The rad9 Gene of Coprinus cinereus Encodes a Proline-Rich Protein Required for Meiotic Chromosome Condensation and Synapsis

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ABSTRACT

The rad9 gene of Coprinus cinereus is essential for the normal completion of meiosis. We examined surface-spread preparations of wild-type and rad9-1 nuclei from the meiotic stages of karyogamy through metaphase I, and we determined the primary sequence, structure, and meiotic expression of the rad9 gene. In wild-type C. cinereus, karyogamy is followed by condensation and alignment of homologous chromosomes. Condensation and axial core development largely precede synapsis, which often initiates at telomeres. A diffuse diplotene phase coincides with dissolution of the synaptonemal complex, and subsequently chromosomes further condense as the cells progress into metaphase I. In contrast, although karyogamy and nucleolar fusion are apparently normal in rad9-1 basidia, only short stretches of synaptonemal complex form. These correlate with stretches of condensed chromatin, mostly at apparent chromosome ends, and regions of presumptive triple synapsis are numerous. rad9-1 basidia enter the diffuse stage of early diplotene, and then 50% of these cells enter metaphase I by the criteria of nucleolar elimination and at least some chromatin condensation. rad9 gene expression is induced after gamma irradiation and during meiosis. The gene has 27 exons and encodes a predicted protein of 2157 amino acids, with a proline-rich amino terminus.

The evolution of meiosis has likely included the refinement of mechanisms that ensure the proper segregation of homologous chromosomes during the first meiotic division. Two prominent, and related, processes are recombination and the formation of a tripartite proteinaceous structure called the synaptonemal complex (SC; Moses 1968). Analysis of the respective roles of recombination and the SC in chromosome segregation has led to the idea that recombination-based processes are important both for initial homol log recognition (Carpenter 1987) and for the maintenance, in the form of chiasmata, of connections between homologs until anaphase I (Baker et al. 1976; Roeder 1990). The role of the SC in most organisms is not homolog recognition per se; SC formation occurs readily between nonhomologous chromosomes (Loïd 1 et al. 1991) and nonhomologous regions of chromosomes (McClintock 1933; Maguire and Riss 1994). Instead, the SC may be essential for the conversion of cross-over events into chiasmata (Engelbrecht et al. 1990; Sym et al. 1993).

The formation and dissolution of the SC during meiosis and the condensation and synapsis of homologs have been examined in detail using electron microscopy (von Wettstein et al. 1984). Components and processes of SC formation have recently been identified and characterized in several organisms (for example, Holm et al. 1981; Dresser and Giroux 1988; Dietrich et al. 1992; Sym et al. 1993; Dobson et al. 1994). In addition to proteins that function directly in SC structure and formation, other gene products with roles in general DNA metabolism are indirectly involved in successful SC formation and are directly or indirectly a part of meiotic recombination and the progression of meiosis. A prominent class of these genes is the rad genes which are required both for DNA repair and for the successful completion of meiosis (Game 1983, 1993). It has been proposed that mitotic DNA repair processes have been recruited during evolution into roles that facilitate the synthesis and segregation of homologs (Alan et al. 1990; Kleckner et al. 1991). It has also been suggested that DNA repair per se is an important component of meiotic DNA metabolism (Bernstein and Bernstein 1991); however, it is difficult to understand how defects in DNA repair would affect meiotic progression and chromosome behavior if the role of DNA repair in meiosis were restricted to base sequence error correction. The available evidence points to a more direct function of certain rad genes in the behavior of meiotic chromosomes, because mutations in these genes result in defects in chromosome condensation, synapsis, and segregation (Zolan et al. 1988; Alan et al. 1990; Pukkila et al. 1992; Valentine et al. 1995; Ramesh and Zolan 1996).

We are studying rad genes in the basidiomycete Copri nus cinereus, in which meiosis is naturally synchronous. Each mushroom cap contains 10 million meiotic cells, which go through meiosis in one wave, tightly coordinated with fruiting body development. At the midpoint
of pachytene, which lasts for ~5 hr, essentially all meiotic cells of the mushroom cap are at this stage (Raju and Lu 1970). Thus, C. cinereus is an excellent experimental organism to use for examining the processes of meiotic chromosome metabolism, the underlying genetic basis for these processes, and the relationship between meiotic chromosome behavior and other aspects of DNA metabolism.

The rad9 gene was initially identified in a screen for radiation-sensitive, meiotic mutants (Zolan et al. 1988). The rad9-1 mutant is characterized by reduced basidiospore production, low spore viability, and failure to complete meiotic divisions (Zolan et al. 1988; Valentine et al. 1995). Zolan et al. (1988) observed stretches of SC in thin-sectioned embedded nuclei of rad9-1 cells, and these were not discernably different, in kind or in frequency, from those observed in wild-type cells. However, because single sections of nuclei were examined, the lengths of the observed SCs and the time course of SC formation and dissolution were not determined.

In a separate study, the rad9 gene was isolated from a chromosome-specific cosmid library (Zolan et al. 1992). The cloned gene was shown to complement the rad9-1 mutation for both radiation resistance and meiosis, and it mapped to the genetically determined rad9 locus.

