SPONTANEOUS ALTERATIONS OF HETEROKARYON COMPATIBILITY FACTORS IN NEUROSPORA¹

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THE original work on heterokaryons in *Neurospora crassa* by BEADLE and COONRADT (1944) demonstrated the potential usefulness of heterokaryotic systems as an experimental tool for the investigation of a number of different genetic problems. In the years following their initial discoveries, it appeared that the experimental use of forced heterokaryons might be somewhat restricted because cultures capable of growth on minimal medium were not always formed and maintained when mutant strains in different genetic backgrounds were combined. The failure of different nuclei to coexist harmoniously in a heterokaryotic mycelium is an incompatibility phenomenon, and this was shown to have a genetic basis by the independent investigations of GARNJOBST (1953, 1955) and HOLLOWAY (1955). As a result of their work, it became clear that not only the formation of heterokaryons but also their maintenance at wild-type growth rates depended, in part at least, upon the presence of homoallelic combinations of certain compatibility genes. They were able to demonstrate that incompatibility was manifested in a variety of different growth phenotypes when these genes were present in heteroallelic combinations. As a practical solution to this problem of incompatibility, investigators have subsequently either resorted to using mutants induced in the same genetic background, or where this has not been possible, a backcrossing program has been used to obtain the necessary homogeneity between strains.

Although a solution is available for solving the practical aspects of this problem, the interaction of genetically different nuclei in the cytoplasm of an incompatible heterokaryon has its counterpart in many biological systems and a great deal has yet to be learned regarding the mechanisms of these interactions. The work of GARNJOBST and WILSON (1956) and WILSON, GARNJOBST and TATUM (1961) has demonstrated that one of the ways such interaction may be manifested is in the form of cytoplasmic incompatibility. Nuclear competition, which usually results in nonadaptive nuclear selection in forced heterokaryons, is another general type of interaction that has been demonstrated in heterokaryotic systems (RYAN and LEDERBERG 1946; GROSS 1952; DAVIS 1960a, b; PITTENGER and BRAWNER 1961). In such cases, growth is erratic and usually ceases, but it may resume again. In most of these combinations, growth stops permanently owing to

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homozygosity of one of the components of the forced heterokaryons. Rarely, however, one observes an incompatible combination which slows down and stops and then resumes growth at a normal rate and remains permanently stable thereafter. This behavior suggested some unique type of nuclear selection and prompted further investigation of these cases.

The studies reported below demonstrate that these stable changes in the growth of forced heterokaryons with heteroallelic combinations of compatibility factors can be attributed to the formation of nuclei with an altered compatibility genotype. In some cases, the changes in compatibility are associated with concomitant genetic loss or lethal mutation. It is not known at present whether all these changes are due to the same phenomenon, but mutation of compatibility factors and somatic recombination both appear to be possible mechanisms. It is not possible, however, to distinguish clearly between these alternatives on the basis of the present evidence.

MATERIALS AND METHODS

The routine techniques used in heterokaryon experiments, in compatibility tests, and in the plating of conidia are adequately described elsewhere (PITTENGER, KIMBALL, and ATWOOD 1955; PITTENGER and ATWOOD 1956; PITTENGER and BRAWNER 1961). In most cases, heterokaryons were simply prepared by superimposing conidia of one strain on those of another in 500 mm-long growth tubes half filled with FRIES minimal medium solidified with 3 percent agar and supplemented with 1.5 percent sucrose. No attempt was made to carefully control the nuclear ratios but the above procedure rarely results in disproportionate ratios in compatible combinations. When it was necessary to recover the homokaryotic components of a heterokaryon, or when nuclear ratios had to be determined, conidial suspensions of heterokaryons were overplated on the synthetic medium of WESTERGAARD and MITCHELL, solidified with 2 percent agar and supplemented with 1 percent sorbose, .04 percent glucose, and the appropriate growth factors. To recover homokaryotic isolates, single colonies from supplemented plates were transferred to supplemented slants and identified as homokaryons by color tests (*al-1* and *al-2* isolates were always used) and by their growth response on minimal and supplemented media.

