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Efficient isolation and mapping of *rad* genes of the fungus *Coprinus cinereus* using chromosome-specific libraries

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ABSTRACT

We have constructed cosmid libraries from electrophoretically separated chromosomes of the basidiomycete *Coprinus cinereus*. These libraries greatly facilitate the isolation of genes by complementation of mutant phenotypes and are particularly useful for map-based cloning strategies. From a library constructed from two co-migrating *C.cinereus* chromosomes, we isolated a clone that complements the *C.cinereus rad9-1* mutation. Examination of this clone showed that it complements both the repair and meiotic defects of this mutant. Restriction fragment length polymorphism mapping using a portion of this clone showed that it maps to the *rad9* locus. In addition, a single copy of transforming DNA is sufficient to complement the *rad9-1* defects. Thus, we believe we have cloned the *rad9* gene itself. We also used a chromosome-specific library and backcrossed isolates to rapidly identify a cosmid clone which is tightly linked to the *rad11* locus and is therefore a suitable starting point for a chromosome walk. These rapid methods of gene mapping and isolation should be applicable to any organism with separable chromosomes.

INTRODUCTION

The basidiomycete *Coprinus cinereus* is an attractive model system for studies of meiosis and DNA repair, because it is a genetically tractable organism in which the process of meiosis is naturally synchronous. We have isolated mutants based on their sensitivity to ionizing radiation (*rad* mutants), and have found that several of these are also defective in meiosis (1; unpublished). We have chosen to use DNA-mediated transformation (2) in order to identify the *rad* genes identified by these mutations.

Nuclear transformation of *C.cinereus* is based on transformation of *trp1-1,1-6* protoplasts with a cloned copy of the wild-type *C.cinereus trp1* gene (2). Transformation efficiencies of at least 1 transformant /10^4 viable protoplasts are readily obtained. Because transformation and DNA repair may share some of the same enzymatic functions, it is reasonable to expect that some *rad* mutants might be defective in transformation. However, the *rad* mutants we have characterized so far all transform at wild-type levels. Thus, we reasoned that it should be straightforward to isolate these genes from genomic libraries. However, we were unable to devise a strategy that would allow the direct selection of transformants that had received the wild-type copy of a *rad* gene. Instead, we screen clone libraries by a two-step process: *trp1-1,1-6*; Rad- strains are transformed with a vector that contains the *trp1* gene (3) and random inserts of other *C.cinereus* sequences. Trp^+ transformants are selected, and then each is screened by a 'chunk test' (1) for its phenotype with respect to repair. Since the *C.cinereus* nuclear genome is approximately 37,500 kb (4), about 5,000 40 kb clones must be screened in order to have a 99% probability of assaying each sequence (5).

To make the recovery of *C.cinereus* genes by this rather laborious procedure more efficient, we decided to take advantage of recent advances in electrophoresis technology. The methods of pulsed field gel electrophoresis have enabled the separation of chromosome-sized DNA molecules from a variety of fungi (reviewed in 6). We have used a contour-clamped homogeneous electric field (CHEF) system (7; BioRad CHEF DRII) to resolve *C.cinereus* chromosomes and have made cosmid libraries from individual chromosomes or chromosome doublets. In this paper we describe our method of making these libraries and its application for the isolation of the *C.cinereus rad9* gene. In addition, we show the power of combining a chromosome-specific library with near-isogenic lines to rapidly identify clones suitable for the initiation of a chromosome walk.

MATERIALS AND METHODS

Strains and culture conditions

The strains described in this work are: Java-6, a wild-type strain used for the mutagenesis and isolation of *rad* mutants (1); Okayama-7 (8), the backcross parent for all the *rad* strains in our lab; MZC3 (ATCC# 12890d), a Rad^+ strain used for restriction fragment length polymorphism mapping (8); *rad9-1*, a *rad* mutant strain isolated from mutagenized Java-6 and backcrossed to Okayama-7 five times (1); *rad11-1*, a *rad* mutant strain constructed in the same manner as *rad9-1*; *acu7*, a mutant strain that cannot utilize sodium acetate as its sole carbon source

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(9); and *rad9-1*; *trp1-1,1-6*, a strain constructed by crossing *rad9-1* with strain 218 (2). Strains were maintained, cultured, and crossed as previously described (1, 2). Radiation sensitivity assays were by the chunk test described in reference 1.

