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Genetics

DNA sequence duplications trigger gene inactivation in *Neurospora crassa*

(genome organization/molecular evolution/DNA methylation/rearrangement induced premeiotically/DNA-mediated transformation)

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ABSTRACT **Transforming sequences are faithfully replicated in vegetative cells of *Neurospora* but are typically subject at high frequency to sequence alterations and methylation in the period between fertilization and nuclear fusion. Previous work showed a correlation between the occurrence of these radical changes, referred to by the acronym RIP, and the presence of sequence duplications resulting from the introduced DNA. Various possible causes for the RIP process were investigated. Introduction of a single copy of a DNA fragment containing the *Neurospora am* gene into a strain having a deletion of this DNA did not lead to RIP, whereas introduction of two or more copies of the same fragment did. A conventional cross of strains having single copies of *am* at unlinked chromosomal locations was used to build a strain duplicated for *am*. Both copies of the duplicated gene were subject to the RIP process in crosses of this strain. We conclude that sequence duplications, *per se*, trigger RIP.**

A genetic mechanism that recognizes duplicated chromosomal segments and destroys them has recently been proposed for *Neurospora crassa* (1). Evidence for this proposal came from examination of the sexual transmission of transforming DNA. A set of transformants, each having a single copy of a complex plasmid (pES174) integrated in the genome, was employed. This plasmid consists of four parts: (*i*) the ζ–η region, an unusual 1.6-kilobase (kb) chromosomal region from *Neurospora* that is normally heavily methylated (2) and that serves as a portable signal for methylation *de novo* (3); (*ii*) ≈6 kb of sequences adjacent to ζ–η that are normally unmethylated in the genome ("flank"); (*iii*) a 3.2-kb segment including a selectable marker for *Neurospora (am)*; and (*iv*) bacterial sequences (pUC8). The *N. crassa* strain used as the transformation host (N24) had a deletion removing the entire *am* (glutamate dehydrogenase) gene (4) and, in place of the methylated ζ–η region, had unmethylated DNA partially homologous to ζ–η (5). Thus, the flank region represented the only significant region of homology between the transforming DNA and the host genome. A key observation was that this segment of the transforming DNA was specifically subject to radical alterations during the sexual phase of the life cycle. The position of restriction sites changed, and, in general, cytosines in the region became methylated. Changes occurred specifically during the period between fertilization and nuclear fusion. The process is referred to by the acronym RIP (rearrangement induced premeiotically). It is not yet known whether the apparent "rearrangements" are due to classical rearrangements or simply multiple point mutations. Interestingly, the copy of the flank region contributed by the transforming DNA and the copy of flank from the host were both subject to the RIP process, even in cases where the two copies were unlinked.

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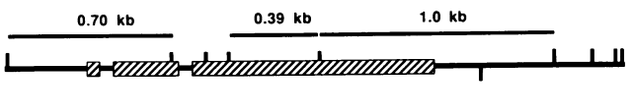

FIG. 1. Restriction map of the 2.6-kb *Bam*HI fragment from the *am* region showing the position of *Sau*3A/*Mbo* I sites (above line) and the *Eco*RI site (below line) (10). The glutamate dehydrogenase coding region, which has two intervening sequences near the 5′ end of the gene, is indicated by the striped rectangles. The *Sau*3A/*Mbo* I fragments detectable by Southern hybridization are indicated (above).

On the basis of these observations, Selker and colleagues (1) suggested that RIP may be a general response of the organism to duplications of chromosomal segments. This would explain observations of non-Mendelian inheritance of transforming DNA in *Neurospora* (6, 7). In addition, it would explain the classical observation that segmental aneuploids are infertile ("barren") in *Neurospora* (see ref. 8).

