Frequent Changes in the Number of Reiterated Ribosomal RNA Genes Throughout the Life Cycle of the Basidiomycete *Coprinus cinereus*

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ABSTRACT

We have examined the stability of the tandemly repeated genes that encode the ribosomal RNA in *Coprinus cinereus*. These genes are contained within two linked *Hin*dIII fragments in a 3.0-Mb chromosome. We monitored the size of these fragments in both mitotic and meiotic segregants using the contour-clamped homogeneous electric field (CHEF) method. No length changes were observed in the smaller *Hin*dIII fragment (100 kb; 10 repeats) among the DNAs prepared from 46 asexual spore derivatives (oidia) or 128 meiotic segregants (basidiospores from 32 tetrads). However, the larger *Hin*dIII fragment (1100 kb; 120 repeats) did exhibit variability. Substantial changes, involving up to 40% of the larger *Hin*dIII fragment were recorded in 7 of 46 oidial isolates (including 4 of 22 transformed derivatives). To learn if the changes were confined to the vegetative portion of the life cycle, we examined transmission of *Hin*dIII variants through three crosses. In the first two crosses (16 tetrads total), no changes were observed in the large *Hin*dIII fragment. However, in the third cross (16 tetrads), each tetrad showed at least one alteration. In half of the tetrads from the third cross, the altered patterns segregated 2:2, suggesting that the changes occurred after mating but prior to premeiotic DNA replication. We conclude that breakage and rejoining reactions within the rDNA are frequent and are not confined to any particular stage of the life cycle. It also appears that certain repeats are sheltered from these events. Finally, marked differences in rDNA stability were observed in the crosses analyzed.

IN *Coprinus cinereus*, the genes encoding the RNA components of the ribosomes are reiterated and organized in one tandemly repeated array (CASSIDY *et al.* 1984). The organization of these genes (collectively called the rDNA) is atypical in that the repeating unit (9.3 kb) includes the 5S gene as well as the 18S, 5.8S and 26S genes and these are all transcribed in the same direction (and thus from the same strand). Meiotic recombination is suppressed within this array (CASSIDY *et al.* 1984), as has been shown for many eukaryotes (PETES and BOTSTEIN 1977; DVORAK and APPELS 1986; RUSSELL, PETERSON and WAGNER 1988). Yet several polymorphisms are known that serve to distinguish the rDNAs of different strains. Within a strain, the 100 repeated copies are usually quite homogeneous. Taken together, these results imply the existence of horizontal information transfer mechanisms, such as unequal crossing over or gene conversion, that result in the spread of new mutations to all of the tandem copies (SMITH 1973; HILLIS *et al.* 1991).

Recently, changes in the number of tandemly repeated copies of the rRNA genes were reported in the ascomycete *Neurospora crassa* (BUTLER and METZENBERG 1989, 1990). These authors concluded that most of these changes occurred during the interval between fertilization and karyogamy (the nuclear fusion that precedes meiosis) and found evidence for both intrachromatid exchange and unequal sister chromatid exchange. They concluded that the arrays were quite stable during mitotic growth.

Mitotic stability has also been documented in the yeast *Saccharomyces cerevisiae*. Using marked rDNA arrays, it was shown that unequal sister chromatid exchange occurs in about 10% of meiosis I divisions (PETES 1980), but in only 0.0002% of mitotic divisions (SZOSTAK and WU 1979). At least part of the genetic basis for mitotic stability was shown to result from the combined actions of topoisomerases I and II (CHRISTMAN, DIETRICH and FINK 1988), which suppress exchange in the interval, presumably by preventing an excess of transcription-induced superhelical arrays (KIM and WANG 1989).

While investigating the fate of transforming DNA in *C. cinereus*, we noticed that many transformants showed alterations in molecular karyotype as judged by orthogonal field alternating gel electrophoresis (OFAGE) (J. N. NORRIS and P. J. PUKKILA, unpublished observations). We have designated such *c*hromosome *l*ength *p*olymorphisms using the acronym CLP. In this study, we have concentrated on the CLPs that involve a 3.0-Mb chromosome, which contains the rDNA locus (*rdn1*). We have determined that these CLPs are in fact the result of alterations in

TABLE 1

Haploid strains

Strain	Relevant genotype	Source
218	*A3B1 trp1-1,1-6 slg1-1 bad1-s rdn1-1100*	BINNINGER *et al.* (1987)
253	*A6B6 acu7-1*	L. A. CASSELTON
306	*A43B43 rdn1-1100*	This laboratory
218-7	*A3B1 trp1-1,1-6 slg1-1 bad1-s rdn1-700*	Oidial isolate of 218
bc3	*A43B43 trp1-1,1-6::trp1-DGH10$^+$ rdn1-1000*	This laboratory
1/91-6B	*A3B1 trp1-1,1-6::trp1-Cc1001$^+$ bad1-s*	This laboratory
1/91-9B	*A43B43 trp1-1,1-6::trp1-Cc1001$^+$ bad1-s*	This laboratory
1/91-11D	*A3B43 trp1-1,1-6::trp1-Cc1001$^+$ bad1-s*	This laboratory
8/90-3A	*A43B1 trp1-1,1-6::trp1-Cc1001$^+$ bad1-2*	This laboratory
7/91-3B	*A43B43 trp1-1,1-6::trp1-Cc1001$^+$ bad1-s rdn1-700*	218-7 × bc3
7/91-7A	*A43B43 trp1-1,1-6::trp1-DGH10$^+$ rdn1-1000*	218-7 × bc3
7/91-7C	*A3B1 trp1-1,1-6 slg1-1 rdn1-700*	218-7 × bc3
7/91-7D	*A3B1 trp1-1,1-6 slg1-1 bad1-s rdn1-700*	218-7 × bc3
8/91-1C	*A3B1 rdn1-500*	7/91-7C × 7/91-7D

rDNA copy number. We have asked if such alterations in rDNA copy number were associated with the transformation process *per se*, or with some other aspect of the asexual cycle. We recovered strains with gross deficiencies in rDNA copy number and used these strains to examine the stability of rDNA arrays during premeiotic and meiotic divisions.

