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Use of RIP to inactivate genes in Neurospora crassa.

Abstract
About two years ago we suggested that a novel genetic mechanism, operating in the period between fertilization and nuclear fusion in Neurospora, scans the genome for sequence dupliations and alters them (Selker E. et al. 1987 Cell 51:741-752).

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Use of RIP to inactivate genes in *Neurospora crassa*.

About two years ago we suggested that a novel genetic mechanism, operating in the period between fertilization and nuclear fusion in Neurospora, scans the genome for sequence duplications and alters them (Selker E. *et al.* 1987 Cell 51:741-752). While this is generally a nuisance for genetic experiments, we suggested that the process (designated RIP) might be "harnessed" by Neurospora researchers for the purpose of directed, *in vivo* mutagenesis of specific chromosomal regions. The idea was simple: cloned DNA corresponding to the sequences to be mutagenized would be introduced into the organism by transformation to render the sequences redundant in the genome. The desired mutants should then be generated at high frequency in a cross. Since making this proposal, we have verified that RIP is triggered by sequence duplications, *per se* (Selker, E. and P. Garrett, 1988 Proc. Natl. Acad. Sci. USA 85:6870-6874), and we and others (personal communications of C. Staben and C. Yanofsky, R. Metzenberg, L. Glass, M. Case, J. Fincham, G. Marzluf and B. Bowman) have demonstrated the efficacy of the proposed mutagenesis technique. For those contemplating the use of RIP for mutagenesis, we thought it might be helpful at this time to list some relevant points.

Nature of RIP-induced mutations. The RIP phenomenon seems to produce exclusively point mutations of one type: G-C to A-T (Cambareri, E. *et al.* 1989 Science 244:1571-1575). RIP typically results in numerous mutations in a single cycle through a cross, resulting in substantial shifts in base composition, Thus it seems unlikely that RIP-induced mutations would be "leaky" or easily revertible. In some respects, RIP-induced mutations should be functionally equivalent to a gene disruption.

Specificity. RIP acts specifically on duplicated sequences, and inactivates both partners of the duplication. Unique transforming sequences are not altered. We know of no case where one element of a duplication, but not the other, was inactivated by RIP. (The process presumably involves "pairing" of duplicated sequences.) Thus for disruption experiments, the introduced copy need not be defective. Nevertheless, if three (or more) copies of a sequence are in the genome, one copy (or more) may escape RIP. Sequences need not be originally from Neurospora to be sensitive. Therefore, if one wished to preserve the function of portions of the transforming DNA not already represented in the genome (for example, as a new selectable marker in Neurospora, or for isolating the mutated sequences by "marker rescue" in *E. coli*), one should pick single-copy transformants for disruption experiments.

Choice of selectable marker The transformation marker should not be similar in sequence to an essential Neurospora gene. Thus Benomyl resistance (ß-tubulin gene) is a poor marker for this purpose. If one wishes to use the transformation marker after the cross, one should use a gene not represented in the transformation host. In such cases we have used a bacterial gene encoding hygromycin B resistance driven by an Aspergillus promoter (Cullen, D. *et al.* 1987 Gene 57:21-26; Staben, C. *et al.*, this issue) or the Neurospora am gene (Kinnaird, J. *et al.* 1982 Gene 20:387-396) in conjuction with the am132 mutant, which has a deletion of the entire gene (Kinsey, J. and B. Hung 1981 Genetics 99:405-414).

Efficiency. RIP is generally very efficient, but the actual frequency of disruption probably depends on a number of factors. The most important is probably the length of the duplicated sequences. Linkage is also a factor. The limited data available suggest that unlinked duplications shorter than about 1 kb are rarely disrupted by RIP (probably <1% of progeny), whereas unlinked duplications of 2.6 kb or longer are disrupted at frequencies on the order of 50%. Linked homologous genes are inactivated at higher frequencies (essentially 100%).

Degree of sequence similarity. Homologous sequences need not be identical to trigger RIP, although the degree of sequence similarity influences the efficiency of the process. Our limited data suggest that sequences differing by greater than 10% are still recognized by RIP. This suggests that it may be possible to extend the use of RIP to genes not yet isolated from Neurospora, but available from related organisms.

Essential genes. Since RIP occurs immediately before karyogamy in tissue having a nucleus from each parent, crosses in which one of the partners has a duplication of an essential gene should, in general, be fertile. Inactivation of essential genes by RIP should yield tetrads with 50% inviable spores. Viable ascospores may contain just the allele from the parent lacking the duplication, or this allele plus an inactivated allele from the duplication strain (assuming at least one copy was unlinked to the natural locus). Indeed, finding just these classes of progeny (none lacking an unaltered copy of the gene) is presumptive evidence that the duplicated segment has an indispensable function. R. Metzenberg has pointed out (personal communication) that it should be simple to "shelter" an inactivated essential gene at the native locus by crossing the strain duplicated for the essential gene with an appropriate duplication-producing translocation strain (see Perkins, D. and E. Barry 1977 Advances in Genetics 19:133-285). One might then be able to demonstrate that the sheltered gene could not be uncovered (by mitotic "breakdown" of the partial diploid), implying that the RIP had generated a lethal mutation.

Mutants lacking obvious phenotypes. An important use of RIP is to test the function of partially characterized sequences. For example, one may wish to test the function of genes isolated on the basis of differential expression. If sexually-produced progeny of a strain duplicated for a cloned DNA segment do not show an obvious phenotype, one should examine tetrads for evidence of an essential gene, and check progeny at the DNA level by Southern hybridization to look for physical evidence of RIP. Generally, but not invariably, sequences altered by RIP become methylated (at cytosines). While methylation *per se* is not a sure sign of RIP, change of methylation in a cross is a good indication of RIP. In order to detect changes in the primary structure of DNA due to RIP, it is useful to employ restriction enzymes insensitive to cytosine methylation. We have found the pair of isoschizomers *Sau*3A (sensitive to cytosine methylation of GATC) and *Mbo*I (insensitive to cytosine methylation of GATC) to be most useful for detecting RIP at the DNA level.

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