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Mre11 Protein Complex Prevents Double-Strand Break Accumulation during Chromosomal DNA Replication

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Summary

Mre11 complex promotes repair of DNA double-strand breaks (DSBs). *Xenopus* Mre11 (X-Mre11) has been cloned, and its role in DNA replication and DNA damage checkpoint studied in cell-free extracts. DSBs stimulate the phosphorylation and 3′–5′ exonuclease activity of X-Mre11 complex. This induced phosphorylation is ATM independent. Phosphorylated X-Mre11 is found associated with replicating nuclei. X-Mre11 complex is required to yield normal DNA replication products. Genomic DNA replicated in extracts immunodepleted of X-Mre11 complex accumulates DSBs as demonstrated by TUNEL assay and reactivity to phosphorylated histone H2AX antibodies. In contrast, the ATM-dependent DNA damage checkpoint that blocks DNA replication initiation is X-Mre11 independent. These results strongly suggest that the function of X-Mre11 complex is to repair DSBs that arise during normal DNA replication, thus unraveling a critical link between recombination-dependent repair and DNA replication.

Introduction

Damage to chromosomal DNA induces a complex cellular response designed to delay cell cycle progression until the DNA is repaired (Hensey and Gautier, 1995; Zhou and Elledge, 2000). Surveillance mechanisms monitor the integrity of the genome. Detection of aberrant DNA and chromosome structures coordinately triggers checkpoint pathways that prevent cell cycle progression and activate DNA repair systems.

Response to DNA damage is critical for cell viability. Unchecked DNA damage can lead to mutations, translocations, and abnormal recombination events during S-phase, and also to chromosome breakage and loss during mitosis. Failure to monitor or to signal damaged DNA is a hallmark of cancer cells (Hartwell and Kastan, 1994). Several cancer-prone syndromes reflect defects in the DNA damage response (Shiloh, 1997). The inherited diseases Ataxia-Telangiectasia (A-T), Ataxia-Telangiectasia like disorder (A-TLD), and Nijmegen Breakage Syndrome (NBS) have similar and overlapping phenotypes, and cells derived from these patients have served as a paradigm for the study of checkpoint pathways (Petrini, 2000). In addition to increased risk for cancer, particularly lymphomas, patients affected with these genetic defects are radiation sensitive. Cells derived from these patients exhibit chromosome fragility and radiosensitivity. They also display radio-resistant DNA Synthesis (RDS). A-T, NBS and A-TLD are caused by mutations in ATM, Nbs1/Nibrin, and Mre11, respectively (Carney et al., 1998; Savitsky et al., 1995; Stewart et al., 1999).

Mre11 forms a tight complex with Rad50 and p95/Xrs2 in *S. cerevisiae* and a similar complex with Rad50 and p95/Nbs1 in humans (Bressan et al., 1999; Dolganov et al., 1996). Biochemical fractionation of human cells indicates that most of Mre11 is in a protein complex that copurifies with Rad50 (Dolganov et al., 1996).

The function of Mre11 is best understood in *S. cerevisiae*, where, along with Rad50, it promotes repair of double-strand chromosome breaks by homologous recombination (HR) and nonhomologous end joining (NHEJ) (Bressan et al., 1999; Haber, 1998; Ogawa et al., 1995). *S. cerevisiae* Mre11 or Rad50 deletion mutants are viable, although they grow slowly (Haber, 1998). C.elegans homozygous for an *mre-11* null mutation are viable (Chin and Villeneuve, 2001). In contrast, deletion of *mre11* in mouse or chicken cells is lethal (Yamaguchi-Iwai et al., 1999; Zhao et al., 2000). Deletion of *rad50* or *nbs1* in mouse is also lethal (Luo et al., 1999; Zhu et al., 2001). Why Mre11, Rad50, and Nbs1 are essential in vertebrates in the absence of external DNA damage is unknown. The similarities between the terminal phenotypes of cells lacking Mre11, Rad50, or Nbs1 suggest that these proteins exert their essential role(s) together in a complex. In vitro, yeast and human Mre11/Rad50 complexes act as Mn^{2+}-dependent double-stranded DNA 3′-5′ exonucleases and single-stranded endonucleases (Moreau et al., 1999; Paull and Gellert, 1998; Trujillo et al., 1998). The nuclease activities of the human complex are enhanced by p95/Nbs1 (Paull and Gellert, 1999). The relationship between these in vitro enzymatic activities and the in vivo DNA repair functions of Mre11/Rad50/Nbs1 remains to be established.

Mre11 complexes form foci at the sites of DNA damage and disperse after repair, consistent with a role in the DNA damage response (Mirzoeva and Petrini, 2001). Of particular pertinence to the work described in this paper, foci containing Mre11 are also detected during S-phase (Mirzoeva and Petrini, 2001). An isoform of histone H2A, H2AX, becomes phosphorylated and colocalizes with these foci following DNA damage (Chen et al., 2000; Paull et al., 2000; Rogakou et al., 1999).

DNA damage induces the phosphorylation of both

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Figure 1. X-Mre11 Is Homologous to Human Mre11

The deduced amino acid sequence of *Xenopus* Mre11 was aligned with the sequence of the human Mre11 protein using the Geneworks program (Intellegenetics). Amino acid identities are boxed in yellow.

Mre11 and Nbs1. Mre11 modification occurs in ATM-deficient cells (Dong et al., 1999), whereas phosphorylation of Nbs1 is ATM-dependent. The role of Nbs1 in the DNA damage checkpoint is controversial. Although Nijmegen breakage syndrome cells show no major defects in cell cycle checkpoint (Girard et al., 2000), elimination of the principal ATM phosphorylation site in Nbs1 attenuates the checkpoint (Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000). Localization of Mre11 to ionizing radiation-induced foci does not require ATM (Mirzoeva and Petrini, 2001).

The role of Mre11 in the DNA damage response is still elusive, in part because it is an essential gene (Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999) and because it is involved in several aspects of the response (Haber, 1998). To circumvent these difficulties, we decided to study the functions of Mre11 in cell-free extracts derived from *Xenopus* eggs. These extracts faithfully recapitulate DNA replication and mitosis, as well as the DNA damage checkpoints that regulate these events. We demonstrate that the *Xenopus* homolog of Mre11 (X-Mre11) is phosphorylated during DNA replication or after exposure to DNA carrying double-strand breaks (DSBs). Mre11 complex is essential to produce normal DNA replication products. Depletion of X-Mre11 complex from extracts leads to the dramatic accumulation of DSBs during DNA replication. However, the ATM-dependent DNA damage checkpoint that operates at the onset of S-phase is unaffected by X-Mre11 complex depletion.

Results

Cloning of *Xenopus* Mre11: X-Mre11

Using a PCR strategy with degenerate oligonucleotide primers designed from regions of the Mre11 proteins conserved among yeast, *Drosophila*, and mammals, we amplified a 470 bp DNA fragment that was subsequently used to isolate a full-length clone from a *Xenopus* cDNA library (see Experimental Procedures). The predicted amino acid sequence of X-Mre11 is 711 amino acids long. As expected from the similarity among Mre11 proteins from *S. cerevisiae, C. elegans, D. melanogaster*, and *H. sapiens*, X-Mre11 is highly homologous to the human protein. A protein alignment between the *Xenopus* and human proteins is presented in Figure 1. They are 70% identical over their whole length, and the phosphoesterase/nuclease N-terminal portions of the proteins are the most conserved (83% identity).