MATERIALS AND METHODS

***C. cinereus* strains and culture conditions:** The origin of the strains used in this study is summarized in Table 1. Strains harboring integrated plasmids are noted as follows. The designation *trp1-1,1-6::trp1-DGH10$^+$* indicates that plasmid DGH10 has integrated into the *trp1-1,1-6* allele and the resulting strain is Trp$^+$. Yeast-malt-glucose medium (RAO and NIEDERPRUEM 1969) supplemented with 100 μg/ml L-tryptophan was used for vegetative growth, oidial germination, and fruit body formation.

Plasmid DNAs: Several plasmid DNAs were used in the DNA-mediated transformation experiments described. Plasmids Cc1001, Cc1002 and Cc1003, which include the tryptophan synthetase gene (*trp1*), have been described (BINNINGER *et al.* 1987; SKRZYNIA *et al.* 1989). Plasmids DGH10 and DGX3 are slightly modified derivatives of pCc1003. Each contains an oligonucleotide insertion in the polylinker region of the vector (C. SKRZYNIA and T. FREEDMAN, unpublished). pACU7(dKpn) is a 4.5-kb deletion derivative of the pHIONA 1 plasmid (MELLON, LITTLE and CASSELTON 1987) and was designed to disrupt the function of the endogenous isocitrate lyase gene following transformation (BINNINGER 1987). In the cotransformation experiments described, the plasmid was first digested with *Bam*HI to release the vector sequences. The number of rRNA genes was monitored using pCc1, a 9.3-kb *Bam*HI fragment cloned into pBR322 that contains the entire rDNA repeating unit (WU, CASSIDY and PUKKILA 1983). Plasmid F41, a 7.2-kb *Bam*HI fragment cloned into pBR322 (BINNINGER 1987) contains the 5′ end of the *trp1* gene and was used as a single copy standard in comparative hybridization experiments.

Transformation and genetic analysis: DNA-mediated transformation was carried out as described (BINNINGER *et al.* 1987). The *acu7* gene is required for growth on acetate as a sole carbon source. The Acu phenotype was scored by comparison of growth on glucose minimal medium (SHAHRIARI and CASSELTON 1974) and acetate minimal medium (KING and CASSELTON 1977). These were prepared as described except 28 mM ammonium chloride was used in place of the ammonium tartrate. To confirm that the Acu$^-$ transformants contained mutations at the *acu7* locus, we demonstrated that the transformants failed to complement the *acu7-1* mutation in strain 253 and failed to yield Acu$^+$ progeny when crossed to strain 253. Oidial isolation was described previously (ZOLAN and PUKKILA 1986). Tetrad analysis was accomplished using a Jena micromanipulator as described (PUKKILA 1992). Six markers were scored in these crosses. The *slg1-1* (*s*low *g*ermination) genotype was inferred from visual inspection of the basidiospore colonies after overnight incubation at 37°. Wild type colonies exhibited complex hyphal branches, while *slg1-1* colonies had only a single germ tube or a small number of branches. Following transfer, no differences in growth rate could be detected. The *trp1* marker was scored by plating on minimal media with and without tryptophan as described (BINNINGER *et al.* 1987). Mating types were scored using four tester strains (1/91-6B, 1/91-9B, 1/91-11D and 8/90-3A). Each had a distinct mating type (*A3B1*, *A43B43*, *A3B43* or *A43B1*, respectively) and each harbored a recessive mutation in a gene required for *ba*sidiospore *d*evelopment (*bad1*). Fully compatible matings result in the production of dikaryotic mycelium, and occur between strains with different mating types. For example, an *A43B1* segregant would show the full mating reaction with only the *A3B43* tester. The mating reactions were confirmed by fruiting tests, since fully compatible matings also result in the production of fruit bodies. The Bad phenotype was scored by inspection of these fruit bodies, as *bad1-s* homozygotes fail to make spores. Finally, the number of rDNA copies at the *rdn1* locus was scored by gel analysis as described below.

Gel analysis: Chromosome-sized DNAs were prepared from protoplasts, digested with *Hin*dIII or *Not*I restriction enzymes, and resolved by the contour-clamped homogeneous electric field (CHEF) method as described (BINNINGER *et al.* 1991). For intact chromosomes, the electrophoretic conditions were 65 V, 144 hr, and 20-min pulse time. For resolution of *Hin*dIII fragments, the electrophoretic conditions were 165 V, 18 hr, and 115-sec pulse time on a CHEF apparatus or 6 V/cm, 9 hr with 60-sec pulses followed by 9 hr with 90-sec pulses on the CHEF Mapper apparatus (Bio-Rad). Neither *Hin*dIII nor *Not*I cuts within the rDNA repeating unit. Fragments containing rDNA sequences were

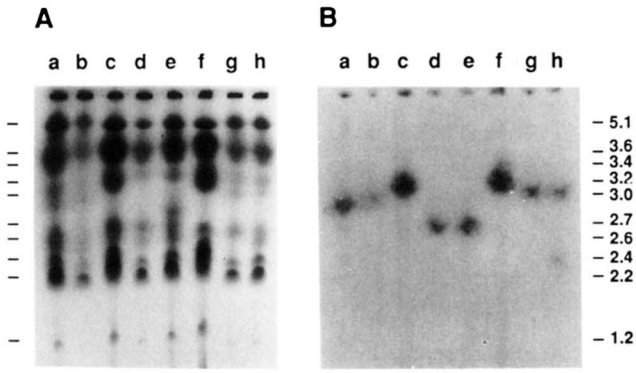

FIGURE 1.—Photograph (A) and autoradiogram following Southern analysis (B) illustrate chromosome length polymorphisms in transformed strains. (A) Chromosome-sized DNAs were resolved using the CHEF method and stained with ethidium bromide. The negative image is shown. (B) DNAs were probed with a ^{32}P-labeled 9.3-kb *Bam*HI fragment from pCc1 (a complete rDNA repeat). Approximate sizes (in megabases) are indicated on the right. Material trapped in the wells is visible at the top of the figure. a, Ac11$^+$; b, 218 (the recipient); c, T88; d, Ac49$^+$; e, Ac1$^-$; f, Ac2$^-$; g, Ac29$^-$; h, Ac18$^+$.

