A CYTOPLASMIC VARIANT OF *NEUROSPORA CRASSA*¹

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OF special significance to cellular heredity in general and extranuclear inheritance in particular is the possibility that certain cellular organelles contain partially autonomous genetic systems. Evidence regarding inheritable systems in chloroplasts and mitochondria has been reviewed by GABOR and GRANICK (1964) with particular reference to *Euglena* and yeast, and GRANICK (1965) has edited a more recent symposium on Cytoplasmic Units of Inheritance. The extranuclear inheritance of respiratory defective petites in yeast had long suggested that mitochondria were involved and the evidence of YOTSUYANAGI (1962) that petites have mitochondria lacking cristae is further evidence of this. Such direct morphological evidence is lacking in most cases but the large number of cytoplasmic mutants of *Neurospora* that have abnormal cytochrome systems (*poky*, MITCHELL and MITCHELL 1952; *mi-3*, MITCHELL, MITCHELL, and TISIERES 1953; *mi-4*, PITTENGER 1956; and *abn-1* and *abn-2*, GARNJOBST, WILSON, and TATUM 1965; DIACUMAKOS, GARNJOBST, and TATUM 1965) have suggested that defective mitochondria are involved in these mutants. On the basis of such extranuclear mutants and the recent discovery of DNA in *Neurospora* mitochondria (LUCK and REICH 1964), there seems to be general agreement, even though rigorous proof is not available, that such extranuclear DNA is involved in the replication and biological functioning of the mitochondria (TATUM 1965; HOROWITZ and METZENBERG 1965).

To what extent the DNA in mitochondria represents a partially autonomous genetic system will have to be determined on the basis of evidence from all levels of cellular organization, but the solution of this problem will most certainly require genetic and biochemical analyses of additional extranuclear mutants. Eventually evidence on this problem will come from a variety of organisms, but at present *Neurospora* may be one of the most useful for studies of this kind. In addition to the presence of mitochondrial DNA and the precedence for extranuclear mutants, *Neurospora* also has other desirable biological attributes for studying extranuclear inheritance. For example, the ability to manipulate heterokaryons, systems in which numerous replicas of genetically different nuclei exist within a coenocytic mycelium, may be useful when applied to heteroplasmons, i.e., systems containing many replicas of alternate states of extranuclear genetic material.

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At present, however, the problem at the genetic level is still one of induction and isolation of new cytoplasmic mutants. This continues to be a difficult task because of the lack of adequate selection techniques. Previous unpublished work in this laboratory showed that it was possible to obtain rare slow-growing isolates following treatment of *Neurospora* conidia with ultraviolet light (UV) or X rays. The slow-growth phenotype was usually dominant in heterokaryons and appeared to be controlled by some extranuclear factor as established by the criterion of heterokaryotic transmission. Most of these were of little experimental value since they behaved as lethals or semilethals as homoplasmons and had to be continually rescued by heterokaryotic rejuvenation with a normal strain.

In the present study, an attempt was made to recover more useful mutants and the schedule of UV treatment was modified to allow a period of time for possible selection and sorting out of mutant types. The treatment is similar to one suggested by PATEMAN (1960) and a period of growth was interspersed between successive UV treatments. A number of presumed extranuclear mutants were recovered and one of them was selected for further study. This mutant, called stopper (*stp*), is characterized by a stop-start phenotype manifested during growth in race (growth) tubes, somatic segregation of conidia into a lethal and nonlethal class and some evidence of an abnormal cytochrome system. The phenotype can be transmitted from one strain to another via heterokaryosis, but does not pass through a sexual cross. Subsequently this stop-start phenotype has been observed to occur spontaneously in continuously growing cultures (McDOUGALL, unpublished; BERTRAND and PITTINGER 1964), and here the phenotype is also cytoplasmically inherited. This same phenotype was previously noted in connection with certain poky and *mi-4* strains (PITTINGER 1956). The early descriptions of these mutants were largely confined to their biochemical properties and maternal inheritance; therefore it seems worthwhile to describe in some detail the somatic behavior of an extranuclear mutant apparently quite typical of those found in *Neurospora*. Since a brief report of this work appeared (McDOUGALL and PITTINGER 1964) two additional papers (GARNJOBST, WILSON and TATUM 1965; DIACUMAKOS, GARNJOBST and TATUM 1965) have described in detail two new extranuclear mutants of *Neurospora* that appear to have many characteristics in common with the mutant reported here.