Strains: Markers used were *al-1* (4637T, albino-1. A reciprocal translocation between linkage groups IR and IIR, inseparable from the albino phenotype); *al-2* (15300, albino-2, linkage group IR); *nic-1* (3416, nicotinic-1, IR); *nic-2* (4540T, nicotinic-2. An insertional translocation of a segment of IR into IIIR, inseparable from the nicotinic-2 phenotype (St. LAWRENCE 1959)); *pan-1* (5531, pantothenic-1, IVR). Further discussion of the markers can be found in BARRATT, NEWMAYER, PERKINS, and GARNJOBST 1954. The fact that two of the markers are associated with rearrangements was incidental to the present study and was not expected to influence the results since the experiments involved heterokaryons. See the Discussion, however, for a possible consequence of the translocations. Duplication-deficiency products of 4637T $\times$ wild are all inviable, but those segregants from 4540T $\times$ wild that contain the duplication are fully viable, though characterized by erratic growth on minimal medium and by greatly reduced fertility in crosses (St. LAWRENCE, personal communication). None of the strains used in the present study were of this duplication type.

HOLLOWAY (1955) made it clear that, because of the diversity of genetic backgrounds that may exist between strains, any attempt to study genetic factors affecting compatibility necessitates the use of a set of standard heterokaryon tester strains with which all other strains may be compared. Two *nic-1 al-2* strains of opposite mating types were carefully selected that were isogenic for all compatibility factors except mating type (PITTENGER 1957). Subsequently, five other strains compatible with these two have been selected as a set of standard heterokaryon testers: (1) *nic-1 al-2* 88a (JK), (2) *nic-1 al-2* 55A (JK), (3) *pan-1 al-1* 1a (JK), (4) *pan-1 al-1* 23A

(*JK*), and (5) *nic-2 al-2 1A (JK)*. These five strains are heterokaryon-compatible in *A + A* and *a + a* combinations, and in crosses with one another they give only compatible progeny when tested with the parents, with other progeny from the crosses, or with any of the appropriate testers. The letter designations *J* and *K* in parentheses indicate compatibility genotypes with which we are concerned and, aside from the mating-type alleles *A* and *a*, they are the only genes pertinent to the discussion which affect compatibility. The complete listing of the factors will only be included when it seems necessary for clarity.

In terms of compatibility, these strains are closely related to wild type 25a originally used by BEADLE and TATUM for many of their mutant isolations. However, in crosses between all of the testers and 25a, a single-gene difference affecting compatibility segregates. Because of the similarity of these strains with 25a, which is one of the progenitors of practically all of the strains in use today (see BARRATT 1962), the compatibility factors in these testers were arbitrarily designated with capital letters even though the dominance relations of none are known.

Another strain used was (6) 74-OR-8-1a (*jk*). Although this strain (subsequently referred to as 74a) is known to differ from the testers by at least four genes affecting incompatibility (PITTINGER, unpublished), the other factors are not relevant to the current problem.

From the crosses listed below several additional strains were isolated. From (4) *pan al-1 23A (JK)* $\times$ (6) 74a (*jk*), came three strains: (7) *pan al-1 12A (jk)*, (8) *pan al-1 13A (jK)*, and (9) *pan al-1 16A (jK)*.

Initially, the compatibility genotypes of Strains 7, 8, and 9 were unknown and when it became necessary to determine the genotype of Strain 7, this was accomplished as follows. From a cross of *pan al-1 12A (jk)* $\times$ *nic-1 al-2 88a (JK)*, 22 *pan al-1 A* progeny were first isolated and tested in heterokaryotic combination with the *nic-1 al-2 55A (JK)* tester. Sixteen were incompatible and six were compatible, suggesting that two factors affecting compatibility were segregating in the cross. These factors were designated *j* and *k* and the two parental genotypes were designated *jk* and *JK*, respectively. From the same cross, unordered tetrads were then collected and by methods similar to those used by GARNJOBST (1953, 1955), four different compatibility genotypes were isolated from among the *nic-1 al-2 a* progeny. They were designated as: (10) *nic-1 al-2 1a (jk)*, (11) *nic-1 al-2 2a (jK)*, (12) *nic-1 al-2 3a (Jk)*, and (13) *nic-1 al-2 4a (JK)*. Fourteen *pan al-1 a*, random progeny from the same cross, were tested in heterokaryotic combination with each of the four strains, Nos. 10 through 13. Each *pan al-1 a* isolate was found to be compatible with only one of the *nic-1 al-2 a* testers. Of the 14 isolates, three were *jK*, five *jk*, three *Jk*, and three *JK*. *pan al-1 a* strains with these four compatibility genotypes were selected from this group. Such results are compatible with the postulated two-factor difference between the parents of the cross. The location of these and their relationship to the *c* and *d* alleles described by GARNJOBST (1953, 1955) have not yet been determined, but they appear to segregate independently of *nic-1*, *al-2*, *al-1 T*, and *pan-1*.

