PREMEIOTIC INSTABILITY OF REPEATED SEQUENCES IN NEUROSPORA CRASSA

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KEY WORDS: repeat-induced point mutation (RIP), DNA methylation, recombination, genome evolution, rDNA

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1 I dedicate this review to David D. Perkins, who by his enthusiasm, altruism, and example has been largely responsible for making Neurospora research enjoyable and rewarding.
INTRODUCTION

What is responsible for the size and organization of a eukaryotic genome? Genomes vary greatly in the amount of repetitive DNA they contain. Lower eukaryotes, such as fungi, tend to have small genomes with few repeated sequences. The genome of Neurospora crassa, for example, consists of only $\approx 4 \times 10^7$bp of DNA, of which <10% is repetitive (20, 41, 57). In contrast, higher plants and animals have genomes that are typically two orders of magnitude larger, and commonly consist of $\approx 50\%$ repetitive sequences. It is tempting to assume that the observed variability reflects different needs of the organisms. It remains possible, however, that the variability simply reflects different degrees of success in resisting the onslaught of "selfish DNA"—sequences that direct their own amplification, but serve no obvious purpose for the organism (17, 58). To assess the relative importance of these possibilities will require information of several sorts.

We need to establish the costs and benefits to an organism of carrying large amounts of repetitive DNA. Presumably, the costs will depend strongly on the biology of the organism (see 14). The success of a filamentous fungus such as Neurospora may depend on its ability to spread extremely rapidly over a food source and gather the resources. If the genome contained a large amount of unnecessary DNA, the increased metabolic cost to the organism could slow its growth. It is easy to imagine that larger nuclei, which would presumably result from a larger genome, would also be detrimental to an organism such as Neurospora that relies on cytoplasmic streaming to move nuclei through incomplete septa between the cells. Other costs of carrying repetitive DNA might be common to all organisms. For instance, any organism should be sensitive to chromosomal translocations involving dispersed repeated sequences. Mutagenesis by insertion of transposable elements might represent another example, although the immediate consequences of mutations in a haploid organism are of course qualitatively different in a diploid.

A full understanding of genome structure will require information on DNA turnover: how repeated sequences arise and become amplified; and what processes oppose their spread. In this review, which is concerned with the
latter part, I summarize and discuss what is known about two processes that act on repeated DNA in the genome of *N. crassa*. The first, premeiotic recombination, eliminates tandem duplications. The second, repeat-induced point mutation, RIP, mutates both linked and unlinked duplicated sequences and often leaves the products methylated. Neither process is unique to Neurospora. Identical or closely related processes have been observed in *Ascobolus immersus* (24, 29) and other filamentous ascomycetes.

Before reviewing premeiotic recombination and RIP, it is helpful to summarize (a) key features of the *N. crassa* life cycle and genome, (b) characteristics of DNA-mediated transformation in *N. crassa*, and (c) early hints of instability of repeated sequences in Neurospora.

**Neurospora Life Cycle and Genome**

The sexual phase of Neurospora and other filamentous ascomycetes differs from that of yeasts in having a prolonged heterokaryotic phase between fertilization and karyogamy (nuclear fusion). The *N. crassa* life cycle is illustrated in Figure 1, and its main features are noted in the legend.

The Neurospora genome consists of seven chromosomes with a total genetic map length of roughly 1000 map units (60). A tandem array of ~170 copies of a 9.3 kb DNA sequence encoding the three large RNA molecules of the ribosome, located near one tip of linkage group V, accounts for most of the repetitive DNA of the organism (7, 41). Unlike most eukaryotic organisms, the ~100 5S rRNA genes of Neurospora are dispersed in the genome (28, 47, 82). The genes share only ~150 bp of sequence similarity (50, 82). Available information suggests that, at least in general, protein coding genes are present as unique sequences in the *N. crassa* genome. Unidentified repeated sequences are occasionally encountered in collections of sequences from the Neurospora genome, however. Some of these possibly represent tRNA genes and relics of transposable elements or retroviruses (37, 71; P. Garrett, J. Kinsey, E. Selker, unpublished results).

**DNA-Mediated Transformation**

Discovery of premeiotic recombination and RIP grew from the development of DNA-mediated transformation in Neurospora. Transformation of Neurospora was first reported by Tatum and associates (48, 49, 83), who used unfractionated genomic DNA. Case et al (13) pioneered the development of an efficient procedure using cloned DNA. Zalkin and other researchers improved the procedure, and DNA-mediated transformation soon became widely applied in Neurospora research (1, 6, 8, 30, 35, 39, 43, 56, 73, 74, 79, 89). Genetic and molecular characterization of the original transformants
Figure 1  Life cycle of *Neurospora crassa*. The organism grows strictly as a haploid of one of two mating types (A or a). The vegetative phase is initiated when either a sexual spore (ascospore) or an asexual spore (conidium) germinates, giving rise to multinucleate cells that form branched filaments (hyphae). The hyphal system spreads out rapidly (>5 mm per hour at 37°C) to form mycelium. Aerial hyphae grow up, and culminate in the production of the abundant orange conidia that are characteristic of the organism. The conidia, which contain one to several nuclei each, can establish new vegetative cultures, or fertilize crosses. If nutrients (principally nitrogen sources) are limiting, Neurospora enters its sexual phase by producing nascent fruiting bodies (protoperithecia), each of which originates from 100–300 vegetative nuclei (33). When a specialized hypha projecting from the protoperithecium contacts tissue of the opposite mating type, a nucleus is picked up and transported back to the protoperithecium. The heterokaryotic cells resulting from fertilization proliferate in the enlarging perithecium. Most of the proliferation involves cells having on the order of five or six nuclei (65; E. Barry, personal communication). In the final divisions, however, the cells are binucleate, containing one nucleus of each mating type. These cells bend to form hook-shaped cells (croziers) and a final conjugate mitosis occurs to produce four nuclei. Septa are laid down to produce one binucleate cell at the crook of the crozier. This cell gives rise to one ascus (65). Genetic analyses have indicated that, in general, the 100 or more asci of a perithecium are derived from a single maternal nucleus and a single paternal nucleus (33, 52). While it is generally assumed that nuclei of the initial fertilized cell undergo 7–10 divisions (22), this may be a gross underestimate (N. Raju, personal communication). Premeiotic DNA synthesis in filamentous ascomycetes occurs prior to nuclear fusion (see 65). When karyogamy finally occurs, the resulting diploid nucleus immediately enters into meiosis. Thus the diploid phase of the life cycle is limited to a single cell. The meiotic products undergo one mitotic division before being packaged as ascospores.
obtained using cloned DNA first demonstrated that Neurospora transformants fall into three classes: unlinked insertions, linked insertions, and replacements (13). Most transformants of *N. crassa* arise by nonhomologous recombination between transforming DNA and chromosomal sequences, whether circular or linear transforming DNA is used (2, 59). Transformants frequently contain multiple copies of the transforming DNA, and two or more copies are commonly integrated at one site (6, 16, 30, 78). Frequently, transformation results in integration of essentially intact transforming DNA (2, 79; J. Kinsey, personal communication).

The least common class of transformants in Neurospora, linked insertions, arise by a single crossover between homologous sequences of a circular transforming DNA molecule and the host chromosome (12, 13, 79). In transformation experiments in which homologous recombination between transforming and host sequences can restore function of a mutated gene that is being selected, replacement of the mutation is observed much more frequently than insertion of the entire plasmid (13, 16).

**Hints of Instability of Repeated Sequences in Neurospora**

Several classical observations are consistent with the idea that the low repetitive DNA content of the *N. crassa* genome is not simply an immediate result of natural selection. Indeed, the organism probably employs several mechanisms to maintain a streamlined genome. As yet, no mechanism to resist redundant DNA has been identified in the vegetative phase of the life cycle but there are reasons to suspect that one or more exist. For one thing, despite numerous attempts, *N. crassa* has never been successfully propagated as a diploid (see 60). This distinguishes Neurospora from some other fungi, including the filamentous ascomycete *Aspergillus nidulans*. Disomic ascospores form occasionally in *N. crassa*, but disomy breaks down rapidly to produce heterokaryons with haploid nuclei (see 60). Partial diploids, constructed by crossing translocation strains, are relatively stable during vege-

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2Although some evidence of unstable autonomous replication of transforming sequences has been observed, stable autonomous replication has not been achieved in Neurospora (16, 59, 67, 85).