Preparation of *C.cinereus* chromosomes

Plugs of intact *C.cinereus* chromosomes were prepared by a modification of a procedure developed by Binninger et al. (10). Protoplasts were prepared from oidia as described (2) except that the enzymes used to degrade the oidial cell walls were novozym 234 (Novo labs) at 10 mg/ml and chitinase (Sigma) at 1−2 mg/ml. Washed protoplasts were resuspended in MM (0.5 M mannitol; 0.05 M maleate, pH 5.5) at a concentration of 6×10^8 cells/ml. A 2% solution of low melting point agarose was prepared in MM, and held at 50°C. Aliquots of protoplasts were mixed with an equal volume of agarose, and the solution was immediately used to fill a BioRad CHEF plug mold. The plugs were chilled at 4°C for 20 minutes, removed with a metal spatula, and added to enough prepared NDS (0.01 M Tris-HCl, pH9.5; 0.3 M EDTA; 2% N-Lauroylsarcosine; 1 mg/ml proteinase K—the Tris-EDTA solution is made, filtered and autoclaved, and the N-Lauroylsarcosine and proteinase K are dissolved in it just before use) to completely cover the plugs. Plugs in NDS were incubated for 24 hours at 50°C, the NDS was removed and replaced with 20 ml Tris-EDTA (as for NDS but without the other components), and the plugs were stored at 4°C. We have found that plugs are stable at 4°C for at least two years.

Gel Electrophoresis

CHEF gels were made in molds sold by the manufacturer. We routinely make a 140 ml gel in a 5×5.5 inch mold. For resolution of small chromosomes we use Beckman LE agarose, and for resolution of large chromosomes we use BioRad chromosomal grade agarose. For both types of gels, we obtain good resolution by running gels at 60V, for about 6 days with a 22 minute pulse time, in 0.5× TBE, which is maintained at about 14°C by circulation through a 4−9°C water bath (Lauda—the requisite temperature of the water bath is determined by the distance between the water bath and the CHEF box, and by the length of tubing used). We change the buffer 2−6 times during a 6-day run. We find that a 6×9 mm piece of plug embedded into the well of a 140 ml CHEF gel is sufficient for chromosome visibility.

Construction of cosmid libraries

Our basic method is outlined in Figure 2. The 10 kb cosmid vector Llc5200 contains the *C.cinereus trp1* gene, a cos site, and an ampicillin-resistance gene, and is described fully in reference 11. The vector was cut with BglII and phosphatased (calf intestinal alkaline phosphatase; Boehringer Mannheim, using procedure described by the manufacturers). Plugs from nine gel lanes were treated in sets of three to make each library. Each set was washed 3× in 3 ml of 10 mM Tris, pH 8, for 30 minutes at room temperature with gentle agitation on a rotary shaker. After the third wash, sets of plugs were washed once in 1× MboI buffer (10 mM Tris, pH 7.8; 100 mM NaCl; 10 mM $MgCl_2$; 1 mM DTT; 100μg/ml BSA). This wash was removed, 3 ml of 1× MboI buffer containing 0.122 units/ml of MboI (Stratagene) was added to each tube, and samples were incubated at 37°C for 20 minutes. Reactions were stopped by adding EDTA to 20 mM, and samples were chilled. DNAs were electroeluted in 0.5× TBE for 13 hours at 70 volts and 2 hours at 100 volts (on ice), concentrated by evaporation, phenol extracted, and extracted with SEVAG (chloroform: isoamylalcohol, 24:1). The NaCl concentration was adjusted to 0.4M, 5 μl of tRNA (2 mg/ml) was added, and the DNA was precipitated with two volumes of 100% ethanol. Pellets were resuspended in 20 μl of TE, and 5 μl was used to assay DNA size and concentration using a 0.5% agarose gel. We estimate that we used about 200 ng chromosomal DNA, and that its size average was about 40 kb. This DNA was ligated to 2 μg of BglII-cut, phosphatased vector, and concentrated by ethanol precipitation. One-half of the ligation was packaged *in vitro* (Gigapack Gold II, Stratagene), and the phage were used to infect bacterial strain NM554 (12) or the SURE strain (13). We followed the manufacturer's instructions for packaging, bacterial preparation, and infection, except that after the packaging was complete we added only 250 μl lambda dilution buffer and 10 μl chloroform. Ten-fold dilutions of this packaging mixture were used to infect prepared bacteria. We estimate that we recovered about 3×10^5 clones/μg insert DNA. Individual colonies were picked into wells of 96-well microtiter dishes, each of which contained 200 μl freezing medium [standard LB (14) plus 1× the freezing medium of Schleif and Wensink (15)] plus 100 μg/ml ampicillin. Plates were incubated at 37°C overnight, and stored at −80°C.