MATERIALS AND METHODS

Strains: In addition to wild type 74-OR-8-1a and an *al-2a* strain (15300, albino-2, linkage group IR), three auxotrophic mutant strains were used: *pan-1 al-1* 23A (5531, pantothenic-1, IIR; 4637T, albino-1, a reciprocal translocation between linkage group IR and IIR inseparable from the albino phenotype); *nic-2 al-2 A* (4540T, nicotinic-2, an insertional translocation of a segment of IR into IIR inseparable from the nicotinic-2 phenotype (St. LAWRENCE 1959); 15300, albino-2); *ad-4; al-2 A* (F4, adenine-4, linkage group IIR; 15300, albino-2). With the exception of 74-OR-8-1a, the strains—henceforth referred to as *al-2*, *pan al-1*, *nic al-2*, and *ad al-2*—were selected because they were heterokaryotically compatible.

Media and growth rate determinations: All crosses and conidial platings were made in petri plates on the medium of WESTERGAARD and MITCHELL (1947), using sucrose as a carbon source. When colonial growth was desired, 1.5% sorbose and 0.1% sucrose were used. Supplemented

medium contained the appropriate requirement in the following concentrations: adenine, 0.2 mg per ml; nicotinamide and calcium pantothenate, 0.005 mg per ml. Growth rate determinations were made in 500 mm-long growth tubes (RYAN, BEADLE, and TATUM 1943) containing 30 ml of minimal medium (VOGEL 1956) supplemented with 1.5% glucose or sucrose and solidified with 1.5% unwashed agar. Special growth tubes, designed for studying perpetual hyphal propagation in *Neurospora* (McDOUGALL and PITTINGER 1962), were also employed when it was desirable to make a growth rate determination over an extended period of time. All growth rate determinations were made at 30°C. Nuclear ratios were calculated using the formula of ARWOOD and MUKAI (1955).

Ultraviolet irradiation and selection of mutants: Six 8-watt General Electric low pressure, mercury vapor, germicidal lamps, placed 15 inches from the conidial suspension, served as the source of UV radiation. An 8 minute UV treatment under those conditions resulted in 10% survival of the phage T3B. The irradiation procedure was essentially that of PATEMAN (1960), which involves successive exposure of a strain to the mutagen. A 10 ml conidial suspension of an *ad al-2* strain containing approximately 2×10^6 conidia was irradiated and a sample of about 1000 conidia was then transferred onto 50 ml of adenine supplemented WESTERGAARD and MITCHELL's agar medium in a 250 ml flask. Following growth and conidiation, conidia were again suspended in 10 ml of sterile water and the treatment repeated. After three such treatments the viability of the conidia was 70%, but following the fourth irradiation, most of the surviving conidia formed only germlings; i.e., conidia whose hyphae branched at least once after four days at 30°C, but did not reach the visible colony status. Less than 5% of these germlings survived when isolated onto adenine-supplemented medium without sorbose. It was, however, possible to rescue a small proportion of the viable germlings by heterokaryotic rejuvenation. This was done by superimposing a suspension of germinated conidia of *pan al-1* on the germling by means of a Pasteur pipette and then transferring the mixture to a slant of minimal medium. Less than 10% of such rejuvenated germlings formed heterokaryons. The *stp* mutant was isolated from such a heterokaryon.

Heterokaryotic transmission: In order to demonstrate heterokaryotic transmission, heterokaryons were prepared on minimal medium in 500 mm-long growth tubes by superimposing conidia of a strain with an auxotrophic marker onto conidia of a second mutant strain suspected of carrying a cytoplasmic defect. Conidia were removed from the distal end of the growth tube and plated on appropriately supplemented media. The presence of complementary albino markers (in certain combinations) made it easy to screen rapidly large numbers of isolates, since heterokaryotic isolates were pigmented whereas homokaryons were albino. Suspected homokaryons were further tested for lack of growth on minimal medium. If, upon reisolation of the homokaryons from a heterokaryon, the mutant phenotype originally associated with one component was now associated with the second component, this was considered evidence for the extranuclear basis of the mutant phenotype.

