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COMPLEMENTATION AT THE AD-4 LOCUS IN NEUROSPORA CRASSA*

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Three cases of complementation between allelic mutants in heterocaryons of *Neurospora crassa* have been reported in which the individual homocaryons lack a specific enzymatic activity.[1–3] More recent and apparently similar phenomena have been observed in which data relating to the involvement of a single enzyme are still lacking.[4, 5]

The present paper presents a detailed analysis of heterocaryon complementation between *ad-4* mutants, each of which has impaired adenylosuccinase activity. The results suggest that the linear structure of both a gene and its products may be revealed by the pattern of interallelic heterocaryon complementation. The enzyme adenylosuccinase, found in wild-type extracts, catalyzes the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP) and fumarate, as well as the splitting of the analogous purine precursor SAICAR to AICAR[6] and fumarate.[1, 7] Adenylosuccinase activity has not been found in appreciable amounts (less than 1 per cent of wild-type activity) in any of the *ad-4* mutants tested, and partial restoration of activity has been detected in all cases examined in which complementation occurs. The degree of complementation varies widely among the different mutant combinations; i. e., growth rates of the heterocaryons on minimal medium range from less than 0.2 to 4 mm/hr (wild-type rate), and enzyme activities range from less than 1 to 25 per cent of wild-type activity. Brief reports of certain of these results have been presented previously.[7, 8]

TABLE 1

CLASSIFICATION OF *ad-4* MUTANTS BY ORIGIN

Strain Used in Obtaining Mutants	Derivation of Strain	No. of Mutants Obtained	Mutagen Used	No. of Complementing Mutants	Mutant Designation
74A		2	None	0	
74A		7	U.V.	1	F31*
74A		19	X-ray	5	F1, F4, F5, F12, F14
5.5A	74A × 73a	1	None	1	F23
5.5A	74A × 73a	3	X-ray	0	
3.1a	74A × 73a	1	None	0	
Y191-R28	F12 revertant	13	X-ray	8	M2-M15
Y191-R13	F12 revertant	7	None	5	M71-M77
Y191-R13	F12 revertant	45	X-ray	21	M27-M70
Y201-R22	F2 revertant	25	X-ray	10	M16-M25
Totals		123		51	

* F mutants listed include only complementing mutants.

Materials and Methods.—The adenine-requiring mutants described in this paper were isolated by the filtration and selective plating technique of Woodward *et al.*[9] The original mutants were derived from the St. Lawrence wild-type strain 74A; other primary mutants were derived from strains 5.5A and 3.1a, which originated from inbreeding of 74A after being crossed with strain 73a. Secondary mutants were obtained from revertant strains Y191-R28, Y191-R13, and Y201-R22. The revertant strains arose by mutation and subsequent reversion in strain 74A at the *ad-4* locus (see Table 1). Mutants were obtained from conidia, either without treatment or following ultraviolet or X-irradiation.

Classification of these mutants into the *ad-4* category is based on their failure to

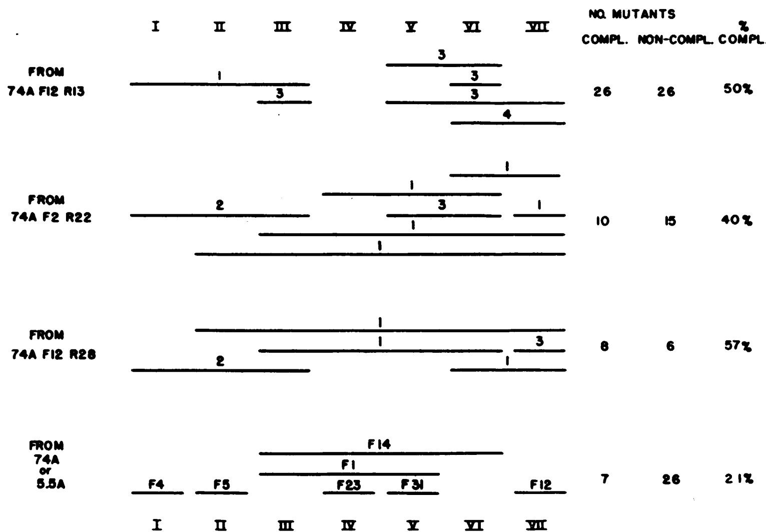


FIG. 1.—Complementation map of the *ad-4* locus based on both primary and secondary mutants. Primary mutants (F designation) are listed individually, since no two have identical complementation patterns. Secondary mutants (upper 3 rows) are listed in groups with numbers above each line referring to the number of mutants exhibiting a particular pattern of complementation. Roman numerals indicate the seven distinguishable functional regions (cistrons).