Preparation of DNA for transformation

A 96-prong replicator was used to inoculate plates of LB-ampicillin medium with colonies from each dish. For each DNA prep, 10 plates were inoculated from one microtiter dish, the colonies were scraped in LB, and cosmid was isolated and purified on CsCl gradients (14). Subpools of DNA were similarly prepared from individual rows of the microtiter dish, and DNA from individual clones was prepared from liquid cultures by a miniprep procedure (14).

Transformations

Transformations were done as described by Binninger et al. (2), with the modification of protoplast preparation as described for CHEF plugs.

Analysis of chromosomes with rare-cutting restriction enzymes

Digestions of chromosome plugs were as described by Binninger et al. (10). The analysis of fragments was by CHEF electrophoresis, using conditions recommended by the manufacturers (BioRad) for the separation of fragments 10 kb to 1 Mb in size. An extra long (14 cm×21 cm) gel bed was used to form a 1% LE agarose gel (210 ml). The pulse time was ramped from 0.5 to 94 seconds over a 28.3 hour run time, and the gel was run at 14°C in 0.5× TBE.

Restriction Fragment Length Polymorphism Mapping

Appropriate *rad* strains were crossed to the Rad^+ strain MZC3. Progeny were isolated and screened for radiation sensitivity. Each isolate was also inoculated into liquid medium, and the cultures were grown at 37°C for one week. Cultures were harvested and lyophilized, and DNA was prepared as described (16) except that one phenol extraction was occasionally substituted for the first SEVAG extraction. DNAs were digested in buffers supplied by the manufacturers (Boehringer Mannheim or New England BioLabs) and separated on 0.7% agarose gels in 1× TBE buffer. Gels were Southern-blotted using alkaline or neutral transfer solutions (14) onto Zetabind (AMF Cuno) or Nytran (Schleicher

and Schuell) and hybridized using Blotto-based buffers (14). Probes were made using random priming (14 or Stratagene Prime-it™), and ranged from 10^8 to 2×10^9 cpm/μg.

RESULTS AND DISCUSSION

Electrophoretic karyotype and mapping

C.cinereus has 13 chromosomes, which range in size from about 1 to 5 Megabases (Mb; 11, 17). Using different conditions, we can achieve separation of either the small or the large chromosomes (Figure 1). Consistent with results previously reported (11), we find that different, interfertile strains of *C.cinereus* have distinct electrophoretic karyotypes.

We have mapped *rad* genes to the electrophoretic karyotype using two approaches. First, two *rad* genes were mapped because they were found to be genetically linked to genes that had already been cloned and which could therefore serve as physical probes for chromosome identification. The *rad9* gene mapped to 3.8 map units from *acu7*, a gene required for acetate metabolism (9), and the cloned *acu7* gene (18) hybridized to chromosome 8 in Java-6, and to the doublet of chromosomes 8 and 9 in Okayama-7 (data not shown). Thus, the *rad9* gene must be located on chromosome 8, 3.8 map units from *acu7*. The *rad11* gene was mapped to chromosome 6, because restriction fragment length polymorphism (RFLP) mapping placed that gene 9.4 map units from the single cluster of ribosomal RNA genes (data not shown), which is located on chromosome 6 (11, 19). In our second approach, several *rad* genes have been mapped by virtue of the fact that the polymorphic chromosomes of Java-6 and Okayama-7 recombine during meiosis to produce marker chromosomes that contain the mutant genes, and hence cosegregate with the mutant phenotypes (M.E.Z., N.K.H., M.Ramesh, N.Yeager Stassen, manuscript in preparation).

