ISOLATION AND CLASSIFICATION OF EXTRANUCLEAR MUTANTS OF *NEUROSPORA CRASSA*[1]

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ABSTRACT

Four extranuclear mutants, [exn-1], [exn-2],[exn-4], and [stp-C], were obtained from N-methyl-N'-nitro-N-nitrosoguanidine-treated conidia and mycelium of *Neurospora crassa*. The three *exn* mutants grow with a pronounced lag from conidia and ascospores and are female fertile, whereas [stp-C] has a stop-start growth phenotype and is female sterile. The mitochondria from all four mutants are deficient in cytochromes $a+a_3$ and b, but contain an excess of cytochrome c. On the basis of growth and fertility, nuclear suppressors and complementation in heteroplasmons, 16 of the extranuclear mutants now available in Neurospora can be divided into three groups. Group I consists of 8 female-fertile variants with both poky-like growth and cytochrome defects. Their slow growth is suppressed by the nuclear factor, f, but not by a second nuclear suppressor, $su-1$([mi-3]). They complement with group III mutants in mixed cytoplasmons. Group II is represented by a single variant, [mi-3]. It is phenotypically modified by the $su-1$([mi-3]) factor, but not by f. Its unique cytochrome spectrum shows a deficiency of cytochrome a, but c and b are present. It complements in heteroplasmons with group I and III mutants. Group III included 7 female-sterile variants with stopper growth phenotypes and the same cytochrome defects as group I. Group III mutants complement both with group I and II isolates, but they are unaffected by either f or $su-1$.

ALTHOUGH much has yet to be learned about the function of the genetic material in mitochondria and chloroplasts, the discovery of DNA in these organelles has focused attention both on the general significance of extranuclear inheritance in cellular heredity and on the origin of the cellular organelles. In studies involving mitochondria, the presence of respiratory-deficient, cytoplasmically-inherited mutants of yeast and Neurospora made these two fungi the organisms of choice for much recent work on protein synthesis, organelle biogenesis, and somatic cell genetics. While the extranuclear mutants of yeast and Neurospora are often referred to as similar, they are in fact quite different in their effects on mitochondrial function; consequently, each organism is uniquely suited for certain types of experimental approaches. In the facultative anaerobe,

Saccharomyces cerevisiae, the respiratory-deficient petite mutants lack certain respiratory enzymes and have anaerobic metabolism even in the presence of oxygen (EPHRUSSI 1953). Furthermore, the mitochondria are structurally aberrant (YOTSUYANAGI 1962). The genetic lesions are such that specific buoyant density changes are found in the DNA of different petites (MOUNOLOU, JAKOB and SLONIMSKI 1966; BERNARDI *et al.* 1970). In contrast, the Neurospora mutant [*mi-1*] or [*poky*] (MITCHELL and MITCHELL 1952) as well as other extranuclear mutants of this fungus, is characterized by low (rather than no) activity of respiratory enzymes (BERTRAND 1969). Furthermore, although respiratory activity may vary drastically, depending on the age of the culture (HASKINS *et al.* 1953), it is strikingly similar in both the mutant and the wild type (EAKIN and MITCHELL 1970). In addition there is no convincing evidence of structural differences in the mitochondria of [*poky*] and wild-type strains. Thus, while the anaerobic petite mutants of yeast function as complete genetic blocks of aerobic respiration, the Neurospora mutants behave simply as "leaky" mutations.

Another striking difference in the two system is that spontaneous and induced extranuclear mutants can be easily isolated in yeast (EPHRUSSI 1953) whereas less than a dozen such mutants have been described in Neurospora. Since the scarcity of mutants in Neurospora can be attributed largely to the lack of a selective technique, we felt it essential to consider additional methods to attempt to recover new mutants. We were anxious not only to recover phenotypically different mutants for genetic and gene-function studies, but also to get a better idea of the spectrum of functionally different mutants that might be recovered in an aerobic organism.

Although unable to find a selective technique for isolating suitable extranuclear mutants, we did isolate five new respiratory-deficient, cytoplasmic mutants. Because all extranuclear mutants, except [*mi-3*], examined by us have similar cytochrome defects, we looked for other ways to distinguish them. By using conventional genetic techniques of phenotypic differences, nuclear suppressors, and complementation in heteroplasmons, we were able to classify 16 extranuclear mutants into three groups.

MATERIALS AND METHODS

Strains: These mutants were obtained from the Fungal Genetics Stock Center: [*poky*] *a* (also called [*mi-1*]; m-1–1.8, maternal inheritance), [*mi-3*] *a* (3754, maternal inheritance), [*SG-3*] *a* (RL 3120–10, slow growth), *f* [*poky*] *a* (3627–3, fast modifier of [*poky*], VR), *f* [*poky*] *A*, and *su-1*[*mi-3*] (383, a nuclear suppressor of [*mi-3*], IL). A culture of [*SG-1*] *a* (RL 3202, slow growth) was provided by Dr. ADRIAN M. SRB of Cornell University. These mutants also were used: [*stp*], (McDOUGALL and PITTENGER 1966); and [*stp-A*] (stop-start growth), [*stp-A18*] (stop-start growth), [*stp-B2*] (stop-start growth), and [*stp-B1*] (slow initial growth), all described by BERTRAND and PITTENGER (1969a).

