Transformation and reversion: Pitfalls imposed by heterokaryosis

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Abstract
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The EcoRI fragment inserted into the EcoRI site of pBR325 is approximately 3.4 kb in size and contains mostly the nontranscribed spacer region, plus external transcribed spacer region, and additional coding sequences approximately 150 bps near the 3' end of 26S rDNA. The desired clone contains the 3.4 kb insert, as well as the 6 kb piece of pBR325 DNA. When this DNA is restricted it generates the following fragment sizes: using HindIII → 5.5 kb, 2.9 kb, 900 bps; using Pst I → 4.9 kb, 2.8 kb, 1.6 kb; using EcoRI → 5.9 kb, 3.4 kb; using BamHI → 9.3 kb using Xho I → 8.9 kb, 400 bps; and using Smal 9.3 kb. (Supported in part by a Department of Energy Grant to SKD.) - - - Molecular Genetics Laboratories of Howard University, Botany Department, Washington, D.C. 20059, and the National Institutes of Health, Bethesda, MD 20014.

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Transformation and reversion: Pitfalls imposed by heterokaryosis.

Selection for reversion of a mutant phenotype often involves the appearance of a revertant nucleus in the same (mitochondrial) cell as an original nucleus, hereafter called parental. The same is true of transformants, which arise in multinucleate spheroplasts in the company of untransformed, parental nuclei. One implication of this is clear enough; namely, that the revertant will appear in greater numbers if it is dominant, especially if conidia are crowded on the selection plate. Another consequence emerged in our laboratory in the course of isolating spermidine-independent revertants of the spe-1 mutant after ultraviolet irradiation.

We irradiated and plated large numbers (ca. 1 x 10⁶) of conidia of an ornithine decarboxylase-deficient spe-1 strain on Vogel's minimal medium. A very large number of revertants appeared, owing to the revertibility of this allele. We picked 26 of them to minimal medium thereby maintaining selection. We then streaked the conidia on minimal medium and picked single conidial isolates for two serial generations, each time maintaining the isolates on minimal medium. The final isolates were plated on spermidine-containing medium as a test for the persistence of parental nuclei among the conidial population. Fourteen of the 26 cultures retained spe-1 nuclei.

This outcome was not wholly surprising, because selection on minimal medium is for prototrophic conidial colonies, not necessarily homokaryotic ones. Moreover, the ratios of heterokaryotic and homokaryotic (revertant) conidia might be expected to remain balanced in many cases, owing to the selection of a heterokaryotic conidium each time (nuclear ratios ranging from 1:2 to 2:1 in bi- and trinucleate conidia). Nevertheless, mere pure cultures might have been expected at this stage, and we therefore investigated the heterokaryotic cultures in detail.

The remarkable fact was that of the 14 impure cultures, 10 were impossible to purify, or yielded homokaryotic revertants that were exceedingly weak, even on supplemented medium. Conidia of many of the heterokaryons, when plated on such media, yielded distinctive germings that failed to grow further. Thus the tendency to select heterokaryons had been enforced by the fact that both the spe-2 and the revertant homokaryons could not grow or grow well on minimal medium. The results suggested that reversion of the spe-1 mutation to partial or complete restoration of ornithine decarboxylase might be associated with the simultaneous loss of an indispensable function. Because the reversion event allowed the heterokaryon to grow well on minimal medium, the revertant homokaryon's weakness or lethality was not due to the incompleteness of the return to wild type catalytic function.

To determine whether the "lethal" event and the reversion event were at the same locus (a test for the location of the latter at the spe-1 locus came later), the standard rationale was applied: all isolates (heterokaryotic and homokaryotic) were mated to a spe-2 strain of the opposite mating type. All of the purified prototrophic homokaryons, as expected, gave viable, prototrophic ascospores. However, so did all of the heterokaryotic cultures. This meant that the revertant nuclei of all heterokaryons contained two mutations, one the reversion to spe-1, the other a lethal or semilethal mutation elsewhere in the genome. The latter was lost during recombination in the cross. Some of the distinctive germings were seen among the progeny, assuring us that the lethals were bona fide, nuclear mutations.) In most, but not all cases, the two mutations were unlinked.

