

## Extreme nuclear disproportion and constancy of enzyme activity in a heterokaryon of *Neurospora crassa*

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### Abstract

Heterokaryons of *Neurospora crassa* were generated by transformation of multinucleate conidia of a *histidine-3* auxotroph with *his-3*<sup>+</sup> plasmid. In one of the transformants, propagated on a medium with histidine supplementation, a gradual but drastic reduction occurred in the proportion of prototrophic nuclei that contained an ectopically integrated *his-3*<sup>+</sup> allele. This response was specific to histidine. The reduction in prototrophic nuclei was confirmed by several criteria: inoculum size test, hyphal tip analysis, genomic Southern analysis, and by visual change in colour of the transformant incorporating genetic colour markers. Construction and analyses of three-component heterokaryons revealed that the change in nuclear ratio resulted from interaction of auxotrophic nucleus with prototrophic nucleus that contained an ectopically integrated *his-3*<sup>+</sup> gene, but not with prototrophic nucleus that contained *his-3*<sup>+</sup> gene at the normal chromosomal location. The growth rate of heterokaryons and the activity of histidinol dehydrogenase—the protein encoded by the *his-3*<sup>+</sup> gene—remained unchanged despite prototrophic nuclei becoming very scarce. The results suggest that not all nuclei in the coenocytic fungal mycelium may be active simultaneously, the rare active nuclei being sufficient to confer the wild-type phenotype.

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### Introduction

In 1946, Ryan and Lederberg found a drastic change in the proportion of nuclear types in a heterokaryon of *Neurospora crassa* containing auxotrophic *leu* nuclei and prototrophic *leu*<sup>+</sup> nuclei that had originated by back mutation of the *leu* allele. When this heterokaryon was grown with leucine supplementation, the degree of selection of the *leu* nuclei was so extreme that the presence of *leu*<sup>+</sup> nuclei could not be demonstrated; the mycelium became a *leu* homokaryon. Davis (1960) found that in a heterokaryon constituted between a *pan-1 m*<sup>+</sup> strain and a *pan-1 m* strain, the nuclear types fluctuated wildly, resulting in a stop–start growth. The behaviour of these heterokaryons was very unusual. However, the [*leu* + *leu*<sup>+</sup>] heterokaryon studied by Ryan and Lederberg could be useful for studying the relationship between the dosage of

a gene (wild-type *leu* allele) and the activity of encoded biosynthetic enzyme (*a*-isopropylmalate synthetase) in coenocytic mycelium. This information could illuminate whether all nuclei in the same cytoplasmic milieu are active simultaneously or most are kept silenced. Because of the long passage of time, the original strain used by Ryan and Lederberg could not be traced. We therefore synthesized [*leu* + *leu*<sup>+</sup>] heterokaryons using strains available from the Fungal Genetics Stock Center, but our heterokaryons did not behave like the one studied by Ryan and Lederberg. However, we fortuitously found extreme change in nuclear types in a heterokaryon that was generated by transformation of a histidine auxotroph with a *his-3*<sup>+</sup> plasmid. We have used several methods to verify that extreme nuclear disproportion occurred in this heterokaryon. This heterokaryon was used to study interactions between nuclei carrying genes in normal and in ectopic chromosomal location, and the relationship between *his-3*<sup>+</sup> nuclear dosage and histidinol dehydro-

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genase enzyme activity. We discuss the implications of the observations for the biology of coenocytic fungal mycelium.

## Material and methods

***N. crassa* strains:** *Neurospora crassa* of the genotype *his-3 al-1; mcm; inl*, and *his-3 al-2* was constructed by intercrossing the following strains: *histidine-3* (FGSC 4496), *albino-1* (FGSC 3714, allele no. JH216); *albino-2* (FGSC 2666, allele no. MN58p), *microcycle microconidiation* (FGSC 7455) and *inositol* (FGSC 498). The *mcm* gene was introduced to induce formation of uninucleate microconidia which facilitate estimation of nuclear ratio by the plating method (Maheshwari 2000). The *al* and *inl* markers were introduced as checks against possible laboratory contamination, but none was ever detected. The constructed strain had a wild-type morphology in agar-grown culture. It was distinguished by white colour, selective production of microconidia in liquid shake culture, and by its requirements of histidine and inositol for growth. Wherever amino acids were added, these were L isomers. Standard *Neurospora* methodology was used (Davis 2000).

