An *mre11* Mutant of *Coprinus cinereus* Has Defects in Meiotic Chromosome Pairing, Condensation and Synapsis

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ABSTRACT

The *rad11* gene of the basidiomycete *Coprinus cinereus* is required for the completion of meiosis and for survival after gamma irradiation. We have cloned the *rad11* gene and shown that it is a homolog of *MRE11*, a gene required for meiosis and DNA repair in numerous organisms. The expression of *C. cinereus mre11* is induced during prophase I of meiosis and following gamma irradiation. The gene encodes a predicted polypeptide of 731 amino acids, and the *mre11-1* (*rad11-1*) mutation is a single base pair change that results in a stop codon after amino acid 315. The *mre11-1* mutant shows enhanced sensitivity to ionizing radiation, but no enhanced sensitivity to UV radiation. It shows a delay in fruitbody formation and a reduction in the number of mushrooms formed per dikaryon. The *mre11-1* mutant also has several meiotic defects. Pachytene chromatin condensation is disrupted, and although some meiotic cells appear to achieve metaphase I condensation, no further meiotic progression is observed. The *mre11-1* mutant also fails to undergo proper chromosome synapsis; neither axial elements nor mature synaptonemal complexes are complete. Finally, meiotic homolog pairing is reduced in the *mre11-1* mutant. Thus, in *C. cinereus*, Mre11 is required for meiotic DNA metabolism.

THE processes involved in meiotic chromosome behavior and recombination bear striking similarity to events involved in certain types of DNA repair. In both meiosis and DNA double-strand break (DSB) repair, homologous sequences are identified and DNA breaks are repaired, often with an exchange of genetic material. In *Saccharomyces cerevisiae*, DSBs initiate meiotic recombination and are generated by a type II topoisomerase-like protein, Spo11 (Keeney *et al.* 1997), in conjunction with the Mre11/Rad50/Xrs2 complex of proteins; this complex also is required for processing of these meiotic DSBs (Johzuka and Ogawa 1995; Nairz and Klein 1997; Tsubouchi and Ogawa 1998). Mutants of *SPO11* in *Caenorhabditis elegans* (Dernburg *et al.* 1998) and *Drosophila melanogaster* (McKim and Hayashi-Hagihara 1998) are deficient in meiotic recombination, implying that certain aspects of meiotic DSB activity may be conserved. Mutations in *S. cerevisiae MRE11*, *RAD50*, or *XRS2* result in defects in meiotic recombination and spore formation, as well as an increased sensitivity to ionizing radiation (reviewed in Haber 1998).

We have chosen to study meiosis and DNA repair in the basidiomycete *Coprinus cinereus.* This fungus grows as a monokaryotic mycelium, and two individuals with compatible mating types can mate to form a dikaryon. Under the proper light and temperature conditions, the dikaryon will form a fruitbody, or mushroom (Zolan *et al.* 1988). Each mushroom contains approximately ten million meiotic cells that undergo meiosis in a naturally synchronous manner. This feature of the biology of *C. cinereus* provides an ideal opportunity to examine the behavior of meiotic chromosomes cytologically. The dynamics of meiotic chromosomes during prophase and metaphase I have been documented thoroughly for wild-type mushrooms (Raju and Lu 1970; Holm *et al.* 1981; Seitz *et al.* 1996).

The *rad3*, *rad9*, *rad11*, and *rad12* complementation groups of *C. cinereus* were identified initially in screens for radiation-sensitive, meiotic mutants (Zolan *et al.* 1988; Valentine *et al.* 1995). Mutant alleles of these genes exhibit meiotic defects and show enhanced sensitivity to ionizing radiation; the four genes comprise a single epistasis group for survival following gamma irradiation (Valentine *et al.* 1995). The phenotypes of *rad3*, *rad9*, and *rad12* mutants have been described in detail (Zolan *et al.* 1988, 1994, 1995; Pukkila *et al.* 1992; Ramesh and Zolan 1995; Seitz *et al.* 1996). These mutants show defects in chromatin condensation and synapsis, and spore production and viability are decreased relative to the levels observed in wild-type mushrooms.

We had shown previously that the *rad11-1* mutant has low spore production and fails to complete the meiotic divisions, and we mapped the *rad11* gene to chromosome 6 of *C. cinereus* (Zolan *et al.* 1992; Valentine *et al.* 1995). We wanted to determine the identity of the *rad11* gene and to explore further the meiotic defects of the *rad11-1* mutant. In this study, we describe the

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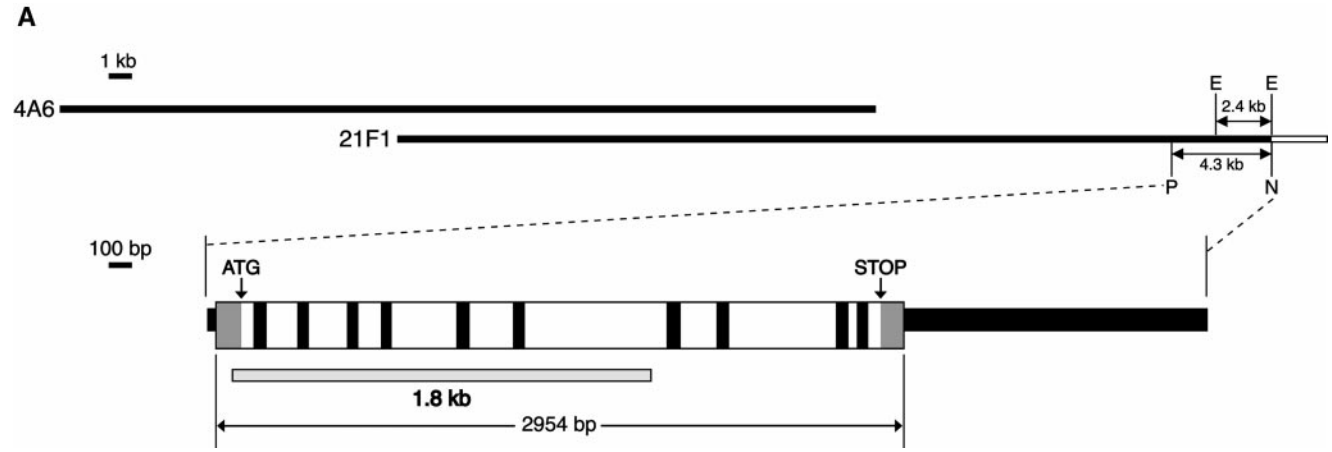

Figure 1.—(A) Gene structure of *C. cinereus mre11*. Cosmids 4A6 and 21F1 from the *C. cinereus* chromosome 6-specific library are indicated as black bars. The 2.4-kb *Eco*RI subclone that contains a portion of *mre11* and the 4.3-kb *Not*I-*Pst*I subclone that contains the entire coding sequence of *mre11* are indicated. The white portion of cosmid 21F1 represents vector DNA. The structure of *mre11* is shown as a large rectangle. 5′ and 3′ untranslated regions are shaded gray, while introns are shaded in black. The 1.8-kb region used as a probe for Northern analysis is depicted below the gene as a light gray bar. (B) An alignment among *mre11* translations from *C. cinereus*, *H. sapiens* (Paull and Gellert 1998), and *S. cerevisiae* (Johzuka and Ogawa 1995). The numbers on the right indicate the amino acid number for each sequence. Amino acids identical to those of *C. cinereus* Mre11 are represented as dots. Gaps introduced to maximize alignment are represented as dashes. Four putative phosphoesterase motifs are shaded black. The glutamine residue that is mutated in *mre11-1* to a stop codon is indicated by an asterisk. A region containing putative overlapping 7-aa nuclear localization signals is boxed. Sequences representing DNA-binding sites in *S. cerevisiae* are shaded gray; the first shaded region represents DNA-binding site A, and the second region is DNA-binding site B.

cloning of *rad11* and its identification as the *C. cinereus* homolog of *MRE11*. We also demonstrate that *rad11-1*/*mre11-1* nuclei display defects in chromatin condensation, homolog pairing, and synaptonemal complex (SC) structure.

MATERIALS AND METHODS

Strains and culture conditions: The dikaryotic wild-type *C. cinereus* strain (J6;5-5 × J6;5-4) used for transformation, gene expression, fruiting, and microscopy experiments has been described previously (Valentine *et al.* 1995). The *rad11-1*/*mre11-1* mutant was generated by UV mutagenesis of Java-6 (Binninger *et al.* 1987) and then was crossed and backcrossed a total of five times to the Rad$^+$ strain Okayama-7 (Wu *et al.* 1983; Valentine *et al.* 1995). Four *mre11-1* dikaryons congenic with the wild-type strain were generated by crossing sibling isolates from the fifth generation of backcrossed *mre11-1* strains: (1) *mre11-1;5-2* × *mre11-1;5-5*, (2) *mre11-1;5-101* × *mre11-1;5-110*, (3) *mre11-1;5-2* × *mre11-1;5-7*, and (4) *mre11-1;5-108* × *mre11-1;5-111*. The transformation strain was constructed by mating an *mre11-1* strain to strain 218 (*trp1-1;1-6*; Binninger *et al.* 1987) and isolating a Rad$^-$Trp$^-$ progeny isolate. This strain was also used to examine fruitbody development in the *mre11-1* mutant by crossing it to *mre11-1;5-2*.

Culture conditions, matings, and fruiting conditions were as described previously (Zolan *et al.* 1988), except for fruitbody development experiments, in which matings were performed directly on 25-mm slant cultures used to induce fruiting.

Gel electrophoresis and Southern hybridization: Genomic DNA was isolated as described in Zolan and Pukkila (1986). Restriction digests were performed as directed by the manufacturer (New England Biolabs, Beverly, MA). Gel electrophoresis was performed using 0.7–1.2% agarose gels in 1× TBE (Tris-HCl, boric acid, EDTA). Extraction of DNA from agarose gels was performed using the QiaexII Gel extraction kit (QIAGEN, Valencia, CA). Southern blots were performed using standard techniques (Zolan *et al.* 1992), and gels were transferred to Magnagraph nylon membrane (MSI). Probes were radiolabeled using the random priming method (Yeager Stassen *et al.* 1997), unless stated otherwise. Hybridizations were performed as described in Yeager Stassen *et al.* (1997), except that after 10–12 hr of hybridization, three 30-min washes at 65° using 0.2× SSC/0.1% SDS were performed, and then blots were exposed to X-ray film at −80°. The film was developed on a Kodak X-Omat automatic developer.

