose that the Mre11 dimeric interface that has been repeatedly observed in the x-ray crystal structures
(i.e., with Leu\textsuperscript{101} engaged in the hydrophobic pocket) is the conformation of the translocation state.

Our current working model for the Mre11 interface has the WT-MR complex sampling both states when not bound to DNA, with the equilibrium lying in the direction of the translocation state. This equilibrium is included in the model to explain the observation that the DNA-dependent ATP hydrolysis rate is much faster than the steady state nuclease rate (i.e., all binding events result in ATP hydrolysis, but only a few result in the excision of a nucleotide from the DNA substrate). Following productive assembly, the MR complex remains in the ‘initiation state’ for the removal of the nucleotide from the 3’ end of the dsDNA. If nucleotide excision occurs in the absence of ATP, a portion of the WT-MR complex dissociates from the DNA, where it can rebind DNA substrate to excise another nucleotide (non-processive translocation). The release of the DNA by the MR complex may involve a conformational change in the Mre11 dimer interface, since the L101D-MR complex appears to stall on the DNA substrate in the absence of ATP, rather than releasing it with the same rate at the WT-MR complex (see Fig. 4). After nucleotide excision in the presence of ATP, ATP hydrolysis by the WT-MR complex triggers a conformational change to ‘translocation state’ and the complex translocates along the nascent ssDNA in preparation for the next nucleotide excision. The translocation step itself could occur following either ATP hydrolysis or ADP/Pi product release. After the translocation step, the WT-MR complex returns to the ‘nuclease initiation state’ to complete the cycle. The L101D-MR complex binds and hydrolyzes ATP (albeit at a slower rate than WT), but because it is unable to form the ‘translocation state’ it dissociates from the DNA rather than moving forward for the next nucleotide removal, hence its lower processivity.
As previously suggested by Hopfner and colleagues (27), a model involving repetitive conformational changes in Mre11, which are driven by Rad50 ATP hydrolysis, is reminiscent of how the NBD of ABC transporters drive the opening and closing of their transmembrane domain to allow substrates into or out of the cell (16). This is consistent with the notion that all members of the ABC protein super-family share a unified functional architecture (19, 27).

In the *S. pombe* system, Nbs1 binding to Mre11 causing several conformational rearrangements in the Mre11 dimer interface (35).

**Acknowledgements**

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**Footnotes**

1 The abbreviations used are: T4 phage, bacteriophage T4; DSB, double-strand break; HR, homologous recombination; gp46, T4 gene product 46; gp47, T4 gene product 47; 2-AP, 2-aminopurine deoxyribonucleotide; MR, Mre11/Rad50; IR, ionizing radiation; NBD, nucleotide binding domain; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; HEX, hexachlorofluoroscein; ABC, ATP binding cassette; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; MR-DNA, Mre11/Rad50-DNA complex; PDBid, protein data bank id; WT, wild-type. 2 Albrecht and Nelson, unpublished observations. 3 Barfoot and Nelson, unpublished observations.

**References**

Table 1

Steady state ATPase Kinetic Constants for WT and L101D MR-DNA Complexes

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>DNA Activation$^b$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad50$^c$</td>
<td>16 ± 0.8</td>
<td>0.146 ± 0.003</td>
<td>N.A.</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Rad50/Mre11$^c$</td>
<td>42 ± 1</td>
<td>0.226 ± 0.006</td>
<td>N.A.</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Rad50/Mre11$^{11111}$</td>
<td>50 ± 1</td>
<td>0.101 ± 0.002</td>
<td>N.A.</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Rad50/Mre11$^{c}$</td>
<td>49 ± 2</td>
<td>3.2 ± 0.1</td>
<td>22</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Rad50/Mre11$^{11111}$</td>
<td>37 ± 1</td>
<td>0.32 ± 0.01</td>
<td>2.2</td>
<td>2.5 ± 0.4</td>
</tr>
</tbody>
</table>

$^a$ Fluorometric assays were performed in 50 mM Tris-HCl, 50 mM KCl, 0.1 mg/ml BSA (pH 7.6) in the presence of 5 mM MgCl$_2$ at 30 °C. Values determined by fitting data to a Hill equation. Errors represent the standard error of the fit. $^b$ Fold increase in $k_{cat}$ between MR-DNA and Rad50. $^c$ Values from reference (18).
Table 2

