STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. V. EVIDENCE FOR ALLELIC COMPLEMENTATION AMONG ad-3B MUTANTS

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PREVIOUS studies (DE SERRES 1956, DE SERRES and KØLMARK 1958) with the class of phenotypically purple, adenine-requiring mutants of Neurospora have shown that such mutants arise as a result of mutation in either one of two separate loci, *ad-3A* and *ad-3B*, on the right arm of linkage group I. The genotype of new purple adenine mutants can be determined most readily by means of heterokaryon tests. Additional tests have shown that all mutants recovered from forward-mutation experiments on wild-type strain 74A are located at one of these two loci (DE SERRES, KØLMARK and BROCKMAN, unpublished). Furthermore, *ad-3A* and *ad-3B* mutations must result from alterations in different functional units or "cistrons" (BENZER 1957), since both heterokaryons in the *cis-trans* test grow at wild-type rate. Whether these two groups of mutants affect the same or different steps in purine biosynthesis has not as yet been determined, but it has been shown recently (BUCHANAN 1960, BERNSTEIN 1961), that both groups are blocked in the conversion of 5-aminoimidazole ribonucleotide (AIR) to 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide (succino-AICAR), a two-step reaction with 5-amino-4-imidazolecarboxylic acid ribonucleotide (carboxy-AIR) as the intermediate (LUKENS and BUCHANAN 1959).

Heterokaryon tests for allelic complementation among samples of X-ray-induced *ad-3A* or *ad-3B* mutants indicated that this phenomenon was either infrequent, or did not occur (DE SERRES 1956, 1960). However, other investigators have shown that the percentage of mutants showing allelic complementation is dependent on their mutagenic origin (CASE and GILES 1960, ISHIKAWA 1961), and that this percentage varies markedly even among mutants of identical mutagenic origin at different loci (CATCHESIDE 1960). Because of this we have begun more extensive tests on larger samples of *ad-3* mutants obtained by a variety of different mutagenic treatments. In the experiments reported in this paper, evidence for allelic complementation has been obtained in heterokaryon tests involving all possible pairwise combinations of 161 *ad-3B* mutants of spontaneous, X-ray and ultraviolet origin.

MATERIALS AND METHODS

The 161 *ad-3B* mutant strains used in the present tests were derived from the

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St. Lawrence wild-type strain 74A, or isogenic derivatives, at Yale University. Spontaneous or induced mutants were obtained by the filtration-concentration method (WOODWARD, DE ZEEUW and SRB 1954). Some mutants (experiment numbers 152, 155, 175, 176, and 230) were obtained from DR. M. E. CASE, Yale University. The origin and number of the mutants is given in Table 1. The mutant identification systems in previous publications (DE SERRES 1956, 1958) proved to be completely impractical for identifying the numerous *ad-3* mutants in our present collection. In the revised identification scheme, the wild-type strain used in the forward-mutation experiment is specified in the first term (74A Yale = 1), the experiment number in the second, and the isolate number in the third term.

Conidia from single colony isolates obtained from platings of the original isolates were used in all experiments. However, 35 *ad-3B* mutants from experiment 175 were induced in a mating type *a* derivative of 74A, and the mating type *A* isolates used were obtained from a cross of the original mutants to 74A.

RESULTS

Heterokaryon tests in liquid minimal medium: All possible pairwise combinations of 161 *ad-3B* mutants were made in duplicate in 10 × 75 mm test tubes in liquid Fries Number 3 minimal medium (BEADLE and TATUM 1945) and incubated at 24° to 26°C. Conidial suspensions ranged from 5 to 50 × 10^6 conidia/ml, and 1 ml of each suspension in minimal medium was added to each tube. Observations were made over a period of 14 to 21 days to permit detection of delayed positive responses. The general procedure developed during these experiments has been described elsewhere (DE SERRES 1962). In the present tests all combinations were made over a period of several months. From 1600 to 4900 tests were made in individual experiments. In this way all combinations were repeated and those showing positive reactions could be verified. A varying num-

TABLE 1

Mutagenic origin of ad-3B *mutants studied (Induced in 74A or isogenic derivatives)*

