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Recombinational Mapping of Capsule Mutations in *Cryptococcus neoformans*

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Seven capsule-negative mutants of *Cryptococcus neoformans* were isolated. All mutations were linked (maximum map distance, 38 U); two mutations were found to be allelic.

Cryptococcus neoformans is a yeast which is pathogenic for humans and animals. Each yeast cell is surrounded by a polysaccharide capsule which has been shown to represent a virulence factor (3, 5, 8). The major capsular polysaccharide in the *d* serotype (discussed here) contains mannose, xylose, and glucuronic acid residues (1) and has been termed a glucuronoxylomannan (4). Acapsular mutants have been described (3, 6, 8), and their preliminary characterization has been reported (6). In the present study we describe linkage relationships among seven capsule mutations.

The life cycle of *C. neoformans*, elucidated by Kwon-Chung (9), involves a haploid yeast phase which ends when cells with opposite mating types are inoculated upon crossing medium. Conjugation is followed by a transient hyphal phase which culminates in meiosis and production of abundant, haploid basidiospores. Convenient collection and analysis of random basidiospores have been described (6). However, unusual products of meiosis have also been described (10). A small proportion of basidiospores (about 2%) give clones which sporulate directly upon inoculation onto crossing medium. Such "self-fertile" strains represent putative diploids (10). An additional, small class of progeny consists of clones which give rise to nonsporulating hyphae upon transfer to crossing medium. A working assumption accounts for the last class as aneuploid, heterozygous disomics for the chromosome bearing the mating-type factors. The mapping procedure in the present study involved determination of proportions of wild-type recombinants in progeny resulting from crosses between capsule mutants. Because recombination often was rare, we attempted to prevent the unusual meiotic products described above from introducing errors into the analysis. We have tried to exclude three alternative explanations for our finding of apparent linkage: complementation in diploids, complementation in disomics, and back mutation.

To determine linkage relationships, pairs of capsule-negative mutants were crossed to each other, random spore analysis (6) was performed, and the proportion of wild-type recombinants was determined by India ink staining. Wild-type colonies produced from these crosses were inoculated onto crossing medium and observed for spore formation (self-fertility) to exclude putative complementing, heterozygous diploids from the estimation of recombinants. Self-fertile colonies were subtracted from the total number of wild-type colonies before calculation of map distances.

To confirm that self-fertile, encapsulated basidiospore colonies were heterozygous diploids, two such colonies selected at random (from a cross, Cap 64 × Cap 70) were transferred to crossing medium, allowed to sporulate, and subjected to random spore analysis. The progeny were 97% of the acapsular phenotype; this result suggests that self-fertile, encapsulated colonies are largely pseudowild-type diploids. The small proportion of encapsulated progeny was consistent with the rate of meiotic recombination usually observed in the cross Cap 64 × Cap 70 (see below).

Encapsulated basidiospore colonies which produced nonsporulating hyphae upon transfer to crossing medium could not be analyzed by allowing sporulation. A priori the encapsulated phenotype could result from recombination or complementation; pseudowild-type colonies could result either from diploidy or from disomy for the chromosome bearing the locus of acapsular mutations. According to a parallel argument, the ability to form nonsporulating hyphae might plausibly result from diploidy or from disomy for the chromosome bearing the mating-type locus. To count such sexually aberrant, encapsulated colonies as recombinants would certainly be

TABLE 1. Mapping of capsule mutations

Cross	No. of colonies observed	No. of wild-type colonies found	No. of encapsulated colonies that were true recombinants[a]	% Wild-type colonies in total population
44 × 59	10,257	2	2	0.019
48 × 59	1,050	207	207	19.7
55 × 59	650	133	122	18.7
44 × 64	1,330	4	4	0.3
48 × 55	1,400	32	29	2.07
44 × 70	1,050	5	5	0.47
60 × 44	1,150	45	45	3.9
55 × 60	650	64	64	9.8
64 × 70	300	10	8	2.6
60 × 64	230	21	21	9.1

[a] Progeny which did not produce spores when transferred to crossing medium.

