Phenotypic lag in macroconidia of *N. crassa his-3*$^+$ transformants and its implication in estimation of nuclear ratios

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A majority of prototrophic macroconidia of two of the three histidine transformants of *Neurospora crassa* tested required histidine for germination. This condition led to an underestimation of the proportion of *his-3*$^+$ macroconidia in the samples and wronged the estimation of ratio of *his-3*$^+$/ *his-3* nuclei by direct plating method. To correctly estimate nuclear ratio, a sample of colonies from histidine-supplemented plating medium was transferred to histidine drop-out slants and the proportion of auxotrophic and prototrophic colonies was determined from their growth response. The amino acid requirement by prototrophic macroconidia was not specific, was limited to colony formation and was not due to low endogenous content of amino acids.

In *Neurospora crassa*, Grigg observed that though macroconidia of a strain contained prototrophic and auxotrophic histidine nuclei, yet they required histidine for optimal germination. (Grigg 1958 Aust. J. Biol. Sci. 11: 69-84). Subsequent growth of colonies was, however, independent of histidine. A specific requirement for histidine was also observed for germination of prototrophic, uninucleate microconidia that were produced from a (*his*$^-$ + *his*) heterokaryon (Grigg 1960 Heredity 14:207-10). This transitory requirement of growth factor by prototrophic conidia was referred to as 'phenotypic lag'. We too observed this phenomenon while examining the relationship between the proportion of *his-3*$^+$ nuclei and the activity of histidinol dehydrogenase in primary transformants of *N. crassa* and realized a potential pitfall in estimating nuclear ratios in heterokaryotic transformants. Our observations also showed that transformants could grow at optimal growth rates despite their highly disparate nuclear ratio. Furthermore, based on observation with macroconidia we improved the sorbose plating medium for testing viability of microconidia.

Transformants. A strain of *N. crassa, his-3 al-1; mcm; inl* was constructed using the following stocks: *his-3* (Y175M614, FGSC 4496), *al-1* (JH216, FGSC 3714), *mcm* (RM124 -2A, FGSC 7455) and *inl* (89601, FGSC 498). The albino and inositol markers were introduced as a check against laboratory contamination. The strain had a wild-type morphology on agar-medium, but in liquid- shake cultures the *mcm* gene was expressed and the strain produced uninucleate microconidia that were used to purify the transformed nuclei (Maheshwari 1999 Fungal Genet. Biol. 26: 1-18). Protoplasts were transformed with plasmid pNH60 containing *his-3*$^+$ gene (Legerton and Yanofsky 1985 Gene 39:129-140). Three *his-3*$^+$ transformed colonies were selected. These grew at a rate comparable to the wild type *N. crassa* (~ 4 mm/ h). Transformation was confirmed by Southern hybridization using the vector or the *his-3* DNA as the probe.

Estimation of nuclear ratio by direct plating and by colony transfer methods. The relevant genotypes of macroconidia produced by heterokaryotic transformants were *his-3* (homokaryotic), *his-3*$^+$ (homokaryotic) and *his-3*$^+$+ *his-3* (heterokaryotic) Macroconidia were plated on sorbose medium (Davis and de Serres 1970 Methods Enzymol 27A:79-143) with or without histidine supplement (all media contained inositol). The proportion of prototrophic macroconidia [*his-3*$^-$ and (*his-3*$^+$ + *his-3*)] was estimated by direct plating on histidine drop-out medium and by colony transfer methods. In the latter method, a sample of colonies (minimum 75 colonies) that appeared on the histidine supplemented medium were transferred to histidine drop-out slants and their genotypes (*his-3*$^+$ or *his-3*) determined from the growth responses (Table 1). In transformant 4T12 (single *his-3*$^+$ integration), the two methods gave similar results, but in 2T5 (double integration) and 3T3 (single integration), the plating method grossly underestimated the proportion of *his-3*$^+$ macroconidia: in 2T5, all transferred colonies grew, showing that it contained only the *his-3*$^+$ nuclear type, and was not heterokaryotic as the results of direct plating method had shown — i.e., the macroconidia (36%) that behaved as *his-3* in the plating method were, in fact, *his-3*$^+$ but required histidine for growing into colonies. In 3T3, the two methods gave just the opposite results.

Genotype of macroconidia that require histidine for germination. To distinguish whether the macroconidia that required histidine for growing into colonies were the homokaryotic or the heterokaryotic type, a transformed nuclear component (*his-3*$^+$; *al-1; mcm; inl*) in 3T3 was extracted by microconidial plating, and combined in a heterokaryon with a *his-3* strain that had orange nuclear marker but was similar otherwise. The proportion of *his-3*$^+$ and *his-3* nuclear components in the reconstructed heterokaryon (*his-3*$^+$; *al-1; mcm; inl* + *his-3 al-1*$^+$; *mcm; inl*) was estimated by direct plating and by colony transfer methods. The plating method showed that only 6% of the colonies were *his-3*$^+$, comprising of 4% albino (*his-3*$^+$) and 2% orange (*his-3*$^+$/ *his-3*). In contrast, the colony transfer method showed that 89% colonies were *his-3*$^+$, comprising of 42% albino and 47% orange. Since the additional colonies that appeared on histidine-supplemented medium included both albino and orange types, it was inferred that a high percentage of homokaryotic as well as heterokaryotic macroconidia required histidine for growth into colonies.

Effect is not specific. In 2T5 homokaryotic transformant (apparently resulting from transformation of a uninucleate protoplast), the increased number of colonies formed on histidine-supplemented medium suggested that, in general, plating efficiency of macroconidia, including of the wild-type strains, may be improved by histidine supplement. However, the three histidine prototrophic strains tested showed only a small improvement in plating efficiency whereas in 3T3 the stimulation was marked (Table 2). Surprisingly, the effect was not specific; of the other amino acids tested, lysine improved plating efficiency of

3T3 macroconidia equally well but arginine and leucine did not.

