GENETIC LOCALIZATION OF THE CLOCK MUTANT AND A GENE MODIFYING ITS BAND-SIZE IN *NEUROSPORA*¹

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MUTANTS of *Neurospora crassa* have been described in which rhythms occur; these include patch (BRANDT 1953; PITTENDRIGH, BRUCE, ROSENSWEIG and RUBIN 1959; STADLER 1959) and timex (SARGENT 1965), in which conidia are formed with a period of about one day. Also, a growth rhythm has been described in clock (SUSSMAN, LOWRY and DURKEE 1964), in which the surface mycelium becomes increasingly dense until growth abruptly ceases except in a few hyphae ("initials") from which the next band is formed.

The original isolate of clock formed bands which were 80 mm or longer on complete medium. However, variants soon appeared in which the size was markedly altered (SUSSMAN *et al.* 1964). Environmental factors such as the composition of the medium (BERLINER and NEURATH 1965; DURKEE unpublished) and temperature (SUSSMAN, DURKEE and LOWRY 1965; BERLINER and NEURATH 1965) were found to alter band size. However, even under identical environmental conditions certain strains differed so markedly in size that genetic control was indicated. The present study was initiated in order to analyze the effect on band size of a modifier gene, named *mad*, and to map the gene.

MATERIALS AND METHODS

Strains used: The standard (wild) strains 4-121A and 4-137a were both reisolated from crosses of strains kindly provided by DR. NORMAN HOROWITZ.

Mutant strains are listed below: (FGSC indicates that the strain was obtained from the Fungal Genetics Stock Center, Dartmouth University, Hanover, New Hampshire.)

Locus symbol	Name	Allele (Isol. No.)	Description
<i>val-1</i>	valine-1	45201	Requires valine (FGSC).
<i>sh</i>	shallow	R2371	Spreading morphological in which hyphae do not penetrate deeply into agar (FGSC).
<i>md</i>	mad	MW84	Spreading morphological.
<i>sp</i>	spray	B132	Spreading morphological in which the aerial mycelium fans outward (FGSC).
<i>inos</i>	inositol	89601	Requires inositol (FGSC).
<i>me-3</i>	methionine-3	36104	Requires methionine (FGSC).
<i>bis</i>	biscuit	B6	Restricted, conidiating colonial (FGSC).
<i>cl</i>	clock	CL11	Banding, non-aerial colonial.

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Maintenance of stock cultures: Stock cultures were grown at 25°C on complete medium (RYAN 1950) prepared without casein hydrolysate and using glycerol as a carbon source. The cultures were stored at 4°C and were subcultured about every 3 months. Silica gel cultures (PERKINS 1962) were prepared from newly received cultures and from strains retained from our isolations. These were used to start new stock cultures after the old ones had gone through several transfers on complete medium.

Crossing technique: Medium for crosses was prepared by inserting strips of filter paper (approx. 20 × 70 mm) in test tubes (16 × 150 mm) containing 5 to 8 ml of crossing medium (WESTERGAARD and MITCHELL 1947) prepared by omitting a soluble carbon source and agar. The protoperithecial parent was inoculated several days prior to fertilization by the other parent. Conidia or hyphal fragments were used as inoculum. The medium was appropriately supplemented if either parent was auxotrophic.

Isolation of ascospores: Two methods of isolating random ascospores were employed. The first method, essentially the same as for ordered isolation, was used when numerous white or grey spores were noted among those discharged onto the tube surface. A sample of spores was transferred to an agar block and arranged in rows. The spores were transferred to isolation tubes (10 × 75 mm) or to phage dishes (Falcon Plastics Corp., Los Angeles 45, California) either before heat-shock or when tiny colonies had formed following heat-shock. This method was used to determine the percent germination. An alternative method, in which spores were spread on agar media in a petri dish, heat-shocked and isolated after germination, was used when few unripe spores were evident among those discharged.

Scoring technique for morphological mutants (The grid technique): It is often difficult to distinguish between morphological mutants once the aerial mycelium has developed and, moreover, the gross morphology of a colony may be quite variable. For example, *cl* (Figure 4) and *md,cl* (Figure 12), or modified *cl*, often resemble each other macroscopically because the former

FIGURE 1.—Single ascospore colonies on complete medium in a phage dish 24 hours after transfer at 25°C, illustrating the grid technique. The cross analyzed was *mad* × *shallow*; squares B3, C1, E2, E3, E5 and F5 are *mads*, A5 and C4 are double mutants and the rest of the colonies are *shallows*. Approximately $\frac{2}{3}$ actual size.

is sensitive to a variety of environmental influences which cause the bands to be shorter and less regular. For example, the composition of the medium, including the nature of the carbon source, affects morphology. However, young colonies of such mutants, 24–36 hours after germination of the ascospores, usually have one or more characteristics by which they may be identified. A method, which we call the grid technique, was devised to permit rapid screening of such colonies (Figure 1). Ascospores are transferred 12 to 24 hours after heat-shock to plastic phage dishes containing complete medium. Each spore is placed at the center of a square so that 32 spores can be grown on one plate. The colonies are scored under a dissection microscope (10–40 $\times$) after incubation for 12 to 24 hours at 25°C. Certain morphological mutants can only be separated into groups by this method and must be transferred to test tubes in which they can be incubated for a longer period.