3Although replacement events formally look like products of double crossovers, in which both strands of DNA are replaced, they could also be products of heteroduplexes resulting from invasion of single strands of the transforming DNA. As expected, the relative frequency of integration of transforming DNA by homologous versus nonhomologous recombination depends on the transforming DNA. The best information available comes from a study by Asch & Kinsey (2) using the *am*<sup>+</sup> gene. They found 22 homologous recombination events among 70 transformants when 9 kb of homology was provided, but only one among 89 when 2–5 kb segments of the 9 kb region was used. It is not clear whether this difference in frequency was due to the length difference of the homology or to a sequence feature in the additional DNA.
tative growth, although they typically break down\(^4\) eventually, generally by loss of the translocated segment (see 53). Duplications of chromosome segments are much less stable in the sexual phase of the life cycle, however. Segmental aneuploids rarely survive a cross. The duplications lead to developmental arrest at or just before karyogamy (66). This phenomenon is discussed more fully below.

Tandem duplications resulting from integration of transforming DNA by homologous recombination are lost at low but detectable frequencies during vegetative growth (30, 77). In general, however, Neurospora transformants are stable when propagated vegetatively. In striking contrast, as first noted by Tatum and associates for transformants obtained by the use of total genomic DNA (48, 49, 86, 87), and later by other researchers for transformants obtained using cloned DNA (12, 16, 30, 77), a large fraction of transformants of *N. crassa* are unstable in the sexual phase of the life cycle. Primary transformants of Neurospora are generally heterokaryotic, containing both transformed and untransformed nuclei. Because of this it was common practice in many laboratories to cross primary transformants to obtain homokaryotic derivatives prior to their characterization (12, 13, 16, 34, 59). In some cases this practice probably complicated the interpretations as a result of discrimination against particular types of transformants. Nevertheless, it became clear that even homokaryotic derivatives of transformants show poor transmission of transformation markers.

**PREMEIOTIC RECOMBINATION**

**Deletion of Transforming DNA**

A set of transformants generated to investigate DNA methylation in *N. crassa* were particularly useful for studying instability of transforming DNA in this organism. These were single-copy transformants generated with the plasmid pES174 (79). This plasmid consists of four parts: (a) the zeta-eta (ζ–η) region, a diverged (14%) direct tandem duplication of a 0.8 kb segment that is extensively methylated in *N. crassa* (79, 80); (b) \(\approx 6\) kb of uncharacterized sequences adjacent to \(\zeta–\eta\), that are normally unmethylated in the genome ("flank"); (c) the \(am^+\) gene of *N. crassa*; and (d) sequences of the bacterial plasmid pUC8 (Figure 2). The transformation host had a deletion of the entire \(am\) gene (\(am_{132}\); 38) and, in place of the \(\zeta–\eta\) region, had one

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\(^4\)Curiously, certain mutations and growth conditions increase the rate of breakdown (54, 55). In 1977, Newmeyer & Galeazzi (53) proposed a mechanism to account for breakdown of duplications that result from quasiterminal translocations or inversions. The basic idea is that both the original rearrangement and breakdown events occur by recombination between repeated sequences located at the breakpoints. Although the model has not yet been properly tested, the finding of a repeated sequence immediately interior to a telomere (71) is consistent with it.
copy of a 0.8 kb segment that is ≈83% identical to this region (31, 81). Thus flank represented the only significant tract of homology between the transforming DNA and the host genome. One transformant analyzed, T-ES174-1, resulted from integration of the plasmid by homologous recombination, generating a local duplication of flank, as illustrated in Figure 2. Deletion of the am+ gene occasionally occurred during vegetative growth: two of six vegetative reisolates from a culture that was initially homokaryotic revealed loss of the gene in a noticeable fraction of the cells (77). Results of Southern hybridizations suggested that deletion occurred by homologous recombination between the two copies of flank.

Greater instability of the transformation marker was observed in crosses of T-ES174-1. Only ≈16% of the progeny from a cross of an apparently homokaryotic isolate of the transformant and an am132 strain were Am+, suggesting loss or inactivation of the gene in two thirds of the chromosomes initially carrying the transforming DNA (77). Normal segregation of the mating type genes, which are linked to the site of integration in T-ES174-1, was seen among progeny of this transformant, suggesting that loss of the marker did not involve lethality. This conclusion was confirmed and extended by analysis of progeny from individual asci. Thirty-five percent of the ascis (16/55) showed Mendelian segregation of Am (4+:4−), and sixty-five percent exhibited complete loss of the marker (9). Southern hybridization analyses on DNA of progeny from completely Am− ascis revealed that the Am, ζ−η, and pUC8 sequences, and one copy of the flank sequences, had been eliminated, apparently by recombination between the two copies of flank. Restriction site polymorphisms between the host and transforming DNA sequences in the flank region revealed that the copy of flank that remains was sometimes predominantly from the host and sometimes predominantly from the transforming DNA. The transforming DNA was not lost by unequal sister chromatid exchange, since no progeny were found with two copies of the

Figure 2 Generation of transformant T-ES174-1 by homologous recombination (79).
transforming sequences, which would be the reciprocal product of this event. Thus the transforming DNA was most likely deleted by intrachromatid recombination, a reversal of the integration reaction (Figure 2). The consistent loss of the transforming sequences from both meiotic products of the affected tetrads suggested that the recombination event had occurred prior to premeiotic DNA synthesis, and thus prior to karyogamy (see Figure 3).

Several nuclear divisions separate fertilization from karyogamy in N. crassa. To determine whether the high frequency of intrachromosomal recombination occurred before or after fertilization, Selker et al (77) took advantage of the fact that, in general, all the asci of a perithecium represent the products of a single fertilization event (see Figure 1 legend). If the recombination event occurred prior to fertilization in a cross between T-ES174-1 and an am strain, all asci from the resulting perithecium would be 0:8 (+:-) for Am. In contrast, if it occurred after fertilization, both 4:4 and 0:8 asci could be generated. Of eight perithecia in which two or more asci were analyzed, three showed just 4:4 asci, two showed just 0:8 asci, and three showed both 4:4 and 0:8 asci. Thus intrachromosomal recombination occurred after fertilization, at least in some of the perithecia. Relatively few asci were scored from most of the perithecia, and deletion of the transforming DNA may have occurred in all of the perithecia. The fraction of 0:8 asci fluctuated widely from perithecium to perithecium, however, suggesting that recombination could occur relatively early or late in the period between fertilization and karyogamy (E. Cambareri & E. Selker, unpublished observations).

The high frequency of this premeiotic intrachromosomal recombination raises the possibility that unlinked repeated sequences might also undergo a high level of recombination prior to meiosis. Recombination between sequences on different chromosomes would of course produce translocations, and recombination between inverted repeats on the same chromosome would give inversions. No evidence of translocations was observed among 16 progeny of strains that had ectopic copies of pES174, indicating that recombination between the unlinked copies of the flank sequences does not occur at frequencies comparable to that detected between the closely linked copies of these sequences (77). A thorough search for premeiotic interchromosomal recombination has not yet been conducted, however.

**Change in rDNA Copy Number**

The tandemly arranged rRNA genes are the only known natural example of linked repeated sequences in Neurospora. In several organisms, including

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*Although it is also conceivable that a nonreciprocal process, possibly associated with replication (e.g. involving polymerase “jumping”), is responsible for the deletion, I refer to the deletion process as intrachromatid (or intrachromosomal) recombination.*
Neurospora, rDNA is unusual in that recombination between homologous chromosomes is suppressed (see 68). The rRNA genes are not recombinationally inert, however. For example, in the yeast *Saccharomyces cerevisiae*, sister chromatid exchanges occur frequently during meiosis (63).

