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A second heterocaryon gene has been identified in Neurospora that affects the maintenance of stable growth rates of heterocaryons, rather than heterocaryon formation, as do the genes C, D, and E. The alleles of the new gene have been tentatively designated HI and hi, for heterocaryon instability. On growth tubes, forced HI + HI and hi + hi heterocaryons grow normally. HI + hi heterocaryons grow at wild type rate initially, then growth slows or ceases. The behavior of HI + hi heterocaryons appears to be unrelated to nutritional requirements of the component strains. Results of conidial platings and single hyphal tip isolations from unstable heterocaryons suggest (1) a greatly reduced number of hi nuclei in conidia and hyphae growing from the conidial inoculum, (2) that migration of hi nuclei is not totally inhibited. Direct observations show that HI and hi conidia germinate and fuse normally. The behavior of HI + hi heterocaryons appears to be independent of initial nuclear ratios, unlike that of the previously described I + i heterocaryons. These results suggest a non-adaptive change in nuclear ratios of the unstable heterocaryons. We have been unable to determine whether this change is a result of selective mitotic inhibition, interference with nuclear migration, or a combination of both. Preliminary genetic analysis suggests that the new gene is on the left arm of linkage group II.

GENE CONTROLLED HETEROCARYON INSTABILITY IN *NEUROSPORA CRASSA*

by

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APPROVAL PAGE

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INTRODUCTION

Heterocaryosis may be defined as the coexistence of two or more genetically different nuclei in cytoplasmic continuity with one another (Davis, 1966). In theory, any multinucleate cell may be heterocaryotic, but frequent hyphal fusions and nuclear exchanges make heterocaryosis a common condition in many fungi (Wilson, 1958). It is often a preliminary requirement for sexual reproduction (Pontecorvo, 1953), and for the parasexual cycle (Pontecorvo, 1956). Under certain conditions, heterocaryosis offers a simple alternative to genetic testing for allelism.

The first artificial heterocaryons were synthesized by Burgeff (1914). Using Phycomyces nitens, he squeezed the contents of a sporangiophore of one mating type into a sporangiophore of the opposite mating type and transferred the second sporangiophore to a nutrient medium where it occasionally regenerated vegetative hyphae which were bisexual heterocaryons.

Hansen and Smith (1932) and Hansen (1938) concluded that the "dual phenomenon" i.e. heterocaryosis, occurred with great frequency in Botrytis cinerea and a number of other fungal species. Single spore isolations revealed that most conidia of the strains were heterocaryotic. Single spores of B. cinerea gave rise to three culture types: M (aconidial); C (conidial); and an intermediate between M and C. Single spores of M or C gave rise only to M or C culture types. Single spores of the MC intermediates produced M, C, and MC intermediate culture types. Comparable results in other species suggested that heterocaryosis is probably the natural state for many fungi.

Beadle and Coonradt (1944) found that they could force heterocaryosis in N. crassa by satisfying the following criteria: The two strains must be of the same mating type; they must each carry at least one recessive gene for a nutritional deficiency; the mutant genes involved must be nonallelic. Inocula of two strains satisfying these criteria were superimposed on minimal agar medium so that heterocaryosis was a prerequisite for growth. They used heterocaryons to test for allelism, and to inhance fertility in mutant strains. Results of their investigations tended to confirm ideas concerning the physiological basis of heterocaryotic vigor proposed by Dodge (1942). They proposed the idea that hyphae containing the most favorable range of nuclear ratios would attain a higher growth rate than hyphae with less favorable ratios, eventually resulting in a hyphal frontier in which nearly all hyphae would contain nuclear ratios in an optimal range. This process has been termed hyphal selection.

Genetics of Heterocaryosis in Neurospora crassa

Holloway (1953) and Garnjobst (1953) demonstrated the presence of genetic factors that controlled heterocaryon formation. Holloway, using Emerson wild types E5297, 5531 pan-1, and 4545 lys, was able to determine the existence of at least four genes controlling heterocaryosis, and to distinguish seven distinct patterns of heterocaryotic growth. No definite correlation has been established between those genes and the genes described by other researchers. Garnjobst discovered some isolates of rib strain Y 30539 that consistently formed heterocaryons with 5531 pan while others never did. The "het+" isolates of Y 30539 rib formed

heterocaryons with all of the standard mutants except 37401 inos. This inos strain would not form heterocaryons with the same standard strains. She determined that two unlinked pairs of alleles (het-C and het-D) were responsible. Later work indicated that a single gene difference at either of the two loci prevented heterocaryon formation. The genetic combinations CD + CD, Cd + Cd, cD + cD, cd + cd, permitted heterocaryon formation; the combinations CD + cD, CD + Cd, Cd + cd, cD + Cd, cD + cd prevented heterocaryon formation (although the combination Cd + cd occasionally exhibited faltering growth). The interactions of the het genes were unrelated to any biochemical deficiencies with which they might be associated (Garnjobst, 1953; 1955). Microscopic examinations of hyphal fusions revealed a protoplasmic incompatibility reaction associated with the mixing of het- protoplasms. The reaction apparently began shortly after protoplasmic flow between the two fused cells. The protoplasm first appeared granular near the point of fusion. It then became increasingly vacuolated until the entire protoplasmic contents of the cell were disorganized. The reaction often affected a number of adjacent cells. Those affected became sharply delimited by septal plugs. Eventually, only remnants of the original protoplasm were left. Similar incompatibility reactions occurred when A and a strains fused (Garnjobst and Wilson, 1956). Microinjection studies indicated that the incompatibility reactions were due to an extractable protoplasmic component which was apparently a protein or protein-RNA complex (Wilson, Garnjobst, and Tatum, 1961; Williams and Wilson, 1966). A third gene, het-e, was found in St. Lawrence wild types and derivatives. A difference at the E locus resulted in a cytoplasmic incompatibility reaction similar in appearance to that described by

Garnjobst and Wilson (1956). The following linkage relations were established for the three genes: d in linkage group IIR, distal to fl (fluffy); c in group IIL, distal to ro-3 (ropy); and e in group VIIL, distal to nic-3 (Wilson and Garnjobst, 1966).

Perkins (1975) used strains containing duplication-generating chromosome rearrangements to study known het genes in N. crassa by creating duplications of these genes in a single strain. Strains with homozygous duplications of the het genes (C/C, E/E, or A/A) appeared normal, while those with heterozygous duplications (C/c, E/e, or A/a) were initially inhibited and released a dark pigment into the agar in the presence of phenylalanine and tyrosine. The heterozygous duplication phenotypes were different from those of incompatible heterocaryons, but, the duplication phenotypes were easily recognizable, and appeared to be analogous to conventional heterocaryon incompatibility. Crosses between duplication generating strains and normal strains produced two kinds of progeny: normal; and inhibited with dark agar; whenever the crosses were heterozygous for any of the het genes studied. The method proved to be valid for use in the search for, and identification of new het genes. Resolution of this technique is apparently limited only by the number and lengths of the various duplications that can be formed. A number of strains, both standard het gene testers and wild types of unknown het genotype, were tested to determine the genotype of each. Results of these crosses suggested the presence of at least one new het gene on the left arm of linkage group II in addition to het-C.

Mylyk (1975) used the same techniques to examine extensively the N. crassa genome for the presence of other het genes. He reported the

presence of five previously unmapped genes (het-5 in group IR, het-6 in group IIL distal to het-C, het-7 in IIIR, het-8 in VIL, het-9 in VIR, and het-10 in group VIIR) which gave heterozygous duplication phenotypes resembling those of heterozygous duplication strains of het-C, het-E, or mating type.

Studies on Genes Affecting Nuclear Ratios

Evidence for nonadaptive changes in nuclear ratios in Neurospora heterocaryons was first observed by Ryan and Lederberg (1946) and Ryan (1946). In studies on adaptation of leucineless mutants back to wild type, certain cultures derived from leucineless strain 33757 were found which had reverted so that they were physiologically identical to wild type. Growth rates of the adapted cultures were the same as wild type and were independent of the concentration of leucine in the medium. Genetic studies indicated that the adaptations were due to back mutations of the same gene or a closely linked gene. On minimal medium, heterocaryons formed with the leucineless and the adapted cultures had growth rates characteristic of the adapted control strains on minimal medium. Heterocaryons formed on minimal medium plus limiting concentrations of leucine grew at the same rate as leucineless controls on limiting concentrations of leucine. Hyphae were isolated from heterocaryons in which the leucineless and adapted strains contained different marker genes for conidial color. When heterocaryotic hyphae were isolated and grown on minimal medium, color markers associated with leucineless nuclei could not be demonstrated. Such hyphae grown on limiting concentrations of leucine did not show color markers associated with the adapted strains. This evidence indicates some selective

activity which acts on the nuclei themselves, and which may be modified by supplementing, with leucine, the medium on which the heterocaryon is growing. They were unable to determine whether this selective activity inhibited or destroyed the unsuccessful nuclei. Attempts to demonstrate an extractable inhibitor of leucine-independent nuclei from both leucine-less strains and heterocaryons were unsuccessful.

Gross (1952) reported successful synthesis of bisexual heterocaryons. He used the conditionally lethal mutants 5531 pan-1 and 4545 lys. The heterocaryons produced were self fertile, but the growth rates (on growth tubes) were severely reduced (approximately 0.5 mm./hr.). Isolations of single hyphal tips and results of conidial platings indicated highly disparate nuclear ratios, which could account for the reduced growth rates of these heterocaryons. Gross believed that the divergent nuclear ratios were due to replication inhibition of A nuclei by a nuclei when the two were in a heterocaryon.

In studies on adaptation in pantothenate-requiring strains of Neurospora, Davis (1960 a; 1960 b) found a modified strain, pan-1, m, derived from 5531 pan-1. The modified strain was apparently able to utilize lower concentrations of pantothenate which would be limiting to the normal pan-1 strain. On growth tubes containing unlimiting concentrations of pantothenate, pan-1 homocaryons grew at rates between 3.5 and 4.0 mm./hr. Homocaryons of pan-1, m, grew at 2.4 mm./hr. Growth rates of heterocaryons on unlimiting pan concentrations were linear with time (as were growth rates of the homocaryons) but, those with lower EMP values grew at lower rates. (The plating technique used measured only the numbers of homocaryotic pan-1 colonies. Also, homocaryotic conidia are often

produced in higher numbers than expected on the basis of random chance. For these reasons, nuclear frequencies in this work were recorded as maximal estimates of pan-1 nuclear frequency. These frequencies were referred to as EMP values). Conidial samples, taken from three widely separated ports along the growth tubes, were plated to determine the EMP values at these intervals. The EMP values were constant (within 8%) throughout the length of the mycelium. When this experiment was repeated on growth tubes containing limiting pantothenate concentrations, growth of pan-1 homocaryons was severely limited, demonstrated by a very short period of growth before they stopped. The growth rates of pan-1, m homocaryons were nearly equal to those of pan-1, m on unlimiting medium. Heterocaryons of pan-1 + pan-1, m exhibited unstable growth rates, consisting of a short period of growth, followed by a period of no growth. Growth then resumed, either at (1) a rate characteristic of pan-1, m homocaryons, (2) a slowly increasing rate that approached that of pan-1, m homocaryons, (3) a fluctuating rate intermediate between those of the component homocaryons, or (4) a high rate, followed by a second cessation of growth. To determine correlations between changes in nuclear frequencies and fluctuations in growth rates, heterocaryons were inoculated at the edges of large Petri dishes containing limiting concentrations of pantothenate. The heterocaryons grew rapidly at first, then stopped. Renewed growth then appeared at sectors along the hyphal frontier. These sectors either continued growing, or, in some cases, stopped a second time. Conidial samples from various points of growing and nongrowing sectors of the mycelium were plated, and the EMP values in each of the sectors was determined. Results indicated that, in general, growth

occurred only when EMP values were below 0.45. Growth stopped when EMP values were above 0.50. It was evident that changes in growth rates of pan-1 + pan-1, m heterocaryons were due to changes in nuclear ratios. Davis assumed that the non-adaptive increase in pan-1 nuclei was the reason for cessation of growth in the heterocaryons, and that further growth involved hyphae having a more favorable ratio of pan-1 to pan-1, m nuclei. He suggested that this phenomenon supports the hyphal selection hypothesis of Beadle and Coonradt (1944). Heterocaryons of pan-1, m + nic-2, al-2 and pan-1 + nic-2, al-2 showed stable growth rates and stable EMP values. These results suggested that the variable growth rates and fluctuating nuclear ratios were peculiar to the pan-1 and pan-1, m genotypes. The evidence also supports the idea that the fluctuating nuclear ratios may be due to nuclear competition for intracellular constituents (probably pantothenate).