Auxotrophs are scored by transferring a minute quantity of mycelium from each colony to a phage dish containing minimal medium (VOGEL 1956), supplemented when necessary.

RESULTS

The grid technique is useful for screening mutants which have dissimilar microscopic morphologies (i.e. branching patterns). Although the parent strains used in this work are easily distinguishable by their branching patterns the progeny of some (e.g. *sp* and *sh*) frequently require careful observation for classification. Both *sp* and *sh*, in the early stages of colony formation, form elaborate fans of hyphae on the agar surface (Figures 9, 11). Forward progression of the colony front is slow until aerial hyphae are produced which grow beyond the front and collapse onto the agar surface. The pattern of formation of aerial hyphae, hence of colony extension, differs in the two mutants so that the edge of a colony of *sp* is ragged while that of *sh* is even (Figures 8, 10).

The mutant *md*, which is a spreading morphological type like *sh* and *sp*, has a distinct branching pattern (Figures 6, 7). In *md*, a main hypha will branch monopodially (i.e. as in standard strains) for a time. Then, quite abruptly, the tip forms numerous branches in rapid succession. In young colonies most of these branches do not continue to grow, but those which do become new main hyphae. The latter also may arise from the lateral branches formed by old main hyphae prior to their deformation. The pattern is best observed during the early stages of colony formation when it is 0.5 to 1.5 cm in diameter. A variation of the same pattern may be seen in established colonies although the densely matted hyphae make observation difficult.

A group of morphological mutants intermediate between the compact restricted types such as *bis* and the spreading, more rapidly growing types such as *sp*, *md* and *sh* is represented by *cl* (Figure 4), which has a growth rate of about 1 cm per day at 25°C. Young *cl* colonies are of smaller diameter and more compact than the colonies of the standard strains of the same age (Figures 2, 3), and their hyphae appear less deformed than those of *bis*, *sp* or *sh* (Figure 5).

The linkage data are given in Tables 1 and 2 and are summarized in Figure 19. Crosses in which germination was poor are not included.

Initially, *cl* was localized by crosses with markers from each of the seven linkage groups. Once the linkage group was determined, 2- and 3-point crosses were used to estimate map distances. Gene order was resolved by several 3-point crosses, the results of only one of which are given.

TABLE 1

Crosses of clock and mad with markers on linkage group V

Zygote genotype and map distance	Parental combinations	Singles region 1	Singles region 2	Doubles regions 1 and 2	Total and percentage germination
$\frac{a \quad b \quad c}{+ \quad + \quad +}$	$abc \quad +++$	$a++ \quad +bc$	$ab+ \quad ++c$	$a+c \quad +b+$	
$\frac{sp \quad +}{+ \quad 8.7 \quad cl}$	173 162	22 10	—	—	367 92%
$\frac{sp \quad inos \quad +}{+ \quad 11.5 \quad + \quad 6.3 \quad cl}$	179 187	33 17	14 13	1 0	442 88%
$\frac{bis \quad + \quad *}{+ \quad 0.9 \quad cl}$	326 347	4 2	—	—	679 >95%
$\frac{me-3 \quad + \quad +}{+ \quad 1.2 \quad bis \quad 1.5 \quad cl}$	135 117	1 2	3 1	— —	259 >95%
$\frac{md \quad + \quad *}{+ \quad 9.5 \quad sp}$	164 149	14 20	—	—	347 71%
$\frac{sh \quad + \quad *}{+ \quad 3.1 \quad md}$	462 496	18 13	—	—	989 >95%
$\frac{val \quad sh \quad +}{+ \quad 6.8 \quad + \quad 4.2 \quad md}$	56 50	3 4	2 2	0 1	118 >95%

* Results of two crosses pooled.

Linkage of *md* to *cl* was indicated by the results of a cross of the *md, cl* double mutant with a standard strain. A difficulty encountered in the positioning of *md* was the uncertainty of the order of the markers *val-1* and *sh*. A 5-point cross was prepared for the purpose of establishing the order of these loci with respect to each other and to *md*. However, two classes of phenotypes recovered from the random analysis of this cross appeared to contain heterogeneous genotypes. The validity of this supposition was established by an analysis of the cross using ordered asci. It was found that *sh* was not expressed in the *sh, sp* double mutant (Figure 18) and in the *sh, md, sp* triple mutant (Figure 17). On the other hand, the *md* gene was expressed in combination with *sp* in that the double mutant grows more slowly than either parent (Figure 16).