Full-length X-Mre11 protein was expressed in *E. coli* and gel purified (see Experimental Procedures). The purified protein was used to generate rabbit polyclonal antibodies. The antiserum recognizes the recombinant protein from *E. coli* lysates (data not shown) and a single polypeptide on Western blots of *Xenopus* egg cytosol (Figure 2A, first lane). When used for immunodepletion, the antibody quantitatively removes X-Mre11 from the extract (Figure 2A, third lane).

X-Mre11 Is a Phosphoprotein

Several proteins involved in the DNA damage response are phosphorylated in response to ionizing radiation. Because radiation generates a variety of aberrant DNA structures, we asked if a defined radiation product, DSBs, could induce X-Mre11 phosphorylation in *Xenopus* interphase extracts. Western blot analysis revealed that a fraction of the X-Mre11 migrated slower on a polyacrylamide gel after exposure to DSBs (Figure 2B, lane 3). This fraction represented phosphorylated X-Mre11, because the band was eliminated by phosphatase treatment. Interestingly, phosphatase treatment converted X-Mre11 from control or DSB-treated extracts into a polypeptide that migrated faster than X-Mre11 (Figure 2B, lanes 2 and 4).

To confirm that X-Mre11 is a phosphoprotein and that its phosphorylation increases following incubation with DSBs, X-Mre11 was immunoprecipitated from γ-^{32}P-ATP-labeled control extracts or extracts incubated with DSBs (Figure 2B, right panel). No ^{32}P-labeled proteins were precipitated by preimmune serum (Figure 2B, PRE). A ^{32}P-labeled polypeptide was immunoprecipitated with X-Mre11 antibodies from extracts treated with

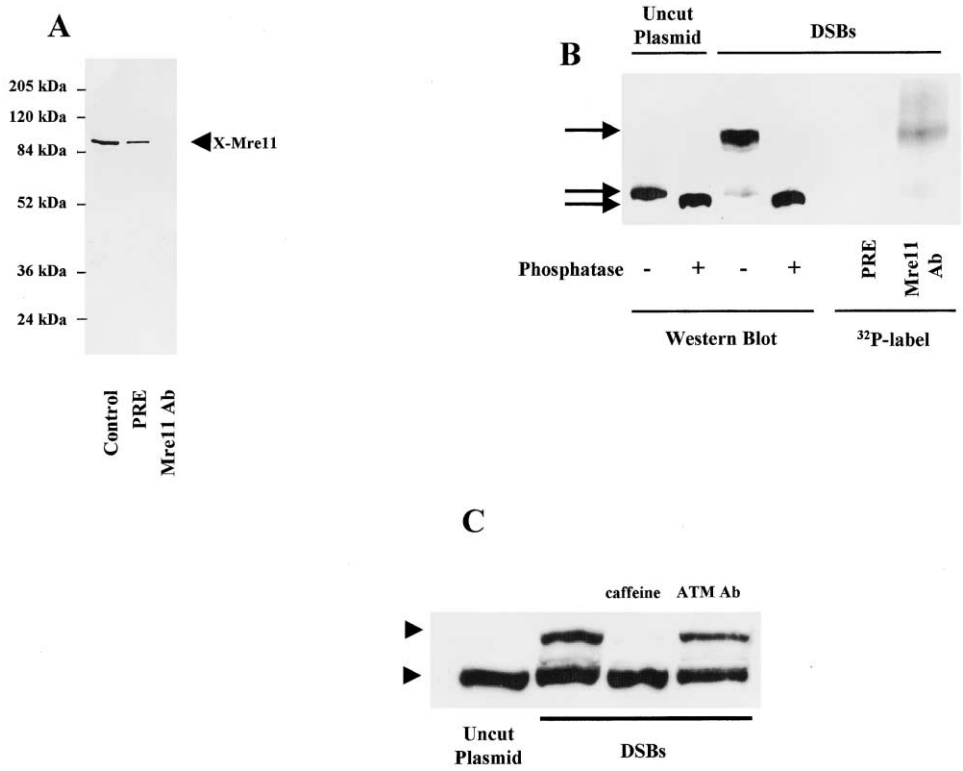

Figure 2. X-Mre11 Is Phosphorylated in a DSBs-Dependent, ATM-Independent Manner

(A) Untreated interphase extract (Control), extract incubated with protein A beads bound to preimmune serum (PRE), and extract incubated with protein A beads bound to anti-X-Mre11 antibodies (X-Mre11 Ab) were probed with a polyclonal serum specific for X-Mre11 protein.

(B) Left panel. X-Mre11 was immunoprecipitated from extracts containing either uncut plasmid (lanes 1 and 2) or DNA containing double strand breaks (DSBs) (lanes 3 and 4; see Experimental Procedures). In lanes 2 and 4, the proteins were treated with λ phosphatase (+). Arrows on the left indicate X-Mre11 isoforms. Right panel. *Xenopus* interphase extracts were labeled with γ^{32}P-ATP, immunoprecipitated with preimmune (PRE) or anti-X-Mre11 antibodies (Mre11 Ab) in presence of DSBs and processed for autoradiography following SDS-PAGE.

(C) *Xenopus* extracts were subjected to PAGE followed by Western blotting with X-Mre11-specific antibody. Interphase extracts were either treated with circular plasmid (uncut plasmid) or with DSBs. Extracts treated with DSBs were incubated with 5 mM caffeine (caffeine) or anti-X-ATM antibodies (ATM Ab), as indicated. Arrows on the left indicate X-Mre11 isoforms.

DSBs (Figure 2B, Mre11 Ab). By aligning the ^{32}P-labeled proteins with the Western blot probed with X-Mre11 antibodies (Figure 2B, left panel), we determined that the ^{32}P-labeled polypeptide corresponds to the hyperphosphorylated X-Mre11 that arose in response to DSBs.

Nbs1/p95 forms a tight complex with X-Mre11 in mammalian cells and is phosphorylated following DNA damage via a pathway that requires ATM protein kinase. We asked if X-Mre11 phosphorylation induced by DSBs was likewise ATM-dependent. To inhibit ATM, we used a specific anti-X-ATM antibody (Robertson et al., 1999) at a concentration that completely abrogates the ATM-dependent DNA damage checkpoint (Costanzo et al., 2000). The antibody failed to block induced X-Mre11 phosphorylation (Figure 2C, lane 4). In contrast, caffeine, which inhibits ATM, ATR as well as the Chk family of protein kinases (Sarkaria et al., 1999), completely abolished induced X-Mre11 phosphorylation (Figure 2C, lane 3). The caffeine-sensitive protein kinase responsible for the DSB-induced X-Mre11 modification remains to be identified, although it is clear from these experiments that it is not ATM.

DSB-Induced Phosphorylation Stimulates X-Mre11-Associated Exonuclease

We then asked if phosphorylation affected the 3′–5′ exonuclease activity of X-Mre11 complex. This enzymatic activity requires the association of Mre11 with Rad50 and is enhanced by Nbs1/p95 (Paull and Gellert, 1998; Paull and Gellert, 1999).

X-Mre11 complexes were immunoprecipitated under native conditions and assayed for 3′–5′ exonuclease activity using different oligonucleotide substrates. End-labeling or internal labeling of the substrates gave identical results (data not shown). As reported for the recombinant human complex, X-Mre11 complex digested double-strand oligonucleotides with blunt or 5′ overhanging ends, whereas double-strand oligonucleotides with 3′ overhangs were not processed (data not shown). X-Mre11 complex precipitated from control extracts was active (Figure 3, column 1). Preincubating the extracts with DSBs, however, increased the enzymatic activity of the X-Mre11 complex by 70% as established by three independent experiments (Figure 3, column 2).