Chromosome walking: A chromosome walk was initiated from cosmid 4A6 from an Okayama-7 chromosome-6-specific library (Figure 1A; Zolan *et al.* 1992). 4A6 DNA was obtained using Wizard columns (Promega, Madison, WI) and homemade solutions prepared as specified by the manufacturer. The DNA was digested with *Pst*I, and T3 and T7 primers were used independently to generate primer extension products of insert DNA immediately adjacent to the vector. [^{32}P]dATP was used in the reaction to create radioactively labeled fragments that were used to probe the chromosome-6-specific library using standard colony hybridization procedures (Sambrook *et al.* 1989). In this way, clones were isolated that showed hybridization with each end of 4A6; this process was repeated for the next steps in the walk. Ultimately, a single step was taken in one direction (21F1; ~17 kb of new sequence; Figure 1A), while two steps were taken in the other direction (4B12 and 21F11; ~50 kb of new sequence); in total, ~100 kb of DNA was covered.

DNA from cosmids 21F1 and 21F11 was digested with *Eco*RI, and subclones representing each end of the walk were ligated into pBluescript SK$^+$ (Stratagene, La Jolla, CA), transformed into the XL1-Blue strain of *Escherichia coli* using the method of Pope and Kent (1996), sequenced, translated, and compared to all polypeptide sequences in the NCBI database using BLAST. A 2.4-kb *Eco*RI subclone of 21F1 showed sequence similarity to the Mre11 family of proteins (Figure 1A). Ulti-

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C. cinereus     MSDYEEDTRPPPNIETADPEDTIKILLATDNHIGYLERDPIRGQDSINTF  (50)
H. sapiens      ----------MSTADAL.D.N.F...V...I.L.FM.K.AV..N.TFV.L  (40)
S. cerevisiae   ----------MDYP..-..R..IT....V..N.N...T.D..WK..     (36)

C. cinereus     REILQLAVKNEVDFILLAGDLFHENKPSRDCLYQTLALLREYTLGDKPIQ  (100)
H. sapiens      D...R..QE.......G.........KT.HTC.E...K.CM..R.V.     (90)
S. cerevisiae   H.VMM..KN.N..MVVQS.....V....KKS...V.KT..LCCM....CE  (86)

C. cinereus     VELLSDPDEGKAAGFS-FPAINYEDPNFNISIPVFSIHGNHDDPQGPGVN  (149)
H. sapiens      F.I..--.QSVNF...K..WV..Q.G.L..............T.AD--    (136)
S. cerevisiae   L.....--.PSQVFHYDE.TNV.......G.S.....AS.DS--        (132)

C. cinereus     GALCALDVLSVSGLLNYMGKFDLPTSDADAATTGIAVRPVLLRKGSTKLG  (199)
H. sapiens      -......I..CA.FV.HF.R--------SMSVEK.DIS....Q.....IA  (177)
S. cerevisiae   -L..PM.I.HAT..I.HF..---------VIESDK.K.V.L.FQ......A  (173)

C. cinereus     MYGVGNVKDQRMHFELRSNRVRMYMPK-DKDEWFNILLVHQNRVKHGPQE  (248)
H. sapiens      L..L.SIP.E.LYRMFVNKK.T.LR..E.ENS...LFVI....S...STN  (227)
S. cerevisiae   L..LAA.R.E.LFRTFKDGG.TFEV.TMREG....LMC....HTG.TNTA  (223)

C. cinereus     YVPEGMFDDSVDLVVWGHEHDCRIIPEPVAGKNYYITQPGSSVATSLADG  (298)
H. sapiens      FI..QFL..FI...I.....E.K.A.TKNEQQLF..S......V...SP.  (277)
S. cerevisiae   FL..QFLP.FL.M.I.....E.IPNLVHNPI..FDVL..........CEA  (273)
                                    *
C. cinereus     EAIEKHVALLEIK-GKEFQLTPIPLRTVRPFVISEVVLEDAAEEEGLDVN  (347)
H. sapiens      ..VK...G..R..-.RKMNMHK...H...Q.FMEDI..---.NHPDIFNP  (323)
S. cerevisiae   ..QP.Y.FI.D..Y.EAPKM.....E.I.T.KMKSIS.---QDVPH.RPH  (320)

C. cinereus     DQMEITKYLKQKVNDLIDQAQALWEERNARSIEAGDEEIPPMLPLVRLKV  (397)
H. sapiens      .NPKV.QAIQSFCLEK.EEML-----E..ERERL.NSHQ.EK-.....R.  (367)
S. cerevisiae   .KDATS...IEQ.EEM.RD.NEETKQKL.DDG.GDMVAEL.K-..I..R.  (369)

C. cinereus     DTTNVTQTSNPI--------RFGQEFQGRVANPRDLLVFHRSKKAGKRGA  (439)
H. sapiens      .YSGGFEPFSVL---------..S.K.VD.....K.IIH.F.HREQKEKTG  (409)
S. cerevisiae   .YSAPSN.QS..DYQVENPR..SNR.V.....GNNVVQ.YKKRSPVT.S-    (418)

C. cinereus     GKVDIDQPELSIDDPDLTVSEKLAKVRVKTLVREYLAAQE----LQLLGE  (485)
H. sapiens      EEINFGKL--------I.KPSEGTTL..ED..KQ.FQTA.KNVQ.S..T.  (451)
S. cerevisiae   K.SG.NGTSI.DR.VEKLF..SGGELE.Q...NDL.NKMQ----.S..P.  (464)

C. cinereus     NGMSDAIQMFVEKDDIHAIQTHVNKSLKTMLKNIKSDEVD--EDDLDDLL  (533)
H. sapiens      R..GE.V.E..D.EEKD..EEL.KYQ.EKTQRFL.ERHI.AL..KI.EEV  (501)
S. cerevisiae   V.LNE.VKK..D..EKT.LKEFISHEISNEVGILSTN.EFLRT..AEEMK  (514)

C. cinereus     AKAKQRQEEEYLEATRAGESAKGKGKA-KATDDDGGAASDDSMLMDIDTG  (582)
H. sapiens      RRFRETRQKNTN.EDDEVRE.MTRAR.LRSQSEESAS.FSADD..S..LA  (551)
S. cerevisiae   .LI..VKRANSVRP.PPK.NDETNFAF----NGN.LDSFRS.N-REVR..  (559)

C. cinereus     GGATFNMSDDDDDDEPPPPPK-------RRAATSRATTTKKAPAKAPAKK  (625)
H. sapiens      E----Q.AN.S..SISAATN.GRGRGRG..GGRGQNSASRGGSQRGR.DT  (597)
S. cerevisiae   SP---DITQSHV.N.SRITHI------------.Q.ESS.PTSKPKRVRT  (594)

C. cinereus     ATTTTARGRGKKAAPPSSDDEVIELDDDEDEISEEEVVAKPVKRTSRAAV  (675)
H. sapiens      GLE.ST.S.NS.T.VSA.RN--MSII.AFKSTRQQPSRNVTT.NY.EVIE  (645)
S. cerevisiae   ..KKKIPAFSDSTVISDAEN.LGDNN.AQ.DVDID.NDIIM.STDEE---  (641)

C. cinereus     LSQSQAPAKKAPAKKKTPARQTQTQLSFAPAGRSSRAAASKARSKMVFDD  (725)
H. sapiens      VDE.DVEEDIF.TTS..DQ.WSS.SS.KI----M.QSQV..GVDFESSE.  (691)
S. cerevisiae   -DA.YGLLNGRKT.T..RPAASTKTA.RRGK..A..TPKTDILGSLLAKK  (690)

C. cinereus     DDDDDD-----------                                   (731)
H. sapiens      ....PFMNTSSLRRNRR                                   (708)
S. cerevisiae   RK---------------                                   (692)
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Figure 1.—(*Continued*)

mately, a 4.3-kb *Not*I-*Pst*I subclone was generated from the same end of 21F1; this fragment contained the entire coding sequence of *C. cinereus mre11* (Figure 1A).

Restriction fragment length polymorphism (RFLP) mapping: *C. cinereus* Okayama-7 and a fourth-generation backcrossed *rad11-1* strain (*rad11-1;4-8*) were mated, the resulting dikaryon was induced to fruit, and spore progeny were isolated. Genomic DNA from parental and progeny isolates was digested with *Hin*dIII, subjected to gel electrophoresis and Southern transfer, and probed with the radiolabeled insert of the 4.3-kb *Not*I-*Pst*I subclone of cosmid 21F1 (Figure 1A).

Transformation rescue assays: Transformations of *C. cinereus* oidial protoplasts were performed as described in Binninger *et al.* (1987) and Zolan *et al.* (1992). A *rad11-1;trp1-1,1-6* strain was transformed with either 3 μg of cosmid 21F1 (which contains the *trp1* marker) or 10 μg of a plasmid construct (pEG1) containing the *mre11* gene. To generate pEG1, a 4.1-kb fragment containing *mre11* plus 689 bases upstream of the initiating Met and 705 bases downstream of the terminal stop codon was amplified from cosmid 21F1 using Vent DNA polymerase (New England Biolabs). Amplifications were performed in a Gene Amp PCR system (model 2400; Perkin-

Elmer, Norwalk, CT). The product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and the *C. cinereus trp1* gene (Skrzynia *et al.* 1989) was cloned into the only *Hin*dIII site of the construct.