It has been noted that strains mutant at other loci on linkage group V than *cl*

FIGURES 2-7.—The appearance of colonies and mycelia of a standard strain and the two mutants, *clock* and *mad* on complete medium at 25°C.

Figure No.	Name	Age (days)	Magnification	Description
2	standard strain	1½	1×	mycelium
3	standard strain		20×	branching pattern
4	clock	2½	1×	mycelium
5	clock		40×	branching pattern
6	mad	2	1×	mycelium
7*	mad		20×	branching pattern

* Arrow a: tip of a main hypha which has ceased growing; arrow b: a lateral branch which has become a main.

TABLE 2

Ordered ascus analysis of a 5-point cross with mad

Parental genotype:	<i>val</i>		<i>sh</i>		<i>md</i>		+		+
	+	3.8	+	2.6	+	6.1	<i>sp</i>	11.2	<i>inos</i>
Number of asci recovered									
Parental tetrads									24
Recombinant tetrads									
Singles, region 1									1
Singles, region 2									1
Singles, region 3									3
Singles, region 4									4
Doubles (4-strand) regions 1 & 4									2
Doubles (2-strand) regions 3 & 4									1
Doubles (3-strand) regions 3 & 4									1
Doubles (3-strand) regions 2 & 4									1
Total number of asci isolated									38

sometimes form growth bands. These include the single mutants *bis* (Figure 13), washed and *md* (Figure 6).

That the size of the bands of *cl* can be reduced by modifier genes of several kinds also has been demonstrated. Thus, the double mutants of *cl* with the linked morphological genes *sp* (Figure 15) and *bis* (Figure 14) formed very small bands. Furthermore, the small *cl* (Figure 12) that was isolated from our cultures and analyzed herein, is shown to be a double mutant of *cl* and *md*, which are linked. Not all modifiers of band size are independently expressed, as our studies of a mutant of *cl*, which has even smaller bands than *md*, *cl*, reveal. Thus, an ordered analysis of recombinants from the cross of such a modified *cl* with a wild strain yielded no wild-types with aberrant morphology, although unmodified *cl* strains were recovered.

Figure 19 is a linkage map derived from the data in this paper. Although discrepancies in the map distances between *cl* and certain other genes exist, its location on linkage group V and its order with respect to the markers tested are clear.

FIGURES 8-18.—The appearance of colonies and mycelia of various single, double and triple mutants on complete medium at 25°C.

Figure No.	Name	Age (days)	Magnification	Description
8	spray	2	1×	mycelium
9	spray		20×	branching pattern
10	shallow	1½	1×	mycelium
11	shallow		40×	branching pattern
12	mad-clock	4	1×	mycelium
13	biscuit	4	1×	mycelium
14	biscuit-clock	8	2×	mycelium
15	spray-clock	4	1×	mycelium
16	mad-spray	8	1×	mycelium
17	shallow-mad-spray	4	1×	mycelium
18	shallow-spray	2	1×	mycelium

FIGURE 19.—Partial maps of linkage group V compiled by PERKINS, GLASSEY and BLOOM (1962) and the present authors. All loci on the map constructed from data given in this paper were positioned by 3- or 5-point crosses.

DISCUSSION

The order of *sh* and *val-1* shown here (Figure 19) is based on more extensive data than those of PERKINS, GLASSEY and BLOOM (1962), whose contrary order depended on only one critical crossover. The shorter map distances between *inos*, *sh* and *val-1* could be due to chance or more likely to strain differences. Close linkage between *cl* and *bis* (SRB 1962) is revealed by these data although allelism is ruled out.

The rhythmic growth of mutants other than *cl* suggests that the periodic formation of growth bands in *Neurospora* is not unique to this locus. Rather, it appears that any of several mutations on linkage group V, which result in a colonial phenotype, may manifest a rhythm. Such a rhythm may be displayed with regard to conidial formation in an unlinked mutant, patch. This potential for rhythmicity in *Neurospora* is not evident in wild-type strains, except under certain environmental circumstances (SUSSMAN *et al.* 1964). It may be that rhythmic growth is a symptom of unbalanced metabolism (genetically or environmentally induced) which leads to the accumulation of a growth-inhibiting substance such as that postulated by SUSSMAN *et al.* (1965) to explain the *cl* mutation. If, however, there were to be selective advantage to rhythmic behavior, as seems to be the case in other organisms, single-gene mutants such as those described here could be a convenient starting point for selection. It is also possible that a rhythm underlies processes in wild-type *Neurospora* which have not yet been studied. Evidence for this possibility is the appearance of a rhythm in wild-type under certain conditions.

SUMMARY

The clock mutant of *Neurospora crassa*, which produces distinct mycelial bands delimited by a difference in hyphal density, is in linkage group V. Another group-V gene, designated mad, modifies the clock phenotype, making the bands shorter and less regular. The most likely gene order is valine-shallow-mad-spray-inositol-methionine-3-biscuit-clock.

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