Spectroscopic examination and manometry: The cytochromes of suspected mutant strains were checked at room temperature by means of a grating spectroscope which was used to examine high intensity light transmitted through 2- to 5-day-old mycelial pads. Sodium hydrosulfite was used as a reducing agent. Warburg manometry of intact and homogenized mycelia was carried out according to the procedure of TISSIERES, MITCHELL and HASKINS (1953).

RESULTS

Heterokaryon IV-6-6 (isolate No. 6 following four UV treatments of 6 minutes each) was one of a number prepared by adding *pan al-1* conidia to a germling from an irradiated *ad al-2* strain. Obviously there was no way of knowing whether any particular germling would have survived had it not been permitted to form a heterokaryon. Conidia of heterokaryon IV-6-6 were plated on minimal medium and individually supplemented plates of adenine and pantothenate. The nuclear

FIGURE 1.—Growth curves of *pan-1 al-1 stp* homokaryons recovered from heterokaryon IV-6-6, together with a normal control on pantothenate supplemented minimal medium at 30°C.

ratio was approximately 3:2 in favor of the *pan al-1* component and a definite segregation for colony size was evident. Both *pan al-1* and *ad al-2* homokaryons were recovered from this heterokaryon or a subculture of it, and their behavior was observed in growth tubes. While some exhibited normal growth rates, others were erratic. Figure 1 shows the type of growth behavior typical of the abnormal *pan al-1* homokaryons derived from heterokaryon IV-6-6. Heterokaryotic isolates and *ad al-2* homokaryons exhibited similar growth patterns. While some isolates started at growth rates comparable to that of the controls (approximately 3.3 mm/hr for *ad al-2* and 4.3 mm/hr for *pan al-1*), they eventually stopped. Growth resumed in the case of some isolates. Others started growth slowly then stopped, and again certain of them resumed growth. Subcultures from isolates that were growing at a normal rate at the distal end of a growth tube usually stopped in the course of growth down a second 500 mm tube. Later experiments demonstrated that cultures exhibiting this abnormal growth behavior were capable of growing over 800 mm at a normal rate before stopping; however, not every isolate was subjected to prolonged testing at this time.

Cultures exhibiting the unusual stop-start phenotype were designated "stoppers" (*stp*). The nuclear constitution appeared to have no bearing on the manifestation of the stop-start phenotype since it was observed in homokaryons of *ad al-2*, *pan al-1* and *nic al-2*, as well as heterokaryons at one time or another. A more useful criterion for identifying such *stp* cultures was later found to be that conidia from such strains usually segregated into size classes during subsequent growth on sucrose-sorbose medium. Germlings and/or microcolonies, which seldom grew when transferred to slants, were found along with a smaller proportion of colonies ranging in size up to normal. Thus, even normally growing strains could be identified as potential stoppers, by segregation of conidia, in the absence of cessation of growth in a 500 mm-long growth tube.

While conidia from many stopper cultures gave rise exclusively to germlings and/or microcolonies, it was possible to isolate normal colonies which presumably lacked the determinant of the *stp* phenotype, from other *stp* cultures. These were normal in the sense that they no longer segregated for colony sizes upon plating their conidia, their growth behavior remained normal and they functioned as a protoperithecial parent in a cross, which *stp* cultures did not. Thus there was considerable variation in the expression of the phenotype referred to as stopper. Another characteristic frequently associated with *stp* mutants was the accumulation of a brown exudate in the aerial mycelia with aging.

Attempts to prevent the expression of the *stp* phenotype by growing the mutant at 25°C or 35°C or by supplementing the medium with yeast extract or casein hydrolysate were unsuccessful.

Heterokaryotic transmission of stp: Heterokaryon IV-6-6 was composed of a normal *pan al-1* strain and an irradiated *ad al-2* strain, yet some *pan al-1* homokaryons derived from this heterokaryon exhibited the *stp* phenotype. It seemed reasonable to assume that the *stp* phenotype was controlled by some extranuclear factor(s) and this was adopted as a working hypothesis. To test the hypothesis, a *pan al-1 stp* strain was made into a heterokaryon with a normal *nic al-2* culture. From that heterokaryon it was possible to obtain conidial-derived *nic al-2* homokaryons that manifested typical *stp* growth curves while others were normal. The conidia of such abnormal isolates formed both germlings and microcolonies when plated, and those changes in the *nic al-2* phenotype were persistent through subsequent subculturing.

