STUDIES ON A CYTOPLASMIC CHARACTER IN *NEUROSPORA CRASSA*

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ABSTRACT

Two morphologically distinctive slow growing strains of *Neurospora crassa* have been isolated and studied. These, abn-1 and abn-2, differ from wild type in that their growth rates are greatly reduced and often irregular, aerial hyphae are absent, conidia are extremely rare, and no protoperithecia are formed. Growth was not improved by addition of any nutrients tested, oxygen consumption was similar to that of wild type, and cytochrome *c* appeared abnormally high, and *b* low or absent. Both abn strains gave rise only to normal progeny in crosses with normal strains. The abn characteristics appear in heterocaryons, and have been transmitted to other genetic strains by means of heterocaryosis followed by plating of conidia. Conidia formed by such heterocaryons typically showed low viability, and gave rise to cultures with great variability in growth rate, morphology, and survival. Even apparently normal derived cultures often later became abnormal or died. It is concluded that the abnormal characteristics are determined primarily by cytoplasmic factors. This conclusion was strengthened by the transmission of the typical characteristics to normal strains by microinjection of cytoplasm from abn cultures, even without demonstrable transfer of nuclei. This constitutes the first time microinjection techniques have been successfully applied to the analysis of a cytoplasmic character in *Neurospora*.

INTRODUCTION

In the course of routine culturing of an inositol-requiring strain of *Neurospora crassa*, it was observed that some transfers appeared morphologically different and grew relatively slowly and poorly. This changed and characteristic growth behavior could not be modified by rapid serial transfer. Interest in this phenomenon was stimulated by the occurrence of an ostensibly similar change in a wild-type culture and, because of the interest of our laboratory in the biochemistry and genetics of morphology of *Neurospora*, by the changed morphological characteristics of these altered cultures. This report describes investigations into these aberrant or "abnormal" strains, which have established that they are not under primary control of nuclear genes, and that the abnormal characteristics can be transmitted to normal strains by heterocaryosis and by transfer of cytoplasm by microinjection.

MATERIAL AND METHODS

Origin of the Abnormal Cultures

The two cultures which suddenly became slow growing and abnormal in appearance were (*a*) a reisolate of the inositol mutant 37401 and (*b*) a transfer of the wild type SY7A. The new traits did not disappear during frequent (serial) transfer of conidia or mycelium to fresh medium; instead, some series of three or four transfers ended in death. Thus the factor(s) responsible appeared to become phenotypically dominant. Growth of the inositolless

isolate in different concentrations of inositol in minimal medium showed that this growth requirement had not been lost. All ascospore germinants examined from crosses of each of the abnormal cultures with the wild type as the protoperithecial parent (10 asci and 100 random ascospores each) appeared normal. As a further test, conidial isolates were obtained to detect the presence of any new mutation that might have arisen.

The conidia were plated on St. Lawrence sorbose medium, plus inositol for (*a*), and incubated at 25° or 30°C. They were distributed on the solidified agar surface in a thin layer of the same medium without sorbose, or they were suspended in distilled water and distributed on the surface of the sorbose medium and/or on a complete nutrient agar medium. From the first plating of conidia a larger number of normal appearing isolates were obtained than from the second, and very few were present in the third plating. A culture producing a moderate number of conidia was chosen from the first plating for the second, etc. Many conidia (±90 per cent) did not germinate, or germinated and then either stopped growing or grew very slowly to visibility. Isolates of each stage of growth from each plating were retained on *Neurospora* complete medium (GSC) of Vogel (1956) for further observation.

The conidial isolates of the original abnormal inositolless and wild-type cultures have been studied in detail and are referred to in this paper as abnormal-1 (abn-1) and abnormal-2 (abn-2). An additional number designates a particular conidial isolate; *e.g.*, abn-1-4.

Of the 113 abn-1 colonies and germinated conidia isolated, only a few became more or less normal, and only 7 of the remainder survived an extensive number of transfers. Two of the 7 died after more than a year of culture, but 5 have been in almost continuous culture to the present time (*i.e.*, more than 3 years and 150 transfers). All conidial isolates that continued to grow, and so could be tested, required inositol for growth and responded as mating type (sex) *a*. The amount of growth in liquid minimal medium containing inositol, and the rate of growth on solid medium, were considerably less than obtained with the normal *inos* culture.

The abn-2 conidial isolates behaved similarly except that no nutritional supplement was required for growth and all responded as sex *A*. Of the 24 isolates retained for observation, 3 continued to grow but only 1, abn-2-1*A*, has survived to date.

Occasionally a sister transfer of an abn-1 or abn-2 culture stopped growing, invariably after a fringe of hyphae had grown out for a short distance from the inoculum. Then either no further growth took place or growth was resumed, sometimes after a considerable period of time.

Stock Cultures

Difco Potato Dextrose Agar (PDA) was selected as the best medium for maintenance of stock cultures. Transfers were made every 5 to 7 days by cutting out a shallow plaque from the middle surface of a test tube slant with a stout needle slightly curved at the tip. The cultures were grown at either 25° or 30°C. They have retained viability when slants were stored at 5°C for 3 months.

The mycelia of abn-2-1*A* have grown after lyophilization, but not those of abn-1-4*a*. To lyophilize successfully it was found necessary to grow mycelia in a liquid medium. Even so, not all tubes proved to contain viable mycelium.

DESCRIPTION OF THE ABNORMAL CULTURES

The branching pattern of the abnormal cultures, as seen on a nutrient agar surface, is in general like that of the wild type, but the hyphae are finer (Fig. 1 *a*) and almost even in size, including most primaries. The mycelium lies flat on the agar surface; aerial hyphae are absent. Short twisted tufts of hyphae occasionally appeared at the top of a slant or only on the inoculum block, especially in the earlier cultures. They appeared to be formed by hyphae emerging from growth under the agar. At times long, attenuated hyphae without septa or branches were observed, a variation not found in the wild type grown under the same conditions.