identified by Southern hybridization and probing with pCc1. When transformed strains were examined, the 9.3-kb rDNA fragment was first resolved from the vector sequences and recovered by electroelution before ^{32}P-labeling. The relative rDNA copy number in each strain was estimated by comparing the sizes of the DNA fragments that contained rDNA copies following *Hin*dIII digestion to molecular weight standards. Both intact chromosomes from *S. cerevisiae* (Bio-Rad) and bacteriophage λ ladders (FMC) were used. The electrophoretic conditions were chosen so that the size classes of interest fell within areas of good resolution on these gels (VOLLRATH and DAVIS 1987). To quantitate the amount of hybridization in particular bands, we used a Zeineh soft laser scanning densitometer (Biomed Instruments).

Statistical analysis: Chi-square values were calculated according to the method of FISHER (1948) to test for independence in the 2 × 2 (contingency) tables.

RESULTS

Chromosome length polymorphisms in transformed strains of *C. cinereus*: We have shown that targeted transformation can be carried out in *C. cinereus* (BINNINGER *et al.* 1991). We have recovered both homologous integration as well as gene replacement events. However, further examination of several transformed strains revealed some additional changes. In particular, we have observed frequent alterations in the molecular karyotype (Figure 1).

To examine the molecular karyotype of a strain, oidia (asexual spores) were harvested from the vegetative mycelium, and the cell walls were removed. The chromosomal DNAs were then resolved using the CHEF method (CHU, VOLLRATH and DAVIS 1986). DNAs from the starting strain (218) are shown in Figure 1A, lane b. These DNAs range in size from 1 to 5 Mb (PUKKILA 1992). Some of the chromosomes comigrate, so 10, not 13, bands are usually observed. In the first experiment, strain 218 (which harbors a double mutation in the gene encoding tryptophan synthetase) was cotransformed with pCc1001 and pACU7(dKpn), and 140 Trp$^+$ transformants were recovered. Homologous replacement of the resident *acu7* locus with plasmid sequences would result in disruption of the isocitrate lyase gene. Three of the 140 Trp$^+$ transformants were also Acu$^-$. Although complementation and segregation analysis confirmed that these transformants contained mutations at the *acu7* locus, Southern hybridization revealed that the endogenous gene appeared to have been silenced by events more complicated that simple replacement of the functional sequences with the deletion derivative (results not shown). While investigating the fate of the transforming DNAs in these three mutant strains, we observed gross alterations in the molecular karyotype in two of the strains (Figure 1A, lanes e and f). Both the 3.0-Mb and the 2.7-Mb chromosomes migrated anomalously in the former (Ac1$^-$) while the 2.7-Mb chromosome migrated anomalously in the latter (Ac2$^-$). In a second experiment, 56 additional Trp$^+$ transformants were examined following cotransformation with these plasmids. Three additional Acu$^-$ isolates were recovered, and one of these (Ac29$^-$) also showed an altered karyotype (Figure 1A, lane g). The 2.6-Mb chromosome migrated anomalously in this mutant.

It is apparent that CLPs occur frequently in these transformants. Although different strains of *C. cinereus* contain many distinguishing CLPs (PUKKILA and CASSELTON 1991; ZOLAN *et al.* 1992), such variation among isolates of a single strain has not been reported previously. It was possible that this genomic instability was caused by disruption or inactivation of the *acu7* locus. We examined nine Trp$^+$ Acu$^+$ transformants from the second experiment, and no changes were observed in seven of these (*e.g.*, Ac 11$^+$, Figure 1A, lane a). However, in one Trp$^+$Acu$^+$ transformant (Ac49$^+$), a CLP involving the 3.0-Mb chromosome was apparent upon visual inspection (Figure 1A, lane d). An additional Trp$^+$ Acu$^+$ transformant (Ac18$^+$) was shown to contain a CLP following the hybridization experiments described below (Figure 1B, lane h). Thus inactivation of the *acu7* locus was not necessary for the recovery of transformants with CLPs.

In the transformants in which *acu7* function was lost, some of the CLPs involved the 2.7-Mb chromosome, which contains the *acu7* locus (PUKKILA and CASSELTON 1991). We have shown that these mobility changes result from either insertions of many copies of the transforming DNA or from translocations (J. N. NORRIS and P. J. PUKKILA, unpublished). Both types of modifications have been seen following DNA-mediated transformation in other filamentous fungi (WERNARS *et al.* 1987; ASCH *et al.* 1992). However,

TABLE 2

Chromosome length polymorphisms involving the *rdn1* locus

Isolates examined	No. with no changes	No. with changes	Isolates with changes	Size of *Hin*dIII fragment(s) in kb[a]
Transformants of 218[b]	17	4	Ac1$^-$[c]	800
			Ac49$^+$	850
			Ac18$^+$	1100 + 600[d]
			DGX3	750
Oidial isolates of 218	7	1	218-7	700
Oidial isolates of 218-4	7	1	218-4H	1100 + 1050[e]
Oidial isolates of 218-7	7	1	218-7H	850

[a] Each isolate also contained a 100 kb *Hin*dIII fragment.
[b] All transformants in Table 2 incorporated *trp1* sequences from either pCc1001, pCc1002, pCc1003 or pDGX3 and became Trp$^+$.
[c] This transformant incorporated pACU7(dKpn) sequences and became *acu7*$^-$.
[d] A 3.0-Mb chromosome and a 2.4-Mb chromosome contained rDNA sequences in this isolate.
[e] A 3.0-Mb chromosome and a 2.9-Mb chromosome contained rDNA sequences in this isolate.

we also noted that in both Acu$^+$ and Acu$^-$ strains, many of the remaining CLPs appeared to involve the 3.0-Mb chromosome, which contains the rDNA. We wished to explore the molecular basis of the CLPs involving this chromosome, since it seemed likely that changes in the number of rDNA repeats were responsible.

