Molecular Analysis of the *Coprinus cinereus* Mating Type *A* Factor Demonstrates an Unexpectedly Complex Structure

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ABSTRACT

We report here the molecular cloning of the *A43* mating type factor from *Coprinus cinereus*, a basidiomycetous fungus. Our molecular analyses revealed an unexpected source of variation in the *A* factor. Though genetic studies have demonstrated that *A* has two subunits, α and β, we located three nonoverlapping fragments in the *A43* region that have *A* factor function following DNA-mediated transformation. The three fragments demonstrate no similarity to one another as judged by restriction enzyme maps and by hybridization on Southern blots. We conclude that the *A43* factor is composed of at least three subunits. When strains carrying different *A* factors are examined by hybridization to the cloned subunits, extensive polymorphism is seen. Both intensity of hybridization and restriction fragment lengths vary between strains. Some strains fail to show any hybridization to a probe. In contrast, other strains from widely separated geographic locations apparently share very similar subunits. From comparative restriction enzyme mapping of *A43* and a mutated *A43* factor, we inferred that a 12-kb deletion in the *A* factor was responsible for the constitutive, dominant phenotype of the mutated *A* factor. The results of transformation experiments support an activator model for the activity of the *A* factor in regulating the *A* pathway.

THE manner in which regulatory networks evolve and their relationship to phenotypic change is of primary interest to evolutionary biologists today (KAUFFMAN 1985; BONNER 1988; CAVENDER 1989). However, genes involved in such networks may have pleiomorphic effects and can be difficult to relate directly to the phenotype of interest (CLINE 1989). The mating system of *Coprinus cinereus* is amenable to evolutionary studies because mating is a relatively simple and well-described developmental pathway (CASSELTON 1978) and because closely related species in the genus demonstrate variation in mating systems (ORTON and WATLING 1979; KEMP 1980). Mating type genes of basidiomycetes can be used to analyze the importance of functional constraints on molecular evolution (BOWIE *et al.* 1990) because they have a feature that is unusual for regulatory genes–they are highly polymorphic and many alleles can be recovered from natural populations (RAPER 1966).

Classical genetic investigations provided a framework for understanding mechanisms for generating that variation and for the function of *C. cinereus* mating type genes. Two unlinked factors, *A* and *B*, control mating and for a completely compatible mating, genetically different *A* and *B* factors must be present in the mating haploids (SWIEZYNSKI and DAY 1960a,b). After mating, a dikaryon with two nuclei per cell is formed and it is this phase that can be induced to form fruit bodies (mushrooms). Remarkably, these important regulatory genes are also highly polymorphic. Approximately 40 *A* and *B* factors have been identified and over 100 may exist worldwide (WHITEHOUSE 1949). In *C. cinereus*, some of this polymorphism can be explained by the observations of DAY (1960, 1963b) that the *A* factor is made up of at least two closely linked subunits, termed α and β. The functions of α and β appear redundant because genetic analysis has shown that an allelic difference at only a single subunit is sufficient for compatibility at *A*. Because the subunits themselves have many allelic forms, recombination between α and β can generate new mating specificities. A unique problem is presented: how have so many alleles evolved such that any two dissimilar alleles recognize non-self and regulate the observed developmental sequence?

The regulatory mechanism of mating type could not be determined by conventional genetic analysis of *Schizophyllum commune* and *C. cinereus* because results failed to distinguish unambiguously between activator and repressor models of gene activity (RAPER 1966; WESSELS 1969; KUHN and PARAG 1972; ULLRICH 1978; CASSELTON 1978). In a repressor model, the *A* factor acts to repress the dikaryon-specific functions in the haploid cell. If two different alleles of at least one subunit are present in the dikaryon, repressor activity would be inhibited and *A* regulated pathways would be expressed. This model predicts that mutations at *A* factor genes should allow constitutive expression of *A* regulated pathways in the haploid and that such mutations should be recessive. Only one

class of mutant *A* factors (*Amut*) were ever obtained when mutations allowing expression of *A* regulated pathways were selected. *Amut* haploids express *A* regulated pathways; however, these mutations are dominant (DAY 1963a; RAPER, BOYD and RAPER 1965; SWAMY, UNO and ISHIKAWA 1984). With an activator model, two different alleles of at least one *A* subunit would be necessary to activate expression of the *A* regulated pathway. The activator model predicts that mutations at the *A* subunit might produce new mating specificities or sterile alleles (KUHN and PARAG 1972). No new specificities were recovered.

We cloned the *C. cinereus A43* mating type factor with the goal of studying its structure, mechanism of regulation, and mode of variation. The structural analysis mapped restriction sites in the 35-kbp cosmid clone and used subcloning and transformation to locate three regions of gene activity. With the goal of understanding the function of the *A* mating-type, we mapped the *A* region in a *A43-mut* strain (SWAMY, UNO and ISHIKAWA 1984) to determine the molecular event associated with the mutant *A* phenotype. To investigate *A* mating type variation in natural populations, we used the *A43* mating type clones to probe other strains carrying different *A* mating type factors. We compared these hybridization results to those reported by MUTASA *et al.* (1990) who cloned a different *A* mating type factor (*A42*).