Other distinctive characteristics noted are the absence of visible flow of protoplasm, except in rare instances, and the occasional presence of plugs in many of the septal pores without evidence of injury (Garnjobst and Wilson, 1956). In addition to the hexagonal crystals of ergosterol (Tsuda and Tatum, 1961), very long crystals have been observed (Fig. 1 *c* and *d*). The latter definitely lie within the hypha, as can be shown by puncturing the cell wall with a microneedle. The crystals are then seen to flow out with the protoplasm.

The cell membrane of abn-1-4*a* and of abn-2-1*A* seems to have the permeability properties of that of the wild type in that growth is only slightly delayed in medium containing 2.5 per cent NaCl.

A few tiny conidia are sometimes formed at the top of the slant in abn-2 in unusually moist air, but they have appeared very rarely in abn-1. Protoperithecia have not been found (3 to 14 days) in either abn-1 or abn-2 on the synthetic crossing medium of Westergaard and Mitchell (1947) or

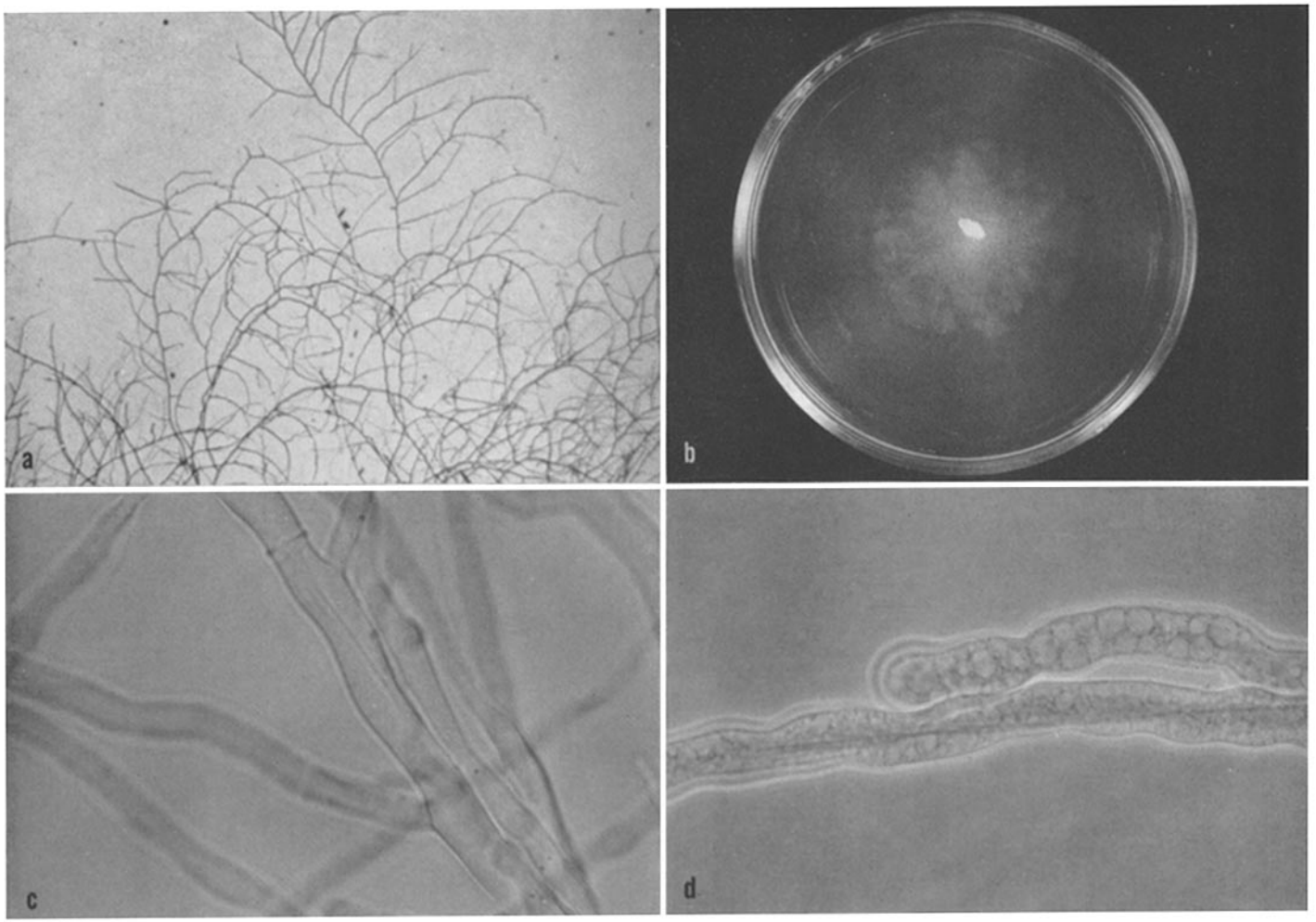

FIGURE 1 Photomicrographs of living material. *a*, mycelium of abn-2 grown in microculture at 30°C; × 12. *b*, 5-day-old plate culture of abn-1 on PDA at 30°C; × 0.6. *c*, hyphae of abn-1 showing crystals within one compartment (cell); × 1200. *d*, hyphae of abn-1 showing very long crystal; phase contrast; × 1200. (Fig. 1 *c* and *d* photographed by Dr. S. Tsuda.)

on Difco Corn Meal Agar. Tests have been made at intervals throughout this study. These were followed by tests for perithecium formation by adding conidia of the opposite mating type after 4, 5, 6, and 7 days of growth on the synthetic crossing medium, each in a separate Petri plate. Although perithecia were formed eventually, this was only after an additional 5 or more days, so the protoperithecia probably were produced by the normal strain. No protoperithecia or perithecia were produced in attempted crosses between isolates of abn-1*a* and abn-2*A*.