Changes in rDNA copy number in transformed strains: The aberrant migration of the chromosome containing the rDNA in many of these transformants was confirmed by Southern transfer and probing with a 9.3-kb *Bam*HI fragment of pCc1 that contains rDNA sequences but no vector DNA (Figure 1B). In these transformants, the chromosome that contains the rDNA sequences ranged in size from 2.7 to 3.0 Mb (Figure 1B). In one Acu$^+$ transformant (Ac18$^+$) an additional block of rDNA sequences was present on a chromosome that is 2.4 Mb (Figure 1B, lane h).

It was possible that these alterations were somehow a consequence of the cotransformation procedure, triggered by the particular plasmid sequences, their free ends, or their sites of integration. Accordingly, we examined seven Trp$^+$ transformants in which either homologous insertion or gene replacement at the *trp1* locus had occurred. Only circular *trp1* plasmids were used for these transformation experiments, and no chance disruption of genes required for chromosome stability could have occurred in these transformants. In six of the seven, no changes were observed (*e.g.*, T88, Figure 1A, lane c). However, a CLP involving the 3.0-Mb chromosome was seen in one of these transformants (DGX3, Table 2).

The changes in mobility of the 3.0-Mb chromosome could have resulted from a variety of causes, including translocation, loss of non-rDNA sequences, or alteration in the number of copies of the tandemly repeated rRNA genes. We utilized restriction enzyme digestion and CHEF electrophoresis to ask if the number of tandemly repeated rRNA genes was altered in any of these transformants. When chromosomal-sized DNAs

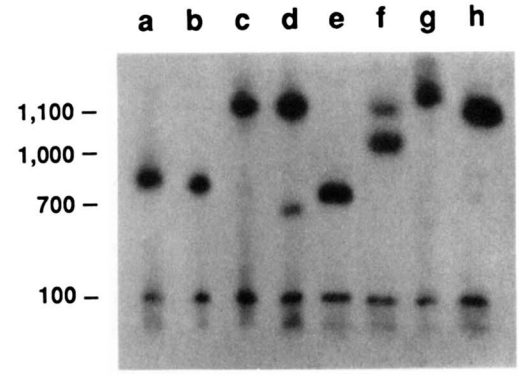

FIGURE 2.—Autoradiogram illustrating changes in length of the large *Hin*dIII fragment that contains most of the tandem rDNA repeats. DNAs were digested with *Hin*dIII, resolved using the CHEF method, and probed with pCc1. Sizes are indicated on the left (in kb). Partly degraded material is visible below the 100-kb bands near the bottom of the figure. a, Ac49$^+$; b, Ac1$^-$; c, Ac2$^-$; d, Ac18$^+$; e, 218-7; f, 218-4H; g, T88; h, 218.

from strain 218 were digested with *Hin*dIII, resolved by CHEF electrophoresis, and stained with ethidium bromide, a single prominent 1,100-kb fragment was seen, with the rest of the DNA cut into pieces 100 kb or smaller (results not shown). Following Southern transfer and hybridization with pCc1, two regions of hybridization were apparent in strain 218 (Figure 2, lane h). The 1100-kb band hybridized strongly, and there was also a 100-kb band that hybridized. When transformants that had no changes in the 3.0-Mb chromosome were examined, the same two bands were observed (*e.g.*, Ac2$^-$, Figure 2, lane c and T88, lane g). However, quite different results were obtained when strains that exhibited size changes in the 3.0-Mb chromosome were examined. Strains such as Ac49$^+$ and Ac1$^-$ that exhibited an increased mobility of the 3.0-Mb chromosome showed a loss of tandemly repeated rRNA genes as indicated by the reduction in size of the largest *Hin*dIII fragment (Figure 2, lanes a and b), while strains such as Ac18$^+$ that exhibited an additional rDNA-bearing chromosome showed an additional fragment that contains rDNA (Figure 2,

lane d). For convenience, we will refer to *rdn1* variants according to the size of the large *Hin*dIII fragment. Thus strain 218 contains an *rdn1-1100* locus. The pattern seen in Ac18$^+$ appears to result from heterogeneity within the colony. According to this view, the majority of the sampled oidia would contain an *rdn1-1100* locus (and a 3.0-Mb chromosome) while the minority would contain an *rdn1-600* locus (and a 2.4-Mb chromosome). Possible origins of this heterogenity are described below.

It was possible that the changes in mobility of the large *Hin*dIII fragments were due exclusively to alterations in non-rDNA sequences in these fragments. We used two approaches to confirm that these size variations reflected changes in rDNA copy number. In the first approach, we used comparative hybridization of genomic DNAs from strain 8/91-1C (*rdn1-500*) and strain 306 (*rdn1-1100*). The DNA samples were digested with *Bam*HI, and appropriate dilutions (using single stranded salmon sperm DNA as a carrier) were electrophoresed and probed with either pF41 (the *trp1* single copy standard) or pCc1 (the rDNA). Hybridization signals in the appropriate bands (7.2 kb for pF41 and 9.3 kb for pCc1) were compared by scanning densitometry. We conclude that strain 306 (*rdn1-1100*) contains 1.8–1.9 times the number of rDNA copies as are present in strain 8/91-1C (*rdn1-500*). To confirm that size variations were typically accompanied by changes in rDNA copy number, we used a second approach. We chose twelve lanes in which *Hin*dIII fragments had been hybridized to the rDNA probe with little background or degradation, and where the size of the large *Hin*dIII fragment varied from 500 to 1100 kb. We measured the hybridization signals by scanning densitometry. We found that in lanes that contained 1000–1100 kb *Hin*dIII fragments, the hybridization signal produced by the large fragment was 8–9 times stronger than the signal from the 100 kb fragment in the same lane. In lanes with 750–900-kb fragments, the ratio was 5–6. In lanes with 500–700-kb fragments, the ratio was 4–4.5. Thus we conclude that changes in size of the large *Hin*dIII fragment are accompanied by changes in rDNA copy number, although we cannot exclude the possibility that some changes in non-rDNA sequences are also occurring.