Butler & Metzenberg (7) have shown in *N. crassa* that tandem rDNA repeats of the nucleolus organizer region (NOR) are subject to copy-number changes at high frequency in the sexual phase of the life cycle. Most progeny of every cross exhibited differences from their parents in rDNA copy number. No sign of interchromosomal recombination was observed. Analyses of progeny from individual asci and from individual perithecia indicated that most alterations occur in the period between fertilization and karyogamy, the stage in which the artificial duplications of the flank region of pES174 transformants exhibited instability. All four progeny having the NOR from a given parent generally showed the same rDNA copy number. Likely evidence of changes both during and after meiosis was also seen, however. In two of 14 asci, the NOR size segregated in a 4:2:2 pattern, suggesting a copy-number change during or after premeiotic DNA synthesis. In one of 14 asci, the NOR size segregated in a 4:3:1 pattern, probably reflecting a change after meiosis.
A small cluster of rDNA repeats (~9), which had been moved to a different chromosome as a result of two sequential chromosomal translocations (61), were no less unstable, indicating that the effect was not dependent on the chromosomal location of the sequences. In 13 of 14 asci, the copy number of this ectopic rDNA was the same for all four progeny that contained it, although the copy number varied from one ascus to another (7). The ectopic rDNA in the exceptional ascus was of one size in two progeny, and one unit larger in two others. A difference of one unit could not occur by a single sister chromatid exchange. Thus this result suggested that intrachromatid recombination was responsible for the copy-number change, at least in this case. At both the normal and the ectopic NOR, most changes in rDNA copy number were reductions, consistent with the idea that many of the changes resulted from intrachromatid recombination, as in transformant T-ES174-1. Magnification of rDNA arrays may occur primarily during vegetative growth, as observed in *N. crassa* by Russell & Rodland (69). In one postmeiotic change in rDNA copy number observed by Butler & Metzenberg, chromosomes exhibiting complementary changes were identified, suggesting that the event occurred by sister chromatid exchange (7).

**PREMEIOTIC MUTATION: DISCOVERY AND MAIN FEATURES OF RIP**

Investigation of the fate of transforming sequences in crosses of pES174 transformants also led to the discovery of RIP. The first clue came from examining progeny derived from crosses of T-ES174-1, the transformant with the local duplication of the flank region (Figure 2). As mentioned above, normal segregation of the transformation marker, Am+, was observed in those asci that did not show intrachromatid recombination. Southern hybridizations revealed, however, that 100% of Am+ progeny had suffered sequence alterations in both copies of the flank region (77). Although the overall length of the sequences appeared at least roughly unchanged, the arrangement of restriction sites in the DNA showed numerous alterations. No changes were detected in the transforming DNA between the copies of flank, i.e. the pUC8, am+, and ζ–η sequences. Use of the isoschizomers *Sau*3A, *Mbo*1, and *Dpn*I, and other restriction enzymes, produced evidence for both changes in the primary structure of the DNA and extensive de novo methylation of cytosines. A survey of random Am+ progeny showed great variability in the alterations (see Figure 4).

Even unlinked copies of flank, resulting from nonhomologous integration of pES174 in the *am*132 strain, were altered in crosses. The changes occurred at a lower frequency, however, and generally appeared less radical (77). Approximately 50% of progeny from any of the four single-copy ectopic
transformants tested exhibited alterations in flank. The transformation marker, \textit{am}^+ showed normal segregation. As in progeny of T-ES174-1, both copies of the duplicated sequences, but none of the other transforming sequences, appeared affected.

\textit{Timing of RIP}

Biological features of Neurospora and inherent properties of RIP allowed us to pin-point when in the life cycle the process occurs (77). The diversity of the alterations in the flank region of random Am$^+$ progeny of the pES174 transformants allowed products of a common event to be distinguished from products of independent events. In the vast majority of asci yielding progeny with altered flank DNA, the products of sister chromatids displayed common alterations. This implied that the changes were determined prior to premeiotic DNA synthesis, and therefore prior to karyogamy and meiosis, as illustrated in Figure 3B). The phenomenon was first dubbed RIP for “rearrangement induced premeiotically” to emphasize this feature (77). In a minority of the asci, restriction pattern differences between pairs of Am$^+$ progeny were observed, which could have resulted from alterations during or after premeiotic DNA synthesis. Such results could also reflect heterologies in the final DNA duplex before premeiotic DNA synthesis, which were later resolved. No indication of meiotic or postmeiotic sequence alterations has yet been observed.

To determine whether RIP occurred before or after fertilization, Selker et al (77) again took advantage of the fact that the 100 or more ascii of a perithecium are usually derived from a single maternal nucleus and a single paternal nucleus (33, 52). Thus, different alterations among progeny from different asci of the same perithecium would indicate that RIP occurs after fertilization. In general, different alterations were observed among progeny of individual perithecia, as shown in Figure 4. These results indicate that RIP occurs after fertilization. Identical alterations of flank sequences were observed among the three completely Am$^-$ asci of one perithecium (number 4 of Figure 4). Results of Southern hybridizations indicated that the pUC8, $\zeta$-$\eta$, and \textit{am} sequences, and one copy of the flank sequences had been lost, presumably by intrachromosomal recombination following RIP. Finding the same pattern of alterations in multiple isolates from a given perithecium indicated that RIP can occur early during proliferation of the heterokaryotic tissue formed by fertilization.

\textit{Proof that RIP is Triggered by Duplications}

These observations led Selker et al (77) to suggest that \textit{N. crassa} has a mechanism that detects and alters sequence duplications in the haploid nuclei of the heterokaryotic tissue resulting from fertilization. Selker & Garrett (78) carried out a set of experiments to test this directly and to check whether the
Figure 4  RIP occurs after fertilization. Progeny altered by RIP from several asci of each of four perithecia (1–4) from a cross T-ES174-1 (T-1) were analyzed by probing Sau3A (S) and MboI (M) digests for the flank region. Sau3A and MboI both recognize and cleave the sequence GATC when it is unmethylated, but Sau3A will not cut at this sequence if the cytosine is methylated. The enzyme MboI is insensitive to cytosine methylation, the only methylation known in N. crassa, but is sensitive to adenine methylation. Results are shown for one isolate per ascus. All the progeny illustrated from perithecia 1–3 except C55 were Am+, and all those from perithecium 4 were Am−. Perithecium 4 represents one of two cases interpreted as RIP followed by deletion observed among six perithecia in which completely Am− asci were found. RIP patterns vary greatly within perithecia 1–3 but not within perithecium 4. Radiolabeled molecular weight markers were loaded in lane L. (Reprinted with permission from Ref. 9.)

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observed instability was in any way dependent on introduction into the cell of foreign DNA (pUC8), or DNA prone to methylation (ζ–η), or on disruption of the chromosome by integration of the transforming DNA. The plan, illustrated in Figure 5, took advantage of the am_{132} deletion (38). In the first phase (Figure 5A), a strain with this deletion was transformed with a 2.6 kb DNA fragment that contained the am gene but no bacterial sequences nor sequences already represented in the host. The idea was to test whether two or more copies of this unmethylated sequence, integrated in the genome without any foreign or methylated sequences, would trigger its inactivation by RIP. RIP occurred, indicating that neither methylated sequences nor non-Neurospora sequences are required for the process. Single-copy transformants, in contrast, were stable through crosses, consistent with the hypothesis that RIP is a response to sequence duplications, and showing that simple integration into the genome does not trigger the process.

Figure 5  Scheme to test if RIP is triggered by sequence duplications. (A) One or more copies of a Neurospora DNA fragment containing the am^{+} gene were introduced into a strain with a deletion covering the entire fragment. The transformants were then crossed and scored for RIP phenotypically and at the DNA level. (B) To test if the observed lack of RIP in some crosses was due to the single-copy status of the transformants, two of them were crossed to build a duplicate-am strain. Evidence of RIP was seen in crosses of this strain, indicating that RIP is indeed a response to sequence duplications (78).
The second phase of the study (Figure 5B) tested the possibility that stability of single-copy transformants was due to something other than their single-copy status (e.g. chromosomal position). Two such strains having single copies of \( am^+ \) at unlinked chromosomal locations were crossed to build a strain having two copies of the gene. Crosses of this duplicate-\( am^+ \) strain showed all the hallmarks of RIP, proving that RIP is triggered by duplications. Approximately 50% of the asci showed inactivation of \( am \), and inactivation always involved both copies of the gene. This provided the first clear evidence that recognition of duplications by the cell involves interaction of the like-sequences (discussed further below). Southern hybridizations revealed sequence alterations, DNA methylation, or both, in many of the inactivated alleles (78). Experiments with strains having the \( am^+ \) gene at its normal chromosomal position plus one copy of \( am^+ \) contributed by transforming DNA gave equivalent results (K. R. Haack & E. Selker, unpublished observations). Similar experiments from several laboratories soon confirmed the suggestion that RIP operates on repeated sequences generally, and verified that the process could be used as a tool for directed, in vivo mutagenesis of specific chromosomal regions (2, 26, 27, 76).