Fig. 2 shows the growth response of abn-1-4*a* and of abn-2-1*A* on PDA in horizontal growth tubes (about 400 mm in length; 20 ml of medium). These isolates are representative of the other abn-1 and abn-2 isolates studied. It will be noted that two types of growth advance have been observed: a regular growth of about 18 to 25 mm per 24 hours (abn-2) or 8 to 13 mm per 24 hours (abn-1), and a "stop and go" type in which the periods of growth and no growth varied in length. Stock cultures on slants also occasionally stopped growing at unpredictable times, but, as pointed out earlier, only very occasionally has growth stopped permanently.

EXPERIMENTAL

Effects of Environment

A variety of environmental conditions have been tested for effects on the growth character of the abn isolates. These included (*a*) a wide range of temperatures, (*b*) continuous flow of air, or air and oxygen 1:1, in growth tubes, (*c*) media at several different hydrogen ion concentrations, (*d*) fractions of, and an omission series of, the complete medium, and (*e*) plate spot tests of 19 different substances. These substances included nutritional factors (hemin, asparagine, glutamine), antagonists (fluorophenylalanine, 5-bromouracil, 2,6-di-aminopurine, azaserine, amethopterin), and antibiotics (actinomycin, puromycin, acriflavin). Wild-type *Neurospora* extract, both dried and undried, added to minimal sucrose agar, and a number of commercially prepared nutrient media (tryptone digest, heart infusion, tomato juice, maize meal, all with agar) also were tested. In addition, Pablum (Mead, Johnson and Company), which contains four different cereals, yeast extract, and added vitamins, was tried. In no case was the growth character of the abn isolates changed significantly.

In summary, abn-1 and abn-2 isolates grew more or less well on a variety of media provided the pH of the medium tested was in the range suitable for growth of the wild type and contained sufficient inositol (for abn-1). The slow growth character, however, remained unchanged.

Respiratory Investigations

Hyphal suspensions and homogenates were examined preliminarily with a hand spectroscope (Mitchell *et al.*, 1953). Both abn cultures appeared to have absorption bands differing from those of wild type and in general similar to those of *mi-1* (*poky*) (Mitchell and Mitchell, 1952), with very high cytochrome *c*. Preliminary examination of mitochondria from abn-1 in the Cary spectrophotometer showed an abnormally high level of cytochrome *c*, little or no *b*, and perhaps an altered *a*.

In order to determine whether the slow growth characteristics of the cultures involve net respiratory deficiencies, Q_{O_2} measurements were made by Warburg manometry. In 17 tests on abn-1-4*a* mycelia grown and examined under different conditions using liquid and solid media, the average Q_{O_2} was 35 μl per mg dry weight per hour, with a range of 26 to 60. These values compare well with those of Giese and Tatum (1946) for wild type, an average of 32 (range 15 to 56). In tests using mycelia of abn-2*A* grown and examined in liquid media, an average Q_{O_2} of 85 was found. It would thus appear that the O_2 consumption of neither abn culture is significantly lower than that of wild type. This finding is similar to that reported for *mi-3* (Tissieres and Mitchell, 1954).

Genetic Analysis

When the *original* abnormal cultures were crossed with Perkins wild type (P*A*, P*a*) as the protoperithecial parent, all progeny examined appeared normal, as stated earlier. The same result was obtained with the conidial isolates, that is, the abn-1 and abn-2 cultures. A total of 206 asci were dissected in order from 13 abn-1 and 3 abn-2 cultures crossed, not more than 2 asci per perithecium being taken. Two hundred or more ascospores also were grown from two crosses, namely, abn-1-4*a* or abn-2-1*A* × wild type (RL 3-8*A*, RL 21*a*) (Rockefeller, Lindegren wild types). As expected, the progeny from the cross of abn-2 grew in minimal medium, whereas from the abn-1 cross a 1:1

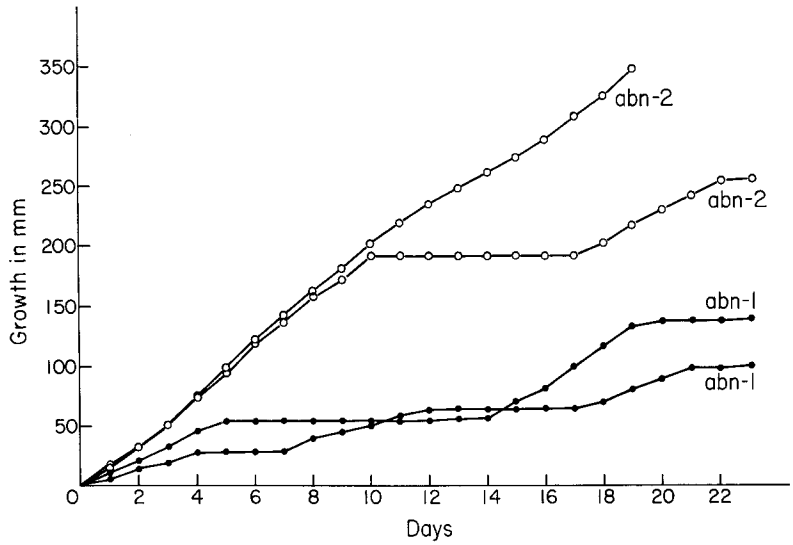

FIGURE 2 Growth of abn-1 and abn-2 on PDA in growth tubes (in duplicate) at 30°C.

segregation of *inos*$^+$ to *inos*$^-$ cultures was obtained. It is possible that nuclei responsible for the abnormal growth were present also but did not participate in the cross. However, the possibility that a mutation had taken place in some cytoplasmic component (Sherman and Slonimski, 1964) was also considered.