DSB stimulation of exonuclease activity correlated with the phosphorylation status of X-Mre11. Caffeine,

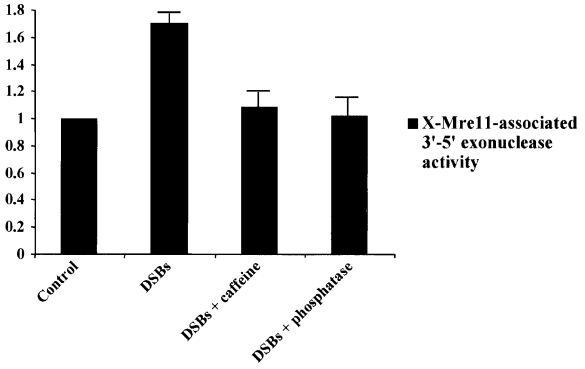

Figure 3. X-Mre11 Exonuclease Activity Is Regulated by DSB-Induced Phosphorylation

X-Mre11 was immunoprecipitated from interphase extracts and incubated with labeled oligonucleotide substrate (see Experimental Procedures) for 60 min. Mre11 exonuclease activity was measured by the amount of degraded substrate. Mre11 exonuclease activity is expressed in arbitrary units; uninduced activity = 1. The first column represents the uninduced activity of X-Mre11 precipitated from an extract treated with circular plasmid (Control). Columns 2 and 3 represent the activity of X-Mre11 precipitated from an extract treated with DSBs or DSBs and caffeine (DSBs + caffeine), respectively. Column 4 represents the activity of X-Mre11 precipitated from a DSB-treated extract that was subsequently treated with λ phosphatase. The data presented are from three independent experiments.

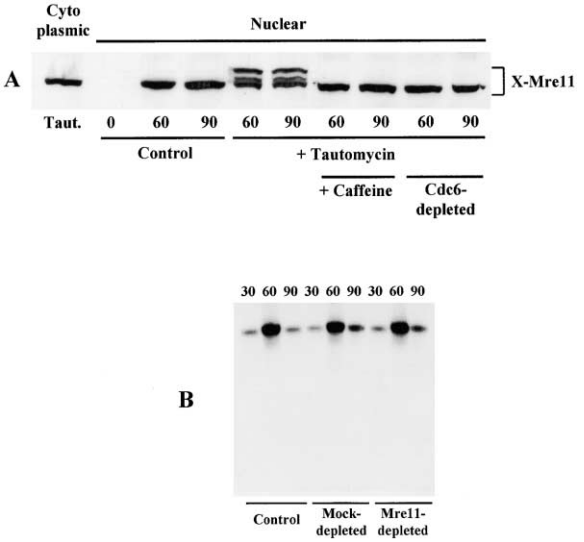

Figure 4. X-Mre11 Is Phosphorylated during DNA Replication, and Is Not Required for DNA Replication Initiation or Elongation

(A) Cytoplasmic and nuclear fractions (see Experimental Procedures) were analyzed by Western blot with anti-X-Mre11 antibodies under the following conditions. Cytoplasm was purified after 90 min incubation with nuclei in the presence of tautomycin (lane 1). Nuclei were incubated for 0, 60, and 90 min in untreated interphase extracts (lanes 2–4). Nuclei were incubated for 60 and 90 min in extracts to which tautomycin was added at 30 min following addition of nuclei (lanes 5 and 6). Nuclei were incubated for 60 and 90 min in extracts treated with tautomycin and caffeine (lanes 7 and 8). Nuclei were incubated for 60 and 90 min in cdc6-depleted extracts in the presence of tautomycin (lanes 9 and 10).

(B) Genomic DNA replication was monitored by 30 min pulses of α-^{32}P-ATP. Incorporation into chromatin incubated with untreated extract (Control), extract incubated with protein A beads bound to preimmune serum (Mock-depleted), or extract incubated with protein A beads bound to anti-X-Mre11 antibodies (Mre11 complex-depleted).

which inhibits X-Mre11 phosphorylation (Figure 2C, lane 3), abolished stimulation (Figure 3, column 3). Moreover, phosphatase treatment of stimulated X-Mre11 complex reduced exonuclease activity to basal levels (Figure 3, column 4). These results establish that DSBs stimulate X-Mre11 complex exonuclease via a pathway that entails phosphorylation of X-Mre11 and/or an associated protein.

X-Mre11 Binds to Chromatin and Is Activated during DNA Replication

To explore further the physiological significance of X-Mre11 phosphorylation, we asked if X-Mre11 was modified during chromosomal DNA replication in the absence of added DSBs. Demembranated *Xenopus* sperm nuclei were incubated in extracts to allow chromatin assembly and DNA replication. The nuclei were then purified, and chromatin-associated X-Mre11 was demonstrated by Western blot. In the absence of incubation, no X-Mre11 was detected in the nuclear fraction (Figure 4A, lane 2). X-Mre11 became associated with the nuclear fraction during replication (Figure 4A, lanes 3 and 4) and remained in association after replication was completed (Figure 4B, lanes 1–3).

To trap phosphorylated intermediates, we added tautomycin, a phosphatase inhibitor, 30 min following addition of nuclei. Under these conditions, at least two hyperphosphorylated forms of X-Mre11 appeared in the nuclear fraction (Figure 4 A, lanes 5 and 6). In contrast, cytoplasmic X-Mre11 isolated from the tautomycin-treated extracts at 90 min was not hyperphosphorylated. Thus, this modification of X-Mre11 depends upon its nuclear localization (Figure 4A, lane 1). It also requires DNA replication. When DNA replication was blocked by depleting extracts of Cdc6, X-Mre11 appeared in the nuclear fraction, but was not hyperphosphorylated (Figure 4B, lanes 9 and 10).

We then asked if the modification of X-Mre11 during DNA synthesis might be due to DSB formation in the replicating DNA. Consistent with this idea, we found that replication-dependent phosphorylation of X-Mre11, like DSB-induced phosphorylation, was sensitive to caffeine (Figure 4A, lanes 7 and 8). Taken together, these results indicate that X-Mre11 associates with chromatin and is phosphorylated, possibly in a DSB-dependent manner, during DNA replication.

X-Mre11 Prevents the Accumulation of DSBs during DNA Replication

A role for Mre11 in DNA replication has been suggested (Petrini, 2000). We therefore asked if X-Mre11 complex depletion affected DNA synthesis. Genomic DNA was pulse-labeled with radioactively labeled deoxynucleotides at various times after addition of nuclei. Control, mock-depleted and extracts quantitatively depleted of X-Mre11 complex, were compared. The kinetics and the levels of incorporation of radionucleotides into genomic DNA, separated by agarose gel electrophoresis, were

identical in the three extracts (Figure 4B). X-Mre11 is, therefore, required neither for DNA replication initiation nor elongation.

We then performed experiments to demonstrate that, in fact, DSBs arise during normal DNA replication, and that, furthermore, activated X-Mre11 complex plays a role in their repair. We designed a TUNEL-based assay to monitor the accumulation of DSBs during DNA replication in the presence or absence of X-Mre11 complex (see Experimental Procedures). Nuclei were replicated in control or in X-Mre11 complex-depleted extracts. At 100 min after incubation, the postreplicative genomic DNA was extracted and end-labeled using terminal transferase (TdT) and radioactive deoxynucleotides (Figures 5A–5C).