To further investigate the function of the rad9 gene in meiosis, we examined the time course of SC formation and dissolution, and the concomitant condensation and dissolution, and the concomitant condensation cycles, of wild-type and rad9-1 cells. We found that the rad9-1 mutant has a novel meiotic defect, and that the two stages of meiotic chromatin condensation are uncoupled in this mutant. In addition, we determined the sequence and structure of the rad9 gene, and found two striking features: the gene is unusually large, with an open reading frame of nearly 6.5 kb; and the amino terminus of the predicted Rad9 protein has a proline content that is more than four times that of the remainder of the protein.

**MATERIALS AND METHODS**

**Strains and culture conditions:** The wild-type dikaryon used in this study has been previously described (Valentine et al. 1995). Two rad9-1 dikaryons, both congeneric with the wild-type control strain, were examined. Each was formed by crossing sibling rad9-1 isolates from the fourth generation of backcrosses into the Rad+ strain Okayama-7 (Wu et al. 1988). Culture conditions, matings, and fruiting conditions were as previously described (Zolan et al. 1988).

**Microscopy:** Surface spreads of C. cinereus chromosomes were prepared as described by Pukkila et al. (1992). Spreads were viewed and photographed using a Philips EM301 transmission electron microscope. For the determination of SC length, photographs were scanned using Photoshop (Adobe Systems, Inc.) and lengths were measured using NIH Image, version 1.56 (Rasband and Bright 1995) on a Macintosh Quadra 800. To observe chromosome condensation, samples were fixed and spread in the same manner as for electron microscopy, but were spread on ProbeOn Plus microscopy slides (Fisher Scientific). Slides were stained with 0.1 mg/ml acridine orange (Sigma A9492) in 70 mM phosphate buffer (22.4 mM Na2HPO4, 47.6 mM KH2PO4, pH 6.5) for 8–12 min at 4°C (Dresser and Giroux 1988). Slides were rinsed with cold 70 mM phosphate buffer, and then samples were mounted with the same buffer. Spreads were viewed and photographed using a Nikon Microphot FXA and Ektachrome slide film (Kodak).

**Nucleic acid isolation, electrophoresis, and hybridization:** RNA was isolated from cap and stipe tissue and analyzed by Northern analysis as described by Yeager Stassen et al. (1996). DNA was isolated as described by Zolan and Pukkila (1988), and gel electrophoresis and Southern hybridization were done as described in Zolan et al. (1992).

**Isolation of rad9 cDNA:** A C. cinereus meiotic cDNA library (Yeager Stassen et al. 1996) was screened using cDNAs that spanned the genomic sequence of rad9. Three cDNA clones, which included 2.3 kb of the 3' end of the gene, 1 kb of the 5' end, and 0.7 kb of the middle of the gene, were recovered. After these were sequenced, reverse transcription and PCR (RT-PCR) were used (as described below) to isolate cDNA representing the remainder of the gene.

**DNA sequencing:** Clones for DNA sequencing were prepared by either the Wizard miniprep procedure (Promega) or by a Qiagen plasmid kit. DNA sequencing was performed by the dideoxynucleotide method (Sanger et al. 1977) with 35S-labeled nucleotides and Sequenase (US Biochemical), by automated sequencing at the University of North Carolina-Chapel Hill automated DNA sequencing facility on a model 373A DNA sequencer (Applied Biosystems) using the Taq DyeDexoxy Terminator Cycle sequencing kit (Applied Biosystems), and by automated sequencing at the Indiana Institute for Molecular and Cellular Biology using a Li-Cor model 4000 DNA sequencer following reaction preparation with SequiTherm thermostable DNA polymerase (Epigenet Technologies). Sequences were assembled using the DNAsis (Hitachi Software Engineering Co.) program. Subclones representing the entire 10.8-kb Smal-SpeI fragment of cosmid 1B11 (Figure 7) were sequenced, using manual sequencing. Several subclones (into Bluescript, Stratagene) were used for this purpose. In addition, oligonucleotide primers (19 or 20 nucleotides) were used as internal priming sites for sequencing reactions. All primers, for both DNA sequencing and PCR (see below) were designed using the primer analysis program Oligo (National Biosciences, Inc.). A single strand of DNA sequence was determined for the 10.8-kb Smal-SpeI genomic fragment. Then, cDNA clones were sequenced such that the second strand of coding region sequence was determined, and the ends of PCR clone B were sequenced. Finally, primers and subclones were used to complete the second strand of all intron sequences. Therefore, double strand sequence information was obtained for the complete region spanned by PCR clone B of Figure 7, and this information was submitted to GenBank (accession no. U34998). The cDNA and genomic sequences were compared to determine the locations and sizes of introns.

**C. cinereus transformations:** Transformations into C. cinereus protoplasts were performed as described in Zolan et al. (1992). For the transformations using clones of the PCR products shown in Figure 7, 0.5 μg of cosmid Llc5200 (Pukkila and Casselton 1991) was mixed with 2 μg of either a single clone or a pool of clones. Alternatively, 1 μg of cosmid was mixed with 5 μg of cloned DNA.