The criteria used for the determination of normality of the cultures described are (*a*) prompt growth (2 to 4 days) from ascospores, (*b*) wild-type phenotype, (*c*) prompt germination of conidia upon transfer, (*d*) the wild-type rate of growth of representative ascospore cultures from each cross as observed in growth tube tests, and (*e*) formation of protoperithecia on synthetic crossing medium.

Two different spontaneous morphological variants have appeared among the progeny from crosses of abn-1-4*a* × P*A*, and one from abn-1-4*a* × *nic-2* (S1441)*A*. In each instance the new phenotype segregated as a single genetic character. None has become prominent in abn-1-4*a*, and perhaps all were lost in transfer or were present in the other parent. No variant appeared among the ascospore cultures (10 asci, 100 random ascospores) from the latest cross of abn-1-4*a* × RL 3-8*A*.

No morphological mutants have appeared among the ascospore cultures of abn-2-1*A* isolates × P*a* or RL 21*a* (protoperithecial parent). However, from the latest crosses of abn-2-1*A* with these wild types, a 1:1 segregation (20 asci, 100 random ascospores) for slow germination and growth from ascospores was obtained. Upon transfer, the slower germinating cultures grew at the normal rate of the wild type. This gene mutation appears to have originated spontaneously in the abn-2-1*A* culture, and its effect is clearly different from that of the abn-2 character and from that of *mi-1* and *mi-3*.

The abn-1 and abn-2 isolates resemble the slow growing strain (*mi-4*) described by Pittenger (1956) in being unable to function as a protoperithecial parent in crosses. For this reason the only possible test for maternal inheritance (Mitchell *et al.*, 1953) of the abn character was to use a heterocaryon (abn + normal) in crosses. Since heterocaryons with abn-2-1*A* as one member of a pair were more successful than those with abn-1-4*a*, only the former isolate was tested in this manner. The results obtained were complex.

Five crosses were made as shown in Table I. When the heterocaryon formed the protoperithecia (crosses 1, 2, and 3), the perithecia were in part normal and in part abnormal, including the perithecial structures and contents. The early stages of perithecium development took place soon after the addition of conidia of the normal strain. Therefore it seems certain that nearly all the protoperithecia were fertilized by the normal conidia. The few slow growing ascospore cultures obtained probably resulted from the presence of the gene mentioned above for slow ascospore germination and early growth. If so, its presence in these ascospore cultures probably shows that abn-2

TABLE I

Results of Crosses with Heterocaryons

Cross	Total ascospores isolated	Normal germination and growth	Slow germination and growth	No growth
1. abn-2-1*A* + S4118 (*m, fl, tryp-2*)*A** × 37401 (*inos*)*a*	176 (17 perithecia sampled)‡	104 (61.8%)	23 (13.2%)	49 (25%)
2. Same as 1	100 random	59	9	32
3. abn-2-1*A* + S4118 ((*m, fl, tryp-2*)*A* × RL 21 (wild type)*a*	100 random	49	4	47
4. RL 21 (wild type)*a* × abn-2-1*A* + S4118 (*m, fl, tryp-2*)*A*	100 random	62	7	31
5. 5531 (*pan-1*)*a* × abn-2-1*A* + S4118 (*m, fl, tryp-2*)*A*	88 (11 perithecia sampled)‡	59 (67%)	2 (2.3%)	27 (30.7%)

* The first member(s) of each cross is the protoperithecial parent; the second, the conidial parent.
‡ Two asci or 10 ascospores per perithecium.

nuclei participated in each cross. The large number of ascospores which did not germinate was thought at first to be due to slow ripening, but ascospores isolated at random at a later time gave similar results. Very few ascospores were shot out, a fact accounted for, in part, by the presence of perithecia with some black spores but without periostia.

As can be seen in Table I, the reciprocal crosses (4 and 5), in which the normal strain formed the protoperithecia, gave results indistinguishable from those described for crosses 1, 2, and 3. That is, no clearcut evidence for the inheritance of the abn character was obtained. The participation in these crosses of a heterocaryon formed with the abn strain apparently led to poor development of the perithecium and its contents.

Transfer of Abnormal Character by Hyphal Fusion

The formation of heterocaryons by abn-1 or abn-2 and mutant strains of normal phenotype seemed to be a useful method of studying the transfer of the slow growth character and the roles of nucleus and cytoplasm in the process. Heterocaryons of similar type were made by Pittenger (1956), who then succeeded in obtaining slow growing cultures from macroconidia formed by the heterocaryon. Examination of these slow cultures showed that some had the nuclear characteristics of the normal strain used, a result that points to cytoplasmic determination of the mutant growth character. In the present experiments both natural and artificial heterocaryons (Wilson, 1963) were investigated.

ABNORMAL-1: Several different biochemical mutants (*a* sex) were selected, all known to form heterocaryons with the normal reisolate from which the abn-1 isolates were derived. When both cultures were inoculated at one end of a growth tube containing minimal sucrose agar (20 ml per tube), a heterocaryon appeared to be formed within 2 days, but growth soon ceased. Such heterocaryons formed on slants produced conidia in sufficient quantity for plating on sorbose medium. Colonies were isolated on slants of complete medium and transfers were made on the same medium, usually at intervals of 5 or 7 days, to observe the type of growth. All slants were incubated at 30°C.