For each transformation, tryptophan prototrophs were selected on regeneration agar (Binninger *et al.* 1987) and screened for enhanced sensitivity to ionizing radiation by a chunk test (Zolan *et al.* 1988). Rescue of the meiotic defects of *rad11-1* was assayed by mating transformants to a compatible *rad11-1* strain and observing mushroom caps macroscopically for spore production. Six rescued transformants were analyzed for the presence of one or more additional copies of *mre11*. Genomic DNA was isolated and digested with *Hin*dIII, which does not digest within *mre11*. The DNA was subjected to gel electrophoresis and Southern transfer. The blot was hybridized with a radiolabeled 1.8-kb genomic fragment of *mre11* (Figure 1A), which was generated by amplifying the region of *mre11* using *Taq* polymerase (Fisher Scientific/Promega) and labeling the product with [^{32}P]dATP.

DNA sequencing and analysis: Plasmids containing subclones were isolated from *E. coli* using either a Qiagen Plasmid Mini kit (QIAGEN) or Perfect Prep Plasmid DNA kit (5 PRIME-3 PRIME, Inc.). Automated DNA sequencing was performed at the Indiana Institute for Molecular and Cellular Biology using a LI-COR model 4000L DNA Sequencer (LI-COR, Inc.) with a SequiTherm Long-Read cycle sequencing kit (Epicentre Technologies Corp.) or an ABi Prism model 377 DNA sequencer (Applied Biosystems, Foster City, CA) with a Thermo Sequenase cycle sequencing kit (Amersham Life Sciences, Inc., Piscataway, NJ). Internal oligonucleotide primers were made by Genosys, Inc. Sequencing data were assembled and edited using Sequencher ver. 3.0 (Gene Codes Corp.), and were translated using DNASIS ver. 2.0 (Hitachi Software Engineering Co., Ltd.). Predicted polypeptides were identified using blastx in BLAST (Altschul *et al.* 1990), and alignments among amino acid sequences were performed using CLUSTAL W ver. 1.74 (Thompson *et al.* 1994). Prediction of nuclear localization sequences was performed using PSORT (Nakai and Kanehisa 1992).

The genomic sequence of *mre11* from *C. cinereus* was determined from cosmid 21F1 (made from strain Okayama-7) and subclones derived from it (see above). The DNA sequences obtained were translated in all six reading frames and compared to all nonredundant polypeptides in the translated NCBI database (GenBank, Bethesda, MD) to identify similar sequences. The mutation in the *rad11-1*/*mre11-1* mutant was determined by amplifying overlapping regions of the *mre11* gene from backcrossed strain *rad11-1;4-8* or the original, unbackcrossed, *rad11* isolate in the Java-6 background and sequencing the PCR products directly, using the PCR primers as sequencing primers. Where polymorphisms between *rad11-1* and Okayama-7 sequences were noted, the relevant regions of *mre11* were sequenced from Java-6 genomic DNA to identify unique base changes in *mre11-1*. The sequence of a fifth-generation isolate of *rad11-2* was determined in the same manner used for *rad11-1*. Additionally, putative *rad11-2* isolates that represented each generation of backcrossing were screened for the mutation present in *rad11-1* by amplifying the affected region by PCR and digesting the product with *Mfe*I, which recognizes only the nonmutated sequence. Each strain used in phenotypic characterization (see above) was checked for the presence of the *rad11-1* mutation.

Isolation of *mre11* cDNA: A *C. cinereus* cDNA λ ZAP library constructed from cap tissue isolated 1 hr before and 1 hr after karyogamy (Yeager Stassen *et al.* 1997) was screened for *mre11*. To obtain the 3′ end of the *mre11* cDNA, primers were designed based on the genomic DNA that encodes the predicted open reading frame of *C. cinereus mre11* and a priming site within the vector (T7 promoter region). Two sequential PCR amplifications were performed. In the first, a primer from the 3′ region of *mre11* was used in conjunction with the T7 primer to amplify from the library: 3 μl of the library was diluted with 2 μl of nanopure water, heated to 100° for 3 min, and then quick-cooled. Samples were centrifuged for 3 min, and 2 μl of the supernatant was used as the template in a PCR reaction. A total of 1 μl of this reaction was used in a second PCR amplification, wherein a nested primer within the *mre11* sequence was used in combination with the T7 primer. PCR products were separated by gel electrophoresis, transferred, and probed with a portion of *mre11* (Figure 1A). Positive products were separated again on agarose gels, excised, purified, ligated into pCR2.1 (Invitrogen), and transformed into *E. coli* XL-1 Blue competent cells. A 1.1-kb clone that hybridized with an *mre11* probe (Figure 1A) was identified and sequenced.

The 5′ end of the *mre11* cDNA was amplified using random amplification of cDNA ends (5′ RACE; Frohman *et al.* 1988). A total of 7 μg of wild-type total meiotic RNA collected from cap tissue at karyogamy was used in a reverse transcription (RT) reaction (SuperScriptII; Gibco BRL Life Technologies, Grand Island, NY). cDNA was purified using the GlassMax system (Gibco BRL Life Technologies), tailed with dCTP (using terminal deoxynucleotidyl transferase; Promega), and 2 μl of the tailing reaction was used to amplify the 5′ end of the *mre11* cDNA, using a poly-G anchor primer and *mre11* primer. Products were separated by gel electrophoresis, and the gel was blotted and probed with a radiolabeled fragment of *mre11* genomic DNA (Figure 1A). Positive products were cloned and sequenced as above, and the 5′ end of the *mre11* cDNA transcript was identified by locating the first base that followed the poly-G tail.

RT-PCR was used to isolate the remainder of the *mre11* cDNA sequence. Poly(A)$^+$ RNA, purified from wild-type caps collected at 6 hr after karyogamy (Yeager Stassen *et al.* 1997), was used in the RT reaction with an oligo(dT) primer to generate cDNA (SuperScript II; Gibco BRL Life Technologies). To amplify the central 2.1 kb of *mre11* cDNA, 2 μl of the 20 μl RT reaction was used as the template in a 100-μl PCR reaction. The products were separated by gel electrophoresis, blotted, and hybridized with a genomic fragment of *mre11* (Figure 1A). The amplified product was cloned and sequenced. Finally, a second RT-PCR reaction using primers in the 5′ region of *mre11* was used to amplify the remainder of the *mre11* cDNA. The product was cloned and sequenced as described above.

Northern analysis: Approximately 0.5 g of cap tissue from 0, 6, and 12 hr after karyogamy was isolated from the wild-type cross J6;5-4 × J6;5-5. Java-6 vegetative dikaryon, Okayama-7 unirradiated vegetative monokaryon, and Okayama-7 vegetative monokaryon isolated 4 hr after irradiation with 40 krad also were collected. Total RNA was isolated as described in Yeager Stassen *et al.* (1997), except that the extraction buffer and ethanol solutions did not contain diethylpyrocarbonate (DEPC), and RNA samples were resuspended in RNase-free H_2O. RNA samples were separated by gel electrophoresis on 1.2% agarose gels containing formaldehyde (Maniatis *et al.* 1982) and were blotted onto nylon membrane. Blots were hybridized with a 1.8-kb genomic fragment of *mre11* (Figure 1A) using QuikHyb (Stratagene). Blots were exposed either to radiographic film or to a phosphorimager screen (Molecular Dynamics) and were visualized using a PhosphorImager scanner and ImageQuant ver. 3.3 software (Molecular Dynamics). Gels were stained with ethidium bromide to confirm equal loading, based on the quantity of rRNA present.

Radiation sensitivity assays: Chunk tests were performed as described by Zolan *et al.* (1988) in a ^{137}Cs irradiator (Mark-1 model 68-A, J. L. Shepard and Associates).

UV sensitivity of *mre11-1* oidia was assayed as described in Ramesh and Zolan (1995), with a dose rate of 2 J/m^2/sec at a distance of 45 cm. Doses used were 0, 50, 100, and 150 J/m^2.

Microscopy: For DAPI (4′,6-diamidino-2′-phenylindole dihydrochloride) staining of basidia, a sliver of cap tissue approximately the thickness of one gill layer was taken from a fruitbody from which the veil cells had been removed. A drop of DAPI (1 μg/ml) was applied to the tissue on a glass slide, a coverslip was added, and the sample was flattened by gently tapping the coverslip with a rubber mallet. Tissue was examined under UV fluorescence on a Nikon Microphot-FXA microscope equipped with appropriate epifluorescence filters.

Surface spreads of *C. cinereus* meiotic chromosomes were performed as described by Pukkila *et al.* (1992). Acridine orange and silver nitrate staining of meiotic nuclei were performed as described in Seitz *et al.* (1996). Analysis of chromatin condensation was performed on a Nikon Microphot-FXA fluorescence microscope. Data were collected on 35-mm color slide film. For examination of silver-nitrate-stained synaptonemal complexes, electron microscopy grids were floated and then analyzed using a JEOL-1010 electron microscope.

Fluorescence *in situ* hybridization (FISH) was performed as described in Li *et al.* (1999). Meiotic nuclei from *mre11-1* caps were examined at 0, 6, and 10 hr after karyogamy. DNA probes were prepared as described in Li *et al.* (1999). Each probe was composed of two overlapping cosmids from interstitial regions of either chromosome 8 or 13. The chromosome 8 probe consisted of cosmids 2G7 and 3F2 from the chromosome-8-specific library (~61 kb in complexity), and the chromosome 13 probe consisted of cosmids 1B2 and 2B9 from the chromosome-13-specific library (42 kb).

Spore production assay: Spore production was determined using the drop method described by Ramesh and Zolan (1995).