Because the effect of nuclear genotype on the recoverability of extranuclear mutants was unknown, different sets of heterokaryon-compatible strains were used. One set included these strains of mixed origin: *nic-1 al-2 2A* (3416, nicotinamide-requiring, IR; 15300, albino, IR), *pan-1 al-1 BF15A* (5531, pantothenate-requiring, IVR; 4637T, albino, translocation IR-IIR, inseparable from albino-1), and *ad-4 al-2 19–1A* (F4, adenine-requiring, IIIR; 15300, albino, IR). Another group of isolates, consisting of five strains heterokaryotically compatible with the

74-OR-8–1a standard wild type, included *ad-4 37–1A*, *nic-1 al-2 237a*, *pan-2 3a* (B3, pantothenate requiring, VIR), *pan-2 al-2 56a*, and *ad4 al-1 2a*. The other strains mentioned were selected from crosses of these above strains. The *f nic-1 al-2 B197A* and *f nic-1 al-2 BK-44a* were selected from *nic-1 al-2 2A* × *f* [*poky*] *a* and *nic-1 al-2 237a* × *f* [*poky*] *A*, respectively.

When necessary the cytoplasmic mutants were introduced into the appropriate, uniform, genetic backgrounds by heterokaryotic transfer.

Media, growth conditions and enzyme assays: Except where otherwise specified, the media, growth conditions, and determination of cytochrome *c* oxidase and succinate: cytochrome *c* oxidoreductase activity have been described previously (BERTRAND, McDOUGALL and PITTENGER 1968; BERTRAND and PITTENGER 1969a).

Isolation of mitochondria and cytochrome spectra: Procedures for isolation of mitochondria and for spectral determination of cytochromes have been presented elsewhere (BERTRAND and PITTENGER 1969a, b; GRIFFITHS, BERTRAND and PITTENGER 1969a). A minor modification was introduced, however, by suspending the mitochondrial fraction for ultrasonic disruption in 0.1 M Tris-HCl-0.01 M EDTA, ph 7.4, containing 30 mg/ml of sodium deoxycholate.

The relative concentrations of the different cytochromes in sonified mitochondrial preparations were calculated as follows from difference spectra obtained with a Cary 16 spectrophotometer:

$$\text{cytochromes } a + a_3 : A_{608} - A_{630}$$
$$\text{cytochrome } b \qquad A_{560} - A_{575} + 0.04\,(A_{550} - A_{575})$$
$$\text{cytochrome } c \qquad A_{550} - A_{575} + 0.04\,(A_{560} - A_{575})$$

The rationale for the calculations has been presented elsewhere (BERTRAND 1969).

Mutagenic treatments: Exposures of conidia to N-methyl-N′-nitro-N-nitrosoguanidine (nitrosoguanidine) were made at room temperature. One ml of the incubation mixture contained 37 μmoles of potassium phosphate, pH 7.1, 33 μg of nitrosoguanidine, and about 10^6 conidia. Samples of this mixture were taken at one-minute intervals for up to five minutes and diluted for plating on Westergaard's sorbose medium. After 3 to 5 days of incubation at 30°C, small colonies were selected from a number of different treatments for further testing in growth tubes to identify abnormal growth.

In an attempt to get more efficient treatment with low levels of the mutagen, a few cultures were grown from a conidial inoculum through 500-mm-long growth tubes containing Vogel's medium with initial concentrations of nitrosoguanidine ranging from 0.1 mg/ml to 50 mg/ml. The effective concentrations of the mutagen probably were much lower than the initial concentration, because the compound is unstable in aqueous solution.

RESULTS

Isolation of [exn-1] *and* [exn-4] *from nitrosoguanidine-treated conidia:* A total of 520 slow-growing strains were selected from conidia that had been treated with nitrosoguanidine to a survival level of less than 30%. The activities of succinate: cytochrome *c* oxidoreductase and cytochrome *c* oxidase first were determined in crude mycelial extracts of each of these strains, in an effort to identify respiration-defective isolates. Depressed activities in either or both enzymes were found initially in 48 isolates, but more than half subsequently grew so poorly that they were unsuited for further analysis. The aberrant growth characteristics of another 16 strains were transmitted to half of the progeny in crosses where the mutant was the fertilizing (male) parent. The slow-growth patterns and/or the respiratory defects of these mutants were assumed to be determined by nuclear factors.