No slow growing or abnormal appearing conidial isolates were obtained from abn-1-4*a* + *pan-1* (5531)*a*; all were normal. Conidial isolates from the heterocaryon abn-1-11*a* + *meth-3*

(36104)*a* gave the most convincing results; that is, slow growing cultures were present among the colonies isolated, as well as many that discontinued growth upon serial transfer. The results of two crosses (slow growing colony isolates × wild type RL 3-8*A*, protoperithecial parent) indeed indicated the presence of the *meth-3* gene in one-half the ascospore cultures (2 pairs per ascus). However, after several transfers of the conidial colony isolates to fresh medium, one grew moderately in liquid minimal medium and the other did not grow in minimal alone or in minimal containing methionine. In the meantime the culture from which the inoculations were made died upon transfer. The conidial colony isolates which later grew in minimal medium undoubtedly contained at least one nucleus with the *inos* mutation as well as the slow growth character, and nuclei with the *meth-3* gene.

Difficulty in separating the components of the heterocaryon was also encountered in other macroconidiating strains tested. In each case the appearance and behavior of some of the conidial isolates were markedly different from those of the controls, including death upon serial transfer. These results do not give complete assurance regarding absence or presence of one or two kinds of nuclei in a conidial isolate, for, as mentioned above, a small number of one kind is not readily detected.

Heterocaryons formed by abn-1-4*a* and microconidiating (*m*, *fl*) nutritionally deficient strains produced both microconidia and macroconidia. It was thought that the plating of these conidia, containing one or few nuclei, might give clear results. Three different strains were used: *tryp-2* (S4118), *nic-2* (S1441), and *lys* (S1383). For good growth of the *tryp* strain, the GSC medium was supplemented with 20 μg L-tryptophan per ml of medium.

Table II gives the results obtained with the three different heterocaryons as compared with controls. Heterocaryons 2 and 3, each composed of the same two strains, and heterocaryon 4 were analyzed in greater detail. The results show good agreement in the ratio of normal and abnormal colony isolates obtained, as well as in the number of deaths occurring before the end of the experiment. Complete accuracy in classifying the isolates probably was not attained. Abnormal appearing isolates sometimes became normal at the next transfer, and *vice versa*. Experience in noting the sequence of change in appearance and behavior was necessary. Also, the presence of the genes *m* and *fl*, though useful in determining the morphology of the normal isolates, might be expected to change the appearance of abnormal ones as compared with abn-1.

The indirect method of crossing the abnormal appearing isolates with the wild type to determine their nuclear composition was tried because many of these cultures were flat colonies or had few or no aerial hyphae. This not only made transfer from complete to minimal media difficult, but gave results that were not clearcut unless the mycelium was transferred several times in sequence. Two asci or 10 to 20 ascospores were isolated per perithecium, and 3 to 5 perithecia per colony isolate were sampled. In addition 100 ascospores or more were usually isolated at random from each cross. Although these tests were not exhaustive, it seemed clear that the abn character had been transferred from abn-1-4*a* to the microconidial strain in at least some cases, a result which suggested the injection experiments described in this paper.

ABNORMAL-2: Heterocaryons of abn-2 isolates with several normal macroconidiating mutant strains also were analyzed as described above. Since the heterocaryons produced macroconidia, the evidence for transfer of the abn character to another strain was again suggestive but inconclusive. For this reason the data are omitted here.

Heterocaryons of abn-2-1*A* and the normal *tryp-2 A* microconidiating strain formed readily and grew for a longer time in growth tubes containing minimal medium than did abn-1-4*a* + *tryp-2*, *m*, *fl*, *a*. The behavior of the normal and artificial heterocaryons of abn-2-1*A* and other strains is described and compared in a later section.

Tests for Transmission by Extracts

Several attempts were made to test for transmission of the abn characteristics by exposing *Neurospora* protoplasts of a normal strain to extracts of abn cultures. Protoplasts of *os* (osmotic), *inos* were prepared by incubation of conidia in minimal medium containing 10 per cent mannitol and 3 per cent β-amylase (Emerson and Emerson, 1958). Extracts of lyophilized abn-1 mycelia and of freshly grown abn-1 were prepared by grinding the material in buffer with sand, centrifuging at 35,000 *g* for ½ hour, and sterilizing by Millipore

TABLE II

Heterocaryons with Microconidiating Strains

Heterocaryon abn-1-4*a*, *inos* +	Conidia germinated	No. colonies isolated	No. transfers (slants)	No. normal isolates	No. abnormal isolates	No. abnormal isolates crossed	Character of isolates recovered from cross
	per cent						
1. *tryp-2, m, fl, a*	2.4	76	1–3	48 (63.1%)	28 (36.8%) (13 died)	1*	*m, fl, tryp*
2. *tryp-2, m, fl, a*	—	100	1–3	64	36 (13 died)	21‡	19 *m, fl, tryp*; 2 heterocaryons¶
3. *tryp-2, m, fl, a*	2.1	114	2–4	71 (62.3%)	43 (37.7%) (17 died)	13§	11 *m, fl, tryp*; 1 *inos*; 1 heterocaryon
4. *nic-2, m, fl, a*	2.7	100	1–2	53	47 (6 died)	14‖	12 *m, fl, nic*; 2 heterocaryons¶
5. *lys, a*	0.8	49	1–2	28 (57.4%)	21 (42.6%)	None	—
6. *lys, a*	1.8	55	1	45 (81.1%)	5 (9.9%)	None	—
Controls							
2, 3. *tryp-2, m, fl, a*	40.0	100	1–2	100	0	—	—
4. *nic-2, m, fl, a*	8.4	50	3	50	0	—	—
5, 6. *lys, a*	48.0	50	1	50	0	—	—

* Died.

‡ 9 died (*m, fl, tryp*) including 3 where of 1 of 2 transfers died.

§ 5 died (4 *m, fl, tryp*; 1 heterocaryon).

‖ None died.

¶ Both *inos* and *m, fl, tryp* recovered from cross.

filtration. Protoplasts were incubated in these solutions and allowed to grow up. Cultures were then serially transferred several times or conidia plated. No changes in any culture could be detected.