Construction and characterization of chromosome-specific libraries

To construct chromosome-specific libraries of *C.cinereus*, we cut CHEF-separated chromosomes out of gels, performed MboI partial digests on the DNA while it was still in the agarose, and electroeluted the digested DNA (our procedure is described in detail in Materials and Methods, and is shown schematically in Figure 2). We estimate that we recovered 3×10^5 colonies per microgram of insert DNA. Colonies were randomly picked, grown overnight in freezing medium, and stored at −80°C. We routinely maintain at least 10 chromosome equivalents of DNA per library. We have so far constructed three libraries from strain Okayama-7 (of chromosomes 6, the 8−9 doublet, and 13) and one library from Java-6 (of chromosome 13).

Characterization of 20 random clones from our four libraries indicated that the insert sizes range from approximately 27 to 43 kb. We did not size-select our insert DNA before ligation, and therefore the possibility exists that some of the inserts in our clones are chimeras of two or more chromosomal fragments ligated together. However, we used a roughly 25:1 molar ratio of vector to insert DNA, which would serve to minimize the chances that two insert fragments would ligate together. Modifications of our procedure, which would ensure that only one insert fragment could be cloned per vector molecule (14) could be used for future library construction. However, we have so far found this potential problem to be minimal, and we test each clone that we wish to use for chromosome walking in three ways. First, we hybridize the entire insert to a Southern blot of separated chromosomes, to ensure that all of its sequences are derived from a single chromosome. Second, we determine that this insert hybridizes to a single fragment produced by a rare-cutting (8 bp recognition sequence) restriction enzyme that does not cut within the insert. Finally, we hybridize the insert to a Southern blot containing, in adjacent lanes, digests of the cosmid

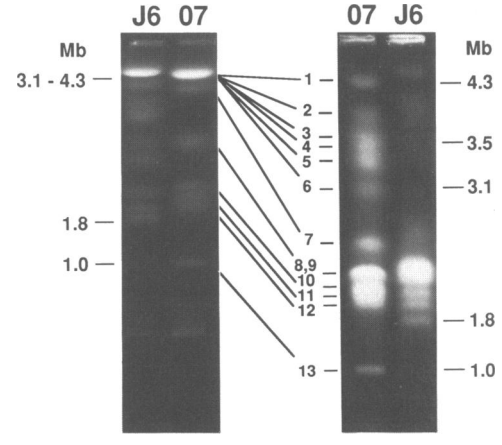

Figure 1. Separation of intact chromosomes of *C.cinereus*. Both gels shown were run at 60V for 6 days, with a 22 min. pulse time. The left gel was 1.0% Beckman LE agarose (160 ml) and the right gel was 0.9% BioRad chromosomal grade agarose (145 ml). Sizes were estimated from multiple gels on which the chromosomes of *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (BioRad) were used as size standards. Estimates were made only from portions of gels in which a log-linear relationship was observed between marker fragment size and distance traveled. Abbreviations used: J6, strain Java-6 (21); O7, strain Okayama-7 (22); Mb, megabase pairs.