The slow growth of two of the remaining isolates clearly was maternally inherited in reciprocal crosses. The slow-growth phenotype of both also could be
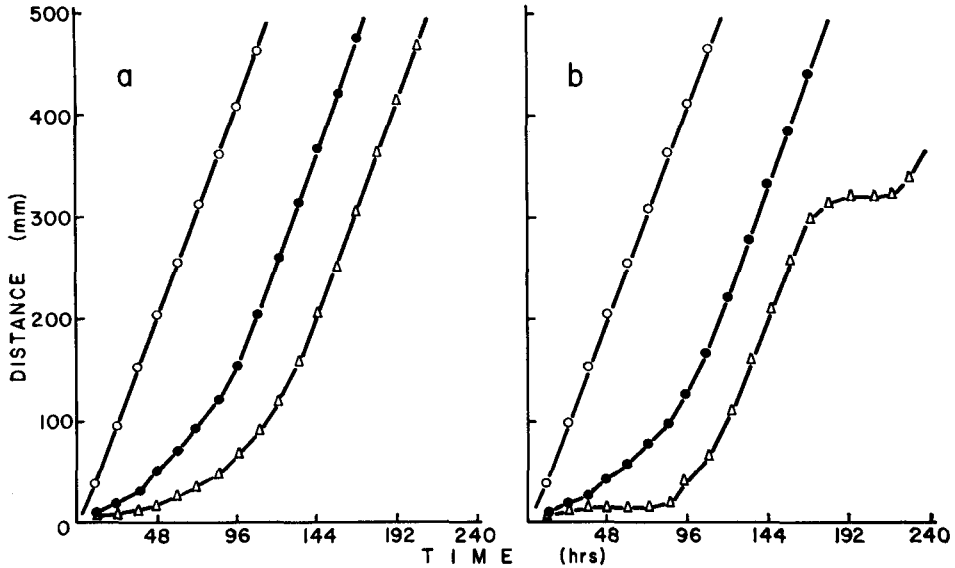
FIGURE 1.—Growth curves typical of a *nic-1 al-2 237a* strain with the following extranuclear mutants: a) [+] (O), [*exn-2*] (●), [*exn-1*] (△), and b) [+] (O), [*exn-4*] (●) and [*stp-C*] (△).

transmitted heterokaryotically to other strains. The two mutants, obtained from independent nitrosoguanidine treatment of conidia of the *pan-2 3a* strain, were designated [*exn-1*] (extranuclear-1) and [*exn-4]* (extranuclear-4). Strains with [*exn-1*] or [*exn-4*] cytoplasm initially grow slowly from conidia or ascospores, but after 4 to 8 days reach normal rates, as shown in Figure 1. The slow growth also is manifested in the formation of microcolonies when conidia are plated on Westergaard's sorbose medium. As discussed later, both strains have abnormal cytochrome systems similar to [*poky*]. In a previous report [*exn-1*] was identified merely by its isolation number as N4–9–4 (BERTRAND and PITTENGER 1968).

Isolation of [exn-2] *following nitrosoguanidine treatment:* Assuming that at low survival rates most induced mitochondrial mutants are in conidia in which the nuclei have already been inactivated by the mutagenic treatment, we tried to rescue possible mitochondrial mutants by heterokaryosis involving untreated conidia of a different genotype. In one such experiment, conidia of *pan-2 al-2 56a* were treated with 33 μg/ml nitrosoguanidine until the surviving spore fraction had decreased to less than five percent. The treated conidia then were combined with an equal number of untreated conidia from an *ad-4 al-1 2a* strain and the mixed suspension was centrifuged. A portion of the conidial pellet was inoculated onto minimal medium at the beginning of a continuous growth tube. The resultant heterokaryon, sampled periodically with inconclusive results, slowed its growth abruptly after growing approximately 13 meters. A mutant strain, [*exn-2*] (extranuclear-2), was isolated from a single conidial isolate from this section of the growth tube and its growth was characterized by a conspicuous lag, as shown in Figure 1a. The mitochondria from the mutant proved to be deficient

in $a + a_3$ and b but contained an unusually high concentration of cytochrome c. These defects were inherited maternally in reciprocal crosses with normal strains and could be transferred to normal strains by means of heterokaryosis.

A spontaneous mutant [exn-3]: Although the [*exn-3*] (extranuclear-3) mutant was not recovered as the result of mutagenic treatment, it was isolated recently as a spontaneous mutant from one of our laboratory stocks and is included here for the record. This mutant, found in a *nic-1 al-2 a* strain, is similar in phenotype to many of the other cytoplasmic mutants. It is maternally inherited in reciprocal crosses, has a slow initial growth rate, and a mitochondrial cytochrome system similar to [*poky*].

A cytoplasmic mutant [stp-C] *isolated from a culture grown on nitrosoguanidine-supplemented medium.* In an experiment set up to determine the maximum concentrations of nitrosoguanidine that would permit growth, a *pan-2 al-2a* strain was inoculated into growth tubes in which the levels of the mutagen in the medium ranged from 0.05 mg/ml to 50 mg/ml. The organism generally did not grow on concentrations of nitrosoguanidine greater than 10 mg/ml. In one growth tube in which the medium had been supplemented with 5 mg/ml of the mutagen, the inoculum initially grew well but after about 300 mm of growth the rate slowed progressively and eventually ceased. We investigated this growth cessation further by breaking the tube near the frontier of the stopped mycelium, and transferring hyphal tips from the stopped culture to fresh medium. No further growth was observed. Conidia from the proximal end of the growth tube were then plated on Westergaard's sorbose medium and only small colonies and conidia with short germ tubes were found after being incubated five days at 30°C. The single conidial isolates obtained from the very small colonies grew in a stop-start pattern in 500-mm growth tubes (Figure 1b); one was selected for further study.