The hypothesis that the RIP process reflects a mechanism that searches the genome for sequence duplications and alters them explained a number of puzzling observations and seemed likely to be correct. Nevertheless, it remained possible that the association between the observed alterations and the sequence duplications was simply coincidental. For instance, the disruption of the chromosome by the transformation event itself might have triggered the RIP process. Alternatively, foreign (pUC8) or heavily methylated (ζ–η) sequences of the transforming DNA might have been responsible. To investigate these possibilities, it was necessary to test the effect of duplicating a *Neurospora* gene without the complication of adding foreign or methylated sequences to the genome. To control for the possibility that RIP was an effect of disrupting the chromosome by integration of the transforming DNA, we decided to test the effect of introducing just native unmethylated *Neurospora* DNA into a strain harboring a deletion covering the entire transforming sequence. We expected this to be possible in *Neurospora*, since sequence homology does not seem to be required for integration of transforming sequences in this organism (see refs. 3, 9). According to our hypothesis, introduction of multiple copies of the sequence would lead to sequence instability, whereas introduction of a single copy would not. Fortunately, a suitable gene and corresponding deletion strain were available. Kinsey had shown that the am_{132} deletion removes about 7 kb and extends well beyond both ends of the *am* gene (J. Kinsey, personal communication), already cloned by Fincham and colleagues (4).

MATERIALS AND METHODS

Experimental procedures were performed as described (1). Southern hybridizations were probed with the entire 2.6-kb *Bam*HI fragment containing *am* (Fig. 1). DNA size standards were from Bethesda Research Laboratories.

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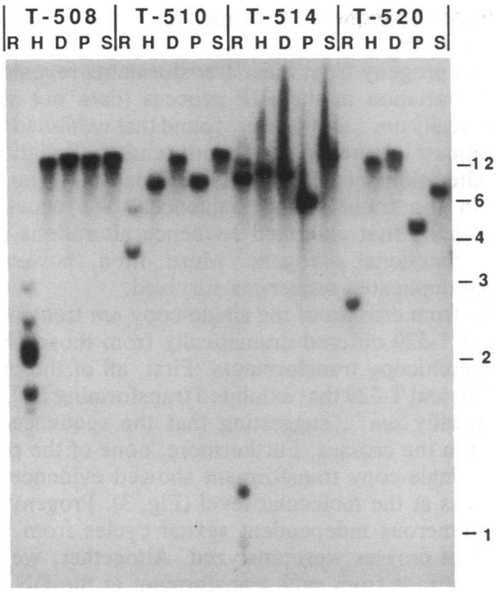

FIG. 2. Characterization of *am* transformants by Southern hybridization. DNA samples isolated from transformants grown in the presence of 5-azacytidine (2) were digested with *Eco*RI (R), *Hin*dIII (H), *Dra* I (D), *Pvu* II (P), or *Sac* I (S), fractionated, and probed for *am*. Positions of selected size standards (kb) are indicated.

RESULTS

RIP Requires Neither Foreign nor Methylated Sequences. In the first phase of the project, we transformed a strain having the am_{132} deletion using a 2.6-kb *Bam*HI fragment containing the entire *am* gene but no sequences represented in the host. Parallel transformations using the *am* fragment either as a linear or as a circular molecule appeared to be equivalent in efficiency. To determine the copy number of the transforming DNA sequences, transformants were characterized by Southern hybridization. Two transformants having single copies of the *am* fragment (T-510 and T-520), one apparently having two copies of the fragment (T-514) and one having approximately four copies of the fragment (T-508), were chosen for the study. Digestion of DNA from each of these transformants with several restriction enzymes that lack sites in the transforming DNA produced just single fragments containing transforming sequences, even using DNA isolated from strains grown in the presence of 5-azacytidine to ensure that the DNA would not be blocked by cytosine methylation (Fig. 2). Apparently, in each case, transforming DNA integrated at one chromosomal position. Digestion of these DNA samples with *Eco*RI, which has a single site in the transforming DNA (Fig. 1), provided a rough indication of copy number of the transforming DNA (Fig. 2).

