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The isolation of mutants affecting ascus development in *Neurospora crassa* and their analysis by a zygote complementation test

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The mutant gene *peak-2* (*pk-2*) in *Neurospora crassa* is located in linkage group V. In the haploid vegetative mycelium it determines dichotomous branching and colonial growth habit. It also affects the sexual reproductive apparatus by determining abnormal, non-linear asci, for the most part eight-spored, in contrast to the linear, eight-spored asci of wild type. In this system, *pk-2* acts as a zygote recessive. That is, asci initiated by zygotes that are homozygous wild type (+/+), or are heterozygous (+/*pk-2*), are morphologically normal, showing the linear arrangement of ascospores; asci initiated by homozygous mutant zygotes (*pk-2*/*pk-2*) are morphologically abnormal, with the arrangement of ascospores differing markedly from linearity (Murray & Srb, 1959, 1962). Controlled reciprocal crosses have shown that heterozygotes develop normal ascus morphology whether the mutant or the wild type has been used as maternal parent (Srb, 1962). Maternal effects, therefore, do not offer a plausible alternative explanation to this apparent instance of zygotic control of ascus morphology. Although the ultimate differentiation of the ascus may be influenced by the meiotic products of the zygote and by their immediate mitotic derivatives, the zygote nucleus at least has a determinative influence on the initiation of ascus development. This conclusion is based on the cytological observation that a characteristic difference in the cellular shape of wild or *peak-2* asci is apparent even before meiosis begins.

The foregoing observations and their interpretation focus on the single diploid cell, the zygote, that is known to exist in the normal life cycle of *Neurospora*. Having, then, a genetic system at least partly under the control of the diploid nucleus, the possibility arises for utilizing some of the procedures of diploid genetic analysis for carrying out studies of the ascus of *Neurospora*. The most obvious possibility is that of diploid complementation analysis. Specifically, if one has two abnormal ascus mutants of independent origin (e.g. mutant-a and mutant-b), and if each acts as a zygote recessive in matings with wild type, then the mating giving a zygote *mut-a*/*mut-b* should provide a test for functional identity or non-identity of the pertinent genetic material. The initial test would be exceedingly simple—mere observation for the appearance of normal or abnormal asci. Normal asci would indicate complementation and be interpreted as the consequence of functional non-identity. Thus, in principle, the zygote complementation scheme just

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proposed would permit a rapid, technically uncomplicated screening for functional non-identity of large numbers of mutants, the effects of which are seen in an important developmental genetic system, the sexual reproductive apparatus of *Neurospora*. An experimental test of the scheme requires that numbers of recessive abnormal ascus mutants be available. The present report describes the isolation of such mutants and their analysis by means of zygote complementation studies.

2. MATERIALS AND METHODS

(i) *Strains of* Neurospora *and culture techniques*

The wild-type strains of *N. crassa* subjected to mutagenic treatment for the production of mutants were the St Lawrence laboratory strains 74*A* and 77*a*, further inbred in our laboratory. These same wild types were used in making crosses with the mutants. The mutant *peak-2*, used as a standard of reference for other, subsequently isolated abnormal ascus mutants, was first described by Murray & Srb (1959), and has been shown to be allelic with *biscuit* (Perkins, 1959). Culture techniques and the complete and minimal media were essentially those of Beadle & Tatum (1945). Crosses were made on slants of Difco corn-meal agar. Platings either of conidia or of ascospores were made on complete medium solidified with agar where the concentration of sucrose was 1·5 % and of sorbose 1·0 %.

(ii) *Mutagenic treatments*

The cells subjected to mutagenic treatment were conidia harvested from 6·5-day wild-type cultures that had been grown on solidified minimal medium. A variety of mutagens was used for the induction of morphological mutants. The mutagens and the conditions of treatment were as follows: ultraviolet light, source Sylvania Germicidal lamp, 40 cm distance, 10–15 min exposure; X-rays from Picker source operated at 220 kV and 15 mA, filtered by 0·50 mm copper and 0·50 mm aluminum, 48 000 r at 2300 r/min dose; β-propiolactone, 0·1 % H_2O solution, 15–17 min exposure; ethane methane sulphonate, 3·0 % in phosphate buffer at pH 8, 1–2 h exposure; dimethyl sulphate, 0·1 % H_2O solution, 10–12 min exposure; *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine, 15 μg/ml H_2O, 2–2·5 h exposure. Conidia exposed to chemical mutagens were washed 2–3 times in distilled H_2O following treatment.