This mutant strain, designated [*stp-C*] (stopper C), did not form fertile proto-perithecia, but when it was used as the male parent in crosses with *nic-1 al-2 2A* the stopper phenotype was not recovered in any of the progeny. The abnormal growth phenotype, however, could be transferred to normal strains by transient heterokaryosis with the stopper mutant, which indicated that the abnormal growth was determined by an extranuclear genetic factor. Mitochondria from the mutant had cytochromes in amounts characteristic of [*poky*] strains.

Mitochondrial cytochrome systems: The mitochondria from 24 to 96-hr-old mycelium of [*exn-1*], [*exn-2*], [*exn-3*], [*exn-4*], and [*stp-C*] strains have very low levels of cytochromes $a+a_3$ and b but contain an excess of cytochrome c (Figure 2b and Table 1). Consistent with the deficiency in cytochromes a, the mitochondria from the mutants have depressed levels of cytochrome oxidase activity (Table 1). However, the activity of mitochondrial succinate: cytochrome c oxidoreductase consistently was nearly twice as high in the mutants as in strains with normal mitochondria. On the basis of the cytochrome data and enzyme activities shown in Table 1, it would appear that the defects in the mitochondrial, electron-transport system of all newly isolated mutants resemble [*poky*].
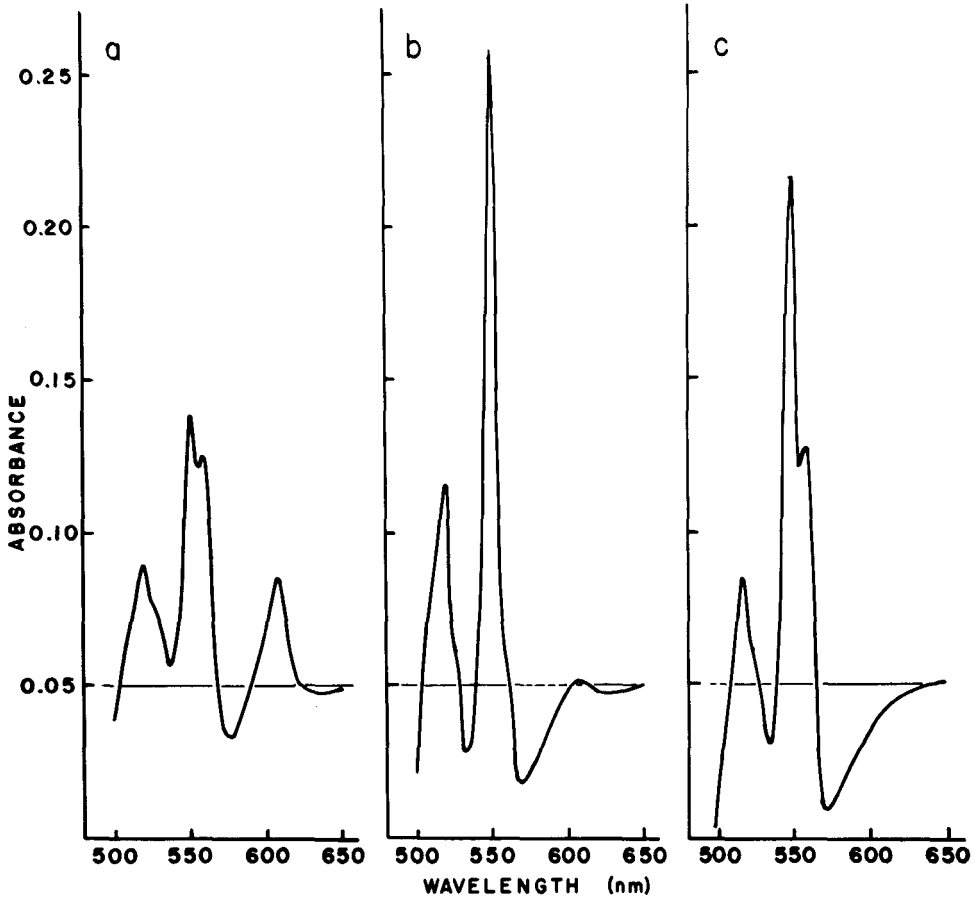
FIGURE 2.—Cytochrome spectra typical of sonified mitochondria isolated from a nic-1 al-2 237a strain with the following extranuclear mutants: a) [+], b) [exn-1], [exn-2], [exn-3], [exn-4], or [stp-C], and c) [mi-3].

A brief classification of the extranuclear mutants of Neurospora: On the basis of growth phenotypes, sexual fertility, and mitochondrial cytochrome abnormalities, the most thoroughly characterized extranuclear mutants of Neurospora can be classified into three fairly well-defined groups (Table 2). Group I includes those mutants that have the [poky] growth phenotypes, are female fertile, and have an excess of cytochrome c and a deficiency (but not absence) of cytochromes a+a_3 and b. BERTRAND (1969) described poky-growth as progressive, to indicate that growth rates increase daily until a constant rate is achieved. The final rate may or may not be the wild-type rate. Maximum rates for different mutants and for different isolates of the same mutant may not be identical, but they are nevertheless progressive. Such progressive growth rates are shown in Figure 1a.