**Transformation:** Protoplasts of the recipient strain, *his-3 al-1; mcm; inl; a*, were prepared by treatment of conidia with cell wall lytic enzyme, and transformed with plasmid pNH60 containing *his-3<sup>+</sup>* gene (Legerton and Yanofsky 1985). Transformants were selected on sorbose plating medium (Davis 2000) supplemented with inositol. Integration of plasmid DNA sequences in selected transformants was confirmed by Southern analysis. The extension growth rate in race tubes and specific growth rate in liquid shake cultures at room temperature of all selected transformants were similar (3.7 mm/h and 0.08/h, respectively).

**Estimation of nuclear ratio:** The nuclear ratio in mycelium was estimated through plating of macroconidia (hereafter referred to as conidia) or microconidia on sorbose plating medium (see Davis 2000). All media contained inositol. Colonies appearing on histidine-supplemented medium were taken as the number of viable conidia. Commonly 200 conidia were plated with three replicates, but in some experiments up to 10,000 were plated. Minimal medium contained inositol.

$\% \text{ his-3}^+(EC) \text{ conidia} = (\text{number of colonies on plate without histidine}) \div (\text{number of viable conidia}) \times 100,$

$\% \text{ his-3 conidia} = 100 - \% \text{ his}^+(EC) \text{ conidia.}$

**Enzyme assay:** The activity of histidinol dehydrogenase in cell-free extracts of shaker-grown mycelia (60 h at room temperature) was measured according to Creaser

*et al.* (1967). The reaction ( $\text{histidinol} + 2 \text{ NAD} \rightarrow \text{histidine} + 2 \text{ NADH}$ ) was monitored by the increase in absorbance at 340 nm due to the formation of NADH.

## Results

### Variability among transformants

Three transformants, 3T3, 4T12 and 2T5, were selected. The proportion of *his-3<sup>+</sup>(EC)* nuclei in the transformants varied (a *Neurospora* gene that has been integrated ectopically by transformation is designated by appending '(EC)' to the gene symbol; Perkins *et al.* 2001). Transformant 3T3 showed phenotypic lag (Pitchaimani and Maheshwari 2000) but transformant 4T12 did not. Transformants 3T3 and 2T5 produced microconidia, but transformant 4T12 did not. The transforming DNA was transmitted to the progeny without inactivation in 3T3 but not in 4T12 and 2T5 (data not shown). Each transformant, therefore, behaved as a genetic variant.

### Alteration of nuclear ratio in transformant 4T12

On the basis of the observation of Ryan and Lederberg (1946) with a [*leu* + *leu<sup>+</sup>*] heterokaryon, we propagated the transformants on histidine medium to determine if auxotrophic *his-3* nuclei would be selected. We estimated nuclear ratio by direct plating of conidia and by colony transfer methods (Pitchaimani and Maheshwari 2000). Transformant 4T12 produced close to 20% prototrophic conidia. The proportion of prototrophic conidia decreased progressively in subcultures made on histidine-supplemented medium. In the fifth subculture, only 0.05% conidia were *his-3<sup>+</sup>(EC)* (table 1). When conidia from this culture were propagated on medium lacking histidine, the proportion of *his-3<sup>+</sup>(EC)* conidia was restored (~20%) quickly. This behaviour was not observed in transformants 3T3 and 2T5. The drastic reduction in prototrophic *his-3<sup>+</sup>(EC)* nuclei in 4T12 was verified by different experimental approaches.

**Table 1.** Progressive reduction in *his-3<sup>+</sup>(EC)* conidia in transformant 4T12 grown on histidine-supplemented medium\*.

Medium	% <i>his-3<sup>+</sup>(EC)</i> conidia in subculture number					
	0	1	2	3	4	5
Without histidine	19	16	14	15	18	16
With histidine	19	15	7	2.0	0.2	0.05

\*Transformant 4T12 was grown in presence or absence of histidine. Conidia were plated on minimal medium or medium supplemented with histidine. The numbers of prototrophic homokaryotic and heterokaryotic colonies were counted. '(EC)' refers to gene integrated ectopically by transformation. Data are average values from two or three experiments.

### Inoculum size test

If the number of prototrophic conidia formed by 4T12 truly decreases in subcultures on histidine medium to 0.05% (or, 1 in 2000 total conidia), then to propagate this growth on medium lacking histidine approximately 2000 conidia would be required to obtain maximum number of successful cultures. To verify this, different volumes from a conidial suspension were put on each of 10 slants of minimal and histidine-supplemented medium. On minimal medium, growth occurred in all tubes even with 50 conidia/slant, whereas on histidine-supplemented medium, only 1 out of 10 tubes showed growth. The number of successful cultures increased with increased inoculum size. When 1600 conidia were added, growth occurred in all 10 tubes. This observation confirmed that the number of transformed nuclei in 4T12 decreased drastically during growth on histidine medium.

