Methods for the identification of genetic alterations by specific revertibility tests in Neurospora crassa

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Methods for the identification of genetic alterations by specific revertibility tests in Neurospora crassa

Abstract
Revertibility tests
Molling, H. V. Methods for the identification of genetic alterations by specific revertibility tests in Neurospora crassa.

The identification of the different types of genetic alterations in a series of allelic mutants is particularly important for understanding gene-enzyme relationships. The approach that is being used to identify the genetic alterations is to study the specific revertibility of individual mutants with the use of chemical mutagens whose mode of action is well expected or known. The use of nitrous acid (NA), ethyl methanesulfonate (EMS), hydroxylamine (HA) and ICR-170 (a monofunctional ocridine mustard) should permit one to distinguish the following three classes of genetic alterations: (1) single base-pair substitutions. Mutants which revert after treatment with either HA and/or NA and in many cases also after treatment with EMS. Some of the single base-pair substitutions are also reversible with ICR-170. (2) Single base-pair insertions and deletions. Mutants which revert only after treatment with ICR-170 and then usually with a very high frequency. In a few cases mutant in this category also give a slight increase over the spontaneous frequency after treatment with NA and EMS. (3) Unidentified genetic alterations. Mutants which revert only spontaneously or stable mutants which do not revert at all.

Preparation of the culture. In all experiments the mutagenic treatment was carried out on suspensions of conidio harvested from 125-ml Erlenmeyer flasks containing 20 ml of glycerol complete medium (10 ml glycerol per liter instead of 20 ml) + adenine sulfate (25 mg per liter). The flasks were inoculated and then incubated for 1 day at 30°C and then for 6-9 days at 25°C. The conidio were harvested by first shaking the cultures with glass beads (4 mm diameter) to break up the chains of conidio; they were then suspended in saline (0.9 %), filtered through a platinum strainer, washed twice by centrifugation and then resuspended in saline. Purple mutants differ from each other with respect to the amount of the Purple pigment in the mycelium. However, when conidio were grown on glycerol complete, supplemented with 250 mg adenine sulfate per liter, purple pigment accumulation is essentially eliminated. The density of the conidial suspensions was measured on a colorimeter (Spectronic 20, Bausch and Lomb, Rochester, New York) at 750 mµ, where the absorption is maximal.

Standard conditions for treatment with NA, EMS, HA and ICR-70.

Treatment of strain: The conidio were suspended in 0.05 M sodium acetate buffer at pH 4.5. One volume of freshly prepared 0.02 M sodium nitrate solution was added to 3 volumes of conidial suspension. The final concentration was 0.005 M NaNO₃, and the treatment was quenched as described above 40 minutes after the start of the treatment.

EMS treatment: The conidio were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by adding enough EMS to bring the final concentration to 0.1 M; the treatment was quenched 300 minutes later.

HA treatment: Before the HA treatment the conidio were suspended in 3 M NaCl and then further diluted five times in the reaction mixture of Brockman and Goben (1965 Science 147: 750). The conidio were suspended in a 0.005 M adenine solution at pH 6.2. The final HA concentration is 1 M. Five minutes before the treatment was quenched, the conidio were centrifuged and decanted and at the quenching time, i.e. 300 minutes after the start of the treatment, the conidio were resuspended in 3 M NaCl. This washing procedure was repeated twice and then the conidio were suspended in the salt solution of Fries' minimal medium adjusted to pH 8.

ICR-170 treatment: ICR-170 is the code number assigned to 2-methoxy-6-chloro-9-(3-ethyl-2-chlorethyl -aminopropylamino) ocridine dihydrochloride by H. J. Brockman and co-workers of the Institute for Cancer Research, Philadelphia. Forward mutations induced by ICR-170 in Neurospora have been analyzed by Brockman and Goben (1965 Science 147: 750). The conidio were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by adding 1 volume of a freshly prepared solution of ICR-170 (250 mg/liter of water) to 49 volumes of the conidial suspension, which gave a final concentration of 10.58 µM/liter. The treatment was quenched as described above 130 minutes after the start of the treatment. The treatment and other manipulations involving ICR-170 on conidio were performed under red light to eliminate the photodynamic effects associated with the ocridine ring. Plates were also incubated in the dark for at least 24 hours to allow sufficient time for the conidio to give rise to small colonies.