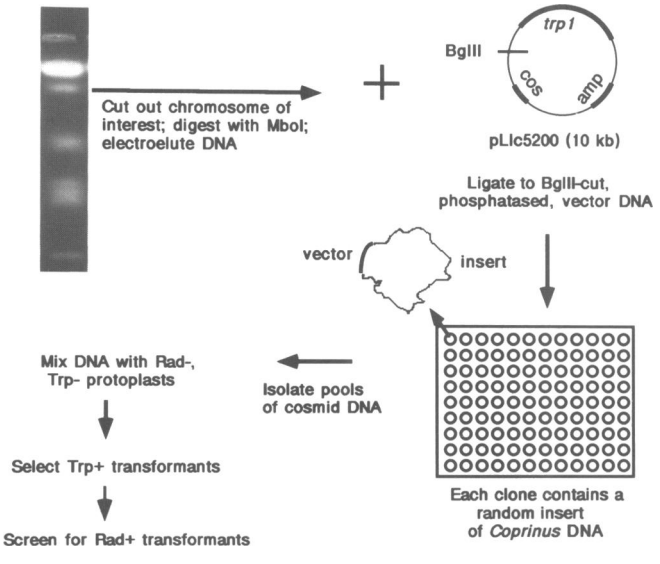

Figure 2. Construction of chromosome-specific libraries. The appropriate chromosome was cut out of a CHEF gel, the DNA was digested in the agarose using MboI, and the DNA was electroeluted. The vector was digested with BglII and phosphatased. Samples were ligated, packaged in vitro, and used to transform bacterial host strains. Ampicillin-resistant colonies were picked and stored in microtiter dishes. For transformations, DNA was isolated from pools of clones or from individual clones and mixed with prepared protoplasts. Transformants were selected for tryptophan prototrophy and then screened for their radiation repair and meiotic phenotypes.

Table 1. Characterization of cosmid clones.

Clone[a]	Insert size (kb)	Chromosome(s)[b]	Restriction fragment[c]	Colinearity[d]
O7-6;4A6	34	6	138 kb, AscI	+
O7-8,9;1B11	37	J6: 8; O7: 8,9 doublet	125 kb, NotI; 315 kb, AscI	+
O7-8,9;5D6	28	J6: 8; O7: 8,9 doublet	310 kb, AscI; 150 kb, SfiI	+
O7-13;1E4	36	13	235 kb, NotI; 135 kb, NotI/AscI double digest	+
O7-13;1D5	32	13	275 kb, NotI; 175 kb, NotI/AscI double digest	+
J6-13;6A4	33	at least five each[e]	Not done (nd)	+
J6-13;6A5	32	13	nd	+
J6-13;6A6	30	13	nd	+

[a]Clones names are given as in this example: O7-6;4A6 means strain Okayama-7, chromosome 6, microtiter dish 4, row A, column 6. Abbreviations used: O7, Okayama-7; J6, Java-6.
[b]Southern blots of CHEF gels were hybridized with clone inserts.
[c]Plugs of Okayama-7 chromosomes were digested with restriction enzymes that have 8 bp recognition sequences. The fragments were then separated by CHEF electrophoresis, and the gels were blotted. Hybridization was with the entire insert of the clone, and sizes are given for cases in which this probe hybridized to only one fragment for that digest.
[d]Colinearity was determined by the side-by-side comparison of restriction digests of the clone with those of the genome from which the clone was isolated, as described in the text. A '+' means that one or more such comparisons were made and found to indicate no rearrangements.
[e]From hybridizations to Southern blots of CHEF gels, estimated distributions are: J6, chromosomes 2,7,8,9,12,13; O7, chromosomes 2,7, 8-9 doublet, 10.

and the genome with the same frequently cutting enzyme, e.g., HindIII. Because the ends of the insert are not HindIII sites, there will be two HindIII junction fragments unique to the clone, each formed from a HindIII site internal to the insert and a HindIII site within the vector. Similarly, there will be two HindIII fragments unique to the genome. All other hybridizing fragments should be internal to the cosmid insert and should—if the insert is a single copy, contiguous and unrearranged segment of the genome—be identical to the fragments of the genome.

Because *C.cinereus* contains little interspersed repetitive DNA (8) the majority of clones should satisfy all three of the above criteria. An analysis of eight clones (Table 1) by two or more of these methods indicated no examples of multiple ligations. One clone analyzed, J6−13; 6A4, hybridized to five or more CHEF-separated chromosomes. Despite this, the insert hybridized to only two cosmid fragments not found in genomic digests, and subfragments of the clone also hybridized to multiple chromosomes. Our interpretation is that this clone contains a repeated sequence and is not the product of multiple ligation events. Our clone analysis (Table 1) also indicated that cross-contamination of libraries with sequences from other chromosomes is not significant. Thus, these libraries should be useful for chromosome walking.

Cloning the *rad9* gene by sib-selection

The standard method for cloning genes from filamentous fungi is the technique known as sib-selection (20). In this method, pools of clones are used to transform a mutant strain. A pool that rescues the mutant phenotype is progressively subdivided, until a single complementing clone is isolated. Using this technique, we have isolated clones that contain the *rad9* gene. The *rad9-1* mutation confers two phenotypes (1). First, *rad9-1* haploid or dikaryotic strains are sensitive to ionizing radiation. Second, a homozygous *rad9-1* dikaryon arrests in meiosis and fails to produce spores. This basidiospore-defective (Bad$^-$; 21) phenotype results in the production of a white mushroom, which is readily distinguishable from the wild-type, black mushroom (the dark color is conferred by the spores).