Microinjection Experiments

The experiments with natural heterocaryons indicated that the abn character usually becomes phenotypically dominant, and suggested that the trait might be cytoplasmic in origin. Attempts were therefore made to transfer the abnormal character to normal strains by microinjection, since this approach offered (*a*) a means of forming a heterocaryon in just one or two cells, and (*b*) a possible way of testing the relative effectiveness of nuclei or cytoplasm in transmitting the abnormal trait.

Except in the preliminary experiments, the normal and abnormal strains used were the same as those mentioned in the preceding sections.

The basic microsurgical techniques employed in these experiments have already been described (Wilson, 1961, 1963). In order to assure the transfer of nuclei, in some of the experiments pipettes with aperture diameters of 2.5 μ were used. In those experiments in which attempts were made to exclude nuclei, pipettes with openings and terminal bores of 0.6 to 0.8 μ were used. In addition, these smaller pipettes were inserted into the donor hyphae next to an ergosterol crystal, so that the aperture was almost immediately partially occluded by the crystal as the protoplasm began to flow into the pipette. This procedure was followed in the hope that entrance of nuclei would be minimized, if not entirely prevented, since it is not possible to see nuclei at all clearly in preparations used in microsurgery. After injection of protoplasm from the abnormal strains into the normal recipient cells was completed, one- or two-cell sections containing the injected cells were excised and transferred to Petri plates containing media

suitable for the growth of the recipient strains. Control hyphal sections were prepared from sister microcultures. After incubation overnight at 30°C, the growth from the injected segments was examined for contamination and to make certain, in the case of the two-cell sections, that both cells had regenerated. Agar blocks containing the growing hyphal segments were transferred to GSC or minimal slants. The cultures derived from the injected hyphal segments were incubated at 30°C and transferred at weekly intervals.

Injection from Abnormal to Normal Strains

Using these microinjection techniques, it appeared possible, in preliminary experiments, to transmit the slow growth character of abn-1 to cholineless (S1022) and to *inos*, and that of abn-2 to *tryp*. Transmission was judged by eventual development of slow growth, followed in most cases by death of the recipient culture in the course of weekly serial transfers on GSC slants.

Natural heterocaryons of abn-2 and *tryp* grow well on minimal slants for a few transfers and produce macroconidia, but the two components separately grow either very slowly on minimal medium (abn-2) or not at all (*tryp*), and neither produces macroconidia. These characteristics of the heterocaryon provide two easily observable criteria of the presence or absence of functional donor nuclei, so abn-2-1*A* and *tryp-2*, *m*, *fl*, *A* were chosen for more extensive microinjection studies.

Table III lists three series of injections of abn-2-1*A* cytoplasm and nuclei into *tryp A* under various experimental conditions, with the effect on subsequent growth of the injected sections as determined by serial transfer on GSC slants. The injected cultures which were heterocaryotic were also grown on a parallel series of minimal slants to facilitate comparison with natural heterocaryons. Such a comparison supports the following conclusions:

1. The abn-2 character predominated in the artificial and natural heterocaryons in about the same length of time.
2. Some injected cells of *tryp* (cells A-1, C-3, and C-5) apparently received no functional donor nuclei, as indicated by failure to grow on minimal medium and to form macroconidia, yet they also ceased to grow on GSC.
3. The onset of abnormal symptoms in the presumably non-heterocaryotic injected cultures was apparently delayed in comparison with the artificial and natural heterocaryons.
4. The abnormal character is more readily transmitted when the donor hyphae are healthy and growing than when they are highly vacuolated and have ceased growing.

Of the injected cultures listed in Table III, C-5 (which never grew as a heterocaryon but did develop an abnormal growth pattern) and D-1 and D-2 (which were heterocaryotic and finally died on GSC slants) were crossed with wild type in order to obtain more information on the nuclear types present.

Culture C-5 was crossed with wild type RL 21*a* and one or two ordered ascospore isolations were made from each of 8 perithecia. All these asci showed a 1:1 segregation of $tryp^+$ to $tryp^-$. In addition, since C-5 occasionally produced microconidia, further testing was possible. Microconidia were plated on sorbose medium and 27 of the resulting colonies were crossed with the wild type. Thirty-seven perithecia were sampled, and the results showed no evidence of the presence of abn-2 nuclei in the C-5 culture. These analyses of microconidia are included in Table IV with the results of the cross of C-5 × wild type, since they all represent samplings of nuclear types present in C-5.

Similar genetic analysis of D-1 and D-2, on the other hand, readily proved these injected cultures to be heterocaryotic.

Injection from Normal to Abnormal

The experimental difficulties involved in making the reciprocal transfer of *tryp* nuclei and cytoplasm to abn-2 cells were such that only a few successful injections could be made. The largest hyphae of abn-1 and abn-2 are approximately 8 μ in diameter. When in contact with the hypertonic solution used to reduce the internal pressure of the recipient hyphae, these hyphae shrink to about 4 μ, necessitating the use of a pipette with an outer diameter of about 3 μ. The pipette had to be inserted nearly parallel to the hypha, instead of perpendicular to the hypha as with preparations of normal cells.