To investigate the methylation state of the transforming DNA, and to obtain further information on the copy number of these sequences, DNA samples from transformants were digested with the restriction enzyme *Mbo* I or *Sau*3A. Both cleave the sequence GATC when it is unmethylated, but *Sau*3A will not cut the sequence if the cytosine residue is methylated, whereas *Mbo* I will. Restriction digests were fractionated and probed with the 2.6-kb DNA fragment used in the transformations. One of the three *Sau*3A/*Mbo* I fragments large enough to be detected by Southern hybridization (Fig. 1) is entirely contained within the coding region. As expected, each of the transformants showed this 0.39-kb fragment (Fig. 3; lanes P_A). The three transformants obtained by transformation with the *Bam*HI fragment in its linear form (T-508, T-510, and T-514) exhibited the 1.0-kb fragment, whereas the transformant obtained using circularized DNA (T-520) did not. (Integration of the *am* fragment in the T-520 presumably disrupted the 1.0-kb fragment.) The 0.7-kb fragment was not observed in transformant T-510, which was not surprising since this fragment extends to the end of the transforming DNA (Fig. 1). Transformant T-508 exhibited multiple distinct bands in *Sau*3A or *Mbo* I digests, and, based on their relative intensities, some of the bands probably

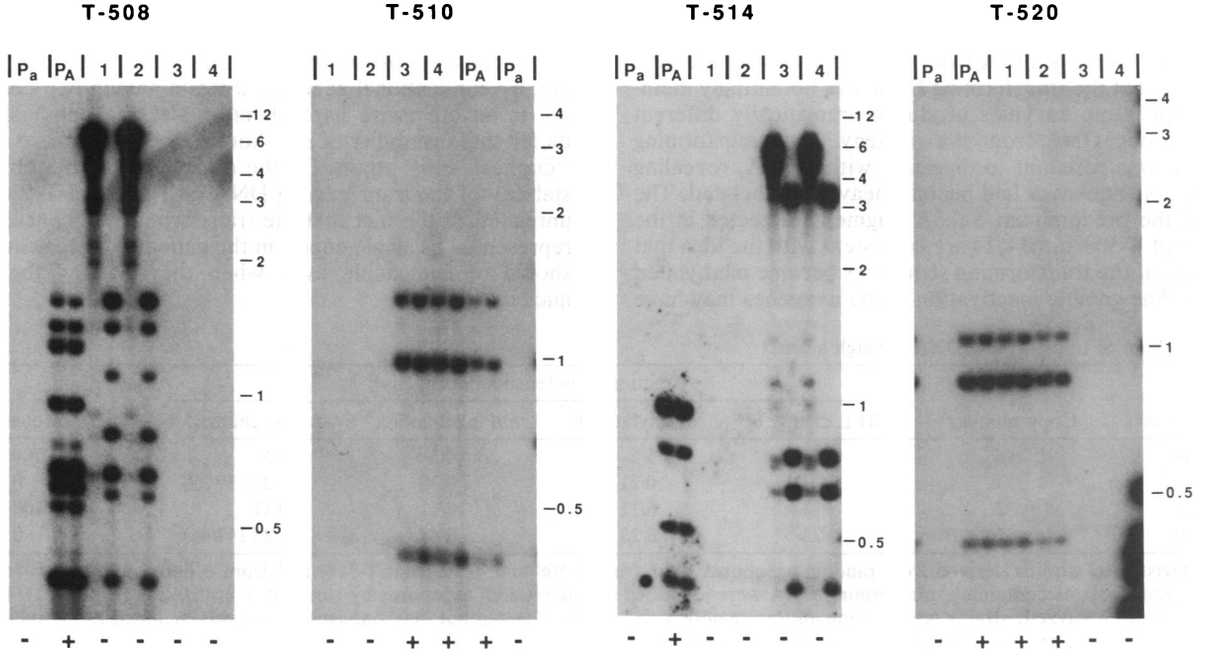

FIG. 3. Fate of transforming sequences through first cross. Transformants were crossed with strain N36 (am_{132}, *in*l, a). Results from a typical tetrad from each of four transformants are shown. (Data are summarized in Table 1.) DNA samples from the parental strains (P_A is transformant; P_a is N36) and four strains representing the meiotic products, in order (1–4), were digested with *Sau*3A (left lane of pair) or *Mbo* I (right lane of pair), fractionated, and probed for the transforming DNA. The *am* phenotype of each strain is indicated below the autoradiograms, and positions of selected size standards (kb) are shown.