Pittenger and Brawner (1961) reported on the alleles I and i which affected the nuclear ratios and growth rate in N. crassa heterocaryons. I + I and i + i heterocaryons exhibited stable growth rates (growth was measured on growth tubes, but little comparative data was actually reported). In I + i heterocaryons, I nuclei underwent a nonadaptive increase which ultimately resulted in a culture homocaryotic for I nuclei. The biochemical deficiency associated with homocaryosis caused growth to stop. The conidial color markers al-1 and al-2, and the auxotroph nic-2 were used to detect any nuclear selection that resulted in highly diverse nuclear ratios. Heterocaryons of al-1 + al-2 exhibit normal to near normal orange color with a variety of nuclear ratios. High proportions of al-1 nuclei result in dark yellow, light yellow, or white conidia; an excess of

al-2 nuclei results in light pink to white conidia. The auxotroph nic-2 was used because it releases a reddish brown pigment into the medium when nicotinic acid is limiting. Thus a heterocaryon of pan-1, al-1 + nic-2, al-2 might show white conidia, indicating a highly diverse nuclear ratio, and a reddish brown pigment indicating a deficiency for nicotinic acid. Conidia were isolated from heterocaryons containing complimentary auxotrophs and the color markers al-1 and al-2. When conidia from a heterocaryon produced a majority of cultures deviating from wild type coloration, the heterocaryon was investigated to determine the existance of nuclear competition. This technique would tend to screen any nonadaptive nuclear increase by one component of a heterocaryon, whether it was due to competition for some intracellular component, or active inhibition of one nuclear type by the other. This is how the strains carrying I and i were discovered. I + I and i + i heterocaryons grew at approximately normal growth rates and showed no systematic increase by one nuclear component, even when started from a variety of nuclear input ratios. When I + i heterocaryons were examined, growth rate and interactions between I and i nuclei depended to some degree, on the initial nuclear ratios. If the initial input ratios of i nuclei exceeded about 70%, the growth rates and nuclear ratios showed no systemic change. When the initial input ratios were less than 70% in favor of the i component, growth stopped and there appeared a sharp decrease in the production of i nuclei, when the nuclear ratios of the proximal and distal ends of the growth tubes were compared. These results demonstrated a nonadaptive change in the nuclear ratios of the heterocaryon, which resulted in cultures that were essentially homocaryotic for I nuclei. Interactions of I and i nuclei appear to be

independent of any particular auxotrophic markers used, i.e. 4545 lys-5, 10575 trip-1, and 3416 nic-1. To determine the behavior of conidial derived I + i heterocaryons, conidia were isolated from I + I, i + i, and I + i combinations. Auxotrophs and color markers have already been mentioned. The majority of colonies resulting from conidia of I + I, and i + i heterocaryons had wild type coloration, while the majority of those resulting from I + i heterocaryons had color phenotypes indicating the presence of a high proportion of I nuclei, regardless of the initial input ratios of the original heterocaryons. The few conidial derived cultures that were not white had a high proportion of i nuclei. Some of the conidial derived I + i heterocaryons were transferred to growth tubes. In some cases, these heterocaryons had a normal color, normal growth rate, and an i nuclear frequency in excess of 70%. Most however, grew varying distances before stopping. These cultures had become homocaryotic for I nuclei. Apparently a nonadaptive increase of I nuclei occurred in conidial derived heterocaryons, unless the nuclear ratio within the conidium approaches 1I : 3i or 1I : 4i. Inheritance studies, especially linkage data, were limited. Thirteen unordered asci, from a cross heterozygous for I gave a 1 : 1 segregation of I and i. Genetic analysis indicated that the locus was in linkage group I or II. The al-1 marker used is associated with a reciprocal translocation occurring between groups I and II, making linkage data difficult to obtain. The results supported the ideas that I + I and i + i heterocaryons undergo no systematic changes in nuclear ratios or growth rates, and that I + i heterocaryons are subject to a nonadaptive increase in I nuclei causing unstable growth or cessation of growth unless the initial proportion of i nuclei is 70% or above.

The genes I and i are found in strains either derived from, or compatible with wild type strain 74-OR-8-1a, which is now known to have the heterocaryon genotype Cde (Wilson and Garnjobst, 1966). The existence of I and i in strains derived from Rockefeller-Lindegren wild types has not been shown.

A number of strains derived from Rockefeller-Lindegren wild types have been found that readily form heterocaryons with other Rockefeller-Lindegren strains, but which do not maintain stable growth rates. These unstable heterocaryons grow at wild type rate initially, then growth slows, or ceases. This growth pattern suggests the presence of a hitherto unknown gene (tentatively designated HI and hi, for heterocaryon instability) in these isolates, which controls the latter stages of heterocaryotic growth. The behavior of the alleles HI and hi in heterocaryons appears to be unrelated to the nutritional requirements of the component strains. Results of single hyphal tip isolations from unstable heterocaryons (HI + hi) suggest: (1) a greatly reduced number of hi nuclei in conidia and hyphae growing from the original conidial inoculum, and (2) that migration of hi nuclei is not totally inhibited. The behavior of HI + hi heterocaryons appears to be independent of initial nuclear ratios, unlike that of the previously described I + i heterocaryons. In the present study, we describe the effects of the alleles in heterocaryons, determine how the alleles inhibit heterocaryotic growth, compare and contrast the characteristics of the new alleles with the characteristics of the alleles I and i, and present some evidence suggesting mitotic inhibition of hi nuclei in the presence of HI nuclei.

MATERIALS AND METHODS

The media used for these experiments are: Vogel's Medium N (Vogel, 1964); Fries medium (Ryan, Beadle, and Tatum, 1943; and Beadle and Tatum, 1945) for microcultures; synthetic crossing medium (Westergaard and Mitchell, 1947); and Glycerol Complete Medium (Tatum, Barratt, Fries, and Bonner, 1950) with the substitution of 0.4% sucrose for half the glycerol. Nutritional supplements were nicotinamide hydrochloride and calcium pantothenate (5 µg/ml) and inositol (50 µg/ml).

The tester strains (Table 1) all have the heterocaryon genotype C, D, E, and were derived from Rockefeller-Lindegren wild types. The mutants include: inl (37401), pan-1 (5531), al-2 (15300), and nic-3 (Y31881).

Growth rates were measured using methods described by Ryan, Beadle, and Tatum (1943).

Mass cultures of conidia were grown in 250 ml Erlenmeyer flasks containing 50 ml of Vogel's Minimal Medium plus the appropriate nutritional supplement. They were incubated for 7 days at 30°C. The conidia were harvested in 30 ml of sterile distilled water and filtered through two filter flasks. The suspensions were diluted 1:1000 and counted on a hemacytometer. The yield varied between 5×10^7 and 10×10^7 conidia/ml for the undiluted suspension.

For platings, a loop of conidia from growth tubes or slants was suspended in a 10 ml sterile distilled water blank and filtered twice through thistle tubes filled with glass wool. The suspensions were counted and diluted to a concentration of 300 conidia/ml, and plated at

Table 1. Tester Strains for Heterocaryon Instability

inl, *HI*

NCRL73(1-4)A *al-2*; *inl*; *HI*

(2-3)A *inl*; *HI*

NCRL74(1-2)a *al-2*; *inl*; *HI*

29 a *inl*; *HI*

inl, *hi*

NCRL74(2-3)A *al-2*; *inl*; *hi*

NCRL74(3-5)A *al-2*; *inl*; *hi*

(2-7)A *inl*; *hi*

(11-7)a *inl*; *hi*

3a *al-2*; *inl*; *hi*

pan-1, *HI*

(2-3)A *al-2*; *pan-1*; *HI*

2A *pan-1*; *HI*

(3-7)a *al-2*; *pan-1*; *HI*

2a *pan-1*; *HI*

pan-1, *hi*

NCRL73(1-8)A *pan-1*; *hi*

NCRL74(1-6)A *pan-1*; *hi*

18A *pan-1*; *hi*

188A *al-2*; *pan-1*; *hi*

21a *al-2*; *pan-1*; *hi*

NCRL74(2-8)a *pan-1*; *hi*

100 conidia/plate. Platings were made in triplicate for each nutritional class, but all slant heterocaryons were double plated (i.e. six plates per class). The plating medium was that described by Brockman and De Serres (1963).

Control of nuclear ratios in conidia inocula was accomplished by a modified version of the technique reported by Pittenger and Atwood (1954) and Pittenger and Brawner (1961). First, the per cent of viable conidia produced by each component strain was determined by plating conidia from 7 day old cultures of each strain grown at 30°C on Vogel's Medium plus the appropriate nutritional supplement. The component strains were then grown in conidial flasks for 7 days under the same conditions. The resulting conidia were harvested according to the procedure already described, and the two suspensions were mixed by volume in ratios adjusted to compensate for differential conidial viability. The calculations were based on the assumption that the average number of nuclei per conidium was the same in the component strains. The mixed suspension was centrifuged at 500 x g, the supernatant fluid decanted, and a portion of the conidial pellet was transferred to the medium with a loop. Heterocaryons with extreme nuclear ratios were formed by microinjections, as described below.

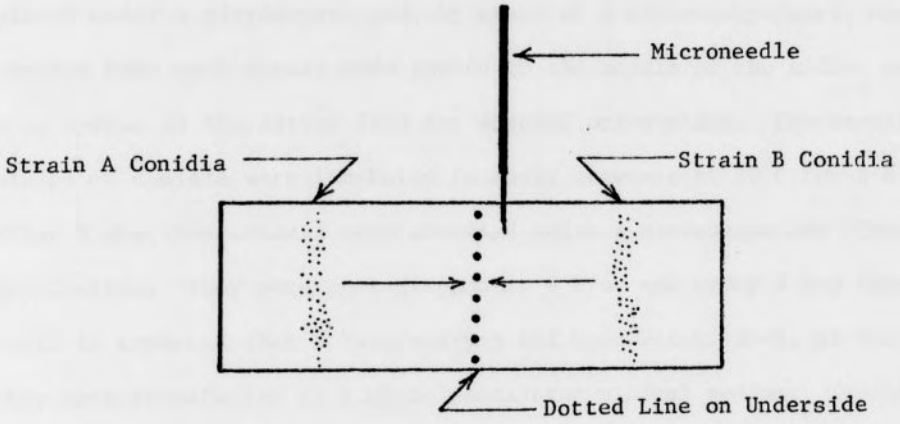
To test the effect of nutritional supplements on unstable heterocaryons, a heterocaryotic plate colony was grown for 18 hrs at 25°C and agar blocks were cut from the frontier region and transferred to growth tubes containing supplemented media. These tubes contained sampling ports through which conidial samples could be removed at various distances from the inoculum site. Blocks of agar and mycelium were removed from the area 1 cm behind the hyphal frontier, incubated 24 hrs at 30°C, and the resulting conidia were plated.