**PCR:** The 9785- and 8890-bp PCR clones shown in Figure 7 were constructed by amplification from cosmid 1B11, which is also shown in Figure 7. Primers used were 23 or 27 nt in length, and the protocol and reagents of the Expand Long
Template PCR System (Boehringer Mannheim) were used. For the amplification, 100 ng of template DNA was amplified in a Perkin-Elmer GeneAmp PCR System 2400, using 1.8 U enzyme in a 50 µl reaction, with the following cycling conditions: 94°, 2 min; followed by 94°, 15 sec; 62°, 30 sec; 68°, 8 min for 10 cycles; followed by 94°, 15 sec; 62°, 30 sec; 68°, 8 min plus 20 sec additional each cycle, for 10 cycles; followed by 68°, 7 min. Amplified fragments were cloned directly (without gel purification) into the pCRII vector (Invitrogen).

For the amplification, 100 ng of template DNA was amplified by reverse transcriptase (BRL) in buffer supplied by the manufacturer, supplemented to 10 mM DTT and 2.5 mM each dATP, dCTP, dGTP and dTTP (Promega), and containing 40 U RNasin (Promega). Samples were held at 100° for 5 min, and then treated with DNAse-free RNase (Boehringer Mannheim) at 400 µg/ml for 30 min at 37°. PCR was then carried out in a 25-µl reaction containing 5 µl of the reverse transcription reaction and using 5 U Taq polymerase (Promega) in buffer supplied by the manufacturer, supplemented to 1 mM MgCl₂, 200 nM for each dNTP, and 1 mM for the appropriate upstream primer. Amplification conditions were: 94°, 2 min; followed by 94°, 15 sec; 55°, 30 sec; 72°, 1 min, for 30 cycles; followed by 72°, 7 min. Reactions were then separated on agarose gels, and sample lanes were blotted and hybridized with a labeled insert of a clone containing the corresponding genomic regions of the rad9 gene. The smallest hybridizing fragments were isolated (using the EluQuick kit, Schleicher and Schuell) and cloned into the pCRII vector.

RESULTS

Meiotic chromosome progression in wild-type C. cinereus: The time course of meiosis in C. cinereus has been examined in intact basidia, by both light (RAJU and LU 1970; PUKKILA et al. 1984) and electron microscopy (HOLM et al. 1981). Recently, cell disruption and surface spreading techniques have been refined (PUKKILA and LU 1985; ZOLAN et al. 1988; PUKKILA et al. 1992), enabling many features of meiotic progression to be monitored at high resolution for numerous cells. To provide a framework for understanding the behavior of meiotic chromosomes in rad mutants, we examined the time course of meiosis in a congenic wild-type strain, using both light and electron microscopy of surface-spread preparations.

Meiosis in C. cinereus begins with karyogamy, which is followed rapidly by the fusion of nucleoli (Figure 1, A and B). We define karyogamy as the time at which roughly half of all basidia have fused nucleoli. In our observations, this occurs 1 hr before the lights are turned on, when cultures are maintained at 25° with a 16-hr light, 8-hr dark cycle. We find that ≥90% of basidia undergo karyogamy in a wave that lasts ~20 min. Thus, the time point defined as karyogamy in our figures will naturally show a range of early meiotic stages (Figures 1, A and B, and 2; see also RAJU and LU 1970). After karyogamy, condensation of chromatin progresses until distinct, condensed chromosomes are apparent (Figure 1C; the stages of condensation intermediate between those shown in B and C are not shown). In previous studies of intact basidia, LU and RAJU (1970) found distinct chromosomes in fusing nuclei. The reason for the discrepancy between their data and ours is not known, although it is possible that our spreading method disrupts early, partial chromatin condensation. We normally see condensation of the level shown in Figure 1C before we observe synapsis (Figure 1D), and synapsis often appears to initiate at telomeres (Figure 1D, arrow). Full pachytene synapsis (Figure 1E) is observed for ~90% of basidia at 6 hr postkaryogamy and is the major class of chromatins observed at 4, 6, and 7 hr postkaryogamy (Figure

FIGURE 1.—Time course of chromatin condensation in wild-type C. cinereus. Samples were fixed, spread, and stained with acridine orange. Photographs are from samples taken at the following times: (A and B) karyogamy (as defined in the text); (C) Karyogamy plus 1 hr; (D) Karyogamy plus 4 hr; (E) Karyogamy plus 6 hr; (F) Karyogamy plus 8 hr; (G and H) Karyogamy plus 9 hr. The arrow in A marks a nucleolus, and the arrow in D marks synapsed telomeres. All size bars indicate a length of 2 µm.
were at or beyond the stage shown in Figure 3C (Figure 4).

Electron microscopy (PUKKILA and LU 1985; PUWLA apparent at about 8 hr postkaryogamy (Figure 3H). Ten approaching synapsis, and it is common for the synapsed meandered, and the average total SC length was 76 ± 9.5 μm (95% confidence limits), in good agreement with the 70 μm average reported by PUKKILA and LU (1985).