Initial attempts to recover *ad al-2* homokaryons from heterokaryon IV-6-6 were unsuccessful. At the time, it appeared that either a lethal nuclear mutation had been induced by the radiation or that the adenine nucleus could not tolerate the extranuclear damage responsible for the *stp* phenotype. As a test of the latter possibility, a *pan al-1 stp* strain was made into a heterokaryon with a normal *ad al-2* strain. Both *stp* and normal *ad al-2* homokaryons were isolated from this heterokaryon. It was later possible to isolate *ad al-2 stp* homokaryons from heterokaryon IV-6-6. The initial failure was attributed to the rarity of *ad al-2* homokaryons, a characteristic often observed even in normal heterokaryons involving this adenine strain.

In every nuclear combination tried that involved a heterokaryon between a *stp* and a normal strain (*ad + pan*, *ad + nic* and *pan + nic*), it was possible to recover homokaryons of both nuclear types and also heterokaryons that were either *stp* or normal. However, it was not always possible to obtain normal and *stp* homokaryons of both nuclear types from any given heterokaryon; in some instances it was necessary to prepare a heterokaryon with an excess of either normal or *stp* cytoplasm, depending on the particular nuclear-cytoplasmic association desired. By so doing it was possible routinely to recover new genotypes with a *stp* phenotype or to "cure" a particular strain of the mutant phenotype.

Heteroplasmon studies: In the course of these studies the *stp* phenotype was first transferred via an *ad al-2* germling to a *pan al-1* strain. The *stp* phenotype was subsequently transferred heterokaryotically from the *pan al-1* strain to the

FIGURE 2.—Growth curves of heteroplasmons composed of three different auxotrophic strains in pairwise combinations on minimal medium at 30°C, together with the respective heterokaryotic and homokaryotic control homoplasmons. Heterokaryons were grown on minimal and the homokaryons on the necessary supplemented media. The combinations are: (a) *ad-4 al-2* and *pan-1 al-1*; (b) *pan-1 al-1* and *nic-2 al-2*; (c) *ad-4 al-2* and *nic-2 al-2*.

Code:

Homoplasmons. A—homokaryon of the first component, normal cytoplasm. B—homokaryon of the second component, normal cytoplasm. C—heterokaryon of A + B. D—homokaryon of first component, *stp* cytoplasm. E—homokaryon of the second component, *stp* cytoplasm. F—heterokaryon of D + E.

Heteroplasmons. G—heteroplasmon of A + E. H—heteroplasmon of B + D.

nic al-2 strain, and also back to the *ad al-2* strain. At each occasion, growth behavior of all possible nuclear and cytoplasmic combinations was observed along with their respective controls. A summary of the data is given in Figures 2a, b and c. It is worth noting that the pantothenic acid requiring mutant acted as an intermediary for the incorporation of *stp* cytoplasm into the adenine- and nicotinic acid-requiring strains; therefore the latter two strains had not previously been in heterokaryotic combination.

In the first two heterokaryotic combinations studied (*ad + pan* and *pan + nic*), the data were consistent in showing that each *stp + stp* combination showed the expected mutant phenotype and each *stp⁺ + stp⁺* combination was normal in growth. Similarly each *stp + stp⁺* grew normally, but asexual segregation in the conidia was not studied in these cases. Initially the latter observation suggested some degree of dominance of *stp⁺*, or possibly the loss of the *stp* factor (or factors). In the last combination studied (*ad + nic*), however, an exception was noted to the otherwise consistent findings: the *nic al-2 stp + ad al-2 stp⁺* combination grew normally for the first five days, gradually slowed, and then abruptly stopped (see Figure 2c G).

The observation that heteroplasmic cultures containing *stp* and normal cytoplasm might exhibit either a normal or mutant phenotype suggested that the expression of either alternative depended on the relative proportion of each. It was possible to check this by preparing heterokaryons between a *stp* culture and a normal strain, with different nuclear ratios. The assumption was made that the nuclear ratios are at least to some extent indicative of the relative amounts of cytoplasm contributed by each of the two components of a heterokaryon, since there is good evidence for this in combinations of *mi-4 + normal* (PITTENGER, unpublished).