In summary, we examined a total of 22 transformants and observed alterations in rDNA copy number in 4 of these. Each transformant contained a 100-kb *Hin*dIII fragment that hybridized to the rDNA probe as well as one or more larger fragments that varied from that found in the starting strain (1100 kb), as summarized in Table 2. Thus we conclude that in this collection of transformants, alteration in rDNA copy number is frequent, and is responsible for the observed CLPs involving the 3.0-Mb chromosome.

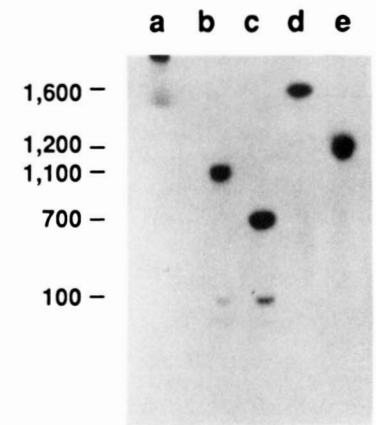

FIGURE 3.—Autoradiogram illustrating linkage of the 1100- and 100-kb *Hin*dIII fragments that contain rDNA. DNAs were resolved using the CHEF method and probed with pCc1. Sizes are indicated on the left (in kb). Material trapped in the wells is visible at the top of the photograph. The strains and enzymes used were as follows. a, 218-3, undigested; b, 218-3, *Hin*dIII; c, 218-7, *Hin*dIII; d, 218-3, *Not*I; e, 218-7, *Not*I.

Structure of the *rdn1* locus: In previous studies, we used restriction fragment length polymorphisms to demonstrate that the tandemly repeated copies of the rRNA genes were located in a single cluster in the genome, and that meiotic recombination was suppressed within the array (CASSIDY *et al.* 1984). Inspection of Figure 2 reveals that the genome contains two *Hin*dIII fragments that hybridize to the rDNA probe. A similar digest is shown in Figure 3, lane b. To establish the location of these fragments in the genome, we carried out two further experiments. We first asked if the 100 kb fragment was in fact an extrachromosomal element that contained rDNA (KIM and WANG 1989). DNA from strain 218-3 (an oidial isolate of strain 218) was electrophoresed without prior digestion, but no DNAs migrating in this region were detected (Figure 3, lane a). We then asked if the 1100- and 100-kb *Hin*dIII fragments were linked. DNA from strain 218-3 was digested with *Not*I, and fragments were resolved by the CHEF procedure. As shown in Figure 3, lane d, only a single fragment was obtained, indicating that both the 1100- and 100-kb *Hin*dIII fragments are present on the same *Not*I fragment. We elected to utilize *Hin*dIII for the remainder of our experiments, since this enzyme allowed us to monitor potential changes within two distinct regions of the *rdn1* locus.

Changes in rDNA copy number in untransformed strains: DNA- mediated transformation is a relatively infrequent process in *C. cinereus*, occurring on average in only 1 out of 10,000 cells. It was possible that the processes of breakage and rejoining, necessary to incorporate exogenous DNA, were activated in a small subset of cells, rendering these prone to other genomic rearrangements such as the CLPs we saw here. Alternatively, the changes in rDNA copy number

could be occurring regularly as part of the asexual cycle in *C. cinereus*. To attempt to distinguish between these alternatives, we isolated individual oidia, which are asexual spores produced by monokaryotic cultures, and allowed these to germinate into individual colonies. Oidia were also the starting material for transformation experiments. We prepared chromosomal sized DNA from eight oidial isolates, and examined the rDNA after *Hin*dIII digestion, Southern transfer, and probing with pCc1. Seven isolates looked like the original strain (Table 2; Figure 3, lane b). However, one colony had undergone a pronounced change in the number of tandemly repeated rRNA genes, in that it had lost 400 kb of the large *Hin*dIII fragment (40% reduction, Figure 2, lane e; Figure 3, lane c). No change in the 100-kb *Hin*dIII fragment was observed. Also, the linkage between the two *Hin*dIII fragments was not altered, since a single fragment was obtained after *Not*I digestion (Figure 3, lane e). This strain was designated 218-7.

It was possible that there were some unsuspected heterogeneities in cells of strain 218 that could be transmitted mitotically and render certain clonal descendants more prone than average to genomic alterations. We asked if oidia derived from strain 218-7 (the isolate containing a CLP) were as likely to exhibit alterations in rDNA copy number as oidia from strain 218-4 (an oidial isolate from strain 218 that looked identical to strain 218). The results are shown in Table 2. One in eight oidial isolates from strain 218-7 exhibited an alteration (218-7H), and one in eight oidial isolates from strain 218-4 also exhibited a change (218-4H; see Figure 2, lane f). In 218-4H, the original band was still present but an additional block of rDNA sequences was also present. This pattern is similar to that observed in strain Ac18$^+$ (Figure 2, lane d) and we assume it also arises from heterogenity within the colony. Thus neither an atypical number of rDNA copies nor alterations in the recent lineage appear to predispose a cell to further CLPs. We conclude that the transformation process is not necessary for the genomic instabilities observed here. Instead, about 15% of asexual spores exhibit heritable changes in the number of rRNA genes carried in the chromosome.