(iii) *Procedure for isolating abnormal ascus mutants and subjecting them to the zygote-complementation test*

No way of screening for abnormal ascus mutants *per se* occurred to us. But inasmuch as pre-existing mutant *peak-2* is characterized by tight colonial growth of the vegetative mycelium, the decision was to attempt to screen for colonial mutants. The hope was that a fair number of colonial mutants might have the abnormal ascus property, but the realization was that many might not. Screening was based upon the observation that, when germinated in standard liquid media in shake culture, conidia of *peak-2* produce colonies roughly in the form of microspheres, whereas under the same conditions wild-type conidia produce colonies of

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larger, less densely packed hyphal aggregates. The process of screening was a simplification of the filtration scheme of Woodward, DeZeeuw & Srb (1954), the principle in the present instance being that an appropriate mesh might pass the microspheres representing colonial mutants while tending to keep back the wild-type mycelia. In practice, mutagenically treated wild-type conidia were incubated in shaking 125 ml. Erlenmeyer flasks containing 20 ml of minimal medium. Periodically, over a span of 24 h and at times dictated by the appearance of visible growth, hyphal clusters were filtered off through a double layer of cheese cloth. The bulk of experiments were carried out at *c*. 25C. At the end of the filtration period, the conidia and small hyphal aggregates remaining in the filtrate were

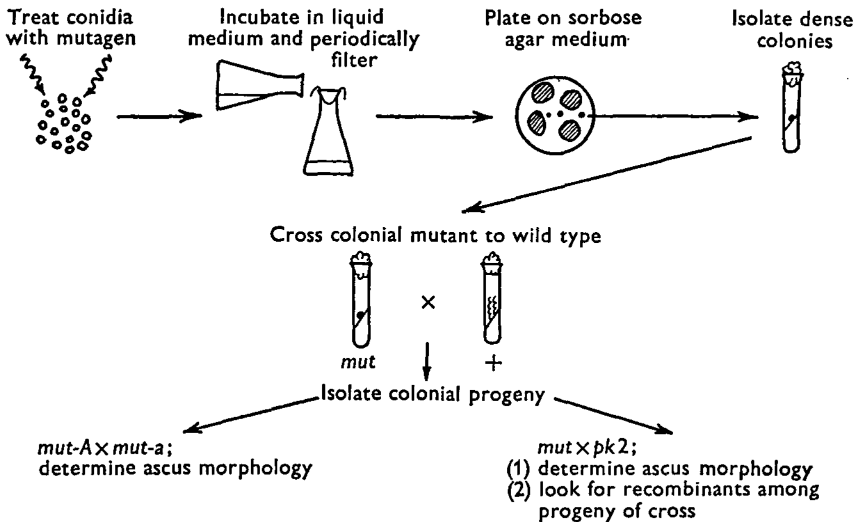

Fig. 1. Summary of procedures for the isolation and preliminary classification of abnormal ascus mutants in *Neurospora crassa*.

plated on sorbose-containing complete medium. Then after 18–20 h, the colonies appearing on the plates were inspected under a stereoscopic microscope for the presence of morphological mutants of the colonial type. On sorbose-containing media, such mutants, when compared to wild type, appear as tighter, denser colonies, or are sometimes recognizable by other variant morphological properties. In fact, all visibly deviant colonies were isolated for further study. Since the mutants sought are visibly detectable, a practicable efficiency for screening demands nothing approaching total elimination of wild types by filtration. Thus, in contrast to the comparable scheme for isolating auxotrophs, the method used here may be carried out with a reduced period of time for filtrations, with favourable consequences for conidial viability. Visible variants were picked into individual culture tubes containing agar complete medium.

The essential first step after isolation of a presumptive mutant is to cross it with wild type. Several purposes are served: (1) results of the cross establish whether the

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variant is genetic; (2) possible heterokaryosis in the initial variant picks is resolved by isolation through an ascospore; (3) inspection of the morphology of the asci arising from the cross with wild type permits the detection of dominant mutants for abnormal ascus; (4) for a given mutant, cultures of each mating type may be recovered, thus permitting a cross to obtain zygotes that are homoallelic for the mutant.