Under RESULTS, some reference will be made to the *I* and *i* alleles previously described by PITTINGER and BRAWNER (1961). All of the strains above, with the exception of 74a, are *I* in genotype. Heteroallelic combinations of *I* and *i* are characterized by the fact that, when the frequency of *I* nuclei is 70 percent or greater, this component shows a nonadaptive increase during growth. This leads eventually to homozygosity of this nuclear type and growth then ceases.

Criteria of compatibility and incompatibility: The term compatible heterokaryon will refer to a forced heterokaryon between *pan al-1* and either *nic-1 al-2* or *nic-2 al-2* of the same mating type which grows on minimal medium at 30°C at a rate of approximately 4 mm/hr or more, and at a constant rate. We have found it operationally expedient to provisionally classify as incompatible any combination which consistently deviates from this norm. By the above definition, a heterokaryon which grows as much as 200 to 300 mm before growth permanently ceases is therefore classified as an incompatible combination. The author is of the opinion that the instability of a forced heterokaryon which eventually leads to the homozygosity of one of the mutant components responsible for the cessation of growth is a valid criterion of incompatibility. The fact that such instability takes longer to be expressed in one genotype than in others may often only be a consequence of the residual genotype. For example, GARNJOBST (1955) has

reported that some *cd/Cd* combinations show only "slight incompatibility," although most are incompatible. The excessive growth of the particular incompatible combinations described in this paper undoubtedly reflects the characteristics of the *nic-2* component as much as it does the incompatibility genotype, for it has been shown that a *nic-2* strain may grow for considerable distances homokaryotically if initially supplied with small amounts of the growth factor by the other nuclear component (PITTINGER 1962). There are numerous factors and mechanisms which may cause deviations from stable wild-type growth rates in forced heterokaryons and some of these, for example, disproportionate nuclear ratios, may be unrelated to incompatibility. However, until the mechanisms responsible for the deviation from normal growth are understood, the position has simply been taken to provisionally classify any combination as incompatible in which the forced heterokaryon in repeated trials fails to grow at a rate comparable to the growth rate of the fastest growing homokaryotic component.

RESULTS

In an attempt to find a reasonable explanation of the changes in growth rates of certain incompatible heterokaryotic combinations, some degree of success was possible only in those cases where growth had either slowed down significantly or stopped completely, and then resumed at a near normal rate. Consequently, although many different heterokaryons were partially analyzed in an attempt to find the reason for their abnormal behavior during growth, in only a few instances was it possible to ascribe their behavior to some specific cause. Some of these cases can be attributed to viable alteration in genetic factors affecting compatibility, but in others genetic loss also appears to be involved.

I. Heterokaryons involving heteroallelic combinations where a change in growth rate can be attributed to a viable genetic alteration

During a routine attempt to recover *pan al-1 A (iJK)* progeny from a cross between *pan al-1 23A (IJK)* and 74a (*ijk*), 50 *pan al-1 A* isolates were recovered and tested with the *nic-2 al-2 1A (IJK)* tester strain. Some of these heterokaryons grew up to 300 mm in growth tubes before growth ceased. At the time, such behavior was interpreted in terms of the expected *I-i* interaction (PITTINGER and BRAWNER 1961); instead, it was later found to be characteristic of many other incompatible combinations involving *nic-2* strains. During the experiment, *pan al-1 A* isolates 12, 13 and 16 in heterokaryotic combination with *nic-2 al-2 1A (IJK)* resumed growth at a normal rate after having once stopped. The heterokaryons were prepared again several times but the resumption of growth was not observed again. Why these combinations occasionally resume growth after having once stopped was then examined further. It is the behavior of heterokaryons involving the three *pan al-1 A(I)* isolates that will be initially considered. (When these studies were originally carried out, the compatibility genotypes of the strains were unknown. These were determined during the investigation as described in METHODS and MATERIALS. Consequently, it should be borne in mind that, although the compatibility genotypes are given to assist the reader in following the discussion, they were unknown at the time the changes in growth were first being investigated.)