Plate cultures for hyphal tip isolations were started from pellets of centrifuged conidia. The numbers of conidia of the two component strains in these pellets were adjusted to give a 1:1 ratio with allowance for differential per cent germination. The cultures were incubated for 12 hrs at 25°C before the hyphal tips were isolated. The isolation procedure was carried out under a transfer hood fitted with an adapter to allow the eyepieces of a dissecting microscope to extend above the hood. The hyphal tips were transferred to 10 x 75 mm slants and incubated at 30°C for at least 48 hrs.

For the diffusable inhibitor tests, a 2X concentration of De Serres sorbose medium (Brockman and De Serres, 1963) was used. Conidial flasks were incubated and harvested in the usual manner, and the resulting conidial suspension contained 50 x 10^7 conidia/ml. Two ml of this suspension were transferred to a 250 ml Erlenmeyer flask containing 50 ml of modified Vogel's liquid with supplement. Because the carbohydrate composition is crucial, this medium was made with 0.1% dextrose, and 0.1% fructose instead of sucrose to avoid sucrose accumulation in the final plating. The cultures were incubated at 30°C for 6 hrs on a shaker. Samples of the cultures were examined under a microscope to assure that most conidia were germinating. The cultures were then filtered through a 5 µm Millipore filter. The filtrate was mixed 1:1 with melted 2X sorbose medium, and this mixture was used for the final plating medium.

Microscope slides for the conidial coalescence experiments were prepared in the following manner. A dotted line was drawn on the underside of the slide, dividing it into the right and left halves (Figure 1). The upper surface of the slide was coated with melted Fries medium (Ryan,

Figure 1. Diagram Showing Microscope Slide Prepared for Conidial Coalescence Experiments



Beadle, and Tatum, 1945). Microneedles were pulled by hand from 1 mm diameter, soft glass capillary tubing. The technique has been described by Chambers and Kopac (1950). Conidia from each component strain were streaked on each side of the agar coated slide. The inoculated slide was placed under a microscope, and, by means of a micromanipulator, one or two conidia from each strain were pushed to the middle of the slide, using the open spaces in the dotted line for spacial orientation. The resulting clumps of conidia were incubated in moist chambers at 30°C for 6 hrs. After 6 hrs, the conidia were examined under a microscope for signs of germination. They were photographed at 6 hrs, and every 3 hrs thereafter until it appeared that a heterocaryon had been established, at which time they were transferred to a plate containing minimal medium. Sterility was maintained by inspection, and the criterion for heterocaryosis was growth on minimal medium.

Microsurgical operations were carried out according to the methods described by Wilson (1961; 1963), and Wilson, et al. (1961), except that the injection apparatus was modified by using a 1 ml syringe connected to the micropipet chuck by Clay-Adams polyethylene tubing (PE 260 fitted with size D male Luer-Lock adapters). The small syringe confers a greater mechanical advantage when injecting material against the high turgor pressure of Neurospora. Crude extract for microinjections was prepared from a 24 hr shake culture of an inl, H1 strain grown in Vogel's minimal plus inositol at 30°C. The mycelium was harvested using a suction funnel and washed in phosphate buffer. The mycelial mat (approximately 12 g wet weight) was chilled and sand ground in equal amounts of sand and 0.1M phosphate buffer, pH 6.8. The crude extract was centrifuged at 500 x g for 20 min to remove the sand.

The cross for genetic analysis was carried out on synthetic crossing medium (SCM) plus pantothenate. The ascospores were isolated in order, and only tetrads with at least three of the meiotic products present were analysed. Testing for heterocaryon instability genotype was done on growth tubes by forming heterocaryons between the isolate being tested, and a known HI or hi tester strain.

The confidence limits, i.e. the probability of detecting intermediates in the sample of eight tetrads if two loci are present, were determined using tables (Dixon and Massy, 1969) based on the binomial probability function

$$P[y] = \binom{n}{y} X^{y}(1-X)^{N-y}$$

where y is the number of intermediates, x is the expected per cent of crossing over, and N is the total number of asci.

RESULTS

Behavior of Unstable Heterocaryons

The effect of the alleles HI and hi on forced heterocaryons is illustrated graphically in Figure 2. At 30°C, homocaryotic HI or hi strains grow at nearly equal, and fairly constant rates (approximately 4 mm/hr), that are linear with time throughout their growth periods. Forced heterocaryons, which are homozygous for HI, such as 73(1-4)A al-2; inl; HI + (2-3)A al-2; pan-1; HI, also grow at approximately 4 mm/hr, much like a wild type homocaryon. Heterocaryons which are homozygous for hi 74(3-5)A al-2; inl; hi + 73(1-8)A pan-1; hi, exhibit a nearly identical growth pattern. Heterocaryons which are heterozygous for the two alleles, 29a inl; HI + 21a al-2; pan-1; hi, 73(1-4)A al-2; inl; HI + 18A pan-1; hi, and 162A al-2; nic-3; hi + 73(1-4)A al-2; inl; HI; may, as illustrated in Figure 2, exhibit any of a number of growth patterns. The most common is characterized by an early growth rate which is linear with time and approaches that of wild type for up to 72 hrs. Between 36 hrs and 72 hrs however, the growth of these heterocaryons may slow down, become very erratic, or cease completely.

Conidial Platings

The possibility that HI + hi heterocaryons might stop because they had become homocaryotic was tested by plating conidia from slant cultures of four stable heterocaryons (as controls) and four unstable (HI + hi) heterocaryons. The results are tabulated in Table 2. Each plating represents the assay of a separate heterocaryotic culture.

Figure 2. Representative Growth Curves for Stable and Unstable Heterocaryons

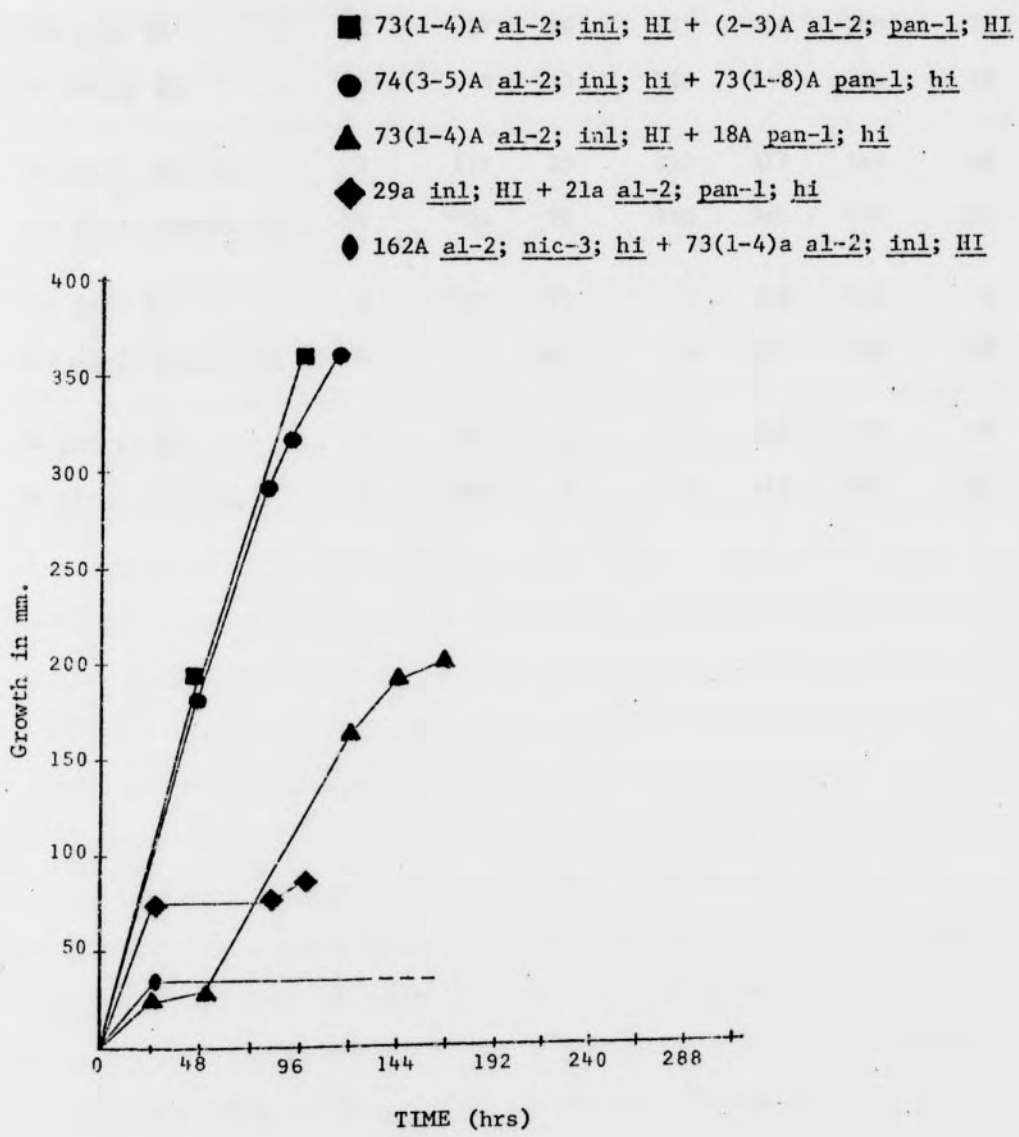


Table 2. Indicated Nuclear Ratios of Stable and Unstable Heterocaryons

Heterocaryon	Culture Number	Homocaryotic Colonies pan-1	Homocaryotic Colonies inl	Heterocaryotic Colonies	Total Count	Control Count	Error
29a inl; HI + 2a pan-1; HI	1	37	70	277	384	358	7%
	2	45	43	347	435	455	4%
3a al-2; inl; hi + 21a al-2; pan-1; hi	3	158	25	334	517	546	5%
	4	33	22	330	385	443	13%
29a inl; HI + 21a al-2; pan-1; hi	5	171	471	6	648	642	1%
	6	0	422	0	422	428	1%
2a pan-1; HI + 3a al-2; inl; hi	7	340	0	1	341	348	2%
	8	604	5	4	613	641	4%