Dissociation constants for dsDNA and ssDNA

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_d$ (µM) dsDNA</th>
<th>$K_d$ (µM) ssDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad50</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Rad50/ATP</td>
<td>0.22 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Mre11</td>
<td>not detectable</td>
<td>not detectable</td>
</tr>
<tr>
<td>Mre11L101D</td>
<td>not detectable</td>
<td>not detectable</td>
</tr>
<tr>
<td>Mre11/Rad50</td>
<td>0.69 ± 0.09</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>Mre11/Rad50/ATP</td>
<td>0.15 ± 0.03</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td>Mre11L101D/Rad50</td>
<td>0.62 ± 0.13</td>
<td>0.96 ± 0.13</td>
</tr>
<tr>
<td>Mre11L101D/Rad50/ATP</td>
<td>0.13 ± 0.03</td>
<td>0.39 ± 0.08</td>
</tr>
</tbody>
</table>

$^a$ Polarization assays were performed in 50 mM Tris-HCl, 50 mM KCl, 0.1 mg/ml BSA (pH 7.6) in the presence of 5 mM MgCl$_2$ at room temperature. Protein concentrations ranged from 0 to 12 µM in each reaction with a DNA concentration of 10 nM. Assays containing ATP had concentrations of 0.25 mM and 0.185 mM for WT and L101D, respectively. Values determined by fitting data to simple equilibrium binding mechanism using SigmaPlot software.
Table 3

*Oligomeric determination of the WT and L101D-Mre11 and Mre11/Rad50 Complex*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Mre11</td>
<td>41,463 ± 769</td>
</tr>
<tr>
<td>L101D-Mre11</td>
<td>40,904 ± 1,679</td>
</tr>
<tr>
<td>WT-Mre11/WT-Rad50</td>
<td>204,717 ± 7,878</td>
</tr>
<tr>
<td>L101D-Mre11/WT-Rad50</td>
<td>227,706 ± 8,614</td>
</tr>
</tbody>
</table>

*Analysis performed using the Sedphat software. Global fits were accomplished with the Simplex fitting algorithm. MW estimates were derived from a Monte-Carlo simulation for non-linear analysis. The MW values reported are the average of the derived estimates of three protein concentrations. Errors are the standard deviations of the derived estimates of the three protein concentrations.*

\(^a^\) Deduced MW for Mre11 is 39,168 Da.

\(^b^\) Deduced MW for the (Mre11\(_2\))/(Rad50\(_2\)) complex is 205,568 Da.
Figure 1. **The MR complex heterotetramer with truncated Rad50 coiled-coil domains.** The ribbon structure is based on PDBid 3THO (25). The Rad50 subunits are colored cyan and green and the Mre11 subunits are colored blue and yellow. The Mre11 dimer interface is indicated. Two molecules of ATP bind at the Rad50 interface and the Mre11 nuclease active site is indicated by the purple spheres representing the active site Mn$^{2+}$ ions.
Figure 2. A) Close-up view of the modeled T4 Mre11 dimer interface. The homology model was generated using the Swiss Model server (39) and based on PDBid 3DSC (36). B) Competition assay for Rad50. Each spectrophotometric ATPase assay consisted of the standard buffer with 0.1 µM WT-Mre11, L101D-Mre11 at the concentration indicated, 0.1 µM Rad50, 1 mM ATP, and 1.0 µM 50 bp dsDNA. C) ssDNA endonuclease activity assays. The four time points for each indicated condition are 0, 8, 16, and 32 minutes. D) Blunt-ended dsDNA exonuclease activity assays. Assays were performed in standard buffer with 0.3 mM MnCl₂ at 30 °C. ATP concentrations of 0.25 mM and 0.185 mM were used for WT and L101D, respectively. Each assay consisted of 1 µM Rad50, 1.25 µM Mre11, and 5 nM DNA substrate. Time points were 0, 1, 2, 4, 8, 16, and 32 minutes.
A steady state exonuclease activity in the presence of ATP. A) Visualization of nuclease products on a 16% Urea-PAGE gel is shown. Each assay consisted of the standard buffer with 0.3 mM MnCl₂, 100 nM Rad50, 105 nM Mre11, and 1.3 µM DNA substrate. Time points for each protein are 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 minutes. The first and final lanes are no protein controls. Approximate mobility of various DNA lengths are shown on the right-hand side of the gel. B) A plot of the $k_{cat}$-exonuclease for either the WT-MR complex (closed diamonds) or the L101D-MR complex (closed squares) with the 2-AP probe at various positions along the DNA substrate relative to the 3' end. Protein, DNA, and ATP concentrations are given in “Experimental Procedures”.