Changes from jk to Jk: Although the heterokaryon between *pan al-1 12A (jk)*

and the tester *nic-2 al-2 A (JK)* is an incompatible one, it characteristically grows up to 300 mm in growth tubes before growth ceases completely. This heterokaryon becomes largely homokaryotic after a short period of growth and always shows a dark brown discoloration in the medium so characteristic of the *nic-2* cultures growing on limiting amounts of nicotinamide. The culture is albino although some pigmentation in the conidia is expected owing to the complementary action of *al-1* and *al-2* when a heterokaryon is formed with anything except a very disproportionate nuclear ratio. Although this combination has been prepared many times, it is unstable and has always stopped growing before reaching the end of the growth tube with the following exception. In one trial including four replications, growth in one of the tubes resumed (Figure 1) and at the distal end of the growth tube the conidia showed some pigmentation as proof of the heterokaryotic condition. A conidial suspension from the proximal end of the growth tube, which showed no pigmentation in the conidia, was overplated in a thin layer of agar on minimal plates as well as on pantothenate- and nicotinamide-supplemented plates to evaluate the nuclear proportions and the extent and characteristics of the heterokaryon formed proximally in the growth tube. When aliquots of a conidial suspension were plated, a total of 266 colonies was found on three pantothenate-supplemented plates, 1197 on three nicotinamide-

FIGURE 1.—Broken Lines: Growth curves of incompatible heterokaryons (Nos. 12, 13, and 16 between *pan al-1* 12A, -13A and -16A, and *nic-2 al-2* 1A) that resumed normal growth after having once stopped. All combinations were white proximally but had wild-type pigmentation distally in the growth tubes. Solid Lines: Growth curves of four of ten incompatible heterokaryons [Nos. 1, 3, 5, and 8 between *pan al-1* 2a (*JK*) and *nic-1 al-2* 1a (*JK*)] which either essentially stopped or stopped growth completely and then resumed growth at a normal rate.

supplemented plates, and none on three minimal plates. An additional plating on minimal medium of an estimated 146,300 conidia also failed to disclose any evidence of compatible nuclei capable of forming heterokaryotic conidia which would grow on minimal medium.

On the other hand, when conidia from the distal end of the same growth tube were plated on the same type of media, 53 colonies were found on the pantothenate-supplemented plates, 199 on the nicotinamide-supplemented plates, and 34 on the minimal plates. The presence of colonies on minimal plates confirmed that the culture was heterokaryotic distally; this was also indicated by the wild-type coloration of the conidia distally in the growth tube.

This apparent change in compatibility reaction was stable; ten heterokaryotic colonies from minimal plates were transferred to minimal growth tubes, and all showed normal growth rates and wild-type pigmentation in the conidia. Apparently one of the components of the heterokaryon had been altered in some way so that the two nuclear components distally were compatible, whereas proximally in the same tube they had been incompatible. To check this hypothesis further, *pan al-1* and *nic-2 al-2* homokaryons were isolated from the supplemented plates on which conidia from the proximal and distal ends of the growth tubes had been plated.

For convenience, the homokaryons from the proximal and distal ends of the growth tube are referred to as *p-pan*, *p-nic*, *d-pan*, and *d-nic*, respectively. Usually at least five homokaryons of each type were tested. Following isolation of these homokaryons, different heterokaryotic combinations were prepared and their behavior in growth tubes was observed. The results of these tests can be summarized as follows: *p-pan* + *p-nic* combinations were all incompatible and growth stopped as it had in the original heterokaryons; likewise *p-pan* and *d-nic* combinations were incompatible; *d-nic* + *d-pan* combinations were all compatible; and the *p-nic* and *d-pan* combinations were also compatible, with growth normal at 4 mm/hr. Thus, it was apparent that the *pan al-1* *A* isolates from the distal end of the growth tubes differed in compatibility reaction from the *pan al-1* *A* isolates originally inoculated into the proximal end. Since there was no evidence of compatible *pan al-1* *A* nuclei in the proximal end, it could be assumed that the change to a compatible genotype was the result of some alteration in a *pan al-1* *A* nucleus subsequent to its inoculation.

In addition to the absence of any compatible heterokaryotic conidia proximally, reconstruction experiments suggested that the change in compatibility reaction actually took place in the growth tubes and was not due to the initial presence of a small number of compatible nuclei (mutations or contaminants). By combining equal proportions of conidial suspensions of *p-pan* and *p-nic*, and to this suspension adding various concentrations of *d-pan* conidia, it was found that a ratio of only one *d-pan* conidium to 10^6 *p-nic* conidia in the *p-nic* + *p-pan* mixture was capable of forming a heterokaryon which was able to grow at a constant rate with no evidence of slowing down or stopping. More extreme ratios gave somewhat erratic results and, although some combinations gave cultures with slow growth rates, they did not stop. Thus, if some *pan al-1* *A* nuclei compatible with

the *nic-2 al-2 A* strain were present in the original inoculum, there is no reason why the resulting culture should either slow down or stop.