A *rad9-1; trp1-1,1-6* strain was constructed and transformed with five pools of 96 clones each from a cosmid library made

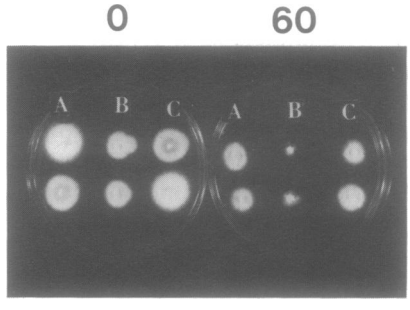

Figure 3. Rad$^+$ transformants of *C.cinereus*. Duplicate plates were treated with 0 or 60 krads of gamma rays. Column **A**, Rad$^+$ control strain; column **B**, Rad$^-$ untransformed strain (*rad9-1, trp1-1,1-6*); column **C**, Rad$^+$ transformant of the strain shown in column B. Each column contains duplicate subcultures of that strain.

from the doublet of Okayama-7 chromosomes 8 and 9. Transformants were selected based on acquisition of tryptophan prototropy (2) and then screened for their transformation from a Rad$^-$ to a Rad$^+$ phenotype. For each pool, roughly 500 transformants were screened, to ensure that each of the 96 clones was sampled at least once. Two pools yielded Trp$^+$Rad$^+$ transformants (Figure 3), and one of these pools was subdivided until a single clone (O7-8,9;1B11) was isolated which complements the Rad$^-$ phenotype of *rad9-1* strains. Thus, from a screen of 480 clones, or about one-tenth the number necessary to screen the entire *C.cinereus* genome, we successfully isolated the *rad9* gene.

Clone O78,9;1B11 complements both the Rad$^-$ and Bad$^-$ phenotypes of *rad9-1* strains (Table 2). The percentage of Trp$^+$ transformants rescued for the repair phenotype ranged, in three experiments, from 52 to 89. These results are in agreement with data reported for another *C.cinereus* gene, the *A* mating type locus, for which 65% of the Trp$^+$ transformants displayed *A* transgene functions (3). Of the Rad$^+$ transformants obtained, 79% to 100% were also Bad$^+$ when crossed to a compatible *rad9-1* strain, confirming our previous prediction (1) that a single genetic change is responsible for both defective phenotypes of rad9-1 mutants.

Table 2. Characterization of transformants obtained with the *rad9* gene (clone O7-8,9;1B11).

Experiment	Rad+ Bad+	Rad+ Bad−	Rad− Bad+	Rad− Bad−	N (Trp+)
1	26 (72%)	6 (17%)	0	4 (11%)	36
2	72 (77%)	0	5 (5%)	17 (18%)	94
3	38 (42%)	9 (10%)	2 (2%)	42 (46%)	91

Rad+ means that the primary transformant was radiation-resistant by the chunk test. Bad+ means that the mating of the primary transformant with another *rad9-1* strain produced a dark mushroom with viable spores.

In two experiments, a portion (10−17%) of the Trp+ Rad+ isolates obtained were Bad− (Table 2). A transgene that complements the *rad9-1* mutation for DNA repair but not for meiosis may be integrated at a site that does not allow proper meiotic expression of the gene. Alternatively, the meiotic expression may be variable in these transformants. For some of the isolates included in experiment 2 (Table 2) initial crosses of the transformants to our *rad9-1* tester strain yielded one or more Bad− mushrooms; however, when these transformants were recrossed with the tester strain, Bad+ mushrooms were obtained. The Rad+ Bad− isolates reported for experiments 1 and 3 have not been similarly retested; they may in fact be variable for basidiospore production also.