wrong if they, in fact, were diploids or were pseudowild-type, heterozygous disomics for a chromosome bearing both capsule and mating-type loci. These two possibilities were evaluated by selection from three crosses between acapsular mutants (Cap 48 × Cap 55, Cap 60 × Cap 44, and Cap 60 × Cap 64) of additional random basidiospore colonies simply for the nonsporulating, hypha-forming phenotype. These were scored for the capsular phenotype. Either of the above two possibilities would lead to the prediction that all basidiospore colonies selected originally for formation of nonsporulating hyphae would be encapsulated. In fact, only one of eight was encapsulated. This result suggests that the nonsporulating, hypha-forming, encapsulated colonies observed in our mapping studies are not pseudowild type either. Colonies exhibiting similar sexual behavior occasionally are recovered as products of crosses between wild-type strains.

The seven capsule-negative mutants were tested for reversion by macroscopic observation for reversion to the glistening, mucoid colonial morphology. No revertants were seen in approximately 10^4 colonies observed for each mutant.

Results of recombination experiments are shown in Table 1; a linkage map consistent with our data is shown in Fig. 1. It was found that the capsule-negative mutations map at several loci in a single linkage group. The experimental variability of the map distance measured between any two sites on different occasions has been roughly 12 to 18% of that distance. Areas 1, 2, and 3 designate different areas of the chromosome where capsule mutations appear to cluster. Two capsule-negative mutations, Cap 59 and Cap 44, mapped much less than 1 centimorgan apart. This narrow region of the chromosome may therefore represent a single gene with mutations Cap 59 and Cap 44 allelic. Evidence for linkage among all seven genetic loci is provided by the calculation that the highest rate of recombination (38 to 39%) was significantly less than that expected for random segregation ($P < 0.01$). In contrast, random recombination was seen when capsule mutants were crossed to either of two auxotrophs (6). The linkage data exhibit a considerable degree of internal consistency; a minor exception to this consistency may be seen in the enlargement in Fig. 1. We have no explanation for this inconsistency. We believe that this ambiguity is best resolved by means of complementation testing. Such a complementation system can probably be based upon the self-fertile diploids which occasionally result from meiosis.

We have excluded two trivial explanations for our linkage data. First, the reversion rate of these mutants was far below the rate of recombination observed between all except the pair Cap 59 and Cap 44. Second, we have found that a small proportion of encapsulated progeny may represent complementing, pseudowild-type diploids. Evidence for this phenomenon is provided by the identification of clones which were self-fertile (as though they contained both mating factor alleles, α and a) and encapsulated, but which segregated acapsular alleles upon sporulation. The number of such presumed diploids was usually much less than 20% of the total number of encapsulated progeny observed, and our results were corrected for the probable rate of diploidization (Table 1).

The significance of the finding of sexually aberrant, encapsulated progeny is not clear. We have presented evidence which suggests that

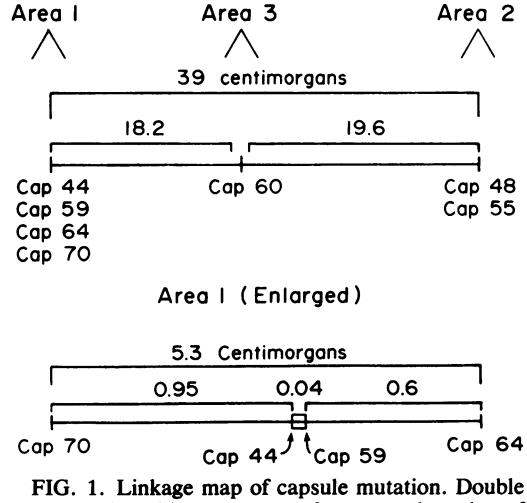

FIG. 1. Linkage map of capsule mutation. Double the values in Table 1 to account for an equal number of "double-negative" recombinants.

these do not represent complementing pseudowild-type colonies. Therefore, it seems reasonable to include them in the mapping calculations. Their exclusion would not change the map by much, however. An attractive hypothesis holds these strains to be disomic aneuploids for the chromosome bearing the mating-type alleles, but we do not yet have additional markers for this chromosome and so cannot test this hypothesis.

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