Of what interest is this story? There are two major points to be made. First, the ultraviolet irradiation used to induce the revertants was mild, calibrated for about 50% or less killing of wild type conidia. The appearance of a very large proportion of multiply mutant nuclei was wholly unexpected, and might be accounted for by peculiarities of the spe-1 phenotype. (It would not be unreasonable to find that a polyamine deficiency, hard to satisfy even in supplemented medium, might be unusually susceptible to DNA damage.) Nevertheless, to the extent that multiple mutation might be seen in reversion of other mutants, our experience underscores the need for a backcross to the mutant in question in order to shed the additional mutational events. If this is not done, pleiotropic effects might be falsely attributed to a reverse mutation. A more troubling consequence ensues in mating a heterokaryotic revertant to wild type to distinguish true reversions from intergenic suppressors: the mutant component of the heterokaryon will emerge among the progeny and will mislead one to the conclusion that reversion is due to a suppressor mutation.

The most important technical arena in which this problem might arise is transformation. Usually, a mutant is used as a recipient of DNA, and selection is then imposed for the positive phenotype. Owing to the apparently relaxed homology requirements for integration in Neurospora, there will be cases in which a plas-
mid or transforming DNA inserts the wild type gene into a resident, indispensable locus. (Alternatively, one tries to purify this strain, but the transformed strain will yield no transformants as ascospores in the cases in which transforming DNA is embedded in a disrupted, indispensable gene.

This is almost certainly a banal lesson to those familiar with the biology of Neurospora. However, those unfamiliar with the complications of heterokaryosis should not have to rediscover them first hand. Because this situation has no counterpart in transformation work in yeast, this description is offered to those who might rely unduly on yeast as a paradigm.

Preparation of a cell-free translation system from a wild type strain of Neurospora crassa.

Cell-free translation systems have proved to be invaluable tools in investigations aimed at the molecular mechanism of protein synthesis and control of gene expression. Unfortunately, the commercially available eukaryotic in vitro translation systems, derived from wheat germ and rabbit reticulocytes are not suitable for translation of all eukaryotic messages. We found it impractical to use them for some Neurospora messages hence the development of an efficient in vitro translation system (IVTS) of fungal origin appeared warranted.

An IVTS based on extracts of the cell-wall deficient ('slime') mutant of N. crassa has been reported by Szczesna-Skorupa et al. (1981, Eur. J. Biochem. 121: 163). Although this strain offers the advantage of a complete absence of a rigid cell wall and facile lysis, difficulties are experienced in achieving uniform growth and reproducible cell densities on account of a heterogeneous population of cells in liquid cultures. An IVTS from a wild type strain should be potentially more useful as those and other artifacts originating from unrelated mutations are avoided.

Preparation of cell-free extract and in vitro protein synthesis

All of the solutions used in the preparation of the lysate were autoclaved and the glassware was washed in chronic acid and treated with diethylpyrocatechol just before use to minimize nuclease activity. Wild type N. crassa (FGSC #262) was grown in Vogel's minimal medium + 2% sucrose at 28° C for 16 h with shaking. The mycelium was harvested on filter paper washed thoroughly and homogenized in 2.5 ml/g cells (wet wt.) of 30 mM Hepes buffer -- 100 mM potassium acetate -- 2 mM dithiothreitol, pH 7.4, with acid-washed sand in a cold mortar. The homogenate was centrifuged for 15 min at 23,000 g at 4° C and upper two-thirds of the supernatant was collected avoiding the top lipid layer, passed through a Sephadex-G25 coarse column, equilibrated with the same buffer. The fractions following the column void volume, with the highest absorbance at 260 nm were pooled and frozen at -76° C in 200 µl aliquots.

For studying endogenous protein synthesis, a 20 µl volume contained the cell lysate supplemented with 0.625 mM ATP, 0.250 mM GTP, 25 mM creatine phosphate, 2.5 µg creatine phosphokinase, 100 µM spermine, 25 µM each of 19 amino acids, 1-2 µl of [35S] methionine (Amersham 10-13 µCi/µl), 0.4 mM Mg acetate and 100 mM K-acetate. Samples were incubated at 21° C for 60 to 90 min, and incorporation of the label into acid-insoluble material was determined by spotting 2-5 µl on filter paper discs.

For translation of exogenously added messenger RNA, 1 µl of the lysate was treated with 12 µg of micrococcal nuclease (Boehringer-Mannheim) in the presence of 1.2 mM CaCl2 by incubation at 20° C for 5 min. The reaction was stopped by addition of EGTA to a final concentration of 2.5 mM. This nuclease-treated lysate was used for protein synthesis with 1 to 5 µg RNA and optimized Mg- and K-acetate (0.35 mM and 20 mM respectively for the RNA enriched in PK specific message) in a final volume of 25 µl as described for the endogenous protein synthesis.