### Hyphal tip analysis

A transitory requirement of amino acid for germination of prototrophic conidia, termed phenotypic lag, has been recognized as a potential pitfall in the estimation of nuclear ratio by the plating method (Grigg 1960; Pitchaimani and Maheshwari 2000). Therefore the reduction in number of prototrophic nuclei in mycelium was substantiated by studying growth response of the hyphal tip, where nuclear division occurs. Fifty single hyphal tips or a hyphal apex with a very small subapical branch were excised from mycelium of transformant 4T12 grown with or without histidine on agar medium in Petri dishes. The average number of hyphal tips taken from the histidine-grown culture that grew on minimal medium was 23 compared to 41 from culture grown without histidine. The results substantiated reduction in *his-3<sup>+</sup>(EC)* nuclei in mycelium grown with histidine supplementation.

### Southern blot analysis

A decrease in number of *his-3<sup>+</sup>(EC)* nuclei should be reflected in reduced intensity of the transforming DNA signal in a Southern blot. The signal was invisible in the transformant after five subcultures in histidine medium (figure 1A, lanes 2 and 3). However, the signal reappeared upon transfer of the histidine-grown transformant to medium lacking histidine (lane 4). Similar results were obtained by probing the same blot with vector DNA (figure 1B). This result was in accord with the observation that drastic reduction occurred in proportion of *his-3<sup>+</sup>* nuclei in mycelium grown on histidine medium.

### Analysis using a genetic colour marker

To visually monitor the change in nuclear ratio in the heterokaryon, the nuclei were tagged using genetic colour markers. A heterokaryon was constructed between transformant *his-3<sup>+</sup>(EC)* *al-1*; *mcm*; *inl*; *a* (albino) and a histi-

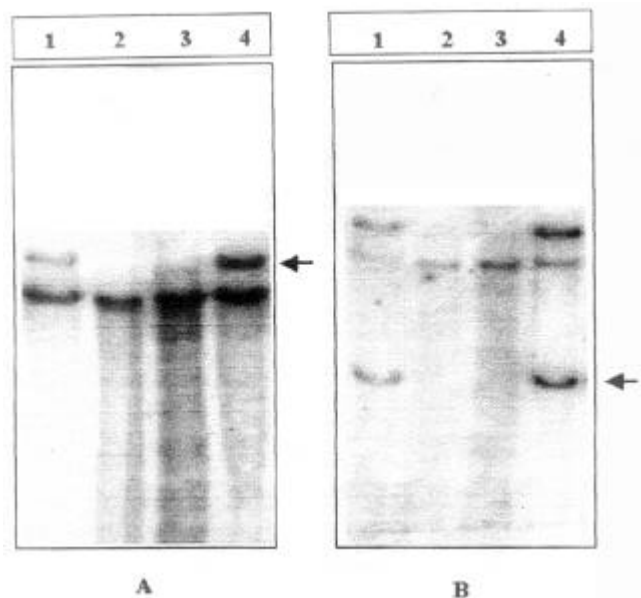
dine auxotrophic strain *his-3* *al-2*; *inl*; *a* (rose-white) (colour gene symbols underlined). The heterokaryon was orange in colour owing to the complementation of *al-1* (allele no. MN58p, FGSC 2666) and *al-2* (allele no. JH216, FGSC 3714) nuclei. It was subcultured four times on minimal medium with or without histidine supplement. It turned into rose-white colour on histidine medium, indicating a reduction in number of *his-3<sup>+</sup>(EC)* nuclei marked with *al-1* gene. When conidia from the histidine-grown heterokaryon were propagated in medium lacking histidine, the orange colour was restored, indicating an increase in the proportion of transformed nuclei.

### Specificity of histidine effect

To determine if the reduction of *his-3<sup>+</sup>(EC)* nuclei was a specific response to histidine, conidia from the histidine-grown transformant were grown in individual tubes containing minimal medium supplemented with selected amino acids. The culture was grown to conidiation and the percentage of prototrophic conidia was estimated by plating. The decrease in proportion of *his-3<sup>+</sup>(EC)* nuclei (i.e. selection of auxotrophic *his-3* nuclei) was a specific response to histidine (table 2).