Culture #1 (29a inl; HI + 2a pan-1; HI) produced 277 heterocaryotic colonies, 37 homocaryotic pan-1; HI colonies, and 70 homocaryotic inl; HI colonies. The total of the heterocaryotic and homocaryotic plate count (358) agrees with the control plate count (384) to within 7%. Culture #2 (same strains) produced 347 heterocaryotic colonies, 45 homocaryotic pan-1; HI colonies, and 43 homocaryotic inl; HI colonies. The total heterocaryotic plus homocaryotic count (435) agrees with the control plate count (455) to within 4%. Culture #3 (3a al-2; inl; hi + 21a al-2; pan-1; hi) produced 334 heterocaryotic colonies, 158 homocaryotic al-2; pan-1; hi colonies, and 25 homocaryotic al-2; inl; hi colonies. The total heterocaryotic plus homocaryotic count (517) agrees with the control plate count (546) to within 5%. Culture #4 (same strains) produced 330 heterocaryotic colonies, 33 homocaryotic al-2; pan-1; hi colonies and 22 homocaryotic al-2; inl; hi colonies. The total heterocaryotic plus homocaryotic count (385) agrees with the control plate count (443) to within 13%. These heterocaryons consistently produced all three classes of conidia, including substantial numbers of heterocaryotic conidia, and roughly equal numbers of each homocaryotic class. The unstable heterocaryons showed two very different trends. Culture #5 (29a inl; HI + 21a al-2; pan-1; hi) produced only 6 heterocaryotic colonies, 171 homocaryotic al-2; pan-1; hi colonies, and 471 homocaryotic inl; HI colonies. The total heterocaryotic plus homocaryotic plate count (648) agrees with the control plate count (642) to within 1%. Culture #6 (same strains) produced no heterocaryotic colonies, and no homocaryotic al-2; pan-1; hi colonies. The homocaryotic inl; HI count (422) and the control plate count (428) agree to within 1%. Culture #7 (2a pan-1; HI + 3a al-2; inl; hi) produced 1 heterocaryotic

colony, 340 homocaryotic pan-1; HI colonies, and no homocaryotic al-2; inl; hi colonies. The total heterocaryotic plus homocaryotic count (341) agrees with the control plate count (348) to within 2%. Culture #8 (same strains) produced 4 heterocaryotic colonies, 604 homocaryotic pan-1; HI colonies, and 5 homocaryotic al-2; inl; hi colonies. The total of the three colony types (613) agrees with the control plate count (641) to within 4%. The plating results for every unstable (HI + hi) heterocaryon indicated that such heterocaryons produced a majority of conidia which were homocaryotic for HI nuclei, a much smaller number of conidia which were homocaryotic for hi nuclei, and very few if any heterocaryotic conidia. These data suggest that unstable heterocaryons either became homocaryotic for the HI component, or there was a selective process which favored the inclusion of HI rather than hi nuclei in conidia. It was also apparent that the hi nuclei are not inactivated or inhibited by their association with HI nuclei. A number of suspected hi vegetative reisolates were tested for their nutritional requirements (on minimal and supplemented minimal medium) and for their hi genotypes (on growth tubes), and in every case, the integrity of their genetic composition was confirmed, i.e. the reisolates were still hi. The observation of a small number of heterocaryotic colonies, produced with some degree of regularity, suggests that heterocaryotic conidia can germinate. This observation is important for two reasons. (1) The efficiency of recovery approaches 100% in two platings. When this is considered together with the observation that all three classes of conidia can germinate, we have some justification for believing that we have accounted for all classes of conidia. (2) If suppression of protein synthesis (or similar biochemical activity) occurred,

it is unlikely that heterocaryotic conidia could germinate to form viable colonies. A conidium containing the combination pan-1; HI + inl; hi probably would not germinate at all, while a conidium containing the combination inl; HI + pan-1; hi would probably exhibit characteristics similar to inositol-less death.

Conidial Platings Along the Length of an Unstable Heterocaryon

If there were a gradual decrease in the proportion of hi nuclei along the length of a growing HI + hi heterocaryon, the trend might be detected by plating conidia at intervals along the length of the mycelium. Such a trend would show up as a decreasing number of heterocaryotic, and homocaryotic hi colonies, and would suggest a gradual or incomplete inhibition of replication of the hi nuclei. If such a trend were not observed, inhibition of nuclear migration, or nuclear selection at conidiation would be more reasonable hypotheses. Two unstable heterocaryons were started on growth tubes made with four sampling ports spaced 1.5 cm apart along the first 7 cm of the tubes. (As the most rapid changes in growth rate occurred during the first 7 cm of growth, it seemed most probable that a trend, if it existed, would be detected in this portion of the mycelium.) Blocks of agar containing hyphae and conidia were removed through the sampling ports every 24 hrs, either until the heterocaryons stopped, or until they grew past the last sampling port. The conidia were plated and the results are tabulated in Table 3. No hi colonies were recovered in any of the platings, suggesting an abrupt decrease in the proportion of hi nuclei, or the possibility of nuclear selection at conidiation. It is also possible that the plating density was not high enough to detect any changes in the hi nuclear frequency, but the

Table 3. Conidial Platings Along the Length of Two Unstable Heterocaryons

Sample Number	Distance from Inoculum	Homocaryotic Colonies		Heterocaryotic Colonies	Error
		pan-1	inl		
First Heterocaryon					
1	10 mm	252	0	0	11%
2	30 mm	213	0	0	13%
3	50 mm	201	0	0	8%
Second Heterocaryon					
1	20 mm	146	0	0	5%
2	40 mm	197	0	0	2%

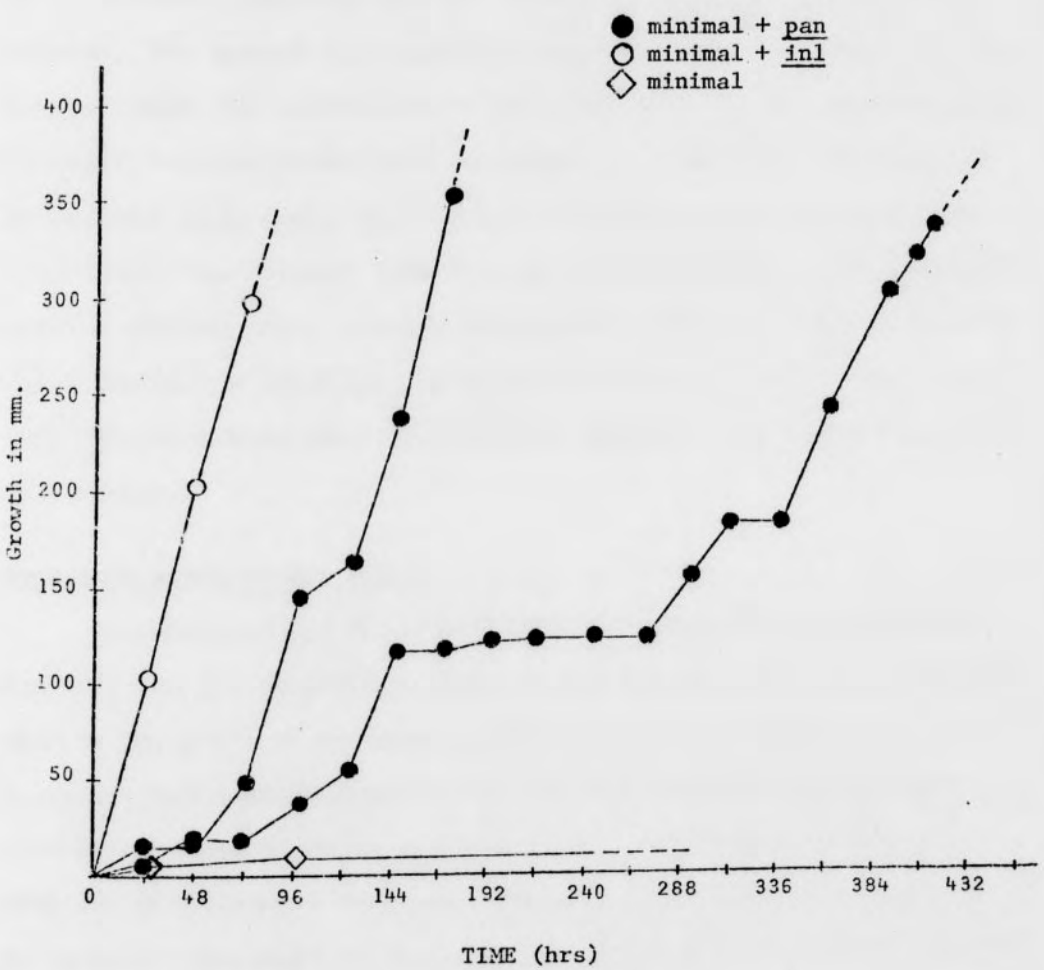
Strains were 2a pan-1; HI + 3a al-2; inl; hi

possibility that nuclear selection at conidiation was affecting the plating results seemed to indicate that a direct assay of single hyphal tips would be more efficient and more appropriate.

Effect of Nutritional Supplements on Unstable Heterocaryons

In order for an assay of single hyphal tips to be reliable, it was necessary to determine whether or not the instability reaction could be reversed by supplying the nutritional supplements necessary for growth of the individual component strains. The experiment was designed so that even if complete reversal of the instability reaction were not observed, recovery of hi nuclei in the frontier region of unstable heterocaryons would indicate that hi nuclei had migrated. The unstable heterocaryon 29a inl; HI + 21a al-2; pan-1; hi was started on a minimal plate, and incubated for 18 hrs at 25°C, thus forcing heterocaryosis. Agar blocks were then removed from the frontier region and transferred to growth tubes containing minimal, minimal + inositol, and minimal + pantothenate. Growth rates were recorded for each group, and representative results are graphed in Figure 3. Hyphae placed on minimal agar grew only 4.0 mm during the first 24 hrs. By 96 hrs, the frontier had advanced only 8 mm, after which it stopped growing. On minimal medium + inositol, both blocks of agar produced mycelia that grew to the ends of the tubes at constant rates that approached that of wild type. The hyphae placed on tubes containing minimal + pantothenate exhibited very slow, faltering growth during the first 48-72 hrs. After 72 hrs, growth of both heterocaryons became quite erratic. Growth rates increased to those of wild type for one or two days, and then slowed abruptly, or ceased for varying periods of time before starting again. One of these heterocaryons appeared to reestablish wild type growth

Figure 3. Behavior of Unstable Heterocaryons on Supplemented Media



rates, but the other one did not. To confirm that mycelia on minimal medium + pantothenate were actually heterocaryotic, conidia from the distal end of the mycelium having the more stable growth rate were plated. (We assumed that this mycelium was more likely homocaryotic for the pan-1; hi component. Erratic growth of the other heterocaryon was probably a result of unstable heterocaryosis.) The results of the platings were as follows: 2 heterocaryotic colonies, 7 colonies on sorbose + pantothenate, 267 on sorbose + inositol, and 288 colonies on sorbose + pantothenate + inositol. The mycelia were apparently heterocaryotic, the instability was only partially, and intermittently reversed, and the HI nuclear population apparently remained predominant throughout the experiment. Isolation of homocaryotic al-2; pan-1; hi colonies, and heterocaryotic colonies from these growth tube cultures indicates that viable hi nuclei were recovered from the original giant colony approximately 25 mm from the inoculum site, suggesting that at least some hi nuclei were able to migrate from cell to cell. The experiment also indicated that scoring single hyphal tips might be productive.