To determine the nature of the change in compatibility reaction of the d-*pan* isolates, both the p-*pan* and the d-*pan* homokaryons were crossed with a heterokaryon tester strain, *nic-1 al-2 88a (JK)*. When 25 *pan al-1 A* progeny from the cross involving the d-*pan* isolate were tested for their compatibility reaction with the *nic-2 al-2 A (JK)* tester, all of the combinations were compatible, indicating that the d-*pan* isolates and the *nic-1 al-2 88a (JK)* strain were isogenic for all compatibility factors.

On the other hand, of 22 random *pan al-1 A* isolates from the cross involving a p-*pan* isolate, only six were compatible with the *nic-2 al-2 1A (JK)* tester. These results showed that the difference in the compatibility reaction of the p-*pan* and d-*pan* isolates had a genetic basis and the 3:1 segregation indicated a two-factor difference between the *pan al-1 A* isolates from the proximal and distal ends of the growth tube. A tetrad analysis of the cross of p-*pan (pan al-1 12A (jk))* with *nic-1 al-2 88a (JK)* confirmed the segregation of two factors affecting compatibility. The factors in the *pan al-1* parent have been designated *j* and *k*. *nic-1 al-2 a* and *pan al-1 a* isolates of all of the four possible combinations of the two factors were isolated for use in additional studies reported below. (This isolation procedure was briefly described in METHODS and MATERIALS.)

Changes from jK to JK: In addition to *pan al-1 12A (jk)*, two other *pan al-1 A* isolates, Nos. 13 and 16, were also observed once among a number of trials to stop growth when in heterokaryotic combination with *nic-2 al-2 1A (JK)*, and to resume growth a short time later (Figure 1). In both cases, employing the same kinds of techniques used with *pan al-1 12A*, it was possible to recover from the

TABLE 1

Numbers of colonies on differentially supplemented media from plating conidia from three single-conidium-derived heterokaryotic cultures, isolated distally from three growth tubes (Nos. 3, 5, and 8) containing heterokaryotic cultures [pan al-1 2a (jK) + nic-1 al-2 1a (jk)] that had ceased growth and then resumed. Homokaryotic colonies are missing on the pantothenate supplemented plates

		Type of medium		
		Minimal	Pantothenate	Nicotinamide
Heterokaryon No. 3	Isolate A	76	75	234
	Isolate B	195	199	433
	Isolate C	44	40	156
Heterokaryon No. 5	Isolate A	133	136	279
	Isolate B	72	65	308
	Isolate C	47	45	222
Heterokaryon No. 8	Isolate A	66	60	191
	Isolate B	127	131	252
	Isolate C	118	111	320

Numbers of colonies represent an average on three plates.

proximal ends of the growth tubes *pan al-1* isolates that were not compatible with the *nic-2 al-2 A* component from either the proximal or distal ends of the same growth tube. Crosses of the *p-pan al-1* isolates to *nic-1 al-2 88a (JK)* showed that both the *p-pan 13* and *p-pan 16* differed from the *nic-1 al-2 88a (JK)* tester by one allele affecting compatibility. That is, the progeny of both of the above crosses segregated 1:1. In heterokaryon tests of the incompatible *pan al-1 a* progeny with *nic-1 al-2 a* tester strains of the genotypes *jk*, *JK*, *Jk*, and *JK*, it was possible to demonstrate that *p-pan 13* and *p-pan 16* were both of the genotype *JK*. However, heterokaryon tests showed that the respective compatible *d-pan* isolates were of the genotype *JK*. Thus, in both cases the *p-pan* and *d-pan* isolates differed by a single compatibility allele; this difference was assumed to result from changes that had taken place within the growth tube.