Mutagenic origin	Experiment number	Mutant numbers	Total of each type
Spontaneous	175	6, 138	3
		142	
Ultraviolet	176	43–48	
	230	50–110	25
X ray	83	2, 5, 10	
	112	1–12	
	152	68	
	155	3–55	
	175	13–2623	
	230	150–257	133
			161

ber of *ad-3A* mutants were also included to show that positive heterokaryon tests could be obtained in each experiment. The composite interaction matrix, with mutants grouped according to mutagenic origin, is given in Figure 1. Only 112 (0.4 percent) positive tests were obtained out of the 25,760 pairwise combniations involving different mutants. Furthermore, all positive tests involve one of the four "leaky" mutants (mutants showing a limited ability to grow initially on minimal medium). All leaky + leaky′ and all nonleaky + nonleaky′ mutant combinations showed no interaction under these test conditions. The interaction matrix defines a complementation map consisting of two complementation subgroups in the *ad-3B* cistron shown in Figure 2.

Genetic tests on the leaky mutants: That the positive tests should involve only leaky + nonleaky mutant combinations was somewhat unexpected. These combinations always showed growth earlier than any of the four leaky mutants alone, but since the leaky mutants can grow on minimal medium in the absence of conidia from other strains, experiments were designed to obtain formal proof of heterokaryon formation.

The growth characteristics of each of the four leaky mutants, 1–112–0008, 1–155–0011, 1–175–0229 and 1–230–0069 were studied first by inoculating growth tubes containing minimal medium with a conidial suspension from each

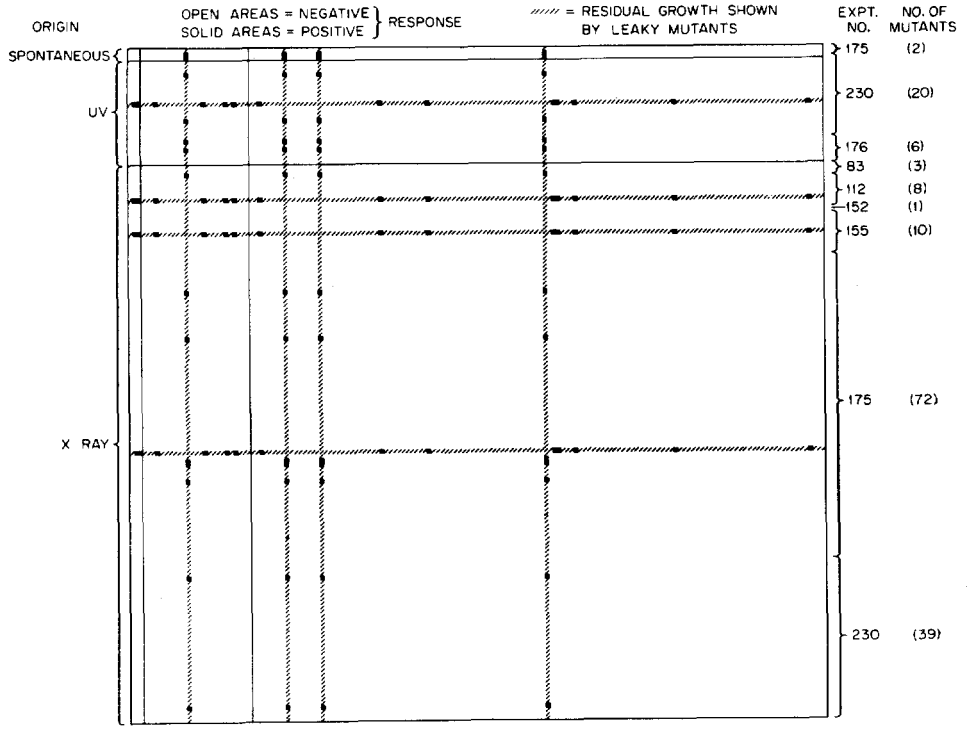

FIGURE 1.—Interaction matrix for all possible pairwise combinations of 161 *ad-3B* mutants in heterokaryon tests.

