Specificity of Repeat-Induced Point Mutation (RIP) in Neurospora: Sensitivity of Non-Neurospora Sequences, a Natural Diverged Tandem Duplication, and Unique DNA Adjacent to a Duplicated Region

Eric J. Foss,* Philip W. Garrett,* John A. Kinsey† and Eric U. Selker*

*Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, and 1Department of Microbiology, Molecular Biology and Immunology, University of Kansas Medical Center, Kansas City, Kansas 66103

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ABSTRACT

The process designated RIP (repeat-induced point mutation) alters duplicated DNA sequences in the sexual cycle of Neurospora crassa. We tested whether non-Neurospora sequences are susceptible to RIP, explored the basis for the observed immunity to this process of a diverged tandem duplication that probably arose by a natural duplication followed by RIP (the Neurospora \( c-7 \) region), and investigated whether RIP extends at all into unique sequences bordering a duplicated region. Bacterial sequences of the plasmid pUC8 and of a gene conferring resistance to hygromycin B were sensitive to RIP in \( N. \) crassa when repeated in the genome. When the entire 1.6-kb \( c-7 \) region was duplicated, it was susceptible to RIP, but was affected by it to a lesser extent than other duplications. Only three of 62 progeny from crosses harboring unlinked duplications of the region showed evidence of changes. We attribute the low level of alterations to deletion of mutable sites. The stability of the of the \( c-7 \) region in strains having single copies of the region suggests that the 14% divergence of the tandem elements is sufficient to prevent RIP. DNA sequence analysis of unduplicated pUC8 sequences adjacent to a duplication revealed that RIP continued at least 180 bp beyond the boundary of the duplication. Three mutations occurred in the 200-bp segment of bordering sequences examined.

IN Neurospora crassa, as in many microorganisms, unique sequences make up the bulk of the genome. The paucity of redundant DNA may be partially due to a process that alters duplicated sequences during the sexual cycle of \( N. \) crassa (Selker et al. 1987; Selker and Garrett 1988; Cambareri et al., 1989). This process, referred to as RIP (repeat-induced point mutation), produces numerous G:C to A:T mutations in both copies of duplicated sequences during the period between fertilization and karyogamy. RIP was discovered by tracing the fate of transforming sequences. When transformants containing linked or unlinked duplications of DNA segments were passed through a cross, restriction sites in the duplicated sequences changed at high frequency. A linked duplication never survived a cross unaltered; unlinked duplications escaped RIP at a frequency of \( \approx 50\% \) (Selker et al., 1987; E. B. Cambareri and E. U. Selker, unpublished data). Sequences altered by RIP were frequently found heavily methylated at cytosines. Crosses of strains that carried single copies of the tested sequences resulted in neither sequence alterations nor de novo methylation. Equivalent results were obtained whether the duplications were created directly by transformation or indirectly by crossing strains having homologous sequences at unlinked chromosomal locations (Selker and Garrett 1988; Fincham et al., 1989).

To further explore the mechanism of RIP, we wished to: (1) investigate if the process extends at all into unique sequences bordering a duplication, (2) determine whether the process is limited to Neurospora DNA, and (3) explore the apparent immunity to RIP of the heavily methylated zeta-eta (\( \zeta-\eta \)) region of Oak Ridge strains of \( N. \) crassa (Selker and Stevens 1985, 1987; Selker et al. 1987). The \( \zeta-\eta \) region consists of a diverged direct tandem duplication of a 0.8-kb segment including a 5S rRNA gene. Sequence comparisons of the \( \zeta \) and \( \eta \) 5S rRNA regions with each other and with other 5S rRNA regions suggested that the approximately 14% divergence between the \( \zeta \) and \( \eta \) tandem repeats resulted from exclusively C:G to T:A mutations. Analysis of a related unduplicated, unmethylated sequence from \( N. \) crassa Abbott 4 supported this idea, and indicated that the \( \zeta-\eta \) region almost certainly reflects RIP of a natural duplication (Grayburn and Selker 1989). The distribution of mutated G:C base pairs in the \( \zeta-\eta \) region parallels the distribution that resulted from RIP of an artificially generated duplication. The mutations occurred primarily at sites with an adenine 3' of the changed cytosine, and rarely at sites with a cytosine at this position (Cambareri et al. 1989; Grayburn and Selker 89). Two explanations came to mind for the apparent immunity of the \( \zeta-\eta \) region to RIP: (1) the RIP mechanism could be blind to the \( \zeta-\eta \) region because of the extensive sequence divergence of the duplicate elements, or (2) all of the sensitive G:C pairs
could have already mutated. To test these possibilities we asked whether duplication of the entire \(\zeta-\eta\) region would render the sequences sensitive to RIP. Our results demonstrate that this was indeed the case, but the frequency of RIP was uncharacteristically low. We conclude that the immunity to RIP of the region is a consequence of insufficient homology between the diverged repeats. The relative resistance to RIP of the reduplicated \(\zeta-\eta\) region may be due to depletion of the preferred substrates for this process.