**Meiotic defects in rad9-l basidia:** Basidia of rad9-l mushrooms undergo karyogamy at the same time, relative to the light cycle, as wild-type cultures. In addition, nucleolus fusion appears to occur with normal kinetics (Figures 5C and 6A are spreads from samples taken at a karyogamy time point). At early time points (Figure 5, B and C) a large proportion of the spreads show a ring-like region of condensation against a noncondensed background. At karyogamy, 51% of spreads have a ring structure, and at 1 hr postkaryogamy, 33% contain a ring. This stage is also observed (in ~5% of the spreads) in wild-type basidia at karyogamy time points, but is not observed thereafter; its persistence in rad9-l indicates that it may be a normal stage that the wild-type passes through quickly, but that lasts longer in the mutant.

Other features of prophase I in rad9-l basidia are consistent with both arrest and abnormalities of the processes of chromatin condensation, chromosome pairing, axial core development, and synopsis. Examination of acridine orange-stained spreads (Figure 5) reveals that full pachytene-like chromosome condensation is never observed. Instead, short stretches of condensed and apparently paired regions are visible, against a diffuse background, at 6 and 8 hr postkaryogamy (Figure 5, arrows in D and F). By 10 hr postkaryogamy, these structures have disappeared (Figure 5G). At this time point, 92% of spreads observed had the diffuse appearance shown in Figure 5G, and 8% had the condensed appearance shown in Figure 5H. At 12 hr postkaryogamy, when wild-type basidia have completed both meiotic divisions (VALENTINE et al. 1995), we scored 213 rad9-l nuclear spreads, and found that 117, or 55%, were condensed as in Figure 5H, and 96, or 45%, were very diffuse, and retained the large nucleolus characteristic of pre-metaphase I spreads. Thus, no rad9-l basidia were observed to undergo a normal level of pachytene condensation. About half arrest at the diffuse stage which follows pachytene, and about half progress into metaphase I. However, the metaphase I stage of rad9-I mutants is not normal; the compaction observed is not as great as that seen in wild-type spreads (compare Figures 1H and 5H), and it is most likely that homologs are not paired.

Higher resolution of the rad9-l phenotype was obtained by silver staining and electron microscopy (Figure 6). By this technique, it is clear that axial core formation is incomplete; in fact, the short stretches of condensed and paired chromosomes seen in the light microscope (Figure 5) are echoed by short stretches of synapsed axial core elements, superimposed on a diffuse background (Figures 6, B–E). The total amount of axial core development and synopsis in rad9-l basidia seems to be established early after karyogamy (Figure 2). Pachytene is followed by a “diffuse stage” (Figure 1F; see also LU and RAJU 1970), which is similar to that described for Saccharomyces cerevisiae (DRESSER and GIROUX 1988), for Sordaria macrospora (ZICKLER 1977), and for many organisms with large chromosomes (WILSON 1925). This stage of decondensation is followed by recondensation (Figure 1G) until metaphase I figures (Figure 1H; note also the loss of the nucleolus) are observed, at 9 hr postkaryogamy. By 12 hr after karyogamy, all basidia have undergone both meiotic divisions (RAJU and LU 1970; VALENTINE et al. 1995).

**Time course of SC formation in wild-type C. cinerea:** Samples prepared as for chromosome condensation studies were spread on plastic-coated slides, stained with silver nitrate, and transferred to grids for viewing by electron microscopy (PUKKILA and LU 1985; PUKKILA et al. 1992; Figure 3). Using this procedure, the lateral elements of the SC are clearly visible, but the central elements are not seen. After karyogamy and nucleolar fusion (Figure 3, A and B), axial core formation occurs rapidly. At 1 hr postkaryogamy, 92% of nuclei examined were at or beyond the stage shown in Figure 3C (Figure 4). For both the karyogamy and 1 hr postkaryogamy samples, spreads such as that shown in Figure 3D were 20% of those observed (Figure 4). These spreads feature long axial cores and one or more regions approaching synopsis, and it is common for the synapsed regions to look like chromosome ends, as shown in Figure 3D. Complete homolog alignment (Figure 3E) is followed by progressive tightening of the SC structure (Figure 3F) until full pachytene synopsis is reached (Figure 3G). The beginnings of SC degradation are apparent at about 8 hr postkaryogamy (Figure 3H). Ten pachytene spreads (the stage shown in Figure 3G) were measured, and the average total SC length was 76 ±
and does not progress (Figure 6E). Measurement of six spreads of samples taken at 6 hr postkaryogamy yielded a range of SC length from 0.7 to 10 \( \mu \text{m} \), with the average length \( 3 \pm 0.6 \mu \text{m} \) (95% confidence limits). At 12 hr postkaryogamy (Figure 6F), most SC elements have degraded, leaving cells that have not entered metaphase I with a very diffuse appearance (Figure 6F). Three other features of rad9-I axial core and SC development are notable. First, most or all of the axial core segments that form are also synapsed (Figure 6, B–E). Second, many of the synapsed regions have the appearance characteristic of chromosome ends (arrows in Figure 6C). Third, numerous tripartite stretches are observed (arrowheads in Figure 6, B–D). While it is possible that these stretches represent canonical tripartite SC regions, the fact that the central element of the SC is not observed in wild-type spreads (Figure 3) makes it more likely that the tripartite stretches observed in rad9-1 cells are instead regions of triple synapsis. This interpretation is supported by the observation of pairing partner switches. An example of such a switch is indicated by the left-most arrowhead of Figure 6B. In this case, the central element switches from synapsis with an element on its left to synapsis with an element on its right. In addition, in most cases of apparent triple synapsis the middle element of the tripartite stretch is