Nature and Specificity of Alterations by RIP

Results from Southern hybridizations indicated that RIP does not, at least in general, result in large changes in the overall length of the affected sequences (77). It was impossible to tell from the Southern hybridizations, however, whether the restriction site rearrangements were due to classical rearrangements (e.g. numerous small insertions, deletions, inversions, or substitutions), point mutations, or a combination of these. Nevertheless, the repeated observation of new fragments of certain sizes among independent progeny suggested that RIP results in point mutations (78). Characterization of two \( \approx 6 \text{ kb} \) segments of DNA, chosen to represent mild and severe alterations by RIP, first revealed the nature of the alterations by the process (10). The mild example was from an unlinked duplication of flank that had been passed once through a cross, and, based on Southern hybridization results, seemed relatively lightly altered by RIP. The severe example was from the linked duplication of flank that had been passed twice through a cross. Its flank DNA exhibited many changed restriction fragments and seemed resistant to RIP in further crosses (see below). Both of the chosen sequences were methylated as a result of RIP.

The altered chromosomal regions were isolated and compared to their native counterparts by heteroduplex analysis to look for gross rearrangements, and by sequence analysis to detect local alterations. No evidence of classical rearrangements was observed by electron microscopy in the \( \approx 6 \text{ kb} \) heteroduplexes. In the sequences from the linked duplication, however, heterodu-
plexes formed under various stringency conditions exhibited evidence of substantial sequence divergence. Small bubbles were observed under conditions expected to allow pairing of molecules having up to 40% mismatch, and roughly half of the heteroduplex was opened under conditions expected to allow pairing of molecules having up to 20% mismatch (10). No bubbles were observed in heteroduplexes formed under very low stringency conditions.

DNA sequence comparisons of segments altered by RIP and their native counterparts revealed one type of mutation: G:C pairs were converted to A:T pairs. In a ≈900 bp segment of the unlinked duplication, ≈10% of the G:C pairs had mutated, and in a ≈600 bp segment from one end of the linked duplication, 31% of the G:C pairs had mutated. Comparison of the melting properties of linearized plasmids having ≈6 kb of either the altered or the native sequence from the linked duplication suggested that ≈50% of the G:C pairs were lost overall (10).

Not all G:C pairs are equally mutable by RIP. The C to T mutations occur primarily at cytosines immediately 5' of adenines, and rarely at cytosines 5' of other cytosines. In the segment of the linked duplication that was sequenced, ≈64%, ≈18%, ≈13%, and ≈5% of CpA, CpT, CpG, and CpC dinucleotides, respectively, changed. The site specificity of RIP seemed equivalent in the segment from the unlinked duplication. It may not be coincidental that the observed low sequence-specificity of RIP, which is unusual for nucleic acid-enzyme interactions, is characteristic of eukaryotic DNA-methyltransferases. For example, the rat methyltransferase modifies ≈99%, ≈14%, ≈6%, and ≈0% of CpG, CpA, CpT, and CpC dinucleotides, respectively, in vitro (84). It is not yet known whether DNA methylation in *N. crassa* has specificity that parallels the site specificity of RIP.

**The Rosetta Stone of RIP: The ζ–η Region**

The ζ–η (zeta-eta) region, which is a diverged tandem duplication of a 794 bp segment including a 5S rRNA gene or pseudogene, was discovered in a study of 5S rRNA genes of *N. crassa* (80). The ζ and η 5S rRNA regions represent the only known exception to the rule that 5S rRNA genes are dispersed in the Neurospora genome (47, 82). A survey of laboratory strains of Neurospora indicated that not all *N. crassa* strains contain the duplication, suggesting that it arose relatively recently (81). Analysis of strains of known pedigree demonstrated, however, that the duplication of the ζ–η region from strain 74A-OR23-1VA had gone through at least six generations. Sequence comparisons between the duplicated elements of the ζ–η region, and between each of these and an unduplicated allele revealed that all of the differences were due to

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6This finding led us to suggest that the acronym RIP be regarded as an abbreviation for "repeat-induced point mutation" (10).
transition mutations (31, 80). All but one of 268 inferred mutations in the region were G:C to A:T changes, suggesting that the region represented a product of RIP.

The distribution of the ζ–η mutations supported the idea that they resulted from RIP. The C to T changes occurred in 74%, 30%, 11%, and 2% of the CpA, CpT, CpG, and CpC dinucleotides, respectively. The data also revealed that the mutations do not occur completely without regard to the identity of the bases immediately 5', or two nucleotides 3', of the changed cytosines: C to T mutations occurred most frequently downstream of adenines, and CpApT sites were favored over all other trinucleotides (31). In the 123 nucleotides upstream of the duplicated region, the duplicated and unduplicated alleles were identical. A single G:C to A:T mutation was found about 20 bp downstream of the duplicated region.

Analysis of the ζ–η region also provided new information about RIP: the process is active on relatively short direct tandem duplications; and duplicate elements that trigger RIP suffer equivalent, but nonidentical, damage. Statistical analysis of the distribution of mutations in the ζ and η halves of the duplication suggested that all the CpA dinucleotides were more or less equally mutable. Visual inspection of the distribution of the mutations in the ζ–η region revealed some higher-order biases, however. For example, the seven CpA dinucleotides that were not altered in either the ζ or η halves of the duplication were all relatively close to the ends of the repeated segment. The ends of the repeats immediately adjacent to the unique DNA were the least mutated regions.

**DNA Methylation Resulting from RIP**

The bulk of the Neurospora genome is unmethylated. It is interesting, therefore, that methylation is a common feature of sequences altered by RIP (77, 78). It is important to note that the methylation resulting from RIP is detected in vegetative cells, cells that are not active for RIP. The same sequences may be methylated at the time RIP occurs, but this possibility has not yet been examined. Methylation, where observed, has usually been very heavy; most or all of the sites examined in the duplicated sequence appear methylated in a large fraction of the molecules. Aside from the tandemly repeated rDNA, which displays some methylation (61), the few methylated patches that have been encountered in wild-type strains show evidence of RIP—polarized transition mutations associated with a repeated sequence. The ζ–η region represents the best-characterized example. Results of restriction analyses suggest that most of the cytosines in the duplication are methylated. No evidence of methylation was found in the adjacent sequences, nor in the unduplicated allele (80, 81; E. Selker, unpublished observations).

Although sequence duplications initially trigger RIP, they are not required...
for the methylation of sequences altered by the process. This conclusion comes in part from the observation of methylation of unique sequences resulting from meiotic segregation. Moreover, a DNA sequence altered by RIP can direct its methylation, de novo. When the $\zeta-\eta$ region was stripped of its methylation by propagation in *Escherichia coli*, and then reintroduced into *Neurospora*, it became specifically and reproducibly remethylated in vegetative cells (79). A comparable experiment using a product of RIP generated in the laboratory produced equivalent results (11). We therefore conclude that the G:C to A:T mutations somehow render the sequences substrates for DNA methylation. A model to account for this has been proposed (75).

**Limits of RIP**

The fact that the *Neurospora* genome is not completely devoid of repeated sequences raised the possibility that different sequences might have different sensitivities to RIP. At one extreme, one could imagine that the process depends on special sites, analogous to promoters or recombinators, that are not present in every DNA segment. Another possibility is that some sequences can confer “immunity” to RIP on neighboring DNA. Finally, it is conceivable that certain regions of the genome might be protected from RIP. Although these possibilities have not yet been thoroughly explored, some relevant information is available.

As far as I know, every sequence of appreciable length (i.e. > 1 kb) that has been duplicated in *N. crassa* and tested, has shown signs of RIP. All of the approximately 15 *Neurospora* genes that have been tested in various laboratories exhibited sensitivity to the process (unpublished observations of E. Cambareri, M. Case, C. Engele, J. Fincham, L. Glass, K. Haack, H. Inoue, B. Jensen, J. Kinsey, G. Marzluf, R. Metzenberg, E. Selker, C. Staben, D. Stadler, C. Yanofsky). Foreign sequences introduced into *Neurospora* in multiple copies were also sensitive to RIP (27).

