Cloning of the rDNA repeat unit: An EcoRI fragment spanning the entire nontranscribed spacer region of Neurospora crassa wild type strain 74A

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Abstract
Cloning of the rDNA repeat unit: An EcoRI fragment spanning the entire nontranscribed spacer region of *Neurospora crassa* wild type strain 74A

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Burnes, P.A., J.-H. Kinnaird and J.R.S Fincham* The nonsense mutant aml7, is suppressible by ssu-1 by tyrosine insertion in residue 313 of NADP-specific glutamate dehydrogenase. It can revert to either Leu313 or Tyr313, consistent with the nonsense codon being either amber (UAG) or ochre (UAA) (Seale et al., 1976 Genetics 86: 262-274). DNA sequencing of the wild type gene (Kinnaird and Fincham, 1983 Gene 26: 253-260) shows codon 313 to be CAG (glutamine). Furthermore, we find that am7 is induced to revert with N-nitroquinoline oxide (NQO), a mutagen reported to be specific for G-C base pairs (Prakash et al., 1974 J. Mol. Biol. 85: 51-65). We conclude that the nonsense codon in am7 is amber (UAG). Since known Neurospora suppressors all suppress the same set of mutants (Seale, 1976 MGGR 148: 105-108) they must all suppress amber. There is no evidence as yet for ochre- or UGA-suppressing mutations in Neurospora. Given the very selective codon usage found so far in strongly and constitutively transcribed Neurospora genes (reviewed in Kinnaird and Fincham, 1983), UAG and UGA nonsense mutants would in any case be expected to be much less frequent than UAG in such genes. With no codons with A in the 3' position, only tyrosine (UAE) can mutate by single base-pair substitution to UAA and only tryptophan (UGG) or cysteine (UGG) to UGA; UAG on the other hand, can arise from the abundant glutamate (GAG), glutamine (CAG) and lysine (AAG) codons.

Chambers, C., R.J. Crouch and S.K. Dutta Cloning of the rDNA repeat unit: An EcoRI fragment spanning the entire nontranscribed spacer region of Neurospora crassa wild type strain 74A.

Nontranscribed spacer regions are known to be variable between species, and thus may be used to identify species differences of the genus Neurospora. This led us to clone this region from a known 74A wild type strain N. crassa strain. pMF2, a plasmid containing the coding sequences for 17S-5.8S-26S rRNA with additional flanking sequences, was constructed by Free et al. in 1979. We used their clone as a probe to clone adjacent sequences. Initial cloning experiments were conducted using pBR325. A clone of 3400 bp containing most of the nontranscribed spacer region of Neurospora crassa rDNA was inserted into the chloramphenicol gene as the termination site, for transcription of rRNA 17S, 5.8S and 26S in Neurospora crassa 74A wild type. To achieve this, plasmid pBR325 was restricted with EcoRI and then treated with bacterial alkaline phosphatase (BAP) at 65°C for 1h. The preparation was then shaken with an equal amount of phenol saturated with 0.1 M Tris-HCl, pH 9.0, followed by the addition of an equal volume of chloroform, shaken again, and then centrifuged at 15,000 rpm for five min. The upper aqueous solution was removed, brought to 1M with NaCl, EtOH precipitated in dry ice for 15 min, then centrifuged again. The pellet was vacuum dried and resuspended in distilled water. Total nuclear DNA isolated from wild-type N. crassa strain 74A (FGSC #987) was restricted with EcoRI. The N. crassa nuclear EcoRI-digested DNA was subjected to electrophoresis on 0.7% agarose gels and the band which corresponded to the nontranscribed spacer sequences was cut out of the gels, electroeluted, and run over a DE-52 column. This DNA was ligated with the BAP-treated EcoRI-digested pBR325 DNA in a ratio of 10:1 to 100:1, and then incubated at 4°C for 1 to 2 days. Escherichia coli strain LE392 was used for the transformation experiments.

Figure 1 explains the cloning strategy and gives the restriction map of both pBR325 and the new plasmid pCC3400 which was made after inserting the rDNA sequences of N. crassa into the EcoRI site of pBR325.

The colonies whose DNA generated the appropriate restriction fragment sizes were rechecked and the DNA blotted onto nitrocellulose paper, then hybridized to an rDNA 32P-labeled probe. This probe contained approximately 600 bps in common with the spacer region which was being cloned. The probe was derived from pMF2 DNA (Frome et al. 137: 1219-1226, 1979). When the plasmid pMF2 DNA is restricted with PstI, two fragments are generated - a 4.36 kb fragment (the cloning vector, pBR322) and a 6 kb fragment (the Neurospora rDNA insert). The 6 kb fragment contains the entire coding regions for 17S to 5.8S to 26S and additional sequences approximately 1 kb which are portions of the spacer regions.
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, 0.5 kb; using PstI → 4.9 kb, 2.8 kb, 1.6 kb; using EcoRI → 5.9 kb, 4.9 kb, 8.9 kb, 900 bp; 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 (multinucleate) 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 germlings 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 homo- karyons 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 germlings 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-