**Figure 3.**—Time course of SC formation in wild-type \textit{C. cinereus}. Photographs are from samples taken at the following times: (A–D) Karyogamy (as defined in the text); (E and F) Karyogamy plus 4 hr; (G) Karyogamy plus 6 hr; (H) Karyogamy plus 8 hr. The arrow in A marks a nucleolus, the arrow in D marks synapsing telomeres, and the arrows in E and F mark synapsed telomeres. All size bars indicate a length of 1 \( \mu \text{m} \).
the same thickness as the outer elements. Were these true tripartite, individual SC stretches, the central elements would likely be much thinner, relative to the outer, lateral elements (Moses 1968; Dresser and Giroux 1988).

**Molecular analysis of the rad9 gene:** We previously constructed a cosmid library of the chromosome 8,9 doublet from strain Okayama-7 and screened it for clones that could complement both the radiation sensitivity and the meiotic defect in rad9-1 (Zolan et al. 1992). We readily obtained complementation and used the process of sib-selection, in which we transformed with progressively smaller pools of clones, to isolate an individual clone, termed 1B11, which contains the rad9 gene (Figure 7). Further evidence that this clone contains the complete rad9 gene, and not an extragenic suppressor, are the observations that the clone maps to the position of the rad9-1 mutation and that it complements this mutation when present in transformants in a single copy (Zolan et al. 1992).

To define the region of cosmid 1B11 that contains the rad9 gene, cotransformations were performed. In these experiments, an intact cosmid vector containing the *C. cinereus* trp1 gene was mixed with gel-purified fragments of 1B11, and the DNA was transformed into rad9-1;trp1-1,1-6 mutants using standard procedures (Binninger et al. 1987; Zolan et al. 1992). Transformants were selected for tryptophan prototrophy and screened for radiation resistance and the formation of spores. The 10.8-kb Spd-Smal fragment shown in Figure 7 complemented both of the rad9-1 mutant phenotypes. The location of the rad9 gene was further confirmed by a series of transformations using overlapping cosmids. One cosmid, 1D7, was found that overlaps 1B11 significantly, but does not complement the defects in rad9-1 strains (Figure 7). In separate transformation experiments, the level of complementation of rad9-1 with cosmid 1B11 ranged from 52 to 89% (Zolan et al. 1992), whereas no complementation, of either the radiation sensitivity or meiotic defects of rad9-1, was obtained for 50 transformants with cosmid 1D7. We therefore concluded that the rad9 gene is probably located near the end of cosmid 1B11 that is truncated in cosmid 1D7 (Figure 7).

Using a probe made from a subclone of cosmid 1B11, which overlaps the end of cosmid 1D7, we examined RNA from vegetative monokaryons and dikaryons, and from fruiting body caps. With this probe, we identified...
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Photographs are from samples taken at the following times: (A) Karyogamy; (B) Karyogamy plus 1 hr; (C) Karyogamy plus 4 hr; (D) Karyogamy plus 8 hr; (E) Karyogamy plus 10 hr; (F) Karyogamy plus 12 hr. The arrow in A indicates a nucleolus, the arrows in C indicate regions which appear to be synapsed telomeres, and arrowheads indicate regions of apparent triple synapsis, as discussed in the text. Numbers of nuclei scored were: 0 hr, 19; 1 hr, 35; 4 hr, 40; 6 hr, 32; 8 hr, 8; 10 hr, 15; 12 hr, 31. All size bars indicate a length of 1 μm.

FIGURE 6.—SC formation in rad9-I. Photographs are from samples taken at the following times: (A) Karyogamy; (B) Karyogamy plus 1 hr; (C) Karyogamy plus 4 hr; (D) Karyogamy plus 8 hr; (E) Karyogamy plus 10 hr; (F) Karyogamy plus 12 hr. The arrow in A indicates a nucleolus, the arrows in C indicate regions which appear to be synapsed telomeres, and arrowheads indicate regions of apparent triple synapsis, as discussed in the text. Numbers of nuclei scored were: 0 hr, 19; 1 hr, 35; 4 hr, 40; 6 hr, 32; 8 hr, 8; 10 hr, 15; 12 hr, 31. All size bars indicate a length of 1 μm.