**NATIVE-REPEATED SEQUENCES**

*rDNA* Even repeated sequences that are native to the *Neurospora* genome can be susceptible to RIP. That the $\zeta-\eta$ region includes relics of 5S rRNA genes suggested that these small genes are sensitive to RIP under some circumstances. The small size (<150 bp) and dispersed arrangement of the $\approx$100 5S rRNA genes of *Neurospora* probably protect them from the process (P. Garrett & E. Selker, unpublished observations). The tandemly repeated genes encoding the three large rRNA molecules are probably protected because of their particular chromosomal position rather than because of something special about their sequence. A single rDNA repeat unit introduced into an ectopic site by transformation was subject to RIP (K. Haack, B. Jensen, & E. Selker, unpublished observations). This suggests that some repeats in the NOR are available for premeiotic pairing.
and therefore raises the possibility that some fraction of the rDNA units of
the NOR are actually susceptible to RIP. Perkins et al (61) noted that a
small cluster of rDNA repeats that had been moved from their normal home
in the NOR by two sequential translocations, exhibit abnormally heavy meth-
ylation. It seems possible that the methylation, which was detected in the
ectopic rDNA after a cross, reflects the operation of RIP. Loss of rDNA
repeats by premeiotic intrachromatid recombination, in conjunction with re-
amplification and natural selection, could effectively rid the NOR of defective
sequences.

TRANSPOSABLE ELEMENTS The primary role of RIP in *N. crassa* may be
to protect the organism from transposons, viruses, and selfish DNA generally.
As would be expected if this were so, the Neurospora genome appears
relatively clean of such sequences. Putative selfish DNA has been found in *N.
crassa*, however. Schechtman identified a \( \approx 1.6 \) kb sequence, "pogo", adja-
cent to the linkage group VR telomere that is also present at several other sites
in the genome and shows some features of a transposon (71, 72). Three
wild-type strains examined showed different restriction fragments hybridizing
with pogo, and their segregation suggested that at least some of the copies
were at different locations in the strains. Analysis of the original copy of pogo
revealed 318 bp imperfect direct repeats separated by an \( \approx 1 \) kb segment (72).
Thirty-three of the 34 nucleotide differences between the terminal repeats are
transitions and methylation is associated with the element (M. Schechtman &
E. Selker, unpublished observations). Thus, the ancestor of pogo was pre-
sumably subjected to RIP.

Kinsey & Helber (37) identified an active transposon, "Tad", in a wild-type
strain of *N. crassa* collected from Adiopodoumé, Ivory Coast. Tad, which is
\( \approx 7 \) kb in length, lacks long terminal repeats, and does not appear related to
pogo. The Adiopodoumé strain was chosen for a transposon search primarily
because it appeared to contain one or more factors that decreased the stabil-
ity of segmental aneuploids and increased the frequency of translocations
(37, 53). Ten or more copies of Tad are present in the Adiopodoumé strain,
but it has not been found in any of more than 400 other wild and laboratory
strains of *Neurospora* screened (36). When crossed into laboratory strains
of *N. crassa*, Tad usually becomes amplified, demonstrating that at least
one copy of the transposon was active. Approximately 1/3 of progeny show
some evidence of alterations of the transposon, however, and sometimes
all copies of the transposon appear inactivated (P. Garrett, J. Kinsey, &
E. Selker, unpublished observations). The finding that Tad does not appear
immune to RIP suggests that either its original host, Adiopodoumé, is defec-
tive for RIP, or that Tad represents a recent intruder into the Neurospora
genome.
PREMEIOTIC INSTABILITY IN NEUROSPORA

Information from analysis of progeny from individual perithecia suggested that RIP can occur in any of the roughly ten cell generations between fertilization and karyogamy. This raised the possibility that duplicated sequences might be sensitive to multiple cycles of RIP. Consistent with this idea, unlinked duplications, which in contrast to linked duplications escape RIP at an appreciable frequency, exhibit less severe alterations than do linked duplications. Thus the severe alterations of linked duplications may reflect the sum of many cycles of RIP.

Cambareri et al (11) showed that sequences altered by RIP can remain susceptible to the process. Duplicated DNA of several single-copy pES174 transformants were followed through seven generations. After a few generations both linked and unlinked duplicated sequences became resistant to RIP. In one series of crosses starting with T-ES174-1, the transformant having a linked duplication of flank, five out of six progeny showed alterations in the second generation, but no further alterations were observed until five generations later, when one of six progeny exhibited evidence of RIP.

Results of an analysis of the stability of hybrids between sequences altered by RIP and their native counterpart, suggest that linked duplications diverge further than do unlinked duplications before becoming resistant to more change. DNA methylation resulting from RIP does not seem to confer resistance to the process. Rather, resistance is primarily due to sequence divergence of the homologous regions. Depletion of preferred substrates for RIP (i.e. C:G pairs in favorable sequence contexts) also plays a role. This conclusion comes from experiments in which one or more copies of a RIP-resistant derivative of flank were introduced into a strain with just the native flank sequence. The resulting transformants were then crossed and scored for RIP (11). When multiple copies of the mutated sequence happened to integrate in a cluster, they were very sensitive to RIP. In contrast, a single-copy transformant showed no evidence of RIP. The altered copy of the flank sequences was apparently unable to trigger RIP of the native copy. When an identical, second copy of the resistant DNA was crossed in from the strain that the resistant sequence was isolated from, RIP occurred, albeit at low frequency (presumably because the copies were unlinked).

Foss et al (27) obtained similar findings using the \( \zeta-\eta \) region, the natural 1.6 kb tandem duplication that must have experienced numerous cycles of RIP (see above). Not surprisingly, this diverged duplication is stable in crosses of strains having single copies of the region (77). In contrast, crosses of strains having two copies of the \( \zeta-\eta \) region, created by crossing single-copy transformants, exhibit evidence of RIP. A relatively low incidence of instability was observed by Foss et al presumably because of depletion of mutable sites, and because the duplicate sequences were unlinked.
THOUGHTS ON THE MECHANISM OF RIP

Detection of Duplications Probably Entails Pairing

How can the cell efficiently detect small sequence duplications, especially when the duplicate copies are at unlinked chromosomal positions? A duplication of a 2 kb segment would correspond to only \( \approx 0.01\% \) of the Neurospora genome. Although detailed information is not yet available, we can start to paint a general picture of the recognition process. Recognition of sequence duplications by RIP is analogous to recognition of homologous sequences by recombination processes, and presumably RIP and recombination are mechanistically related. The temporal correlation of RIP and high frequency intrachromosomal recombination is consistent with this idea. Furthermore, observations on the pattern of inactivation of genes imply that RIP relies on pairing of duplicate sequences, as in recombination. In nuclei containing two copies of a sequence, RIP mutates either both sequences or neither sequence, but if three or more copies are present, the process frequently results in a combination of altered and unaltered copies (26, 78). All available data are consistent with the idea that RIP invariably operates on repeated sequences in a pair-wise manner.

Studies on recombination, especially in the yeasts *Schizosaccharomyces pombe* and *S. cerevisiae*, provide ample precedent for pairing of unlinked homologous sequences (see 64). In both mitotic and meiotic cells, reciprocal exchange and nonreciprocal information transfer occur nearly as frequently between gene-sized segments of homologous sequences embedded in nonhomologous chromosomes as between homologous sequences at allelic positions (32, 44). Although the products of RIP and intrachromosomal recombination appear among sexual progeny in Neurospora at frequencies at least an order of magnitude greater than the frequency of recombination between sequences of comparable size in normal meiotic yeast cells, the mechanism of pairing in all these processes may be identical. The vastly reduced amount of substrate for pairing in premeiotic (haploid) nuclei relative to meiotic (diploid) nuclei may effectively focus the pairing machinery on repeated sequences. Support for this idea comes from work of Wagstaff et al (90) using haploid yeast cells in which meiotic processes had been artificially activated. The frequency of intrachromosomal recombination observed in these haploid cells (\( \approx 30\% \)) was approximately tenfold higher than that characteristic of standard (diploid) meiotic cells. When considering the high frequency of RIP and premeiotic recombination in *N. crassa* it is notable that the pairing process in this fungus is probably extended over a much longer period than in yeasts; efficient pairing may occur throughout the entire period between fertilization and karyogamy.

A collection of mutants defective in RIP would help to firmly establish the
relationship between premeiotic sequence recognition and meiotic processes. Identification and characterization of such mutants may be complicated, however, because they may be sterile in homozygous crosses and because RIP only occurs in tissue that has nuclei from both parents. No mutants defective in RIP have yet been identified. The mutation \textit{mei-2}, which abolishes chromosome pairing and meiotic recombination in homozygous crosses (but produces enough ascospores for analysis), is not impaired in RIP (H. Foss, N. Raju, A. Schroeder, & E. Selker, unpublished observations). Apparently efficient pairing of homologous sequences in preparation for RIP and chromosome pairing in preparation for crossing over are not synonymous.