Endogenous contents of amino acids. To determine if the stimulatory effect of amino acids was because macroconidia of transformants had low amino acid content, the amino acid composition of 3T3 macroconidia was compared with those of a strain (*his-3⁺; al-1; mcm; inl*) whose macroconidia germinated equally well with or without the histidine supplement. Washed con (~ 3 x 10^8) from 7-day-old cultures were extracted twice with 3 ml of 70% ethanol at 60°C. The clarified extracts were dried under vacuum, the residual material was extracted with diethyl ether to remove lipids, dissolved in water and taken for automated amino acid analysis. Surprisingly, the concentration of individual amino acids in macroconidia of duplicate cultures, even though of similar age, was variable (Table 3). Nonetheless, the average values showed that excepting cysteine, methionine and leucine which were 2- to 3-fold lower in 3T3, the other amino acids were 2- to 4-fold higher compared to that in the *his-3⁻* strain. A low amino acid content was, therefore, not the reason for phenotypic lag.

Importance of the present observations. The observations emphasize that in any estimation of nuclear ratio in heterokaryons, the results of direct plating method should be validated by the colony transfer method. The phenotypic lag in 2T5 and 3T3 macroconidia was analogous to some types of dormant spores of bacteria and fungi wherein their germination is triggered by an organic compound (Sussman and Halvorson 1966 Spores: Their dormancy and germination, Chapter 7, Harper & Row). In the transformants this condition likely results from a transgene position effect causing a silencing of some germination-specific genes. Finally, although amino acid activation of macroconidia of the two histidine transformants is a special case, this observation was extended to microconidia – another type of asexual vegetative spores produced by *N. crassa* whose germination has been very low or erratic. The finding that certain amino acids markedly improve the plating efficiency of microconidia of *N. crassa* is being reported separately.

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Table 1. Estimation of nuclear ratios in histidine transformants by direct plating and colony transfer methods

Transformant	Average no. of colonies / plate± s.d[a].		% Prototrophic and auxotrophic macroconidia					
	Without Histidine	With Histidine	By plating[b]			By colony transfer		
			his-3⁺	his-3	Ratio his-3+:his-3	his-3⁻	his-3	Ratio his-3+:his-3
3T3	35 ± 17	145 ± 47	25 ± 08	75 ± 08	1 : 3	74 ± 03	26 ± 03	3 : 1
4T12	34 ± 12	161 ± 27	22 ± 08	78 ± 08	1 : 4	19 ± 01	81 ± 01	1 : 5
2T5	83 ± 21	130 ± 03	64 ± 22	36 ± 22	2 : 1	100 ± 00	0	-

[a]200 macroconidia (haemocytometer count) were plated in all experiments. All media contained inositol. Colonies appearing on histidine supplemented medium is taken as the number of viable macroconidia. Each value is average of at least three experiments, each with three replicate platings.

[b]Under *his-3⁻* column both prototrophic homokaryotic and prototrophic heterokaryotic macroconidia are included. % *his-3⁻* macroconidia = (number of colonies on plate without histidine) ÷ (number of viable macroconidia) × 100. % *his-3* macroconidia = 100 - % *his-3⁺* macroconidia.

Table 2. Effect of amino acid supplementation on plating efficiency of *N. crassa* macroconidia[a]

Strain	Number of colonies formed / plate ± s.d				
	Control	Histidine	Lysine	Arginine	Leucine
74-OR23-IV A	121 ± 20	151 ± 15	162 ± 07	147 ± 11	78 ± 16
mcm (RM124 -2*A*)	140 ± 12	179 ± 16	163 ± 14	131± 13	104 ± 24
al-1; mcm; inl (PM2-21*a*)	112 ± 14	133 ± 13	125 ± 09	121 ± 05	101± 01
Transformant 3T3	35 ± 17	145 ± 47	125 ± 35	30 ± 09	19 ± 07

[a]Data are average values from three experiments. In all experiments 200 macroconidia were added / plate. All plating media contained inositol except when 74 -OR23-IVA and *mcm* were used. Each value is average of three experiments, each with three replicates.

Table 3. Amino acid composition of *N. crassa* macroconidia

Amino acid	nmoles/10^6conidia[a]					
	al-1; mcm; inl			Transformant 3T3		
	Sample #1	Sample #2	Average	Sample #1	Sample #2	Average
Asp	0.254	0.187	0.221	0.664	1.151	0.908
Thr	0.147	0.100	0.124	0.279	0.596	0.438
Ser	0.207	0.150	0.179	0.358	0.891	0.625
Glu	0.692	0.202	0.449	0.761	1.584	1.173
Pro	0.201	0.056	0.129	0.195	0.398	0.297
Gly	0.939	0.514	0.727	0.865	1.736	1.301
Cys	0.089	0.038	0.064	0.025	n.d.	0.025
Val	0.080	0.053	0.067	0.165	0.354	0.260
Met	0.130	0.050	0.090	0.026	0.023	0.025
Leu	0.130	0.079	0.105	0.029	0.048	0.039
Tyr	0.060	0.028	0.044	0.099	0.124	0.112
Phe	0.961	0.038	0.671	0.296	0.857	0.577
His	0.058	0.023	0.041	0.057	0.122	0.089
Lys	0.194	0.157	0.176	0.211	0.930	0.571
Arg	0.085	0.028	0.057	0.154	0.201	0.178

[a]Concentrations compared on basis of 10^6 conidia. n.d., not detected