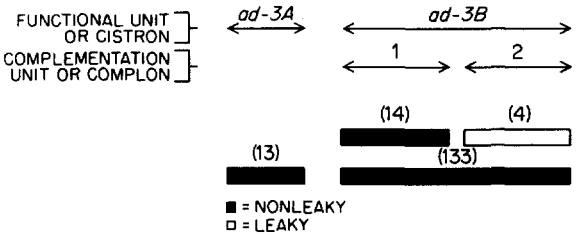

FIGURE 2.—Complementation maps of the *ad-3A* and *ad-3B cistrons* based on the present and previous (DE SERRES 1956, 1960) heterokaryon tests on the purple, adenine-requiring mutants of *Neurospora crassa.*

strain. Such tests showed that a variable lag period (directly portional to inoculum density) was followed by progressively more rapid growth until wild-type growth rates were finally attained. This change in the rate of growth was accompanied by a simultaneous loss of the purple pigmentation that is characteristic of *ad-3* mutants. Conidia from the *ends* of such growth tubes, however, inoculated into minimal medium showed the same lag period and initial slow rate of growth as the original inoculum. Such tests showed conclusively that the change in growth rate and loss of pigmentation was not due to reversion but to adaptation of the mutant of minimal medium.

Proof of heterokaryon formation in mutant combinations showing "positive tests": The more rapid growth observed in certain combinations with nonleaky mutants does not, in itself, constitute proof of heterokaryon formation. Since growth under such conditions could be due to other types of interaction, tests were made to obtain direct evidence for heterokaryon formation. For these experiments three cultures of the leaky mutant 1–112–0008 grown on minimal medium in pairwise combination with each of the nonleaky mutants 1–175–0108, 1–175–0244, and 1–230–0083, were selected for study. If heterokaryons had been formed, then conidia from these cultures should produce three types of colonies when plated in medium supplemented with adenine (with 25 to 50 conidia per plate): (1) the leaky adenine-requiring homokaryon, (2) the nonleaky adenine-requiring homokaryon, and (3) the adenine-independent heterokaryon. With this procedure, heterokaryotic colonies can arise only from heterokaryotic conidia and in no other way. Adenine-requiring single colony isolates can be further identified by means of heterokaryon tests with both the leaky and nonleaky "parental" strains. The analysis of 50 colonies isolated from conidial platings of each of these three cultures is given in Table 2. This analysis shows that the single colony isolates arise from (1) heterokaryotic conidia, and (2) conidia homokaryotic for either the leaky or nonleaky components. The more rapid growth of certain leaky mutant + nonleaky mutant combinations can be attributed to heterokaryon formation and to interaction of the differentially defective gene products in each component.

Properties of mutants in the same complementation subgroup in the ad-3B *cistron:* The heterokaryon tests show that the present group of 161 mutants can

TABLE 2

Number of colonies of each type obtained from plating conidia from combination of leaky mutant 1–112–0008 and three different nonleaky mutants showing "positive" heterokaryon tests on minimal medium

		Number of isolates of each colony type			
Leaky strain	Nonleaky strain	Leaky	Nonleaky	Heterokaryon	Total
1–112–0008	1–175–0108	18	7	25	50
1–112–0008	1–175–0244	4	25	21	50
1–112–0008	1–230–0083	19	10	21	50

be subdivided into at least three subgroups: the first with 133 noncomplementing mutants, a second with four leaky mutants, and a third group of 14 nonleaky mutants.

WOODWARD, PARTRIDGE and GILES (1958) have demonstrated a correlation between the distance separating two mutants on the complementation map and the time required for heterokaryon formation and growth. In general they found that there is a direct relation between the map positions of a given pair of mutants and the rate of response. In the analysis of *ad-3B* mutants, marked variability in the response time could be interpreted as presumptive evidence for a greater complexity in the organization of the *ad-3B* cistron than indicated by the interaction matrix of the present sample of mutants. Marked variation in the time of heterokaryon formation was observed between different combinations of mutants in the same experiment. However, since there could have been a ten-fold difference in the conidial concentration of individual suspensions in our experiments, the evidence for variation was not considered conclusive.

To obtain more precise data on these points, the behavior of individual mutant combinations was studied on Fries minimal medium in growth tubes. Suspensions of conidia from each strain were adjusted to the same concentration. A drop of suspension from leaky mutant 1–112–0008 and each of the 14 nonleaky mutants was used to make pairwise combinations on the agar surface at one end of each growth tube. All pairwise combinations were made in triplicate and the tubes were marked to record total growth at 24° to 26°C at daily intervals. The growth of these heterokaryons was compared with wild type 74A, and leaky mutant 1–112–0008 in combination with *hist-3* mutant 1–152–0009 and *ad-3A* mutant 1–112–0013. With a constant conidial inoculum the agreement among triplicate tubes was excellent and the mean growth rate for each heterokaryon over the total length of the growth tube is given in Table 3. Each heterokaryon eventually achieved wild-type growth rate and the time required was found to be specific for each combination. The data show a marked diversity in the mean growth rates; the combination 1–112–0008 + 1–175–0108 grows at least as rapidly as wild type or any of the nonallelic combinations, whereas the combination of the leaky mutant with 1–230–0253 grows only slightly faster than the leaky mutant itself. Additional experiments on the other three leaky mutants in com-