RESULTS

Isolation of *mre11* from *C. cinereus*: The *C. cinereus rad11* gene is required for successful completion of meiosis and for survival after exposure to ionizing radiation (Valentine *et al.* 1995). We had shown previously, using RFLP and chromosome length polymorphism mapping, that *rad11* is located on chromosome 6 of *C. cinereus* (Zolan *et al.* 1994). Using a *C. cinereus* chromosome-6-specific cosmid library, we had isolated a cosmid (4A6; Figure 1A), which is 1.6 map units from *rad11* (Zolan *et al.* 1992). Because a single map unit in *C. cinereus* can be as small as 30 kb (Dutta 1974; Holm *et al.* 1981), a bidirectional chromosome walk was initiated from this cosmid. Cosmids that hybridized with either end of 4A6 were identified and the process was repeated for subsequent steps in the walk. DNA fragments from the distal ends of the walk were subcloned and sequenced, and the DNA sequence from the distal end of cosmid 21F1 (Figure 1A) was found to encode a polypeptide with similarity to Mre11 homologs from *Homo sapiens*, *Mus musculus*, *S. cerevisiae*, *Schizosaccharomyces pombe*, and *C. elegans*.

mre11 mutants in *S. cerevisiae* exhibit enhanced radiation sensitivity and meiotic defects (*e.g.*, Ajimura *et al.* 1993). Therefore, several experiments were performed to determine whether *mre11* is *rad11*. First, RFLP mapping was used to ask whether *mre11* maps to the *rad11* locus. No recombination between an RFLP pattern specific to *mre11* and the radiation-sensitivity phenotype was revealed in 50 progeny of a cross between a wild-type strain (Okayama-7) and *rad11-1* (data not shown). This indicated that the region of DNA containing *mre11* is two or fewer map units from the locus encoding *rad11*.

Second, transformation experiments were performed to determine whether *mre11* from *C. cinereus* is able to rescue the meiotic defects and radiation sensitivity of the *rad11-1* mutant. A *rad11-1;trp1-1,1-6* strain was transformed with either cosmid 21F1 or a plasmid containing the *mre11* coding sequence plus ~0.7 kb upstream and downstream of the gene (pEG1); both constructs contained the *trp1* marker. Tryptophan prototrophs (184 from the cosmid transformation and 97 from the pEG1 transformation) were screened for radiation sensitivity and gross spore production levels. Transformation with either construct resulted in rescue of both defects of *rad11-1*. A total of 79% of cosmid transformants and 82% of pEG1 transformants were resistant to 40 krad of γ-radiation as measured by growth of a small mycelial patch (chunk test; Zolan *et al.* 1988). Of these transformants, 83% of cosmid transformants and 76% of subclone transformants were competent for spore production; complementation of the sporeless phenotype was observed in crosses of transformants to a compatible *rad11-1* strain. To confirm that the phenotypic rescue of *rad11-1* was due to introduction of *mre11*, six transformants exhibiting rescue of the radiation-sensitivity phenotype were analyzed for the presence of additional copies of *mre11* in their genomes. In each, at least one new band was detected using a probe specific to *mre11* (Figure 1A; data not shown). Thus, the rescue of both phenotypes of *rad11-1* in these transformants likely was due to the presence of introduced *mre11* DNA.

In a third approach to determine whether *mre11* is *rad11*, we asked if the *mre11* gene is altered in the *rad11-1* mutant. The genomic sequence of *mre11* from the Okayama-7 strain of *C. cinereus* was determined (GenBank accession no. AF178433). A single base change (C to T transition) was identified in the *rad11-1* mutant 1256 bases downstream of the predicted translational start site of *mre11*. This mutation was identified in a fourth-generation backcrossed isolate (see materials and methods), and the original, unbackcrossed *rad11-1* mutant also carries the same C to T transition. The *mre11* gene also was sequenced in the wild-type strain (Java-6) that was mutagenized to create *rad11-1*. The base change in *rad11-1* is unique to the *rad11-1* strain and is not a polymorphism present between Java-6 and Okayama-7. Thus, three lines of evidence, RFLP mapping, transformation rescue, and direct sequencing, have shown that *C. cinereus rad11* is *mre11*.

Previous studies in *C. cinereus* had indicated that our collection of mutants contained two mutant alleles of *rad11*. Fifth-generation backcrossed isolates of two

TABLE 1

***mre11* introns**

No.	Position relative to ATG	Size (bp)	5′ Splice junction[a]	Internal sequence[b]	3′ Splice junction[c]
1	52	57	GCTGgtctgt	tcagtggtcccgtcaaaagcaataaggtctgatgaattggtggttttg	tagACCC
2	240	50	TGAGgtattg	actcccaatgtgtctctgcatggtcagggttaacggtgccc	aagGTCG
3	454	49	TCTCgtacgt	gatgacgatattaccgctttgtccagtcattcacgtcaaa	cagATTC
4	600	46	AGTCgtacgt	ccacgttatcttgccccccactacgactgacgctctc	cagAACG
5	924	57	ACCGgtgggc	tatttcataatggttaggccttgcactgttgttgacctggcctttctt	cagTGTG
6	1167	51	AAAAgtaggt	tgatactttatttttccccaacttcaccattctcaccgaagcg	cagACAC
7	1827	61	AGACgtgcgc	ccttgtgttccccaatgccctatttttttgaattgacgcgtttcaacttgctc	tagACAT
8	2042	54	CAAAgtgtgt	ttctctttcttgcacttattaaaacccactctaatgcctttgtta	aagGGAA
9	2555	54	TGAGgtgcgt	tcgaagaattttgtggaggaggagtttgctcacaagtgtggtttt	cagTCAA
10	2645	49	GGTGgtaagt	ttcatcggcttcagttgaatacagctcgctcatgcttttg	cagTTTG

[a] Uppercase letters denote exon sequence. Lowercase letters designate the beginning of the intron.
[b] Underlined sequences are those that are similar to the branchpoint consensus sequence CTRAY or a variation, TTRAY (Keller and Noon 1984; Brown 1986).
[c] Lowercase letters are the final bases of the intron. Uppercase letters denote the beginning of the following exon.

strains in our collection failed to complement one another, and their mutations mapped to the same locus on chromosome 6; these mutants were named *rad11-1* and *rad11-2* (Valentine *et al.* 1995). However, analysis of a fifth-generation isolate of *rad11-2* showed that the sequence of *mre11* in this strain is identical to that of *mre11* in *rad11-1*, including the C to T mutation at the same position. Examination of the presumptive unbackcrossed progenitor of this strain showed no defect in the *mre11* gene. We conclude that *rad11-1* was duplicated inadvertently during the backcrossing procedure. Thus, all fifth-generation isolates of *rad11-1* and *rad11-2* have been collectively renamed *mre11-1*. All strains used for the experiments presented in this work were examined and have the identical mutation in *mre11*.

Gene structure and expression of *mre11* in *C. cinereus*: To determine the structure of *mre11* in *C. cinereus*, the cDNA sequence was obtained and aligned to the genomic sequence for strain Okayama-7. The *mre11* coding sequence consists of 2193 bases interrupted by 10 introns ranging in size from 46 to 61 bp, and the average size is 53 bp (Table 1; Figure 1A). The number and average size of the introns are consistent with those of introns from other *C. cinereus* genes [*e.g.*, *trp1* (Skrzynia *et al.* 1989), *rad9* (Seitz *et al.* 1996), and *rad51* (Yeager Stassen *et al.* 1997)]. The 5′ splice sites of the introns correlate well with the consensus sequence determined for filamentous fungi (GTRNGT, Table 1; Gurr *et al.* 1987; Edelmann and Staben 1994). The 3′ splice sites are also similar to the consensus sequence, YAG; in introns 2 and 8, the Y was replaced by an A, which is not uncommon for fungal genes (Gurr *et al.* 1987; Seitz *et al.* 1996). Each intron was examined for a branchpoint consensus sequence (CTRAY) derived from the examination of various multicellular eukaryotes, including fungi (*Neurospora crassa*; *e.g.*, Kinnaird and Fincham 1983; Woudt *et al.* 1983), plants (Brown 1986), and mammals (Keller and Noon 1984). Each *mre11* intron contained at least one sequence that corresponded well to this consensus sequence (underlined in Table 1). In many cases, a T was present in the first position, which is common in the introns of many organisms (*e.g.*, Keller and Noon 1984; Brown 1986). The consensus branchpoint sequence that can be derived from these 10 introns is YTNAY. In every intron, one consensus sequence was found that is predicted to initiate between 12 and 20 bases from the 3′ splice junction (Table 1).

The transcriptional start site of *C. cinereus mre11* was determined by 5′ RACE. The first base following the G-tail sequence used in the procedure was located 108 nt upstream of the predicted translational start site (Figure 1A); this methionine codon was selected because it was the only one present in the proper reading frame in the first 165 amino acids (aa) of the predicted Mre11 polypeptide sequence. The 3′ end was determined by sequencing a cDNA clone obtained by amplification from a *C. cinereus* cDNA library (see materials and methods) and identifying the last base before the poly(A) tract. This was found to be 123 nt from the translational stop site (Figure 1A).

Expression of *mre11* during meiosis and following gamma irradiation was examined by Northern analysis. Meiosis is naturally synchronous in *C. cinereus*; therefore, we examined the expression of *mre11* in wild-type cap tissue from different meiotic stages. A 2.4-kb transcript was observed at karyogamy (K; Figure 2A, lane 1) and, of the timepoints examined, was most abundant at 6 hr after karyogamy (K + 6; the pachytene stage of prophase I; Figure 2A, lane 2). Analysis of phosphorimager data indicated a fourfold induction of *mre11* transcript levels

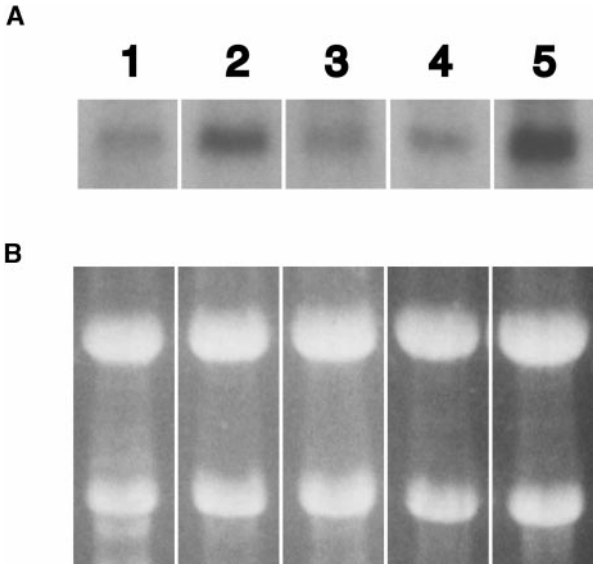

Figure 2.—Northern analysis of *mre11* expression. (A) Wild-type meiotic RNA hybridized with a 1.8-kb fragment of *mre11* genomic DNA (Figure 1A). Lane 1, cap tissue isolated at karyogamy. Lane 2, cap tissue at 6 hr after karyogamy. Lane 3, cap tissue at 12 hr after karyogamy. Lane 4, total RNA isolated from wild-type vegetative monokaryon tissue. Lane 5, total RNA isolated from wild-type vegetative monokaryon tissue 4 hr after irradiation. (B) Gel that was blotted for the hybridization shown in A, stained with ethidium bromide.

between K and K + 6. A twofold reduction in transcript was observed between K + 6 and K + 12 (Figure 2A, lanes 2 and 3). A transcript also was observed in vegetative monokaryon tissue, and a twofold induction of transcript level was observed in vegetative tissue harvested 4 hr after irradiation (Figure 2A, lanes 4 and 5). The gel was stained with ethidium bromide prior to blotting to confirm equal loading of the samples, based on the quantity of rRNA present (Figure 2B).