**MATERIALS AND METHODS**

Pertinent information on the primary strains used is listed in Table 1. Strains were grown on Vogel's minimal medium (Davis and DesGarees 1970) with 2% sucrose and other supplements required to allow growth of auxotrophs. All strains tested for RIP were homokaryotic. Crosses were performed on Westergaard's medium following standard techniques (Davis and DesGarees 1970). DNA was isolated from N. crassa strains and analyzed by slot-blot and Southern hybridizations as described previously (Selker, Jensen and Richardson 1987; Selker et al. 1987).

DNA sequencing was performed on double-stranded DNA following the protocol described by Krafft et al. (1988). Plasmid pEC26 was used as the template to sequence the unique sequences of pUC8 (Viera and Messing 1982) adjacent to the duplicated flank sequences of Neurospora transformant T-ES174-1 (T-l) that were altered by two passages through the sexual cycle. This \(\approx 4\)-kb plasmid was derived from pEC24 (Cambareri et al. 1989) by circularization of a HindIII fragment including essentially all of pUC8 and \(\approx 1.5\) kb of the flank region. The sequences reported were determined independently on both strands. To sequence out from the flank region, we used a 17-nucleotide primer (5' TGCTGAGCACTGATGTC) complementary to nucleotides 52-68 (numbering from the first nucleotide of the EcoRI site connecting pUC8 and flank) of the published flank sequence (Cambareri et al. 1989). To sequence the other strand, we used a 16-nucleotide primer (5' ACGGTCTCCGCGC) matching pUC8 sequences 204-219 nucleotides from the EcoRI site. As a control for sequence alterations not associated with crossing the transformant, T-l, for this we used pPG21 as the sequencing template. This \(\approx 3.5\)-kb plasmid was derived from pEC25 (Cambareri et al. 1989) by circularization of a fragment extending from a SacI site \(=0.5\) kb from the EcoRI site at the edge of the flank region to a SacI site \(=0.2\) kb beyond the junction of pUC8 and the am region (see Figure 5A). As an additional control, we sequenced a \(=0.2\)-kb segment of pUC8 sequences in pEC26 and pPG21 that was most distal to the flank duplication (segment a in Figure 5A). This was accomplished using a 20-nucleotide primer (5' GATTGTACTGAGAGTGCACC) matching pUC8 sequences \(=210-230\) bp from the HindIII site of pUC8.

To sequence the pUC8 DNA adjacent to the duplicated flank region of strains LG3:3-4, LG3:6, LG3:6-2 and LG3:6-4 (Cambareri, Singer and Selker 1991), we isolated the DNA region of interest after amplification using the polymerase chain reaction (PCR) with the flank and pUC8 primers described above. The PCR cocktails included 0.5 \(\mu\)g Neurospora DNA, 100 pmol of each primer, 200 \(\mu\)M each of dNTPs, 1 unit "Replinase" DNA polymerase (New England Nuclear), and the buffer supplied with the polymerase. Thirty cycles of 1-min sequential incubations at 94\(^\circ\), 52\(^\circ\) and 72\(^\circ\) were performed.