Meiotic transmission of rDNA copy number: All of the changes in rDNA copy number discussed above occurred in haploid, monokaryotic strains. It was of interest to examine rDNA copy number stability in other stages of the life cycle, since previous workers had concluded that most of the changes occurred following fertilization but prior to meiosis (BUTLER and METZENBERG 1989, 1990). Accordingly, we crossed strain 218-7 (*rdn1-700*) with a compatible strain bc3 (*rdn1-1000*) that had been backcrossed three times to strain 218. As illustrated in Figure 4, the rDNA segregated in strictly Mendelian fashion in

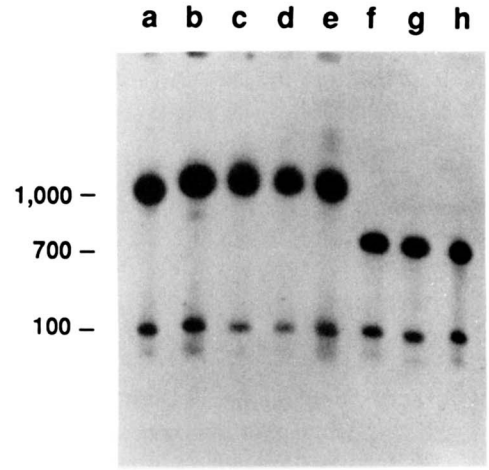

FIGURE 4.—Autoradiogram illustrating Mendelian inheritance of rDNA size variants. DNAs were digested with *Hin*dIII, resolved using the CHEF method, and probed with pCc1. Sizes are given on the left in kb. Material trapped in the wells and partially degraded material (below the 100-kb band) are visible. Note that lanes a and h are the parental strains, while lanes d–g contain DNAs from one tetrad. a, bc3; b, 7/91-6C; c, 7/91-6D; d, 7/91-7A; e, 7/91-7B; f, 7/91-7C; g, 7/91-7D; h, 218-7

the six tetrads that were examined. Figure 4 shows the starting strains (lanes a and h), DNAs from each member of a complete tetrad (lanes d–g), and two of the four samples from an additional tetrad (lanes b and c). Each meiotic segregant we examined inherited the 100-kb *Hin*dIII fragment, and one or the other parental form of the large *Hin*dIII fragment. Thus no changes were observed in rDNA copy number in isolates that had undergone mating, fruit body formation, basidiospore formation or basidiospore germination. This was a surprising result, since BUTLER and METZENBERG (1989) failed to observe even a single ascus in which the rDNA had been transmitted in strictly Mendelian fashion among the 14 asci that they characterized. Although the sample size is small, the difference is highly significant ($\chi^2 = 19.9$, $P < 0.0001$).

The six tetrads in the first cross derived from a single fruit body. To ask if rDNA stability was typical in fruit bodies with this amount of rDNA, we carried out a second cross between two meiotic segregants of the first cross, 7/91-7A (*rdn1-1000*) × 7/91-7D (*rdn1-700*). These had inherited the distinctive parental DNA sizes. We isolated 10 additional tetrads from two fruit bodies of this second cross. The rDNA continued to be stably transmitted through this generation. As summarized in Table 3, each segregant in the 10 tetrads received the 100-kb *Hin*dIII fragment plus a parental sized *Hin*dIII fragment.

To ask if the spore DNAs used in these two crosses came from products of typical meiotic divisions, we examined the segregation of five additional markers (*trp1*, *slg1*, *bad1*, *A* and *B*). These markers are unlinked, except for *slg1-1*, which is linked to *A3*. Each

TABLE 3

Meiotic transmission of rDNA and analysis of tetrads

Parental *Hin*dIII fragments	No. tetrads examined	No. with nonparental rDNA	No. with exchanges between indicated gene and centromere[a]			
			A (15 cM)	*B* (10 cM)	*trp1* (5 cM)	*bad1* (10 cM)
1000 + 700	6	0	0	0	0	1 (8 cM)[c]
1000 + 700	10	0	1 (5 cM)	1 (5 cM)	1 (5 cM)	1 (5 cM)
700 ± 700	16	16[c]	5 (16 cM)	1 (3 cM)	2 (6 cM)	3 (9 cM)

[a] Expected map distance (from published values for *A*, *B* and *trp1*, and analysis of 27 tetrads for *bad1*) for each interval is indicated in parentheses.

[b] Numbers are determined from the best arrangement (to minimize exchanges) of second division segregation (SDS) frequencies, and cM = 1/2 %SDS.

[c] Of these, 1 segregated 4:0, 2 segregated 3:1, 7 segregated 2:2, 3 segregated 2:1:1, and 3 segregated 1:1:1:1.

marker segregated 2:2, as expected for normal meiotic products. We also observed exchanges in the four gene-centromere intervals that we monitored (Table 3).

It was of interest to determine if the rDNA stability would be observed in fruit bodies with more severe rDNA deficiencies. Accordingly, we examined 16 tetrads from two fruit bodies of cross 7/91-3B (*rdn1-700*) × 7/91-7C (*rdn1-700*), which contained less rDNA that the previous crosses. These two strains were also haploid segregants from the 218-7 × bc3 cross, but each inherited the same parental *Hin*d III fragments. When tetrad segregant DNAs were digested with *Hin*dIII, each contained the 100-kb *Hin*dIII fragment. However, numerous changes in the size of the larger *Hin*dIII fragment were observed (Table 3). The larger *Hin*dIII fragment ranged in size from 500 to 1100 kb. In general, the bands were broader than those obtained from the previous isolates, and multiple bands were sometimes observed. These results suggest that some changes may have taken place during vegetative growth of the colony. We confirmed that all these spore DNAs also came from products of typical meiotic divisions by the same tetrad analysis described above. The difference in the number of tetrads with nonparental rDNAs in crosses 1 and 3 is highly significant ($\chi^2 = 22$, $P \ll 0.0001$), and the difference between cross 2 and 3 is also highly significant ($\chi^2 = 26$, $P \ll 0.0001$). We conclude that the 100-kb *Hin*dIII fragment is always transmitted without alteration. Furthermore, in some crosses but not all, the larger *Hin*dIII fragment is also transmitted without alteration.