MATERIALS AND METHODS

Strains: Table 1 lists haploid strains used in this study. Strains other than those collected by G. MAY were kindly provided by the person listed as the source. Mating tests demonstrated that all the strains listed here have different *A* mating type factors from one another. Table 1A lists laboratory strains used in this study. Ok7 (Okayama7, *A43 B43*) was derived from a Japanese dikaryon and genomic DNA of Ok7 was used to construct the cosmid library. Though the *A* mating type of Ok7 was originally designated *A2* (SWAMY, UNO and ISHIKAWA 1984), the *A2* mating type had previously been assigned to another strain (PR2301, ATCC 26054). The strain *A43-mut B43* was derived by nitrosoguanidine mutation of strain 5026 and has the constitutive phenotype in the haploid that is characteristic of mutant *A* mating types (DAY 1963a; SWAMY, UNO and ISHIKAWA 1984). *A43-mut* (with *B6* or *B42*) mates with its progenitor strain, 5026 (*A43 B43*). Strain 5132 was isolated from the same fruit body as 5026 but carries the alternate alleles, *A7* and *B7*. Strain 218 was used as the *A3* Trp$^-$ recipient for transformation assays. It carries the *trp1-1,1-6* double mutation which does not revert at a detectable rate (BINNINGER *et al.* 1987). Strain T2-1 was the *A43* Trp$^-$ recipient strain used for control transformations.

Table 1B lists natural isolates that were derived from dikaryons (fruit bodies) collected from natural populations. North Carolina strains, NC3, NC5 and NC6 represent dikaryons collected as fruit bodies from one location near Chapel Hill, North Carolina. The parental haploids of some of these NC dikaryons were isolated by the veil cell method of COWAN (1964) and these isolates have a "v" in the strain designation. Other NC haploids, indicated with a "b," were obtained from single basidiospore cultures.

Library construction: The cosmid vector, LLC5200, includes the *C. cinereus* gene *TRP1* (SKRZYNIA *et al.* 1989) and has been described previously (PUKKILA and CASSELTON 1991). Partial *Mbo*I fragments of genomic DNA isolated from strain Ok7 (*A43, B43*) were size fractionated and fragments 38–50 kbp were ligated into the polylinker *Bam*HI site. After ligation, cosmid clones were packaged with Gigapack II Gold (Stratagene) and transfected into competent *Escherichia coli* DH5 cells (Bethesda Research Laboratories). Five thousand independent transformant colonies were picked and the library was ordered into 52 microtiter plates with 96 wells each. With a genome size of approximately 37,500 kbp, a library of 5,000 clones of 40 kbp each was designed to represent the genome 5× over and give a 98% probability that any gene is present at least once in the library (CLARKE and CARBON 1976).

Transformation and sib-selection: Transformations of *C. cinereus* haploids were carried out according to published procedures (BINNINGER *et al.* 1987). To find *A*, we transformed the *A3* Trp$^-$ recipient, 218 (Table 1) with clones from the Ok7 *A43* library. We screened for the presence of false clamps, a specialized morphology produced in strains that are expressing the *A* regulated pathway (Figure 1, b and c) (SWIEZYNSKI and DAY 1960a). Untransformed haploid strains or transformants that contain only the resident *A* factor had only cross-walls between adjacent cells (Figure 1a). When *A* regulated pathways were expressed, false (unfused) clamps were observed at the position of the cross-walls (Figure 1, b and c). In the dikaryon, fused clamp connections were found (Figure 1d).

We initially transformed with pools of DNA composed of 96 clones from microtiter plates and once *A* was located in plate 203, used the "elimination" scheme of METZENBERG and KANG (1987) to locate the *A* clone. Because plate 203 had 2 clones of *A*, an incorrect well (A2) was indicated by the results of the 7 prescribed transformations. We then devised a binary search scheme and located an *A* clone in well E12. Using a fragment internal to this clone as a hybridization probe onto colony lifts, we confirmed that the well C2 also had an *A43* factor. We used the clone from well E12, designated c203-E12, for the work reported here.

To locate regions of c203-E12 with *A* mating type activity, we subcloned *Pst*I and *Hin*dIII fragments into pUC9 (Bethesda Research Laboratories), pBluescript KS$^-$ (Stratagene) or pCc1003 (SKRZYNIA *et al.* 1989) plasmids. The plasmid pCc1003 is a construct of pBluescript KS$^-$ with a 5-kbp *Bgl*II fragment carrying the *C. cinereus TRP1* gene ligated into the *Bam*HI site (SKRZYNIA *et al.* 1989). These constructs were transformed into the *A3* Trp$^-$ recipient as above.

Mapping and Southern blot analyses: Genomic DNAs were prepared by a mini-prep method (ZOLAN and PUKKILA 1986) and approximately 1 μg of genomic DNA was loaded per lane on agarose gels. Analyses of cloned and genomic DNAs by SOUTHERN (1975) blots were made as previously described (MAY and TAYLOR 1988). The blots were hybridized to ^{32}P-radiolabeled DNA probes labeled by random priming (FEINBERG and VOGELSTEIN 1983; U.S. Biochemicals). For mapping c203-E12 and linkage analysis, we used the following high stringency conditions: 65°, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 0.01 M EDTA) in a standard hybridization buffer (MANIATIS, FRITSCH and SAMBROOK 1982). For genomic analyses of strains with different *A* factors, we used reduced stringency conditions of 55°, 8 × SSPE. Posthybridization wash temperatures matched those of the hybridization and four washes were made, two for 30 min each with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) with O.1%

TABLE 1

Haploid strains

Strain	Mating types[a]	Geographic origin	Source
A. Laboratory strains			
Ok7	*A43, B43*	Japan	D. MOORE
5026	*A43, B43*	Japan	T. ISHIKAWA
5132	*A7, B7*	Japan	T. ISHIKAWA
A43-mut	*A43-mut*		T. ISHIKAWA
218	*A3, B1 (trp1-1,1-6)*	Britain	This laboratory
A3 tester	*A3, B43*		This laboratory
T2-1	*A43, B43 (ade8, trp1-1,1-6)*		This laboratory
A43 tester	*A43, B1*		This laboratory
B. Isolates from natural populations			
Java(a)		Java	Basidiospore of ATCC 42722
144x	*A53, B53*	Britain	Veil cell, dikaryon collected by J. NORTH
Scot-L-v1		Scotland	Veil cell, dikaryon collected by R. KEMP
Scot-L-v2		Scotland	Veil cell, dikaryon collected by R. KEMP
NC3-v1		North Carolina	Veil cell of dikaryon
NC3-b12		North Carolina	Basidiospore of dikaryon
NC5-b3		North Carolina	Basidiospore of dikaryon
NC5-b6		North Carolina	Basidiospore of dikaryon
NC6-v1		North Carolina	Veil cell of dikaryon
NC6-v7		North Carolina	Veil cell of dikaryon

[a] Auxotrophic markers, when present, are listed under the mating types.