reflect the superimposition of several copies of a common fragment. We estimate that approximately four copies of the *am* fragment integrated together in this transformant. At the other extreme, transformants T-510 and T-520 each displayed just two *Sau*3A/*Mbo* I fragments, beside the 0.39-kb internal fragment, indicating that each of these carried single copies of the *Bam*HI fragment. Transformant T-514 exhibited three bands in addition to the 0.39-kb fragment, suggesting that it resulted from integration of two copies of the fragment. Right-junction fragments were not detected for the transforming DNA in T-510 and T-514, presumably because of the very short region of homology between these fragments and the probe. T-514 has two copies of the 1.0- and 0.39-kb internal fragments (Fig. 1).

To test the introduced sequences for the RIP process, we crossed each transformant with a tester strain (N36) having the am_{132} deletion and analyzed the fate of the transforming DNA. *Neurospora* is a coenocytic organism. Consequently, primary transformants typically carry untransformed nuclei, which in a cross give rise to tetrads lacking transforming DNA. Hybridization experiments using the *am* fragment as probe indicated that about one-half of the tetrads from crosses of transformants T-514, T-510, and T-520 and about one-quarter of the tetrads from crosses of transformant T-508 contained transforming DNA (data not shown). The first indication that the RIP process does not depend on integration of methylated sequences or foreign DNA came from scoring the *am* phenotype of progeny from these transformants. Progeny of the multicopy transformants T-508 and T-514 were almost invariably phenotypically am^-, even though a reasonable fraction of them contained transforming DNA (Table 1). To confirm this indication that the *am* sequence is susceptible to RIP, we analyzed progeny at the molecular level. Southern hybridization results from a typical tetrad for each of the transformants are shown (Fig. 3). Progeny of both multicopy transformants (T-508 and T-514) exhibited changes in restriction fragments, diagnostic of the RIP process. Differences observed with *Mbo* I between DNA of primary transformants and progeny indicated that the nucleotide sequence of the transforming DNA had been altered.

Although *Sau*3A and *Mbo* I produced identical restriction profiles in digests of DNA from the primary transformants, indicating that the transforming DNA was not initially methylated, the same enzymes produced dramatically different profiles using DNA from the progeny. The transforming DNA became resistant to digestion with *Sau*3A, revealing that the *am* sequences had become heavily methylated. The sizes of the predominant *Sau*3A fragments detected in the progeny of T-508 and T-514 are consistent with the idea that all copies of the transforming sequences became methylated (Fig. 3). The genetic inactivation of the *am* genes may have been due to the sequence alterations, or to the DNA methylation, or both.

Random progeny from these transformants revealed considerable variation in the RIP process (data not shown). Phenotypically am^- strains were found that exhibited a range of alterations: sequence alterations and methylation, sequence alterations but little or no methylation, or no visible change in the transforming sequences. We occasionally found progeny that exhibited sequence alterations but retained a functional *am* gene. More often, however, no functional duplicated sequences survived.

Results from crosses of the single-copy *am* transformants T-510 and T-520 differed dramatically from those obtained with the multicopy transformants. First, all of the progeny from T-510 and T-520 that exhibited transforming DNA were phenotypically am^+, suggesting that the sequences were unaltered in the crosses. Furthermore, none of the progeny of either single-copy transformant showed evidence of the RIP process at the molecular level (Fig. 3). Progeny representing numerous independent sexual cycles from several independent crosses were analyzed. Altogether, we examined >20 tetrads from each transformant at the DNA level and ≈40 tetrads phenotypically. The data for all four transformants are summarized in Table 1. We conclude that RIP occurred at high frequency in the multicopy *am* transformants but was not evident in the single-copy transformants.

