ISOLATION AND CHARACTERIZATION OF PERITHECIAL DEVELOPMENT MUTANTS IN NEUROSPORA

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Manuscript received May 31, 1977
Revised copy received September 15, 1977

ABSTRACT

The isolation and characterization of mutants that block perithecial development in *Neurospora crassa* are described. Several classes of mutants have been isolated after UV mutagenesis, and those that block perithecial development when used as the female (protoperithecial) component of a cross have been further characterized. These mutants fall into 29 complementation groups. Twelve of the 33 mutants block development at the protoperithecial stage; no other clustering of block points is observed. Many of the mutants show an altered vegetative growth rate as well; in several mutants this lower growth rate cosegregates with the female sterile phenotype. Only one mutant also blocks development of the perithecium when used as the conidial parent. None of the mutants are temperature sensitive; two can be suppressed by growth on a complete crossing medium. There is no indication that the mutants are at or in the mating-type locus, nor are any of the mutants mating-type specific. Genetic mosaics have been formed using mixtures of mutant and marked wild-type nuclei; no mutants are cell autonomous by this criterion. The significance of these results in terms of "developmental" mutants isolated in other organisms and in relation to models of eukaryotic development is discussed.

THIS communication reports the first phase of a study of perithecial development in the eukaryote *Neurospora crassa*. Perithecial development is initiated when a strain (either mating type *A* or *a*) has exhausted the medium upon which it resides. Protoperithecia are then formed (Figure 1). These structures are fertilized by attachment of a cell of the opposite mating type to the trichogyne (Figure 1). The transition from the immature fruiting body, the protoperithecisum, to the mature perithecium involves a seven-fold increase in the diamenter of the protoperithecium, a dramatic increase in the level of pigmentation, and the formation of several new and distinct tissue types, including the ascospores (Figures 1 and 2).

The perithecium is the sexual structure and as such is completely dispensable for the vegetative growth of the organism. The perithecium is composed of tissue

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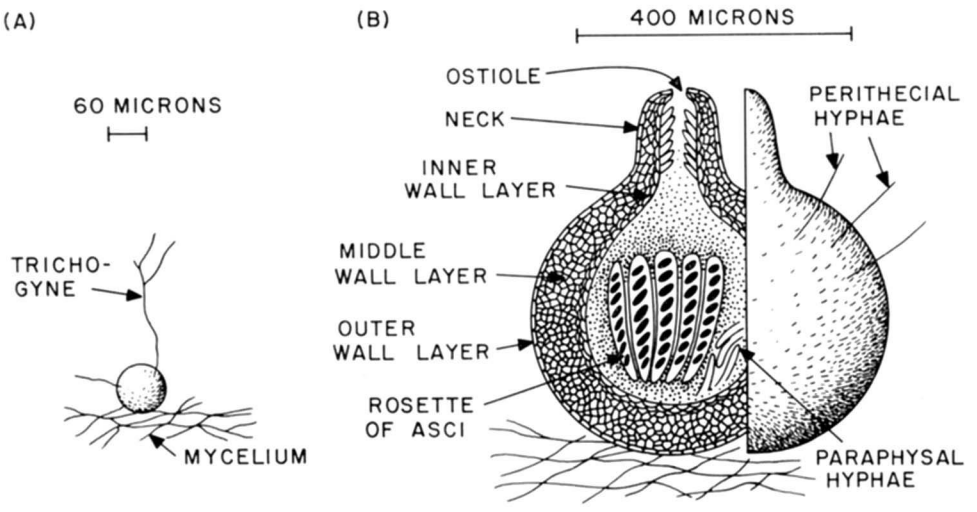

FIGURE 1.—A diagram of a protoperithecium and a fecund perithecium.

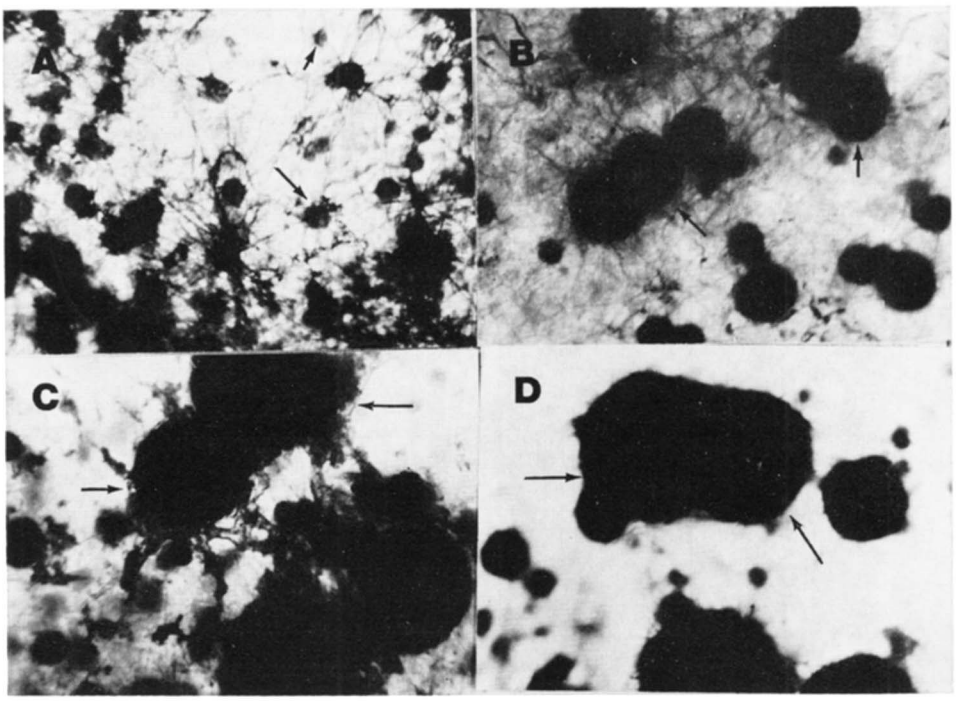

FIGURE 2.—Photographs of fruiting bodies of the female fertile *pyr-3 A* strain used to select the steriles (60X). (A) At time of crossing (0 hr), (B) 24 hr, (C) 60 hr, (D) 268 hr. Arrows point to protoperithecia (A) or perithecia (B–D).

that is derived almost exclusively from the female (protoperithecial) component in a cross (MITCHELL and MITCHELL 1952; HOWE and BENSON 1974; JOHNSON 1974, 1975; HOWE and JOHNSON 1976); thus strains that are defective as the female component of a cross are almost invariably effective as the male or conidial component. These two facets of the life cycle of Neurospora when combined with the ability to cross either mating type, *A* or *a*, as either male or female provide a unique opportunity for the isolation of absolutely defective developmental mutants which, although not conditional lethals, can still be conveniently maintained by vegetative growth, easily analyzed genetically by crossing as the male, and examined for allelism in heterokaryons.

Moreover, the independence of the vegetative and sexual phases makes possible the easy distinction of two different types of mutants affecting development. In Neurospora, as in *Drosophila melanogaster* and presumably all higher eukaryotes, developmental mutants can arise either as a result of mutations in genes specific for the affected developmental process or in nonspecific genes that "mimic" specific mutations. This latter category is due to the fact that many developmental events are uniquely sensitive to alterations in basic metabolism. Thus, many developmental mutants have been traced to alterations in steps in intermediary metabolism, *e.g.*, rudimentary, *r*, in Drosophila (NORBY 1970); or in Neurospora, *lys-5* (asco) (STADLER 1956), *cys-3*, (MURRAY 1965), *pyr-3* (RADFORD 1971), all of which affect perithecial development as well as being auxotrophic mutations; or *col-2* (BRODY and TATUM 1966), which causes a colonial vegetative growth phenotype as a pleiotropic effect of an altered G6PD. One advantage of a life cycle like Neurospora's is that vegetative growth rate can be directly quantitated independently of perithecial development; the measurements can then be used to determine whether the developmental mutant shows vegetative growth rate alterations. These measurements, when combined with an examination of the vegetative morphology, allow one quickly to distinguish specific mutations from mutations that are not specific for perithecial development.