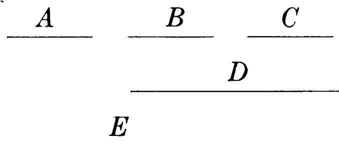
grow on minimal or hypoxanthine-supplemented medium, accumulation of a weak purple pigment when grown on limiting adenine, and their inability to complement with *ad-4* testers (those mutants that do not complement with other *ad-4* mutants). In addition, genetic tests have been carried out on 20 of these mutants, confirming their *ad-4* classification in all cases.[10]

Of 25 mutants assayed for adenylosuccinase activity, no more than 1 per cent of wild-type activity was detected in any mutant extract, and only those with no detectable growth on minimal medium were used in the complementation studies. Assays for adenylosuccinase activity were carried out by following the change in absorption at 280 mμ on a recording spectrophotometer as the reaction proceeds

from AMP-S to AMP. Enzyme extractions were prepared as described in an earlier report[1] except that tris HCl buffer was used in these experiments.

Heterocaryon tests were made by mixing conidial suspensions of two strains on minimal agar Petri plates, as described by de Serres.[11] More comprehensive observations have led to the demonstration of complementation between certain pairs of strains previously reported as negative on the basis of tests of short duration.[1]

Results.—Of the 123 *ad-4* mutants that have been isolated, 51 complement in a heterocaryon with at least one other allelic mutant. Such complementation results in adenine-independent growth of the heterocaryon and partial restoration of enzyme activity. The pattern that emerges when pairwise combinations are tested for complementation can best be illustrated in diagrammatic form (Fig. 1). Sixteen different complementation types are observed among the 51 mutants; the relationships of these mutants indicate the existence of seven distinguishable functional regions (cistrons) arranged in a linear order to form what may be designated a *complementation map.* The actual ordering of mutants on this map is based on mutants having overlapping non-functional regions. Non-functional regions are indicated by solid lines in Figure 1. For example, the ordering of three hypothetical cistrons A, B, and C could be deduced by obtaining mutants (D) which complement only with A (thus overlapping B and C on the map) and others (E) which complement only with C (overlapping A and B). Mutants A, B, and C all complement with each other, while mutants D and E provide evidence for linearity in either the order ABC or CBA:



The entire complementation map of the *ad-4* locus has been deduced by this method. Any overlap in the complementation map is indicative of a lack of complementation within a period of 10 days (see Table 2). In some cases, heterocaryons were established by utilizing nutritional mutant markers; this procedure gave results that were no different from those obtained without the use of such "forcing" markers.[1]

It seems significant that the most widely separated alleles on the complementation map form heterocaryons most rapidly and have, in general, the highest adenylosuccinase activities as measured from crude extracts. Heterocaryons between alleles separated by 2–5 functional cistrons produce up to 25 per cent of wild-type activity (Fig. 2). If alleles are separated by less than 2 functional cistrons, there is a decrease in enzyme activity down to less than 1 per cent of wild type for some combinations involving adjacent-cistron damage. Furthermore, adjacent-cistron combinations generally have the slowest growth rates on minimal medium and require, on the average, a longer time for growth to commence from mixed conidial inocula on minimal agar (Table 2).

Data from secondary mutants derived from F12 revertants (M2-M15 and M27-M77) have not been included in these tables. Complementation data and reverse

TABLE 2*

CLASSIFICATION OF MUTANT COMBINATIONS ON THE BASIS OF TIME REQUIRED TO GIVE A POSITIVE GROWTH RESPONSE AT 25° C.