RIP Is Triggered by Sequence Duplications. Our analysis of the primary transformants and their immediate progeny told us that the RIP process is not a response to foreign sequences or methylated sequences. Results of these experiments suggested that copy number of the transforming sequences was the determining factor for the process. Nevertheless, it remained possible that the correlation between copy number and instability was coincidental. For example, one could imagine that disruption of "chromatin structure" might trigger sequence alterations. By this model, our single-copy transformants would represent cases in which integration did not significantly disturb the chromatin.

To address this possibility and to directly test our hypothesis that copy number, *per se*, triggers RIP, we carried out the experiment diagramed in Fig. 4. Transformants harboring single copies of *am* at unlinked sites were crossed to generate, by conventional genetics, a strain having two copies of *am* in an otherwise haploid nucleus. Our hypothesis predicted that instability of *am* should appear in crosses of this "duplicate-*am*" strain. On the other hand, if the observed stability of the transforming DNA of T-510 and T-520 were unrelated to the fact that the transforming sequences were represented as single copies in the genome, these sequences should remain stable even when they occupy the same nucleus.

Table 1. Fate of transforming DNA through a cross

Transformant	Copy number†	Fraction of independent progeny*				RIP frequency, %
		RFL change‡	Methylation§	*am* inactivation¶	No change‖	
T-508	≈4	5/5	5/5	4/5	0/5	100
T-510	1	0/21	0/21	0/39	21/21 (39/39)	0
T-514	2	9/11	6/11	10/11	0/11	100
T-520	1	0/25	0/25	0/44	25/25 (44/44)	0

*Characteristics of strains derived from random ascopores (four from T-508 and eight from T-514) and from ordered ascopores (rest) are summarized. Isolates containing transforming DNA were identified among random ascopores by slot blot hybridization (see ref. 1). For the ordered spores analyzed, strains representing all four meiotic products were tested but only one isolate tetrad is included in the tabulation. No more than two tetrads were analyzed from any given perithecium.

†Estimate from Southern hybridization data.

‡*Mbo* I restriction fragment length alterations in transforming DNA.

§Cytosine methylation at GATC sites detected by comparison of *Sau*3A and *Mbo* I digests.

¶*am* phenotype tested as described (3).

‖Fractions not in parentheses represent progeny tested phenotypically (for *am*) and at the DNA level; fractions in parentheses represent these progeny plus those scored just phenotypically.

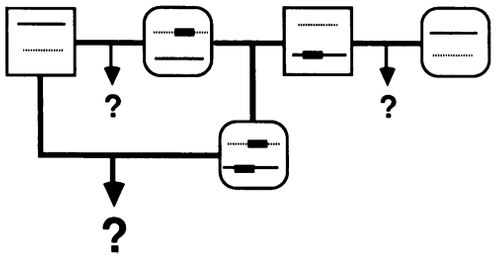

FIG. 4. Scheme to test hypothesis that the RIP process is triggered by sequence duplications. Transformants having single copies of the 2.6-kb *am* fragment (filled rectangles) at unlinked positions (only two of the seven *Neurospora* chromosomes are illustrated) are employed. In a preliminary cross (not illustrated), the transformants (of the same mating type) are crossed with N36 to isolate homokaryotic derivatives of opposite mating types (represented by boundaries with sharp or rounded corners). Though not anticipated, RIP would be detected in these preliminary crosses, which, along with the illustrated intercross (resulting in a strain with two copies of the transforming DNA) and outcrosses (smaller question marks), serve as controls. If the hypothesis were correct, RIP would occur in the cross of the strain having two copies of the transforming DNA (larger question mark).