A single variant, [mi-3] is the only representative of group II. Growth of this extranuclear mutant is progressive, somewhat intermediate between poky-like growth and wild type (MITCHELL, MITCHELL and TISSIERES 1953). The [mi-3]

TABLE 1

Relative cytochrome contents (absorbancy/10 mg mitochondrial protein), activities of cytochrome c oxidase (min^{-1}/ mg mitochondrial protein), and succinate: cytochrome c oxidoreductase (μmoles cytochrome c reduced/min/mg mitochondrial protein) in mitochondria from normal and mutant strains

Mutant	Culture age (hours)	$a+a_3$	Cytochrome content b	c	Cytochrome oxidase	Succ.:cyt. c oxidoreductase
[+]	23	0.0351	0.0861	0.1086	56.0	0.532
	36	0.0331	0.0790	0.0877	48.2	0.507
[exn-1]	36	0.0018	0.0126	0.2346	0.7	1.004
	72	0.0103	0.0524	0.1611	13.3	0.912
[exn-2]	36	0.0027	0.0168	0.2374	0.5	1.490
	72	0.0083	0.0444	0.1571	11.2	1.031
[exn-3]	36	0.0016	0.0142	0.2339	0.3	1.138
[exn-4]	36	0.0041	0.0183	0.2493	4.1	1.100
[stp-C]	64	0.0091	0.0505	0.1861	14.6	1.224
	96	0.0082	0.0557	0.1905	13.3	1.048
[poky]	36	0.0038	0.0229	0.2192	4.1	1.006
	72	0.0097	0.0514	0.1657	9.7	0.811
[mi-3]	36	0.0009	0.1384	0.2485	0.9	0.653

mutant is female fertile and has a unique cytochrome system; mitochondria have no detectable cytochrome $a+a_3$ but contain both cytochromes b and c, with the ratio of c to b being higher than it is in the wild type.

The mutants in group III have a stop-start growth phenotype, as shown for [stp-C] and [stp-A] in Figures 1b and 3c, respectively. The mutants are female sterile and conidia are usually of low viability, but mitochrondria have the same
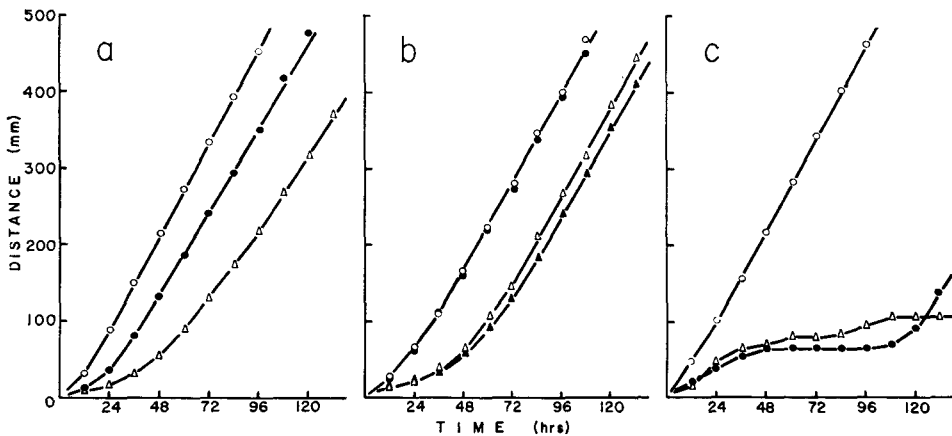

FIGURE 3.—Growth curves illustrating the interaction of f with [exn-1] and [stp-A]. a) f nic-1 al-2 Bl-97A with [+] (O) and [poky] (●) cytoplasm, and f^+ nic-1 al-2 237a [exn-1] (△); b) f (O and ●) and f^+ (△ and ▲) containing nic-1 al-2 [exn-1] progeny in a tetrad from f^+ nic-1 al-2 237a [exn-1] × f nic-1 al-2 Bl-97A [+]; and c) f^+ pan-1 al-1 BF15A with [+] (O) and [stp-A] (●) cytoplasm, and f nic-2 al-2 Bl-97A [stp-A] (△).