Demembranated sperm nuclei (Figure 5A, columns 1 and 2), postreplicative nuclei replicated in a control extract (Figure 5A, columns 3 and 4), or in mock-depleted extracts (Figure 5A, columns 5 and 6) were not significantly end-labeled with TdT. In contrast, nuclei replicated in extracts depleted of X-Mre11 complex were extensively end-labeled (Figure 5B, column 8). Labeling was TdT-dependent (Figure 5A, column 7) and was resistant to aphidicolin, indicating it was not due to contaminating DNA polymerase (Figure 5A, column 10). To establish that the increase in end-labeling was due to the removal of X-Mre11 complex, human Mre11/Rad50/Nbs1 complex, purified from baculovirus-infected cells (Paull and Gellert, 1999), was added to depleted extracts prior to replication. The added complex reduced end-labeling to control levels (Figure 5B, columns 1 and 2). Depletion of X-Mre11 complex does not prevent TdT access to DSBs. Mre11/Rad50/Nbs1 complex, added to the in vitro reaction on postreplicative nuclei prior to TdT, did not inhibit TdT labeling (Figure 5A, column 9).

Nuclei isolated during DNA replication in control extracts were not end-labeled by TdT (Figure 5B, columns 3–5). Thus, single-stranded DNA fragments generated during DNA replication (Okazaki fragments) are not a substrate for TdT under our experimental conditions. Inhibiting DNA replication with 6-dimethylaminopurine (6-DMAP, a protein kinase inhibitor), by depletion of Cdc6 or with actinomycin D (a primase inhibitor), blocked end-labeling in the depleted extracts (Figure 5B, lanes 6–8). These data demonstrate that MRE11-complex prevents the accumulation of DSBs in replicating genomic DNA.

Inactivation of *mre11* in chicken cells leads to rapid apoptosis (Yamaguchi-Iwai et al., 1999). We wished to assess whether the accumulation of DSBs in depleted extracts was a consequence of apoptosis. First, we showed that ZVAD, a potent peptide inhibitor of caspase, did not inhibit end-labeling in depleted extracts (Figure 5C, compare columns 1 and 2). We next demonstrated that truncated PARP (t-PARP), a substrate for caspase-3 cleavage (Hensey and Gautier, 1997) was stable in control, mock-depleted, or depleted extracts (Figure 5D). As expected, t-PARP was completely cleaved following addition of cytochrome C. Finally, we quantified the concentration of DSBs following induction of apoptosis. Nuclear end-labeling in extracts treated with cytochrome C was 7-fold higher than in depleted extracts (Figure 5C, column 4). This establishes that the accumulation of DSBs in nuclei replicated in the absence of X-Mre11 complex is not a consequence of apoptosis.

We confirmed the accumulation of DSBs in depleted extracts with an assay unrelated to TdT end-labeling. Histone H2AX, phosphorylated at serine residue 139 (phospho-H2AX), is found in association with DSBs in chromatin (Rogakou et al., 1998). We obtained an antibody against mammalian phospho-H2AX and demonstrated that it recognized the *Xenopus* homolog. Sperm chromatin was partially digested with restriction endonuclease NotI to create DSBs. The chromatin was incubated for 1 hr in interphase extract, then purified and probed with the phospho-H2AX antibody. Phospho-H2AX was detected in the NotI-treated sample (Figure 5E, lane 1), whereas undigested chromatin showed no signal (Figure 5E, lane 2). We then isolated nuclei that were replicated in mock-depleted or depleted extracts. As is clear from Figure 5D, left panel (compare lanes 3 and 4), postreplicative nuclei isolated from extracts depleted of Mre11 complex contained phospho-H2AX, and therefore, DSBs. The concentration of DSBs induced by NotI as assessed from the TUNEL and the immunological assays were comparable (Figure 5C, lanes 1 and 3). Phosphorylation of histone H2AX in depleted extracts was inhibited in a dose-dependent manner by human Mre11/Rad50/Nbs1 complex added prior to DNA replication (Figure 5E, right panel, lanes 1–4). Phosphorylation of histone H2AX was dependent upon DNA replication; Cdc6 depletion entirely eliminated the phospho-H2AX signal (Figure 5E, right panel, lane 5).

Taken together, these data establish that DSBs arise during genomic DNA replication and that X-Mre11 protein complex blocks their accumulation.

X-Mre11 Is Not Required for the DNA Damage Checkpoint that Inhibits S-Phase Entry

In mammalian cells, Mre11 associates with Rad50 and Nbs1/p95. Phosphorylation of Nbs1/p95 by ATM following DNA damage is important for the DNA damage-induced S-phase checkpoint (Gatei et al., 2000; Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000). We have described an in vitro system based upon partially purified *Xenopus* extracts that recapitulates the ATM-dependent DNA damage checkpoint (Costanzo et al., 2000). Using this system, we asked if X-Mre11 plays a role in the checkpoint. Initiation of DNA replication is monitored after mixing 2 fractions (M and B) prepared from untreated extracts or extracts treated with DSBs (M* and B*). Both fractions are required to initiate replication of chromosomal DNA.

A DSB-induced checkpoint, as indicated by the absence of DNA replication, was seen in mixtures containing fractions M* and B* (compare lane 3 and 5, Figure 6). M* and B* fractions prepared from X-Mre11 complex depleted extracts (Figure 2A) likewise failed to support DNA replication, indicating a functional checkpoint (Figure 6, lane 6). As previously described, DNA replicated efficiently in the presence of fractions derived from ATM-inhibited extracts treated with DSBs (Figure 6 lane 7 and Costanzo et al., 2000). This establishes that X-Mre11 complex is not required for the ATM-dependent checkpoint that prevents initiation of DNA synthesis following DNA damage.