\textbf{RIP Probably Involves a Processive DNA-cytidine Deaminase}

The two chains of a segment of DNA affected by RIP sometimes show significant differences in the number of G to A (or C to T) changes. In the sequenced segment from the unlinked duplication of flank sequences, 34 G to A changes, but only 13 C to T changes, were found on one strand. A statistical evaluation that took the composition of the original sequence into consideration indicated that this difference is nonrandom. A more striking example was found in a segment of the \textit{am} gene from an unlinked duplication that passed once through a cross: 28 C to T changes occurred in a 350 nucleotide segment of one strand, and none occurred on the other (M. Singer, R. Eyre, & E. Selker, unpublished observations). That a given strand showed just C to T or G to A changes, but not both, in spite of the fact that the original composition of the two chains were comparable, implies that RIP results from a single type of mutation and that RIP operates on one chain of DNA at a time. Analysis of a unique sequence immediately adjacent to a duplicated region that was altered by RIP showed that the process sometimes extends a short distance beyond the duplicated region (27). Taken together, these observations suggest that the RIP machinery acts in a processive manner.

In principle, G:C to A:T mutations could occur by (a) directed misincorporation during DNA replication, (b) misincorporation following an excision process (e.g. involving a glycosylase), or (c) direct chemical conversion of the bases. Although we do not know whether the G:C to A:T mutations of RIP result from G to A or C to T changes, the latter seems more plausible from a mechanistic standpoint. Cytosine and 5-methylcytosine (5mC) are prone to spontaneous deamination yielding uracil and thymine, respectively (see 21, 46). Under physiological conditions, spontaneous deamination of C and 5mC in double-stranded DNA probably occurs at rates considerably below $1 \times 10^{-10}$ per second, and organisms have repair processes to fix G-U (see 19, 45) and G-T (see 91) mismatches before mutations become established. The high frequency of G:C to A:T changes by RIP thus suggests that the process involves enzymatic deamination of C or 5mC.
Figure 6 Possible mechanisms of G:C to A:T mutations involving enzymatic deamination. Hypothetical enzymatic reactions are shown by dashed arrows. (A) The mutations of RIP could result from enzymatic deamination of cytosine to form uracil (x) followed by DNA replication if excision of uracil by DNA-uracil glycosylase were switched off or were incomplete. (B) Alternatively, the mutations could result by cytosine methylation (y) followed by deamination (z), avoiding the uracil intermediate. (C) Enzymatic methylation of cytosine (solid arrows) involves a nucleophilic attack at the 6 position of cytosine to form a reactive 5,6-dihydrocytosine intermediate (bracketed). The resulting negative charge at the 5 position activates this previously inert carbon to accept a methyl group from S-adenosylmethionine (SAM). The dihydrocytosine intermediate is also very sensitive to displacement of the 4-amino group. Thus C to T changes could occur directly without involving a 5-methylcytosine (mC) intermediate. On mechanistic grounds, deamination would more likely follow methylation of the dihydrocytosine intermediate (as illustrated) than vice versa (D. Santi, B. Branchaud, personal communications).

residues (10). The normal repair processes might be turned off in ascogenous tissue, or simply overwhelmed by RIP (Figure 6). Although enzymatic deamination of cytosine nucleosides and nucleotides are known, and evidence exists for RNA-cytosine deaminases (see 4, 18), no example of the postulated DNA-cytosine deaminase has been described. Curiously, the enzymatic mechanism of cytosine methylation is thought to go through an intermediate (5,6-dihydrocytosine) that is prone to spontaneous deamination. The dihydrocytosine intermediate is at least 10,000-fold more prone to deamination than cytidine (see 23, 70, 93). Thus, the C to T changes
of RIP may be catalyzed by one enzyme without releasing a 5-mC intermediate, as illustrated in panel C of Figure 8. Furthermore, a common enzyme might be responsible for deamination in ascogenous tissue and for methylation in vegetative cells. Conceivably, the different outcomes might reflect different substrate concentrations, physical conditions, and/or cofactors in the respective tissues. For example, if ascogenous tissue should have an unusually low level of S-adenosylmethionine, this could prolong the lifetime of the dihydrocytosine intermediate, and thus result in deamination.

_Detection and Mutation of Duplications May Be Temporally Separated_

The search for homologous sequences in RIP may be relatively time consuming, while the mutagenesis may be rapid. Since all sequences are present in duplicate in the G2 phase of the cell cycle, recognition of duplications probably just occurs in G1. Although it is conceivable that the pairing machinery carries with it the enzyme(s) responsible for the mutagenesis, it seems more likely that the mutagenesis machinery would be associated with the replication machinery. The apparent processivity of RIP could simply reflect the inherent processive nature of DNA replication. Homologous sequences sharing a minimum length (e.g. 1–2 kb) would form stable pairs, which would accumulate during G1. The replication machinery, with its accessory RIP-apparatus, would then modify and dissolve the paired sequences.

**EARLY SIGHTINGS OF PREMEIOTIC INSTABILITY**

_Instability of Transformation Markers_

As mentioned above, genetic instability was noticed in early transformation studies. In 1973, Mishra et al (48, 49) reported that a large fraction of _Inl_+ strains, obtained by treating an _inl_ (inositol-requiring) strain with massive quantities of DNA from a wild-type strain, showed non-Mendelian transmission of _inl_+ in crosses. Approximately one half of the _Inl_+ strains transmitted the marker to 2% or less of their progeny, and about one third of the strains transmitted the marker to ~20–30% of their progeny. The remaining _Inl_+ strains showed normal transmission of _inl_+. (These might have resulted from replacement or true reversion of the _inl_ mutation in the recipient.) All _Inl_+ strains obtained in experiments without DNA treatment, or with DNA treatment using DNA from the inositol-requiring strain, exhibited normal transmission of the marker. Poor transmission did not depend on which strain was used as the female. Furthermore, non-Mendelian transmission was not limited to the first cross, but was seen again when _Inl_+ _F_1 progeny were crossed, indicating that poor transmission could not be attributed to heterokaryosis. Based on these findings, an "exosome" model of transformation
was proposed in which the introduced DNA did not integrate into the genome, and was therefore readily lost during meiosis (48). Analysis of progeny from individual asci revealed that, invariably, the introduced marker was transmitted to either zero or four of the eight isolates. This finding was taken as evidence that the mechanism removing exosomes was either not active or was fully active in meiosis.

The observed instability most likely resulted from RIP. The strains that transmitted \textit{\textit{\textit{inl}}+} to roughly 25\% of their progeny behaved like single-copy transformants having the introduced DNA at sites unlinked to the \textit{\textit{\textit{inl}}} gene of the host. The strains that transmitted the marker at lower frequencies behaved as if they had linked copies of the transforming gene. Based on what is known about the fate of transforming DNA in Neurospora, it seems unlikely that a sizable fraction of the transformants had linked duplications, however. The extremely poor transmission of \textit{\textit{\textit{inl}}+} may have been due to lethality resulting from RIP acting on critical sequences that happened to be taken up by the transformants.

Working with a similar set of transformants, Szabó & Schablik (86) obtained more or less equivalent results. In crosses of the \textit{\textit{\textit{Inl}}}+ transformants with an \textit{\textit{\textit{Inl}}}– strain, most of the transformants were barren on medium lacking inositol but fertile on medium containing inositol, consistent with the idea that the \textit{\textit{\textit{inl}}}+ gene was inactivated premeiotically. Crosses between a wild-type strain and the \textit{\textit{\textit{Inl}}}+ transformants were fertile on either medium, presumably because of complementation of the inactivated alleles by the \textit{\textit{\textit{inl}}}+ gene of the wild-type.

In 1984, Grant et al (30) demonstrated instability of a transformation marker, \textit{\textit{\textit{am}}}+, in strains obtained by transformation of an \textit{\textit{am}} deletion strain using the cloned gene. None of 33 \textit{\textit{Am}}+ strains obtained transmitted the gene to more than 2\% of their progeny in crosses. Results of Southern hybridizations demonstrated that the strains had multiple copies of the transforming DNA, arranged mostly in clusters of tandem arrays. Two possible explanations for the results were suggested by the authors—either the \textit{\textit{am}}+ sequences were chromosomally integrated but then eliminated during meiosis, or they were present on autonomously replicating plasmids that were lost in the cross. Although restriction analysis revealed changes that appeared partially attributable to loss of autonomous plasmids, in retrospect, it seems likely that the transforming DNA was all integrated in the genome. Loss of the transforming marker probably resulted from a combination of RIP and intrachromosomal recombination. Occasional transformants that showed Mendelian segregation of \textit{\textit{am}}+ were obtained in transformations of a strain with a point mutation in the \textit{\textit{am}} gene. Such transformants probably arose by clean replacement of the mutation.

