Collateral damage: Spread of repeat-induced point mutation from a duplicated DNA sequence into an adjoining single-copy gene in *Neurospora crassa*

MEENAL VYAS and DURGADAS P KASBEKAR*

Center for Cellular and Molecular Biology, Hyderabad 500 007, India

**Corresponding author (Fax, 91-40-27160591; Email, kas@ccmb.res.in)*

Repeat-induced point mutation (RIP) is an unusual genome defense mechanism that was discovered in *Neurospora crassa*. RIP occurs during a sexual cross and induces numerous G : C to A : T mutations in duplicated DNA sequences and also methylates many of the remaining cytosine residues. We measured the susceptibility of the *erg-3* gene, present in single copy, to the spread of RIP from duplications of adjoining sequences. Genomic segments of defined length (1, 1·5 or 2 kb) and located at defined distances (0, 0·5, 1 or 2 kb) upstream or downstream of the *erg-3* open reading frame (ORF) were amplified by polymerase chain reaction (PCR), and the duplications were created by transformation of the amplified DNA. Crosses were made with the duplication strains and the frequency of *erg-3* mutant progeny provided a measure of the spread of RIP from the duplicated segments into the *erg-3* gene. Our results suggest that ordinarily RIP-spread does not occur. However, occasionally the mechanism that confines RIP to the duplicated segment seems to fail (frequency 0·1–0·8%) and then RIP can spread across as much as 1 kb of unduplicated DNA. Additionally, the bacterial *hph* gene appeared to be very susceptible to the spread of RIP-associated cytosine methylation.

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1. Introduction

The genome of the filamentous fungus *Neurospora crassa* is unusual in that it is totally devoid of any active transposable elements (Galagan *et al* 2003). The absence of transposable elements is thought to be due to the operation of a genome defense process called repeat-induced point mutation (RIP). RIP occurs in the haploid nuclei of the premeiotic dikaryon that forms during a sexual cross and induces multiple G : C to A : T mutations and, frequently, methylation of cytosine residues in duplicated DNA sequences that are > 500 bp and share > 80% similarity (Selker 1990). The extent of methylation and severity of RIP are correlated: greater the number of RIP-induced mutations, heavier the methylation (Singer *et al* 1995a). DNA methylation interferes with gene expression by preventing transcription elongation (Rountree and Selker 1997). Only one *N. crassa* strain, isolated from Adiopodoume in Ivory Coast, was found to harbour an active transposable element called *Tad* (Kinsey and Helber 1989). All other Neurospora strains examined (including those of species other than *N. crassa*) contained only RIP-inactivated relics of *Tad* (Kinsey 1990). Strikingly, the Adiopodoume strain also was one of only seven strains that were identified as dominant suppressors of RIP following a screen of more than 400 *N. crassa* wild isolates (Bhat *et al* 2003). This finding supported the idea that RIP protected the genome against the proliferation of transposable elements.

Keywords. DNA methylation; epigenetic silencing; genome defense; RIP

Abbreviations used: ORF, Open reading frame; PCR, polymerase chain reaction; RIP, repeat-induced point mutation.

The only repeated sequences in the Neurospora genome not affected by RIP are the ribosomal RNA genes. The three large ribosomal RNA subunits are encoded by a 9·3 kb DNA sequence. Approximately 170–200 copies of this gene are arranged in a tandem array near one tip of LG V, constituting the nucleolus organizer region (NOR). The immunity of these genes to RIP is a result of their position and is not an inherent property of the rDNA itself as evidenced from the fact that these sequences become susceptible to RIP on being relocated elsewhere in the genome (Galagan *et al* 2003; Selker 1990). There are also ~ 70 copies of the 5S rRNA genes dispersed throughout the genome. They are approximately 120 bp in size. Since RIP is not induced by duplications smaller than 380 bp (Watters *et al* 1999), the resistance of the 5S rRNA genes is most likely due to of their small size.

Although RIP is targeted specifically to repeated DNA, it has been found to occasionally 'spill' across the boundary of the duplicated target sequence into the adjoining non-duplicated sequences. Foss *et al* (1991) sequenced ~ 200 bp of single-copy sequences adjacent to a ~ 6 kb linked duplication before and after passage of the duplication through two generations of crosses. They found three polarized transition mutations (C to T) in the 'after' sequence, about 20, 50 and 180 bp from the edge of the duplication. This level of mutation was considerably below that seen in the duplicated segment (19 mutations in the first 200 bp from the edge and 41 in the next 200 bp), and no mutations were seen in a single-copy segment of similar length located > 2 kb from the duplication. These results suggested that there is a sharp decrease in the mutation frequency as the RIP machinery approaches the edge of the duplication and spills over into the unduplicated sequence. In contrast, Irelan *et al* (1994) reported that a bacterial *hph* gene (conferring resistance to the antibiotic hygromycin) located as a ~ 2 kb single copy sequence between the elements of a ~ 2 kb duplication was inactivated in a cross at much higher frequencies (12–14%). However, it was not clear what proportion of the inactivation was due to the spread of RIP mutations (and therefore irreversible) and what due to the spread of only the RIP-associated cytosine methylation (and therefore reversible). RIP-associated methylation also can extend beyond the duplicated region into flanking sequences (Irelan and Selker 1997; Prakash *et al* 1999). Nevertheless, Irelan *et al* (1994) found RIP mutations as much as 930 bp from the edge of the nearest element of the duplication and Southern analysis indicated that mutations might occur even 4 kb from the duplication boundary.