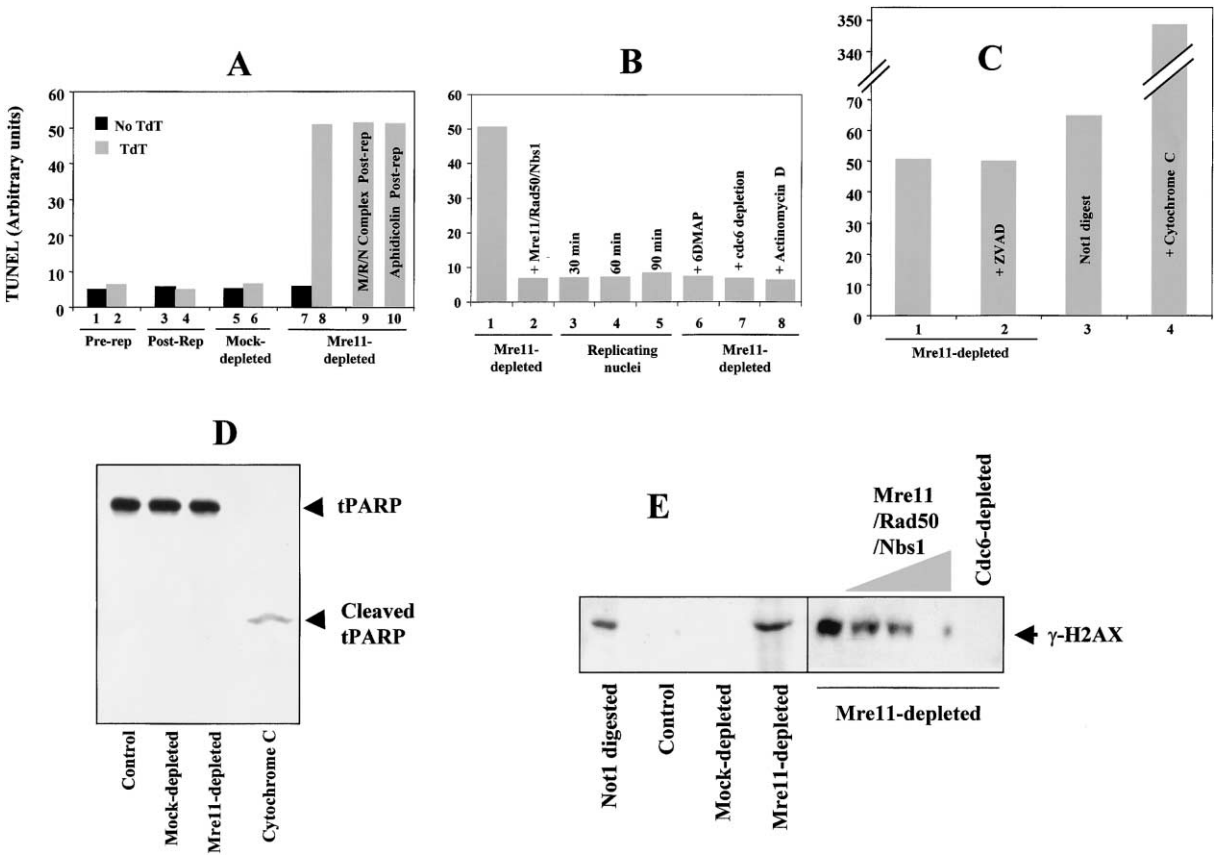

Figure 5. X-Mre11 Prevents the Accumulation of DSBs that Arise during DNA Replication

(A–C) DSBs were detected by TUNEL assay measuring incorporation of γ^{32}P-dGTP into genomic DNA.

(A) Labeling of prereplicative nuclei (columns 1 and 2), postreplicative nuclei isolated from a control extract (columns 3 and 4), a mock-depleted extract (columns 5 and 6), and a X-Mre11 complex-depleted extract (columns 7 and 8). Controls were incubated without terminal transferase (No TdT, columns 1, 3, 5, and 7). Nuclei replicated in X-Mre11 depleted extracts were purified and incubated with TdT in the presence of 500 nM Mre11/Rad50/Nbs1 complex (column 9). Labeling of postreplicative nuclei replicated in X-Mre11 complex-depleted extracts in the presence of aphidicolin (column 10).

(B) Labeling of nuclei replicated in a X-Mre11 complex-depleted extracts (column 1) and in a X-Mre11 complex-depleted extract supplemented with 500 nM human Mre11/Rad50/Nbs1 (Paull and Gellert, 1999) recombinant complex prior to replication (column 2). Labeling of nuclei during replication in untreated extracts at 30 min (column 3), 60 min (column 4), and 90 min (column 5). Labeling of nuclei incubated in a X-Mre11 complex-depleted extracts in which DNA replication was inhibited by 6DMAP (column 6), by Cdc6-depletion (column 7), or by addition of actinomycin D (column 8).

(C) Labeling of nuclei replicated in X-Mre11 complex-depleted extracts (column 1) and in a X-Mre11 complex-depleted extract in the presence of ZVAD. Labeling of nuclei partially digested with Not1 (column 3). Labeling of nuclei incubated in extract in the presence of cytochrome C (column 4).

(D) Cleavage of radiolabeled tPARP in various extracts. tPARP cleavage in normal extracts (Control), extracts treated with preimmune serum (PRE), X-Mre11-depleted extracts (Depleted), and in Cytochrome C-treated extracts (Cyto C).

(E) Chromatin fractions were analyzed by Western blot with γ-H2AX antibodies under the following conditions: Left panel. Chromatin partially digested with Not1 (lane 1). Chromatin extracted from nuclei replicated in a control extract (lane 2), a mock-depleted extract (lane 3), and a X-Mre11 complex-depleted extract (lane 4). Right panel. Chromatin extracted from nuclei replicated in a X-Mre11 complex-depleted extract (lane 1) and in a X-Mre11 complex-depleted extracts supplemented with increasing amounts of human Mre11/Rad50/Nbs1 recombinant complex (lanes 2: 125 nM, 3: 250 nM and 4: 500 nM). Chromatin extracted from nuclei incubated in Cdc6-depleted extract (lane 5).

Discussion

Regulation of Mre11 Activity

We isolated the *Xenopus* homolog of Mre11 (X-Mre11) and showed that it is similar to other Mre11 family members, from yeast to human (Haber, 1998; Petrini et al., 1995; Tavassoli et al., 1995). The Mre11 phosphoesterase motifs, which are important for the catalytic activity of the protein (Bressan et al., 1998; Haber, 1998; Moreau et al., 1999), are particularly well conserved.

Our data suggests that the molecular architecture of the complexes formed with X-Mre11 is also conserved. When X-Mre11 immunoprecipitates were probed with an antibody against mouse Rad50, we observed a 150 kDa crossreacting protein similar in molecular weight to mouse Rad50 (data not shown). This observation suggests that, like its mammalian counterpart, X-Mre11 is present in a complex with other proteins, including X-Rad50. Therefore, immunodepletion removes an X-Mre11 complex.

The *Xenopus* and human Mre11 complexes (Paull and Gellert, 1998) display 3′–5′ exonuclease activity with

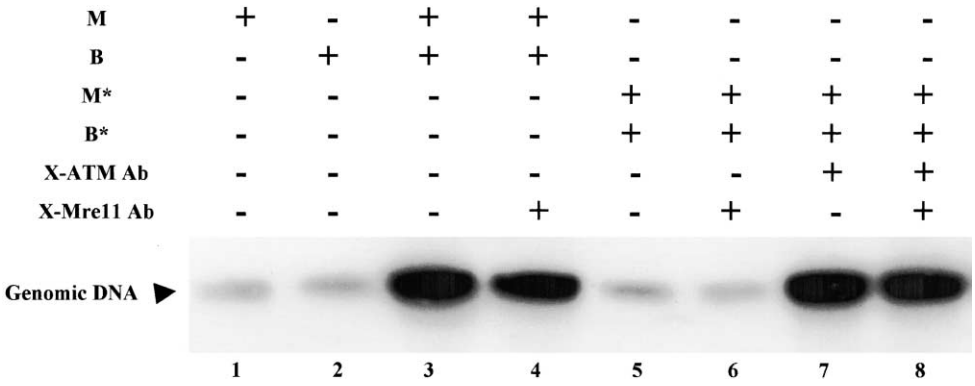

Figure 6. X-Mre11 Is Not Required for the DSB-Induced, ATM-Dependent Checkpoint

DNA replication was assayed in reconstituted extracts consisting of 6-DMAP-treated chromatin and fractions M and B prepared from different sources as indicated. Fractions M and B are derived from extracts treated with circular plasmid DNA. Fractions M* and B* are derived from extracts treated with DSBs. M and B (lanes 3 and 4), as well as M* and B* fractions (lanes 5–8), were prepared from extracts in which X-ATM was inhibited with specific antibodies (X-ATM, lanes 7–8) or extracts in which X-Mre11 complex was depleted (X-Mre11 Ab, lanes 4, 6, and 8), as indicated.

similar substrate specificity (data not shown). Both exonucleases process blunt or 5′ overhanging DSBs, but cannot degrade DSBs with 3′ overhangs.

The X-Mre11-associated exonuclease is stimulated by addition of DSBs to the extracts in the absence of any other aberrant DNA structures. Importantly, stimulation is accompanied by and dependent upon phosphorylation of X-Mre11 complex. Caffeine that prevents X-Mre11 phosphorylation, or phosphatase treatment of activated X-Mre11 complex, reduce exonuclease activity to basal levels. Mammalian Mre11 was shown to be phosphorylated following ionizing radiation, although, in this case, the precise nature of the inducing factor(s) is unknown (Dong et al., 1999). In contrast to the irradiation-induced phosphorylation of Nbs1, X-Mre11 phosphorylation induced by DSBs is not dependent on X-ATM. Thus, X-Mre11 phosphorylation occurs in the presence of specific antibodies against X-ATM (Robertson et al., 1999). This agrees with the observation that Mre11 phosphorylation and focus formation is normal in DNA-damaged A-T cells (Dong et al., 1999; Mirzoeva and Petrini, 2001). The kinase responsible for X-Mre11 phosphorylation is not known. However, the reaction is sensitive to 5 mM caffeine. This pattern of inhibition is similar to the mammalian ATM-related (ATR) kinase.