TABLE 2

Characteristics of the extranuclear mutants of Neurospora

Group	Mutant	Growth phenotype	Female fertility	Mitochondrial cytochromes			Suppression		Mutagen
				$a+a_3$	b	c	by f	by $su\text{-}1$	
I	[*poky*]	poky	+	deficient	deficient	excess	+	−	spontaneous
	[*SG-1*]	poky	+	deficient	deficient	excess	+	−	acriflavin
	[*SG-3*]	poky	+	deficient	deficient	excess	+	−	spontaneous
	[*stp-B1*]	poky	+	deficient	deficient	excess	+	−	spontaneous
	[*exn-1*]	poky	+	deficient	deficient	excess	+	−	nitrosoguanidine
	[*exn-2*]	poky	+	deficient	deficient	excess	+	−	nitrosoguanidine
	[*exn-3*]	poky	+	deficient	deficient	excess	+	−	spontaneous
	[*exn-4*]	poky	+	deficient	deficient	excess	+	−	nitrosoguanidine
II	[*mi-3*]	intermediate	+	deficient	normal	excess	−	+	spontaneous
III	[*stp*]	stopper	−	deficient	deficient	excess	−	−	UV-light
	[*stp-A*]	stopper	−	deficient	deficient	excess	−	−	spontaneous
	[*stp-A18*]	stopper	−	deficient	deficient	excess	−	−	spontaneous
	[*stp-B2*]	stopper	−	deficient	deficient	excess	−	−	spontaneous
	[*stp-C*]	stopper	−	deficient	deficient	excess	−	−	nitrosoguanidine
	[*abn-1*]	stopper	−	deficient	deficient	excess	··	··	spontaneous
	[*abn-2*]	stopper	−	deficient	deficient	excess	··	··	spontaneous

cytochrome defects as do the group I mutants. The [mi-4] mutant, first described by PITTENGER (1956), but now lost, as well as [abn-1] and [abn-2] (GARN-JOBST, WILSON and TATUM 1965), which we have not examined, would also belong to group III.

In our experience both growth and fertility can be altered drastically in response to different genetic backgrounds; even [poky] in some nuclear backgrounds may have a stopper phenotype (PITTENGER 1956; BERTRAND and PITTENGER 1969a). Consequently, we were interested in other criteria to distinguish mutants of groups I, II, and III. MITCHELL and MITCHELL (1956) already had shown that [mi-1] and [mi-3] respond differently to a nuclear factor, f, which acts as a suppressor of the progressive growth rate by eliminating the lag, and also is known to suppress [SG-3] and [stp-B1] (GRIFFITHS, BERTRAND and PITTENGER 1968b) and [SG-1] (GILLIE 1970). Extending this observation to other mutants created no problem in the case of the female-fertile strains. We have observed a 1:1 segregation of suppressed and unsuppressed growth in asci from crosses in which [exn-1], [exn-2], [exn-3], and [exn-4] mutants were the female parents in crosses with an f strain as the fertilizing parent (Figure 3b). Thus, in the presence of f the initial lag in the growth of all group I mutants is eliminated.

Because group III mutants were female sterile, we could not introduce the f factor into the nuclear genotype by sexual crosses. Instead we had to achieve the necessary cytoplasmic-nuclear combinations by heterokaryotic transfer. For example, [stp-A] and [stp-C] were introduced into an f nuclear background by making heteroplasmons between f+ pan-1 al-1 BF15A [stp-A] and f nic-1 al-2 BI-97A [+] as well as between f+ pan-2 3a[stp-C] and f nic-1 al-2 BK 44a [+]. From such heterokaryons, slow-growing isolates were initially selected as possible [stp-A] or [stp-C] mutant homoplasmons. Later nicotinamide- and panto-thenate-requiring homokaryons were selected from conidial platings and further tested in 500-mm growth tubes to determine their growth phenotypes.

When such stopperlike homokaryons were recovered in both f+ pan and f nic isolates, we concluded that [stp-A] and [stp-C] mutants were not affected by the f supressor gene. To confirm the f genotype of the stopperlike nicotinamide isolates, they were crossed as the male parent to f+ [poky] female parents. The suppressed and unsuppressed pokylike growth phenotypes were found to segregate 1:1 in asci from these crosses. In such a way we confirmed that all stopperlike mutants, in contrast to group I isolates, are unaffected by the f genotype. However, the [abn-1] and [abn-2] strains (GARNJOBST et al. 1965) have not been analyzed by the f test although in other respects they resemble group III mutants.

The distinctiveness of the mutants in the three groups was corroborated by the interactions of the individual variants with su-1([mi-3]), a nuclear suppressor of [mi-3]. This suppressor, which originally was described as su⁻ by GILLIE (1970), effects normal growth and a wild-type cytochrome system in strains with [mi-3] cytoplasm (BERTRAND 1971). Using procedures similar to those described for f, we have found that su-1([mi-3]) does not modify the abnormal

growth and cytochrome phenotypes of any one of the mutants of groups I or III. Therefore, the suppressor is specific for [mi-3] (Table 2).

<div align="center">DISCUSSION</div>

Before we started these experiments it was apparent that the primary problem was not mutant induction in mitochondria, but rather the recognition and isolation of the mutants. Unsuccessful in overcoming the inherent problem brought about by the large number of mitochondria in the coenocytic hyphae and conidia, we nevertheless added five respiratory-deficient strains to the small list of extranuclear mutants of Neurospora. Four of them were isolated from strains treated with nitrosoguanidine, which could have acted as a mutagen or selective agent, but because of the very low recovery there is no good evidence that any of the four were induced rather than spontaneous. Furthermore, with the methods currently employed we could have predicted with reasonable certainty that the majority of the new mutants would have to be either dominant and/or have a superior rate of division. The five new mutants certainly have this property of suppressiveness, or we could not have transferred them heterokaryotically and recovered them so easily in new nuclear genotypes. However, what we originally did not know about these suppressive variants was whether they were representative of a highly select group of a few different mitochondrial cistrons or were in fact generally representative of mutants of the mitochondrial genome. Our current attempt to distinguish among mutants has helped to clarify this problem.