Perkins *et al* (1997) studied the occurrence of RIP in long segmental duplications (typically > 100 kb) that are obtainable as segregants from crosses between some interstitial or quasi-terminal translocation strains and normal sequence strains and found evidence that RIP mutations might have occurred in loci outside the duplicated segment. Somewhat surprisingly, they also obtained a few mutations in loci that were unlinked to the duplication. Subsequently, results from our laboratory suggested that the *col-18* mutation obtained by Perkins *et al* (1997) from a cross parented by the duplication strain *Dp(VIR > III) OY329* is linked to the duplication but not covered by it (Bhat and Kasbekar 2001). Thus it had become increasingly apparent that single-copy Neurospora genes are indeed susceptible to RIP effects emanating from nearby duplicated sequences.

We sought to measure the susceptibility of a single-copy Neurospora gene to the spread of RIP from a series of adjoining duplications. For this purpose we chose the *erg-3* gene, which encodes the ergosterol biosynthetic enzyme sterol C-14 reductase, as the single copy target. RIP-induced null mutants of *erg-3* are viable and ascospores with mutations in *erg-3* produce colonies with a distinct morphology on Vogel's – sorbose agar medium, which makes them very easy to score under a dissection microscope (Prakash *et al* 1999; Noubissi *et al* 2000). The *erg-3* gene is roughly in the middle of a large contig of 58655 bp whose sequence was determined in the *N. crassa* genome project (Galagan *et al* 2003). Its protein coding sequence is between nucleotides 23338 (third base of the stop codon on the inverted complementary strand) and 24897 (first base of the start codon, Papavinasasundaram and Kasbekar 1994). We used PCR to amplify defined segments of this contig and used the amplified DNA to construct duplication strains by transformation. Crosses made with these duplication strains would induce RIP in the duplicated segments and the frequency of *erg-3* mutants in the progeny would provide a measure of RIP spread into the adjoining single-copy sequence.

2. Materials and methods

2.1 *Strains and growth conditions*

The *N. crassa* strains of the standard Oak Ridge background 74-OR23-1 *A* (FGSC 987) and OR8-1 *a* (FGSC 988) and the mutant strain *erg-3 a* (FGSC 2725) were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City, KS 66160–7420, USA. Crossing and maintenance of Neurospora strains was essentially as described by Davis and De Serres (1970). Hygromycin resistance was scored by streaking conidia onto 1·5% agar plates containing Vogel's N medium plus 'sorbose' (0·05% fructose, 0·05% glucose and 2% sorbose) and supplemented with hygromycin B (Sigma) at 200 μg/ml made from a 100 mg/ml aqueous stock solution. Only strains expressing the *hph* gene could grow on hygromycin medium.

2.2 *Oligonucleotide primers, polymerace chain reaction and construction of plasmids used for transformation*

The primers used for the polymerace chain reaction (PCR) amplification of segments of the contig were made in the CCMB oligonucleotide synthesis facility and are described in table 1. Primers designated F or T represent those from the 5′ or 3′ side of the *erg-3* open reading frame (ORF), respectively, and C or N indicate primer sequences from the coding or non-coding strand, respectively. The table 1 also indicates the position of the 5′ base of the primer in the numbering scheme in which the *erg-3* coding sequence is located between nucleotides 23338 and 24897. The reaction conditions used for the PCR were a 4 min denaturation at 94° followed by 30 cycles of 1 min denaturation at 94°, 1 min annealing at 55° and 1 min elongation at 72°. The amplified DNA was purified by gel electrophoresis and cloned into the vector p*CSN 43* which carries the bacterial *hph* gene for resistance to the antibiotic hygromycin (Staben *et al* 1989).