Scoring of Single Hyphal Tips

Conidial platings of unstable heterocaryons grown on growth tubes suggested that (1) an abrupt decrease in the numbers of hi nuclei occurred early in the growth of the heterocaryons (as if the instability were due to partial migration inhibition), or, (2) that there was some nuclear selection favoring HI nuclei at conidiation. To determine if nuclear selection at conidiation were occurring, and, in particular, to identify any hi nuclei that might be in the hyphae, single hyphal tips were isolated from small colonies (3-10 mm radius) of 2a pan-1; HI + 3a al-2; inl; hi,

and transferred to minimal agar slants, with and without pantothenate and/or inositol supplements. The results are shown in Table 4. None of the 127 hyphal tips on minimal slants produced visible cultures. Eighty of 102 hyphal tips on minimal + pantothenate + inositol slants did produce visible cultures. These results suggest that the hyphae had either become homocaryotic, or, that one group of nuclei had been inactivated. Also, the processes causing the instability reaction had proceeded to completion within 3-10 mm from the center of the inoculum. One hundred new hyphal tips were incubated on minimal slants for 24 hrs at 30°C, after which an appropriate concentration of calcium pantothenate was added to 50 of the slants. One culture developed from a hyphal tip on a minimal slant, while 22 cultures developed from hyphal tips on minimal + pantothenate slants. This experiment shows that a substantial number of functional pan-1; HI nuclei existed within the hyphal tips. Previous plating studies and growth tube experiments indicated that unstable heterocaryons could be revived and grown if conidia or portions of the hyphal frontier were transferred to minimal agar containing nutritional supplements necessary to support homocaryotic growth of the hi component. This enabled us to score for either type of nucleus in the hyphal tips. Fifty hyphal tips were placed on minimal + inositol slants, and 50 tips on minimal + pantothenate slants. Only one visible culture had grown on a minimal + inositol slant at the end of 12 days. (Its pale pink conidia indicated that it was probably heterocaryotic although nearly homocaryotic for the hi nuclei.) There were 38 orange cultures growing on minimal + pantothenate. (Orange cultures may be heterocaryotic, but conidial color and the larger number of viable cultures growing on minimal + pantothenate suggest that they are largely

Table 4. Nuclear Composition of Single Hyphal Tips

Test Number	Medium	Number of Hyphal Tips	Number of Cultures Produced
1	Minimal	127	0
	Minimal + Pantothenate + Inositol	102	80
2	Minimal	50	1
	Minimal + Pantothenate at 24 hrs	50	22
3	Minimal + Inositol	50	1
	Minimal + Pantothenate	50	38
4	Minimal + Inositol	50	0
	Minimal + Pantothenate	50	47

homocaryotic for the pan-1; HI component.) These data indicated that the pan-1; HI nuclei were functional, and that they were the dominant nuclear type. As only 38 of 50 hyphal tips were recovered on minimal + pantothenate slants, the experiment was repeated to rule out the possibility of losing hi nuclei due to mechanical injury of the hyphal tips. The second time, none of the 50 tips placed on minimal + inositol slants produced visible cultures, while 47 of 50 tips on minimal + pantothenate did produce visible cultures. These results tend to confirm that there were only very small numbers of hi nuclei in hyphal tips 3-10 mm from the inoculum site. When it occurs, resulting growth on minimal + inositol appears to be too abundant to be due to residual precursor pools of necessary nutrients within the hyphal tips. Later, during microsurgical experiments, three to five cell hyphal segments placed on minimal agar plates did not grow nearly as well. The observation that hi colonies, or heterocaryotic colonies are recovered from hyphal tips suggests that the hi nuclei in the hyphal tips are biochemically active with the exception of those reactions related to mitotic processes, or possibly processes affecting nuclear migration. It is also probably safe to assume, based on available data, that hi nuclei are not destroyed by an association with the HI strains. The experiments appear to rule out nuclear selection at conidiation as a cause for previous plating results, and although the occurrence of differential inhibition of nuclear migration can not be completely eliminated, this evidence does little to support this hypothesis because localized high concentrations of hi nuclei have not been found. The experiment does not rule out the possibility of differential germination rates of HI and hi conidia when they are in close association with one another. Also, both types of

conidia may germinate, followed by a differential rate of fusion between HI and hi hyphae.

Test for a Diffusable Germination Inhibitor

Testing for the production of a diffusable germination inhibitor was done by plating conidia from strains 2a pan-1; HI and 21a al-2; pan-1; hi on sorbose agar made with a filtered minimal + pantothenate liquid medium in which the HI strain had previously been grown for 6 hrs at 30°C. As the carbohydrate composition of sorbose medium is crucial, the minimal + pantothenate medium was modified by using fructose and glucose instead of sucrose. Control platings of the HI and hi strains were made on regular sorbose medium. On the experimental plates, strain 2a pan-1; HI produced 138 colonies from 300 conidia (46% conidial viability), and 21a al-2; pan-1; hi produced 249 colonies (83% conidial viability). Unfortunately, control plates were lost. However, previous conidial viability studies gave 65% viability for the HI strain, and 47% viability for the hi strain. These results strongly suggest that there is no diffusable germination inhibitor involved in the unstable heterocaryons.

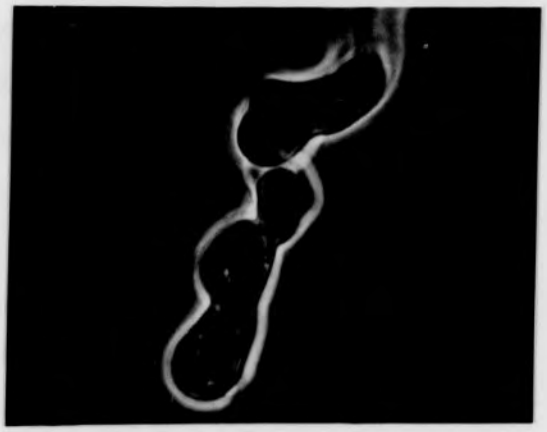
Conidial Germination and Coalescence of Stable and Unstable Heterocaryons

Using a micromanipulator, clumps of two and four conidia (half of them from each of two different strains) were formed on microscope slides coated with Fries minimal agar, so that any abnormalities in the formation of unstable heterocaryons might be observed. The slides were incubated in moist chambers at 30°C, and examined at about 6 hrs for signs of germination. Photomicrographs (Figure 4) were taken at 3 hr intervals after germination. Germinated conidia were transferred to a plate of minimal

Figure 4. Heterocaryons Formed by Four Conidia

HI + HI

hi + hi



HI + hi

Conidia photographed at approximately 9 hrs. Arrows point to original conidia (C), and to fusions (F). Magnification 1300X.

medium. The tabulated results are included in Table 5. It was difficult to determine exactly when coalescence first occurred, but under the conditions of this experiment, (conidia from each strain touched one another) coalescence appeared to occur at the same time, or even before formation of a germ tube. Continuity of the protoplasm was often not evident until after germ tube formation. However, it appeared that conidial coalescence was a prerequisite to heterocaryon formation because fusions always occurred between conidia, and never between young hyphae. Although this experiment is not well suited for statistical analysis, we encountered no unusual difficulty in establishing either stable or unstable heterocaryons. Four heterocaryons of the strains 21a al-2; pan-1; hi + 3a al-2; inl; hi were recovered from 24 attempts. These heterocaryons all arose from clumps of four conidia. We were unable to form this type of heterocaryon by using only one conidium from each strain. Five heterocaryons were formed from 17 attempts using strains 74(1-2)a al-2; inl; HI + (3-7)a al-2; pan-1; HI (two conidia from each strain). One heterocaryon was also recovered from six attempts using one conidium from each strain. Two unstable heterocaryons resulted from 19 attempts (four conidia) using the strains (3-7)a al-2; pan-1; HI + 3a al-2; inl; hi. Unstable combinations seemed to germinate and coalesce just as well as stable combinations. No morphological or intracellular abnormalities were observed that could be associated with either of the stable combinations, or with the unstable one. This experiment has not revealed any defects in germination or fusion that might be due to the HI + hi instability. Newly formed stable heterocaryons, transferred to minimal plates, covered the entire surface of the plates in 48 hrs. The unstable heterocaryons stopped growing before the

Table 5. Conidial Germination and Coalescence of Stable and Unstable Heterocaryons

Heterocaryon	Attempts	Successes	Ratio
21a al-2; pan-1; hi + 3a al-2; inl; hi	24	4	4/24
74(1-2)a al-2; inl; HI + (3-7)a al-2; pan-1; HI	17	5	5/17
(3-7)a al-2; pan-1; HI + 3a al-2; inl; hi	19	2	2/19

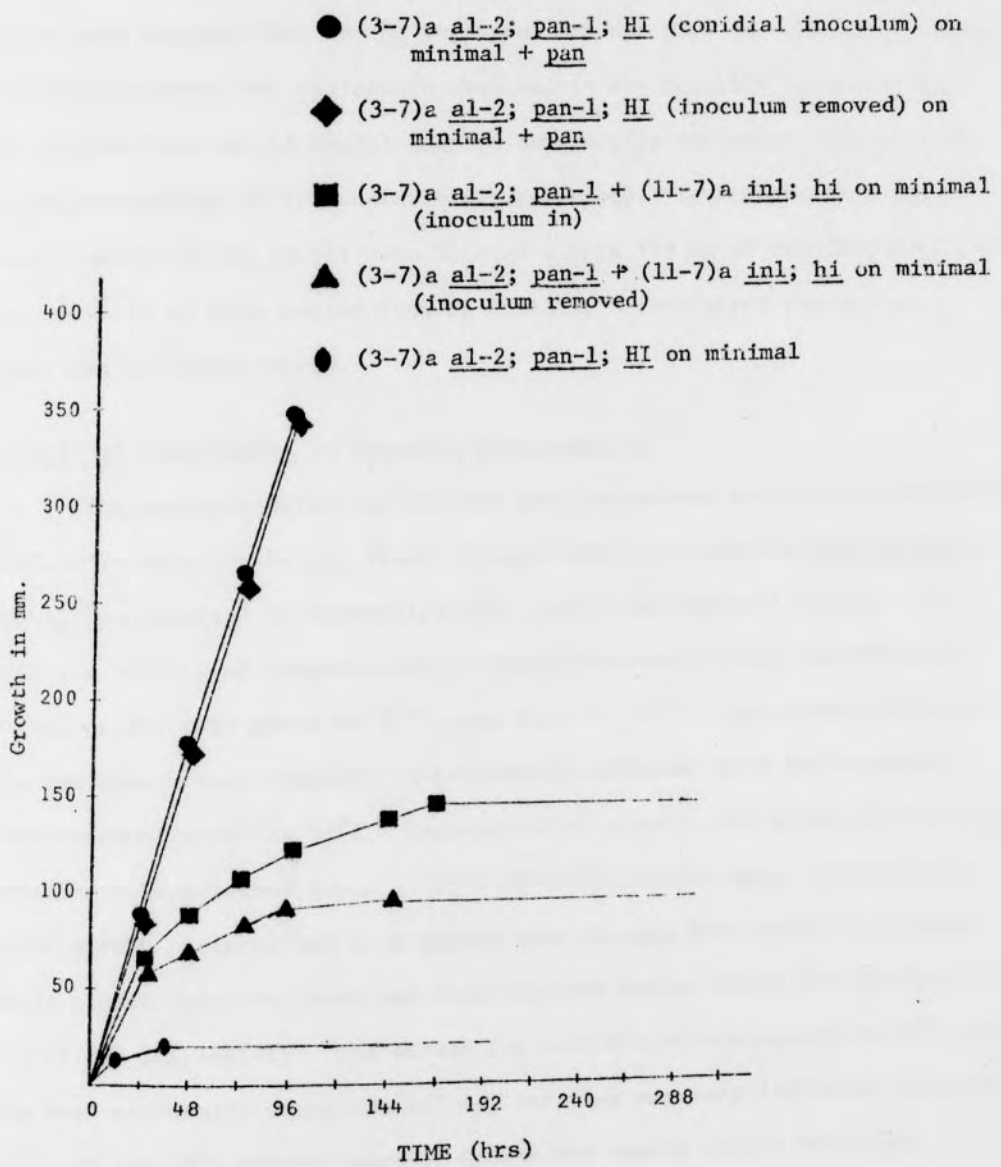
Conidia were placed on minimal agar in clumps of four, using a micromanipulator.

plates were completely covered. The results of this experiment suggest that unstable heterocaryons are formed (from conidia) in much the same way as stable heterocaryons. Apparently, HI conidia do not inhibit germination of hi conidia, and neither is the unstable combination a result of differential conidial coalescence.