Finally, we showed that X-Mre11 is phosphorylated in control extracts. Addition of DSBs induces phosphorylation at additional sites, as evidenced by a shift in apparent molecular weight on SDS-PAGE gels

DNA DSBs Are Generated during DNA Replication

Although DSBs are known to arise during DNA replication in bacteria, experimental evidence for DNA breakage during eukaryotic replication has not been reported (Cox et al., 2000; Seigneur et al., 1998; Seigneur et al., 2000).

Our data show that DSBs are generated during the normal course of DNA replication in *Xenopus* egg extracts. First, we find that only the nuclear fraction of Mre11 is hyperphosphorylated during replication. Recall that DSBs induce X-Mre11 phosphorylation, which, like replication-induced phosphorylation, is sensitive to caffeine. Second, when X-Mre11 complex is depleted from the extracts, we observe a dramatic accumulation of DSBs as demonstrated by two different assays for DSBs. Finally, accumulation of DSBs is strictly dependent on DNA replication. We propose that these DSBs are normally very transient and are rapidly removed by activated X-Mre11 complex.

Comparison between TUNEL labeling of postreplicative nuclei in depleted extracts with that of chromatin digested with NotI indicates that each chromosome accumulates, on the average, 5–10 DSBs (data not shown). It is possible that additional breaks occur during replication that are eliminated by alternative repair pathways. The accumulation of DSBs during replication in X-Mre11 complex-depleted extracts is consistent with the effects of depletion on the morphology of condensed chromosomes during mitosis. When postreplicative nuclei were driven into mitosis by addition of metaphase-arrested *Xenopus* extract, normal chromosomes and metaphase spindles formed in nuclei replicated in control extracts whereas nuclei replicated in depleted extracts showed fragmented chromosomes and assembled grossly abnormal spindles (data not shown).

In theory, DSBs could be generated in several ways during replication. They could arise if a replication fork passed through a nicked DNA template or from failure to repair DNA adducts induced by DNA modifying agents. Finally, Holliday junctions formed at stalled replication forks could produce DSBs (Scully et al., 2000; Seigneur et al., 1998; Zou and Rothstein, 1997). Aberrant DNA structures that lead to collapsed replication forks are known to occur at palindromic DNA sequences. Hypothetically, these forks will restart if they are in close proximity to a replication origin, or if the replication block is resolved by Sister Chromatid Exchange (SCE). SCE requires the activity of Mre11, BRCA1, Nbs1, and Rad50 (Scully et al., 2000). Inactivation of any of these genes inhibits recombination-dependent SCE repair and generates DSBs.

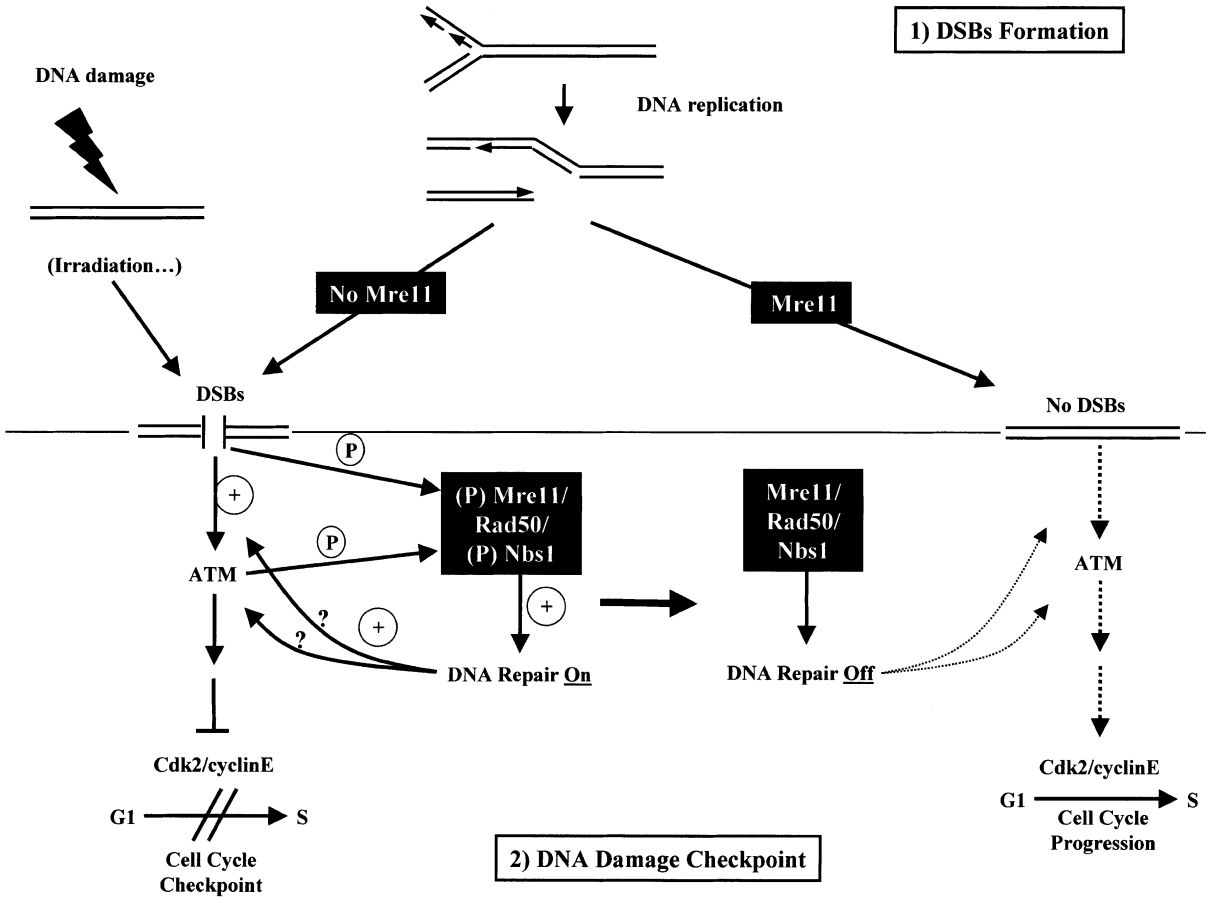

Figure 7. Our Preferred Model for Mre11 Function in DNA Synthesis and the Response to DNA Damage

(1) Top half: Mre11 prevents the accumulation of DSBs during DNA replication. DNA DSBs can arise following external DNA damage or as a consequence of DNA replication.

(2) Bottom half: proposed role for the Mre11/Rad50/Nbs1 complex in the maintenance of the DNA damage checkpoint.

DSBs activate the ATM-dependent checkpoint pathway leading to cdk2 inactivation and inhibition of DNA replication initiation (Costanzo et al., 2000). DSBs also trigger the activation of the Mre11/Rad50/Nbs1 complex through both ATM-dependent and independent phosphorylation. The Mre11/Rad50/Nbs1 complex is essential for the repair of DSBs. We hypothesize that Mre11-driven repair reinforces the ATM-dependent checkpoint (possibly through Nbs1 function) and maintains this checkpoint signaling active during repair. After DSB repair, Mre11 complex is no longer hyperphosphorylated; DNA repair and the ATM-dependent checkpoint are downregulated.