Plating medium. To estimate the viability of the treated and untreated conidio, they were plated in Westergaard's minimal medium supplemented with sorbose (15 g/liter), glucose (0.5 g/liter), fructose (0.5 g/liter), Casamino acids (200 mg/liter), a vitamin solution as in glycerol-complete medium (1 ml/liter) and adenine sulfate (25 mg/liter). To estimate the number of revertants the conidio were plated in the same substrate used for scoring survivors but supplemented with 0.2 mg adenine sulfate per liter instead of 25 mg/liter.

In the plates used to determine survival the density of the conidio was 5-10/µl of substrate in a total volume of 100 ml. For scoring of revertants after NA, EMS or ICR-170 treatment, the conidio were plated to a density of 10⁶ conidio/ml and 2 x 10⁵ conidio/ml each in a total volume of 100 ml. For scoring of revertants after the HA treatment, the density of the conidio was 2 x 10⁹/ml in a total volume of 500 ml. The plating was done in 15 x 200 mm intergrid plates.

Statistical test. The test for significance is done according to Birnbaum (1954 J. Am. Stat. Assoc. 49: 254), in this test the number of revertants is considered as having a Poisson distribution. The probability is calculated by assuming that the
Isolation of subcellular fractions of Neurospora mycelia.

The following procedure for the isolation of subcellular fractions from Neurospora mycelia has been developed in our laboratory during the past two years.

**Cultures:** Two 125 ml Erlenmeyer flasks containing 20 ml of Vogel's minimal medium supplemented with 1.5% sucrose and 1.5% agar are inoculated, cultured first at 30°C for 3 days in the dark and then at 25°C with continuous illumination. Conidia are harvested in 50-100 ml of distilled water at 50-100°C and transferred to a 5-gallon carboy containing 15 liters of sterile Vogel's minimal medium supplemented with 1.5% sucrose. After 3 days' incubation with continuous aeration at 25-30°C, the mycelia are collected on a double layer of cheesecloth in a sieve 40 cm in diameter. The mycelial cells are washed thoroughly with a 20-mesh sieve with water and sterilized in an oven at 160°C for at least 3 hours. The jar is rotated 288 rpm at a speed of at least 1-1/2 hours.

The supernatant is poured from the jar and set aside. The beads are washed at least 6 times with a total (1 to 1.5 volumes) of sucrose-EDTA and the washed are combined with the supernatant.

**Isolation of subcellular fractions:** Large pieces of unbroken mycelial and residual beads are removed from the homogenate by vacuum filtration through a single layer of double-napped pajama flannel and placed on a 30-cm Buchner funnel. The filtrate, designated M_1_, is centrifuged at 2,000 x g for 10 minutes in the VRA rotor of a Lourdes Ultracentuge. The crude nuclear precipitate (N) is set aside. The supernatant is centrifuged at 16,000 x g for 20 minutes. The mitochondrial precipitate (M) is collected and centrifuged at 16,000 x g for 20 minutes. The slight precipitate is combined with the supernatant.

**Additional purification of nuclear and mitochondrial fractions:** Fraction N is suspended in 10-12 ml of a solution containing 0.5 M sucrose and 10 mM CaCl_2_ and filtered through double-napped flannel. The filtrate is centrifuged at 500 x g for 2 minutes and the fluff layer of nuclei, together with the supernatant, is decanted from the hard pellet of cell-wall debris and unbroken cells. The supernatant is centrifuged at 5,000 x g for 15 minutes with a syringe and discarded. The nuclear pellet (N2) remains at the bottom.

**Additional purification of the crude mitochondrial fraction (M):** Fraction M is obtained by a "double-shelf" technique. 1.2 ml of 1.2 M sucrose is layered upon 5 ml of 1.5 M sucrose. 5 ml of a suspension of crude mitochondria (in 0.5 M sucrose) are layered above the double layers. After centrifugation at 16,000 x g for 30 minutes, mitochondria are removed from the central layer with a syringe fitted with a wide-gauge, blunt-tipped cannula. The suspension is diluted to 0.5 M sucrose and the double-shelf centrifugation is repeated.

**Discussion:** Probably no one procedure can be devised that is ideal for the isolation of all of the various subcellular organelles from one cellular homogenate. The procedure described is therefore a compromise. For example, CaCl_2_ is necessary for the stability of nuclei, (Reich and Andrews, 1961 Biochim. Biophys. Acta 53:574). However, mitochondria tend to aggregate in the presence of divalent cations. Similarly, the preservation of ribosomes with Mg^{++} leads to the sedimentation of aggregated mitochondria with the nuclear fraction.