![Figure 1](image_url)  
**Figure 1.** —Structure of pES174 (Selker, Jensen and Richardson 1987). This plasmid includes the glutamate dehydrogenase gene (am) and adjacent sequences from N. crassa (heavy dashed line), the \(\zeta-\eta\) region (wide solid line), a diverged duplication of a 794-bp segment including a 55 rRNA gene (open box), about 6 kb of adjacent sequences (flank), and the bacterial sequences of pUC8 (wavy line). One degree equals 37.5 bp.

The products were then extracted with chloroform/isooamy alcohol (49:1); precipitated with ethanol and ligated into the Smal site of pBluescript SK\(^{+}\) (Stratagene) using standard techniques. Ligation products were introduced into Escherichia coli strain DH5\(^{+}\)F\(^{ad}^{*}\) (BRL) by transformation. Plasmid DNA isolated from transformants was analyzed first by restriction analysis and then by sequencing using the flank primer. Two independent clones were sequenced for each Neurospora strain. The pUC8 sequences adjacent the flank sequences of U-Gl (Cambareri et al. 1989), which had been previously cloned (Cambareri et al. 1989), were sequenced using the pUC8 primer described above.

Southern hybridizations were performed as previously described (Selker, Jensen and Richardson 1987). The following DNA segments were used as probes: pUC8, entire 2.7-kb plasmid (Viera and Messing 1982) linearized with EcoRI; flank, 4.8-kb EcoRI-BamHI fragment from pES174 (see Figure 5A); \(\zeta-\eta\) region, 0.8-kb BamHI-BamHI fragment from pES174 (see Figure 5A; detects entire 1.6-kb \(\zeta-\eta\) region); am region, 2.7-kb BamHI-BamHI fragment from pJR2 (Kinsey and Rambosk 1984; this fragment covers 2.4 kb of the EcoRI-HindIII am region in pES174).

**RESULTS**

We used several previously characterized single-copy N. crassa transformants obtained with the plasmid pES174 as starting material for this study (Selker, Jensen and Richardson 1987). This plasmid, illustrated in Figure 1, consists of bacterial sequences (pUC8), the glutamate dehydrogenase gene (am) of N. crassa, the \(\zeta-\eta\) region, and approximately 6 kb of DNA adjacent to the \(\zeta-\eta\) region ("flank"). The transformation host, N24, has a deletion (am,\(\zeta,\eta\); Kinsey and Hung 1981) that removes all of the am region represented in pES174, and in place of the \(\zeta-\eta\) region has the unique, unmethylated theta (\(\theta\)) region of a Mauriceville strain (Selker, Jensen and Richardson 1987; Grayburn and Selker 1989). Thus the only region of pES174 with homology in the host genome is flank. Previous Southern hybridization experiments suggested that this region, alone, was altered in single copy transformants, such as T-ES174-1 (T-l), T-
ES174-3 (T-3), T-ES174-5 (T-5) and T-ES174-9 (T-9) (SELKER et al. 1987) when they were crossed. For part of the present study we built strains harboring two copies of pES174 sequences by crossing pairs of these single-copy transformants. We then asked whether all four regions of the integrated DNA were sensitive to RIP in the duplication strains.

The transforming sequences of T-3, T-5 and T-9 had each integrated at a different position in the genome. Their chromosomal locations are unknown, except in T-5, where the sequences are linked to mating type A. The locations of the crossover events within the plasmids are roughly known. In both T-3 and T-5, integration occurred in the flank region of the plasmid, whereas in T-9 integration occurred very close to the am-9 junction (SELKER, JENSEN and RICHARDSON 1987). To obtain strains of appropriate mating types for our experiments, Am+ progeny of crosses from each of these crosses which were analyzed, but the other regions of the plasmid remained unaltered, as expected (data not shown). The chosen strains, designated T-3' and T-9', respectively, did not show alterations in the flank sequences. These strains were then each crossed with T-5 to build strains with a duplication of the pUC8, am, and G-9 sequences, and a triplication of flank. DNA was extracted from random isolates with the Am+ phenotype, digested with BamHI, and analyzed by Southern hybridization to identify those containing the desired duplication (Figure 2). The plasmid sequences of the three transformants are associated with distinctive BamHI fragments, facilitating identification of the duplication progeny. The duplication strains D:3'' + 5' and D:9'' + 5' were selected for subsequent study.