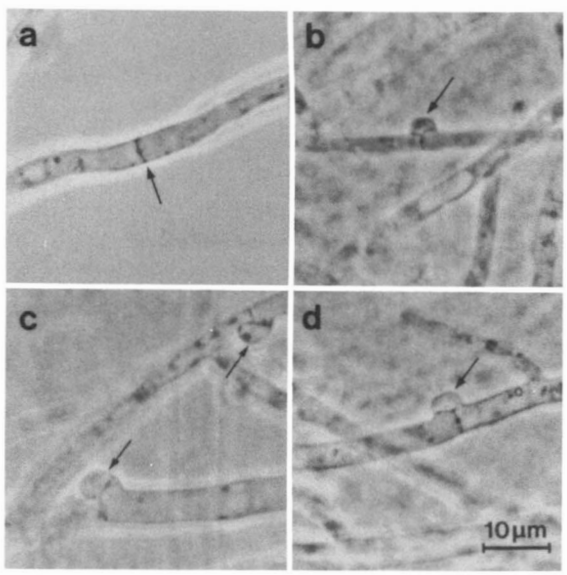

FIGURE 1.—Light micrographs illustrating the morphology of different cell types in the development of a dikaryon. (a) Haploid cells with cross-wall separating adjacent hyphal segments (arrow). (b) *A3, B1* + *A43, B1* heterokaryon with false (unfused) clamp cell (arrow). (c) *A3/43* transformants with false clamp cells (arrows). (d) *A43, B43* + *A3, B1* dikaryon with fused clamp (arrow). Note that the unfused clamp (b) exhibits three cross-walls in a "Y" shape while the fused clamp (d) has only two cross-walls.

SDS followed by two washes for 15 min each with 0.2 × SSC, 0.1% SDS.

Orthogonal-field-alternation gel electrophoresis (OFAGE) analysis for linkage: Plugs with chromosomal size *C. cinereus* DNAs were prepared as described by BINNINGER *et al.* (1991). Briefly, protoplasts of haploid oidia (asexual spores) were produced using Novozyme 234 (Novo Industri A/S) in stabilization buffer (0.5 M maleate, 0.5 M mannitol, pH 5.5) and the protoplasts were embedded in Bio-Rad LGT agarose (prepared in stabilization buffer and held at 37°). After the agarose solidified, the plug was deproteinated by incubating overnight at 50° in NDS buffer (0.01 M Tris-HCl, pH 9.5, 0.5 M EDTA, 2% Sarkosyl, 2 mg/ml proteinase K).

Restriction enzyme digestions of DNA in agarose blocks were made as follows. Agarose plugs, cut to the appropriate size for the well, were rinsed three times in 0.01 M Tris-HCl, 0.5 M EDTA, pH 9.5, and then rinsed three times in 0.01 M Tris-HCl, 0.001 M EDTA (TE). A 0.1 M solution of phenylmethylsulfonyl fluoride (PMSF, Sigma) in isopropyl alcohol was quickly mixed into TE to give a final concentration of 0.02 M PMSF. Plugs were incubated in the 0.02 M PMSF at room temperature for 10 min and then rinsed three times in TE. PMSF is toxic and these steps were carried out in an exhaust hood. The plugs were preincubated in restriction enzyme buffer for 30 min at 37°. Fresh enzyme buffer and 10 U. of restriction enzyme were then added and gel slices were incubated at 37° for 2 hr.

Plugs prepared as above were run in gels utilizing OFAGE (CARLE and OLSON 1984). Chromosome gels were run at 60 V, 20-min switch times, 5° for 6 days. Plugs previously digested with *Not*I or *Sfi*I restriction enzymes were run overnight at 170 V, 1-min switch times, 5° for 18 hr. A commercial ladder (FMC) was used as the size standard. DNA fractionated on OFAGE gels was blotted and probed as described above. Because the *PAB1* gene is located 0.5 map unit distal to the *A* factor (NORTH 1987), we used the cloned *C. cinereus PAB1* gene, *cPAB1-1* (MUTASA *et al.* 1990) to establish physical linkage.

Matings: Matings were made on YMG/T media by placing blocks of inocula 1–5 mm apart. Matings were repeated at least once. Transformants of the *A3* recipient strain were