The long-range intent of the present study is to isolate large numbers of perithecial developmental mutants, to determine which of these may show blocks in specific processes, and ultimately to use these specific mutants to elucidate the molecular mechanisms that control Neurospora development. The present communication describes the genetic characterization of 33 mutants that fail to produce ascospores as the female component of a cross.

A number of other investigators have reported on female sterility in Neurospora. Some reports were on isolated instances of female sterility (DODGE 1935, 1946; MITCHELL, MITCHELL and TISSIERES 1953; WESTERGAARD and HIRSCH 1954; STADLER 1956; SRB 1957; HOROWITZ *et al.* 1960; FITZGERALD 1963; MURRAY 1965; MITCHELL 1966; TERENZI and REISSIG 1967; VIGFUSSON 1969; TAN and HO 1970; BHATTACHARYA and FELDMAN 1971; RADFORD 1971; ITOH and MORISHITA 1971; Ho 1972). Other more extensive studies have been made on a number of developmental mutants affecting the female sexual cycle (MYLYK 1971; MYLYK and THRELKELD 1974; WEIJER and VIGFUSSON 1972),

the male (conidial) sexual cycle (VIGFUSSON and WEIJER 1972; WEIJER and VIGFUSSON 1972) or development of the ascospores (review in SRB, NASRALLAH and BASL 1973).

The current study differs from earlier studies in several ways: (1) all these mutations are newly induced by UV mutagenesis; (2) quantitative techniques have been developed for the analysis of the mutant defects; (3) several novel approaches have been employed for determining the time and place of mutant action.

MATERIALS AND METHODS

Strains and media

The following strains were used in this study: *pyr-3(KS-43) A* (pyrimidine auxotroph); *ad-2(STL-2) A* (adenine auxotroph); *col-4 A, a* (colonial morphology); *74 A* (wild type); *inl a* (inositol auxotroph); *per-1 A* (light perithecia, HOWE and BENSON 1974; HOWE and JOHNSON 1976); *ff-1 A, a* (female sterile mutant, TAN and Ho 1970); *al-2 ad-2 inl a* (multiple linkage group markers including albino).

Media included: Minimal Medium (Min), (VOGEL 1964); Crossing Medium (Wx), (WESTERGAARD and MITCHELL 1947) supplemented with 2% sucrose; Wx supplemented with 0.1% sucrose and 0.5% sorbose (Wx S); Min in which 0.1% dextrose, 0.1% glycerol, and 2% sorbose were used as sole carbon sources (Min S) and 2% corn meal agar (CM). Supplements were added at 5 mg/l for vitamins and 50 mg/l for other compounds. For most experiments on perithecial development standard Wx was supplemented with 100 mg/l adenine (ad) or uridine (*ur*) as required. This increased level of supplements gave a better crossing reaction of the *pyr-3* and *ad-2* strains.

Mutant hunts

The method of isolating female sterile mutants was a modification of that of BHATTACHARYA and FELDMAN (1971). Parental strains were recloned periodically. Fresh conidia were suspended in sterile water at 10^6 conidia/cc, and treated with a Hanovia Germicidal ultraviolet bulb 683 A1 B at 75 ergs mm^2/sec for 10 tto 15 minutes. This treatment resulted in 30% to 90% kill in different experiments.

The mutagenized conidia were suspended at 45° in 0.8% plain agar at an expected concentration of 10 viable conidia/ml. Ten ml aliquots were immediately poured over filter papers which had been placed on the surface of plates containing 25 ml of Wx S, ad or ur.

The plates were incubated at 25° in the dark for 5 to 8 days. At this time colonies were apparent in the top agar. Most of the colonies were large enough both to have reached the surface of the top agar (a condition necessary for protoperithecial formation) and to have grown through the filter paper leaving a colony in the agar below the filter paper. At that time the filter paper and top agar were transferred to fresh plates of plain 1.5% agar. Both the original (master plate) and the new plate (replica) were incubated at 25° in the dark for an additional 5 days.

The replica plates were then crossed with 2 ml of a suspension containing 10^6 fresh *inl a* conidia/ml. The suspension was carefully spread over the surface of the plates with a glass spreader and the excess water poured off. The plates were incubated two more weeks at 25° in the dark and then examined. Individual colonies were picked from positions on the master which corresponded to colonies on the replica which had failed to form visibly mature perithecia or had formed visibly altered perithecia.

The putative mutants were transferred to 13 × 100 mm tubes of Wx plus supplements. Three successive tests for female fertility were performed on the putative mutants as the final stage of the screen.

Fertility determinations

In all crosses the female parent is listed first. Fertility was recorded in either of two ways. For critical analyses the strain in question was grown on Wx plus supplements in a 9 cm petri plate at 25° in the dark. Conidia growing over the edge of the plate were removed periodically by wiping with alcohol. On day 8, a 2 ml suspension of fresh conidia of the opposite mating type (at least 10^6 conidia per ml) was pipetted onto the plate and spread carefully over the surface with a glass spreader (0 days). The fertility of the strains was assayed with the aid of a stereo dissecting scope 14 days after crossing. The presence of perithecia, their size, and their number were recorded.

For less critical experiments the stocks in question were transferred to 13 × 100 mm tubes, incubated at 25° for 8 days and fertilized with 0.3 ml of a suspension of conidia of the opposite mating type. On the fourteenth day the fertility was determined by examining the walls of the tube for ejected ascospores.

For initial work, *ff-1 a* and *ff-1 A* were used as the male parents in these determinations. Later, *per-1(PBJ-1)* was stubstituted because of its increased efficiency as the male parent. The use of a female-sterile or *per-1* strain as male is important because the male parent may grow and form perithecia even if the plate is completely covered with a mycelium of the other parent. Such an event might completely obscure otherwise clear tests of female fertility.

Fertility was quantitated by counting the number of fruiting bodies and determining their size and color. Numbers of fruiting bodies were determined by counting at least five fields chosen at random and averaging the number of fruiting bodies produced per field. Perithecia were defined as any fruiting bodies that show increased levels of pigmentation and the formation of perithecial hyphae (JOHNSON 1975). Fecund perithecia were defined as perithecia having ostioles [95% of the perithecia which have ostioles contain ascospores (JOHNSON 1975)] or a clump of ejected spores at the ostiole of the perithecium. The distribution of the perithecial sizes was determined with the aid of an ocular micrometer. Extensive analyses were based on measurements of 100 perithecia chosen at random. For most studies the color of the perithecium was designated qualitatively as light brown, brown, dark brown or black.

Complementation tests

Complementation tests between mutants selected in the *ad-2* background and mutants selected in the *pyr-3* background were performed by mixing conidia of the desired mutants on Wx with no supplements; tests between two *ad-2* or between two *pyr-3* were performed on Wx plus appropriate supplements. Heavy suspensions of conidia and/or mycelia from both desired strains were mixed in the tube or on the plate. Fertility was determined as described above. The production of ascospores was taken as an indication that the mutants complement.

Formation of mosaic perithecia and measurement of nuclear ratios

Mosaic perithecia were formed as described in an earlier communication (JOHNSON 1976b). Briefly, the procedure is to mix conidia produced by the *per-1* (produces white perithecia) strain with conidia from the desired mutant strain, which is *per-1+* (produces black perithecia). The *per-1* mutation is completely autonomous for ascospore formation (HOWE and JOHNSON 1976) and at least partially autonomous in the other perithecial tissues (JOHNSON 1976b). The presence of black pigment therefore signals the presence of the *per-1+* nucleus in that particular tissue; this is then interpreted to signify that that particular mutant can participate in the formation of that tissue type.