RIP is not limited to Neurospora sequences: Strains D:3'' + 5' and D:9'' + 5' were backcrossed to N36 to determine which sequences of pES174 were susceptible to RIP. Random progeny that contained at least one copy of the transforming DNA were identified by slot-blot hybridizations on cell lysates (data not shown). It was not important to distinguish between strains having single and double copies of pES174 since both copies could have been altered. DNA samples from progeny containing the plasmid sequences and from both parents were digested with Sau3A and MboI. These enzymes both recognize the sequence GATC, but Sau3A is sensitive to cytosine methylation, whereas MboI is not. Thus fragments that appear in Sau3A, but not in MboI digests are indicative of cytosine methylation, which is frequently associated with RIP. Novel MboI fragments represent changes in the DNA sequence. Southern blots were probed separately for all four regions of the plasmid and inspected for differences in restriction patterns between parents and progeny indicative of RIP.

Changes in the restriction patterns of the progeny from D:9'' + 5' are evident in the autoradiograms shown in Figure 3. All but 2 (lanes b and l) of 13 progeny showed alterations of pUC8. Analysis of the cross of D:3'' + 5' produced similar results: 5 of 13 progeny showed alterations of pUC8 sequences (data not shown). We conclude that pUC8 sequences are sensitive to RIP when duplicated. Evidence of RIP was also seen in the am and flank sequences (Figure 3).

As a further test for the effect of RIP on non-Neurospora sequences, we tested the bacterial hph gene, which confers resistance to hygromycin B. We transformed strain J511 with the plasmid pH25, which includes the hph gene driven by the Aspergillus nidulans trpC promoter (CULLEN et al., 1987) and is known to function in N. crassa (STABEN et al. 1989). Hygromycin resistant transformants were made homo- karyotic by repeated isolation of microscopic single conidial colonies after streaking conidia on selective medium. They were then crossed to a (hygromycin B sensitive) wild-type strain (74A-OR23-1VA). Progeny from these crosses were screened for segregation of hygromycin B resistance. The cross of one transformant, T-hyg-1, showed apparently Mendelian segregation of hygromycin resistance; 16 of 40 progeny were resistant to the drug. In contrast, crosses of transformants T-hyg-4, -2 and -3 showed poor transmission of hygromycin resistance, transmitting the trait in 0, 2 or 6 progeny of 40 tested, respectively. When the four transformants were examined at the DNA level by Southern hybridization, using a probe for the hph gene, the strain that transmitted hygromycin resistance in a Mendelian way exhibited a single.

![Figure 2](image-url)
FIGURE 3.—Alterations in pES174 sequences in progeny from cross of pES174-duplication strain D:9"+5'. Genomic DNA (0.5 mg) of the parental strains D:9"+5' (Pn) and N36 (Pc), and their progeny (a–m), were cut with Sau3A or Mbol (left and right lanes, respectively, under each heading) and probed for pUC8, am, the z-η region, or flank sequences, as indicated. Alterations in Mbol or Sau3A restriction patterns of progeny DNA, relative to those of the parents, are indicative of changes in primary sequence or methylation, respectively. All regions of the plasmid except the f-v region show alterations in at least some of the progeny. The scale on the left shows sizes in kb.