Growth Tube Tests for Clustering of hi Nuclei in the Inoculum and for Biochemical Activity of hi Nuclei

Recovery of only small numbers of heterocaryotic and homocaryotic al-2; inl; hi hyphal tips within 3-10 mm of the inoculum suggested the possibility that differential nuclear migration might cause clustering of most of the hi nuclei within the conidial inoculum. To test for this clustering, the growth rates of unstable heterocaryons of the strains (3-7)a al-2; pan-1; HI + (11-7)a inl; hi were compared with the rates of similar heterocaryons from which the inocula were removed after 12 hrs incubation. Representative results of this and related experiments are given in Figure 5. Unstable heterocaryons had noticably lower growth rates, and underwent more rapid changes to lower growth rates at 24 hrs when their inocula were removed, than when the mycelia were left intact. That these differences in growth rates were not due to mechanical injury was demonstrated by cutting the inoculum from one of two homocaryons of the HI strain growing on growth tubes containing supplemented minimal agar. There was little difference in the growth of the two homocaryons (see Figure 5). A minimal + pantothenate agar block was inoculated with conidia of the HI component, and transferred to a growth tube containing minimal agar. These conidia germinated, but the homocaryon grew only 20 mm in 36 hrs before stopping. (This growth may be exaggerated due to diffusion

Figure 5. Effect of Cutting Inoculum From Unstable Heterocaryons

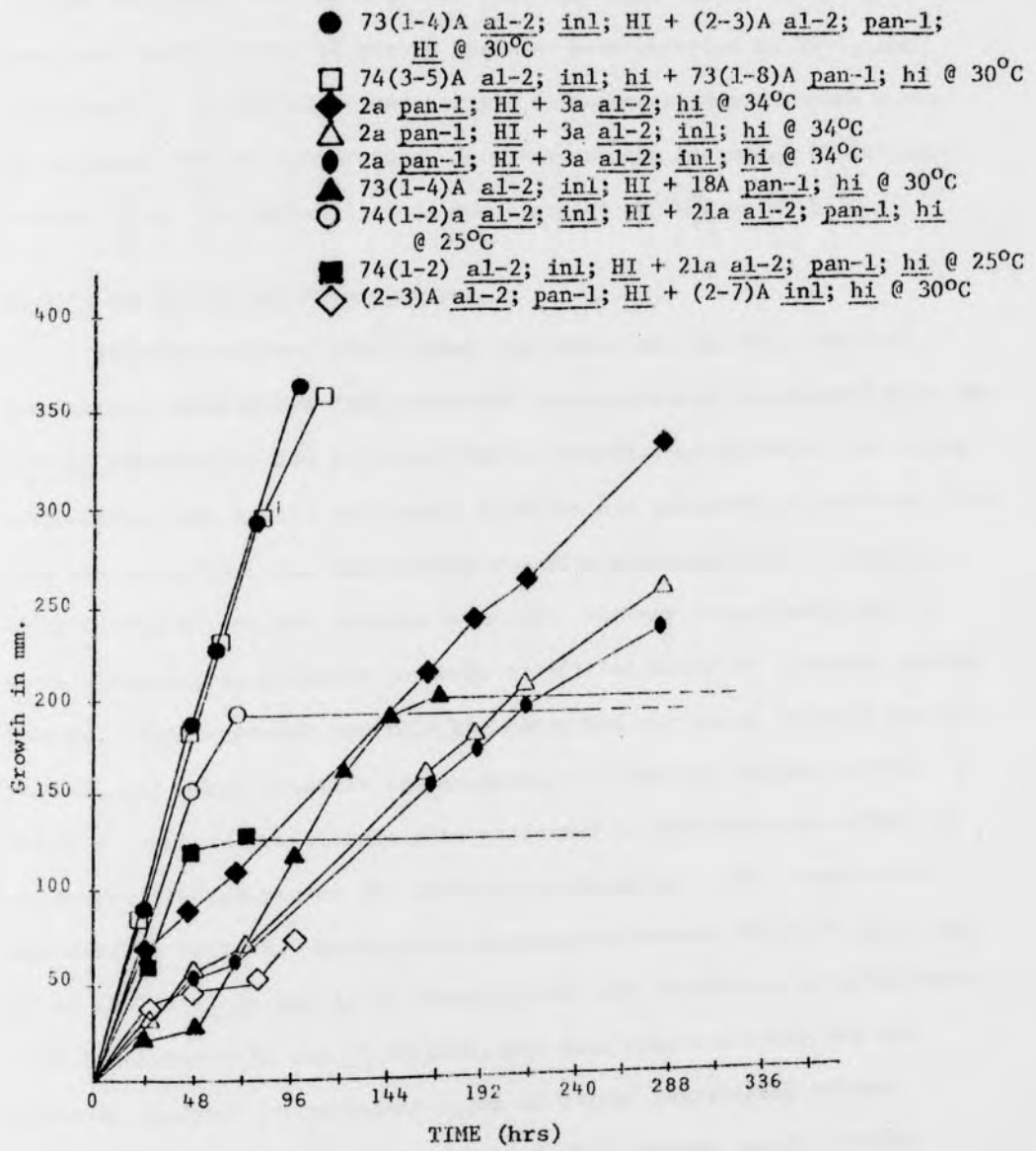


of pantothenate from the inoculum block.) Unstable heterocaryons usually grow further than 20 mm. It is obvious that mechanical injury did not cause a lag in growth of the homocaryotic HI component, and doubtful that it caused the observed changes in growth of the unstable heterocaryons. It is also obvious that the HI component did not grow far enough on minimal medium to account for the growth observed in the unstable heterocaryons. It follows that the hi nuclei exerted some active influence on a portion on the metabolism of the unstable heterocaryons. It appears that significant numbers of hi nuclei were located within 7-8 mm of the inoculum, and that removal of this region from an unstable heterocaryon removed an important nutrient source.

Effects of Temperature on Unstable Heterocaryons

The apparent effect of the new gene on nuclear activity is somewhat similar to that of the cdc (cell division cycle) mutants in Saccharomyces cerevisiae reported by Hartwell, et al. (1974) and Hartwell (1974). To test the effects of temperature on unstable heterocaryons, two unstable heterocaryons were grown at 25°C, and three at 34°C. The growth rates of the two groups were compared to previously collected data for unstable heterocaryons grown at 30°C. Representative results are shown in Figure 6. Unstable heterocaryons grown at 25°C and 30°C exhibit some variations in their growth patterns but both groups show changes from relatively rapid early growth rates to lower and less constant rates, which are characteristic of the instability. The curves for unstable heterocaryons at 34°C show the most conformity among themselves, but they are very different from the 25°C and the 30°C growth curves. Growth was nearly linear with time throughout most of the experiment, with the important exceptions of changes

Figure 6. Effect of Temperature on Growth of Unstable Heterocaryons



to lower growth rates during the first 72 hrs, and slow growth rates (1.0 mm/hr) during the remainder of the experiment. Two of the heterocaryons stopped just short of the end of the tubes, and one actually reached the end. This slow growth pattern, which occasionally alternates with more rapid spurts of growth has also been observed at 30°C, and therefore, it is not clear whether the differences in the growth curves are actually due to temperature, or other unknown factors. It is clear however, that the instability is not reversed at 25°C or at 34°C.

Studies on Artificial Heterocaryons

Results obtained from hyphal tip isolations and from conidial germination experiments indicated that the reaction(s) associated with the HI + hi instability did not occur before conidial germination, but after coalescence, and before the hyphal frontier had advanced as little as 3 mm from the inoculum. The instability reaction(s) probably occur shortly after fusion of the two strains involved. Nuclear transplantation, by microinjection, is probably the only artificial means to simulate hyphal fusions. This approach controls the donor and recipient roles of the two strains, and makes possible the production of extreme nuclear ratios. A number of nuclear transplants were performed to determine the effect of reciprocal transplants on the instability phenotype. The results are tabulated in Table 6. Reciprocal transplants between 74(1-2)a al-2; inl; HI and (3-7)a al-2; pan-1; HI demonstrated that artificial heterocaryons could be formed with two HI strains, and that heterocaryosis was not prevented by donor or recipient roles of either nutritional mutant. Artificial heterocaryons could also be formed between two hi strains (3a al-2; inl; hi and 21a al-2; pan-1; hi). Unstable heterocaryons could

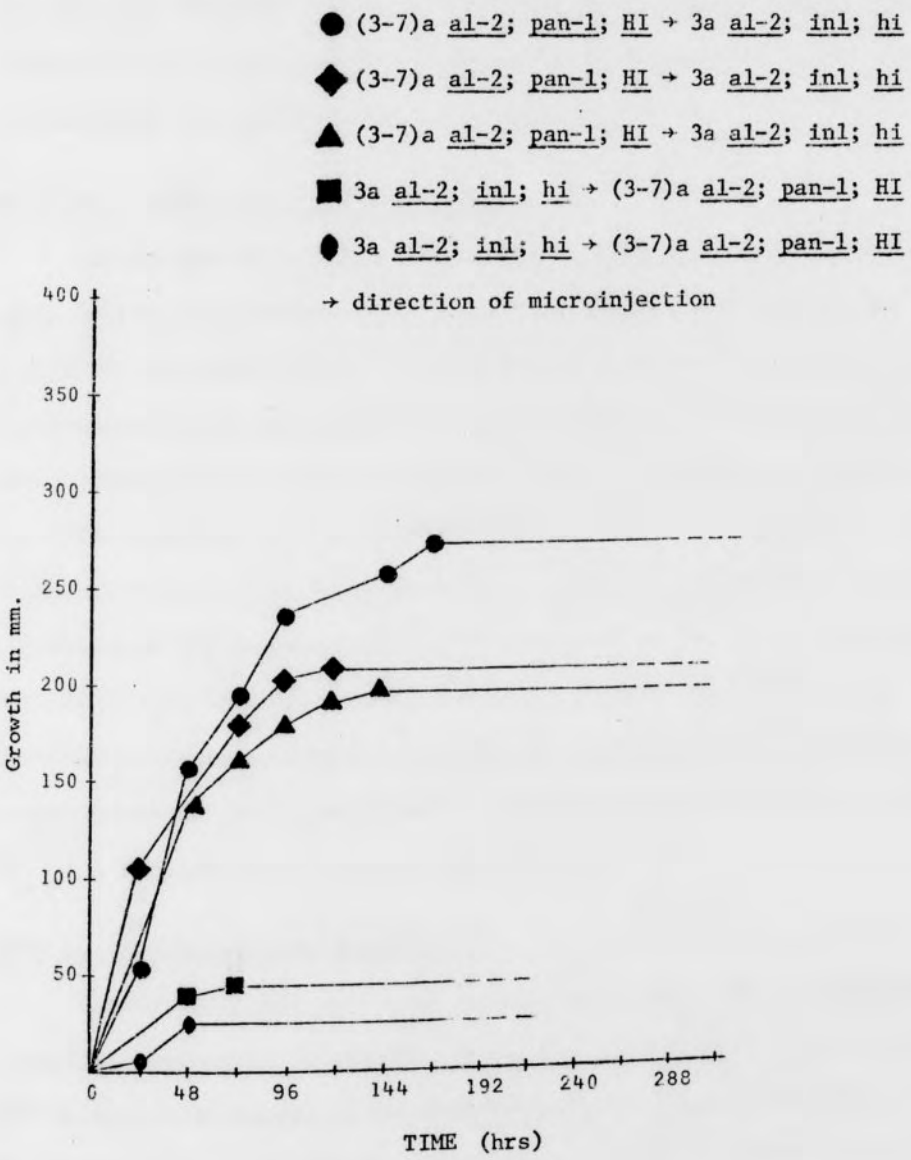
Table 6. Formation of Heterocaryons by Nuclear Transplantation