Since the RIP process operates prior to meiosis, instability was not expected in crosses to build strains having two copies of the gene, and none was detected. All progeny having transforming sequences were phenotypically am^+, and none exhibited altered restriction fragments. The transforming DNA of T-510 and T-520 appeared to segregate independently, producing the required strains having the *am* region from both transformants. Results from a typical tetrad are shown in Fig. 5*A*. When such a strain, carrying two copies of the *am* gene at unlinked positions, was crossed, all hallmarks of RIP were seen. Four of six tetrads isolated from a cross of this duplicate-*am* strain and the am_{132} deletion strain lacked am^+ isolates. Both copies of the *am* gene must have been inactivated in these tetrads. In contrast, no loss of the *am* marker was observed among 22 tetrads from backcrosses of the single-copy strains selected to build the duplicate *am* strain. Examination of representatives of each meiotic product at the DNA level revealed further unmistakable evidence of the RIP process. An example is shown in Fig. 5*B*. Tetrads 4 and 5 exhibited novel *Mbo* I fragments, showing that sequence alterations had occurred. *Sau*3A digests revealed evidence of DNA methylation.

We also crossed the duplicate-*am* strain (C7-3.4), as well as the strains used to build it, with strains carrying the wild-type *am* gene. The phenotypic ratios (am^+:am^-) of tetrads from the cross of a strain having the *am* region of just T-510 gave a distribution expected for independent segregation of the *am* gene of the wild type and that of T-510 (Table 2). All 12 tetrads from the cross of a strain having the *am* region of just T-520 gave a 4:0 ratio of am^+ to am^- isolates, suggesting genetic linkage of the transforming DNA of T-520 and the normal *am* locus. The cross involving the duplicate-*am* strain produced all 2:2 tetrads. Considering the apparent linkage of T-520 transforming DNA and the normal *am* locus, these results indicated that inactivation of *am* had occurred (Table 2). Examination of sample tetrads at the DNA level confirmed that the duplicated *am* sequences of strain C7-3.4 had been altered by the RIP process as in the cross with the *am*-deletion strain. For example, in tetrads 7 and 11, illustrated in Fig. 5*C*, T-520 transforming DNA showed obvious alterations, and the transforming DNA from T-510 and T-520 exhibited heavy methylation. In both tetrads, the two copies of the transforming DNA segregated to the same spores and yet the spores were am^-, indicating that both of the *am* genes had been inactivated. In tetrad 16, T-510 and T-520 transforming sequences segregated to different spores; both