DISCUSSION

We have shown that the number of tandemly repeated copies of the rDNA is not fixed in *C. cinereus*, but instead frequently undergoes expansion or contraction. These changes are most easily observed when clonal descendants of a single nucleus are examined, such as when DNA is isolated from oidia produced by a single transformed cell. The changes can be extensive, involving addition or removal of up to 500 kb of DNA. This difference represents 17% of the chromosome that contains the rDNA, and the resulting CLP was easy to detect. Our analysis indicated that 18% of transformants we examined underwent such an alteration. Previous studies have drawn attention to genome modifications such as DNA methylation (BULL and WOOTTON 1984; MOOIBROEK *et al.* 1990) and translocations (ASCH *et al.* 1992) that occur following DNA-mediated transformation of filamentous fungi. It is clear that CLPs involving the rDNA-bearing chromosome should be added to this list. In the particular transformants that we examined, the rDNA variations had no obvious phenotypic effect. However, it seems likely that in some genetic backgrounds, rDNA copy number could affect the phenotype. Thus, it would appear to be essential to ask if the rDNA copy number is altered before concluding that a novel phenotype in a transformed strain is due to the planned sequence gain or disruption.

It is also clear that the number of rRNA genes can change during vegetative growth without any involvement of DNA-mediated transformation. The results are consistent with those obtained by RUSSELL and RODLAND (1986). They studied the number of rRNA genes in certain strains of *N. crassa* in which the initial size of the nucleolus organizer was reduced (RNO strains). They observed that the rRNA gene copy number could increase during vegetative growth, restoring the wild-type level. As pointed out by BUTLER and METZENBERG (1989), vegetative changes may also have occurred prior to the post-fertilization changes that they studied. It also appears that vegetative changes are frequent in the aneuploid strains studied by BUTLER (1992).

In our experiments, expansions and contractions of the rDNA were monitored by isolating chromosomal-sized DNA molecules from the oidia (asexual spores) produced by a particular strain. Such preparations usually gave one large *Hin*dIII fragment of characteristic size for the particular strain. However, when single oidia from the strain were allowed to germinate

and DNA was prepared from their clonal descendants, the discrete bands we obtained often differed in size from that seen in the parental strain. In most isolates, the patterns we saw would be consistent with the occurrence of the expansions or contractions at the time of oidial germination. If sequences were lost by intrachromatid exchange prior to DNA replication and no further changes occurred, the resulting colony would contain a large *Hin*dIII fragment of reduced size. Such a pattern was seen in Ac1$^-$, Ac49$^+$, DGX3 and 218-7. If the intrachromatid exchange occurred following DNA replication, the resulting colony could show one parental and one variant *Hin*dIII fragment, and the relative proportions would reflect the number of oidia harvested that harbored each chromosome. Such patterns were seen in Ac18$^+$ and 218-4H. If sister chromatid exchange occurred following DNA replication, the resulting colony would either show two variant *Hin*dIII fragments or one variant fragment (if one product did not contribute to oidia formation). The latter pattern was seen in 218-7H. In some of the tetrad segregants from the 7/91-3B X 7/91-7C cross, multiple bands were observed. Since the above models predict a maximum of two bands per segregant, additional changes must have occurred during vegetative growth of the colony to give rise to the multiple bands.

We have also shown that the number of rDNA repeats can change during premeiotic and meiotic divisions. Our analysis of segregation patterns of the large *Hin*dIII fragment in 16 tetrads from the 7/91-3B × 7/91-7C cross are consistent with results obtained by BUTLER and METZENBERG (1989). In both studies, a portion of the tetrads (11/14 in their study, 7/16 in our study) exhibited 2:2 segregation patterns, consistent with premeiotic occurrence of the expansions or contractions. The remaining segregation patterns in both studies are consistent with meiotic or postmeiotic changes.

Two of our findings were not expected. First, we never saw changes in the 100-kb *Hin*dIII fragment (about 10 rDNA repeats) in the 174 isolates we examined. In contrast, only 2 of the 14 asci studied by BUTLER and METZENBERG (1989) contained the parental-sized interstitial NOR (about 9 rDNA repeats). Under our gel conditions, small *Hin*dIII fragments differing by one repeat unit would have been shifted about 1 mm in mobility, and consequently some changes could have escaped our notice. However, any intrachromatid or unequal sister chromatid exchanges that involved both a repeat within the 100-kb fragment and also a repeat in the large *Hin*dIII fragment should have been detected easily, since the resulting strain would have lost the internal *Hin*dIII site. It appears that breakage and rejoining events that mediate changes in copy number are unlikely to occur within the terminal repeats of the array in *C. cinereus* that lie within the 100-kb *Hin*dIII fragment.

It was also surprising to observe strict Mendelian segregation of rDNA size variants in two crosses. Initially, the size variants we used to monitor transmission resulted in an overall rDNA deficiency of roughly 25%. It is clear that the deficiency *per se* is not responsible for the stable transmission of size variants because in the third cross, the overall rDNA deficiency was 33% but extensive changes in rDNA amount were observed. We observed that different fruit bodies of identical genotype behaved uniformly with respect to rDNA stability, even though they were grown and collected in independent experiments. Since our sample size is small, further experiments will be necessary to establish that the rDNA stability/instability phenotype has a genetic basis and is not the consequence of some unsuspected heterogeneities in culture conditions. We can eliminate one simple genetic model already. The stability cannot be the result of accidental homozygosis of a recessive mutation in the 218-7 × bc3 cross, because two segregants from this cross (7/91-3B × 7/91-7C) exhibited numerous rDNA size changes in the next generation.

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LITERATURE CITED

ASCH, D. K., G. FREDERICK, J. A. KINSEY and D. D. PERKINS, 1992 Analysis of junction sequences resulting from integration at nonhomologous loci in *Neurospora crassa*. Genetics **130:** 737–748.

BINNINGER, 1987 A transformation system for the basidiomycete fungus *Coprinus cinereus*. Ph.D. Thesis, University of North Carolina at Chapel Hill.

BINNINGER, D. M., C. SKRZYNIA, P. J. PUKKILA and L. A. CASSELTON, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835–840.

BINNINGER, D. M., L. LE CHEVANTON, C. SKRZYNIA, C. D. SHUBKIN and P. J. PUKKILA, 1991 Targeted transformation in *Coprinus cinereus*. Mol. Gen. Genet. **227:** 245–251.