Component Strains	Attempts	Successes	Ratio
74(1-2)a al-2; inl; HI → (3-7)a al-2; pan-1; hi	18	8	8/18
(3-7)a al-2; pan-1; HI → 74(1-2)a al-2; inl; HI	19	4	4/14
3a al-2; inl; hi → 21a al-2; pan-1; hi	7	2	2/7
3a al-2; inl; hi → (3-7)a al-2; pan-1; HI	19	2	1/19
(3-7)a al-2; pan-1; HI → 3a al-2; inl; hi	13	4	4/13
74(1-2)a al-2; inl; HI → 21a al-2; pan-1; hi	10	4	4/10
21a al-2; pan-1; hi → 74(1-2)a al-2; inl; HI	6	1	1/6

Arrow (→) indicates direction of nuclear transplant

be formed by transplanting HI nuclei into hi recipient strains, and donor and recipient roles of the associated nutritional mutants did not prevent heterocaryosis. However, if hi nuclei were injected into HI recipient strains, the production of unstable heterocaryons was very low. Only three heterocaryons were recovered from 25 injected cells. At no time has a protoplasmic incompatibility reaction, of the type described by Garnjobst and Wilson (1956), been observed. When artificial heterocaryons (HI → hi and hi → HI) were placed on growth tubes, there were marked differences between the growth patterns of each group. When HI protoplasm was injected into an hi recipient, early growth varied from 2 mm/hr to 4 mm/hr followed by a rapid decrease in growth rates at 48 hrs, and cessation of growth between 197 hrs and 275 hrs (Figure 7). These growth curves resemble curves obtained most often when heterocaryons are started from simultaneous, superimposed conidial inocula. Total growth varied between 197 mm to 275 mm down the growth tubes. Growth curves were very different for artificial heterocaryons formed by injecting hi protoplasm into HI recipients. Growth rates for these heterocaryons were never normal, 0.21 mm/hr and 0.83 mm/hr initially, and neither heterocaryon ever grew 50 mm in total length. The very slow, inhibited growth may have accounted for the low recovery rate of hi → HI heterocaryons. This result is probably due to the fact only small amounts of hi protoplasm are injected, and thus small numbers of hi nuclei are injected into the recipient cell. This cell is then removed in a segment of three to five cells, and placed on a minimal plate. Thus the nuclear ratio heavily favors the HI component (or any recipient). It probably takes much longer for small numbers of injected HI nuclei to produce enough of the active agent to inactivate all or most

Figure 7. Growth Curves for Artificial HI + hi Heterocaryons



of the hi nuclei in the recipient cells. When small numbers of hi nuclei are injected into an HI recipient, the small numbers of hi nuclei are probably inactivated much more rapidly. These experiments also indicate that the inactivation reaction(s) are unidirectional against the hi nuclei, and that the ultimate outcome of the HI + hi instability, unlike the interaction of I and i nuclei described by Pittenger and Brawner (1961), is unaffected by highly diverse nuclear ratios.

An Attempt to Extract an Active Agent

Extraction of an active agent from a mycelial culture of 74(1-2)a al-2; in1; HI was attempted using 0.1 M phosphate buffer at pH 6.8. The ability of the crude extract to inhibit regeneration was tested by a series of microinjections into strain 21a al-2; pan-1; hi. The microinjections were alternated with control punctures without injection. A comparison of the times required for injected and control cells to regenerate is tabulated in Table 7. As shown in Table 7, there was no difference in the time required for regeneration of injected and control cells, suggesting that the active agent may be involved in processes not immediately concerned with regeneration, e.g. mitosis itself, or that the active agent is only produced in heterocaryons. It is, of course, also possible that the agent was not extractible by our method.

Genetics of Heterocaryon Instability

Genetic data for this gene are not extensive. The heterocaryon instability phenotype is usually observed as a change to a lower growth rate in the later stages of heterocaryosis, well after protoplasmic transfer, so scoring of progeny phenotypes would be a lengthy process. In an attempt

Table 7. Effects of Microinjected HI Extract on Regeneration of hi Recipient Strains

	Regeneration Times				Failed to Regenerate	Killed Cells	Total
	20 min.	50 min.	60 min.	90 min.			
Injected Cells	1	2	3	7	15	2	30
Control Cells	0	1	4	3	18	1	27

HI extract derived from 74(1-2)a a1-2; in1; HI. Recipient strain was 21a a1-2; pan-1; hi. Control Cells were punctured but not injected. Killed Cells died due to mechanical injury or failure of wound healing mechanism.

to simplify and speed up genotype identification, a testing scheme was adapted from that of Pittenger and Brawner (1961), who used the conidial color markers al-1 and al-2. When these markers are incorporated into heterocaryons, the conidia exhibit wild type coloration, unless the nuclear ratio heavily favors one nuclear type. Using both of these albino mutants would probably have been better than using al-2 and its wild type allele alone, but al-1 is associated with a reciprocal translocation, and has the heterocaryon genotype C, d, e, so an attempt was made using al-2 and its wild type allele. The hope that unstable heterocaryons on slants would exhibit the color of the HI component (e.g. (3-7)a al-2; pan-1; HI + (11-7)a inl; hi would produce white conidia) was unrealized. The conidial color of such heterocaryons ranged from white or pink, to shades of orange indistinguishable from wild type color. Conidial color was, therefore, unrealiable, and it was necessary to compare the growth rates of two heterocaryons, each formed by the isolate being tested, and one HI or hi tester strain.

Twelve ordered tetrads were isolated from the cross 2a pan-1; HI x 21a al-2; pan-1; hi (2a pan-1; HI was the protoperithecial parent) and eight tetrads containing at least three of the meiotic products were scored. The isolates were tested for mating type, auxotrophic genotype, and heterocaryon instability genotype. The ratio of HI to hi genotypes did not deviate from the expected 1:1 ratio. Five of the eight tetrads exhibited second division segregation, suggesting a centromere distance of about 30 units.

The confidence level obtained from eight asci, is 72.5% probability that two loci might occur within fifteen units of each other. Scoring of 15 asci would have resulted in a 79% confidence level two loci are within

ten units of each other. However, using growth tubes to test for the instability phenotype in so many isolates becomes quite cumbersome and time consuming. This problem could best be attacked using the duplication generating strains used by Perkins (1975) and Mylyk (1975) for heterocaryon and vegetative incompatibility genes. Dr. Perkin has applied the techniques using the duplication generating strains of two of our isolates and has kindly furnished us with the following information. "My first results indicate that HI is in IIL covered by NM149 duplications but not by P2869 duplications. (P2869 was called Y163?9i . . .) This would put it quite close to het-c." (Personal Communication, October 22, 1974)

DISCUSSION

A new heterocaryon gene has been found in Neurospora crassa that controls the maintenance of stable growth rates of heterocaryons rather than heterocaryon formation, as do the genes het-c, het-d, and het-e investigated by Garnjobst (1953; 1955), Garnjobst and Wilson (1956), Wilson (1958), Wilson, Garnjobst and Tatum (1961), Wilson and Garnjobst (1966), and Williams and Wilson (1966). The alleles of the new gene have been tentatively designated HI and hi for heterocaryon instability.

On growth tubes, HI + HI and hi + hi heterocaryons grow at constant rates approaching those of wild type (4.2-4.4 mm/hr at 30°C). HI + hi heterocaryons may exhibit a number of different growth patterns. Growth usually proceeds at nearly wild type rates for 12 hrs to 72 hrs, and then slows to a subnormal rate, and finally ceases before reaching the ends of the tubes. Occasionally, growth does not stop, but proceeds to the ends of the growth tubes at subnormal rates. Growth may proceed in erratic starts and stops during the later stages. Growth may also stop within 12 hrs and not start again. Experiments using various nutritional mutants (inl; pan-1; and nic-3) suggest that the behavior of unstable heterocaryons (HI + hi) is independent of any nutritional requirements of the component strains.

The ultimate outcome of the interaction of the HI and hi alleles is not reversed by higher or lower temperatures. Unstable heterocaryons grown at 25°C had growth patterns that closely resembled those of heterocaryons grown at 30°C. One unstable heterocaryon incubated at 34°C grew at a rate

approaching that of wild type for 24 hrs, then slowed to a subnormal rate which it maintained to the end of the growth tube. The other heterocaryons (same strains) never grew at wild type rates, but did grow at subnormal rates to within about 1 cm of the ends of the growth tubes where they stopped without using the remaining surface of the medium. Incubation at 34°C apparently prolonged growth of these unstable heterocaryons but it neither prevents the changes to lower growth rates, nor reverses the cessation of growth that often occurs.

When conidia from stable heterocaryons (HI + HI or hi + hi) were plated, both nuclear components were easily recovered. When conidia from unstable heterocaryons (HI + hi) were plated, the resulting colonies were predominantly homocaryotic for the HI component strain. Only small numbers of colonies have been recovered which were homocaryotic for the hi component strain, and very few colonies which were heterocaryotic. Several of these homocaryotic hi colonies were scored for their nutritional and heterocaryon instability phenotypes, and in every case, the integrity of both nutritional and instability genes has been maintained, i.e. these nuclei did not undergo genetic adaptation. The observation that only small numbers of homocaryotic hi colonies and heterocaryotic colonies are recovered suggests three possibilities. First, the mycelium has most probably become virtually homocaryotic for the HI component. Second, the hi nuclei may have been biochemically inactivated so that they may be present, but unable to express themselves. Third, differential nuclear selection at conidiation, favoring inclusion of HI nuclei, might produce these results as an artifact, but it is unlikely that events occurring during conidiation would stop mycelial growth of the heterocaryons. We

favor the first hypothesis because the homocaryotic HI counts agree very closely with the control plate counts, suggesting that we have accounted for all, or nearly all, viable conidia. There is also evidence that the homocaryotic HI strains cannot grow far enough on minimal medium to account for the amount of growth that usually results from an unstable heterocaryon. The third possibility is unlikely because assays of single hyphal tips give results that are statistically comparable to those obtained by conidial platings.