The z-η region is resistant, but not immune to RIP: In contrast to the apparent immunity of the z-η region to RIP in crosses of T-3, T-5, T-9 and other strains harboring single copies of this diverged tandem duplication (Selker et al. 1987), alterations were observed in occasional progeny from strains having two copies of pES174 sequences, and thus two copies of the entire z-η region. Three of 52 progeny from D:3''+5' or D:9''+5' (two of thirteen from D:3''+5', and one of 49 from D:9''+5') showed changes in z-η sequences. An example is shown in Figure 4. We have
TABLE 1
N. crassa strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>MT</th>
<th>Description</th>
<th>pUC8</th>
<th>c-7</th>
<th>flank</th>
<th>hyg</th>
<th>Source</th>
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<tr>
<td>74A-OR23-1V</td>
<td>A</td>
<td>wild-type</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>J511</td>
<td>a</td>
<td>am*</td>
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<td>1</td>
<td>1</td>
<td>0</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>SELKER, JENSEN and RICHARDSON (1987)</td>
</tr>
<tr>
<td>N36</td>
<td>a</td>
<td>am1/2 parent</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>SELKER, JENSEN and RICHARDSON (1987)</td>
</tr>
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<tr>
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<td>N24/pES174</td>
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<td>1</td>
<td>2</td>
<td>0</td>
<td>SELKER, JENSEN and RICHARDSON (1987)</td>
</tr>
<tr>
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<td>N24/pES174</td>
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<td>1</td>
<td>2</td>
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<td>SELKER, JENSEN and RICHARDSON (1987)</td>
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<tr>
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<td>F1 of T-3 × N36</td>
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<td>1</td>
<td>2</td>
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<td>T-3a in SELKER et al. (1987)</td>
</tr>
<tr>
<td>T-9'</td>
<td>a</td>
<td>F1 of T-9 × N36</td>
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<td>2</td>
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<td>T-9a in SELKER et al. (1987)</td>
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<td>3</td>
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<tr>
<td>D:9* + 5'</td>
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<td>F1 of T-9' × T-5</td>
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<td>3</td>
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<td>This study</td>
</tr>
<tr>
<td>T-hyg-1</td>
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<td>1</td>
<td>1</td>
<td>4</td>
<td>This study</td>
</tr>
</tbody>
</table>

no explanation for the apparent difference in sensitivity of the reduplicated c-7 region in crosses of the different strains. It is clear, however, that the c-7 region is sensitive to RIP when represented in the genome as two identical copies. In addition, as expected for a sequence already severely altered by RIP, it appears less sensitive to the process than virgin duplicated sequences (i.e., pUC8, am, and flank).

RIP can extend into unique sequences bordering a duplicated sequence: In order to gain insight into the mechanism and consequences of RIP, we wished to determine whether or not RIP ever extends beyond the border of a duplication. The fact that no evidence of sequence alterations in unique DNA adjacent to duplicated sequences has been observed by Southern hybridization analyses suggested that little, if any, mutation occurs outside of the duplicated segments. To explore the range of RIP in more detail, we sequenced ≈200 bp of unique sequences adjacent to a linked duplication of flank in transformant T-1 (Figure 5A), and in the same region after the duplication had been passed through two generations of RIP. The sequence of the DNA rescued from the original transformant exactly matched the sequence of the original plasmid. In contrast, three mutations were found in the DNA rescued from the Lg2:1:2 (CAMBARERI, SINGER and SELKER 1991), the second generation derivative of T-1 (Figure 5B). All three were polarized transitions characteristic of RIP, and

all three occurred in 5'CpA dinucleotides (5'TpG on the opposite chain), the site most susceptible to RIP (CAMBARERI et al. 1989; GRAYBURN and SELKER 1989). The mutations, which occurred about 20, 50 and 180 bp from the edge of the duplication all resulted in C to T changes on the chain shown. We
also examined a segment of pUC8 DNA that was most distant from the duplicated region (segment a in Figure 5A). The sequence of this region in the original transformant was identical to that in the second generation derivative, both matching the known sequence of pUC8 (data not shown).

Strain LG2;1:2 was chosen to check for extension of RIP into unique sequences because the linked duplicated sequences in this strain were known to be extensively mutated (CAMBARERI et al. 1989). We also checked the same region in two other closely related strains (LG6;3:4 and LG6;3:6) which stemmed from the same first generation cross of T-1 that led to strains (LG6;3:4 and LG6;3:6) which stemmed from the original transformant. The sequence is numbered from the EcoRI site connecting the duplicated and unique sequences. In contrast, strain LG2;1:6, the seventh generation derivative of T-3 (CAMBARERI, SINGER and SELKER 1991), showed a C to T mutation at position 52 in the sequence presented in Figure 5B, one of the CpA sites that was also found changed in one of the linked duplication strains (LG2;1:2). We conclude that extension of RIP into unique sequences adjacent to duplicated sequences is not uncommon, and is not limited to linked duplications.