Figure 3.
Figure 4. **Steady state exonuclease activity in the absence of ATP.** A) Visualization of nuclease products on a 16% Urea-PAGE gel is shown. Each assay consisted of the standard buffer with 0.3 mM MnCl$_2$, 200 nM Rad50, 210 nM Mre11, and 1.3 µM DNA substrate. Time points for each protein are 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 minutes. The first and final lanes are DNA standards with lengths of 50, 40, 30, 20, and 10 nts. B) A plot of the $k_{cat}$-exonuclease for either the WT-MR complex (closed diamonds) or the L101D-MR complex (closed squares) with the 2-AP probe at various positions along the DNA substrate relative to the 3’ end. Protein, DNA, and ATP concentrations are given in “Experimental Procedures”.
Figure 5. **Pre-steady state exonuclease activity in presence of ATP visualized with urea-PAGE.** 

*A*) Assay using standard 50 bp substrate with biotin/streptavidin blocking the 3’ end of ds50-R. Each assay consisted of the standard buffer with 0.3 mM MnCl$_2$, 3 µM Rad50, 3.15 µM Mre11, and 100 nM DNA substrate. Time points for each protein are 0, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, and 120 seconds. 

*B*) Assay using 50 bp substrate with biotin/streptavidin blocking the 3’ end of ds50-R and a phosphorothioate linkage at position 26 on ds50-F. The conditions and time points were identical to those in *A*) except that the 40 second time point was omitted from WT-MR complex reaction.
Figure 6. Pre-steady state exonuclease activity in presence of ATP monitored using stopped-flow fluorescence and 2-AP containing DNA substrates. The assays were carried out at 30°C in the standard buffer with 0.3 mM MnCl$_2$. The blue and red traces are the exonuclease activity using the 50 bp DNA substrate with the 2-AP probe located at the 1$^{st}$ and 12$^{th}$ positions, respectively, relative to the 3’ end of the DNA.  
A) The exonuclease activity of the WT-MR complex. Each assay consisted of 1 mM ATP, 200 nM DNA, and 3 µM WT-MR complex.  
Figure 7. Proposed two-state model for the Mre11 dimer interface based on studies of the T4 MR complex (gp46/47). Details of the model can be found in the text of the Discussion. A legend can be found in the lower left-hand corner. The small purple circle found on each Mre11 subunit represents the nuclease activity site. The ‘initiation state’ of the MR complex is depicted as having a parallel Mre11 dimeric interface and the ‘translocation state’ is depicted with the Mre11 dimer interface in close contact at one end (V-shaped).
Supplemental Figure 1. Circular dichroism spectra of WT- and L101D-Mre11 proteins. The experiment was carried out as described in “Experimental Procedures”.
Supplemental Figure 2. **Oligomeric determination of the WT Rad50/L101D Mre11 complex.**

Representative analytical equilibrium ultracentrifugation results for one complex concentration (initial $A_{280}: 0.6$). Equilibrium concentration gradients were formed at velocities of 11 600 ($\circ$), 13 800 ($\square$), and 15 800 rpm ($\Delta$). Solid lines represent the global fits produced. Inset: fit residuals.
Chapter IV: Conclusions

Summary

The data presented in Chapter II provides for the first time a biochemical characterization of T4 Mre11 and Rad50 complex, which had been the only remaining proteins involved in T4 phage recombination and DSB repair left uncharacterized. The MR complex in T4 phage is shown to have conserved properties with its homologs in other organisms. It has ATPase, endonuclease, and exonuclease and DNA binding activities.

The ATPase activity of Rad50 is quite low by itself ($k_{cat}$ of 0.15 s$^{-1}$). However, upon the addition of Mre11 and dsDNA the activity is increased by ~22 fold and the Hill coefficient increases from 1.4 to 2.4 suggesting that Mre11 and DNA act as activators for Rad50’s catalytic activity and may promote communication between the two nucleotide binding domains of Rad50.