Location of Damaged Sites on Complementation Map	Number of Days after Mixing Conidia before Growth Commences									
	2	3	4	5	6	7	8	9	10	More than 10
Adjacent cistrons	16	8	5	3	3	2	0	0	1	2
1-cistron separation	16	3	2	1						
2-cistron separation	15	1								
3-cistron separation	17									
4-cistron separation	13									
5-cistron separation	5									

* Numbers in the body of the table indicate the number of separate heterocaryon combinations of a given class which start growing within the time indicated. Replicate experiments with any given heterocaryon agree within 1 day in nearly all cases (see text).

mutation experiments indicate that a restriction was imposed on F12 at the time of its mutational origin. This restriction prevents complete restoration of enzyme activity by reverse mutation and limits its enzyme restoration by complementation. Among a total of 68 F12 revertants assayed, none was found with enzyme activity equivalent to wild type. On the other hand, four other *ad-4* alleles have all given rise to revertants that are quantitatively equivalent to wild type. The revertants from F12 used in obtaining secondary mutants were derived from a category having the lowest adenylosuccinase activity (3 per cent of wild type). There is also evidence of a second restriction's having occurred at the reversion step. Two secondarily derived mutants were found to yield only revertants with activities limited to 3 per cent of wild type from a total of 21 examined. In addition, no heterocaryon involving any two such secondarily derived mutants has manifested more than 3 per cent of wild-type enzyme activity, even when the two damaged sites are separated by 3 functional cistrons on the complementation map. Such a restriction is not observed when mutants of secondary origin complement with mutants derived directly from 74A. However, the use of secondarily derived mutants makes it possible to infer on the basis of the complementation pattern a restoration of the original F12 damage, presumably at the reversion step, and subsequent damage in other cistrons.

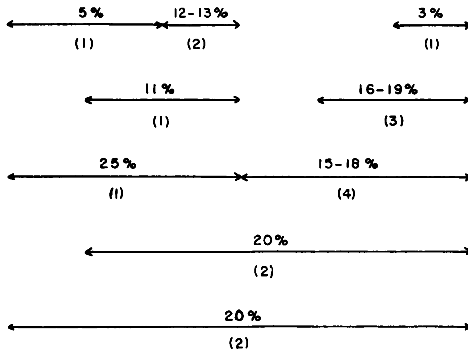


FIG. 2.—Adenylosuccinase activities in heterocaryons expressed as per cent of wild type. Figures below each line indicate the number of different mutant combinations assayed, all of which are separated by the same distance on the complementation map. Roman numerals indicate the seven functional regions.

Certain mutant combinations form heterocaryons that are temperature-sensitive to varying degrees. All temperature-sensitive heterocaryons involve mutants whose combined damage includes the four cistrons, III, IV, V, and VII. Those combinations that result in temperature-sensitive heterocaryons are listed in Table

TABLE 3

GROWTH RATES IN MM/HR OVER 99-HOUR PERIOD ON MINIMAL + HISTIDINE AGAR

Strains	Rate at 25°	Rate at 35°	Blocked Cistrons
F15	3.26	0.10	III IV V VI
F14-M4 heterocaryon	2.14	0.05	III IV V VI VII
F1-F12 heterocaryon	2.95	0.70	III IV V VII
F14-F12 heterocaryon	0.62	0.05	III IV V VI VII
F14-M14 heterocaryon	0.94	0.58	III IV V VI VII
F14-M9 heterocaryon	2.17	1.10	III IV V VI VII
F1-M14 heterocaryon	3.65	2.86	III IV V VII
74A wild type	3.53	3.62	

3, which shows comparative growth rates at 25° and 35° C. F15, a temperature-sensitive mutant, is also listed.

A final and very pertinent question pertains to the linear order as determined by genetic mapping of the locus as compared to the order deduced from the complementation map. Unfortunately, a complete recombinational analysis has not yet been possible because of poor fertility in crosses between *ad-4* mutants. However, the data obtained thus far indicate that certain alleles, widely separated on the complementation map, are also the most widely separated, as judged by the frequency of prototroph recovery from crosses between them.[10]

Discussion.—Perhaps the most striking feature of complementation between allelic mutants is the possibility of describing their relationships in terms of a linear complementation map. A reasonable assumption appears to be that such a map reflects a linear organization of the gene and gene products. On the basis of this assumption and utilizing the quantitative data on adenylosuccinase activity in the heterocaryons, a working hypothesis has been devised to explain the mechanism of complementation at the *ad-4* locus. Nuclear recombination has not been detected in these *Neurospora* strains during vegetative growth, and this hypothesis involves the assumption that interaction occurs between gene products in the cytoplasm.