A second step, which is not essential but that has proved useful, is to cross the presumptive mutant with *peak-2*. Inspection of asci from such a mating reveals whether or not the mutant complements *pk-2*, which is used as the initial standard of reference for abnormal ascus mutants. A sample of progeny of the mating, obtained by plating ascospores, tells whether the newly isolated mutant recombines readily with *pk-2*.

The third step, taken after a cross of the presumptive mutant with wild type and after isolation of mutant progeny, is to cross mutant progeny of opposite mating type in order to obtain homoallelic zygotes. Inspection of the asci that develop from these zygotes reveals whether an abnormal ascus mutant has been found. The basic steps in the procedure for isolating abnormal ascus mutants and analysing them by the zygote complementation test are summarized in Fig. 1.

Finally, when recessive abnormal ascus mutants have been identified and verified, they are involved in a matrix of pairwise crosses with other independently isolated mutants. A cross between recessive abnormal ascus mutants that produces linear asci from the zygotes is designated as showing complementation; a cross where the zygotes produce abnormal asci is designated as non-complementing.

3. RESULTS

(i) *Preliminary analysis of the mutant isolates*

Following mutagenic treatments of wild-type conidia, 2372 mutants showing colonial type morphology of the vegetative mycelium were isolated. The results of the prelimminary tests to determine whether the mutant genes also affected morphology of the ascus are summarized in Table 1. Among those mutants that could be carried through each of the steps in preliminary analysis, 637 gave linear asci when homoallelic zygotes were produced and also gave linear asci in the cross with *pk-2*. These mutants were of no further interest for present purposes and were set aside. Aberrant asci were produced by 156 mutants both in the cross that gave rise to homoallelic zygotes and in the cross with *pk-2*. These mutants were classified as aberrant ascus mutants functionally indistinguishable from *pk-2* by the zygote complementation test. For twenty-eight mutants the results showed linear asci in the cross with *pk-2*, but abnormal asci arising from the homoallelic zygotes. These mutants, therefore, are abnormal ascus mutants functionally distinguishable from *pk-2* by the zygote complementation test. In a large number of instances crosses of mutant × mutant were infertile, with the consequence that the effect of the homoallelic zygote on ascus development could not be tested. In seventy-five of these instances, the cross of mutant × *pk-2* produced morphologically aberrant

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asci. Presumably these mutants are abnormal ascus mutants functionally indistinguishable from *pk-2* by the complementation test. After the cross of each of these mutants with *pk-2*, progeny ascospores were plated. No wild-type colonies were found. Thus the mutant genes fail to recombine with *pk-2* or do so only at a low frequency, and the case for allelism is strong. Finally, in 1476 instances where the cross mutant × mutant was infertile, the cross with *pk-2* gave linear asci. Mutants in this category either are not abnormal ascus mutants or they are abnormal ascus mutants that complement with *pk-2*.

Table 1. *The search for abnormal ascus mutants of* Neurospora crassa: *results of tests of 2372 mutants having morphological abnormalities of the vegetative mycelium*

mut × *mut* (linear); *mut* × *pk-2* (linear)	637
mut × *mut* (non-lin.); *mut* × *pk-2* (non-lin.)	156
mut × *mut* (non-lin.); *mut* × *pk-2* (linear)	28
mut × *mut* (infert.); *mut* × *pk-2* (non-lin.)	75
mut × *mut* (infert.); *mut* × *pk-2* (linear)	1476

Each mutant was subjected to each of two tests. The cross mutant × mutant permits inspection of asci formed from homoallelic mutant zygotes; the cross mutant × *peak-2* gives asci that can be scored for complementation or the lack of it between *pk-2* and the mutant being tested. In the tabulated results, *mut* is a mutant gene under test, *pk-2* the pre-existing recessive abnormal ascus mutant used as a standard of reference. The three categories of result for a given cross are: linear = linear, or normal, asci produced; non-lin. = non-linear, or aberrant, asci produced; infert. = infertile cross, no asci produced.

Table 2. *The search for abnormal ascus mutants of* Neurospora crassa: *results of efforts to obtain asci from homoallelic zygotes for* 240 *mutants that had been infertile under routine procedures*

mut × *mut* (linear)	45
mut × *mut* (non-lin.)	46
mut × *mut* (infert.)	149

Each of the 240 mutants chosen for retesting had given linear asci in a cross with *pk-2*. The conventions used for summarizing the data are the same as in Table 1.