Two heterokaryons were prepared between *pan al-1 stp* and *ad al-2 stp⁺*, using the technique of PITTENGER, KIMBALL and ATWOOD (1955). In the first heterokaryon (A) conidia were combined in a pellet in a 10:1 ratio in favor of the *ad al-2 stp⁺* component. The second heterokaryon (B) was prepared with a

TABLE 1

The average growth rate and the proportion of nuclear types at successive intervals for heterokaryon (A) prepared by combining conidia of ad al-2 and pan al-1 stp in a ratio of 10:1

Successive intervals of growth (mm)	Average growth rate (mm/hr) during interval	Nuclear ratio	
		<i>ad</i>	<i>pan</i>
450	5.0	.48	.52
485	4.8	.54	.46
395	4.9	.76	.24
630	4.9	.65	.35
575	4.7	.81	.19
235	4.8	.62	.38
Total 2770			

Growth was on Vogel's minimal medium at 30°C.

conidial ratio of 20:1 in favor of the *pan al-1 stp* homokaryon. Each heterokaryon was placed in a growth tube having ground glass interchanges so that the heterokaryons could grow for an extended period of time (McDOUGALL and PITTENGER 1962). The results of this experiment are presented in Tables 1 and 2.

Heterokaryon A behaved as predicted. Although this conidial mixture was made up at an input ratio of 10:1 in favor of the normal *ad al-2 stp*⁺ component, the nuclear ratio established was initially about 1:1. The growth rate of the heterokaryon was normal throughout the experiment. Samples of conidia taken during the course of three weeks growth formed only normal sized colonies when plated. However, there was evidence that suggested the presence of the *stp* factor in this culture. In a routine check to determine whether the *pan al-1* strain which went into the heterokaryon with *stp* cytoplasm was now normal, 13 *pan al-1* homokaryons were isolated from the culture after it had been growing one week. Nine of those homokaryons grew at constant rates (four of which approximated that of the normal *pan al-1* control), three slowed down during growth but did not stop, and one stopped after only 13 mm of growth. Thus, even though the parental culture grew at a normal rate for the 21 days it was observed, and formed normal sized colonies exclusively, the growth of some of the *pan al-1* homokaryons suggested that the *stp* factor was still present.

Heterokaryon B, constituted from the same suspensions used to make up heterokaryon A, behaved quite differently in the 53 days it was observed. It grew 467 mm the first four days; however, growth then gradually slowed from 5.1 to 1.7 mm/hr and finally ceased for five days. The heterokaryon then resumed growth, only to go through the same cycle of stopping for five days and starting two more times. Despite the initial normal growth rate, over 90% of the conidia plated from a sampling port only 55 mm from the origin of the inoculation formed germlings. No normal sized colonies were observed when conidia were plated from seven different sampling ports of the culture, although at one such plating the germinated conidia exhibited more growth than did the usual germlings.

It was interesting to observe that even after stopping three different times for a total of 15 days, the culture was still able to grow at a normal rate (5 mm/hr) for seven days before it slowed down and stopped for the fourth time; then after three days, growth resumed at a reduced rate. At this point the experiment was terminated. Although it was impossible to determine the exact proportion of normal to *stp* cytoplasm in heterokaryons A and B, such results suggested that the growth behavior of those cultures was related to the proportion of abnormal cytoplasm.

Inheritance studies: Transmission of the *stp* phenotype through heterokaryosis is evidence for the extranuclear basis of the factor or factors involved (JINKS 1964). Attempts to demonstrate the maternal inheritance of *stp* were not completely successful because *stp* cultures were usually sterile when used as protoperithecial parents, though they could be crossed as conidial parents. Seven complete asci from different perithecia were analyzed from a cross of *al-2a* × *pan al-1A stp*. Segregation for all nuclear markers was normal, but no evidence of

TABLE 2

The average daily growth rate and the proportion of nuclear types at various intervals for heterokaryon (B) prepared by combining conidia of ad al-2 and pan al-1 stp in a ratio of 1:20

mm growth in 24-hr intervals	Average growth rate (mm/hr)	Nuclear ratio	
		<i>ad</i>	<i>pan</i>
111	4.6	..	..
123	5.1	.22	.78
122	5.1	..	..
111	4.6	..	..
92	3.8	..	..
74	3.1	..	..
40	1.7	..	..
Stopped for 5 days	..	..	..
17	0.7	.26	.74
108	4.5	..	..
105	4.4	..	..
81	3.4	..	..
51	2.1	..	..
7	0.3	..	..
Stopped for 5 days	..	..	..
7	0.3	..	..
75	3.1	.36	.64
99	4.1	..	..
106	4.4	..	..
89	3.7	..	..
63	2.6	..	..
31	1.3	..	..
Stopped for 5 days	..	..	..
77	3.2	.59	.41
115	4.8	..	..
125	5.2	..	..
122	5.1	.43	.57
115	4.8	..	..
117	4.9	..	..
120	5.0	..	..
114	4.8	.34	.66
46	1.9	..	..
71	3.0	..	..
77	3.2	..	..
60	2.5	..	..
13	0.5	..	..
Stopped for 3 days	..	..	..
42	1.8	..	..
65	2.7	.39	.61
Total 2791			