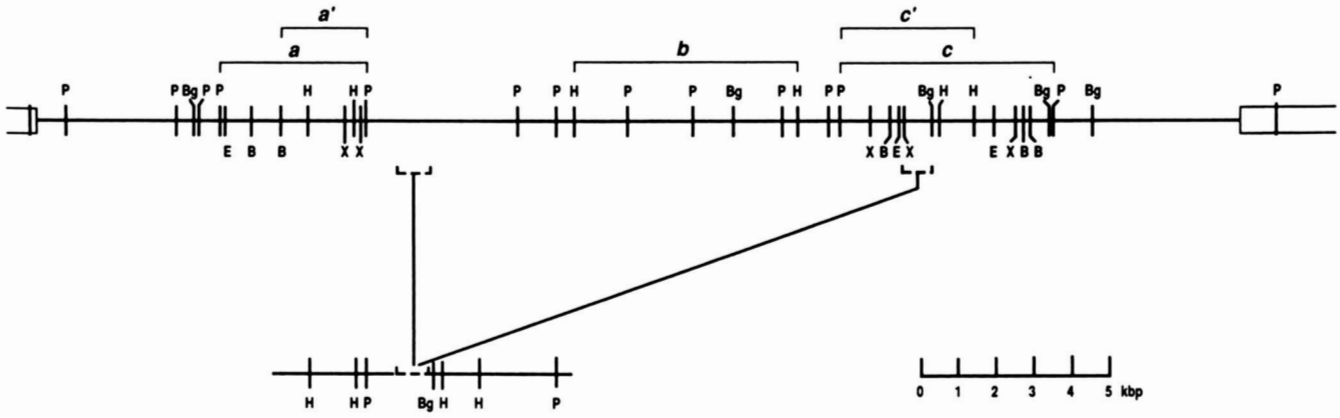

FIGURE 2.—Diagram of restriction endonuclease sites mapped onto the clone c203-E12. *Bgl*II (Bg), *Hin*dIII (H) and *Pst*I (P) sites have been located for the entire *C. cinereus* genomic insert which is *ca.* 35 kbp in length. Additional *Bam*HI (B), *Eco*R1 (E) and *Xho*I (X) sites have been mapped in the region of *A(a)* and *A(c)* for c203-E12. For subunits *A(a)* and *A(c)*, the smallest subclones with *A43* activity are designated by *A(a')* and *A(c')*. The deletion mapped in *A43-mut* is shown below. The dotted lines represent the region to which the right border could be mapped (see RESULTS). The position of the left border was inferred by the length of a 1.75-kbp *Hin*dIII/*Pst*I fragment in *A43-mut* that hybridized to the 2.5-kbp *Hin*dIII/*Pst*I fragment making up the left side of *A(c)* in *A43* and that also hybridized to the 5.7-kbp *Hin*dIII fragment to the left of *A(b)*.

crossed to the *A3* tester as well as Ok7. Transformants resulting from control experiments with the *A43* recipient strain T2-1 were crossed to the *A43* tester and 218. Three to five days after inoculation, a compatible mating could be judged by the appearance of rapidly growing dikaryons with fused clamps (Figure 1d) on the outer edge of the mating colony.

RESULTS

We isolated a 35-kbp cosmid clone, c203-E12, which has *A43* mating type factor activity in transformation assays (Figure 2). This clone was isolated by a sib-selection procedure from a cosmid library of the *C. cinereus* haploid strain carrying the *A43* mating type factor. It has the following characteristics:

1. c203-E12 is located on the same chromosome as *A* and is linked to *PAB1*. Genetic analysis located the *A* mating factor on one of the largest chromosomes and finer mapping located *A* at 0.5 map unit from the *PAB1* locus (NORTH 1987; DAY 1960). We established this physical linkage by showing that *cPAB1-1* and c203-E12 both hybridized to the largest chromosome of *C. cinereus* on chromosomal DNA blots (data not shown) and to the same *ca.* 250-kbp *Not*I or *Sfi*I fragments resolved by OFAGE (Figure 3).

2. Comparison of genomic *Pst*I fragments and those of clone c203-E12 demonstrate that the clone is not rearranged. Fragments that do not correspond in size between the two digests are either flanking fragments or the *TRP1* locus in the Ok7 genomic DNA (Figure 4).

3. Transformation analyses demonstrated that when c203-E12 DNA was introduced into a recipient strain carrying a different mating factor, *A3*, the two phenotypic changes expected of a cell with two different *A* mating types were observed in 65% of the Trp^+

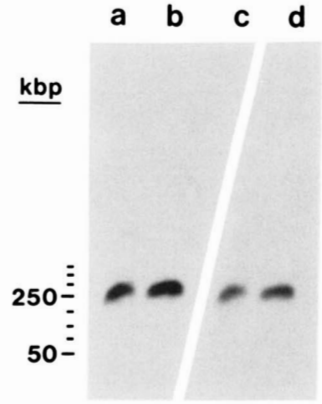

FIGURE 3.—Autoradiogram illustrating physical linkage of the *A43* region to *PAB1*. Lanes a and b are a SOUTHERN (1975) transfer from an OFAGE gel with fractionated Ok7 genomic DNA digested with *Not*I and *Sfi*I restriction endonucleases, respectively. The blot was probed with radiolabeled pUC9 clone, pE12P5-3, which represents *A(a)*. Lanes c and d represent hybridizations of the same blot with a *cPAB1-1* radiolabeled clone after stripping the previous pE12P5-3 probe.

cells. First, the majority of *A3* cells transformed with the *A43* clone had false clamps (FC^+, Figure 1c). This is the expected change of morphology (SWIEZYNSKI and DAY 1960a). Second, *A3/43* transformant cells with false clamps were compatible with both the *A3* and *A43* testers. This change in the mating phenotype of the *A3* recipient is the result of introducing the *A43* clone.

Structure of *A43*: We located three nonoverlapping fragments which gave *A* mating type activity (Figure 2, Table 2). We have provisionally labeled these *A(a)*, *A(b)* and *A(c)* because the correlation with the α and β subunits described by DAY (1960) is not yet clear. *A(a)* is a 3.8-kbp *Pst*I fragment cloned into pUC9 (pE12P5-3) and pCc1003 (bs/t3.8-6). *A(b)* is a 5.7-