Soon thereafter, Dhwale & Marzluf (16) and Case (12) reported sexual instability of the \textit{\textit{qa-2}}+ gene introduced into a \textit{\textit{qa}} (quinic acid requiring) strain
by transformation. Approximately 74% of the transformants in one study showed poor transmission of the marker (16). In an extensive survey of Qa-2+ transformants that arose in a variety of ways, Case (12) observed loss of gene expression in pairs of meiotic products, as reported for the Inl+ transformants obtained using total Neurospora DNA (48), as well as for transformants obtained using pooled DNA from a cosmid collection (25). Results of Southern hybridizations showed that loss of qa-2+ function was due to inactivation rather than loss of the transforming DNA (12). Subsequent analysis of DNA from representative strains confirmed that inactivation resulted from RIP (E. Foss, M. Case, & E. Selker, unpublished observations).

In retrospect, possible manifestations of RIP and premeiotic intrachromosomal recombination are evident in the first report of Neurospora transformation using cloned DNA (13). The transformants were obtained by treating a qa-2 strain with plasmid DNA including the qa-2+ gene and the adjacent gene, qa-4+ (13, 74). Putative transformants were crossed before analysis to obtain homokaryotic isolates. Two of 14 Qa-2+ strains obtained in this way integrated at or very near the native qa-2 locus. Both showed inactivation of qa-4+. It is likely that the qa-4+ gene was inactivated by RIP. Interestingly, two out of three sexual isolates of these two "linked-insertion" type transformants lacked all bacterial sequences. Presumably the sequences were lost by premeiotic intrachromosomal recombination.

**Recurrent Mutation of Cya-8**

There is no reason to think that RIP is limited to duplications created by transformation. As discussed above, the $\zeta-\eta$ region apparently represents a case of RIP of a natural tandem duplication. D. Perkins & V. Pollard (personal communication) have collected a set of observations that may reflect the operation of RIP on an unlinked duplication involving two structural genes. First, they noted that $\approx 20\%$ of progeny from crosses in which one or both partners carry the eas mutation (easily wettable conidia) grew very poorly. Characterization of representative progeny demonstrated that they all suffered from mutations at a single locus unlinked to eas, and Bertrand showed that they were deficient in cytochrome aa1. Mutations in the new locus, cya-8, occurred only in the nucleus that contained the eas mutation, and occurred in the period between fertilization and karyogamy. These observations are consistent with the idea that the eas mutation resulted from an insertion that included cya-8 sequences. Standard laboratory strains having this mutation would thus have two copies of cya-8 sequences, and would therefore be subject to RIP.

**Barren Phenotype of Segmental Aneuploids**

Strains containing duplications of segments of chromosomes can be constructed readily in Neurospora by crossing appropriate translocation strains
As first noted by St. Lawrence in 1953 (see 60), partial diploids display great instability in the sexual cycle. In general, they produce "barren" perithecia, i.e. perithecia that produce no or few ascospores (60). Infertility of segmental aneuploids in Neurospora contrasts with the fertility of duplication strains in the homothallic fungus A. nidulans (3), and with the fertility exhibited by the vast majority of Neurospora strains having balanced rearrangements, i.e. those not having duplications (60).

The barren phenotype of segmental aneuploids has been thoroughly studied by Perkins and associates (see 60, 66, 88). Raju & Perkins (66) noted that the degree of barrenness appears to be independent of the genic content and size of the duplication and that barren perithecia are usually more or less normal in size and abundance. A careful cytological study of crosses involving nine different segmental aneuploids revealed that perithecial development is initiated but sexual development is arrested before meiosis (66). All strains seemed blocked during crozier formation or about the time of karyogamy. Each strain showed some variability in the stage of arrest, however, and some variability between strains was also observed. This study clearly indicated that duplications, per se, lead to developmental abnormalities prior to meiosis. The authors noted: "The question remains unanswered, what type of genetic element could be responsible for barrenness, that is distributed so ubiquitously through the chromosomes as to be present even in short duplicated segments, and whose dosage effect is so potent that it interferes with karyogamy or meiosis."

Most likely the barren phenotype of segmental aneuploids is an indirect result of RIP. A typical duplication covers 10–50% of a chromosome and probably includes a number of critical genes. Presumably, the RIP machinery efficiently detects these large duplications, and then wreaks havoc throughout them. Duplications of chromosome segments are occasionally transmitted through a cross without apparent alterations (88). It would be interesting to know if the nontranslocation chromosome of the duplication is generally (or always) from the nonduplication parent, as one would expect if RIP caused serious damage in the duplicated sequences. Limited analysis of survivors from crosses of duplication strains has not revealed mutations in genes covered by the duplication at frequencies typical of RIP (D. Perkins, M. Singer, E. Cambareri, & E. Selker, unpublished observations). Nevertheless, using a chromosomal duplication covering just two known genes, Perkins (personal communication) has found evidence of RIP among the rare progeny from such a cross. The relatively low frequency of mutations recovered could indicate that RIP operates inefficiently on large duplications. Alternatively, it

7The barren phenotype itself is to varying degrees unstable, as might be expected considering that duplications are somewhat unstable during vegetative growth. Loss of the barren phenotype is invariably associated with breakdown of the duplication.
PREMEIOTIC INSTABILITY IN NEUROSPORA

It is interesting to consider how RIP could result in the barren phenotype. The general expectation is that so long as only one partner in a cross has a duplication, the mutations resulting from RIP would be complemented by the unaffected genes of the other nucleus. This appears to be generally the case for small duplications created by transformation. It is not uncommon, however, to observe poor fertility in crosses involving a strain with a duplication of just 3–10 kb. Furthermore, one characteristic of barren perithecia, namely the poor development of beaks (the structure surrounding the opening through which the ascospores are shot), is also frequently seen in crosses of strains harboring small duplications (E. Cambareri, K. Haack, P. Garrett, & E. Selker, unpublished observations). RIP might block sexual development in several ways. One possibility is that genes having nucleus-limited functions required during sexual development are widely distributed in the genome. In some cases production of defective products by genes mutated by RIP could poison the cell. Insufficient dosage of gene products may be serious in some cases as well. Finally, it seems possible that general deleterious effects might result from RIP. For example, changes in chromatin structure and/or new DNA methylation in the duplicated regions could affect DNA replication.

EVOLUTIONARY IMPLICATIONS OF RIP AND PREMEIOTIC DELETION

All observations to date suggest that RIP inactivates both copies of duplicated genes. Nevertheless, because of the temporal position of RIP in the Neurospora life cycle, duplication of an essential gene need not lead to death of the organism. Since RIP occurs in cells having nuclei from each parent, except where both parents harbor the same duplication, complementation may prevent cell death. Moreover, in the case of unlinked duplications, a sizable fraction of duplications escape recognition. Linked duplications are recognized very efficiently, but premeiotic deletion will rescue one member of a direct tandem duplication in many of the progeny. A substantial number of ascospores from a cross in which one parent has a duplication of an essential gene will be dead, of course, but this should not represent a large sacrifice on the part of the species.

Generation of Diversity

GENE FAMILIES RIP may be responsible for the single-copy status of Neurospora genes that are represented as small families of identical or closely related genes in other organisms. The histone genes represent one example (92). Since gene duplications are generally regarded as a starting point for the
evolution of new genes, it might seem that RIP should limit diversity. RIP may actually promote diversity, however. In general, 25% of progeny from a cross in which one parent harbors a small unlinked duplication should contain one native and one mutated copy of the sequence (Figure 7). Unlinked duplicated sequences appear to become resistant to subsequence cycles of RIP after fairly limited divergence (i.e. 10–20%). Sequences altered by RIP might therefore provide useful raw material for the evolution of new genes. Thus, while RIP may suppress the generation of gene families composed of very similar members, the process may spur the development of gene families with divergent members.