Special efforts were made to resolve the ambiguity of the large category of mutants that gave linear asci when crossed to *pk-2* but that produced no asci at all when crosses were made to test the effect of homoallelic zygotes. From this category, 240 mutants were chosen for further study. Parental cultures were re-isolated after crosses with wild type, and the cross mutant × mutant was attempted on various media and under various conditions thought to favor fertility. No generally applicable formula was found for achieving crosses with mutants that previously had been recalcitrant. The results are summarized in Table 2. For 149 of these mutants all efforts to obtain asci from homoallelic zygotes failed. Among the remainder, 45 of the mutants gave linear asci when homoallelic zygotes were produced. These mutants were discarded since they are not abnormal ascus mutants. The remaining 46 mutants gave aberrant asci from homoallelic zygotes. Since they produced linear asci in crosses with *pk-2*, they are abnormal ascus mutants that complement *pk-2*.

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(ii) *Special categories of mutants*

In the course of testing abnormal ascus mutants, three were found to behave as dominants. They failed to complement with *pk-2* or with any other abnormal ascus mutants. In crosses with wild type (producing + /*mut* zygotes), abnormal asci were produced. Results of recombination studies indicate that these dominants result from mutations at the *peak* locus rather than at some other locus at which mutation may produce abnormal ascus mutants. The dominance of these mutants is not complete, since within a perithecium arising from a cross wild type × dominant *peak* some heterozygotes give rise to linear and others to aberrant asci. When crosses are made using pairs of parents chosen either from normal or aberrant asci, the results are essentially the same. The results indicate that at least for the most part the variation in phenotype within a perithecium does not have a genetic basis.

A very small fraction of the colonial mutants turned out to be temperature sensitive, having a markedly colonial growth habit at 30 C and above, and a normal or nearly normal growth habit at 25 C. Among these colonial mutants, some give ascus abnormality as well as abnormality of the vegetative mycelium. Others do not.

(iii) *The establishment of complementation groups*

In order to test further the functional identity or non-identity of those mutants that had been established as recessive abnormal ascus mutants, a large sample of the mutants was selected for crossing in all pairwise combinations. Again, production of normal asci by a crossed pair was taken as evidence of complementation, production of aberrant asci as non-complementation. The summarized results are seen in Table 3, where the matrix indicates the existence of seven complementation groups. Sufficient genetic analysis has been carried out to show that when members of different groups are hybridized, wild-type recombinants are found among the progeny and that at least some of the complementation groups are located in different chromosomes. A more precise analysis waits to be done.

Table 3. *Assignment of recessive abnormal ascus mutants of* Neurospora crassa *into functional groups by means of the zygote complementation test*

The matrix summarizes the results of crossing 197 mutants in all pairwise combinations. The roman numerals for designating the complementation groups are arbitrarily assigned, but 'I' designates the group in which *pk-2* is included. The arabic numerals in parentheses show the number of independently isolated mutants belonging in the groups. (NL = non-linear asci; L = linear asci.)

	I (179)	II (12)	III (1)	IV (1)	V (1)	VI (1)	VII (2)
I	NL	L	L	L	L	L	L
II	—	NL	L	L	L	L	L
III	—	—	NL	L	L	L	L
IV	—	—	—	NL	L	L	L
V	—	—	—	—	NL	L	L
VI	—	—	—	—	—	NL	L
VII	—	—	—	—	—	—	NL

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Between members of different complementation groups, complementation is complete, in so far as can be told by the observational test applied. In other words, the asci produced by a complementing pair of mutants present the same morphological picture as those produced by a cross of wild type × wild type or of wild type × recessive mutant. More refined tests, of course, might reveal incomplete complementation. Within the large complementation group of which *pk-2* is a member, incomplete complementation is shown by a small number of hybrid combinations. In such instances the perithecia include a mixture of linear and non-linear asci, as found when a dominant *peak* is crossed with wild type. Here again, preliminary hybridization experiments indicate that for the most part the mixture is not based on genetic variability. Presumably these mixed perithecia represent the effects of intragenic complementation operating near the threshold of a morphogenetic response. Apparent intragenic complementation in this system will be reported in detail in a separate publication.