Conditions as in Table 1.

the *stp* phenotype appeared in any of the progeny. Failure to recover mutant progeny when *stp* was used as a male parent indicated that it was not determined by a nuclear factor. Repeated attempts were then made to cross a *stp* mutant as a

female parent. In only one case did a cross of *pan al-1A stp* $\times$ *al-2a* produce a few perithecia and a total of 57 viable random spores were obtained. None, however, showed the *stp* phenotype in growth tubes nor did any segregate for germlings and/or microcolonies when their conidia were plated.

Four of the pantothenic acid-requiring random spores recovered from the above cross were backcrossed as protoperithecial parents to a *pan al-1 stp* strain. At least one ascus was taken from each cross and growth rates of the single-spore isolates determined. The segregation for mating type was normal and again no *stp* mutant was recovered from any of the asci. Thus there is no evidence that the *stp* phenotype has ever passed through a cross.

Spectroscopic and manometric studies: *Poky*, *mi-3*, *mi-4*, *abn-1* and *abn-2* are all extranuclear *Neurospora* mutants which are known to manifest characteristic cytochrome absorption bands that differ from wild type (MITCHELL, TISSIERES 1953; HASKINS, TISSIERES, MITCHELL and MITCHELL 1953; PITTENGER 1956; GARNJOBST, WILSON and TATUM 1965). SRB (1963) has also reported alterations of cytochrome bands in cytoplasmic mutants of *Neurospora*. An examination of the cytochromes of the strains used in this study by the use of a grating spectroscope revealed the following: the *a* and *b* cytochrome bands were not discernible in any of the strains studied. This included the *stp* mutant, wild type 74*a* and all auxotrophic mutants previously referred to, as well as *poky* mutants 3627-1 and 3627-2 obtained from the Fungal Genetics Stock Center. These results agree with earlier findings when observations were made at room temperature (HASKINS, TISSIERES, MITCHELL and MITCHELL 1953).

Observations concerning cytochrome *c*, which bands at 550 m μ , were variable. The *stp* mutant at times gave a very intense band at 550 m μ , which was identical with that of *poky* (3627-2). When *stp* cytoplasm was passed via heterokaryosis from one homokaryon to another, the recipient homokaryon also manifested an intense cytochrome *c* band in some cases. An intense cytochrome band at 550 m μ was observed only in cultures which showed the *stp* phenotype or in cultures derived from a heterokaryon in which the *stp* factor(s) was known to be present. Control cultures never manifested an intense cytochrome *c* band. Since *poky* itself did not always show its characteristic absorption spectrum, the variation obtained in some experiments might be attributed in part to the insensitivity of the technique and/or differences in the quantity of the *stp* factor(s) present in a particular culture.

A comparison of the oxygen uptake of both intact and homogenized mycelia of control versus *stp* cultures by Warburg manometry gave inconclusive results. Characteristically *stp* mutants grow erratically, as evidenced from preceding data. Oxygen uptake values of *stp* mutants likewise showed variation, and overlapped control values in some instances, just as the growth rate of a *stp* mutant was normal at times. Slow growing *stp* cultures invariably gave low oxygen uptake values, making it difficult to distinguish between cause and effect. On the other hand, if a *stp* culture was growing at a rate comparable to the control when harvested (as evidenced by the amount of growth in the flask), oxygen uptake values were also comparable.