Seven abn-2 cells were successfully injected with *tryp* protoplasm (series E) and transplanted, first to plates containing PDA plus tryptophan, then to slants of the same medium. Cells E-1 and E-7 died in the slants; E-2, E-3, E-4, and E-5 formed vigorous cultures with macroconidia; but the

TABLE III

Transfer of Nuclei and Cytoplasm by Microinjection from abn-2-1A to tryp-2

Cell section	Growth on minimal medium	Weekly serial transfers on GSC slants							
		1	2	3	4	5	6	7	8
Series A									
1	−	++	++	++	++	+	−	−	−
2	−	++	++	++	++	++	++	++	++
3	−	++	++	++	++	++	++	++	++
4	−	++	++	++	++	++	++	++	++
Series C									
1	++	++	++	−	−	−	−	−	−
2	++	++	++(b)	−	−	−	−	−	−
3	−	++	++	++	++	−(b)	−	−	−
4	−	++	++	++	++	++	++	+	++
5	−	++	++	++	++	++	+	−	+(b)
Series D									
1	++	++	++	++	+	−	−		
2	++	++	+	−	−	−	−		
3	− (died)								
4	++	++	++	++	+	+	−		
5	++	++(b)	+	−	−	−	−		
abn-2 + *tryp**									
1	++	+(m)	−(m)	−(m)	−	−	−		
2	++	++(m)	+(m)	+(m)	−	−	−		
tryp control sections									
Series A and C									
1	−	++	++	++	++	++	++	++	++
2	−	++	++	++	++	++	++	++	++
3	−	++	++	++	++	++	++	++	++
Series D									
1	−	++	++	++	++	++	++	++	++
2	−	++	++	++	++	++	++	++	++
3	−	++	++	++	++	++	++	++	++

Key

++, good growth. (b) extensive browning of medium.

+, scanty growth. (m) minimal medium.

−, no growth.

Series A. Donor microcultures 3 days old. Hyphae highly vacuolated. Very few nuclei visible. Pipette opening 2.5 × 4.0 μ. Injected sections incubated at 30°C on minimal medium overnight, then transferred to GSC slants.

Series C. Donor microcultures 1 day old. Hyphae non-vacuolated and actively growing. Large numbers of nuclei present. Pipette for C-1 and C-2, 0.6 μ inner diameter; for C-3, C-4, and C-5, approx. 0.8 μ inner diameter. Injected sections transferred directly to GSC slants.

Series D. Donor microcultures 1 day old. Hyphae non-vacuolated and actively growing. Many nuclei present. Pipette 2.5 μ inner diameter. Injected sections incubated at 30°C on minimal medium overnight, then transferred to minimal slants. This procedure resulted in loss of D-3, which apparently was not heterocaryotic.

* abn-2 + *tryp-2*, natural heterocaryons grown on minimal medium.

TABLE IV

Nuclear Types Found in Abnormal Cultures Derived from Injected Cells

Experiment	Cultures from injected cell	No. nuclei sampled*	Nuclear types found in recipient culture	
			Donor	Host
1. abn-2-1 *A* injected into *tryp-2, m, fl, A*	C-5	35‡	−	+
	D-1	13	+	+
	D-2	17	+	+
2. *tryp-2, m, fl, A* injected into abn-2-1*A*	E-2	10	+	+
	E-3	10	+	+
	E-6	21	−	+

* Cultures crossed with RL 21*a* (wild type) as protoperithecial parent.

‡ See text for explanation.

growth from E-6 was identical with that of abn-2 controls, both in rate and in appearance. Injected cultures E-2, E-3, E-4, and E-5 were tested by weekly serial transfer on minimal GSC, PDA, and PDA plus tryptophan at 25° and 30°C. After 10 weeks all these cultures still resembled wild type in appearance and rate of growth on the slants, except E-3, which after the sixth transfer grew much more slowly than the others until the fourth day after inoculation.

In spite of the early normal appearance (almost normal in E-3) of these apparent heterocaryons, two types of evidence suggest that the abnormal character was present.

Growth tubes containlng GSC were inoculated with the original growth of the injected segments (first transfer in the case of E-2 and E-3). As the growth reached the far end of the tube, an agar block containing mycelium was cut out and transferred to a new growth tube before conidia had formed. Fig. 3 gives the growth curves of three such serially inoculated tubes. Some of these curves show plateaus where the injected cultures grew very slowly or stopped completely for periods of several days after transfer to new tubes. These plateaus do not include the lag period immediately after transfer, because the starting point for measurement was marked after a new mycelial frontier had been established. This behavior was not shown by *tryp* or the wild type control sections, but was found in many growth tube tests of abn-1 and abn-2 on PDA and PDA plus tryptophan (Fig. 3). The average growth rate of E-2 was approximately that of wild type (100 mm/day), and considerably faster than that of the *tryp* control sections (57 mm/day). E-6, which was identical in appearance with abn-2, grew about 10 mm/day for 9 days and then died.

Protoplasm from E-4 was again injected into *tryp*, and 5 injected sections were tested on serially inoculated growth tubes. One injected culture had the appearance of a heterocaryon; the rest looked like *tryp*. The apparent heterocaryon at first grew at wild type rate, but its growth rate decreased near the end of the first tube, and it died after progressing 140 mm down the second tube. Two of the non-heterocaryotic injected cultures grew at the rate of normal *tryp* through the first tube, and also died after a short period of growth in the second tube. The remaining 2 non-heterocaryotic injected cultures and the 2 *tryp* control sections grew at the rate of 50 mm/day through all three tubes.

Abn-2-1*A* cultures E-2, E-3, and E-6 were crossed with wild type (Table IV), and, as expected from the analysis of the first series of injections, E-2 and E-3 both contained the genes *m*, *fl*, and *tryp-2*; the progeny of E-6(*A*) × wild type RL 21*a* were all wild type.

DISCUSSION

At least 8 different strains of *Neurospora crassa* have been described and investigated which differ from the wild type in rate and vigor of growth. In none has a specific growth requirement been associated with the phenomenon. Seven of these strains are characterized by changes in the cytochrome pattern as compared with wild type, and their growth pattern is marked by slow and often irregular growth from conidia or ascospores, or from both. The results obtained with these strains have recently been reviewed by Srb (1963) and by Jinks (1964). Therefore only a brief summary is given here.