Growth rates

The rate of linear growth was determined by inoculating conidia at one end of a 20 cm growth tube (RYAN 1950) containing 14 ml of Min + ad or ur. Every 12 hr the tube was marked at the front of the growing mycelium. Growth rates were determined after a constant rate of growth was obtained, usually about 24 hr after inoculation at 25°. The "original" *ad-2* and *pyr-3* strains were used as controls and all growth rates are presented as percent of the control growth rate.

RESULTS

Isolation and preliminary classification of mutants

About 130,000 mutagenized conidial colonies were screened for the ability to produce perithecia when used as the female (protoperithecial) parent in a cross. Altogether, 76 strains were found to be altered in their ability to function as the female component (Table 1).

Among these were 33 completely female-sterile mutant strains (class A). These failed to produce ascospores within three weeks after crossing when used as the female component of a cross. These mutants are the major class studied and will be described in more detail later.

Another set of mutants (class B) was composed of seven isolates representing at least five independent mutations at the *per-1* locus (HOWE and BENSON 1974; JOHNSON 1974, 1975; HOWE and JOHNSON 1976). The seven strains all produced light perithecia and ascospores instead of the black structures produced by wild type.

Classes C, D and E include mutant strains that seemed to be defective in some aspect of perithecial morphogenesis but were not completely sterile. Some isolates

TABLE 1

*Classification of putative mutants**

Class	Number mutants	Description	Isolate numbers
(A)	33	female sterile	ABH8, ABI3, ABI4, ABJ7, ABK1, ABP5, ABP6, ABP11, ABR3, ABR7, ABR8, ABT1, ABT5, ABT9, ABT10, PBI8, PBJ3, PBJ4, PBJ6, PBM4, PBM7, PBM8, PBN1, PBN4, PBR3, PBS1, PBS4, PBS7, PBS8, PBS9, PBT1, PBT2, PBT3
(B)	7	light perithecia (*per-1* type I)	ABI1, ABT8, PBE1, PBJ1, PBP1, PBT4, PBT5
(C)	16	slow protoperithecial development, normal perithecial development	ABD2, ABD14, ABH14, ABJ2, ABJ10, ABP1, ABP2, ABP7, ABR2, ABR4, ABT4, PBP2, PBP3, PBP6, PBS5, PBS6
(D)	12	perithecia in clumps	ABD18, ABD29, ABH7, ABO1, ABP3, ABP8, PBM5, PBN5, PBR2, ABR10, ABT12
(E)	3	slow proto- and perithecial development	ABK3, ABP4, PBJ7
(F)	2	female sterile and abnormal vegetative	PBM2, PBS3
(G)	3	female sterile but defective vegetative growth	ABJ8, PBR1, ABS3
TOTAL	76		

* Descriptions of phenotypes of perithecial defective mutants are presented. Isolates starting with the letter "A" were obtained in the *ad-2* background, and those with "P" in the *pyr-3* background.

changed the normal time course of perithecial development (classes C and E), while others altered the normal distribution of perithecia on the mycelium (class D) (JOHNSON 1975).

Two classes of mutant strains showed severe effects on both the sexual and the vegetative phases. The strains were either completely female sterile and showed extremely restricted colonial growth (class F), or were inviable vegetatively over prolonged periods so that they could not be maintained (class G).

Position of mutant block points

The female-sterile mutants (Table 1, class A) were examined to determine where in the wild-type developmental sequence the mutant block point(s) occurred. Both parental strains from which these mutants were selected form protoperithecia within seven days after inoculation. Normal protoperithecia are light brown spheres about 60 μm in diameter (Figures 1, 2A, 3A). Within twelve hr after being brought into contact with cells of the opposite mating type (crossing), normal protoperithecia respond by increasing diameter, accumulating pigment, and forming perithecial hyphae. By 24 hr, these changes are readily noticeable (Figures 2B, 3B). By 60 hr, enlargement has ceased (Figures 2C, 3C). Pigment continues to accumulate until late into the sexual cycle (JOHNSON 1975); other development events, including spore and neck formation, occur

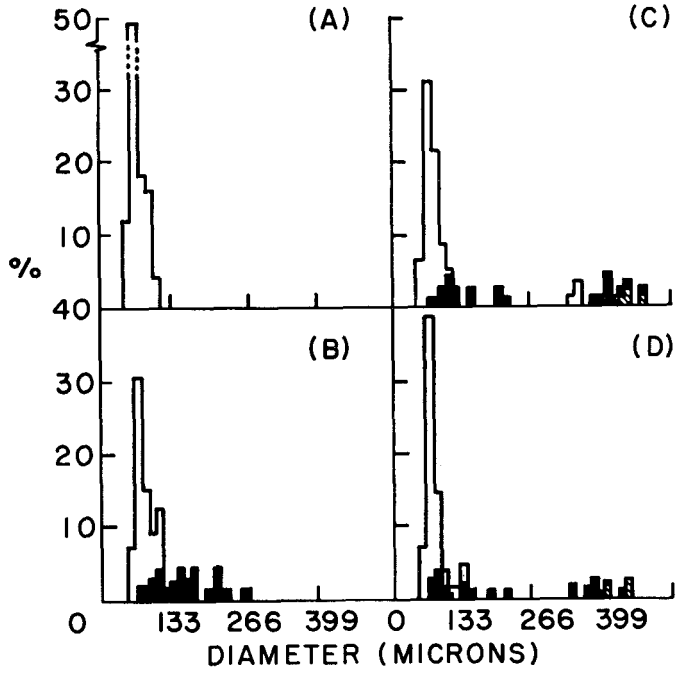

FIGURE 3.—Histograms show the distribution of diameters of fruiting bodies at different stages in development for the *ad-2* parental strain. Open areas indicate protoperithecia, solid bars are perithecia and hatched areas are fecund perithecia. (A) 0 hr protoperithecia, (B) 24 hr perithecia, (C) 60 hr perithecia, (D) 115 hr perithecia.

TABLE 2

*Block points of isolates**

Isolate number (1)	Number of protoperithecia (number/mm²) Typical value $\bar{X} \pm SD$ (2)	$\bar{\bar{X}} \pm SD_{\bar{x}}$ (3)	Number of repeats (4)	Diameter of perithecia (μm) Typical value $\bar{X} \pm SD$ (5)	$\bar{\bar{X}} \pm SD_{\bar{x}}$ (6)	$\bar{X}$90th percentile $\pm SD$ (7)	Number of repeats (8)	Diameter of uncrossed perithecia (μm) $\bar{\bar{X}} \pm SD_{\bar{x}}$ (9)	Number of repeats (10)
ad-2 w.t.	7.1 ± 5.2	10.3 ± 5.2	12	310 ± 170	300 ± 40	500 ± 60	4	74 ± 3	3
ABH8	1.03 ± 0.06	4.0 ± 4.4	9	330 ± 80	280 ± 70	430 ± 30	4	63 ± 3	3
ABI3	0	0	4						
ABI4	0	0.08 ± 0.08	6						
ABJ7	0	0.01 ± 0.02	6						
ABK1	4.3 ± 0.7	17.8 ± 7.9	7	140 ± 30	150 ± 50	260 ± 130	4	200 ± 10	2
ABP5	0.07 ± 0.11	0.5 ± 0.7	3	(36)					
ABP6	3.2 ± 0.9	4.6 ± 5.7	4	100 ± 20	160 ± 80	300 ± 210	2		
ABP11	0.7 ± 0.4	1.6 ± 2.6	5	(88)					
ABR3	0	0.6 ± 1.0	3						
ABR7	0	0.6 ± 1.0	6						
ABR8	0.03 ± 0.06	0.01 ± 0.01	3	(60)					
ABT1	1.1 ± 1.0	1.8 ± 1.9	7	200 ± 50	180 ± 60	260 ± 30	3		
ABT5	0	0	3						
ABT9	0	0.07 ± 0.16	9						
ABT10	0.4 ± 0.5	0.6 ± 0.6	8	50 ± 16	49 ± 4	77 ± 10	3		
pyr-3 w.t.	3.7 ± 3.2	5.1 ± 3.4	6	340 ± 160	330 ± 90	490 ± 40	7		
PBI8	0	0	9						
PBJ3	0	0	6						