Although the data in Table 1 include abnormal ascus mutants isolated after treatments of wild type with each of the mutagens listed in Materials and Methods, only mutants isolated in the series of experiments with chemical mutagens have been sufficiently tested to be included in the matrix of Table 3. In its present stage, the work provides no basis for attempting correlations between the complementation properties of a mutant and the mutagen that induced it.

4. DISCUSSION

The developing ascus is the most striking morphogenetic system in *Neurospora*. In it goes on the majority of specifically identifiable events in the sexual reproductive process. That it has been the object of selection and other modifying influences of evolution is seen, for example, in the characteristic differences between the asci of *N. crassa* and *N. tetrasperma* (Dodge, 1927). The results reported here show the possibility of accumulating for study at least one category of mutants representing loci involved in the development of the ascus. The results show further that the mutants may be assigned to functional groups on the basis of a zygote complementation test, and that at least seven such groups exist.

Although various observations, including ones by Dodge, Singleton & Rolnick (1950) and by Mitchell (1966), implicate activity by the zygote nucleus in the development of the ascus, they do not preclude supplementary activity by the meiotic products of the zygote and their immediate mitotically derived daughter nuclei. In any case, the zygote complementation studies emphasize the existence in the developing ascus of a regular set of relationships of dominance and recessiveness in the genetic material. At this point, their relationships appear to be no less regular, or irregular, than those characterizing the genetic systems of organisms for which the majority of the life cycle is diploid. The presumption, therefore, must be that evolutionary processes act upon dominance relationships in the diploid cell of *Neurospora*.

The many abnormal ascus mutants isolated for this work may not, and almost

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certainly do not, provide a representative sample of genetic deviations affecting ascus morphology. The scheme for obtaining the mutants depends upon selecting for deviations in growth habit of the vegetative mycelium. The results have shown that not all colonial mutants produce asci with abnormal morphologies. The likelihood is that not all mutant genes that determine aberrant morphology of the ascus need have visible effects on the vegetative hyphae. In general, the mutants under study, including with certainty alleles of *pk-2*, have the attributes of the 'sorbose-type colonials' shown by de Terra & Tatum (1963) to have cell walls whose component polysaccharides differ from those characteristic of wild type. Even if subsequent study were to show that all the mutants reported here were cell-wall mutants, one still could readily visualize morphogenetic components in addition to the wall as being involved in the development of the ascus. Nevertheless, the present material affords a promising start for analysis of genetic participants in ascus morphogenesis.

Even confining oneself to those mutants provided by the selection device that was employed, questions arise about the representativeness of certain of the information. The most obvious gap in knowledge arises because asci from homoallelic zygotes could not be obtained for a large fraction of the mutants. Whether the infertility of certain crosses designed to obtain homoallelic mutant asci is due to the effects of large deletions, or to other cause, remains to be determined. Thus, at this time the reason for the disproportionate number of mutants falling into the complementation group including *pk-2* is not readily determinable. The disproportion, however, cannot be due solely to the procedural step that permits early classification of mutants that fail to complement *pk-2*.

Finally, since the wild-type strains of *N. crassa* produce conidia the majority of which are multinucleate, the assumption is that many induced mutant nuclei are not recognized by the screening process because of their occurrence in heterokaryotic cells. Perhaps the majority of mutant isolates comes from the 20 to 30 % of conidia that in our wild-type strains are uninucleate. Whether this situation induces a bias on the kinds as well as numbers of mutants recovered after mutagenic treatment is not clear. On *a priori* grounds, the expectation is that dominant mutants would be in a more favourable position for recovery under the conditions of the mutation experiments. In fact, dominants were isolated very infrequently by comparison with recessive abnormal ascus mutants, suggesting that the actual frequency of occurrence of dominant mutants is relatively low in this system. A pertinent question is whether mutants that are dominant with respect to ascus morphology are dominant also in a heterokaryotic system in the vegetative mycelium.

SUMMARY

A large number of mutants that affect the morphology of the ascus of *Neurospora crassa* has been isolated by means of a screening technique that utilizes the filtration principle to separate colonial type mutants from wild type. In relationships with each other and with wild-type alleles, the mutant genes exhibit zygote

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dominance and recessiveness. A zygote complementation test for placing the recessive mutants into functional groupings is described. Results obtained by use of the test place the mutants into seven functional groups. Dominance relations in the developing ascus of *Neurospora* are discussed.

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