As new mutants were isolated, we attempted to define genetic criteria that might be used to distinguish them. Because the first two extranuclear mutants of Neurospora, [mi-1] and [mi-3], had different cytochrome spectra (MITCHELL, MITCHELL and TISSIERES 1953), we had hoped (as more mutants became available) eventually to recover all possible permutations of cytochrome abnormalities, but were unable to do so. In fact all subsequently isolated extranuclear respiratory mutants closely resemble [mi-1]; that is, they have a 1.5- to 2-fold increase in cytochrome c in the mitochondria and a deficiency of cytochromes $a+a_3$ and b in young cultures (Table 2). The possible significance of this common "mutant" cytochrome spectrum became more apparent as additional mutants with this phenotype were isolated. It became increasingly clear that the absence or deficiency of cytochrome $a+a_3$ and b did not mean that [mi-1], for example, could not synthesize these cytochromes, because in fact older mutant cultures produce significant amounts of those cytochromes (HASKINS et al. 1953; BERTRAND 1969; RIFKIN and LUCK 1971). Thus, we had to assume that in mutant strains, regulatory mechanisms are prominently involved in the cytochrome phenotype. The significance of this, and particularly the apparent derepression of cytochrome c, became even clearer with the observations that inhibitors of either the electron transport chain or mitochondrial protein synthesis resulted in the overproduction of cytochrome c in wild-type strains. WOODWARD, EDWARDS and FLAVELL (1970) have observed the increase in cytochrome c in wild-type cultures

grown in the presence of antimycin A, and the chloramphenicol effect has been noted by BERTRAND (unpublished). We suggest, then, that the excess of cytochrome c in extranuclear mutants does not necessarily indicate any genetic identity; rather it indicates that the mutants probably are functionally related in that they affect either the electron transport system or some component of the mitochondrial protein synthesizing system. However, because the Group II mutant, [mi-3], has the only unique cytrochrome spectrum, we assume that it is genetically different from the others.

It is significant that genetic criteria define at least three groups of extranuclear mutants in Neurospora. That these mutant groups represent different extranuclear genetic lesions is suggested from at least three lines of evidence. (1) The mutants from group I, II, and III retain their group-specific phenotypic properties even when the cytoplasmic factors are transferred to a common nuclear background (BERTRAND and PITTENGER 1969a, 1972). (2) Nuclear modifiers are group specific, i.e., the nuclear suppressor f is specific for group I mutants and su-1([mi-3]) is specific for group II. (3) Mutants from different groups form complementing heteroplasmons. We have reported elsewhere (BERTRAND and PITTENGER 1972) that mutants from group I can form normally growing heteroplasmons with group III mutants, but mutants from within the same group do not form complementing heteroplasmons with one another. The [mi-3] + [stp-B1] heteroplasmon occasionally has been observed to be at rates higher than either homoplasmon; however, because the synergistic effect is not as great as [mi-3] + group III combinations, we are reluctant to judge its significance.

It should be pointed out that while this classification of extranuclear mutants is based solely on genetic methodology, the group differences are not inconsistent with biochemical evidence. RIFKIN and LUCK (1971) for example, have suggested that [mi-1] is defective either in synthesis or assembly of the small ribosomal subunit. They found no evidence for such a defect in group II and III mutants, namely [mi-3] and [abn-1].

Because we still do not understand either the mechanics of complementation between mitochondrial mutants or, the mechanisms by which the f gene eliminates progressive growth of group I mutants without affecting either the cytochrome defects (MITCHELL and MITCHELL 1956) or the ribosome defect (RIFKIN and LUCK 1971), it is clear we have a great deal to learn about interactions between the genetic systems within the same cell. Nevertheless, certain observations do seem particularly relevant. The injection of [abn-1] mitochondria (DIACUMAKOS, GARNJOBST and TATUM 1965) and [mi-1] mitochondria (WILSON 1969) into wild-type strains, and the eventual expression of the mutant phenotype in the recipient cultures, certainly indicates that the mitochondria contain the information for the characteristic abnormal growth and cytochrome defects observed in mutants of group III and I. Because mutants from these groups also form complementing heteroplasmons with one another, the expression of these extranuclear genes can not be restricted to the organelle of which they are a part. Such complementation of different extranuclear mutants (expressed in hetero-

sis) is possible only if genetically different mitochondria sharing a common cytoplasm exchange either genetic information or gene products or, if mitochondrial genes are expressed extramitochondrially. Although there is no evidence in Neurospora that such complementation results from the formation of wild-type recombinants (PITTENGER 1956), we cannot rule out that possibility since recombination among extranuclear mutants in yeast has been observed (THOMAS and WILKIE 1968; COEN *et al.* 1970). The evidence supporting alternative explanations is inconclusive.

LITERATURE CITED

BERNARDI, G., M. FAURES, G. PIPERNO and P. P. SLONIMSKI, 1970   Mitochondrial DNA's from respiratory-sufficient and cytoplasmic respiratory-deficient mutant yeast. J. Mol. Biol. **48:** 23–42.