The behavior of the seven isolates reported as Rad− Bad+ (Table 2) is of special interest, because in these strains a transgene silencing/reactivation process, as has been observed in some transgenic plants (22), apparently takes place. For each case, the primary transformant is Rad−. However, when that strain is mated to our *rad9-1* tester, the resulting mushroom is wild-type. Further examination of one of these mushrooms showed that 66% of its progeny were Rad+, and that the integrated vector and transgene sequences cosegregated with the Rad+ phenotype, implying strongly that the Rad+ phenotype was derived from the transgene and was not the result of a reversion event. Thus, our interpretation is that the transgene is inactive in the primary transformant, but becomes stably reactivated at some point during the growth of the dikaryon or during the development of the mushroom. In this Rad− Bad+ primary transformant, the site of integration of the transgene was chromosome 8, the location of the endogenous gene. However, the integration event was not an insertion into the endogenous *rad9* sequence; hybridization of a portion of the clone to an AscI digest of this transformant showed that the endogenous 315 kb AscI fragment was not affected by the transformation and that a new AscI fragment was formed (data not shown). For the second Rad+Bad− primary transformant obtained in experiment 3 (Table 2), no *trp1* sequences were integrated, nor was a new AscI fragment formed (data not shown). Therefore, for this isolate we cannot distinguish between gene replacement and genetic reversion.

Analysis of CHEF-separated chromosomes of four Rad+ Bad+ transformants from experiment 3 showed that the *rad9* transgene was integrated into variable positions; integrations into chromosomes 1, 2 and 5 were observed (data not shown). Therefore, clone O7-8,9;1B11 probably contains all the coding and regulatory sequences required for *rad9* gene expression. In addition, the intensity of hybridization of a probe made from a fragment of O7-8,9;1B11 was the same when the transgene was compared with the endogenous gene, indicating that a single copy of transforming DNA is sufficient to rescue the *rad9-1* mutant phenotypes (data not shown).

To further confirm that clone O78,9;1B11 does contain the wild-type *rad9* gene, we performed RFLP analysis using a 5 kb XbaI fragment from the clone (Figure 4). A cross between a *rad9-1* strain and strain MZC3 (a Rad+ strain) was made, and the progeny were analyzed for both radiation sensitivity and RFLP type. Seventy-two progeny were analyzed, and no recombinants were obtained.

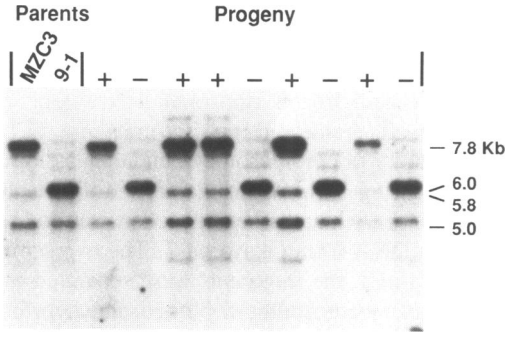

Figure 4. RFLP mapping of clone O7-8,9;1B11. A 5 kb XbaI fragment from the clone was used as a probe against DNAs from a *rad9-1* strain, strain MZC3, and progeny of a cross between these two strains. Samples were digested with SalI, the fragments were separated on 0.7% agarose gels, and the gels were blotted and hybridized.

Taken together, our data indicate that we have cloned a complete copy of the *rad9* gene. This clone, O7-8,9;1B11, complements both mutant phenotypes of *rad9-1* strains, maps to the chromosomal site of the *rad9* locus, and rescues *rad9-1* mutants when integrated into different chromosomal sites. Using a fragment from the clone, we performed colony hybridizations to seven pools of 96 clones from the library of chromosomes 8 and 9 of Okayama-7. We obtained a total of six overlapping clones, including O7-8,9;1B11. If we estimate the sizes of chromosomes 8 and 9 to be roughly 2.5 Mb each (Figure 1), then this doublet library is of 5 Mb of chromosome. Seven pools of 96 clones, with insert sizes averaging 35 kb, represent 23.5 Mb of insert DNA, or nearly five chromosome equivalents. Therefore, the representation of a single copy sequence six times in that number of pools is consistent with expected results; we believe we have constructed representative, random libraries.