TABLE 3

Mean growth rate and time required for heterokaryons between various nonleaky ad-3B *mutants and leaky mutant 1–112–0008 to achieve wild type growth rate on minimal medium in growth tubes (24° to 26°C)*

Time (hours) to achieve wild type growth rate	Mean growth rate, mm/hour	Mutant in combination with 1–112–0008	
	4.36	1–175–0108	
0–24	4.09	1–175–0234	
	4.05	1–230–0083	
25–48	4.04	1–230–0098	
	3.98	1–175–0235	
	3.71	1–230–0138	
49–72	3.28	1–230–0096	
	3.01	1–175–0244	
	2.93	1–230–0150	
73–96	2.85	1–175–0142	
	2.56	1–083–0010	
	2.20	1–175–0063	
97–120	2.19	1–176–0044	
	2.05	1–230–0253	
121–144	1.97	1–112–0008	
		Controls	
	4.17	1–152–0009	*(hist-3)*
0–24	4.14	1–112–0013	*(ad-3A)*
	4.14	74A	(alone)

bination with the same 14 nonleaky mutants showed no significant difference in the behavior of these three strains from mutant 1–112–0008.

DISCUSSION

Correlation with mutagenic origin: One striking feature of the present experiments is the overall low incidence of mutant combinations showing allelic complementation. However, there is an apparent correlation between the mutagenic origin of the *ad-3B* mutants and the percentage showing positive tests in these experiments as follows: spontaneous, 67 (2/3), ultraviolet-induced, 20 (5/25); and X-ray-induced, 8 (11/133). The sample sizes of the first two classes of mutants are small, but the fact that a higher percentage of mutants showing allelic complementation was found among the UV-induced mutants parallels the data of CASE and GILES (1960). The correlation that they found between mutagenic origin and the percentage of *pan-2* mutants showing allelic complementation is as follows: spontaneous, 50 (3/6); ultraviolet-induced, 47 (8/17); and X-ray-induced, 23 (12/52). The very low percentage of complementing mutants among those of X-ray origin explains the negative tests obtained earlier on smaller samples of *ad-3B* mutants exclusively of X-ray origin (DE SERRES 1956).

The finding that at least one member of each pair of complementing *ad-3B* mutants is leaky is of particular interest. In many screening programs, it is not uncommon for mutants showing *any* growth on minimal medium to be discarded or set aside. If such mutants had been eliminated from the present sample, the evidence against allelic complementation among *ad-3B* mutants (no positive responses in all pairwise combinations of 157 nonleaky mutants) would have been overwhelming and this paper would have had a different title! Apparently not all leaky mutants show allelic complementation, however; none of the tests on various pairwise combinations of leaky *ad-3A* mutants have given positive tests (DE SERRES, unpublished).

Genetic tests on leaky mutants: The ability to detect complementation between many of the leaky-nonleaky mutant combinations has been enhanced by making heterokaryon tests in test tubes in liquid media. Each test tube is, in effect, a miniature growth tube and each combination can be observed for a period of several weeks. The marked differences between the type of growth and pigmentation of heterokaryon involving leaky mutants and leaky mutants alone under these conditions are not as marked or as readily reproducible on the surface of agar in petri plates (DE SERRES 1956). Furthermore, the delayed positive responses shown by some of the combinations could not have been detected with the older test conditions.

The mechanism of allelic complementation: Another noteworthy feature of the data is the marked diversity in the time of formation and the rate of growth of various mutant combinations. Of particular interest is the fact that there are three *ad-3B* mutant combinations that grow as fast as wild type or any of the nonallelic mutant combinations (Table 3). Futhermore, the growth of these combinations mimics that of the two nonallelic combinations and wild type in that growth commences with no significant lag. Thus, with some of these combinations of mutants there is clearly *no apparent difference in allelic* vs. *nonallelic complementation* with regard either to the time of heterokaryon formation or the subsequent rate of growth. Any hypothesis concerning the mechanism of allelic complementation must account not only for this similarity but also the extreme diversity in the time of heterokaryon formation, and the marked differences in the subsequent rates of growth.