Changes from jk to JK. From the cross of *pan al-1 12A (jk)* with *nic-1 al-2 88a (JK)*, a variety of genotypes were recovered and several unsuccessful attempts were made, using a variety of combinations, to determine the frequency of alterations involving combinations of single-gene differences such as *JK + JK*, *Jk + JK*, *jk + JK*, etc., as compared to changes in combinations of two gene differences, i.e., *jk + JK*. Although several technical problems have prevented us from obtaining any meaningful answer to this question, one additional example of an alteration of compatibility was encountered. In a combination of *pan al-1 1a (jk)* and *nic-1 al-2 2a (JK)*, which grew from 150 to 300 mm before stopping, two new examples were found of alterations from a *jk* genotype to a *JK* genotype. In one growth tube, for example, the mixed inoculum grew initially at a rate of 2.5 mm/hr, slowed to a rate less than 1 mm/hr, and then growth suddenly increased to a rate averaging 3.1 mm/hr. *pan al-1 a* isolates were recovered from both ends of the growth tube and tested with various compatibility genotype testers. The isolates recovered distally were compatible with a *JK* tester and incompatible with a *jk* tester. On the other hand, the *p-pan* isolates were still incompatible with a *JK* tester and compatible with a *jk* tester. Although these strains were not analyzed by progeny testing, the data suggest that the *pan al-1 1a (jk)* culture inoculated proximally in the tube had undergone some alteration resulting in a *JK* genotype. A second example was analyzed in a similar fashion and gave the same result.

II. Heterokaryons involving heteroallelic combinations where a change in growth rate can be attributed to genetic loss

In the analyses of incompatible heterokaryons that temporarily stopped growing and subsequently resumed growth, it was not at all uncommon to find that one of the nuclear components could not be recovered as a homokaryon distally. Such cases appear to have special significance, not only because of the apparent lethal condition associated with one of the nuclear components, but also because this lethality seems to be associated with the change in incompatibility reaction. This type of change has not been uncommon, but until this phenomenon was discovered in strains of known compatibility genotypes, it was not possible to carry out the type of analysis described below.

This analysis involved a heterokaryon between *nic-1 al-2 1a (jk)* and *pan al-1 2a (jK)*. Ten different growth tubes were inoculated with a conidial mixture of the two homokaryons. Growth in all ten stopped, but in four (Nos. 1, 3, 5, and 8) growth resumed after variable periods, as shown in Figure 1. Conidia from the distal ends of the tubes were plated on three types of media henceforth called minimal (M), pantothenate-supplemented (P), and nicotinamide-supplemented (N). The average number of colonies on the plates for heterokaryons 1, 3, 5, and 8 were as follows: No. 1, M-14, N-172, P-16; No. 3, M-29, N-129, P-33; No. 5, M-4, N-172, P-2; No. 8, M-8, N-117, P-7. *nic-1 al-2 1a (jk)* homokaryotic colonies were prevalent on the N-plates and were easily recovered for further analysis. The plate counts suggested there were no *pan al-1* homokaryons present and this was confirmed by isolation of all of the colonies from the P-plates. Such a situation is not necessarily unexpected if the nuclear ratios are disproportionate. In any event, cultures recovered as colonies from the P-plates from heterokaryons 3, 5, and 8 (No. 1 was not analyzed further) were wild type in color and suggested a proportionate ratio of the *al-1* and the *al-2* components. Three such cultures from each heterokaryon were again plated on various supplemented media. The plate counts are given in Table 1. There was still no evidence of any *pan al-1* homokaryons. All of the colonies from one P-plate from at least one of the three cultures plated from each of the three different heterokaryons were isolated, and none were homokaryotic for *pan al-1 a*.

Cultures from heterokaryotic conidia recovered distally from the growth tubes of all three heterokaryons grew at normal and constant rates and, on replating, all again failed to produce any viable *pan al-1* homokaryons. The *nic-1 al-2* homokaryons recovered distally from the growth tubes were retested in heterokaryons and in each case all behaved as if they were still *jk* in genotype. Since such cultures were now compatible distally, and since there was no evidence for change in the *nic-1 al-2 (jk)* component, it was tentatively assumed that the *pan al-1* component had changed from *jK* to *jk*. This particular change from *K* to *k* had not previously been observed. However, in addition to the change in compatibility, consideration also had to be given to the second change, that is, the alteration from normal viability to a condition which shall be referred to as a recessive lethal condition of the *pan al-1* nuclear component. Such results can be satisfactorily explained by assuming that genetic loss of the *K* allele is involved and this deficiency is responsible for the homokaryotic lethal condition of the *pan al-1* nucleus. Furthermore, the presumed loss of the *K* allele also would result in a heterokaryon homoallelic for the *k* allele, thus removing the heteroallelic condition responsible for the incompatibility.