2.3 *Transformation and construction of duplication strains*

The *erg-3 a* strain was grown in Vogel's medium N supplemented with 1·5% glucose for 10 days and the conidia were harvested in sterile water and filtered through a cheese cloth. The conidia were pelleted by centrifugation, washed twice in 30 ml of 1 M sorbitol and resuspended in 1 M sorbitol at 3×10^9/ml. Approximately 500 ng of plasmid DNA was added to 40 µl of the conidial suspension and electroporated in a 0·2 cm cuvette (BTX) in a BioRad Genepulser. The conditions used were 1·5 KV, 25 µF and 600 Ω. After the pulse, 960 µl of 1 M sorbitol was added and 500 µl of the transformation mix was plated on a Vogel's-sorbose plate containing 220 µg/ml hygromycin. Transformants could be picked after incubation at 30° for 3–4 days. The transformants were crossed to the wild-type strain 74-OR23-1 *A* to obtain the *hph*-marked transgenes in the *erg-3*$^+$ background.

Each primary transformant represents a unique transgene that duplicates the genome segment in that transgene. Duplications of segments from the 5′ and 3′ side of the *erg-3* ORF are designated by the letters F and T, respectively. The distance between the *erg-3* ORF and the closest boundary of the duplication is indicated by the designations A, B, C and D, and are respectively, 0, 0·5, 1 and 2 kb. Numerals 1, 1·5, 2 and 2·5 indicate the length of the duplicated segment. To control against possible transgene position effects we examined more than one transgene per duplication (except TB1·5). A schematic representation of the duplicated segments vis-a-vis the *erg-3* ORF is given in table 2 and the primers used for the amplification of each segment are also indicated. Southern analysis was done to confirm the presence of each transgene in the *erg-3*$^+$ segregants (data not shown).

2.4 *Ascospore collection and determination of erg-3 mutation frequencies*

Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Generally ascospores began to be shot within 16–18 days. Since it is known that RIP frequency increases with the age of the cross (Singer *et al* 1995b), *erg-3*

Table 1. Primers used to amplify genomic segments form the neighbourhood of *erg-3*.

Primer	Sequence	Start base
FC1	ATG CTG GCT GTG ATG CTG TA	24902
FN1	GGT GAT GAT TGT GAC TCG TGG	25896
FC2	CTG CCC CTC ATG TCC AAT CT	25409
FN2	TAC CAG GCA CCG ACC TTG	26398
FC3	ACC ACC ACC AAC CAT ACC ACG AGC	25901
FN3	GAA AAC ACC ACC AAT GAC GTA CAC	26898
FC4	CTA CCA ACG CAC CGT CGT GAA GCA	26898
FN4	AAC CCT CCG GTT CCG AAC GTT TTG	27897
TC1	GGA ACA ATC TAG AAT AGG TAG AGC A	22343
TN1	CCG ATG GAT AAT GTT CTG TGG	23343
TC2	CGG CTT CTC CCTGATACTAGC	21843
TN2	CAG TGC GTC TCC ATCCAG	22843
TC3	TAT TGG ATT CCC AGA TGT TAA GGA	21127
TN3	GTC TAC ACC TAC CGC GAC AAC ACC	22105
TC4	CAT TGC ATC AGA GAT GGT CGC CTC	20129
TN4	ACC CTC CCG TCC CAT AAT ACC GAT	21128

mutation frequencies were determined in ascospores harvested after 31 days. Ascospores were harvested by washing the lids with ~ 1 ml water. The proportion of colonies with the *erg-3* colony morphology was determined under a dissection microscope.

3. Results and discussion

3.1 *Spread of RIP*

The 46 duplication strains were crossed with the wild type strains 74-OR23-1 *A* or OR8-1 *a*. The duplicated segments were from the 5′ or 3′ side of the *erg-3* ORF and were 1 kb, 1·5 kb or 2 kb long. Seventeen duplications were of type A (that is, the boundary of the duplicated segment lay within 5 bp of the *erg-3* ORF), 18 were of type B, five type C and six type D (in which the ORF and the duplications were separated by 500 bp, 1 kb or 2 kb, respectively). It was expected that RIP would be induced in the duplicated DNA segments and the frequencies of *erg-3* mutant progeny would provide a measure of the spread of RIP from the duplicated segment into the single-copy *erg-3* gene.

Mutant *erg-3* progeny were recovered in seven crosses, five involving type A duplications, and one each with duplications of types B and C (table 2). These seven crosses yielded *erg-3* mutation at frequencies in the range 0·1–0·8%. Mutation frequencies below 0·1% are not easy to score, therefore we have presumed that the *erg-3* mutation frequencies in the remaining 12 crosses with type A, B and C duplication strains are probably very slightly below our detection limit. Our results show that RIP can indeed spread across a distance of as much as 1 kb of single-copy DNA, although all the observed frequencies were considerably below those seen in crosses duplicated for the *erg-3* coding sequence (range 2–20%, Noubissi *et al* 2000).