Characteristics of *C. cinereus* Mre11: The predicted *C. cinereus* Mre11 polypeptide (CcMre11) consists of 731 aa and has a molecular mass of 80.6 kD and a pI of 4.86, values that agree well with those of Mre11 from *H. sapiens* (81 kD, Petrini *et al.* 1995; pI of 5.4) and *S. cerevisiae* (72 kD, Johzuka and Ogawa 1995; pI of 5.4). The polypeptide shows high levels of similarity with other homologs of Mre11. Like other members of the family, CcMre11 is well-conserved throughout the N-terminal portion of the polypeptide but is quite divergent in the C terminus (Figure 1B). Pairwise alignments were performed between aa 18–478 of CcMre11 and the equivalent regions of three Mre11 homologs. MUS23 from *N. crassa* (Watanabe *et al.* 1997) shares 50.5% identity with CcMre11, Mre11 from *H. sapiens* (Paull and Gellert 1998) shares 47.1% identity, and Mre11 from *S. cerevisiae* (Johzuka and Ogawa 1995) shares 44.5% identity with CcMre11. The well-conserved putative phosphoesterase motifs required for the nuclease activities of Mre11 are also present in CcMre11 (Figure 1B, shaded in black; Sharples and Leach 1995). A region containing putative overlapping 7-aa nuclear localization sequences was identified in CcMre11 from aa 598–607 (Figure 1B, boxed). The C to T transition identified in the *mre11-1* mutant (discussed above) changes the predicted glutamine residue (aa 316; Figure 1B, asterisk) into a stop codon. If the mutant gene is translated, this change should result in a truncated polypeptide less than half the length of the wild-type protein.

Mitotic phenotypes of the *C. cinereus mre11-1* mutant: The *mre11-1* mutant was isolated in a screen for strains that showed both enhanced sensitivity to ionizing radiation and defects in meiosis (Valentine *et al.* 1995). The mutation in *mre11-1* is recessive; full complementation of both phenotypes is achieved when *mre11-1* is crossed to wild-type strains (Valentine *et al.* 1995). The *mre11-1* mutant demonstrates enhanced sensitivity to ionizing radiation. Following irradiation with 40 krad of gamma rays, small patches of *mre11-1* mycelia inoculated on complete medium show no new growth after 2 days, whereas the growth of wild-type cultures is not inhibited. In more sensitive assays, we have shown that uninucleate mitotic spores (oidia) have 0.1% survival following 40 krad of radiation treatment (Valentine *et al.* 1995). To investigate whether *mre11-1* is sensitive to UV radiation, oidia from *mre11-1* and wild-type strains were screened for survival following UV irradiation. The *mre11-1* mutant did not show a level of survival lower than that of the wild-type strain; *e.g.*, at 100 J/m^2, both strains showed ~5% survival. These results are similar to those obtained for other members of the *C. cinereus* epistasis group that includes *mre11-1* (Zolan *et al.* 1988; Ramesh and Zolan 1995).

A dikaryon of *C. cinereus* can be induced to produce mushrooms under certain light and temperature conditions (Zolan *et al.* 1988). In this process, the dikaryotic mycelium forms hyphal knots, some of which differentiate into fruitbody initials. These in turn grow in size to form a mature mushroom. The culminating event of fruitbody formation is the successful completion of meiosis and spore formation in the mushroom cap; however, fruitbody development is a mitotic event that occurs even in the absence of meiosis (Zolan *et al.* 1988; Pukkila 1994).

We have found that fruitbody development is slower, and fewer mushrooms are formed, in the *mre11-1* mutant than in a wild-type strain. The number of mushrooms generated per mating inoculum and the number of days required for fruitbody maturation were determined for 10 independent crosses between either congenic wild-type strains (J6;5-4 × J6;5-5) or compatible *mre11-1* strains. In wild-type crosses, the number of mushrooms produced ranged from two to five, with half of the matings yielding more than three mushrooms each. In contrast, the majority of matings (80%) between *mre11-1* strains produced one or two mushrooms

each, and no mating produced more than three mushrooms. The time required for fruitbody maturation was longer in the *mre11-1* mutant as well. For wild-type crosses, the first day mature fruitbodies (opened mushrooms) were observed occurred on day 10 or 11 postinoculation. In contrast, most *mre11-1* mushrooms appeared between 12 and 14 days postinoculation, and one mating required 20 days to fruit. This effect was not due to obvious differences in growth rate between wild-type and *mre11-1* monokaryons nor to differences in the kinetics of mating between the two types of crosses (data not shown). The delay of fruiting in *mre11-1* appeared to occur in the initiation of fruitbody formation, as the maturation of a fruitbody initial to a full mushroom occurs with the same kinetics in *mre11-1* as in wild-type cultures; once a fruitbody initial appears, it usually takes 2–3 days to develop into a mature, opened mushroom.

Meiotic progression and chromatin condensation in the *mre11-1* mutant: We have exploited the natural synchrony of meiosis in *C. cinereus* to examine the meiotic defects of the *mre11-1* mutant. DAPI staining of *mre11-1* basidia revealed that karyogamy, the beginning of meiosis, occurred at approximately the same time as in wild-type cells (Seitz *et al.* 1996); roughly 50% of nuclei showed nucleolar fusion 1 hr prior to the lights coming on, on a 16-hr light/8-hr dark schedule.

Meiotic prophase I is characterized by chromatin condensation and SC formation; full SC structures characterize the pachytene stage (Von Wettstein *et al.* 1994). Wild-type *C. cinereus* meiotic nuclei are essentially all in pachytene at K + 6 (Raju and Lu 1970; Pukkila *et al.* 1992; Seitz *et al.* 1996; Li *et al.* 1999); the chromosomes appear as highly condensed, visibly paired figures by acridine orange staining (Figure 3A; Seitz *et al.* 1996). In the *mre11-1* mutant, acridine-orange-stained nuclei exhibited variable, but always incomplete, levels of condensation at K + 6, as assessed by the visualization of individual chromosomes. The majority of nuclei showed limited condensation superimposed upon a diffuse background, and short regions of apparent pairing were occasionally visible (Figure 3B). However, in some nuclei (~25% of all nuclei examined), the chromosomes appeared distinct and condensed with limited visible pairing, reminiscent of the leptotene stage in wild-type nuclei (Figure 3C; Seitz *et al.* 1996). The ratio in which these phenotypes occurred was not consistent; on some slides, most nuclei appeared to be of the diffuse type, while on others, the condensed yet well-spread phenotype was predominant. The less-condensed nuclei did not seem to spread as well as those that were more condensed; this has also been observed for alleles of *rad12* (Ramesh and Zolan 1995). No nuclei achieved wild-type levels of chromatin condensation at K + 6.

Metaphase occurs in wild-type nuclei by K + 9 (Figure 3D). This stage is characterized by a greater level of condensation than that observed at K + 6, and it is

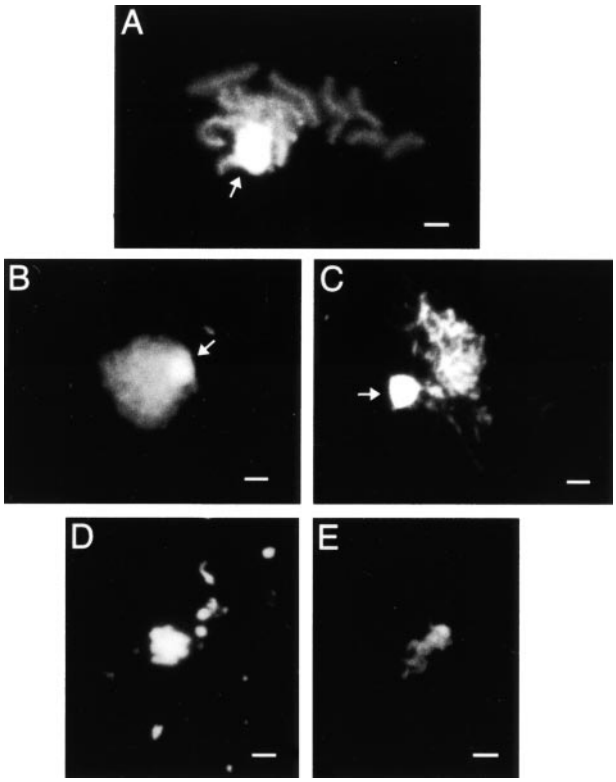

Figure 3.—Chromatin condensation in wild-type and *mre11-1* meiotic nuclei. Meiotic nuclei from various timepoints were spread and were stained with acridine orange. (A) A pachytene wild-type nucleus at 6 hr after karyogamy (K + 6). (B) An uncondensed *mre11-1* nucleus at K + 6. (C) A condensed, but unpaired, *mre11-1* nucleus at K + 6. (D) A wild-type metaphase nucleus at K + 9. (E) An *mre11-1* metaphase-like nucleus at K + 12. Arrows in A–C indicate the nucleolus. Bars, 1 μm. Numbers of *mre11-1* nuclei scored were as follows: K + 6, 173; K + 12, 14.

preceded by a brief "diffuse stage" at diplotene in which the chromatin appears less condensed than at the pachytene stage (Lu and Raju 1970; Seitz *et al.* 1996). Between K + 8 and K + 10, acridine orange staining of *mre11-1* nuclei revealed a slight increase in condensation from the levels observed at K + 6; by K + 11, nuclei appeared more diffuse than those at K + 8 to K + 10 (data not shown). By K + 12, many nuclei appeared to achieve a more highly condensed, metaphase-like state (Figure 3E). The fraction of nuclei in metaphase varied among samples; one preparation had 30% metaphase-like spreads, while a different slide contained virtually 100% metaphase-like spreads.