DISCUSSION

The characteristics of the *stp* mutant—somatic segregation in asexual spores, heterokaryotic transmission, vegetative persistence, and failure to be transmitted through a sexual cross—are consistent with the interpretation that the genetic basis of the mutant is determined by an extranuclear factor. Somatic segregation of *stp* was evident in both homokaryotic and heterokaryotic cultures, as manifested in the growth behavior of the conidia. At one extreme there were cultures whose conidia in most instances formed only lethal germlings or microcolonies, while other cultures formed both germlings and normal appearing colonies. Cultures derived from normal appearing colonies were heterogeneous; some segregated on plating for both germlings and normals, a few gave primarily germlings, and still others gave only normal colonies. An isolate was classified as normal when it maintained a normal growth rate in a 500 mm-long growth tube, formed only normal colonies when its conidia were plated, and was female fertile. It is difficult to prove, however, that such cultures are completely free of the *stp* factor. If the average number of such factors in a conidium is large, it may be virtually impossible to obtain a true homoplasmon.

The ability to transfer a phenotypic difference associated with one strain to a second strain via heterokaryosis is one criterion considered as evidence for extra-chromosomal inheritance. Heterokaryotic transfer of the *stp* phenotype was effected from an *ad al-2 stp* strain to a *pan al-1* strain and from *pan al-1 stp* isolates to both an *ad al-2* and a *nic al-2* strain. The resulting *nic al-2 stp* and *ad al-2 stp* isolates had all the *stp* characteristics associated with the original *ad al-2 stp* strain although these two strains had not been directly associated in heterokaryosis. It is difficult to explain such behavior on the basis of any genetic factor other than an extranuclear one.

A difference in reciprocal crosses is expected of cytoplasmically inherited traits, but it was not possible to obtain conclusive evidence on this point owing to the female sterility of strains carrying the *stp* factor. Failure to recover *stp* progeny from a cross between a *stp* male parent and a normal protoperithecial parent is nevertheless consistent with the proposed extranuclear basis of the *stp* phenotype. In a single instance a few perithecia were found when a *stp* culture functioned as the female parent in a cross, but no *stp* mutants were found among the progeny. Taking this result at face value, it could be concluded that *stp* was similar to the "minute" cytoplasmic variant of *Aspergillus* (FAULKNER and ARLETT 1964) or the AC-7 mutant described by MUNETA and SRB (1959), neither of which are normally transmitted through a sexual cross, although AC-7 can be transmitted paternally under certain circumstances. The preferred interpretation with *stp*, however, is that the single instance of fertility can be attributed to a mosaic cytoplasm. In other words, protoperithecia which functioned in this cross contained enough normal cytoplasm to function normally whereas the cytoplasm containing a greater proportion of the *stp* factor was infertile, as were most *stp* cultures. JINKS (1956) has suggested on the basis of his studies of cytoplasmic changes and sexual differentiation in *Aspergillus* that a complete and

fully integrated cytoplasm is essential for a successful differentiation of the sexual apparatus.

The fact that the *stp* phenotype is vegetatively persistent (some isolates have been maintained in culture for three years) suggests that the factor(s) responsible has genetic continuity and represents an altered form of some replicating normal cytoplasmic component. Likewise the diverse stopper phenotypes observed with the same nuclear genotype and the ability to recover *stp* and *stp*⁺ homokaryons from conidia of some *stp* cultures indicate that such strains are indeed heteroplasmons containing normal and altered forms of some cytoplasmic component. The excess of cytochrome *c* often observed in *stp* cultures suggests that mitochondria are involved.

It is of interest that this same stop-start phenotype has been noted in some *poky* cultures, and in *mi-4* (PITTENGER 1956), and in *abn-1* and *abn-2* (GARNJOBST, WILSON, and TATUM 1965). Likewise the acriflavin treated culture from which stable slow-growth (SG) cultures were isolated also showed the stop-start phenotype (SRB 1963). With the exception of SG, these extranuclear mutants have cytochrome abnormalities suggesting the possibility of mitochondrial abnormalities. DIACUMAKOS, GARNJOBST, and TATUM (1965) have reported that the injection of mitochondrial fractions isolated from *abn-1*, which has an aberrant cytochrome system, produces drastic changes in the phenotype of normal recipients. However, both nuclei and cytoplasm were required in order to restore a normal phenotype to *abn-2* by microinjection, and there was evidence that the abnormal character persisted (GARNJOBST, WILSON, and TATUM 1965).