The evidence for a deficiency or perhaps the loss of an entire chromosome may be somewhat speculative, but it is compatible with the data. First, it has not been possible to recover the *pan* mutant from crosses involving a *nic-1 al-2* strain with the compatible heterokaryotic culture recovered distally from the growth tubes. Heterokaryon No. 3 mentioned above was crossed to *nic-1 al-2 55A* and the progeny were plated on nicotinamide-supplemented medium and examined for mutants, that is, for pantothenate-requiring isolates. If a lethal mutation were present and segregating independently from the *pan* mutant, or even linked with

it, one would expect to find some pantothenate-requiring ascospore segregants, recognizable by their mutant phenotype, on the plates. Twenty plates, each containing several thousand viable ascospores, were examined. Every spore that even resembled a mutant was isolated, but only *nic-1 al-2* progeny were recovered. There was no evidence that the *pan al-1 A* component had even participated in the cross. Such results might be expected if a rather large deficiency were present in the *pan al-1* nucleus, but would not be anticipated if only a typical recessive lethal mutation were involved. There is good evidence, from the behavior of several different pseudowild types originating through 3-1 segregation of the ring-of-four chromosomes from a translocation heterozygote, that large chromosome deficiencies which are nonlethal in a heterokaryon are not recovered from crosses (MITCHELL, PITTENGER, and MITCHELL 1962; PITTENGER 1954).

The most convincing evidence suggesting genetic loss of the *K* allele is that it has been possible to show that the *pan al-1* nucleus which was originally of the genotype *jk* is now compatible not only with *nic-1 al-2* strains which are *jk*, but also with those which are *jK*. Since incompatibility has been shown to result from heteroallelic combinations of certain alleles (GARNJOBST 1955; HOLLOWAY 1955), one would predict that the original *jK* strain, if it lacks the *K* allele owing to loss of genetic material (and is designated as *j*) would form a compatible heterokaryon with testers that are either *jk* or *jK*.

The experimental manipulation of lethal nuclei within heterokaryotic systems has precedents in the experiments conducted by ATWOOD and MUKAI (1954) in testing for homology of heterokaryon mutants with indispensable functions. The following experiments were undertaken to demonstrate that the proposed *pan al-1 (j)* strain was compatible in a heterokaryon with either *nic-1 al-2 (jk)* or *nic-1 al-2 (jK)*. To accomplish this, *pan al-1 (j)* nuclei were transferred from the heterokaryon where they were originally present to a new combination with *nic-1 al-2 (jK)*, as shown in the flow diagram which follows.

A' *pan al-1* + + (*j*)
 C *pan al-1 nic* + (*jk*)
 D + + *nic al-2* (*jK*)

—Trikaryon plated on minimal medium and single colony isolates were picked. A' + D cultures were distinguished from A' + C + D by colony counts on differentially supplemented media (See Table 2)

By this procedure, it was possible to recover *pan al-1* (*j*) + *nic-1 al-2* (*jK*) heterokaryons and then to demonstrate that they were indeed capable of growing at normal and constant rates on minimal medium in growth tubes. Their genotype were confirmed by plating conidia on differentially supplemented media as shown in Table 2. These results are similar to those presented in Table 1 for (*j*) + (*jk*) combinations and, together with the growth rates, indicate that the (*j*) homozygous lethal type was compatible with both (*jk*) and (*jK*) genotypes.

This evidence, together with that already presented, can be interpreted to mean that the lethal factor in the *pan al-1* nucleus represents a genetic loss of the *K* locus. Such an explanation is preferred because it requires only a single event to account for both the lethality and the compatibility due to loss of one of the incompatibility alleles in the heterokaryon. On the other hand, if mutation and genetic loss were both involved, two separate events within the same nucleus must be postulated in addition to multiple alleles affecting compatibility. That is to say, in addition to a lethal change, the mutation of *K* could be to an allele different from *k*, but one which is compatible with both *K* and *k*. A multiple series of alleles affecting compatibility is feasible, but the postulated interaction needs confirmation.

A complete analysis was performed only on heterokaryons 3 and 5 above. However, homology tests (ATWOOD and MUKAI 1954) were carried out with heterokaryons 3 + 5, 3 + 8, and 8 + 5; in all cases, the lethal present appeared to be the same. A similar situation, where a change to compatibility is accompanied by failure to recover one of the nuclear types as a homokaryon, has been noted a number of times, but because of the variety of incompatibility genes involved, analyses similar to the one presented above have not been performed. It is tempting to speculate, however, that they may be similar in origin to the one analyzed.