BERTRAND, H., 1969   Isolation and characterization of cytoplasmic mutants in *Neurospora crassa.* Ph.D. Dissertation. Kansas State University. ——, 1971   A nuclear suppressor of the cytochrome defects of the [*mi-3*] extranuclear mutant of *Neurospora crassa* (Abstr.) Can. J. Genet. Cytol. **13:** 626.

BERTRAND, H. and T. H. PITTENGER, 1968   Nitrosoguanidine induced cytoplasmic mutants of Neurospora. (Abstr.) Genetics **60:** 161–162. ——, 1969a   Cytoplasmic mutants selected from continuously growing cultures of *Neurospora crassa.* Genetics **61:** 643–659. ——, 1969b   Cytochrome spectra of three cytoplasmic mutants. Neurospora Newsl. **14:** 6. ——, 1972   Complementation among cytoplasmic mutants of *Neurospora crassa.* In press.

BERTRAND, H., K. J. McDOUGALL and T. H. PITTENGER, 1968   Somatic cell variation during uninterrupted growth of Neurospora in continuous growth tubes. J. Gen. Microbiol. **50:** 337–350.

COEN, E., DEUTSCH, P. NETTER, E. PETROCHILO and P. P. SLONIMSKI, 1970   Mitochondrial genetics I. Methodology and phenomenology. Symp. Soc. Exptl. Biol. **24:** 449–496.

DIACUMAKOS, E. G., L. GRANJOBST and E. L. TATUM, 1965   A cytoplasmic character in *Neurospora crassa.* The role of nuclei and mitochondria. J. Cell Biol. **26:** 427–443.

EAKIN, R. T. and H. K. MITCHELL, 1970   Alterations of the respiratory system of *Neurospora crassa* by the *m-1* mutation. J. Bacteriol. **104:** 74–78.

EPHRUSSI, B., 1953   *Nucleo-cytoplasmic relations in microorganisms.* Clarendon Press, Oxford.

GARNJOBST, L., J. F. WILSON and E. L. TATUM, 1965   Studies on a cytoplasmic character in *Neurospora crassa.* J. Cell Biol. **26:** 413–425.

GILLIE, O. J., 1970   Methods for the study of nuclear and cytoplasmic variation in respiratory activity of *Neurospora crassa,* and the discovery of three new genes. J. Gen. Microbiol. **61:** 379–395.

GRIFFITHS, A. J. F., H. BERTRAND and T. H. PITTENGER, 1968a   Cytochrome spectra of cytoplasmic mutants in Neurospora. Neurospora Newsl. **13:** 16–17. ——, 1968b   Nuclear suppression of maternally inherited phenotypes in *Neurospora crassa.* (Abstr.) Genetics **60:** 185.

HASKINS, F. A., A. TISSIERES, H. K. MITCHELL and MARY MITCHELL, 1953   Cytochromes and the succinic acid oxidase system of poky strains of Neurospora. J. Biol. Chem. **200:** 819–826.

McDOUGALL, K. J. and T. H. PITTENGER, 1966   A cytoplasmic variant of *Neurospora crassa.* Genetics **54:** 551–565.

MITCHELL, M. B. and H. K. MITCHELL, 1952   A case of maternal inheritance in *Neurospora crassa.* Proc. Natl. Acad. Sci. U.S. **38:** 442–449. ——, 1956   A nuclear gene suppressor of a cytoplasmically inherited character in *Neurospora crassa* J. Gen. Microbiol. **14:** 84–89.

MITCHELL, M. B., H. K. MITCHELL and A. TISSIERES, 1953 Mendelian and non-Mendelian factors affecting the cytochrome system in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **39**: 606–613.

MOUNOLOU, J. C., H. JAKOB and P. P. SLONIMSKI, 1966 Mitochondrial DNA from yeast "Petite" mutants: specific changes in buoyant density corresponding to different cytoplasmic mutations. Biochem. Biophys. Res. Commun. **24**: 218–224.

PITTENGER, T. H., 1956 Synergism of two cytoplasmically inherited mutants of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **42**: 747–752.

RIFKIN, M. R. and D. J. L. LUCK, 1971 Defective production of mitochondrial ribosomes in the poky mutant of *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S. **68**: 287–290.

THOMAS, D. Y. and D. WILKIE, 1968 Recombination of mitochondrial drug-resistance factors in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **30**: 368–372.

WILKIE, D., 1964 *The cytoplasm in heredity*. Methuen and Co., London.

WILSON, J. F., 1969 Mitochondrial transplantation studies in *Neurospora crassa*. XI Int. Bot. Congress (Abstracts) p. 240.

WOODWARD, D. O., D. L. EDWARDS and R. B. FLAVELL, 1970 Nucleocytoplasmic interactions in the mitochondrial structure and function in Neurospora. Symp. Soc. Exptl. Biol. **24**: 55–69.

YOTSUYANAGI, Y., 1962 "Etudes sur le chrondriome de la levure. I, II". J. Ultrastruct. Res. **7**: 121, 141.