Figure 7  Fate of duplicated sequences altered by RIP. A cross between a wild-type strain (top right) and a strain harboring an unlinked duplication of a chromosomal segment is illustrated. Single and duplicate copies of the segment are indicated by boxes. In the period between fertilization and karyogamy, both copies of the duplicated sequence are altered by RIP (hatching) in the nucleus harboring the duplication. The homologous sequence in the other nucleus (empty box) is unaltered and, in general, should complement mutations resulting from RIP. The four possible combinations of chromosomes harboring the altered or unaltered homologues are shown. If the duplicated segment included essential genes, two of the meiotic products (c and d) should be inviable. One of the two viable products (b) would receive one altered and one unaltered sequence.
"SPONTANEOUS" MUTATION  The existence of processes such as somatic hypermutation of antibody genes (40) and RIP suggests that we should reexamine the meaning of "spontaneous" (in the sense of accidental) mutation. Cells can cause mutations to occur. Thus, some mutations thought to result from environmental insults to DNA, chemical instability of the nucleotides, or mistakes in DNA replication or repair, may actually result from enzyme-catalyzed conversions. The postulated DNA-cytosine deaminase of RIP may be more widespread than RIP itself. Considerable evidence in both prokaryotes and eukaryotes indicates that cytosines that are subject to methylation are unusually mutable (see references in 80). While it is assumed that this is due to failure to repair G·T mismatches resulting from spontaneous deamination of 5-mC residues, such mutations could be due to enzymatic deamination. A DNA-cytosine deaminase might normally work in conjunction with a G·T to G·C correction system, such as that recently discovered in animal cells (91), to demethylate DNA (mC:G → T·G → C·G). Any mismatches left uncorrected would lead to mutations. Furthermore, since the unstable 5,6-dihydrocytidine is thought to be an intermediate in enzymatic methylation (70, 93), the methylase itself should act as a deaminase and may directly catalyze C to T changes.

Mutation and Elimination of Repeated Sequences

Presumably both premeiotic recombination and RIP control the spread of repeated sequences. Deletion by premeiotic recombination should minimize the number of tandemly repeated sequences in the genome, and RIP is ideally suited to counter all types of selfish DNA. However, RIP does not directly eliminate selfish genes; it simply inactivates them, and thus generates pseudogenes. Although sequences altered by RIP could be preferentially deleted from the genome, no evidence for this idea is available. Sequences altered by RIP might confer a selective disadvantage on an individual beyond that expected from loss of function. If so, RIP would both inactivate selfish DNA and given natural selection a tool to eliminate it from a population.

Control of Genome Structure

In addition to inactivating genes, mutations resulting from RIP suppress subsequent pairing of the affected sequences. Thus RIP might thwart synopsis of nonhomologous chromosomes in meiosis. RIP should also prevent recombination between dispersed repeated sequences, which would result in translocations and other rearrangements. The observation that linked sequences that have been altered by RIP are poor substrates for intrachromosomal recombination supports this idea (11).

Chromosomal rearrangements that occur in spite of RIP may create duplications, either directly, or after recombination involving parental and
rearranged chromosomes (see 60). As discussed above, the resulting segmental duplications are nearly sterile, probably because of RIP. Thus, RIP should indirectly rid a population of certain chromosomal rearrangements. This should help to preserve the gross organization of the genome (78), and may be responsible for the rarity of chromosomal rearrangements among Neurospora strains collected from nature (62). Although no evidence is available suggesting that RIP operates outside of the perithecium, the intolerance of diploidy and disomy exhibited by Neurospora might be due to a low frequency of a RIP-like process operating in vegetative tissue.

PREMEIOTIC INSTABILITY IN OTHER FUNGI

RIP may be limited to a subset of the filamentous ascomycetes. The process has not been detected in basidiomycetes (5, 51), and duplicate sequences are not inactivated in the well-studied yeast *S. cerevisiae*, or in the yeast *S. pombe* (E. Selker, unpublished observations), neither of which have a heterokaryotic phase in their life cycle. In contrast, duplicate sequences are inactivated in the heterothallic filamentous ascomycete *Ascobolus immersus*, which, like *N. crassa*, has an extended stage in which haploid nuclei of different strains share a common cytoplasm (24, 29). As in *N. crassa*, inactivation in *A. immersus* occurs in the interval between fertilization and karyogamy, appears to involve pairing, acts on both linked and unlinked sequences, and results in heavy methylation. *A. immersus* also shows high frequency premeiotic intrachromosomal recombination. The two organisms exhibit some differences, however. In *A. immersus*, a linked duplication sometimes survives a cross unaltered. Furthermore, in some cases, genes inactivated in *Ascobolus* appear to revert, especially if the organism is treated with the drug 5-azacytidine, which interferes with DNA methylation. The RIP process may result in considerably fewer mutations in *A. immersus* than in *N. crassa*. Results of Southern hybridizations are consistent with this idea (29), but no sequence information is yet available. The inactivation process of *A. immersus* could even involve only methylation, although this would imply that this fungus, unlike Neurospora, has an efficient mechanism to maintain methylation patterns during vegetative propagation (see 75).

Preliminary evidence suggests that RIP also operates in the plant pathogens, *Gibberella pulicaris* (Y. Salch & M. Beremand, personal communication) and *Gibberella fujikuroi* (M. Dickman & J. Leslie, personal communication), both of which are also heterothallic filamentous ascomycetes with a dikaryotic phase preceding karyogamy. In contrast, RIP does not occur, at least at high frequency, in two other heterothallic filamentous ascomycetes, *Magnaporthe grisea* (F. Chumley & B. Valent, unpublished observations) and *Cochliobolus heterostrophus* (O. Yoder, personal communication), both of which are also plant pathogens.
RIP has not been found in the two homothallic filamentous ascomycetes that have been examined, *Sordaria macrospora* (42) and *Aspergillus nidulans* (C. Scazzochio, personal communication), nor in the functionally homothallic ascomycete *Podospora anserina* (15). Nevertheless, all three organisms show high frequency deletion of tandem repeats. Considering that RIP inactivates both members of a duplication, it seems reasonable from an evolutionary standpoint that RIP would be limited to heterokaryotic cells in outbreeding organisms (77). All the nuclei in the premeiotic tissue of a homothallic fungus usually come from a single parent. If that parent happened to have a duplication of an essential gene, RIP might result in cell death (see Figure 7). The situation would be comparable to the operation of RIP in vegetative cells. Premiotic intrachromosomal recombination, which eliminates just one member of a tandem duplication, is more conservative than RIP, and should not cause problems for a homothallic organism.

**SUMMARY**

Maintenance of a streamlined genome is probably important to a free-living fungus. The period between fertilization and karyogamy in the life cycle of Neurospora and related fungi provides an ideal time for "genome-cleaning". Premiotic intrachromosomal recombination deletes tandem repeats at high frequency in both homothallic and heterothallic filamentous ascomycetes. This eliminates excess copies of tandemly repeated genes and at the same time favors their homogenization. Heterothallic fungi such as Neurospora also take the bolder steps of mutating and modifying both copies of duplicated sequences, linked or unlinked, by RIP. Because these organisms are outbreeders, and because RIP operates immediately prior to meiosis in cells having nuclei from both parents, the process does not cause much lethality or loss of genetic information. RIP should effectively counter selfish and redundant DNA, and at the same time generate raw material for evolution. In addition, RIP should both prevent chromosomal rearrangements by causing divergence of dispersed repeated sequences and rid a population of duplication-generating rearrangements. Thus, this form of genetic instability potentially stabilizes the gross organization of the genome.

**ACKNOWLEDGEMENTS**

I thank Ed Barry, Bruce Branchaud, Ira Herskowitz, Jack Kinsey, Mike Lynch, Bob Metzenberg, Dot Newmeyer, David Perkins, Daniel Santi, Frank Stahl, Charles Yanofsky, and present and past members of my laboratory for stimulating discussions. I am grateful to Ed Cambareri, Jette Foss, Jeff Irelan, Jack Kinsey, Bob Metzenberg, Vivian Miao, Rik Meyers, Dot Newmeyer, David Perkins, and Jeannie Selker for comments and useful suggestions on the developing manuscript. I am also grateful to all my colleagues who kindly
communicated their unpublished results. The work from my laboratory was supported by National Science Foundation Grant DCB 8718163 and Public Health Services grant GM-35690 from the National Institutes of Health. Part of this work was done during the tenure of an Established Investigatorship of the American Heart Association.

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