Figure 6.—SC formation in rad9-I. Photographs are from samples taken at the following times: (A) Karyogamy; (B) Karyogamy plus 1 hr; (C) Karyogamy plus 4 hr; (D) Karyogamy plus 8 hr; (E) Karyogamy plus 10 hr; (F) Karyogamy plus 12 hr. The arrow in A indicates a nucleolus, the arrows in C indicate regions which appear to be synapsed telomeres, and arrowheads indicate regions of apparent triple synapsis, as discussed in the text. Numbers of nuclei scored were: 0 hr, 19; 1 hr, 35; 4 hr, 40; 6 hr, 32; 8 hr, 8; 10 hr, 15; 12 hr, 31. All size bars indicate a length of 1 μm.

a transcript of ~6.6 kb, which is induced after gamma irradiation (data not shown) and during meiosis (Figure 8; the probe used for this northern was a rad9-cDNA clone insert, but identical results were obtained using genomic DNA clones). By doing northern hybridizations with small fragments from across the region, we determined that the 6.6-kb transcript was roughly centered in the 10.8-kb SpeI-SmaI fragment shown in Figure 7 and that another transcript, of ~2.7 kb, was detected by sequences at the SpeI end. Both transcripts were also present in the rad9-I mutant (data not shown), although transcript levels were not compared between mutant and wild-type strains.

We sequenced the entire 10.8-kb genomic region and found, using the BLAST procedure (Altschul et al. 1990), that the 2.7-kb transcript encodes an ATP-dependent RNA helicase of the DEAH box family (M. E. Zolan, unpublished data; Linder et al. 1989). The two closest homologs of the C. cinereus helicase are the PRP2 gene of S. cerevisiae (Chen and Lin 1990) and the PRH1 gene of Schizosaccharomyces pombe (Inoue et al. 1992), both of which function in RNA splicing. We estimate that the 10.8-kb SpeI-SmaI fragment contains ~65% of the C. cinereus helicase homolog. Because this fragment contains the complete coding sequence of the 6.6-kb transcript and completely complements the defects in rad9-I strains in transformation experiments, we reasoned that the 6.6-kb transcript is most likely the actual rad9 gene.

To definitively rule out the helicase homolog as the source of complementation of the rad9-I mutation, we used PCR to amplify, from cosmid 1B11, the regions shown as A and B in Figure 7. In cotransformation experiments with the trp1 vector, clones of each fragment complemented the defects of rad9-I strains. Because fragment B includes only 325 bp upstream of the rad9 translational start site (Figure 7), and none of the helicase coding sequence, we conclude that the 6.6-kb
transcript encoded at the end of cosmid 1B11 is indeed derived from the rad9 gene.

To determine the structure and coding sequence of the rad9 gene, the cDNA sequence was determined and compared with the genomic sequence. The rad9 open reading frame is 6471 bp in length and is interrupted by 26 introns (Figures 7 and 9), which range in size from 47 to 60 bp. The introns are found in all three possible codon phases (Figure 9), with an even distribution of interruptions between codons (46%) and within codons (54%). Exons usually begin with a purine residue and end with a guanine, and all intron ends are defined by the 5’ GT and 3’ AG observed for virtually all spliceosomal introns. The 5’ splice-site consensus sequence, GTNNGT (Figure 9), agrees with that generally found for filamentous fungi (Gurr et al. 1987; Edelmann and Staben 1994), as does the 3’ splice site, YAG. In two cases, the Y at the 3’ splice site was replaced by an A, as has been observed for other fungal genes (Gurr et al. 1987).

The 26 rad9 introns do not consistently display internal sequences similar to either the YGCTAAC branch point consensus described for filamentous fungi (Gurr et al. 1987) or the YNYRAY eukaryotic consensus (Sharp 1987). However, 22 of the 26 introns contain the sequence CTNA (underlined in Figure 9), which is similar to the NYRA core of YNYRAY. In 21 of these introns, the A residue of the CTNA sequence is located 12 to 20 bases upstream of the 3’ splice junction. This positional conservation, coupled with the preservation of the CTNA sequence itself, is possibly indicative of functional properties conferred by this consensus. If CTNA does represent a partially conserved branch point in C. cinereus introns, then the associated adenosine residue may serve as the attacking nucleophile at the 5’ splice site, as seen in other cases of eukaryotic splicing (Sharp 1987).

The predicted Rad9 protein has 2157 amino acid residues, a molecular weight of 239 kD, a pI of 7.3, and a proline-rich amino terminus (Figure 10). The first 430 amino acids are 17.7% proline, whereas the remainder of the protein is 4.3% proline, which is consistent with the average composition of eukaryotic proteins (4.6% proline; Doolittle 1986). Analysis of hydropathy (Kyte and Doolittle 1982) for Rad9 indicated that
there are no membrane-spanning domains. There are four sites in the Rad9 protein that are consensus sequences for phosphorylation by mitogen-activated protein kinase (MANSOUR et al. 1994; these are underlined in Figure 10). Additionally, the size and basic amino acid composition of the sequence PVKKGSRKKK (positions 607-616, Figure 10) lead us to speculate that this region may function in nuclear localization (GARCIA-BUSTOS et al. 1991).

Database searches (using the BLAST algorithm; ALT-SCHUL et al. 1990) identified sequences with similarity to two different regions of the Rad9 protein. Significant matches (with random hit probabilities of 7.8 e-32 and 5.9 e-38, respectively), were to predicted proteins of unknown function in *S. pombe* (chromosome I cosmid, Genbank No. 250113) and *S. cerevisiae* (chromosome IV cosmid, Genbank No. SC9395). Sequence alignments revealed that these proteins are probably homologous; for each pair-wise comparison, there is 23% identity (excluding the 21-29 small gaps), over a region of ~1000 amino acids. However, the *S. pombe* protein, at 1583 amino acids, and the *S. cerevisiae* protein, at 1494 amino acids, are significantly smaller than the Rad9 protein, and neither contains a dramatically proline-rich amino terminus.