By K + 12, wild-type basidia have completed the two meiotic divisions and contain four nuclei (Raju and Lu 1970; Valentine *et al.* 1995). By K + 15, most *mre11-1* basidia exhibited a single diffuse or condensed nucleus (data not shown), as was seen by Valentine *et al.* (1995) at K + 12 using propidium iodide staining. Some cells contained deformed or stretched nuclei, and a few contained two nuclei, suggestive of anaphase-like activity; however, no further meiotic progression was ever ob-

served (data not shown; Valentine *et al.* 1995). The *mre11-1* mutant also makes few spore initials as observed by electron microscopy, and often only a single spore is initiated instead of four (Valentine *et al.* 1995). Macroscopically, mushroom caps appear to be devoid of mature spores, and quantitation of spore production confirmed that essentially no mature spores are made in *mre11-1* mushrooms.

Synaptonemal complex formation and homolog pairing in *mre11-1*: Surface-spread nuclei of *mre11-1* were stained with silver nitrate and observed by transmission electron microscopy to examine the SC, a proteinaceous structure that forms between homologs during prophase I. In wild-type *C. cinereus*, axial elements (AE), which are components of the SC, form completely and subsequently synapse in the mature SC (Holm *et al.* 1981; Seitz *et al.* 1996). Wild-type meiotic nuclei exhibit maximal synapsis at pachytene (K + 6; Figure 4A; Seitz *et al.* 1996). In contrast, neither AEs nor mature SC was complete in the *mre11-1* mutant at the same timepoint. Some synapsis was evident in the majority (62%) of nuclei at K + 6, but not all AEs present were synapsed

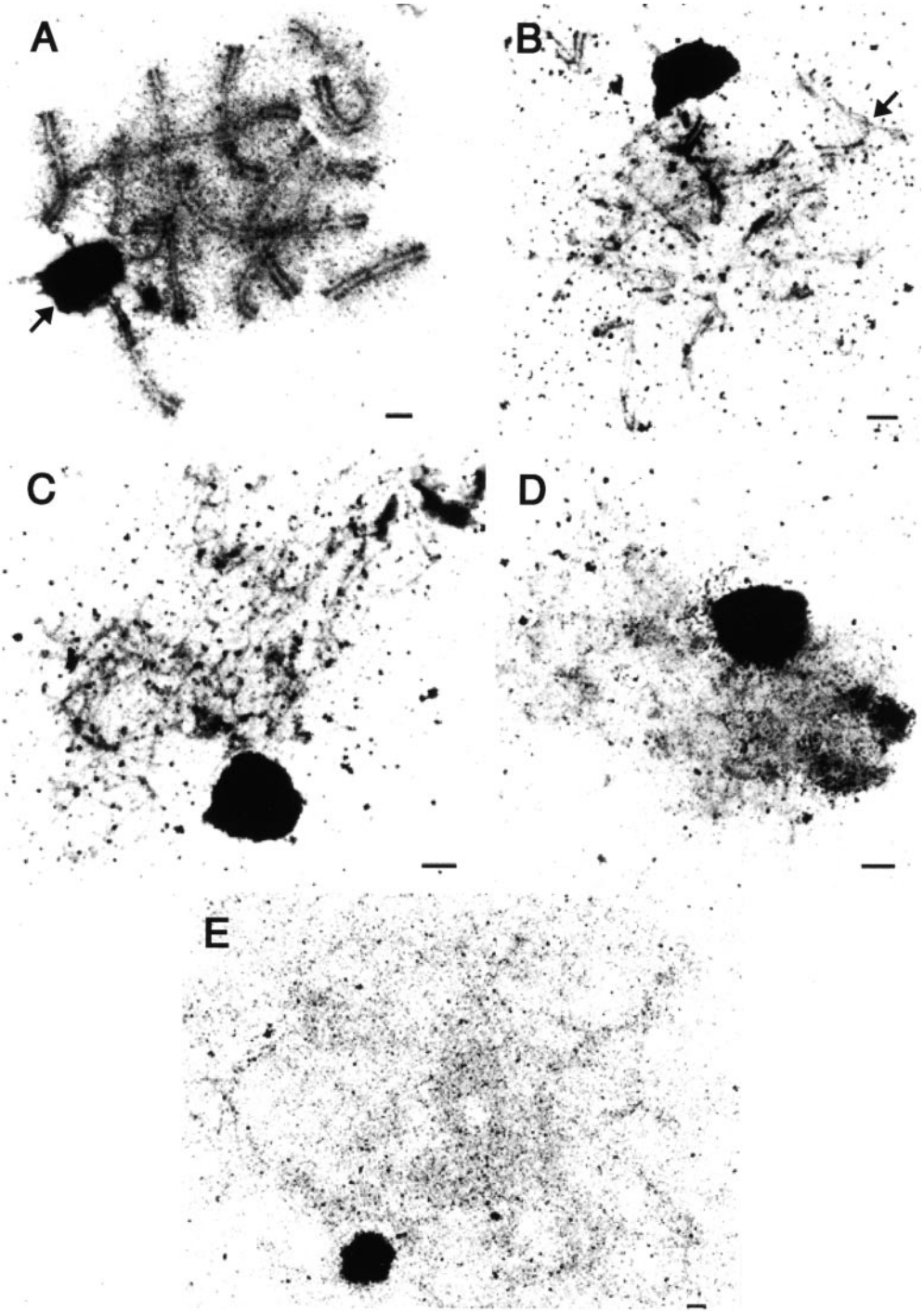

Figure 4.—SC formation in wild-type and *mre11-1* nuclei. (A) A wild-type pachytene nucleus at 6 hr after karyogamy (K + 6). The arrow indicates the nucleolus. (B–D) *mre11-1* nuclei at K + 6, as described in the text. The arrow in B indicates aberrant synapsis. (E) An *mre11-1* nucleus at K + 10. Bars, 1 μm. Numbers of *mre11-1* nuclei scored were: K + 6, 82; K + 10, 13.

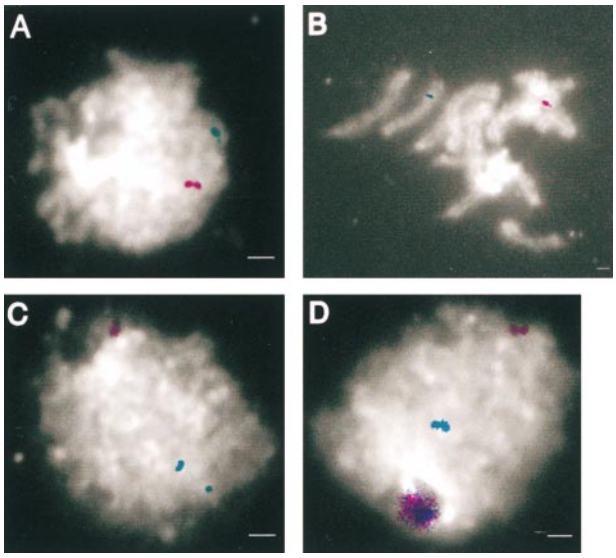

Figure 5.—FISH analysis of wild-type and *mre11-1* meiotic chromosomes. Chromosome 8 is indicated in blue, and chromosome 13 is in red. (A) A wild-type nucleus at 1 hr prior to karyogamy (K − 1), just after nucleolar fusion. (B) A wild-type pachytene nucleus at 6 hr after karyogamy (K + 6). (C) An *mre11-1* nucleus at K + 6 in which the locus on chromosome 13 is paired, but that on chromosome 8 is not. (D) An *mre11-1* nucleus at K + 6 in which both chromosomes 8 and 13 exhibit paired signals. The nucleolar signal present in this sample is background we occasionally observe. Bar, 1 μm.

(Figure 4B). Additionally, regions of synapsis involving three different AE fragments were occasionally observed (data not shown). In some cases, possible pairing partner switches or aberrant synapsis was observed (arrow, Figure 4B). Some nuclei (26%) showed no synapsis, exhibiting only limited AE formation against a diffuse background (Figure 4C); these nuclei resembled wild-type leptotene nuclei (Seitz *et al.* 1996), but the AEs were generally less complete and were more wispy in appearance. A small percentage (13%) of nuclei had no discernible structure at K + 6 (Figure 4D). By K + 10, no SC was visible in any nucleus; only a diffuse haze was observed (Figure 4E). Thus, as in wild-type nuclei (Seitz *et al.* 1996), any SC that forms in *mre11-1* nuclei is dispersed or degraded by late prophase.

Some synapsis occurs in more than half of all *mre11-1* nuclei. However, because SC formation is not dependent upon homology (McClintock 1933; Loidl *et al.* 1991; Maguire and Riess 1994), the synapsis data do not show explicitly whether pairing associations between homologous chromosomes are defective in the *mre11-1* mutant. FISH was used to assay homolog pairing in *mre11-1*. DNA probes from either chromosome 8 or 13 were hybridized to spread meiotic nuclei of *mre11-1* and were labeled with FITC and rhodamine, respectively. The chromosomes were counterstained with DAPI, and the spreads were examined by fluorescence microscopy. In previous studies of homolog pairing in wild-type nuclei, we defined pairing for each probe as one observable focus of staining or two foci situated 1.1 μm or less from one another (Li *et al.* 1999). In wild-type nuclei, pairing occurs rapidly, such that chromosome 8 was paired in 93% of meiotic nuclei examined just after nuclear and nucleolar fusion (Figure 5A), and >95% of probes from either chromosome 8 or 13 were paired at K + 6 (Figure 5B; Li *et al.* 1999).