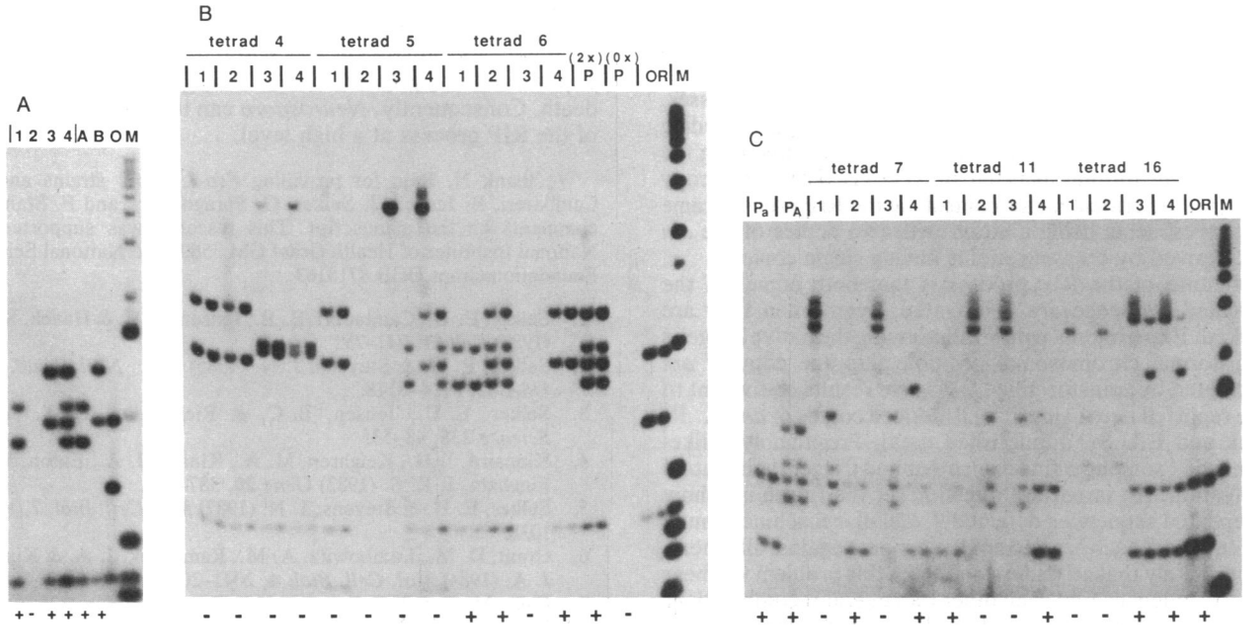

FIG. 5. The RIP process is triggered by sequence duplications. (*A*) Building a strain having two unlinked copies of *am* gene by crossing a pair of transformants, each having a single *am* gene. Homokaryotic derivatives of transformant T-510 (*A*) and T-520 (*B*), obtained in a preliminary cross (Fig. 3), were crossed. Progeny representing the four meiotic products were analyzed for *am* expression (phenotype indicated below autoradiogram) and at the DNA level by using *Mbo* I. Results from just one tetrad, the parental strains, and an Oak Ridge (O) strain having the wild-type *am* region are shown. The bottom 6 size standards (M) are 0.34, 0.40, 0.51, 1.0, 1.6, and 2.0 kb. (*B*) Strain C7-3.4 ("2×P" in *B*, "P_A" in *C*), having two copies of the *am* gene derived by crossing strains having single copies of this gene (*A*), was crossed with strain N36, lacking the *am* region ("0×P" in *B*) or with strain *Fsp-1, Fsp-2, a* (P_a in *C*), having the wild-type *am* region. Progeny from individual tetrads were analyzed for *am* expression (phenotype indicated below autoradiogram) and at the DNA level by using *Sau*3A (left lane of each pair) or *Mbo* I (right lane of each pair). A strain having the wild-type *am* region was included as a control (OR). The three tetrads illustrated in *C* each came from independent perithecia. The bottom 10 size standards (M) are 0.22, 0.30, 0.34, 0.40, 0.51, 1.0, 1.6, 2.0, 3.0, and 4.0 kb for *B*.

Table 2. Phenotypic ratios (am^+:am^-) among tetrads from crosses of *Fsp-1*, *Fsp-2*, am^+ with strains having one (T-510-5.6 and T-520-6.3) or two (C7-3.4) copies of am^+ gene

Strain	Phenotypic ratio				
	4:0	3:1	2:2	1:3	0:4
T-510-5.6	2	8	3	0	0
T-520-6.3	12	0	0	0	0
C7-3.4	0	0	10	0	0

showed sequence alterations as well as DNA methylation. As expected, the *am* gene contributed by the wild-type strain was not subject to RIP.

The same set of tetrads illustrates a feature of the RIP process. Cytosine methylation, when associated with the altered sequences, is characteristically coextensive with the length of the repeated sequences. Segregation of transforming DNA of T-510 and T-520 in tetrad 16 identifies each of the two methylation bands with one copy of the transforming DNA. Based on the size of the methylation bands, it appears that the transforming sequences of T-510 and T-520, which span *Sau*3A fragments totaling ≈2.7 and ≈3.0 kb, respectively (Fig. 3), became methylated at every *Sau*3A site in the repeated sequence (Fig. 5*C*). The scarcity of methylation in the genome overall (11) highlights such methylated patches. We are hopeful that analysis of the frequent association of DNA methylation with the RIP process will give us clues to the cause and consequences of DNA methylation in *Neurospora*.