A critical feature of this hypothesis is the observation that no *ad-4* interallelic (intralocal) heterocaryon has yielded more than 25 per cent of wild-type activity. A maximum of 25 per cent of wild-type activity was also reported for glutamic dehydrogenase from heterocaryons between *Neurospora am* mutants.[2] If some type of cytoplasmic recombination occurred at the RNA or polypeptide level, functional enzyme could be formed by exchanges resulting in the union of two nondefective parts of two gene products damaged at different sites. The reciprocal product of such an exchange would carry both damaged sites. If this type of recombinational mechanism is operative and exchange is random, only one of the four possible combinations would result in the formation of a functional enzyme. The yield of 25 per cent of wild-type activity can be explained by such a mechanism, assuming that a nuclear ratio of 1:1 is approximated in the heterocaryon and also that the sum of normal plus defective enzyme formed in a heterocaryon between *ad-4* mutants is equivalent to the total normal enzyme formed in wild type.

Recent reports have revealed the possibility that recombination at the polypeptide level may indeed occur in some in vitro systems. In the case of ribonuclease[12] it has been found that under certain conditions the modified enzyme dissociates into two fragments—a small peptide and a large component—both of which alone are inactive but which, when mixed under the appropriate conditions, give

restored enzyme activity. Dissociation and recombination has also been reported in mixtures of CO-hemoglobin A and ferrihemoglobin A.[13] This recombination results in the detection of an additional component of intermediate electrophoretic mobility. Furthermore, in a recent study of human haptoglobins, Bearn and Franklin[14] showed the presence of a haptoglobin in heterozygotes which differed from haptoglobins formed by either homozygote in having a peak of intermediate sedimentation rate as well as intermediate electrophoretic mobility, in addition to the peaks characteristic of both homozygotes.

Additional support for some type of random exchange mechanism is furnished by the observation that the time required for growth to commence from conidial mixtures between two mutants can be modified within certain limits by the conidial concentrations used. The conidial concentration should influence directly the relative number of fusions between the two conidial types. A small conidial population size would, on the basis of random exchange between gene products, be less likely to start forming functional enzyme in a given time because of a decrease in the total number of possibilities for exchange. The tendency for heterocaryons between mutants damaged in adjacent cistrons to have less enzyme activity than those between mutant combinations more widely separated on the complementation map could be explained by the same type of restriction as that encountered when linkage distances between mutant loci are less than 50 crossover units. In the case of heterocaryons, this would mean that when damage in two different mutants becomes close enough in terms of position within the locus, the probability of exchange is so low that not enough functional enzyme is formed, compared to the degradation rate, to give a growth response in a given mixed conidial population. Occasionally, in the weakest heterocaryons between *ad-4* mutants (those requiring 6 or more days for growth to commence), growth is delayed more than usual or never commences at all, depending on the conidial concentration. Such sporadic results are never observed in the stronger heterocaryons and would not be expected on the basis of the increased probability for exchange between more widely separated defective sites. The theoretical probabilities for exchange dependent on distance appear to be a reasonable explanation for the quantitative correlation between distance on the complementation map and enzyme activity or growth measurements observed in heterocaryons.

Earlier observations indicated that a heterocaryon between F12 and a nonallelic mutant (an interlocal heterocaryon) yielded 50 per cent of wild-type adenylosuccinase activity.[1] This result can be explained most simply on the assumption that such heterocaryons contain only half as many functional genes for a given locus as does wild type. This type of complementation would not require cytoplasmic interaction as has been suggested for interallelic complementation, but if such interaction occurs, the recombination hypothesis would still predict a 50 per cent yield.