The next highest match in BLAST searches was between the proline-rich amino terminus of Rad9 and various proline-rich proteins, such as the plant cell wall protein extensin (KIELISZEWSKI and LAMPORT 1994). However, these BLAST probability scores (2.4 e-16-1.5 e-18) are less supportive of homology than those obtained for the yeast genes, and, unlike Rad9, extensins are proline-rich throughout their protein sequences. Attempts at alignments of Rad9 with extensins (not shown) further indicated that these proteins are probably not evolutionarily homologous.

**DISCUSSION**

**Correlation of information from light and electron micrographs of wild-type spreads:** Our data indicate that the condensed and paired figures shown in Figure 1E are representative of fully aligned chromosomes. The diffuse stage of chromatin decondensation corresponds to the beginning of dissolution of the SC and loss of axial core structure (Figures 1F and 3H; also compare Figures 2 and 4). A logical interpretation of these data is that SC elements contribute to the compaction observed at pachytene and that the dissolution of axial cores leads to the diffusion of chromatin observed at early diplotene (Figure 1F). While it is clear that chromosomes do not completely decondense after

**Figure 9.—Intron sequences of the rad9 gene. Upper case letters represent flanking exon sequences and lowercase letters indicate intron sequences. No, intron number; Pos, intron position relative to the ATG shown in Figure 7; Ph, intron phase (0, between two codons; 1, between the first and second nucleotides of a codon; 2, between the second and third nucleotides of a codon). Underlined sequences are the conserved CTCN sites discussed in the text.**
Figure 10.—Amino acid sequence of the predicted Rad9 protein. Arrows indicate the positions of introns. Numbers above the amino acid sequence indicate exon number, and numbers in parentheses indicate exon sizes. Asterisks indicate that the lengths of the first and last exons are for their coding segments only; 5’ and 3’ UTR regions are not included because their lengths were not established. Potential target sites for phosphorylation by mitogen-activated protein kinase (amino acid positions 382-385, 390-393, 425-428, and 1502-1505) and a potential nuclear localization signal (positions 607-616) are underlined.

Defects in rad9-1 basidia: The defects in meiosis of rad9-1 are seen consistently in light and electron micrographs (Figures 5 and 6). With both types of analysis, the short stretches of condensation and synopsis against the diffuse background are the hallmark of the rad9-1 phenotype. Many of the synapsed stretches of rad9-1 chromatin resemble telomeres, and it is possible that the ring-like regions of condensation and synopsis observed for rad9-1 are synapsed with other, available condensed chromatin regions. Evidence for aberrant synopsis in rad9-1 comes from our observation (Figure 6, B-D) of regions of triple pairing. The use of telomeric and interstitial probes in fluorescence in situ hybridization (LOIDL et al. 1994; WEINER and KLECKNER 1994) will help to determine whether the small amount of synopsis observed for rad9-1 is in fact homology-based or fortuitous.

A striking feature of rad9-1 nuclei is their failure to develop during prophase I. We hypothesize that the defect in rad9-1 is manifest very early in meiosis. The processes of karyogamy and nucleolar fusion appear normal, and the condensation of telomeres may initiate as for wild-type cells, but further condensation and axial core development are not possible. This defect may be a direct consequence of the absence of functional Rad9 protein in these cells, or the Rad9 protein may be indirectly required for chromatin condensation; for example, it may have a role in the resolution of interlocks that is required for the progression of condensation and axial core development (HOLM et al. 1981).

It is apparent, however, that meiosis continues, at least to some extent, in rad9-1 mutants. The small amount of condensation observed gives way, by 8 hr postkaryogamy, to a more diffuse state (Figures 5G and 6F). Interestingly, about half of all rad9-1 basidia progress into the metaphase I stage of the cell cycle, with shrinkage of the nucleolus and at least some compaction of the chromatin (Figure 5H), although it is not possible to determine from our data whether homologs are in some way paired in rad9-1 metaphase I. The chromatin of the remaining half of rad9-1 basidia remains...
diffuse and gradually becomes more and more dispersed in appearance. These data are consistent with our previous observations, made on intact basidia (Valentine et al. 1995), which showed that about half of rad9-1 basidia arrest in prophase I and about half continue to metaphase I or anaphase I. Because no rad9-1 basidia exhibit full pachytene-stage chromosome condensation, but fully half show at least some compaction at metaphase I, we conclude that metaphase I condensation is not dependent on successful pachytene condensation. Therefore, these two processes may be distinct, differently regulated events. In a large survey of recessive sexual-phase mutants in wild strains of N. crassa, Raju and Leslie (1992) described several mutants which are similar to rad9-1 in that the initial meiotic defect is observed in zygotene, but the cell cycle processes such that either meiotic arrest or cell death occurs at a later stage.