Pairing was determined for *mre11-1* nuclei at K + 1, K + 6, and K + 10. At each timepoint, a given probe was paired in about half of all nuclei examined, although at each timepoint, the locus on chromosome 13 was more likely to be paired than that on chromosome 8 (Table 2). Approximately 75% of nuclei demonstrated pairing for at least one of the two chromosomes (Figure 5C; Table 2), but only 30% showed pairing for both chromosomes examined (Figure 5D; Table 2). These results demonstrate that homolog pairing interactions stable to spreading are reduced, but not abolished, in the *mre11-1* mutant.

DISCUSSION

MRE11 homologs have been identified in a wide range of organisms, including *S. cerevisiae* (Ajimura *et al.* 1993), *S. pombe* (Tavassoli *et al.* 1995), *N. crassa*

TABLE 2

FISH of *mre11-1* meiotic nuclei

			Percentage pairing[d]			
Cross[a]	Timepoint[b]	*n*[c]	Ch. 8	Ch. 13	Both Ch. 8 and Ch. 13	Either Ch. 8 or Ch. 13
2	K + 1	116	49	57	28	78
1	K + 6	67	54	67	36	85
2	K + 6	112	48	63	33	78
2	K + 10	91	41	51	32	68

Ch., chromosome.
[a] *mre11-1* crosses and FISH probes are identified in materials and methods.
[b] Number of hours after karyogamy (K).
[c] Number of nuclei examined.
[d] Percentage of total nuclei in which listed chromosome(s) demonstrated pairing.

(Watanabe *et al.* 1997), and humans (Petrini *et al.* 1995). Mre11 shows sequence similarity to the SbcD endonuclease from *E. coli* (Sharples and Leach 1995), and it has been shown recently to have several activities related to processes of DSB repair (primarily by the mechanism of nonhomologous end-joining), telomere maintenance, and meiotic recombination (reviewed in Haber 1998). In conjunction with Rad50 and Xrs2 (in *S. cerevisiae*) or p95, the product of the *NBS1* gene implicated in Nijmegen breakage syndrome (in humans; Carney *et al.* 1998), Mre11 is thought to act as a nuclease and/or structural component in the repair of DSB lesions (Bressan *et al.* 1998; Trujillo *et al.* 1998; Usui *et al.* 1998). Mre11 also is required for the formation and processing of the DSBs that initiate meiotic recombination in *S. cerevisiae* (Johzuka and Ogawa 1995; Nairz and Klein 1997; Tsubouchi and Ogawa 1998; Moreau *et al.* 1999).

The *mre11* gene of *C. cinereus*: We isolated the *mre11* gene from *C. cinereus* and showed that *mre11* corresponds to the genetically defined *rad11* locus. By identifying *C. cinereus rad11* as *mre11*, we have linked *rad11* and, by association, the other *C. cinereus rad* genes with meiotic function to pathways involved in DNA repair. The coding sequence of *mre11* in *C. cinereus* is 2193 bases in length, and it is interrupted by 10 introns (Figure 1A). The *C. cinereus mre11* transcript is 2.4 kb in size and is induced during meiosis and after treatment with ionizing radiation (Figure 2). During meiosis, a transcript is present at the K timepoint but is more abundant at K + 6. Defects in homolog pairing are observable at K in the *mre11-1* mutant (*e.g.*, Table 2), confirming that Mre11 is required early in meiosis. It is unclear whether the *mre11* transcript levels reflect protein accumulation accurately; Mre11 protein could be more abundant prior to K + 6, even though transcript levels appear to peak at that timepoint. This idea is not without precedent, as it has been shown that transcript levels of *S. cerevisiae RAD50* fluctuate independently of Rad50 protein levels (Raymond and Kleckner 1993). Alternatively, while certain activities of Mre11 may be needed early in prophase, other functions, such as recombination initiation, could peak during pachytene in *C. cinereus*.

At 4 hr following gamma irradiation, the *mre11* transcript level was induced only twofold over that of an unirradiated control culture (Figure 2A). The actual peak of induction following irradiation may occur at a different time; *rad51* expression peaks at 2 hr after irradiation (Yeager Stassen *et al.* 1997). However, observed induction also depends on the baseline expression level, and an *mre11* transcript was observed in the unirradiated monokaryon culture. This suggests that *mre11* may be required for normal mitotic growth and, thus, is an essential gene in *C. cinereus*. If this is true, then *mre11-1* likely is not a null mutant allele. Examination of both wild-type and mutant Mre11 protein levels will assist us in understanding how *mre11* expression is regulated in meiosis and following irradiation.

In BLAST analysis, the *C. cinereus* Mre11 protein showed highest identity (50.5% over 461 aa) to an Mre11 homolog in *N. crassa*, MUS23 (Watanabe *et al.* 1997). However, MUS23 is truncated at the C terminus relative to other Mre11 homologs and may be functionally dissimilar to them (Watanabe *et al.* 1997, and see below). Among other homologs, CcMre11 shows highest similarity to Mre11 from mammals. It shares the conserved features common to all Mre11 proteins: the four phosphoesterase motifs required for the nuclease activities of Mre11 and acidic residues at the C terminus (Figure 1B; *e.g.*, Sharples and Leach 1995; Watanabe *et al.* 1997).

The *C. cinereus mre11-1* mutant likely does not make a full-length protein; it is predicted to truncate after aa 315 (Figure 1B). Usui *et al.* (1998) have shown in *S. cerevisiae* that a region at the C terminus (DNA-binding site B; Figure 1B) is required for meiotic DSB formation. A second region, DNA-binding site A (Figure 1B), is located between aa 410 and 420 in *S. cerevisiae* and is required for DSB processing. Both DNA-binding site A and functional phosphoesterase sequences appear to be necessary for nuclease activity in *S. cerevisiae* (Usui *et al.* 1998). Although the phosphoesterase motifs are not altered in the *C. cinereus mre11-1* mutant, neither DNA-binding region is predicted to exist in the mutant polypeptide, based on alignments to ScMre11 (Figure 1B). Therefore, it is unlikely that *mre11-1* is capable of forming or processing DSBs. It is also important to note that a region of CcMre11 containing a putative nuclear localization signal (Figure 1B) would be missing in the *mre11-1* mutant. A similar sequence in the HD1 *A* mating-type protein of *C. cinereus* has been shown empirically to function in nuclear localization (Spit *et al.* 1998). Therefore, Mre11 may not be targeted properly to the nucleus in *mre11-1*. However, Usui *et al.* (1998) have demonstrated that the first half of Mre11 in *S. cerevisiae* contains one or more regions that allow binding to Rad50. Because the first half of Mre11 should be present in the *mre11-1* mutant, the mutant protein might achieve nuclear localization via an association with CcRad50. Thus, it is unclear whether the mutant Mre11 protein is capable of being targeted to the nucleus.

Mitotic phenotypes of the *C. cinereus mre11-1* mutant: The *C. cinereus mre11-1* mutant has two demonstrated mitotic phenotypes, enhanced sensitivity to ionizing radiation and defects in fruitbody development. All *S. cerevisiae MRE11* mutants identified show some degree of sensitivity to ionizing radiation or methyl methanesulfonate; where examined, these mutants have been shown to have defects in DSB repair (reviewed in Haber 1998). Therefore, the *C. cinereus mre11-1* mutant likely is defective in repairing radiation-induced DSBs, although the mechanisms used for DSB repair in *C. cinereus* have not been characterized. The *C. cinereus mre11-1* mutant does not show enhanced sensitivity to UV radiation.

This renders *mre11-1* more similar in phenotype to *mre11* mutants from *S. cerevisiae*, which do not demonstrate UV sensitivity (*e.g.*, Ajimura *et al.* 1993; Nairz and Klein 1997; Tsubouchi and Ogawa 1998), than to UV-sensitive mutants of *MRE11* homologs identified in two other fungi, *N. crassa* (*mus-23*; Watanabe *et al.* 1997) and *S. pombe* (*rad32*; Tavassoli *et al.* 1995). The *mus-23* and *rad32* genes are shorter than the other *MRE11* homologs, including *mre11* from *C. cinereus*. It has been proposed that, in addition to their roles in the repair of DSBs, *mus-23* and *rad32* may also function in a nucleotide excision repair pathway for UV-induced lesions that has been identified only in *N. crassa* and *S. pombe* (Watanabe *et al.* 1997; Wilson *et al.* 1999). Although this pathway has not been sought in *C. cinereus*, the phenotypic similarity of *mre11-1* to the *mre11* mutants of *S. cerevisiae* may imply that the mitotic repair function of CcMre11 is more similar to that of Mre11 in *S. cerevisiae* than to those of MUS23 in *N. crassa*, despite the higher sequence similarity of CcMre11 to MUS23.

