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The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*

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ABSTRACT

Post-transcriptional gene silencing (PTGS) involving small interfering RNA (siRNA)-directed degradation of RNA transcripts and transcriptional silencing via DNA methylation have each been proposed as mechanisms of genome defence against invading nucleic acids, such as transposons and viruses. Furthermore, recent data from plants indicates that many transposons are silenced via a combination of the two mechanisms, and siRNAs can direct methylation of transposon sequences. We investigated the contribution of DNA methylation and the PTGS pathway to transposon control in the filamentous fungus *Neurospora crassa*. We found that repression of the LINE1-like transposon, *Tad*, requires the Argonaute protein QDE2 and Dicer, each of which are required for transgene-induced PTGS (quelling) in *N.crassa*. Interestingly, unlike quelling, the RNA-dependent RNA polymerase QDE1 and the RecQ DNA helicase QDE3 were not required for *Tad* control, suggesting the existence of specialized silencing pathways for diverse kinds of repetitive elements. In contrast, *Tad* elements were not significantly methylated and the DIM2 DNA methyltransferase, responsible for all known DNA methylation in *Neurospora*, had no effect on *Tad* control. Thus, an RNAi-related transposon silencing mechanism operates during the vegetative phase of *N.crassa* that is independent of DNA methylation, highlighting a major difference between this organism and other methylation-proficient species.

INTRODUCTION

In many organisms, transposon-related sequences make up a large portion of the genome. There is evidence that some of these sequences have evolved to play important roles in heterochromatin formation, centromere function and gene regulation (1–3). On the other hand, the cell has developed mechanisms to limit the expansion of these elements in order to prevent excessive genome instability. The relatively recent discovery and subsequent dissection of post-transcriptional gene silencing mechanisms (RNAi, cosuppression, quelling) that can act to silence repeated sequences has led to a model in which a double-stranded RNA intermediate, homologous to the targeted gene is processed by the RNAseIII molecule Dicer into siRNAs of 21–25 nt in length (4). siRNAs are subsequently used as guides by the RNA-induced silencing complex (RISC) to degrade homologous transcripts (5). This general model is conserved in a wide range of organisms, thus one proposed explanation for the retention of post-transcriptional gene silencing (PTGS) has centred on a role in limiting the expansion of naturally occurring repeated sequences, such as transposable elements (6). A number of observations support this hypothesis: in *Caenorhabditis elegans* and *Chlamydomonas*, several genes are essential to both RNAi- and transposon control-pathways (7–9); siRNAs against transposon sequence have been cloned in both *Arabidopsis* and *Drosophila* (10,11). These findings all suggest that there is an ongoing control of transposon proliferation in the host.

In addition to PTGS, transcriptional gene silencing (TGS) mechanisms such as DNA and histone methylation have also been implicated in transposon control. Large swathes of the human and *Arabidopsis* genomes are methylated, and these methylated areas are rich in transposon-related sequence

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(12–14). Moreover, mutations which block DNA methylation lead to increased transcript levels and increased transposition rate of both DNA transposons and retrotransposons (12,15–18). Remarkably, in fission yeast and plants, it has been demonstrated that heterochromatin formation is directed by siRNAs in an Argonaute complex with similarities to the RISC involved in RNAi, suggesting that the processes of PTGS and TGS are intertwined (2,16,19–21).

The phenomenon of 'quelling' in *Neurospora* displays most of the characteristic features of PTGS observed in other organisms (22). In addition to quelling however, *Neurospora* possesses additional homology-dependent gene silencing mechanisms such as repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD) (23,24). In particular, RIP is able to inactivate duplicated DNA sequences by causing multiple C:G to T:A mutations and cytosine methylation at the pre-meiotic, dikaryotic sexual phase of the life cycle. That the *Neurospora* genome is littered with RIP-mutated relics of a wide range of transposon families is testament to the efficiency of RIP. Indeed, the *Neurospora* genome does not reveal the presence of a single active transposon (25).

Despite this, transposition of the LINE1-like, non-LTR retrotransposon *Tad* has been detected in an African strain, Adiopodoumé, which contains up to 40 copies of this element (26,27). Subsequent analysis showed that this strain was RIP-proficient and that the *Tad* elements in this strain were fully susceptible to RIP (28,29). To search for an explanation as to why *Tad* remained active in the RIP-proficient Adiopodoumé strain, we considered the possibility that additional silencing mechanisms, such as quelling, might also be employed in transposon control in *Neurospora*. To explore this hypothesis, we examined whether Adiopodoumé was quelling-proficient and also monitored the rate of *Tad* transposition in diverse quelling-defective mutants. A role for DNA methylation in limiting the expansion of artificially introduced *Tad* elements in *Neurospora* has also been documented (30). Therefore, in light of the above observations (in *Schizosaccharomyces pombe* and *Arabidopsis*) of a link between PTGS and TGS mechanisms, we investigated the relative effects of quelling and DNA methylation on transposon control in *Neurospora*, and whether these two mechanisms are connected in this species.