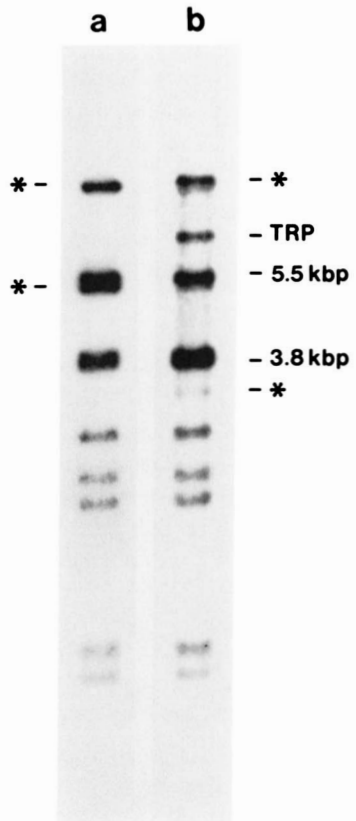

FIGURE 4.—Autoradiogram illustrating that clone c203-E12 is not rearranged. Lane a is a *Pst*I restriction digest of clone c203-E12 and lane b is a *Pst*I digest of Ok7 genomic DNA. The vector (LLC5200) contains *TRP1* and so hybridizes to a 6.5-kbp *TRP1* band in the genomic DNA. Flanking sequences are marked by * and are the only other fragments not corresponding in the two digests.

kbp *Hin*dIII fragment cloned into pBluescript (bs-E12H3–5) and pCc1003 (bs/tE12H3–2). *A(c)* is a 5.5-kbp *Pst*I fragment cloned into pUC9 (pE12P2–6) and pCc1003 (bs/tE12P5.5–24). Each of the bs/t subclones was introduced into the *A3* recipient by transformation. As shown in Table 2, Trp^+ transformants for each of the *A43* subcloned fragments exhibited altered morphology (FC^+) and mating behavior at a high frequency. *A(a)*, *A(b)* and *A(c)* did not cross-hybridize at reduced stringency (Figure 5, a, b, c Japan *A43* lane) and showed no relationship to one another in restriction maps (Figure 2).

In control transformation experiments we introduced the same bs/t subclones into the *A43* Trp^- recipient strain to obtain Trp^+ transformants. Changes in morphology and mating were not observed (Table 2).

Smaller subclones were prepared for the *A(a)* and *A(c)* regions (Figure 2). The smaller fragment carrying *A(a)* activity, *A(a')*, is a 2.2-kbp *Bam*HI/*Pst*I fragment (Figure 2) cloned in pUC9 and designated pdB1. Its mating type activity was determined by making co-transformations with pCc1003 (cloned *C. cinereus TRP1*). When cotransformed into the *A3* recipient,

TABLE 2

Transformation and mating results

DNA fragment[a]	Recipient mating type	% Trp^+ FC^+[b]	% Trp^+ FC^+ that are compatible[c]
A(a)	*A3, B1*	72 (93)	83 (12)
	A43, B43	0 (10)	
A(b)	*A3, B1*	68 (31)	95 (21)
	A43, B43	0 (11)	
A(c)	*A3, B1*	42 (62)	96 (26)
	A43, B43	0 (18)	

[a] Transforming DNA is bs/tP3.8-6 for *A(a)*, bs/tE12H3-2 for *A(b)* and bs/tE12P5.5-24 for *A(c)*. These clones are described in the RESULTS.

[b] Percent of Trp^+ with FC^+, all transformants are Trp^+. Values in parentheses represent the number of observations.

[c] Transformants of the *A3 B1* recipient were crossed to the *A3* tester (Table 1). Transformants of the *A43 B43* recipient, all FC^-, were crossed with the *A43* tester (Table 1). None mated.

5% of the Trp^+ transformants (7/150) were FC^+. Three of the four FC^+ Trp^+ transformants made with pdB1 were compatible with the *A3* tester. Cotransformation experiments with any of the *A43* mating type fragments and pCc1003 were characterized by a lower recovery rate of FC^+ colonies than for experiments in which *TRP1* was part of the plasmid construct even though the same recipient strain was used. Apparently the introduction of *A43* mating type genes into this recipient decreases the viability of regenerating protoplasts and an excess of Trp^+ single transformants is recovered unless the mating type gene is covalently linked to the selected marker. The smaller fragment carrying *A(c)* activity, *A(c')*, is a 3.4-kbp *Pst*I/*Hin*dIII fragment (generated by partial digestion) cloned in pCc1003 and was designated bs/tE12P/H3.4. 50% of the Trp^+ transformants had false clamps (22/44) and 95% (19/20) of the FC^+, Trp^+ colonies was compatible with the *A3* tester.

Mapping *A43-mut*: We mapped the *A* region of an *A43-mut* strain (SWAMY, UNO and ISHIKAWA 1984) and located a large deletion in the region of *A43* (Figure 2). The deletion left the *A(a)* fragment intact, deleted the *A(b)* fragment entirely and partially deleted the *A(c)* fragment. The right end of the deletion, in *A(c)*, was mapped by comparing *Bam*HI, *Bgl*II, *Pst*I/*Hin*dIII and *Xho*I restriction enzyme digest patterns of the *A43-mut* and the *A43* strains (Ok7 and 5026). Ok7 and 5026 were identical for all digests. The right end of the deletion is indicated by a dashed line because the *Xho*I site and associated 0.8-kbp *Xho*I fragment were deleted but the *Bgl*II site and associated 3.1-kbp fragment were left intact. The position of the left end of the deletion was inferred by the size of the 1.75 *Pst*I/*Hin*dIII fragment which hybridized both to a probe from the left end of *A(c)* and to a probe adjacent to *A(a)*.

Mating behavior of *A* transformants and *A43-mut*: The *A43-mut* and one class of *A* transformants (*A(a)*

and *A(c)*) had false clamps and mated by donating nuclei to the tester but did not accept nuclei from the tester. In contrast, the *A(b)* transformants, also with false clamps, both accepted and donated nuclei. In general, the *A(b)* transformants tended to be less stable mitotically and had a lower regeneration rate than transformants of *A(a)* and *A(c)*. Most haploid strains of *C. cinereus* produce asexual spores (oidia). Following mating, oidial production usually ceases in the dikaryon although *Amut Bmut* strains continue to produce oidia. We observed that some of the FC^+ transformants continued to produce oidia but these seemed to be transformant colonies with few false clamps.