DISCUSSION

The RIP process is not simply a response to disruption of the chromosome by integration of transforming DNA nor is it due to introduction of foreign, or unusual, sequences into the genome. When a single copy of the *am* gene was inserted into the genome of a *Neurospora* strain having a deletion removing this region, the gene was completely stable, phenotypically and at the DNA level. In contrast, transformants harboring multiple copies of the same fragment (which contained just *Neurospora* sequences) exhibited great instability in crosses. Invariably, the instability occurred in the stage preceding nuclear fusion and meiosis, consistent with results using the complex transforming plasmid pES174 (1). Proof that copy number is the critical factor controlling the RIP process came from experiments using a strain with two copies of the *am* gene, derived by crossing strains having single copies.

A feature of the RIP process is that both copies of the duplicated sequence are inactivated, even when they are unlinked. Experiments with strains having the native *am* gene at its normal chromosomal position plus one copy of *am* contributed by transforming DNA gave results equivalent to those reported here using just introduced copies of *am* (K. R. Haack and E.U.S., unpublished data). Presumably, "like-sequences" somehow find each other and trigger their mutual inactivation. An important question for the future is, "how are repeated sequences detected?" Cellular machinery must efficiently test each segment of the genome against all others. It seems likely that an understanding of this problem will bear on how sequences find each other for general recombination. In general recombination and the RIP process, homologous segments of just several kilobases are sufficient to initiate the respective processes, even when the sequences are unlinked. It is interesting that a high frequency of intrachromosomal homologous recombination was detected at the same stage as RIP in *Neurospora* (1).

We do not yet know the nature of the rearrangements resulting from the RIP process. It seems probable, however, that the RIP mechanism does not randomly scramble sequences. This conclusion is based on the frequent observation of similar "RIP-patterns" among progeny suffering independent rearrangements. Tetrads representing three perithecia (derived from independent fertilizations) from the cross of the duplicate-*am* strain with the am^+ strain provide an example (Fig. 5*C*). An equivalent situation was noticed in the analysis of progeny from primary transformants harboring multiple copies of the *am* fragment (not shown) as well as in previous experiments with pES174 transformants (1). These common alterations probably reflect point mutations which eliminate restriction sites or create new ones.

Whatever the nature of the sequence alterations resulting from the RIP process, the fact that they result in genetic inactivation and occur at high frequency suggests that the process is a powerful evolutionary force. Duplication of any chromosomal segment may unleash RIP. Duplications result, for example, from crosses of strains having terminal or insertional translocations (see ref. 12). Similarly, the RIP process may effectively counter "selfish DNA" including transposons and other amplified or mobile sequences. Sequences need not be native to *Neurospora* to be susceptible to the RIP process (E. Foss, P.W.G., and E.U.S., unpublished data). Nevertheless, it seems likely that some sequences, such as those of rRNA genes, are "immune," or at least resistant, to RIP. Conceivably, protection could be conferred by the nucleotide sequence or by the chromosomal position of a privileged repeated sequence.

It is interesting to consider the possibility that a process equivalent to the RIP process operates in organisms beside *Neurospora*. The mechanism responsible for the process must scan the genome for duplicated sequences and then inactivate them. Thus it could not work in a diploid nucleus such as in a somatic cell of a higher organism or in a nucleus undergoing meiosis. Although both copies of a duplicated gene are inactivated by RIP, duplication of an essential gene does not necessarily lead to cell death in *Neurospora*. The reason for this has to do with the temporal position of the RIP process in the life cycle. Cells experiencing the process have two genetically distinct nuclei, one from each parent. Except in an unlikely situation in which both nuclei harbor the same duplication, complementation should generally prevent cell death. Consequently, *Neurospora* can tolerate the operation of the RIP process at a high level.

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