TABLE 2—Continued

Isolate number (1)	Number of protoperithecia (number/mm²) Typical value $\bar{X} \pm SD$ (2)	$\bar{\bar{X}} \pm SD_{\bar{x}}$ (3)	Number of repeats (4)	Diameter of perithecia (μm) Typical value $\bar{X} \pm SD$ (5)	$\bar{\bar{X}} \pm SD_{\bar{x}}$ (6)	^{X}90th percentile $\pm SD$ (7)	Number of repeats (8)	Diameter of uncrossed perithecia (μm) $\bar{\bar{X}} \pm SD_{\bar{x}}$ (9)	Number of repeats (10)
PBJ4	0.2 ± 0.3	0.3 ± 0.5	9	360 ± 130	360 ± 40	540 ± 50	3		
PBJ6	7.3 ± 1.0	9.2 ± 3.6	7	130 ± 30	120 ± 10	170 ± 20	7		
PBM4	0.02 ± 0.02	0	3	(35)					
PBM7	12.9 ± 8.8	9.7 ± 4.6	3	110 ± 30	110 ± 2	140 ± 10	3		
PBM8	0.4 ± 0.2	1.5 ± 1.6	7	180 ± 60	190 ± 20	280 ± 30	4		
PBN1	1.8 ± 1.2	1.3 ± 0.7	2	110 ± 40	140 ± 40	170 ± 10	2	90 ± 10	2
PBN4	0.06 ± 0.09	0.2 ± 0.3	3						
PBR3	6.4 ± 2.6	3.5 ± 1.5	7	180 ± 100	200 ± 20	290 ± 80	6	150 ± 60	4
PBS1	5.8 ± 0.8	5.6 ± 4.6	7	110 ± 20	100 ± 10	130 ± 20	4		
PBS4	1.3 ± 1.1	3.2 ± 2.7	7	270 ± 100	280 ± 130	390 ± 160	5		
PBS7	0	0	2						
PBS8	0	0	2						
PBS9	5.3 ± 2.9	4.0 ± 2.0	7	180 ± 150	220 ± 70	310 ± 100	4	190 ± 90	4
PBT1	1.0 ± 0.7	2.0 ± 2.4	8	180 ± 50	190 ± 30	320 ± 100	3		
PBT2	8.3 ± 3.2	12.0 ± 5.6	9	140 ± 30	130 ± 20	160 ± 20	4		
PBT3	0	0	3						

* Measurements of the number of protoperithecia produced and the diameter of the perithecia (protoperithecia) are shown. Typical values (columns 2 and 5) present the mean and standard deviation in one standard experiment. Columns 3, 6, and 9 represent the mean of the means and their standard error. Columns 4, 8, and 10 present the number of repeats of these tests.

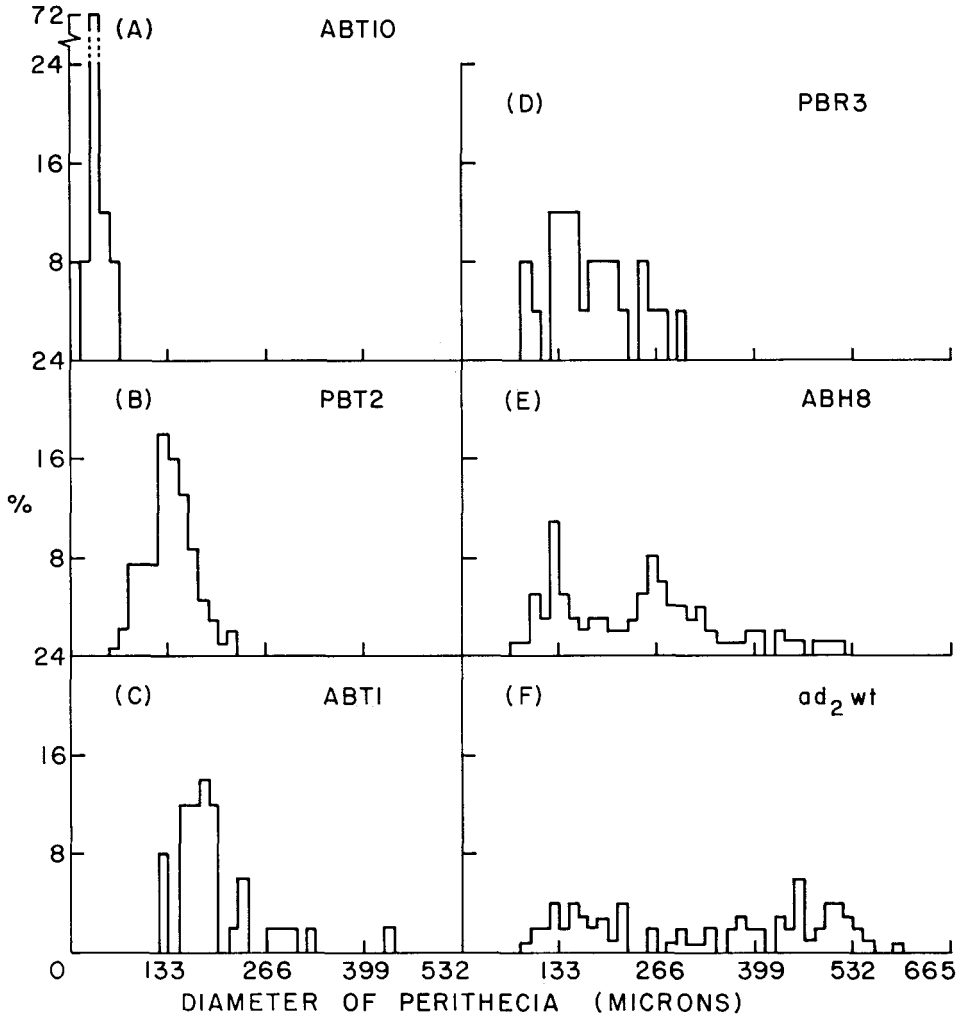

FIGURE 4.—Histograms show the distribution of diameters of perithecia of several mutants strains: (A) ABT10, (B) PBT2, (C) ABT1, (D) PBR3, (E) ABH8, (F) *ad-2*, female-fertile parental strain. All measurements were made between eleven and fourteen days after crossing.

during this period. Development culminates at 11 days when mature ascospores begin to be discharged (Figures 1, 2D, 3D).

Preliminary experiments suggested that only three mutant strains produced normal-sized perithecia. Diameter was therefore chosen as a convenient and appropriate parameter with which to quantitate the relative positions of the mutants' block points. The largest group of mutants is comprised of those that produce no protoperithecia. Twelve mutants fall into this category (Table 2). Another six strains (ABP5, ABP11, ABR8, ABT10, PBM4 and PBN4) sometimes produce a few protoperithecia, which in some cases are smaller than those of wild type (50–60 μm). The remaining fifteen strains routinely differentiate

reproducible quantities of protoperithecia; some mutants undergo further development showing increased diameter, the accumulation of pigment and the production of perithecial hyphae. The block points of these mutants have been measured (Table 2) and are displayed in Figure 4. Relative block points are displayed diagrammatically in Figure 5.

A complicating factor in this analysis is that, under these conditions, as shown in Figures 3C and 3D, only 10% of the perithecia in typical wild-type control cultures actually produce ascospores (fecund perithecia); the remaining perithecia stop development early and soon lose competence to complete the normal sexual developmental cycle (JOHNSON 1975, and manuscript in preparation). To compensate for this phenomenon, two different methods of determining block points have been employed: (1) the average diameter of the whole range of perithecia has been measured, and (2) the diameter of the perithecia that are at the ninetieth percentile of diameter in each particular test has been determined. This latter classification gives an estimate of the block point of that portion of the population of perithecia that would normally complete the entire developmental cycle in a wild-type control. A comparison of the relative block points as determined by average perithecial size on the one hand, or by size of perithecia at the ninetieth percentile on the other, shows that the two methods result in similar relative values and a nearly identical ordering of the block points (Figure 5).