The type A duplications cover non-coding segments of the *erg-3* gene that are immediately upstream or down stream, of the start and stop codons. Therefore in crosses with such duplications *erg-3* mutations can potentially be generated either by RIP in the non-coding segments or by the spread of RIP from the duplication into single-copy ORF sequence over distances of as little as 20 bp beyond the duplication boundary. Despite this proximity, the *erg-3* mutation frequencies were below detectable levels in crosses with 12/17 type A duplication. Moreover, the mutation frequencies in the five crosses which produced any *erg-3* mutants did not differ very much from that of the B and C type crosses which yielded *erg-3* mutants. We interpret

Table 2. Frequency of *erg-3* mutants induced by the spread of RIP.

Primers	Frequency of *erg-3* mutants
ATG TAG *erg-3*	
TC1–TN1 TAI	< 0·1, 0·1, 0·2
FC1–FN1 FA1	< 0·2, 0·6, 0·8
TC1–TN2 TA1.5	< 0·2, < 0·2
FC1–FN2 FA1·5	< 0·2, < 0·2
TN1–TC3 TA2	< 0·2, < 0·2, < 0·3
FC1–FN3 FA2	< 0·3, < 0·2, < 0·3, 0·8
TN2–TC2 TB1	< 0·2, < 0·3, < 0·3
FC2–FN2 FB1	< 0·2, < 0·2, < 0·3
TN2–TC3 TB1·5	< 0·2
FC2–FN3 FB1·5	< 0·1, < 0·2, < 0·2, < 0·3, 0·7
TN2–TC4 TB2·5	< 0·1, < 0·2, < 0·3
FC2–FN4 FB2·5	< 0·3, < 0·3, < 0·3
TN3–TC3 TC1	< 0·2, < 0·2, < 0·3
FC3–FN3 FC1	< 0·1, 0·2
TN4–TC4 TD1	< 0·1, < 0·2, < 0·2
FC4–FN4 FD1	< 0·2, < 0·2, < 0·2

these results to suggest that ordinarily RIP does not spread into single-copy DNA. However, such control occasionally fails, and when this happens RIP can spread across as much as 1 kb of single-copy sequences. In other words, the spread of RIP might be more a function of the probability of failure of the mechanism(s) that normally confine RIP to duplicated sequences. In which case the duplication size (at least in the 1–2 kb range) or its distance from the target gene (in the range 0–1 kb) might not be as important. Consequently, sequences located just a few base-pairs away as well as those separated by as much as 1 kb can both become equally susceptible to alteration by RIP-spread.

Another conclusion that can be drawn from our results is that the non-coding segments of the *erg-3* gene must contain relatively few sites whose mutation can disrupt function. An earlier study in our laboratory revealed that the *STR1* gene, an *erg-3*-homologue from the phytopathogenic fungus *Nectria haematococca* MP VI, can be expressed in *N. crassa* even if only 70 bp of its 5′ non-coding sequence are present (G Srinivas and D P Kasbekar, unpublished results). It would seem that 70 bp was insufficient for a 5′ untranslated mRNA region and a promoter. In other words, very little more than an intact ORF might be required for wild-type function. In which case most of the *erg-3* mutants induced in crosses involving type A duplications probably arose from alteration of the *erg-3* ORF by RIP-spread, rather than by RIP mutation of the non-coding regions.

3.2 *Spread of RIP-associated cytosine methylation*

We performed a cross that was homozygous for one of the FA1 transgenes (the one that produced *erg-3* mutants at a frequency of 0·8% in the cross with the wild type). In this homozygous cross, RIP-spread resulted in the production of *erg-3* mutants at a frequency of 2%, which is roughly twice that of the hetrozygous cross. Of 40 *erg-3* segregants examined, 26 were resistant to hygromycin and 14 were hygromycin-sensitive. Seven of 14 hygromycin-sensitive segregants examined were all found to have silenced the *hph* gene in a reversible manner. We assume that the reversible silencing of *hph* is caused by the spread of RIP-associated cytosine methylation within the transgene. The silencing of *hph* appeared to occur in 35% of progeny inheriting a RIP-mutated *erg-3* allele. Since the *hph*-marked transgene was unlinked to *erg-3*, we can conclude that 70% of the nuclei in which RIP-spread inactivates *erg-3*, also cause inactivation of *hph* by spread of RIP-associated methylation. Although 0·7% of the *erg-3*$^+$ segregants also were expected to be hygromycin-sensitive (0·02 × 0·7 × 0·5), none of the 520 *erg-3*$^+$ segregants examined showed the hygromycin-sensitive phenotype. It has been reported previously that the *hph* gene can become spontaneously methylated even during vegetative growth (Pandit and Russo 1992), therefore we suspect that this also makes *hph* very susceptible to the spread of RIP-associated cytosine methylation.

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