Structure of *A* in strains with different *A* mating types: When radiolabeled subcloned fragments were hybridized to genomic blots at reduced stringency, a variety of patterns was observed (Figure 5). Each blot includes genomic DNA used as the source of the clone (Japan *A43* is Ok7) and also genomic DNA from the transformation recipient strain (Britain *A3* is 218). A range of hybridization signals was observed, from very weak hybridization (*e.g.*, Figure 5c, Britain *A3* lane) to bands of very similar size and hybridization intensity to that of the cloned sequence (*e.g.*, Figure 5a, lanes Java(a), NC 6-v1, NC 3-v1). The majority of lanes have only a single hybridizing band.

Where smaller cloned fragments, *A(a')* and *A(c')*, were used as hybridization probes, fewer bands hybridized. When the blot shown in Figure 5a was probed with the *A(a')* cloned fragment, only the bands which hybridized strongly to the larger probe (Japan *A43*, Java (a), NC 6V-1, NC 3V-1) still hybridized (data not shown). We have not examined smaller probes derived from the *A(b)* region. When the blot shown in Figure 5c was probed with the *A(c')* cloned fragment, most bands were still observed but hybridization to the Britain *A3* and NC6-v1 bands and to the lower band in strain NC3-b12 was not seen.

One of the striking results of the blots shown in Figure 5 is that many natural isolates carry at least one subunit which is very similar to a subunit found in *A43*. For those haplotypes, Figure 5d diagrams the similarities and differences in restriction fragments patterns and hybridization intensities among the bands hybridizing to cloned probes. Bands judged to be *A43*-like had a similar size and strong hybridization signal (compare to Figure 5, a, b, c). Thus, isolates from widely scattered geographic locations have *A43*-like subunits although these are not necessarily associated with one another as they are in the Japan *A43* haplotype.

DISCUSSION

We have cloned the *A43* mating type factor of *C. cinereus* in order to explore its function in cell-type determination and its evolution. We cloned the *A43* mating-type factor based on the false clamp phenotype expected of cells with two different *A* factors but a common *B* factor (SWIEZYNSKI and DAY 1960a). Our functional assays confirmed that it is the presence of two unlike alleles that allows mating to proceed, not the presence of two like alleles which prevents mating. The analysis of *A43* yielded surprising results and these findings bear on the structure, function and evolution of *A*.

Structure of *A* and nomenclature: The finding that the *A* mating-type factor of *C. cinereus* is made up of at least three dissimilar subunits was unexpected. Although DAY (1960) analyzed many tetrads and basidiospores, he never observed more than two nonparental classes of *A* mating factors. He cautiously concluded that there were at least two subunits and named them α and β. This terminology followed the convention set by experiments with *S. commune* where the α and β subunits are further apart and more easily recombined (RAPER, BAXTER and MIDDLETON 1958). The difference in our results and those of DAY (1960) could have one of several explanations. The two parental *A* factors Day used, *A5* and *A6*, may have differed at only two of the three subunits or they may have had only two subunits. Alternatively, because the *A* subunits are so close together, recombination rates in one of the intervals between subunits may have been too low to be detected. The entire region of *A* is heavily rearranged in different strains and this may lower rates of recombination. In *S. commune*, recombination rates between α and β are highly variable and not entirely explained by physical distance (RAPER 1966; SIMCHEN 1967).

We are using the nomenclature *A(a)*, *A(b)* and *A(c)* to describe the three independently functioning parts of our cosmid clone because it is not yet possible to relate these findings to those of DAY (1960). The three fragments we describe undoubtedly correspond to three subunits of *A* since each can activate the *A* regulated pathway following transformation. Recombination or walking experiments may turn up other subunits in this region. Our results suggest that transformation and hybridization methods can be used to determine allelic relationships among the subunits of the *C. cinereus* mating factors. For example, the *A(a)* subunit from *A43* could be introduced into the strain Java (a) to determine if the apparent similarity in restriction fragments corresponds to functional similarity. Such unambiguous criteria could be used to designate which sequences are alleles of α, which are alleles of β and which are alleles of the previously unexpected γ (and perhaps δ, etc.). Molecular and genetic analyses of the *A* mating type factor in *S. commune* suggest that it too is composed of more than two genes (NOVOTNY *et al.* 1991).