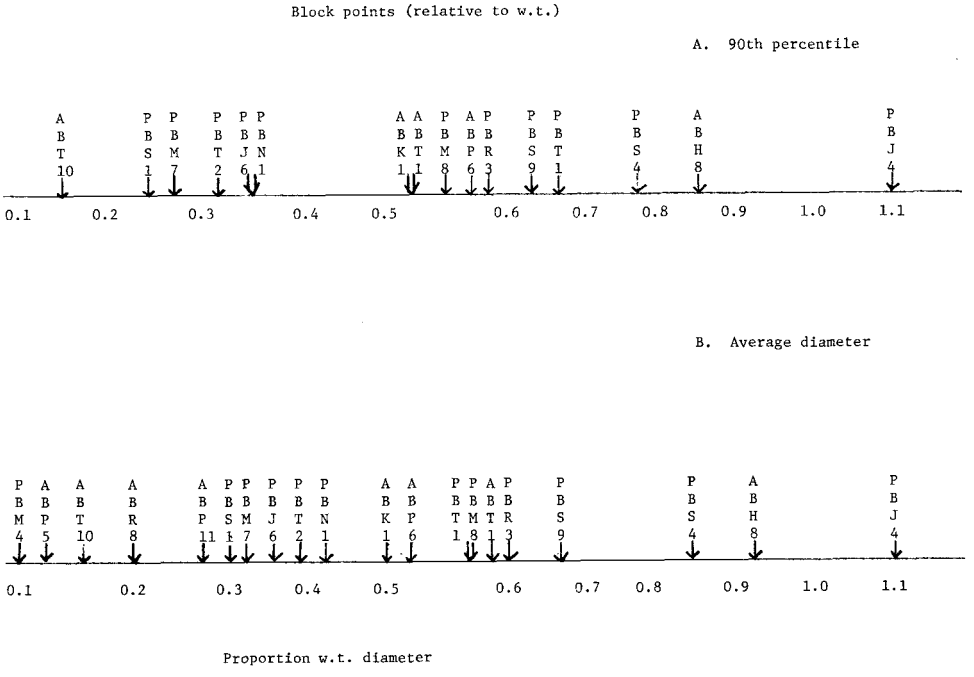

FIGURE 5.—Relative block points of mutants blocking after protoperithecial formation. (A) Assigned relative to wild type by average perithecial diameter, (B) assigned by average diameter of perithecia at the ninetieth percentile, includes mutants producing only a few perithecia.

A class-interval test was performed on the data, as shown in Figure 5. The test divided the distribution of block points into five to eleven equally sized intervals and tested whether the block points were distributed at random among these intervals. These tests failed to show any evidence of clustering of mutant block points. However, if the additional twelve mutants that produce no protoperithecia are added to the distribution, a significantly nonrandom distribution is observed ($p < 0.001$).

Three mutants (ABK1, PBR3, PBS9) produce perithecia in uncrossed conditions (Table 2). The diameters of these "perithecia" are similar to diameters obtained when the mutant strains are crossed.

Tests for dominance

Heterokaryons involving the developmental mutations were forced using *ad-2* and *pyr-3* as complementary forcing markers. Thirty of the mutant strains were uniformly recessive when such heterokaryons were forced with wild type. Three strains (ABH8, PBI8, and PBT3) acted as "partial dominant" mutations; *i.e.*, in heterokaryons with wild type these mutants often did not produce ascospores when used as the female component of a cross. This effect seems to be due either to "preferential growth" of the mutant nuclei (PBI8 and PBT3) such that 70–90% of the nuclei in the heterokaryon were mutant or to a "true dominance" where even at equal nuclear ratios the mutant phenotype was observed (ABH8).

Complementation tests

These tests were carried out for all pairwise combinations of mutant strains as described in MATERIALS AND METHODS. The results suggest that the 33 mutations fall into 29 complementation groups (Figure 6). Two groups contain two mutants each (PBN1 and PBN4, and PBR3 and PBS9). These two groups may represent repeats of the same mutation because they were in both cases picked from the same parental clone and the mutants' block points are similar. Members of the other group of three noncomplementing mutants (ABT1, PBJ3 and PBS4) are

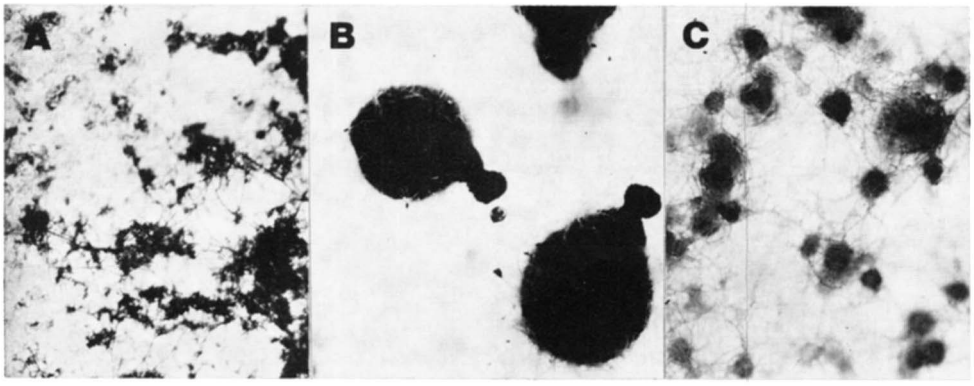

FIGURE 6.—Photographs of (A) ABT1 strain, (B) heterokaryon ABT1 + PBJ6, (C) PBJ6 strain (60X).

independent isolates. The three mutations block the developmental process at quite distinct points in the sexual cycle.

Effects on other phases of the life cycle

The mutant strains were used as the male (conidial) parent in a cross to *col-4 a*. All of the strains except PBJ6 produced abundant ascospores. None of the strains under these conditions produced any noticeable change in the morphology of the perithecia or of the ascospores. Several of the crosses ejected more white ascospores than did wild-type crosses; this seems to be due to chromosome rearrangements in several instances, but not in all (JOHNSON 1975). In general, then, the mutations do not affect the male sexual cycle, with the exception of PBJ6, which when used as either the female or male component of a cross, blocks perithecial development at similar stages (JOHNSON 1975, 1976a, and manuscript in preparation).

The linear growth rate and the morphology of the vegetative phase of each of the mutants were also compared with wild type (Table 3). Many of the mutants show altered linear growth rates and the majority of the strains have altered vegetative growth patterns ranging from a complete absence of conidia to an alteration in the pattern of conidiation. ABH8, ABK1, PBN1, PBS1, and PBT2 have both wild-type growth rates and qualitatively wild-type morphology. Representative mutants with slow growth were crossed to determine whether slow growth rate segregated independently of female sterility. In all cases, slow growth and sterility were linked. In seven mutant strains (PBI8, PBJ3, PBM4, PBM8, PBN4, PBS7, and PBS8) sterility cosegregated with slow growth (< 5 centimorgans); in PBT3 they were linked, but a few isolates that showed wild-type growth rates remained sterile.

Mosaic analysis of tissue specificity of genetic defect

All of the mutant strains were examined using forced heterokaryons of mutant nuclei and wild-type nuclei carrying either *per-1(ABI-1)* or *per-1(PBJ-1)*. Both *per-1* genes are Type I alleles that block the development of black pigment in all perithecial tissues (HOWE and JOHNSON 1976). Since the mutant strains contained the *per-1*$^+$ allele and this locus is expressed autonomously (JOHNSON 1976b), the presence of black pigment in any particular perithecial tissue was indicative of the presence of the female-sterile nucleus in that tissue.