### Nuclear selection in heterokaryons containing transformed nuclei

Is the reduction of prototrophic nuclei a specific property of the heterokaryon containing transformed nuclei? To



**Figure 1.** Southern analysis of transformant 4T12 grown in liquid medium. DNA was digested with *EcoRI* and probed with the insert region of pNH60 containing *his-3* gene (A) or with vector pRK9 (B). Lane 1, 4T12; lanes 2 and 3, 10 and 20 µg DNA from histidine-grown transformant; lane 4, after subculturing on medium without histidine. The signal specific for *his-3<sup>+</sup>* DNA or vector pRK9 is indicated by an arrow.

determine this, nuclear selection was studied in two heterokaryons, both of which contained nuclei with the same mutant *his-3* allele (linkage group IR) but which differed in the locus of the wild-type gene. Heterokaryon SS-2 contained nuclei with the *his-3<sup>+</sup>(EC)* allele introduced at an ectopic position. In heterokaryon SS-4, the *his-3<sup>+</sup>* allele was at the normal chromosomal location. The proportion of *his<sup>+</sup>(EC)* conidia in SS-2 decreased 60-fold, whereas the *his-3<sup>+</sup>* conidia in SS-4 were decreased only 1.25-fold (table 3). This showed that *his-3* nuclei interacted differently with *his-3<sup>+</sup>* and *his-3<sup>+</sup>(EC)* nuclei. Would the number of *his-3<sup>+</sup>* nuclei decrease when present together with *his-3<sup>+</sup>(EC)* nuclei? A strain containing *his-3<sup>+</sup>* in the normal chromosomal position was fused to 4T12 transformant [*his-3 al-1; mcm; inl; a + his-3<sup>+</sup>(EC); al-1; mcm; inl; a*]. The white conidia of 4T12 and the rose-white conidia of a strain *his-3<sup>+</sup> al-2; inl; a* were mixed in equal proportion and placed on minimal medium. Single hyphal tips from the putative heterokaryotic mycelium formed were isolated on slants of minimal agar. The hyphal-tip-derived cultures produced orange conidia owing to the complementation of *al-1* and *al-2* genes, confirming that a three-component heterokaryon (SS-1) was formed (table 4). SS-1 was subcultured four times on medium with or without histidine and the relative proportions of nuclear components were estimated by conidial plating. On minimal medium only the prototrophic conidia formed colonies. The relevant genotypes of the three-component hetero-

karyon [*his-3 + his-3<sup>+</sup>(EC) + his-3<sup>+</sup>*] and of histidine-prototrophic conidia produced by it are (phenotypes in parenthesis): (i) *his-3<sup>+</sup>(EC) al-1* (white), (ii) [*his-3<sup>+</sup>(EC) al-1 + his-3 al-1*] (white), (iii) *his-3<sup>+</sup> al-2* (rose-white), (iv) [*his-3 al-1 + his-3<sup>+</sup> al-2*] (orange), (v) [*his-3<sup>+</sup>(EC) al-1 + his-3<sup>+</sup> al-2*] (orange), and (vi) [*his-3 al-1 + his-3<sup>+</sup>(EC) al-1 + his-3<sup>+</sup> al-2*] (orange). The number of white cultures was very small or nil compared to rose-white cultures, showing that there was a selection in favour of *his-3<sup>+</sup>* nuclei over *his-3<sup>+</sup>(EC)* nuclei. Similar behaviour was observed in heterokaryon SS-3 (table 4).

#### Histidinol dehydrogenase activity in heterokaryon

Since the proportion of *his-3<sup>+</sup>(EC)* nuclei in 4T12 transformant could be altered drastically, this transformant provided a system to determine whether the activity of the encoded protein (histidinol dehydrogenase) is correlated with the dosage of encoding nuclei. In transformant 3T3 grown with histidine, the proportion of *his-3<sup>+</sup>(EC)* nuclei decreased by 3.5-fold but histidinol dehydrogenase activity was not affected (table 5). In transformant 4T12, after five subcultures on histidine medium, the proportion of *his-3<sup>+</sup>(EC)* conidia decreased from nearly 20% to 0.05% (i.e. 400-fold) but histidinol dehydrogenase specific activity was unchanged.