The nuclease activity of the MR complex is shown to be in the Mre11 subunit and ssDNA endonuclease, dsDNA exonuclease, and dsDNA endonuclease activity are all observed. The dsDNA exonuclease activity seen is in a 3’ to 5’ direction which does not agree with the suggested in vivo activity of the MR complex. This has similarly been observed in S. cerevisiae (1). This disagreement in polarity may result from the absence of accessory proteins required for the 5’ to 3’ activity and/or metal dependence of the nuclease reaction. We have shown that Mg$^{2+}$-dependent nuclease assays containing UvsY and gp32 may allow the MR complex to produce a 3’ overhang through a 5’ to 3’ endonuclease activity. This phenomenon is not yet completely understood and may require further investigation to fully comprehend. Similar to the activation seen in Rad50 ATPase activity,
there is an increase in Mre11 nuclease activity in the presence of Rad50. Mre11 does
degrade DNA without Rad50, but the activity is ~100 fold lower. This implies a
bidirectional communication between the subunits of the MR complex.

The introduction of a leucine to aspartic acid mutation at the dimer interface of Mre11
has provided interesting insight into the importance of this dimeric interaction. Initially, we
found that the mutation had little effect on some of the properties of the MR complex. Our
studies have shown that the L101D-Mre11 mutant is able to bind to Rad50 with a similar
affinity as wild-type. Also, the L101D-MR complex appears to have the same stoichiometry
(M₂/R₂) as the wild-type complex. Similarly, the L101D mutation appears to have no impact
on the DNA binding properties. Both wild-type and L101D-Mre11 had no measureable
binding to dsDNA. However, upon addition of Rad50 binding was seen and the Kᵰ values
were similar between the two complexes (0.69 and 0.62 µM for wild-type and L101D-MR
complexes, respectively). The addition of ATP brought about tighter binding (0.15 and 0.13
µM for wild-type and L101D-MR complexes, respectively). Binding properties for ssDNA
followed similar trends.

Major differences were seen, however, in the ATPase and the exonuclease assays.
The L101D-MR complex had a near-normal ATPase rate in the absence of dsDNA.
However, Rad50 only had a 2.2 fold increase in ATPase activity upon the addition of
L101D-Mre11 and dsDNA suggesting that the L101D mutation has somehow disrupted the
communication between Mre11 and Rad50 in the presence of DNA. In the exonuclease
assays performed the L101D-MR complex appeared to have a greatly reduced processivity
compared to wild-type. This does not appear to come from the loss of exonuclease activity
due to nuclease rates at or above wild-type rates on 2-aminopurine assays probing the 1st position.

The possibility of multiple Mre11 conformational states has been previously proposed based on structural data (2, 3). Based on our data we have proposed a model for the MR complex that has Mre11 utilizing at least two states which we have termed the “nuclease initiation” and “translocation” states. The nuclease initiation state is stabilized by the L101D mutation and the translocation state is inhibited by the L101D mutation. Prior to DNA binding the MR complex is in equilibrium between the nuclease initiation and translocation states with the equilibrium favoring the translocation state. Once productive assembly is reached the MR complex removes a nucleotide from the 3’ end of the dsDNA and, if ATP is present, a conformational change to the translocation state occurs. The complex then moves along the DNA to position itself near the next nucleotide. It is not yet clear if the switch to the translocation state occurs after ATP hydrolysis or ADP/Pi release. After completion of the translocation step the complex returns to the nuclease initiation step. The L101D-MR complex is stabilized in the nuclease initiation step and it unable to form the translocation state. Therefore it must dissociate after each nuclease reaction and rebind to the DNA giving it its lower level of processivity. In the absence of DNA the MR complex partially dissociates from the DNA and is able to rebind to excise another nucleotide.

**Future Direction**

It has been shown in the literature that the MR complex is essential for the initial resection in eukaryotic DSB repair (4, 5). However, it recently has been shown that resection of unmodified DNA ends does not require the active MR nuclease complex and that the MR complex may play a structural role in this type of resection (4, 5). RNase H1 has been shown
to remove RNA primers during Okazaki fragment processing and it has also been suggested that it is involved in DNA resection (6, 7). This has prompted our lab to explore the possibility that RNase H1 is involved in DSB resection in T4 phage. We plan to perform the in vitro assays necessary for investigating this possibility while our collaborators at the Kreuzer lab at Duke University plan to perform the in vivo work.

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References