The cistron concept: In the general application of the *cis-trans* test (LEWIS 1951) to other systems, pairwise combination of mutants in the *trans* configuration has often shown that mutations affecting the same enzyme have different functional defects. However, it is becoming increasingly apparent both that (1) the unit of function or *cistron* (BENZER 1957) defined by this test on allelic mutants is not identical in each instance, and that (2) these functional units are not the same as those defined by the *cis-trans* test on nonallelic and biochemically unrelated mutants. For example, the most recent study on a series of allelic mutants (*leu-2* locus) known to affect a single enzyme has shown that the complementation patterns (GROSS 1962) define at least 18 functional units. The reluctance of this investigator and many others to apply the term *cistron* to the complementation subgroups may be interpreted as (1) an indication of the gen-

eral acceptance of the term *cistron* as a synonym for the gene, the classical unit of function, and (2) general recognition of the facts that the growth response of allelic mutants in the *trans* configuration is (a) often quite different from non-allelic mutants when measured in terms of linear growth rate, and (b) markedly different when measured in terms of the level of specific enyzme activity (FINCHAM 1959, WOODWARD 1959, ISHIKAWA 1961).

The *cis-trans* test was devised by LEWIS (1951) to define the fundamental unit of function or "gene" in the absence of biochemical data. In this context, the application of BENZER's term *cistron* to the complementation subgroups defined by the *cis-trans* test on series of allelic mutants (WOODWARD, PARTRIDGE and GILES 1958, CATCHESIDE and OVERTON 1958) is misleading. This problem has been considered by a number of investigators in view of the inadequacy of the term *cistron*. PRITCHARD (1960) advised a return to the older term, *functional unit*, and DEMEREC and HARTMAN (1959) proposed a new term, *complementation unit*. We have abbreviated the latter term to *complon* to provide an operational term for the subgroups on a complementation map similar to BENZER's *recon*, *muton* and *cistron*.

One feature common to most complementation maps is the existence of a class of mutants that are noncomplementing. The variety of mechanisms of mutation that can give rise to this type of complementation pattern have not been completely determined, but genetic fine structure analyses of the *pan-2* (CASE and GILES 1960) and *ad-8* loci (ISHIKAWA 1961) have shown that such mutations can map as single site mutants and apparently are randomly distributed. It would appear from both of these studies that there are a large number of point mutations within each locus that can completely inactivate the fundamental unit of function.

Operationally, then, in heterokaryon tests on mutants with identical biochemical requirements or phenotype, the fundamental unit of function or *cistron* is designated by the class of noncomplementing mutants on a complementation map: the *complons* are designated, by those complementing mutants (with non-overlapping or overlapping defects) that are united in a unique way by the non-complementing group.

Whereas heterokaryon tests on mutants at the *arg-1* (CATCHESIDE and OVERTON 1958), *td* (LACY and BONNER 1961), *am* (FINCHAM 1959), *ad-4* (WOODWARD *et al.* 1958), *ad-8* (ISHIKAWA 1961), *iv-2* and *iv-3* (BERNSTEIN and MILLER 1961), *leu-2* (GROSS 1962), *pan-2* (CASE and GILES 1960) and *tryp-1* and *tryp-3* (AHMAD and CATCHESIDE 1960) loci have defined but a single *cistron* at each locus, the complementation map of each *cistron* consists of a number of *complons* varying from two to 18. Similar studies on mutants in the *ad-3* region (DE SERRES 1956) have defined two *cistrons* (designated *ad-3A* and *ad-3B*), and the evidence presented in this paper indicates that the complementation map of the *ad-3B* *cistron* can be subdivided into at least two *complons*.

SUMMARY

Heterokaryon tests of all possible combinations of 161 *ad-3B* mutants of independent origin were made in duplicate in test tubes containing liquid minimal medium. Only 112 (0.4 percent) of the 25,760 pairwise combinations involving different mutants were positive. All combinations showing positive tests involved one of four leaky mutants and 14 nonleaky mutants. Growth tube studies on the heterokaryons revealed a wide range of growth rates from wild type to only slightly faster than the rate of the leaky mutant itself. These tests show that the complementation map of the *ad-3B cistron* consists of at least two complementation subgroups or *complons*.

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