Other hypotheses have been considered as possible mechanisms to account for the partial restoration of enzyme activity in heterocaryons between mutants which lack as homocaryons the same enzyme activity. For example, one nucleus might make all the functional enzyme, in effect receiving from the other nucleus some type of activator. Another possibility is that the product of one nucleus may compete so strongly for an inhibitor of the modified enzyme formed by the other nucleus as

to render the latter capable of carrying out its catalytic function. Evidence is still lacking to indicate definitively whether the enzyme in complementing interallelic heterocaryons is, in fact, a hybrid derived in part from both nuclei or whether one nuclear type produces all the functional enzyme.

More than 50 per cent of the *ad-4* mutants obtained to date fail to complement with any other *ad-4* mutants. On the complementation map these could be represented by a solid bar across the entire map and interpreted as having been damaged in all seven cistrons of the locus. If this were true, however, they would not be expected to occur with such a high frequency. The probability that these are deletion-type mutations for the whole locus is very low, since many of these mutants revert to adenine-independence at a high rate. The unexpected finding that more than twice as many secondary mutants (those derived from F12 revertants) complement with other *ad-4* mutants as do primary mutants may indicate either that a certain amount of non-specific damage or that a specific type of damage is necessary to render a mutant capable of complementation. Secondary mutants have been subjected to three separate dosages of X-ray irradiation (36,000 r each) and have been altered at the *ad-4* locus on each occasion, while primary mutants received only one such dose in their production. The complete absence of interallelic complementation found in some mutants is not irreversible. The primary mutant F2 does not complement with any other *ad-4* mutant, but 40 per cent of the secondary mutants derived from a back mutant of F2 do complement in at least one combination. Furthermore, the over-all pattern exhibited by this group of secondary mutants extends across the entire complementation map (see Fig. 1).

Mutants that do complement but have more than one non-functional cistron cannot easily be visualized as having been damaged in more than one site, since they also occur quite frequently and in most cases are capable of undergoing reversion with restoration of enzyme activity. In such cases of apparent widespread damage within the locus, a single critical site within one cistron may be damaged, resulting in a type of position effect extending over several cistrons. There is evidence from recombination data at the *pan-2* locus that genetic localization is possible even with mutants blocked in several cistrons.[15]

The data from temperature-sensitive heterocaryons indicate that these mutants have damage in common and support the idea that the complementation map is a valid diagrammatic representation of the *ad-4* locus. If the enzymes from such heterocaryons prove to be thermolabile, the data would suggest a direct correlation between the map and the enzyme molecule.

Summary.—One hundred and twenty-three mutants at the *ad-4* locus have been tested for complementation in heterocaryons. Fifty-one of the mutants gave at least one positive response. Although these mutants lack adenylosuccinase activity, partial enzyme activity is restored when complementing mutants are combined in heterocaryons. The relationships of the complementing mutants at the *ad-4* locus are such that these mutants can be arranged in a unique linear sequence which may be designated a complementation map of this locus. This arrangement is possible because of the apparent presence in certain alleles of continuous multiple non-functional regions which overlap non-functional regions of other alleles. The complementation map of the *ad-4* locus is divided into seven functional regions (cistrons). There is a marked correlation between distance on the complementation

map and the strength of the heterocaryon as judged by enzyme activity, growth rate, or time required for growth to commence from mixed conidial inocula. Thus heterocaryons between mutants damaged in adjacent cistrons have low adenylosuccinase activities, and activities increase with increasing distance to the maximum value of 25 per cent of wild type for mutants separated by 2–5 cistrons.

Possible interpretations of these results are discussed and the hypothesis is proposed that interallelic complementation involves the formation of defective gene products by different mutant nuclei in a heterocaryon. Cytoplasmic recombination of such gene products either at the primary (RNA) or secondary (polypeptide) level, perhaps by a process analogous to crossing over, could yield enzymatically active protein.

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[6] Abbreviations used are SAICAR = 5-amino-4-imidazole(N-succinylocarboxamide) ribotide; and AICAR = 5-amino-4-imidazolecarboxamide ribotide.

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*COHOMOLOGY THEORY OF VARIETIES OVER RINGS**

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1. *Introduction.*—Some recent developments in algebraic geometry, e. g., the theory of deformations of complex analytic structures by Kodaira and Spencer, investigations on the principles of degeneration and of connectedness by Chow and on elliptic modular functions by Igusa, suggest the importance of the notion of varieties considered not merely over fields but more generally over rings. This