The specific meiotic phenotype of rad9-1 basidia is, to our knowledge, unique. Numerous mutants, characterized in fungi, plants, and animals, fail at the completion of axial core formation or synapsis (for recent reviews, see Raju 1992; Hawley et al. 1993; Staiger and Cande 1993; Pukkila 1994). Phenotypes reported in fungi range from the almost complete absence of axial core development, in severe rad50 strains (Alani et al. 1990), to the full axial core development and alignment without synapsis seen in zip1 disruptions (Sm et al. 1993). However, the rad9-1 phenotype, of short axial core elements, essentially all of which are synapsed, a preponderance of telomere-like regions, and a correlation between condensation and synapsis, is previously unreported.

The rad9 gene: The rad9 gene contains 26 introns, which is the largest number reported, to our knowledge, for a fungal gene. The size range of these introns, 47–60 bp, is consistent with the small size (usually <100 bp) of introns found in other filamentous fungi (Gurr et al. 1987), but is unusually tight, even for C. cinereus (Yeager Stassen et al. 1996). Intron frequency for rad9 (4.0 introns/kb of coding sequence) is less than the average value (6.3 introns/kb) for other genes of C. cinereus (L. Baunsgaard, J. Vind and H. Dalboge, unpublished data, GenBank No. X70789; O. Ishibashi and K. Shishido, unpublished data, GenBank Nos. D13295 and D13226; Skrzynia et al. 1989; Tymon et al. 1992; Logsdon et al. 1995). The intron splice junctions of rad9 fit those previously reported for filamentous fungi (Gurr et al. 1987). Furthermore, potential internal branch points have been identified (Figure 9); however, experimental support is necessary to demonstrate any functional significance of these regions.

The features of Rad9 necessary for its dual roles in meiosis and the survival of gamma irradiation are not yet known. The identification of a putative nuclear localization signal (Figure 10) suggests that Rad9 could be a nuclear protein, which would be consistent with a role in DNA metabolism. The cosmid 1D7, which does not complement rad9-1 mutants for either gamma irradiation survival or meiosis, is missing 650 bp from the 3' end of the rad9 transcript (Figure 7). Therefore, this portion of the gene is required for Rad9 function in both cellular events. We have not yet determined the mutational site in the rad9-1 mutant. The behavior of this mutant, which is strongly defective in meiosis but only slightly sensitive to gamma irradiation (Zolan et al. 1988; Valentine et al. 1995), is reminiscent of the rad50-1 mutants described by Alani et al. (1990), in that they exhibit a "separation of function" phenotype. In screens of >20,000 mutagenized cultures (Zolan et al. 1988; Valentine et al. 1995), only one rad9 mutant was recovered. We are currently screening directly for lack of complementation of the meiotic defect in rad9-1, as an alternative strategy for the isolation of additional rad9 alleles; if the primary type of viable rad9 mutant is similar in phenotype to rad9-1, then, because the gene is so large, this new screen should readily yield new mutants.

The peak of accumulation of rad9 transcript is at ~6 hr after karyogamy (Figure 8 and data not shown), although defects in the rad9-1 mutant are apparent by 1 hr postkaryogamy (Figures 5 and 6). The rad9 gene product is therefore likely to be necessary from early in meiosis through at least pachytene, the stage of all wild-type basidia at 6 hr postkaryogamy. However, as previously described for RAD50, regulated levels of transcript accumulation do not necessarily correspond to varying levels of protein product (Raymond and Kleckner 1993), and therefore the precise temporal relationship between rad9 expression and Rad9 function can only be determined by direct analysis of the Rad9 protein.

The Rad9 protein is unrelated in sequence to any other protein found in previous screens for genes necessary for meiosis or DNA repair. Although homologs of Rad9 exist in S. cerevisiae and S. pombe, the functional and evolutionary relationships among these genes and the C. cinereus protein are not yet known. In our screens for radiation-sensitive, meiotic mutants (Zolan et al. 1988; Valentine et al. 1995), we have deliberately followed the path set by Gane (1983, 1993) and others (Baker et al. 1976) in the investigation of genes with roles in both DNA repair and meiosis. This laboratory has so far isolated and sequenced two genes, rad9 and rad51, the C. cinereus ortholog of the S. cerevisiae RAD51 gene (Yeager Stassen et al. 1996). The C. cinereus rad9 gene is not a homolog of any of the genes denoted by the S. cerevisiae rad mutants, nor was a rad51 mutant found in our genetic screens, and yet homologs of these genes are present in both organisms. In Neurospora crassa, alleles of the mei-3 gene, an ortholog of RAD51 (Cheng et al. 1993; Hatakeyama et al. 1995), have been found in screens for strains exhibiting mutation sensitivity (Delange and Mishra 1981). Therefore, the different biology of ascomycetes and basidiomycetes
could make the recovery of certain classes of mutants more likely. The analysis of diverse organisms will hence provide a broad perspective on the meiotic process, and the characterisation of genes with meiotic function in multiple organisms will facilitate an understanding of the genus-specific and fundamental aspects of meiotic pathways.

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LITERATURE CITED


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