**DISCUSSION**

The RIP process of Neurospora detects and mutates duplicated sequences in the period between fertilization and karyogamy. This process was discovered by following changes in restriction patterns of a 6-kb region of Neurospora DNA ("flank") that is adjacent to a naturally occurring diverged duplication, the η-η region (SELKER et al. 1987). Analysis of the fate of the Neurospora am gene, when duplicated and passed through a cross demonstrated that the duplication, per se, renders sequences susceptible to RIP (SELKER and GARRETT 1988). We show here that this process is not limited to Neurospora DNA. Duplications of sequences foreign to Neurospora led to gene inactivation, and extensive alterations in restriction patterns in crosses. Many progeny exhibited new bands in common. Presumably each new band is due to destruction of a restriction site, or creation of a new site, by a single G:C to A:T mutation. Inspection of the am gene sequence (KINNAIRD and FINCHAM 1983) reveals, for example, that the new 0.78-kb MboI fragment detected in this region can be attributed to destruction of a single site. As expected, alterations of pUC8 sequences by RIP is frequently accompanied by cytosine methylation (SELKER et al. 1987; SELKER and GARRETT 1988; FINCHAM et al. 1989).

The η-η region is almost certainly the result of tandem duplication of a 794 bp segment including a 5s rRNA gene, followed by RIP (SELKER and STEVENS 1985, 1987; GRAYBURN and SELKER 1989). Sequence comparison of the η-η region and its presumptive
unduplicated ancestor suggested that the tandem duplication lost \( \approx 75\% \) of the G:C base pairs at sites that are most susceptible to RIP (Grayburn and Selker 1989). The \( \xi-\eta \) region appears "immune" (or very resistant) to RIP except when reduplicated. This suggests that the approximately 14\% divergence between the tandem elements is sufficient to prevent recognition of the duplication by the RIP machine. Curiously, after two generations of RIP, the closely linked flanked sequences of T-ES174-1 appear more than 20\% divergent (Cambareri et al. 1989; Cambareri, Singer and Selker 1991). Thus, it seems possible that the short length and/or the direct juxtaposition of the duplicated elements of the \( \xi-\eta \) region limited RIP.

Although sensitive to RIP when it was reduplicated, the \( \xi-\eta \) region was still relatively resistant to the process. Only one out of 49 random progeny from D:9' + 5' \times N36 and two of 13 isolates of D:3' + 5' \times N36 showed signs of RIP. It seems likely that the reason for this is that most of the readily mutable sites had already been changed.

We show for the first time in this paper that RIP can extend into unique sequences adjacent to a duplicated region. Three mutations occurred in a 200 bp segment immediately adjacent to a \( \approx 6 \) kb linked duplication that had gone through two crosses. This level of mutation is considerably below that in the duplication. Nineteen mutations occurred in the first 200 bp of the duplication, and 41 occurred in the next 200 bp (Cambareri et al., 1989). The observations that RIP can extend beyond the edge of a duplication and that G to A or C to T mutations on the two strands of some affected sequences are non-randomly distributed (Cambareri et al., 1989; M. Singer, R. Eyre and E. Selker, unpublished observations) are consistent with the idea that its mechanism operates in a processive manner. RIP may operate by deamination of cytosine or 5-methylcytosine residues. According to this interpretation, the three mutations detected outside of the duplication all occurred on the same strand. Curiously, the closest mutations in the adjacent duplicated region would have occurred on the opposite strand. These mutations presumably occurred in a separate round of RIP. It will be interesting to learn what is responsible for the gradual reduction in the frequency of mutations by RIP, as its machinery approaches and then passes the boundary of a duplication.

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LITERATURE CITED


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