In all cases, the perithecia produced by these heterokaryons contained black pigment. All perithecial tissues, including the rosette of ascospores, contained black pigment, indicating the presence of mutant nuclei within those tissues. The mutant nuclei, therefore, were able to participate in the formation of all perithecial tissues through the aegis of the female-fertile *per-1* nuclei.

Temporal specificity of developmental arrest

Another way of finding out about the specificity of the mutant block points is to ask whether the diameter of the perithecia produced by these mutants increases at wild-type developmental rates prior to the point at which arrest

TABLE 3

*Effects on vegetative and male characteristics**

Mutant isolation number	Relative growth rate	Vegetative morphology	% white spores as male
ad-2	1.00 ± 0.04	wild type	4
ABH8	1.07 ± 0.04	wild type	4
ABI3	0.88 ± 0.76	altered	3
ABI4	1.10 ± 0.06	altered	2
ABJ7	0.97 ± 0.10	altered	3
ABK1	1.03 ± 0.06	wild type	3
ABP5	1.10 ± 0.10	altered	0
ABP6	0.79 ± 0.38	altered	5
ABP11	0.90 ± 0.12	altered	4
ABR3	0.66 ± 0.06	altered	4
ABR7	0.90 ± 0.16	altered	3
ABR8	0.87 ± 0.12	altered	5
ABT1	0.40 ± 0.46	altered	0
ABT5	1.05 ± 0.08	altered	2
ABT9	1.04 ± 0.04	altered	34
ABT10	1.04 ± 0.08	altered	3
pyr-3	1.00 ± 0.02	wild type	5
PBI8	0.91 ± 0.04	altered	1
PBJ3	0.44 ± 0.04	altered	0
PBJ4	0.53 ± 0.00	wild type	11
PBJ6	0.81 ± 0.00	wild type	1–20*
PBM4	0.59 ± 0.12	altered	3
PBM7	0.89 ± 0.10	altered	21
PBM8	0.49 ± 0.04	altered	33
PBN1	1.03 ± 0.08	wild type	15
PBN4	0.52 ± 0.04	altered	11
PBR3	0.92 ± 0.00	altered	6
PBS1	0.99 ± 0.04	wild type	4
PBS4	0.92 ± 0.00	altered	2
PBS7	0.68 ± 0.00	wild type	0
PBS8	0.51 ± 0.06	altered	20
PBS9	0.99 ± 0.04	altered	0
PBT1	1.00 ± 0.06	altered	6
PBT2	0.92 ± 0.06	wild type	0
PBT3	0.63 ± 0.00	altered	4

* Vegetative effects of the mutants are shown. These include: linear growth rate relative to the original female-fertile auxotroph, conidial and growth morphology, and proportion of ejected ascospores which are white.

occurs. Nonspecific effects might cause a slowing down of the rate of development prior to the actual arrest point.

The diameters of perithecia were measured at several points throughout development. Most of the mutants were "specific" in that development occurred at normal rates up until the block point (Figure 7). The only exception to this rule was ABT1 which appears to develop somewhat more slowly than wild type even prior to its block point.

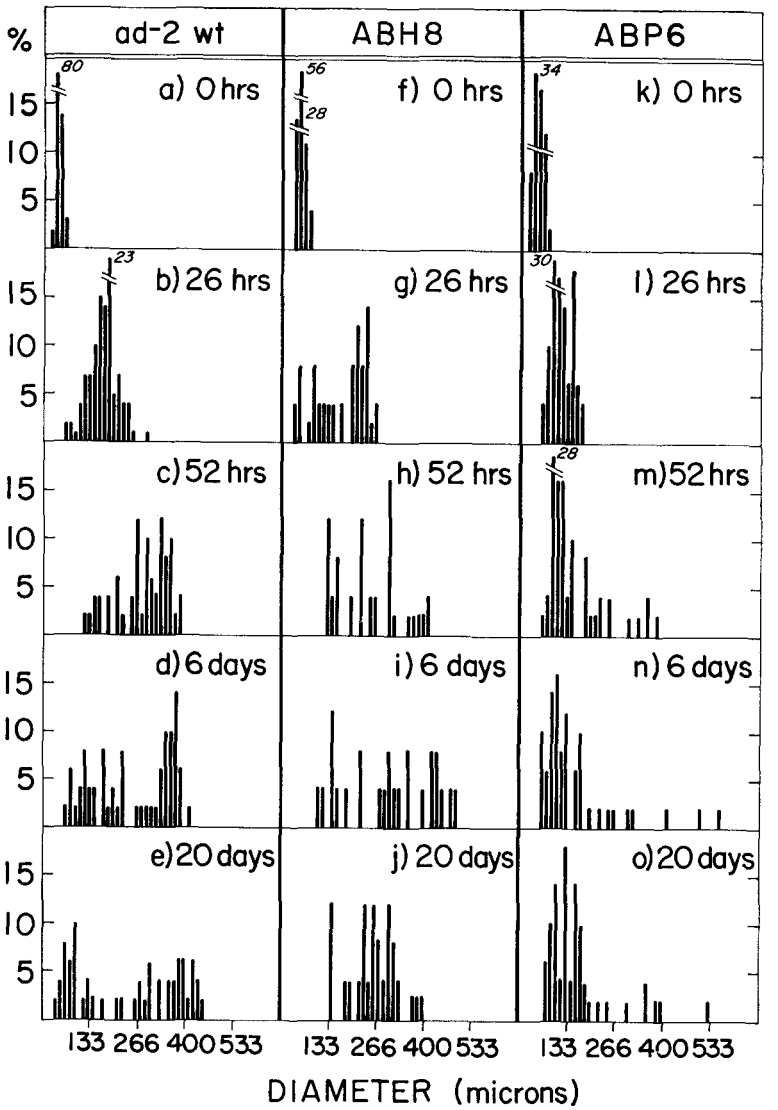

FIGURE 7.—Histograms show the distribution of diameters of fruiting bodies at different stages of development for the female-fertile *ad-2* strain and for female-sterile strains ABH8 and ABP6 which were selected from the *ad-2* parental strain. (a) *ad-2* at 0 hr after fertilization, (b) *ad-2* at 26 hr, (c) *ad-2* at 52 hr, (d) *ad-2* at 6 days, (e) *ad-2* at 20 days, (f) ABH8 at 0 hr, (g) ABH8 at 26 hr, (h) ABH8 at 52 hr, (i) ABH8 at 6 days, (j) ABH8 at 20 days, (k) ABP6 at 0 hr, (1) ABP6 at 26 hr, (m) ABP6 at 52 hr, (n) ABP6 at 6 days, (o) ABP6 at 20 days.

Attempts to supplement the developmental defect

In Neurospora, many auxotrophic mutations also cause the mutant strain to be female sterile even when supplemented with the appropriate growth requirement (MURRAY 1965; RADFORD 1971; MYLYK and THREKLELD 1974). The

mutants were grown on Wx supplemented with yeast extract, or casamino acids, or beef heart extract to determine whether the female sterility could be overcome by a complete medium. No improvement in the fertility of the mutants was observed on these media. However, growth on corn meal agar allowed two isolates to produce fecund perithecia (ABH8 and PBM8).