TABLE 2

*Conidial plating results (numbers of colonies) used to confirm the genotypes of four heterokaryotic isolates presumed to be pan al-1 (*j*) + nic-1 al-2 (*jK*) which were recovered from conidia of the trikaryon [pan al-1 (*j*) + pan al-1 nic-1 (*jk*) + nic-1 al-2 (*jK*)]*

Isolate	Type of medium			
	M	P	N	PN
1	26	30	696	710
2	197	200	1331	1391
3	27	30	496	494
4	7	4	106	92

DISCUSSION

The extreme changes which occur in the growth rates of the incompatible heterokaryotic combinations described above can be attributed to permanent alterations in the compatibility reaction between the component strains of the heterokaryon. That such compatibility changes have a genetic basis has been shown in several cases by demonstrating that one of the homokaryotic components from the distal end of the growth tube is genetically different from the corresponding strain isolated proximally from the same tube. Some alterations have been shown to involve genetically viable changes, but in others a recessive lethal is present. It has not been possible, however, to establish the exact nature of the lethal condition by breeding tests. The mechanism(s) responsible for these alterations unfortunately cannot be determined on the basis of evidence now at hand. Mutation of compatibility factors and somatic recombination both appear to be possible mechanisms, but the data do not distinguish clearly between these alternatives. Even a third mechanism, somatic nondisjunction leading to chromosome loss, would explain the recessive lethal changes. At present, however, there is no compelling reason to believe that all of the observed alterations are the result of a single mechanism.

Although the evidence for somatic recombination in heterokaryons of *Neurospora* is conflicting (WEIJER and DOWDING 1960; CASE and GILES 1962; PITTINGER and COYLE 1963), it is still not possible to rule out its occurrence in heterokaryons. If a single mechanism is preferred to account for the above results, somatic recombination appears as likely an explanation as any at the present time. If one assumes that nuclear fusions and subsequent haploidization occur in heterokaryotic cultures, equal and unequal reassortment of chromosomes during haploidization could account for the respective viable and homozygous lethal changes that have been encountered. It is also possible that unequal reassortment of chromosomes during haploidization involves the linkage groups associated with the reciprocal translocation present in the *pan al-1* strain. In the case of *al-1* (4637T), a deficiency for IIR and a duplication for IR would result in the loss of the *D* (*het-2*) gene in IIR. This might suggest that what we have called *K* is in fact the *D* gene described by GARNJOBST (1955). Such an explanation is not at all inconsistent with the results, and it is quite possible that the lethal mutations so frequently encountered could be the direct consequence of the presence of the translocation in the stocks.

Mutations could conceivably account for all observed results if one is willing to make certain assumptions. A mutational event seems a most likely explanation of the viable compatibility changes. The recessive lethal changes could also have a mutational origin if one first assumes that multiple alleles for compatibility exist in *Neurospora*. The change, for example, from *k* to *K*, could then in fact be interpreted as a change from *k* to *k'* if one assumes *k'* to be compatible with both *k* and *K* and if one further assumes that *k'* is homozygous lethal. This combination of assumptions does not appear too likely, but such an explanation cannot be excluded by the present data.

The advantage of the incompatibility system described above is that even a very rare event, be it mutation, somatic recombination, or somatic nondisjunction, is potentially capable of being recovered because of the marked selective advantage bestowed upon certain nuclei in which the change in compatibility takes place. Just how efficient this system is for detecting rare events remains to be seen. Nevertheless, with this system and with the appropriate marked stocks now being developed, one should eventually be able to recover altered types and to determine whether the changes are ever accompanied by recombination of linked and unlinked markers. Since such experiments should eventually make it possible to distinguish between these various alternatives, there is little to be gained by further discussion regarding mechanism until the more definitive experiments are performed.

SUMMARY

Genetic analyses have been made on forced heterokaryons of *Neurospora crassa* that show erratic growth patterns, where the rate of growth gradually decreases and eventually stops. The analysis of genotypes of nuclei recovered from the distal ends of growth tubes of such incompatible heterokaryons, where growth resumed after an indeterminate lag, has shown that the resumption of growth is due to the formation of a new compatible genotype. In these cases, the heterokaryon-compatibility reactions of nuclei carrying the same biochemical markers from the proximal and distal ends of a growth tube are different. In some cases, the nuclear alterations are viable, but in others one of the nuclear components of the heterokaryon is lethal as a homokaryon. It is not known whether the changes originated by mutation, by somatic recombination or by nondisjunction.

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