The Essential Function(s) of Mre11

Inactivation of any one of the three components of the Mre11/Rad50/Nbs1 complex in mouse or in chicken cells abolishes normal cell proliferation (Luo et al., 1999; Xiao and Weaver, 1997; Yamaguchi-Iwai et al., 1999; Zhu et al., 2001). Therefore, Mre11 complex is essential for cell viability even in the absence of externally induced DNA damage. It has been difficult to determine the essential role of Mre11 complex because inactivation of any one of the three genes induces a terminal apoptotic-like phenotype. Depletion of X-Mre11 complex from *Xenopus* extracts, however, did not trigger apoptosis, presumably because of the small number of DSBs generated. By extension, our data suggest that in the absence of Mre11 complex in chicken or mouse cells, cell death occurs as a secondary consequence of the loss of genomic integrity. The phenotype that we observe in *Xenopus* is reminiscent of ATR inactivation in mouse, which generates chromosome breaks unaccompanied by caspase activation (Brown and Baltimore, 2000). We propose, therefore, that one essential function of Mre11/Rad50/Nbs1 complex in chicken or mouse cells is to prevent DNA replication-dependent DSB formation. Interestingly, Mre11-containing complexes accumulate at stalled DNA replication forks cells from the xeroderma pigmentosum variant (XPV) (Limoli et al., 2000). Based upon its established role in DNA repair, we propose that Mre11 complex repairs DSBs as they arise during replication (Figure 7). A fraction of X-Mre11 is nuclear and is hyperphosphorylated during replication. Phosphorylation enhances the nuclease activity of X-Mre11 complex, an activity that is likely to play a role in DSB repair.

mre11 is not essential in *C. elegans* or in *S. cerevisiae* (Chin and Villeneuve, 2001; Ogawa et al., 1995). This might reflect differences in the way yeast and higher eukaryotic chromosomes are replicated, or the presence of redundant systems that repair DSBs. Alternatively, DSBs might arise more frequently in larger and more complex genomes containing repetitive DNA sequences.

We have unraveled a critical link between recombination-dependent DNA repair and DNA replication by demonstrating the requirement for Mre11 complex during DNA replication in the absence of induced DNA damage.

Mre11 and the DNA Damage Checkpoint

Cells from A-TLD or NBS patients, which harbor hypomorphic mutations in *mre11* and *nbs1*, respectively, exhibit radio-resistant DNA synthesis (RDS) (Petrini, 2000). The RDS phenotype, found also in cells from A-T patients (Carney et al., 1998; Stewart et al., 1999), possibly reflects a defect in one or several DNA damage checkpoint(s) operating at the time of initiation of or during DNA replication. It is not clear if Nbs1 is involved in the DNA damage checkpoint. Although NBS cells show no major defects in cell cycle checkpoint (Girard et al., 2000), phosphorylation of Nbs1 is important for the G1 to S DNA damage checkpoint (Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000).

In contrast, we have shown that the DSB-induced, ATM-dependent checkpoint that down-regulates cdk2 and prevents origin activation and firing (Costanzo et al., 2000) is not dependent on Mre11. This is similar to *C. elegans*, where *mre11* mutants undergo the DNA damage checkpoint that induces apoptosis (Chin and Villeneuve, 2001).

First, it is possible that Nbs1 complexes that lack Mre11 might participate in the mammalian DNA damage checkpoint, whereas complexes that contain both proteins would function in DSB repair. In such a model, depletion of Mre11 would leave Nbs1 and the checkpoint intact but block repair of DSBs. Evidence for such multiple protein complexes awaits the isolation and characterization of the Nbs1 *Xenopus* homolog.

Alternatively, Nbs1 in complex with Mre11 might be required not for the initiation but for the maintenance of the checkpoint. In *Xenopus*, initiation of DNA replication is synchronous, and the rapid ATM-dependent checkpoint activation does not require Mre11/Rad50/Nbs1 complex. In mammals, were replication initiation is asynchronous, Nbs1, and possibly Mre11/Rad50/Nbs1 complex, might be needed at a later stage during S-phase. Nbs1 might promote a feedback mechanism that reinforces the ATM-dependent checkpoint until repair is completed (Figure 7). When subjected to irreparable DNA damage, *S. cerevisiae* first activates a DNA damage checkpoint, then resumes cell cycle progression: a phenomenon called adaptation. Mutants defective in adaptation have been isolated that remain arrested in cell cycle after DNA damage. These mutants resume cell cycle progression when either Mre11 or Rad50 are inactivated (Lee et al., 1998), consistent with the idea that Mre11 complex maintains rather than activates the DNA damage checkpoint. This hypothesis is attractive, because it reconciles genetic data with other experimental analysis in different species.

Experimental Procedures

Cloning of the *Xenopus* Homolog of Mre11

To isolate X-Mre11 cDNAs, we synthesized degenerate primers that correspond to coding sequences for amino acids highly conserved among the Mre11 proteins from human, mouse, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. The primers F3 (5′-GAY YTB TTY CAY GAR AAY AAR CC) and R4 (5′-TGD ATN ACR AAN ARR TTR AAC CA) represent codons 60–67 and 210–217 of the human sequence. First strand cDNA was synthesized from *Xenopus laevis* oocyte mRNA using a polyT primer and Super Script II reverse transcriptase (Gibco BRL). Amplification with R3 and F4 PCR primers was carried out for 30 cycles of 30 s at 95°C, 40 s at 54°C, and 60 s at 72°C. A fragment of the expected size (470 bp) was obtained and was cloned into pBluescript KS+. The sequences of the inserts in several clones were determined and shown to correspond to X-Mre11. The same PCR fragment was labeled by random priming and used to screen a *X. laevis* tadpole head cDNA library in the λZAP vector (Hemmati-Brivanlou et al., 1991). Plaque lifts were prepared and hybridized as previously described (Bibikova et al., 1998), and phagemids carrying the inserts were excised according to the supplier's protocol (Stratagene). Two clones containing the complete coding sequence, plus 5′ and 3′ untranslated regions were identified and sequenced (GenBank Accession # AF134569).

Antibody Production

The coding region of X-Mre11 was amplified from the phagemid using Pfu DNA polymerase (Stratagene) and the primers 5′-CAG CCG GCA CAT ATG AGT TCT TC and 5′-TAA AAC GGA TCC TAT TTA TCT ACG GCC. The former incorporates the ATG codon into an NdeI site at the 5′-end of the cDNA, and the latter puts a BamHI site beyond the termination codon. The resulting PCR product was cloned between the NdeI and BamHI sites of pET16b (Novagen); this links X-Mre11 to an N-terminal His_{10} tag. Upon transformation into *E. coli* BLR (DE3) and induction with IPTG, this construct led to the production of substantial amounts of insoluble X-Mre11 protein.

The pellet from lysed bacteria was dissolved in 6 M urea, and the His-tagged X-Mre11 was enriched by binding to and elution from a His-bind column (Novagen). The protein was subjected to SDS-PAGE and negatively stained with $CuCl_2$. Gel slices containing X-Mre11 were excised and sent to Covance Research Products Inc. (P.O. Box 7200, Denver, PA 17517) where rabbit antibodies were raised using standard protocols. The resulting antiserum and preimmune serum were used for Western blots at 1:2000 dilutions and for immunoprecipitations as described later.