Some of the female-sterile mutants isolated in this study were also observed to produce fecund perithecia when confronted with contaminating colonies of other genera of fungi. Similar synergistic effects have been reported in *Polysphondylium violaceum* (WARREN, WARREN and COX 1976); in *Dictyostelium discoideum* some aggregation mutants can be helped through the development process by the addition of wild-type amoebae to the plate (LOOMIS 1975). Therefore, the entire array of mutants was tested for possible synergistic effects by growing the mutant strains in the presence of heterokaryon-incompatible, female-fertile strains. A *col-4 per-1(PBJ-1) a* strain was inoculated in the middle of a plate at the same time as the mutant (mating type *A*) was inoculated at the edge. The *col-4* marker restricts growth so that the edges of the two mycelia meet at a distinct region in the center of the plate; *per-1* serves as a perithecial color marker to distinguish which of the two strains has served as the maternal parent (JOHNSON 1974; HOWE and BENSON 1974; HOWE and JOHNSON 1976). The *a* mating type serves two functions: (1) as both the male and female parent of the *A* strain and (2) as a heterokaryon-incompatibility factor that inhibits functional hyphal fusion (this was necessary because earlier complementation tests had shown that 30 of the mutants are recessive in heterokaryons). No synergistic effects were observed under these conditions. All black perithecia were of the expected mutant phenotype, while the light-colored perithecia were fecund.

Relationship of female-sterile mutants to mating type

The developmental mutants selected from *pyr-3* wild type were checked to see whether any of these mutations might be at or tightly linked to the mating-type locus. Only PBJ6 was linked to mating type, at a distance of 16 map units. Furthermore, none of the mutants were mating-type specific; all also blocked development when in the *a* mating type. Thus, there is a noticeable absence of any association of these developmental mutants with mating type.

DISCUSSION

I have isolated 76 mutant strains of *Neurospora crassa* that are altered in their production of perithecia when used as the female component of a cross. Of these, 33 are absolute female steriles and fail to produce ascospores within two weeks after crossing under conditions in which the fertile parental strains always form and eject ascospores. The remaining 43 strains may include examples of leaky mutations in the sexual cycle or in some phase of the sexual cycle, as well as examples of other types of perithecial developmental mutants reported in the

literature, *e.g.*, the scattered perithecial strains isolated by WHEELER and MCGAHEN (1952).

Since the mutants require Min + ur or ad for growth, all auxotrophs are immediately eliminated. A majority of the developmental mutants show either altered growth rates or altered vegetative morphology or both. Segregation analysis of a sample of these mutants indicated that in some cases (PBI8, PBJ3, PBM8, PBN4, PBS7, PBS8) slow growth and female sterility are linked, while in another case (PBT3) the phenotypes were separable by recombination. There is no obvious correlation between growth rates and the position of developmental arrest. None of the mutants in these crosses showed any genetic or functional linkages to the mating-type locus, with the exception of PBJ6, which is located at a distance of 16 map units from mating type. This is quite different from the situation in both yeasts and in Basidomycetes, where a number of developmental mutants map into the mating-type loci and/or are mating-type specific (HAWTHORNE 1963; RAPER and RAPER, 1973; MACKAY and MANNEY 1974a, b; MEAD and GUTZ 1976). All of the mutants except PBJ6 have no effect on perithecial development when used as the male parent. This observation is not surprising since most, if not all, of the perithecial tissue proper (as opposed to the ascospores) is derived solely from the female parent (HOWE and BENSON 1974; JOHNSON 1974, 1975; HOWE and JOHNSON 1976). However, the function blocked in PBJ6 seems to be required in both the male and female parents for successful crossing (JOHNSON 1975, 1976a, and manuscript in preparation).

An attempt was made to determine whether mutants might show tissue-specific defects and to locate the tissues in which the mutant might be defective. Mosaics were generated and analyzed to see whether the mutant nucleus failed to participate in the development of any particular tissue(s). No evidence for such a failure was found. Apparently the presence of female-fertile nuclei allows the mutant nuclei to participate in the formation of any perithecial tissue. This observation may be due either to nonautonomy of the mutant defects [*per-1*, the marker used in these experiments, has been shown to be at least partially cell autonomous (JOHNSON 1976b)] or to the presence of multiple nonsister nuclei in most of the cells of the perithecium, rather than to a lack of tissue specificity in the mutants.

Another experiment to determine the temporal specificity of the developmental mutants involved measuring perithecial diameter at several points during development to see if mutant perithecia developed normally prior to their block points. All but ABT1 perithecia did develop normally.

Tests for dominance of these mutants are difficult to perform in Neurospora because the tests must be performed in heterokaryons where the ratios of the two mutant nuclear types can fluctuate widely. However, at least 30 of the mutants are recessive. Only ABH8 is clearly a dominant mutation.

Complementation tests suggest that the mutants fall into 29 distinct complementation groups. Two groups each contain two possibly nonindependent mutants, and one group contains three independently isolated mutants. This

latter group is interesting in that the three putative alleles (ABT1, PBJ3, and PBS4) block perithecial development at very different points. Attempts have been made to test this tentative assignment of allelism by recombinational analysis. In these tissues, a mutant is combined in a forced heterokaryon with a female fertile *per-1* (spore color) strain; this heterokaryon is then used as the female component of a cross to one of the other two putative alleles. Repeated attempts have so far failed to yield progeny from the cross of the two female-sterile nuclei; only the female-fertile component of the heterokaryon can be recovered. The evidence of allelism of these genes could be interpreted in several ways; for example: (1) the genes could be alleles of a "complex" locus such that different alleles affect development at different points; (2) the genes could be "*cis*-dominant"—they cannot be complemented by gene products from another nucleus, but appear recessive in complementation tests because the other nucleus provides all the gene products needed for development; (3) the genes could code for proteins required in high quantities; when one nucleus in a heterokaryon is defective in these genes the entire culture would not be able to make enough of this important gene product. The fact that ABT1 is the only mutant that fails to develop perithecia at wild-type rates prior to the block point may also have some bearing on the interpretation of these tests for allelism.

The best estimates for the total number of genes that are necessary only for perithecial formation are between 200 and 400. These numbers were obtained by assuming that the mutations represent independent mutational events; each gene is equally likely to be hit by a given event. These numbers are not accurate because this assumption is clearly not true for other systems that have been examined in detail.

The protoperithecial stage appears to be the part of the female developmental cycle most sensitive to mutational blocks. At least twelve mutants produce no protoperithecia. No other clustering of block points for different mutants was observed, suggesting that the developmental sequence from 0 to 60 hours is uniformly sensitive to the action of mutant alleles. No mutants that blocked development later than 60 hours were obtained. One possible explanation for this is that the screen used to isolate these mutants may have been fairly ineffective in detecting mutants blocked late in perithecial development. This is unlikely, however, since new mutant hunts using *per-1* instead of *per-1*$^+$ as the parental strain from which the developmental mutants were selected also failed to yield a different spectrum of mutations. In the *per-1* hunts, very late blocks (after 60 hours and up to 260 hours after crossing) should have been detectable (JOHNSON 1975). Of course, this lack of clustering of mutant block points does not necessarily mean that new gene functions are being turned on uniformly throughout later time periods. The time of mutant gene function must be prior to and may be well before the point at which the development of the mutant is blocked; moreover, the size of the perithecium may not have an integral relationship to the time of block. These arguments seem unlikely for four reasons. (1) The protoperithecia produced by these mutant strains begin perithecial development normally and

proceed normally until the point at which the mutants are blocked (Figure 7). (2) Ordering the block points either by mean diameters or by diameters at the ninetieth percentile gives nearly identical ordering (Figure 5). (3) Diameters of perithecia remain uniform when varying numbers of conidia are used to make a cross [unlike the parental strains that show a two-fold increase in perithecial diameters under similar conditions (JOHNSON 1975)]. (4) Preliminary observations on the intensity of pigmentation, the number of perithecial hyphae present on the surface of the perithecium, the extent of development of the internal tissues, and the quantities of a stage-specific protein (SRB, NASRALLAH and BASL 1973; NASRALLAH, personal communication) are compatible with the theory that the mutations uniformly block all further perithecial development. These facts argue that the diameters of the blocked perithecia are genetically determined and that the size of the perithecium and the other developmental parameters which are occurring are integrally related to each other. These observations would not necessarily be expected if the developmental gene product were needed at an earlier stage of development.