The characters of 2 of these variants (C115, C117) are due to single nuclear genes; 2 are maternally inherited (*mi-1* (*poky*), *mi-3*) and were shown to differ from each other (Mitchell *et al.*, 1953); and 2 (*mi-4*, *stp*) are unable to function as the protoperithecial parent but, in common with the other 4 strains, only normal progeny result when the normal parent forms the protoperithecia

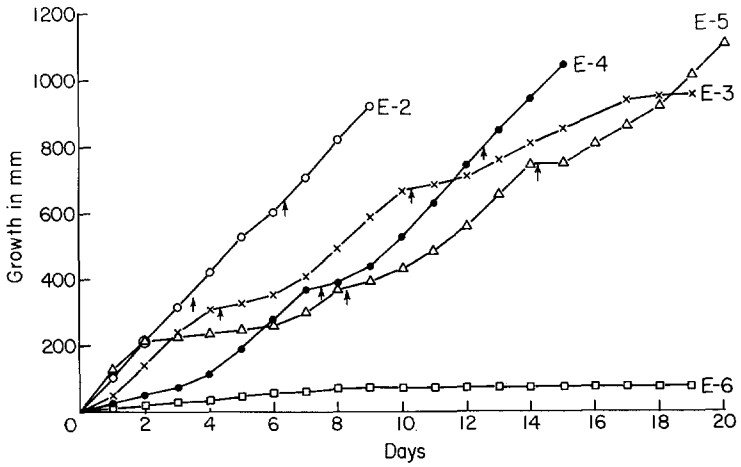

FIGURE 3 Growth on complete medium of abn-2 after injection with *tryp-2*, *m*, *fl* nuclei and cytoplasm. E-2, E-3, E-4, and E-5 at 30°C; E-6 at 25°C, no growth after 9th day. Arrows indicate transfer to new growth tube.

in crosses (Pittenger, 1956; McDougall and Pittenger, 1964). *AC-7* differs from all others in that under certain circumstances paternal as well as maternal transmission of the slow growth character can be detected in crosses (Srb, 1963). In an additional strain, *SG* (Srb, 1958, 1963), the slow growth character is inherited maternally, but spectroscopically the cytochromes appear the same as in the wild type.

The abn-1 and abn-2 variants described in the present paper most nearly resemble *mi-4* (Pittenger, 1956), in that (*a*) growth is slow and sometimes irregular, (*b*) cytochrome abnormalities, in general similar to those in *mi-1*, have been detected, and (*c*) the characters are not transmitted through a cross in which normal protoperithecia are involved. The abn variants differ from *mi-4* in that the rate of growth is considerably slower, no protoperithecia are formed, and conidia are either absent or rare, and when formed are few and very small.

The abn strains appear to differ markedly from *mi-1* in that no "curing" on continued vegetative growth has been observed.

In view of the constancy and predominance of the abn strains, as observed over a three year period of regular culture, it is perhaps surprising that cultures formed by heterocaryosis with normal strains or by microinjection of abn cytoplasm show a great variability. This is manifested in the striking fluctuations in growth rates of such cultures, and in the great diversity of growth rate and survival of cultures derived from conidia of such cultures. These conidia, even from apparently vigorous cultures, derived by heterocaryosis or injection show both very low viability (under 0.01 per cent) and extreme variability in the surviving colonies. In these characteristics the *derived* abn strains resemble *mi-4*.

A reasonable hypothesis would seem to be that the relative constancy of the abn characteristics in our strains represents a corresponding homogeneity in a cytoplasmic entity or constituent which determines these properties. In cultures formed by cytoplasmic exchange, a heterogeneous mixture of the normal and abn cytoplasmic components would be present and subject to random fluctuations in relative numbers or activity, and also subject to segregation in the formation of conidia. To this might reasonably be ascribed the low viability of conidia and the great variability of colonies derived from the viable conidia.

In regard to the indicated cytoplasmic determination of the abn characteristics, some aspects warrant additional discussion. Three types of evidence have been presented which indicate cytoplasmic control. (*a*) When the normal strains were used as protoperithecial parents in crosses with either abn-1 or abn-2, the progeny were all normal. (*b*) Natural heterocaryons of abn-1 or abn-2 with genetically marked normal strains produced some viable conidia in the period before the abn character became phenotypically dominant. When the conidia were plated, some of the resulting colony

isolates showed the abn character associated with the marker of the normal strain. (*c*) Tryptophan cultures apparently injected with abn-2 cytoplasm only (that is, cultures which gave no evidence of being heterocaryotic) also developed the abn character.

No direct tests for maternal inheritance could be made, since abn-1 and abn-2 do not form protoperithecia. However, an attempt was made to use heterocaryons of normal and abn strains as the protoperithecial parent. Although there was evidence of slower and poorer development of some perithecia and asci in such crosses, no conclusive evidence of genetic transmission of the abn character could be obtained.

Although the abn character appears to be cytoplasmically determined, the results of some of the experiments suggest that the nucleus does have a role in the establishment and maintenance of the character. First, the abnormality has not been transmitted to a wild type, either by natural heterocaryosis or by microinjection. Second, limited evidence suggests that the abn character developed more quickly in injected recipient cells if functional donor nuclei were included. Third, it may be significant that the one abnormal cell apparently injected with normal cytoplasm *only* did not show even a temporary reversion to normal appearance or normal growth rate.

The long duration of the rejuvenation of abn-2 produced by injection of normal *tryp* nuclei and cytoplasm is surprising, especially in view of the rapidity with which the abn-2 character becomes phenotypically dominant when abn nuclei and cytoplasm are injected into *tryp*. No explanation is available for this behavior.

This work was supported in part by research grant CA-03610 from the National Cancer Institute and by research grant G-18880 from the National Science Foundation.

Received for publication, December 2, 1964.

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