We are using the term "haplotype" to describe the

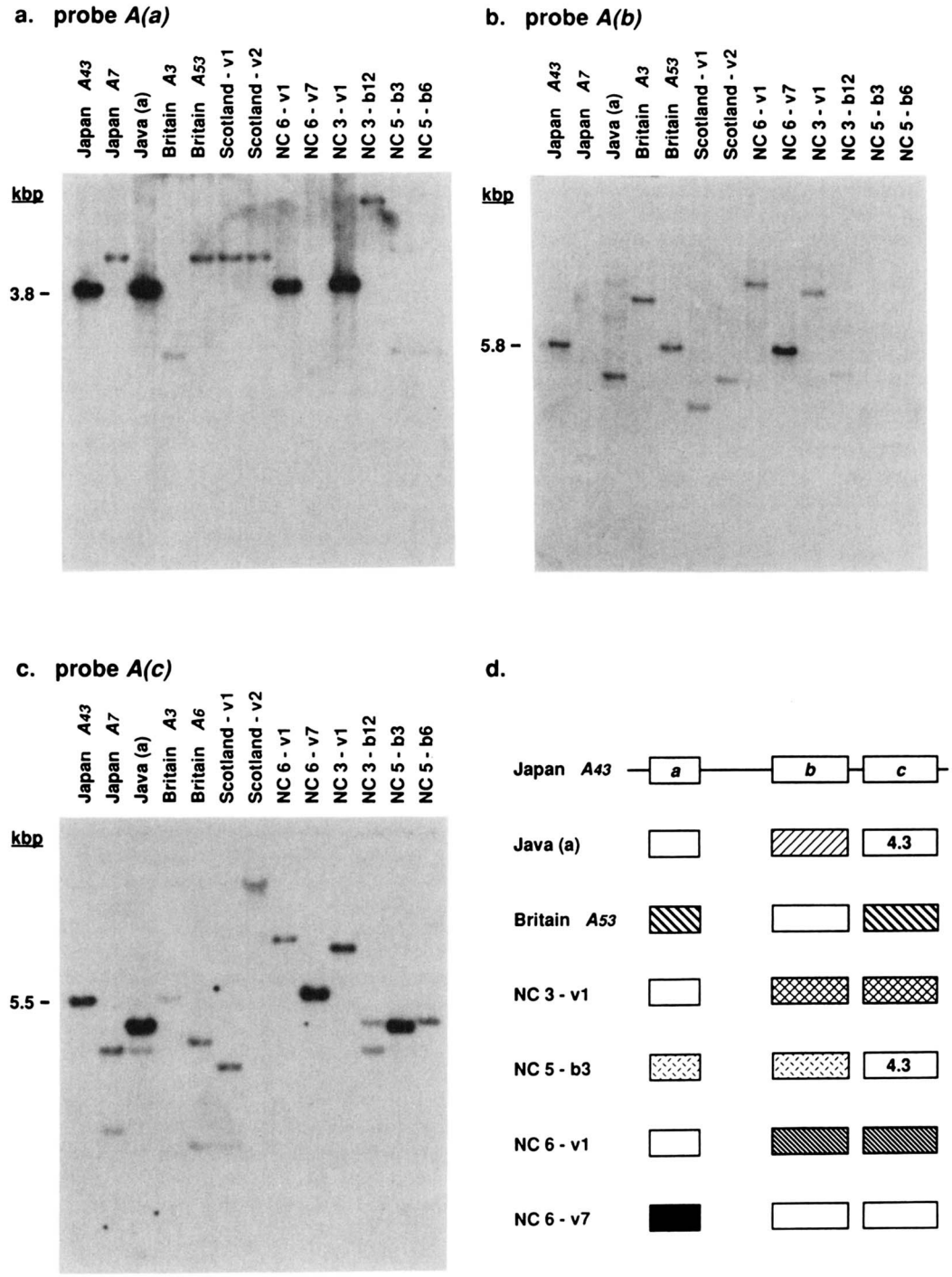

FIGURE 5.—Autoradiograms and diagram illustrating Southern analyses of the *A* region of 13 haploid *C. cinereus* strains carrying different *A* mating factors (Table 2). Hybridizations of these blots with probes indicated were carried out under reduced stringency conditions described in the MATERIALA AND METHODS. (a) *Pst*I digest of genomic DNAs blotted and probed with clone pE12P5–3 (3.8 kbp, *A(a)*) region. (b) *Hin*dIII digest of genomic DNAs blotted and probed with clone bsE12H3–5 (5.8 kbp, *A(b)* fragment). (c) *Pst*I digest of genomic DNAs blotted and probed with clone pE12P2–6 (5.5 kbp, *A(c)*). (d) Diagram summarizing restriction fragment variability of those natural isolates that carry at least one subunit similar to *A(a)*, *A(b)* or *A(c)*. Those fragments with a similar size and hybridization signal to that found in Japan *A43* are indicated by an open box. Those of a different size but with strong hybridization signals are indicated with an open box and the size observed. Those fragments with very different patterns of hybridization are indicated by shading (a distinct type of shading was used for each strain).

set of subunits making up any particular *A* factor. The term haplotype is appropriate because, like the animal major histocompatibility complex (KLEIN 1986), the *A* factor is a complex of genes with related function and the evolutionary relationship of the parts making up the complex is not necessarily known. In *C. ciner-*

eus, the problem of how different forms of a given subunit can evolve and function is especially interesting because models for gene action require that these different alleles must interact.

Models of *A* activity and analysis of *A43-mut*: Although our results do not exclude models in which each *A* subunit acts to repress *A* regulated pathways in the haploid cell, the results do place serious constraints on repressor models. We found that the introduction of a single subunit (either *A(a)*, *A(b)* or *A(c)*) resulted in expression of the *A* regulated pathway. Either each subunit must have its own target gene or genes, or any particular cell must express only one of the three subunits. The latter possibility can be discounted because each of the three subunits allowed expression of *A* regulated pathways in a majority of the transformants examined (with possible exception of *A(c)*). The dominance of *A43-mut* is not predicted by simple repressor models.

Our results do support models in which two different alleles of an *A* subunit interact to activate dikaryon-specific functions after hyphal fusion. Two types of molecular models have been proposed. Both require that mating type products form dimers or higher order aggregates. The models suggest that a heterotypic aggregation causes a conformational distortion such that a new activity is achieved (KRONSTAD and LEONG 1990; METZENBERG 1990; SCHULZ *et al.* 1990). The models predict that null mutations should produce a sterile phenotype which was observed following disruption of the *b* locus in *Ustilago maydis* (KRONSTAD and LEONG 1990). Though it is not at all obvious why a large number of distinct heterotypic combinations should serve as activators, there are several examples of heterotypic dimers acting as regulatory elements (*e.g.*, MURRE *et al.* 1989; DRANGINIS 1990). Our results also suggest that the number of interacting forms may not be as high as was assumed previously. Because *A* is made up of three subunits, only five alleles at each subunit would be required to give 125 *A* haplotypes which is close to the predicted number for the species (WHITEHOUSE 1949).