X-Mre11 Detection

Xenopus interphase extracts were prepared according to Murray (1991). Extracts were treated with 10 ng/μl of circular pBR322 or with 10 ng/μl of pBR322 digested with HaeIII and with 5 mM caffeine at 20°C. Extracts were electrophoresed on 10% SDS-PAGE and blotted with X-Mre11 specific antiserum. Analysis of nuclear X-Mre11 was performed in presence of 3 μM tautomycin. Fifty microliters of extract containing 8000 nuclei/μl were diluted in nuclei isolation buffer (100 mM KCl, 25 mM HEPES (pH 7.8), 2.5 mM $MgCl_2$, and 0.01% Triton X-100) and purified through a 30% sucrose cushion. Samples were spun for 15 min at 6000 × g at 4°C; the pellet were washed once with the same buffer and processed for electrophoresis.

X-Mre11 Activity

5′-Labeled Oligonucleotide Templates

The Tp74 oligo described by Paull and Gellert (1998) was 5′-labeled with γ ^{32}P-ATP by using T4 polynucleotide kinase according to standard procedures. Unincorporated nucleotides were removed by separation through a G50 sephadex spin column equilibrated with 50 mM Tris/1 mM EDTA (pH 8.0). The labeled oligonucleotide was then annealed to its complementary oligonucleotide by cooling from 90°C to 4°C overnight. The double-stranded oligonucleotide was purified on an 18% polyacrylamide gel. Gel slices containing the template were excised and extracted with Tris-EDTA (pH 8.0) overnight at room temperature.

3′-Extended Oligonucleotide Templates

5′-labeled double-stranded oligonucleotides were extended at their 3′ end by incubation with 50 U of terminal transferase (Gibco) in presence of 50 μM dATP in 100 μl reaction consisting of 100 mM potassium cacodylate (pH 7.0), 1 mM $CoCl_2$, and 0.2 mM DTT, for 30 min at 30°C. After heat inactivation for 10 min, the reaction mixture was passed through a G50 Sephadex spin column.

Exonuclease Activity Assay

Exonuclease activity assay was modified from (Paull and Gellert, 1999). Briefly, 100 μl of interphase extract was incubated with 1 μg of digested plasmid or with 1 μg of digested plasmid and 5 mM caffeine for 30 min at 20°C. The extracts were then diluted 5-fold in PBS 0.1% Triton X-100 and incubated for 2 hr with 25 μl of Protein A-sepharose 4B (Pharmacia), coupled to 100 μl of anti-X-Mre11 serum. The beads were washed extensively in buffer containing 0.5 M NaCl, 0.5% Triton X-100, 40 mM Tris (pH 7.4), and 2 mM DTT. The final washes were performed in the reaction buffer consisting of 25 mM MOPS (pH 7.0), 150 mM KCl, 10% polyethylene glycol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 2 mM DTT. The reaction buffer was brought to 37°C and added to 25 μl of control beads or to 25 μl of immunoprecipitates in a 40 μl reactions containing 0.08 pM of the 5′-labeled double-stranded oligonucleotide or the same double-stranded oligonucleotide with the 3′ homopolymeric tail. Reaction mixtures were incubated at 37°C for 60 min. Exonuclease digestions were complete: internal labeling of the oligonucleotide with dNTPs or end-labeling with T4 kinase gave identical results (data not shown). The beads were then collected and the supernatant was incubated with 0.2% SDS, 5 mM EDTA and 0.1 mg/ml proteinase K for 15 min. The reactions were then spotted on DE81 filters (Whatman). The filter were washed in 0.5 M (NH4)HCO3 and dried under light. Radioactivity associated with undigested double-stranded oligonucleotides on the filters was measured by liquid scintillation counting (Kiss-Laszlo et al., 1996). Some immunoprecipitates from extract treated with digested DNA were incubated with λ phosphatase for 30 min at 30°C, spun again, and washed with high salt buffer and reaction mix buffer.

X-Mre11 Complex Depletion/Cdc6 Depletion

For X-Mre11 complex depletion, 50 μl of interphase extract were incubated with 25 μl protein A sepharose beads coupled to 50 μl preimmune serum or with 50 μl of X-Mre11 antiserum for 60 min at 4°C. For cdc6 depletion, 50 μl of interphase extract were incubated with 25 μl protein A sepharose beads coupled to 10 μl of cdc6 antiserum (Costanzo et al., 2000) for 60 min at 4°C.

Recombinant Mre11/Rad50/Nbs1 Proteins

Human Mre11/Rad50/Nbs1 complex was purified from baculovirus-infected cells according to published protocols (Paull and Gellert, 1999). The recombinant trimeric complex was used at a concentration of 500 nM, unless specified otherwise.

DNA Replication

DNA replication was performed according to Costanzo et al. (1999). Briefly, nuclei were assembled for 15 min then incubated in interphase extracts at a concentration of 1000 nuclei/μl. Aliquots of the reaction were pulsed labeled with α-^{32}P-dCTP from 0–30 min, 30–60 min, and 60–90 min at 20°C. Reactions were stopped with 5% SDS, 80 mM Tris, 8 mM EDTA (pH 8.0) and digested with 1 mg/ml proteinase K for 30 min. DNA was extracted with phenol/chloroform and electrophoresed on 0.8% agarose gel.

TUNEL Assay

50 μl of untreated interphase extracts, of extract treated with preimmune serum or of X-Mre11-depleted extract were incubated with 10,000 nuclei/μl for 120 min at 20°C. Extracts were diluted in 1 ml of a buffer consisting of 100 mM KCl, 25 mM HEPES (pH 7.8), 2.5 $MgCl_2$ and 0.4% Triton X-100. Samples were layered on a sucrose cushion made with the same buffer without Triton and spun for 20 min at 6000 × g in a HB-6 rotor (Sorvall). The pellet was washed and incubated at 37°C for 4 hr in a buffer containing 90 U of Terminal transferase, 100 mM potassium cacodylate (pH 7.0), 1 mM $CoCl_2$, 0.2 mM DTT, 25 μCi dGTP (3000 cu/mM), and 50 μM dGTP. Control reactions were incubated in the same buffer but TdT was omitted. Reaction mixtures were then treated with 0.1 mg/ml proteinase K and the DNA was phenol/chloroform extracted and electrophoresed on 0.5% agarose gel at 100 volt for 60 min. The gel was fixed in 20% TCA, dried, and then exposed for autoradiography. The labeled band was excised from the gel and quantified by scintillation counting. 6-DMAP was used at 3 mM concentration, aphidicolin at 100 μg/ml, and actinomycin D at 10 μg/ml.

Phosphorylated Histone H2AX Detection

50 μl of mock-depleted or X-Mre11-depleted interphase extract were incubated with 10,000 nuclei/μl for 90 min at 23°C. Postreplicative chromatin was isolated by diluting the extracts in chromatin isolation buffer containing 1 mM NaF, 1 mM Na vanadate, and 0.125% Triton X-100. Samples were layered onto a sucrose cushion in chromatin isolation buffer lacking Triton X-100, then spun at 6000 × g for 20 min at 4°C. Positive control was prepared by incubating sperm nuclei for 30 min in interphase extract to decondense the chromatin. The chromatin was then isolated and digested for 2 hr with NotI. Digested chromatin was isolated and incubated in interphase extract for 60 min.

Chromatin was boiled in Laemmli buffer and processed for SDS-PAGE electrophoresis. Anti-phosphorylated H2AX antibody was used for Western blot at 1/6,000 dilution.

Checkpoint Assay

X-Mre11 checkpoint function was assayed as previously described (Costanzo et al., 2000), except that M and B or M* and B* fractions were prepared from X-Mre11-depleted extracts as described previously.

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