Although the physiological basis of the cessation of growth in the cytoplasmic mutants of *Neurospora* is unknown, it seems likely that it is often related in some way to a high proportion of an abnormal cytoplasmic factor. Depending on the method of preparation of a particular heteroplasmon, it was possible to show either normal or abnormal growth behavior (see Tables 1 and 2). Most of the heteroplasmons studied in fungi are unstable, and usually it is the gradual loss of the normal component that is responsible for the breakdown (JINKS 1964). JINKS (1964) favors the interpretation that the instability of such heteroplasmons is attributable to a superior rate of replication of the abnormal component. Thus cessation of growth, regardless of the eventual biochemical explanation, may be merely the end result of the suppressiveness of the abnormal factors which cannot function as homoplasmons in a heterokaryotic culture. It is also conceivable that an accumulation of some selfintoxicating substance, when the proportion of the *stp* factor reaches a certain level, could bring about the cessation of growth. Subsequent dilution and/or elimination of this substance would permit renewal of growth. The factors responsible for stopping are not lost, however, since stopping and starting may be repeated a number of times at fairly regular intervals (see Table 2). It is of interest to note that cessation of growth may have a different basis in different mutants if morphological observations have any significance. In many cases with *stp*, growth slows down appreciably and the mycelium becomes very sparse before growth ceases. When growth resumes, usually after considerable delay, it can often be seen to start some distance behind

the leading frontier of mycelial growth. In other mutants (BERTRAND and PITTINGER 1964) growth is often quite luxuriant right up to the time growth ceases; when it resumes after a short time it appears to do so at the frontier and not proximal to it.

Although a superiority in the replication rate of the abnormal extranuclear factor may be the explanation for the suppressiveness or dominance of a number of cytoplasmic mutants, this may be an over simplification of the behavior of *stp*. If the rate of division of the cytoplasmic particle is involved, it must be dependent to some extent on the relative proportion of the abnormal factors in the heteroplasmon, as is suggested by the results presented in Table 2. There was no evidence for a superior rate of replication of *stp* in the case of heterokaryon A, since it grew normally for the three weeks it was observed without any indication of an increase in the proportion of the *stp* factor. Thus the rate of multiplication of *stp* and *stp*⁺ factors may depend on their relative proportions in the culture. There is precedence for such behavior with nuclear genes in heterokaryons (PITTINGER and BRAWNER 1961). With other strains having a stop-start phenotype associated with an extranuclear factor, BERTRAND (unpublished) has found that there is not necessarily any correlation between the cessation of growth of a culture and the proportion of conidia giving rise to stopper cultures that are recovered just before the culture stops or immediately after it resumes growth. Thus, in some cases, cessation of growth appears to involve more than a mere increase in the proportion of the mutant factor.

Certain observations are consistent with the notion that the severity of the effect of *stp*, or its rate of replication, may be quite different depending upon the physiological condition of the organism. For example, conidia plated from a section of a continuous growth tube in which growth was proceeding at a normal rate, and continued to do so for several days, may result in the exclusive production of lethal germlings. This might be interpreted to mean that the same proportion of *stp* factors produce very different effects depending on whether they are present in a germinating conidium or in an actively growing mycelium where physiological processes would be expected to be more nearly in equilibrium. Though it seems less likely, it may be that this difference is related in some way to the restriction in the proportion of *stp* and *stp*⁺ factors that can be present in a single conidium, thus making it impossible to obtain the same ratio of the two factors that exist in the much larger domain of the mycelium.

SUMMARY

Following ultraviolet irradiation of an *ad-4 al-2* strain and subsequent heterokaryotic rejuvenation of a resulting slow growing germling with a *pan-1 al-1* strain, homokaryons of both nuclear types were isolated which exhibited an erratic stop-start phenotype when cultured in growth tubes. This mutant phenotype was vegetatively persistent through subsequent subculturings and was designated stopper (*stp*). Since it was possible to transmit the phenotype by heterokaryosis to other strains, the mode of inheritance of *stp* is believed to be cyto-

plasmic. On occasions *stp* isolates showed an accumulation of cytochrome c and characteristically gave rise to a variety of colony sizes; when their conidia were plated, germlings ranged from normal to lethal. Somatic segregation made it possible to recover diverse expressions of the *stp* phenotype having the same nuclear genotype. The *stp* mutant has never passed through a sexual cross when used as either a male or female parent, although the latter was almost always infertile. Stopper isolates may be interpreted to possess a mosaic cytoplasm consisting of mutated *stp* factors and their normal counterparts. The growth behavior of cytoplasmic mixtures between *stp* and a normal strain showed differences which appear to depend on the relative proportion of each in the heteroplasmon.

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