Our analysis of *A43-mut* underscores the necessity of ascertaining the molecular basis for the constitutive, dominant activation of the *A* regulated pathway in *A43-mut* and other *A* factor mutations. The *A43-mut* strain harbored a 12-kbp deletion that leaves the *A(a)* fragment intact, deletes the *A(b)* fragment, and partially deletes the *A(c)* fragment. This haploid strain expressed the *A* regulated pathway constitutively and continued to do so after mating with *A43*. It may be that an altered *A(a)* subunit or the truncated *A(c)* subunit in the *A43-mut* strain can form aggregates with the altered conformation required for activator function. The constitutive, dominant phenotype of *Amut* strains has been interpreted in this way (METZENBERG 1990). Alternatively, it may be that both *A(b)* and *A(c)* are functionally null and *A(a)* is unaltered in *A43-mut*. Such a result would suggest that the three subunits interact in the haploid, and would require consideration of more complicated models of *A* factor function.

Mating behavior of *A* transformants: The phenotype of one class of *A* transformants and *Amut* strains implies that nuclear migration may not be entirely under the control of the *B* mating type as was classically described (SWIEZYNSKI and DAY 1960b). One class of strains with false clamps (including *A(a)* and *A(c)* transformants and *A43-mut*), mate with *A43* testers if these strains are also compatible at *B*. However, strains in this class mate only by donating nuclei to the tester rather than both accepting and donating nuclei. In *S. commune*, strains with mutated *A* factors behave similarly (RAPER, BOYD and RAPER 1965). CASSELTON (1978) has suggested that dikaryon formation can be divided into an establishment phase in which parental nuclei are brought together and a maintenance phase in which clamp connections are seen and conjugate nuclear division takes place. The observation that *A* controls clamp initiation and conjugate division suggests that *A* functions late in development, in a phase maintaining the dikaryotic condition. WESSELS and NIEDERPRUEM (1967) and WESSELS (1969) posed similar possibilities based on evidence of enzymatic activities during the mating process. Precocious *A* activity may block migration though the mating pair is compatible at *B*. In contrast, a second class of strains with false clamps (including *A(b)* transformants and apparently some of DAY's *Amut* strains [DAY 1963a]), will both donate and accept nuclei when mated with testers. It will be of interest to determine if *A* activity is different in these two classes of transformants.

Evolution: Variation at the *C. cinereus A* mating type factor is generated on two levels. On the level of the entire haplotype, we found that the three parts of *A* are shuffled into different combinations to produce different *A* mating haplotypes. The North Carolina isolates were most interesting because they were collected within a meter of each other on a single day yet we found fragments similar to *A(a)*, *A(b)* and *A(c)* that were not associated with one another as they are in *A43*. The short physical distance between subunits and the extensive rearrangements apparent across the entire region can explain DAY's (1960, 1963b) observation of low recombination rates. Given that recombination rates are low, these data contrast with the prediction that low recombination rates evolved to prevent inbreeding (SIMCHEN 1967) and suggest instead that rare recombination events might have a positive selective value.

On the level of the individual fragments making up

A, each shows tremendous variability on Southern blots probed with *A43* fragments. This variability is demonstrated both in terms of restriction fragment lengths and hybridization intensities. In other filamentous fungi, quite a range of hybridization and sequence relationships between mating type alleles have been described. In *Neurospora crassa*, the two alternate forms of the mating type locus, *A* and *a*, are so dissimilar in both coding and flanking regions (GLASS, GROTELUESCHEN and METZENBERG 1990; STABEN and YANOFSKY 1990) that the term "idiomorph" has been coined to replace allele (METZENBERG and GLASS 1990). Three *Aα* mating type loci of the homobasidiomycete, *S. commune* have been cloned and sequenced. Both the subunits and the flanking regions of these different *Aα* loci are highly dissimilar (GIASSON *et al.* 1989) though sequencing has turned up some similarities in the open reading frames (ORFs) making up *Aα* loci (NOVOTNY *et al.* 1991). In *C. cinereus*, another mating-type, the *A42* factor from Java has been cloned; little or no hybridization of this mating type DNA to the genomic DNA of two other strains with different *A* mating types (*A5* and *A6*) was observed and a 9-kbp region of nonhomology was reported (MUTASA *et al.* 1990). The discrepancy with our results is not clear but could it could be due to the particular DNA used as the probe (*e.g.*, the *A42* subunit probes may be more highly diverged from most other subunits) or the strains being probed. In contrast to situations in which the mating type alleles have been described as idiomorphs, the *b* mating type genes of *Ustilago maydis* can be clearly described as being allelic with one similar size ORF and both highly variable and highly similar regions within the ORFs of different *b* mating types (KRONSTAD and LEONG 1990; SCHULZ *et al.* 1990). We believe that *C. cinereus A* mating type factor may represent an evolutionary intermediate condition where a range of similarities exists between the cloned sequence and other *A* mating types. Judging by the hybridization intensities and the fact that in most cases only a single band is discerned, we should be able to recover those intermediate forms, make sequence analyses and describe evolutionary mechanisms of change.

Determination of those evolutionary mechanisms will help determine whether or not there is positive selection for a mating system with both multiple genes and multiple forms of those genes. Explanations for the evolution of multiple allele mating systems has focused on the level of inbreeding or outbreeding allowed, often with the assumption that outbreeding should be favored (*e.g.*, RAPER 1966). Alternative explanations such as limitations on the time available for mating (IWASA and SASAKI 1987) or the ability to regulate outcrossing rates depending on ecological conditions (UYENOYAMA 1988) have been less well explored. These hypotheses await testing on the evolutionary and population level. The genus *Coprinus* is particularily well suited for such studies because closely related species use very different mating systems (ORTON and WATLING 1979).

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