## Discussion

Three instances of extreme nuclear disproportion in *Neurospora* heterokaryons have been reported (Ryan and Lederberg 1946; Davis 1960; present study). A difference between the behaviour of the [*leu + leu<sup>+</sup>*] heterokaryon studied by Ryan and Lederberg (1946) and that of the [*his-3 + his-3<sup>+</sup>(EC)*] heterokaryon studied here is that, whereas in the former the reduction in prototrophic (*EC*) nuclei occurred immediately, in the latter it occurred gradually, requiring a few subcultures. Ryan and Lederberg (1946) explained their observation by postulating inactivation of the *leu<sup>+</sup>* nuclei by *leu* nuclei. Interestingly, Ryan (1946) found that selection of the mutant nuclei

**Table 2.** Specificity of histidine in reducing the proportion of *his-3<sup>+</sup>(EC)* nuclei in transformant 4T12\*.

Amino acid supplement	No. of colonies per plate	% Prototrophic conidia
–	47 ± 8	30
Histidine	2 ± 0.7	1.5
Arginine	73 ± 16	48
Lysine	78 ± 7	53
Leucine	66 ± 2	38

\*Transformant was subcultured thrice on histidine-supplemented medium before use. Two hundred conidia were added per plate. Solution of amino acid was added at 1.3 mM before autoclaving.

**Table 3.** Selective reduction in proportion of transformed nuclei in heterokaryon 4T12 grown on histidine medium\*.

Heterokaryon	Relevant genotype	Subculture	% Prototrophic conidia produced	
			Minimal medium	+Histidine medium
SS-2	[ <i>his-3<sup>+</sup>(EC) + his-3</i> ]	1	30 ± 00	18 ± 1
		4	25 ± 8	0.3 ± 0.4
SS-4	[ <i>his-3<sup>+</sup> + his-3</i> ]	1	71 ± 13	60 ± 9
		4	70 ± 12	48 ± 3

\*Heterokaryons were subcultured in absence or presence of histidine.

**Table 4.** Competition between *his-3<sup>+</sup>* and *his-3<sup>+</sup>(EC)* nuclei in a three-component heterokaryon\*.

Heterokaryon and genotype	Subculture	% Prototrophic conidia in culture					
		With histidine			Without histidine		
		White	Rose-white	Orange	White	Rose-white	Orange
SS-1	1	4 ± 3	54 ± 6	42 ± 4	3 ± 3	59 ± 12	38 ± 12
[ <i>his-3 al-1</i> + <i>his-3<sup>+</sup>(EC) al-1</i> + <i>his-3 al-2</i> ]	4	1 ± 1	44 ± 2	55 ± 1	1 ± 1	51 ± 7	48 ± 8
SS-3	1	1	50 ± 6	49 ± 7	0	61 ± 2	39 ± 3
[ <i>his-3 al-1</i> + <i>his-3 al-2</i> ]	4	0	39 ± 7	61 ± 7	0	43 ± 18	57 ± 18

\*Data are average values from three experiments. One hundred prototrophic colonies from minimal medium were grown to conidiation and scored on the basis of their phenotype as white (*his-3<sup>+</sup>(EC)*), rose-white (*his-3<sup>+</sup>*) or orange (*[his-3<sup>+</sup>(EC) + his-3<sup>+</sup>]*) (see text).

did not occur if the prototrophic nuclear component contained a wild-type allele that had not arisen by back mutation of the leucine mutant—an observation that we have confirmed (data not presented). Davis (1960) concluded that competition between *pan* and *pan-m* nuclei was a function of the nuclear types and the concentration of pantothenic acid in the medium. Pittenger and Brawner (1961) reported that genes in some strains of *N. crassa* are expressed only in heterokaryons, where they affect the division rate of nuclei, leading to extreme nuclear disproportion. That genetic background strongly affects nuclear selection is supported also by the present observation. Nonadaptive increase in mutant *his-3* nuclei occurred only when in association with transformed nuclei, but not when associated with nuclei with a wild-type *his-3<sup>+</sup>* allele at the normal chromosomal location. Moreover, among the histidine transformants tested, nuclear disproportion was not found in transformants 2T5 and 3T3. The behaviour of nuclei in heterokaryons can be very different from that in sexual cross. For example in matings between Spore killer (*Sk-2K*) and Spore killer sensitive (*Sk-2S*) strains, the progeny are almost exclusively Spore killer. By contrast, the heterokaryons between these strains produce conidia that are almost exclusively *Sk-2S* (Barry 2002).