Mapping the *rad11* gene using near-isogenic lines and a chromosome-specific library

The *rad9* gene was straightforward to clone, since its Rad− phenotype was readily complemented *in trans* in primary transformants. However, other genes of interest may be less easy to isolate if the level of complementation is lower or if the mutant phenotype causes poor transformation. In such a case, a map-based cloning method may be appropriate. One such approach involves the screening of near isogenic lines (NILs) for RFLP or randomly amplified polymorphic DNA (RAPD; 23) markers that have remained linked to a mutant locus. A backcrossed line

and the backcross parent are compared with different probes, to find a polymorphism. Any probe detecting a polymorphism represents sequences from the original mutant that are retained through backcrosses. Further mapping of these sequences can quickly lead to clones of regions closely linked to desired genes. These methods have been highly successful in tomato and other plants (24, 25).

Our modification of the above technique makes use of chromosome-specific libraries combined with NILs. We are interested in cloning the gene *rad11*, which, like *rad9*, is required for both radiation-damage repair and meiosis in *C.cinereus*. *rad11* mutants arrest in early prophase I of meiosis and are sensitive to ionizing radiation (M.E.Z., unpublished observations). The *rad11* gene is linked to the ribosomal RNA gene cluster in *C.cinereus*, which is located on chromosome 6 (11). We constructed a library of chromosome 6, and then assayed random clones by comparing DNA from a *rad11* strain (isolated in a Java-6 background), which had been backcrossed to Okayama-7 five times, with DNA from Okayama-7. The two samples were digested with BamHI, the fragments were separated on agarose gels, and the gels were Southern blotted and hybridized with probes made from 20 different clones from the Okayama-7 chromosome 6 library. Three of these clones detected an RFLP between the two DNA samples. Each of the three was further tested by RFLP mapping, again using BamHI as the diagnostic enzyme. Clone O7-6;4A7 contained sequences 18.5 map units from *rad11*, O7-6;4B8 mapped 14 map units from *rad11*, and O7-6;4A6 mapped only 1.6 map units from the *rad11* gene.

Because we used only one enzyme to screen for polymorphisms between a *rad11* strain and the backcross parent, we may have rejected other clones that are linked closely to the *rad11* gene; we might have found retained RFLPs for some of the other 17 clones analyzed if we had used more restriction enzymes. However, our method is highly efficient; by using entire cosmids as probes, we were screening approximately 35 kb of chromosome at a time. We could repeatedly screen the same RFLP blots and rapidly rule out clones, such as A7 and B8, which are only loosely linked to the *rad11* gene. We are now in a fine position to begin a chromosome walk to *rad11*, as clone O7-6;4A6 is only 1.6 map units from this gene. Based on an estimated minimum genetic length of 1,300 cM (26), and a genome size of 37,500 kb (4), the average relationship between physical distance and genetic distance in *C.cinereus* should be 29 kb. Therefore, our walk to the *rad11* gene from clone O7-6;4A6 should be roughly 50 kb. Observed measurements of the relationship between physical distance and recombination frequency in *C.cinereus* have ranged from 100 kb/map unit, for the distance between the A mating type factor and the gene *pab1* (27), to 1.7 kb/map unit for sequences near the *rad12* gene (M.Ramesh and M.E.Z., unpublished observations).

CONCLUSIONS

We have shown that chromosome-specific libraries greatly facilitate gene mapping and cloning in the fungus *C.cinereus* . By starting a screen for complementing DNA fragments using only sequences from the correct chromosome, the number of clones that must be examined can be reduced by a factor of 10 or more. In addition, the combination of chromosome-specific libraries with the use of backcrossed lines makes the isolation of clones tightly linked to desired genes very efficient. A complementary approach to ours was recently described by Brody et al. (28), who divided existing libraries of *Aspergillus nidulans* into chromosome-specific pools by hybridization with probes prepared from isolated chromosomes. These methods are readily applicable to any organism with separable chromosomes, including fungi (6) and many protists (e.g., 29). For organisms with larger genomes, flow-sorted chromosomes have recently been shown to be suitable starting material for chromosome-specific libraries (30). In addition to their use in specific gene mapping and cloning, these powerful tools will facilitate the development of complete physical maps of specific chromosomes. The karyotypes of many fungal species are quite variable (Figure 1; 6, 11), and an understanding of this variation, and ultimately of the evolution of fungal genomes, will require long range, contiguous physical maps of chromosomes.

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