Heterocaryon instability was not reversed by supplying the growth medium with nutritional supplements necessary to grow the component strains individually. When agar blocks from the frontier region of a plate culture of an unstable heterocaryon were placed on minimal medium containing the nutritional supplement necessary to support the HI component strain, the hyphal tips produced a culture that grew at wild type rate, just as if HI conidia had been inoculated on to this medium. When the medium contained the supplement that supported growth of the hi component strain, the instability was only partially reversed, and growth rates were erratic. This strongly suggests that the interaction of the two genes is independent of the utilization of any of the usual growth factors. This experiment also indicated that some hi nuclei were capable of migrating from the inoculum region to the frontier region, a distance in this case of approximately 25 mm to 30 mm from the inoculum region. It appears that heterocaryotic, or homocaryotic hi hyphal tips can be revived if placed on medium containing the appropriate nutritional supplements.

Results of conidial platings of unstable heterocaryons suggested that they probably stopped growing because the mycelium had become

homocaryotic for the HI component. Homocaryosis could develop in a number of ways. Differential migration rates of the two types of nuclei, or, differential rates of nuclear replication might be responsible.

If the alleles HI and hi affect the conidial viability, then fewer hi conidia might germinate, and the resulting heterocaryon would be nearly homocaryotic from the beginning. A number of tester strains were plated, to determine the per cent conidial viability for each, and no correlation between heterocaryon instability genotype and conidial viability was detected. Germinating HI conidia might produce a diffusible product that inhibits the germination of hi conidia, or hi conidia might germinate but fail to fuse with conidia or hyphae of the HI strain. I believe that the evidence eliminates all but two of the possibilities (either differential nuclear migration, or differential rates of nuclear replication). When conidia were plated from various points along the length of an unstable heterocaryon, no heterocaryotic or homocaryotic hi colonies were recovered from any of the conidial samples. Failure to detect a decreasing trend in the numbers of hi nuclei suggests an abrupt decrease in the numbers of these nuclei, or, that the nuclei are excluded during conidiation. It is also possible that the plating density was not high enough to detect existing hi nuclei, but an assay of single hyphal tips allowed a more direct and more efficient approach to all three questions, than would increasing plating densities of conidia.

It became crucial to find any hi nuclei existing within the mycelium. Apparently there are few if any hi nuclei in the conidia of unstable heterocaryons at any point along the entire length of the mycelium. Assays of single hyphal tips removed from a frontier as close as 3 mm from the

inoculum of a plate culture indicated that there were only small numbers of hi nuclei in the hyphae; and that the nuclear ratios that occur in conidia are probably the same as those that occur in hyphae This assay indicated that an unstable heterocaryon becomes homocaryotic (or nearly so) very early in the life of the mycelium, and that the biochemical processes causing the instability reaction are already near completion by the time the frontier has advanced 3 mm from the inoculum. The abrupt decrease in the hi nuclear count is probably not due to a failure of hi conidia to germinate in the presence of HI conidia. Conidia from an hi strain germinated and produced colonies on sorbose medium made with liquid minimal medium in which conidia from an HI strain had previously germinated and grown for 6 hrs. This tends to rule out the existance of a diffusable germination inhibitor produced by the HI conidia. The processes of conidial germination and heterocaryon formation from conidia were examined in detail by observing the formation of both stable and unstable heterocaryons, from clumps of two and four conidia, under a microscope. When heterocaryons were formed from small conidial clumps, protoplasmic exchange was accomplished by direct conidial coalescence, rather than by fusion of germ tubes, or fusion of one germ tube with a conidium of the other component strain. Although it was often difficult to determine exactly when protoplasmic exchange between two conidia first occurred, conidial coalescence always occurred early in the formation of the heterocaryon. Based on these observations, it appears that conidial coalescence is a prerequisite for heterocaryosis under these coniditions.

No particular difficulty was encountered in obtaining heterocaryons from stable or unstable combinations of conidia, and no morphological or

intracellular differences in the germination-coalescence processes were observed which could be associated with the formation of unstable heterocaryons. Apparently hi conidia do germinate and fuse normally with HI conidia during formation of unstable heterocaryons. It appears that biochemical reactions causing the instability occur after conidial coalescence, and before the resulting hyphae have grown 3 mm. In most cases, it was necessary to have four conidia to form a heterocaryon successfully. One heterocaryon was formed between the strains (3-7)a al-2; pan-1; HI, and 74(1-2)a al-2; inl; HI from two conidia. It is probable that the higher incidence of successes using four conidia stems from the fact that most of the strains used in the coalesce experiment had approximately 50% conidial viability. This means that two conidia from each strain are necessary to increase the probability that one from each strain will germinate.

When the inoculum region was removed from unstable heterocaryons after 12 hrs on growth tubes, the change to lower growth rates at 24 hrs was more pronounced, and the subnormal growth rates during the later stages were lower than when the mycelia were left intact. A control test demonstrated that the effect of mechanical injury was negligible. When the pan-1, HI component was placed on minimal medium, the total amount of growth was not enough to account for the amount of growth of either group of unstable heterocaryons, whether the inocula had been removed or not. Removal of the inoculum region seems to have removed a significant number of hi nuclei from the mycelium, thus reducing the growth rate. The evidence strongly suggests that the majority of the hi nuclei are located within the inoculum region, which suggests differential nuclear migration as a cause for progressive homocaryosis. However, it may also be possible for rapid

inhibition of hi nuclear replication to produce similar results by reducing the number of hi nuclei available to each succeeding cell, thus increasing the proportion of HI nuclei in each new cell. It is clear however, that hi nuclei do control synthesis of vital nutrients in unstable heterocaryons, and that it is progressive homocaryosis that inhibits growth of these heterocaryons regardless of the actual cause of homocaryosis itself.

At least two previous cases of nonadaptive changes in nuclear ratios of Neurospora heterocaryons have been reported in which the nuclear ratios, and the behavior of the heterocaryons was related to the nutritional requirements of the component strains. Lederberg (1946), and Ryan and Lederberg (1946) reported that heterocaryons of leu (33757) and a back mutation of leu underwent changes in nuclear ratios favoring the nuclei of the adapted back mutation when the heterocaryons were grown on minimal medium. When similar heterocaryons were grown on medium supplemented with leucine, the nuclear ratios favored the leu nuclei. Growth rates also changed to resemble the rates of the individual component strains. Davis (1960) reported a similar effect caused by a mutation in the membrane transport systems involved in the uptake of pantothenate from the medium. The pan-1, m strain absorbed limiting concentrations of pantothenate more efficiently than the pan-1 strain. If heterocaryons of these two strains were grown in medium containing limiting concentrations of pantothenate, the nuclear ratios tended to favor the pan-1, m nuclei, and growth rates tended to resemble those of the pan-1, m homocaryons. If the medium contained unlimiting pantothenate concentrations, pan-1 was the predominant nuclear component. Heterocaryons of pan-1 + pan-1, m also start and stop growing at irregular intervals depending on the changes in the nuclear

ratios. Although the HI component of an unstable heterocaryon may escape the effect of unstable heterocaryosis when the appropriate nutritional supplement is added, plating results have indicated that inhibition of hi nuclei cannot be completely reversed by adding either supplement.

Studies on artificial heterocaryons have shown that reciprocal nuclear transplants between two HI strains or two hi strains may be successful regardless of the donor or recipient roles of the associated nutritional deficiencies. However, when HI nuclei were transplanted into hi recipient strains, the resulting artificial heterocaryon grew at or near wild type rates for up to 48 hrs (after an initial lag) then slowed to subnormal rates, and stopped growing between 120 hrs and 168 hrs, or after as much as 275 mm total growth. When hi nuclei were injected into HI recipient strains, total growth of the resulting heterocaryon did not exceed 50 mm. These observations suggest a unidirectional inhibition of hi nuclei. They also suggest that since only very small numbers of donor nuclei can be injected into the recipient strain, small numbers of hi nuclei are inhibited much more rapidly when the direction of the transplant is hi → HI, than when the direction of the injection is HI → hi. It is particularly important to note that, unlike the I + i heterocaryons described by Pittenger and Brawner (1961), the ultimate fate of an unstable heterocaryon is unchanged by manipulating the initial nuclear ratios. At no time was a protoplasmic incompatibility reaction such as that described by Garnjobst and Wilson (1956) observed.

Attempts to extract an active agent from an HI strain have been unsuccessful. It is possible that the agent is not extractible in phosphate buffer, that the agent is only present during heterocaryosis, or

that our assay (inhibition of regeneration of injected cells) would not detect the agent. Attempts to detect an active agent by injecting whole protoplasm from HI + hi heterocaryons into an hi strain have also been unsuccessful. As heterocaryon instability is a delayed phenotype, it may be very difficult to design the appropriate assay. It is also possible that hi codes for a receptor site (involved with nuclear replication or nuclear migration) that has a reduced affinity for a substrate or inducer common to both strains. Homocaryotic growth would then be normal because the corresponding receptor site coded by HI would not be competing for the substrate or inducer. In an unstable heterocaryon, competition for the substrate would favor the HI receptor site with its higher substrate affinity. In this situation, as the nuclear ratios become more diverse, the severity of competitive inhibition increases. If the postulated receptor sites were located on the nuclear membrane, or within the nuclei themselves, differential nuclear activity could be explained quite easily.

Only preliminary genetic data can be presented at this time. There was no deviation from a 1:1 ratio of HI:hi progeny in eight ordered tetrads. Because the instability is detectable only on growth tubes, and only in the later stages of heterocaryotic growth, conventional genetic analysis is quite cumbersome and time consuming. Recently, Perkins (1975) and Mylyk (1975) have used duplication generating strains to map heterocaryon incompatibility genes. Dr. David Perkins has subjected two of our testers to this technique and has kindly reported to us that HI is probably in linkage group IIL close to het-c (Perkins, personal letter, October 22, 1974). Since I may also be in linkage group II (Pittenger and Brawner, 1961) we cannot rule out the possibility that the new gene is I acting

differently in a different genetic background, or that it may be an allele of I.

The apparent effect of the new gene on nuclear activity is somewhat similar to that of the cdc (cell division cycle) mutants in Saccharomyces cerevisiae reported by Hartwell et al (1974) and Hartwell (1974). Approximately 150 temperature-sensitive mutants (defining 32 genes, each of whose products is essential for the completion of one step in the cell division cycle) have been isolated and characterized. The steps involved include those involved with budding and DNA synthesis among others. However, the new gene probably resembles more closely the nuclear replication mutants found in Aspergillus. Orr and Rosenberger (1976a; 1976b) reported finding eleven temperature-sensitive mutants in A. nidulans which could undergo conidial germination and even some cell elongation at the non permissive temperature, but could not undergo nuclear replication so that only one, or at most two, nuclei could exist in a cell. This approach was possible because Aspergillus conidia are usually mononucleate. Multinucleate conidia, and the apparent inability to differentiate the nuclei of the two strains in hyphae, have been major hindrances in studies of the new gene. The absence of thymidine kinase in Neurospora crassa (Grivell and Jackson, 1968) has prevented use of the usual autoradiographic techniques. Future work on this gene may be possible by coupling microinjection with autoradiography.

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