A change in nuclear ratio can occur if nuclear divisions in a heterokaryon are asynchronous or if the prototrophic nuclei are inactivated (silenced). In heterokaryons of animal cells, generated by fusion of cultured cells in different phases of the cell cycle, experiments demonstrated that a diffusible *trans*-acting factor produced by the dividing nuclei induces mitosis in nuclei in other phases of cell cycle (Rao and Johnson 1970). Although nuclei in a common cytoplasm would be expected to divide synchronously, except in the hyphal tips nuclear division is asynchronous (Loo 1976; Serna and Stadler 1978; Raju 1984). Results of Southern analysis (figure 1) showed that extreme nuclear disproportion in transformant 4T12 was due to selective reduction in the number of *his-3<sup>+</sup>(EC)* nuclei. A possibility is that ectopic integration of *his-3<sup>+</sup>*

**Table 5.** Dosage of *his-3<sup>+</sup>(EC)* nuclei and activity of histidinol dehydrogenase in histidine transformants\*.

Transformant	Growth supplement	% <i>his-3<sup>+</sup>(EC)</i> conidia	Histidinol dehydrogenase (specific activity)
3T3	Nil	84 ± 11	1.7 ± 0.08
	Histidine	67 ± 3	1.1 ± 0.19
4T12	Nil	19 ± 1	1.0 ± 0.30
	Histidine	0.05 ± 0.0	0.9 ± 0.20

\*4T12 transformant was grown in presence or absence of histidine. Data are average values of at least three experiments. One unit of histidinol dehydrogenase = amount of enzyme that produces 1 mmole of histidine (2 NADH) per min at 25°C. Specific activity = units per mg protein.

gene in 4T12 disrupted a gene involved in DNA synthesis or in nuclear division, leading to lower survival rates. For example, *N. crassa* mutants *uvs-3* and *uvs-6* are abnormally sensitive to histidine (Newmeyer 1984; Schroeder *et al.* 1997). However this cannot be a general explanation for shifts in nuclear ratio, as in the heterokaryon studied by Ryan and Lederberg (1946). Another possibility is that the vector DNA (figure 1B) along with the transgene acts as genetic load when the selection pressure is off.

In *N. crassa* heterokaryons involving biochemical mutant genes, the observed changes in nuclear ratio have been of nonadaptive type (Ryan and Lederberg 1946; Davis 1960), the mutant nuclei being selected over the wild-type nuclei when the culture was grown with the nutritional supplement. The nonadaptive change in nuclear ratio, without compromising the growth rate of heterokaryon, is at variance with the hypothesis that heterokaryosis is a unique mechanism in fungi for dealing with short-term adaptation through overall change in nuclear ratio (Burnett 1976).

The condition of auxotrophic nuclei outcompeting prototrophic nuclei of certain genotypes is analogous to the phenomenon of suppressivity in naturally occurring senescent strains of *Neurospora* (Griffiths 1998). In these

strains, senescence is due to mitochondrial plasmids, which integrate into the mitochondrial genome. For unknown reasons, the variant mitochondria have a replicative advantage and displace the normal mitochondria.

Though the molecular basis of the observed behaviour in the 4T12 transformant is not known, advantage was taken of the 4T12 transformant to determine if enzyme activity is correlated to nuclear ratio in heterokaryon—the objective of this investigation. Despite a 400-fold reduction in *his-3<sup>+</sup>*(*EC*) nuclei in mycelia grown on histidine, the activity of encoded enzyme remained unaltered. The present observation contrasts with the observations of Flint *et al.* (1981), who reported that activity of the enzyme specified by *arg<sup>+</sup>* locus in [*arg<sup>+</sup>* + *arg*] heterokaryons was related to nuclear ratio. These workers mixed conidia of two strains in different proportions to generate heterokaryon but did not give the actual nuclear ratio in the heterokaryon formed.

How can one reconcile the constancy of enzyme activity in heterokaryon with highly disproportionate nuclear ratio? We are led to the view that most nuclei in coenocytic fungal hypha are kept silenced, a very small number of active nuclei at any given time being sufficient to confer the wild-type phenotype. Fluorescence staining showed that hyphal compartments of *N. crassa*, particularly in the distal region of the hypha, are filled with nuclei. If a majority of nuclei in the vegetative hypha are silenced, then what may be the significance of the multinuclear condition? A possibility is that the fungal mycelium stores nitrogen and phosphorus in an organic form (DNA), which is recycled and translocated to the tip during its foraging through nutrient-limited substratum.

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