Obviously, temperature-sensitive mutations would allow the direct determination of the time of action of the mutant gene product because the mutant could be shifted between permissive and nonpermissive conditions that would allow the delimitation of the temperature-sensitive period. Attempts to revert a few of these mutants to temperature-sensitive forms are planned.

With the exception of PBR3 and PBS9, the diameters of the perithecia produced by these mutants show a smaller variance than the parental strains (Table 2). This would be expected if all but these two strains had specific blocks in the developmental process that would cause less variance than the nonspecific blocks that keep many perithecia in fertile strains from developing. Thus, the limited data give no evidence for the clustering of mutant block points that would be expected if entire new batteries of genes were being turned on simultaneously during development.

I would like to thank DAVID STADLER, BENJAMIN HALL, ADRIAN SRB, MARY BASL, and ANNA GENG for many helpful discussions. ADRIAN SRB, DAVID STADLER, and DEBRA FERGUSON were most helpful in the preparation of the manuscript. This work was supported by Public Health Service Predoctoral Fellowship GM-47532, Public Health Service Training Grant GM-01035, and Public Health Service Grant GM-12953.

LITERATURE CITED

BHATTACHARYA, L. and J. F. FELDMAN, 1971 A rapid screening procedure for female-sterile mutants. Neurospora Newsl. **18:** 11.

BRODY, S. and E. L. TATUM, 1966 Phosphoglucomutase and morphological changes in *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S. **58:** 923–930.

DODGE, B. O., 1935 The mechanics of sexual reproduction in Neurospora. Mycologia **27:** 418–438. ———, 1946 Self-sterility in "bisexual" heterokaryons of Neurospora. Bull. Torrey Botan. Club **73:** 410–416.

FITZGERALD, P. H., 1963 Genetic and epigenetic factors controlling female sterility in *Neurospora crassa*. Heredity **18:** 47–62.

HAWTHORNE, D. C., 1963 A deletion in yeast and its bearing on the structure of the mating type locus. Genetics **48**: 1727–1729.

HO, C. C., 1972 Mutations blocking development of the protoperithecium in Neurospora. Neurospora Newsl. **19**: 15–16.

HOROWITZ, N. H., M. FLING, H. L. MACLEOD and N. SUEOKA, 1960 Genetic determination and enzymatic induction of tyrosinase in Neurospora. J. Mol. Biol. **2**: 96–104.

HOWE, H. B., JR. and E. W. BENSON, 1974 A perithecial color mutant of *Neurospora crassa*. Mol. Gen. Genet. **131**: 79–83.

HOWE, H. B., JR. and T. E. JOHNSON, 1976 Phenotypic diversity among alleles at the *per-1* locus of *Neurospora crassa*. Genetics **82**: 595–603.

ITOH, T. and K. MORISHITA, 1971 Effects of cytoplasm on protoperithecium formation in *Neurospora crassa*. Japan. J. Genet. **46**: 7–15.

JOHNSON, T. E., 1974 Some mutations affecting perithecial and spore pigmentation. Neurospora Newsl. **21**: 19–20. ——, 1975 Perithecia development and pattern formation in *Neurospora crassa*. Ph.D. Thesis, University of Washington, 210 pages. Order No. 75–28,369. ——, 1976a Analysis of a perithecial developmental mutant in *Neurospora crassa* that is male and female sterile. Genetics **83** (abstr.): s36–37. ——, 1976b Analysis of pattern formation in Neurospora perithecial development using genetic mosaics. Devel. Biol. **54**: 23–36.

LOOMIS, W. F., JR., 1975 *Dictyostelium discoideum, A Developmental System*. Academic Press, New York.

MACKAY, V. and T. R. MANNEY, 1974a Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. I. Isolation and phenotypic characterization of nonmating mutants. Genetics **76**: 255–271. ——, 1974b Mutations affecting sexual conjugation and related processes in *Saccharomyces cerevisiae*. II. Genetic analysis of nonmating mutants. Genetics **76**: 273–288.

MEAD, J. H. and H. GUTZ, 1976 Mating-type mutations in *Schizosaccharomyces pombe*: Isolation of mutants and analysis of strains with an h^- or $h+$ phenotype. Genetics **83**: 259–273.

MITCHELL, M. B., 1966 A round-spore character in *Neurospora crassa*. Neurospora Newsl. **10**: 6.

MITCHELL, M. B. and H. K. MITCHELL, 1952 A case of "maternal" inheritance in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **38**: 442–449.

MITCHELL, M. B., H. K. MITCHELL and A. TISSIERES, 1953 Mendelian and non-Mendelian factors affecting the cytochrome system in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **34**: 606–613.

MURRAY, N. E., 1965 Cystine mutant strains of Neurospora. Genetics **52**: 801–808.

MYLYK, O. M., 1971 A genetic study of female sterility in *Neurospora crassa*. Ph.D. Thesis, McMaster University.

MYLYK, O. M. and S. F. H. THRELKELD, 1974 A genetic study of female sterility in *Neurospora crassa*. Genet. Res. **24**: 91–102.

NORBY, S., 1970 A specific nutritional requirement for pyrimidines in rudimentary mutants of *Drosophila melanogaster*. Hereditas **66**: 205–214.

RADFORD, A., 1971 Attempts to restore fertility to *pyr-3* inter-allelic crosses in Neurospora. Microbial Genet. Bull. **33**: 15.

RAPER, J. R. and C. A. RAPER, 1973 Incompatibility factors: Regulatory genes for sexual morphogenesis in higher fungi. In: *Basic Mechanisms in Plant Morphogenesis*. Brookhaven Symp. in Biol., **25**: 19–39.

RYAN, F. J., 1950 Selected methods of Neurospora genetics. Methods Med. Res. **3**: 51–75.

SRB, A. M., 1957 Exotic growth forms in Neurospora. J. Heredity **48**: 146–153.

SRB, A. M., J. B. NASRALLAH and M. BASL, 1973 Genetic control of the development of the sexual reproductive apparatus of Neurospora. In: *Basic Mechanisms in Plant Morphogenesis.* Brookhaven Symp. in Biol. **25**: 40–50.

STADLER, D. R., 1956 A map of linkage group VI of *N. crassa.* Genetics **41**: 528–543.

TAN, S. T. and C. C. Ho, 1970 A gene controlling the early development of protoperithecium in *Neurospora crassa.* Molec. Gen. Genet. **107**: 158–161.

TERENZI, H. F. and J. L. REISSIG, 1967 Modifiers of the *cot* gene in Neurospora: the gulliver mutants. Genetics **56**:321–329.

VIGFUSSON, N. V., 1969 Sterility mutants in *Neurospora crassa.* (Abstract) Can. J. Genet. Cytol. **11**: 483.

VIGFUSSON, N. V. and J. WEIJER, 1972 Sexuality in *Neurospora crassa.* II. Genes affecting the sexual development cycle. Genet. Res. **19**: 205–211.

VOGEL, H. J., 1964 Distribution of lysine pathways among fungi: Evolutionary implications. Am. Naturalist **98**: 435–446.

WARREN, A. J., W. D. WARREN and E. C. COX, 1976 Genetic and morphological study of aggregation in the cellular slime mold *Polysphondylium violaceum.* Genetics **83**: 25–47.

WEIJER, J. and N. V. VIGFUSSON, 1972 Sexuality in *Neurospora crassa.* I. Mutations to male sterility. Genet. Res. **19**: 191–204.

WESTERGAARD, M. and H. HIRSCH, 1954 Environmental and genetic control of differentiation in Neurospora. Proc. Symp. Colston Res. Soc. **7**: 171–183.

WESTERGAARD, M. and H. K. MITCHELL, 1947 Neurospora V. A synthetic medium favouring sexual reproduction. Am. J. Bot. **34**: 573–577.

WHEELER, H. E. and J. W. MCGAHEN, 1952 Genetics of Glomerella. X. Genes affecting sexual reproduction. Am. J. Bot. **39**: 110–119.

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