Our data also indicate a role for *C. cinereus* Mre11 in fruitbody development. The *mre11-1* mutant is a poor fruiter, requiring longer periods of incubation, which result ultimately in fewer mushrooms than are seen for wild-type crosses. Notably, only the initiation of fruiting, and not the process of fruitbody maturation from an initial, is affected in the *mre11-1* mutant. Mre11 has been implicated in several general activities of vegetative cells. A *rad32* mutant of *S. pombe* was demonstrated to have a 300-fold increase in minichromosome loss, relative to wild-type levels (Tavassoli *et al.* 1995). Mre11 also has been implicated in telomere maintenance (Boulton and Jackson 1998; Nugent *et al.* 1998), detection of DNA damage (Lee *et al.* 1998; Nelms *et al.* 1998), and interactions between sister chromatids during G2 (Moore and Haber 1996; Haber 1998). Although *mre11-1* may not be a null mutant, deficiencies in any of these activities caused by the *mre11-1* mutation might affect fruitbody formation in the mutant indirectly. For example, although no obvious differences in vegetative growth rate were observed between *mre11-1* and wild-type cultures, the nuclear density or cell number may be lower in the *mre11-1* mutant, resulting in a reduced ability of the mycelium to undergo differentiation into fruitbodies. Alternatively, if a vegetative culture must pass a DNA damage checkpoint before allowing fruitbody differentiation to begin, the *mre11-1* mutant may require more time or may be less likely to pass such a checkpoint, resulting in fewer mushrooms that initiate more slowly than in wild-type cultures.

Meiotic phenotypes of the *C. cinereus mre11-1* mutant: The *C. cinereus mre11-1* mutant is defective in meiosis, as examined by light and electron microscopy and by FISH. Chromatin condensation, synapsis, and homolog pairing are all defective in the mutant (Figures 3–5; Table 2). Although no nuclei exhibit wild-type levels of any of these processes, the phenotypes are quite variable. Several explanations for this are plausible. First, the *mre11-1* mutation may be leaky. Although the mutant protein is predicted to be truncated to approximately half the length of the wild-type product, we have no evidence to suggest that it is entirely nonfunctional. If some product is present, there may be variability from cell to cell in the amount of functional protein present. Also, stochastic variability in meiotic progression may occur among cells in the absence of wild-type Mre11. Second, some phenotypic variability may derive from the assays themselves. We have used two-dimensional microscopy to examine structures and processes that occur inside the three-dimensional cell. The effect of spreading forces on weak interactions may result in observable differences among spread nuclei, when in reality these may represent very similar conditions in an intact cell.

In spite of the variability of chromatin condensation observed during prophase I (Figure 3, B and C), the *mre11-1* mutant is able to progress to an event resembling metaphase I (Figure 3E). Thus, in *mre11-1* nuclei, as was seen for the *C. cinereus rad9-1* mutant (Seitz *et al.* 1996), the processes of prophase and metaphase condensation have been uncoupled. Though the majority of *mre11-1* nuclei appear to achieve metaphase, the number within a given experiment is variable. This is not true for *rad9-1* nuclei; though none of the nuclei condensed properly during prophase I, consistently half of all nuclei in each experiment appeared to achieve metaphase condensation (Seitz *et al.* 1996). Both of these phenotypes are in contrast to those of *C. cinereus rad12* mutants, in which significant prophase I condensation occurs, but all nuclei arrest in diffuse diplotene (Ramesh and Zolan 1995). If the observed postdiplotene condensation in *mre11-1* nuclei truly represents metaphase, then the missing activity of Mre11 is not required for metaphase condensation.

Like chromatin condensation, synapsis is defective in the *mre11-1* mutant. In most nuclei, some synapsis is evident, but it is never complete (Figure 4B). The SC is a protein structure that in wild-type nuclei forms between aligned homologous chromosomes during prophase I. However, SC can form between regions of chromatin that are nonhomologous (Loidl *et al.* 1991; Maguire and Riess 1994). Our analysis did not address directly whether regions of synapsis, as observed by electron microscopy, occur between paired homologs, as analyzed by FISH. However, the presence of pairing partner switches and aberrant synapsis (Figure 4B) implies that at least some nonhomologous synapsis is occurring.

This interpretation is similar to that proposed by Nairz and Klein (1997) for the *mre11S* mutant in *S. cerevisiae*. The authors concluded that because more SC structures were visible than could be accounted for by the amount of homolog pairing, some SC must be forming between nonhomologous chromosomes. The *mre11S*

mutant can form, but cannot process, DSBs (Nairz and Klein 1997). Nairz and Klein (1997) suggested that a lack of DSB processing during meiosis might uncouple synapsis from the homology search. Their model suggested that, in the absence of DSBs, no synapsis can occur. Unprocessed DSBs would lead to partial, nonhomologous synapsis (as in the case of *mre11S*), while fully homologous synapsis would require the processing of DSBs. If programmed DSBs also are features of meiosis in *C. cinereus*, then the *mre11-1* mutant would be predicted to make no DSBs because it is a truncation (see above). However, some synapsis (in certain cases, quite extensive synapsis; Figure 4B) does take place. Therefore, either some meiotic DSBs are made in the *mre11-1* mutant, or the model for the role of DSBs in synapsis proposed by Nairz and Klein (1997) does not apply to *C. cinereus*.

Our analysis showed that homolog pairing stable to spreading is defective, but not abolished, in *C. cinereus mre11-1* nuclei; 30% of nuclei demonstrate pairing of single loci on two chromosomes (Table 2). Additionally, the level of pairing is constant throughout meiosis, from shortly after karyogamy (K + 1) until K + 10. For this study we elected to use probes representing single loci on two different chromosomes, chromosome 8, which is ~2.5 Mb in size, and chromosome 13, which is ~1 Mb. In nuclei in which only one chromosome was paired, it was more likely to be chromosome 13 (Table 1). This may reflect a difficulty in forming or maintaining pairing associations on the larger chromosome (chromosome 8), or it may be due to the nature of the region of DNA probed on each chromosome; however, in wild-type nuclei neither chromosome demonstrated a bias in pairing ability at these loci (Li *et al.* 1999).

By examining a single locus on a given chromosome, rather than employing a chromosome painting approach in which a large region or the entire length of a chromosome is probed, we were able to examine unambiguously the uncondensed chromatin of the *mre11-1* mutant. However, by relying on a single locus per chromosome, we most likely have underestimated the total amount of pairing on a given chromosome (Weiner and Kleckner 1994; Kleckner 1996); this was shown to be the case in our analysis of wild-type nuclei (Li *et al.* 1999). Additionally, if pairing interactions occur in *mre11-1* nuclei, but are weak, they may be susceptible to disruption by the spreading forces used in these experiments. This effect likely is not due to the uncondensed or partially condensed state of the *mre11-1* chromatin, as pairing interactions in uncondensed wild-type nuclei at early meiotic timepoints appear to be stable to spreading (Figure 5A; Li *et al.* 1999). For these reasons, the percentage of *mre11-1* nuclei examined that have portions of chromosome 8 and 13 paired may be >30%.

Our results for pairing in the *C. cinereus mre11-1* mutant are generally consistent with those obtained for related mutants in *S. cerevisiae*. Weiner and Kleckner (1994) showed that a *rad50*Δ mutant paired at ~25% of the level seen in a wild-type strain, and a *rad50S* mutant paired with ~37% efficiency. The authors suggested that homolog pairing and meiotic DSB activity likely are coordinated early in meiosis, and that meiotic recombination events may help to stabilize pairing interactions. By parallel analysis, DSBs may not be formed at all in the *C. cinereus mre11-1* mutant (assuming that programmed DSBs are a feature of meiosis in *C. cinereus*), resulting in a decreased ability of the mutant to stabilize pairing interactions. An alternative explanation is that homolog pairing occurs with wild-type frequency in the *C. cinereus mre11-1* mutant, but the interactions in the mutant are more susceptible to disruption by spreading.

Our pairing data for *mre11-1* in *C. cinereus* differ, however, from those presented by Nairz and Klein (1997) for the *S. cerevisiae mre11S* mutant. They found that pairing in a homozygous *mre11S* mutant at a pachytene timepoint is reduced to ~4% of the level seen in wild-type cells, which is lower than the 30% pairing at K + 6 seen for the *C. cinereus mre11-1* mutant. It is unclear why these values are so different. One possibility is that the two mutations confer differences in pairing efficiency. Alternatively, probe choice may reflect variations in the ability of different regions of the chromosomes examined to pair efficiently. A third possibility is that pairing interactions within nuclei from the two organisms may show different susceptibilities to spreading techniques. However, we cannot rule out the possibility that Mre11 function is more critical for homolog pairing in *S. cerevisiae* than in *C. cinereus*.

The precise correlation between DSB activity and early meiotic chromosome behavior may vary among organisms. In *S. cerevisiae*, a *rad50*Δ mutant fails to form DSBs, and it is defective in homolog pairing and SC formation (Alani *et al.* 1990; Loidl *et al.* 1994; Weiner and Kleckner 1994). An *S. cerevisiae spo11*Δ mutant also is deficient in SC formation and homolog pairing (Giroux *et al.* 1989; Loidl *et al.* 1994; Weiner and Kleckner 1994). However, *spo11* mutants in *C. elegans* and *D. melanogaster* make apparently normal SC, and the *C. elegans spo11* mutant is proficient for homolog pairing (Dernburg *et al.* 1998; McKim *et al.* 1998). It has been suggested that DSB activity in *S. cerevisiae* is likely to influence the stabilization of homolog pairing and may also trigger SC formation, while other mechanisms may accomplish these events in animals (Weiner and Kleckner 1994; Prinz *et al.* 1997; Dernburg *et al.* 1998). Thus, a dichotomy for the role of meiotic recombination in other meiotic chromosomal events has emerged between *S. cerevisiae* and animals.

Meiotic nuclei from the *mre11-1* mutant of *C. cinereus* phenotypically resemble *mre11*, *rad50*, and *spo11* mutants from *S. cerevisiae*, all of which are defective to some degree in homolog pairing and SC formation. However,

the requirement for DSB activity in pairing and synapsis may be different in *C. cinereus* than in *S. cerevisiae*. The *C. cinereus mre11-1* mutant may fail to form DSBs, yet it is still capable of some homolog pairing and SC formation. This differs from what has been observed for *mre11*, *rad50*, and *spo11* null mutants in *S. cerevisiae*, in which a lack of DSB formation is accompanied by comparatively less pairing and/or synapsis. Given that we do not know the exact role of either Mre11 or DSBs in meiotic recombination in *C. cinereus*, it is possible that the precise relationships among recombination initiation, pairing, and synapsis in *C. cinereus* may be distinct relative to those previously characterized in other systems.

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