BULL, J. H., and J. C. WOOTTON, 1984 Heavily methylated amplified DNA in transformants of *Neurospora crassa*. Nature **310:** 701–704.

BUTLER, D. K., 1992 Ribosomal DNA is a site of chromosome breakage in aneuploid strains of Neurospora. Genetics **131:** 581–592.

BUTLER, D. K., and R. L. METZENBERG, 1989 Premeiotic change of nucleolus organizer size in Neurospora. Genetics **122:** 783–791.

BUTLER, D. K., and R. L. METZENBERG, 1990 Expansion and contraction of the nucleolus organizer region of Neurospora: Changes originate in both proximal and distal segments. Genetics **126:** 325–333.

CASSIDY, J. R., D. MOORE, B. C. LU and P. J. PUKKILA, 1984 Unusual organization and lack of recombination in the

ribosomal RNA genes of *Coprinus cinereus*. Curr. Genet. **8:** 607–613.

CHRISTMAN, M. F., F. S. DIETRICH and G. R. FINK, 1988 Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II. Cell **55:** 413–425.

CHU, G., D. VOLLRATH and R. W. DAVIS, 1986 Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:** 1582–1585.

DVORAK, J., and R. APPELS, 1986 Investigation of homologous crossing over and sister chromatid exchange in the wheat *Nor-B2* locus coding for rRNA and *Gli-B2* locus coding for gliadins. Genetics **113:** 1037–1056.

FISHER, R. A., 1948 *Statistical Methods for Research Workers*. Oliver & Boyd, Edinburgh.

HILLIS, D. M., C. MORITZ, C. A. PORTER and R. J. BAKER, 1991 Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science **251:** 308–310.

KIM, R. A., and J. C. WANG, 1989 A subthreshold level of DNA topoisomerases leads to the excision of yeast rDNA as extrachromosomal rings. Cell **57:** 975–985.

KING, H. B., and L. A. CASSELTON, 1977 Genetics and function of isocitrate lyase in Coprinus. Mol. Gen. Genet. **157:** 319–325.

MELLON, F. M., P. F. R. LITTLE and L. A. CASSELTON, 1987 Gene cloning and transformation in the basidiomycete fungus *Coprinus cinereus*: Isolation and expression of the isocitrate lyase gene (*acu-7*). Mol. Gen. Genet. **210:** 352–357.

MOOIBROEK, H., A. G. J. KUIPERS, J. H. SIETSMA, P. J. PUNT and J. G. H. WESSELS, 1990 Introduction of hygromycin B resistance into *Schizophyllum commune*: preferential methylation of donor DNA. Mol. Gen. Genet. **222:** 41–48.

PETES, T. D., 1980 Unequal meiotic recombination within tandem arrays of yeast ribosomal DNA genes. Cell **19:** 765–774.

PETES, T. D., and D. BOTSTEIN, 1977 Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast. Proc. Natl. Acad. Sci. USA **74:** 5091–5095.

PUKKILA, P. J., 1992 Methods of genetic manipulation in *Coprinus cinereus*, in *culture Collection and Breeding of Edible Mushrooms*, edited by S. T. CHANG, P. G. MILES and J. A. BUSWELL. Gordon & Breach, Philadelphia (in press).

PUKKILA, P. J., and L. A. CASSELTON, 1991 Molecular genetics of the agaric *Coprinus cinereus*, pp. 126–150 in *More Gene Manipulations in Fungi*, edited by J. W. BENNETT and L. L. LASURE. Academic Press, New York.

RAO, P. S., and D. J. NIEDERPRUEM, 1969 Carbohydrate metabolism during morphogenesis of *Coprinus lagopus* (*sensu* Buller). J. Bacteriol. **100:** 1222–1228.

RUSSELL, P. J., R. C. PETERSON and S. WAGNER, 1988 Ribosomal DNA inheritance and recombination in *Neurospora crassa*. Mol. Gen. Genet. **211:** 541–544.

RUSSELL, P. J., and K. D. RODLAND, 1986 Magnification of rRNA gene number in a *Neurospora crassa* strain with a partial deletion of the nucleolus organizer. Chromosoma **93:** 337–340.

SHAHRIARI, H., and L. A. CASSELTON, 1974 Suppression of methionine mutants in *Coprinus* I. Complementation and allele specificity as criteria of suppressor gene action. Mol. Gen. Genet. **134:** 85–92.

SKRZYNIA, C., D. M. BINNINGER, J. A. ALSPAUGH II and P. J. PUKKILA, 1989 Molecular characterization of *trp1*, a gene coding for tryptophan synthetase in the basidiomycete *Coprinus cinereus*. Gene **81:** 73–82.

SMITH, G. P., 1973 Unequal crossover and the evolution of multigene families. Cold Spring Harbor Symp. Quant. Biol. **38:** 507–513.

SZOSTAK, J. W., and R. WU, 1979 Insertion of a genetic marker into the ribosomal DNA of yeast. Plasmid **2:** 536–554.

VOLLRATH, D., and R. W. DAVIS, 1987 Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. Nucleic Acids Res. **15:** 7865–7876.

WERNARS, K., T. GOOSEN, B. M. J. WENNEKES, K. SWART, C. A. M. J. J. VAN DEN HONDEL and H. W. J. VAN DEN BROEK, 1987 Cotransformation of *Aspergillus nidulans*: a tool for replacing fungal genes. Mol. Gen. Genet. **209:** 71–77.

WU, M. M. J., J. R. CASSIDY and P. J. PUKKILA, 1983 Polymorphisms in DNA of *Coprinus cinereus*. Curr. Genet. **7:** 385–392.

ZOLAN, M. E., and P. J. PUKKILA, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6:** 195–200.

ZOLAN, M. E., J. R. CRITTENDEN, N. K. HEYLER and L. C. SEITZ, 1992 Efficient isolation and mapping of *rad* genes of the fungus *Coprinus cinereus* using chromosome-specific libraries. Nucleic Acids Res. **20:** 3993–3999.

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