We found that the expansion of an experimentally introduced *Tad* element was particularly pronounced in a *qde-2* null background, lacking an Argonaute protein thought to constitute part of the RISC in *Neurospora* (31). This was evidenced both in terms of increased transcript levels and increased copy number of the *Tad* elements. Importantly, a similar effect was confirmed when we introduced a *qde-2*$^{-}$ allele into progeny of a strain harbouring a natural invasion of TAD. Additionally, siRNAs against *Tad* sequence were found, suggesting that a quelling-like mechanism is responsible for the taming of transposable elements in *N.crassa*. In support of this, *Tad* transcripts accumulated heavily in strains lacking both of the *Neurospora* DICER-LIKE proteins. However, in contrast to quelling, QDE1 (an RdRP) and QDE3 (a DNA helicase) were not required suggesting that, although similar, the quelling- and transposon control-pathways do not entirely overlap (32,33). Notably, we found that the major bulk of introduced *Tad* elements were not significantly methylated, and furthermore the absence of the DNA methylation machinery had no obvious effect on *Tad* expression, indicating that in *Neurospora*, unlike several other organisms, there is no RNAi-directed DNA methylation mechanism for transposon control and instead suggesting that the major determinant is post-transcriptional.

MATERIALS AND METHODS

N.crassa strains: The wild-type (WT) strain 74-OR23A and Adiopodoumé were obtained from the Fungal Genetics Stock Center, University of Kansas, Kansas City (strains FGSC 987 and FGSC 430, respectively). The *dim-2*$^{-}$ strain (34) was a gift from Eric Selker, University of Oregon. The *qde* and *dcl* mutant strains used in this study were obtained by insertional disruption by plasmid insertion, have been previously described in detail elsewhere, and were as follows: 627 (*qde-3*$^{-}$), 820 (*qde-2*$^{-}$), 107 (*qde-1*$^{-}$) DCL1ko (*dcl-1*$^{-}$), DCL2ko (*dcl-2*$^{-}$) (31–33,35). The double Dicer mutant strain was obtained by the crossing of *dcl-1*$^{-}$ and *dcl-2*$^{-}$ strains.

Transformation and growth conditions

Strains were grown in Vogel's minimal medium for *Neurospora* (NMM), as described elsewhere (36). Ascospores from crosses were heat-activated to induce germination at 60°C for 30 min, as previously described (37). For *Tad* transformation experiments, spheroplasts were prepared from the mutant and wild-type strains using the method of Orbach *et al.* (38). Each strain was co-transformed with 1 μg of the plasmid pCSN44.1, which contains the *hph* hygromycin-resistance gene as a selectable marker (39), and 5 μg of the plasmid pTad1-1, containing a full-length cloned *Tad* element (40). Transformations were plated on NMM containing 0.2 mg/ml hygromycin and at least 10 individual colonies from each transformed strain were then purified by three serial platings to purify for homokaryons. Purified colonies were then transferred to solid slant NMM media and arbitrarily referred to as the first asexual 'generation'. Subsequent generations were propagated by serial transfer of conidia after three days of vegetative growth on slants. Each generation was then grown in liquid NMM for three days, with shaking, at 28°C to produce sufficient mycelia for nucleic acid extraction. Differences in the *Tad* load between the transformed mutant strains were compared using the Student's *t*-test (two-tailed).

Northern and small RNA analysis

Small RNA purification was performed as described previously (41). A riboprobe was transcribed using T3 RNA polymerase (Promega) on a *Tad* PCR template that contained a T3 promoter site at the 5′ end. To produce a template for ORF1, the primers used were ORF1aT3 (5′-CGCGAATTAACCCTCACTAAAGGGAGAAACTGCTTT-3′) and ORF1b (5′-CCAAGGCAGCAACAGTAC-3′), while for ORF2 the primers used were ORF2aT3 (5′-CGCGAATTAACCCTCACTAAAGGGATACTGTATTGGAACGTG-3′) and ORF2B (5′-GGTCTCGTCTGCGAAGCCG-3′), where T3 promoter sites (including adaptor region) are underlined. Prior to hybridization, labelled transcripts were hydrolysed to an average size of 50 nt, 15 volumes of 80 mM sodium bicarbonate and

120 mM sodium carbonate were added to the transcriptional reaction and incubated at 60°C for 3 h. To stop the hydrolysis, 20 µl of 3 M sodium acetate (pH 5.0) was added. Prehybridization and hybridization were at 35°C in 50% deionized formamide, 7% SDS, 250 mM NaCl, 125 mM sodium phosphate (pH 7.2), and sheared, denatured, salmon sperm DNA (100 mg/ml). After overnight hybridization, membranes were washed twice in 2× SSC and 0.2% SDS at 35°C for 30 min and once in 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 60 mM sodium chloride and 10 µg/ml RNase A at 37°C for 1 h to remove unspecific background. Northern analysis of *Tad* expression was performed using standard protocols (42). A PCR product comprising nucleotides 54–1569 of *Tad* used as a template to create a ^{32}P-labelled DNA probe. In each blot, ~5 µg of total RNA was loaded per lane. Equal loading was ensured by controlling ethidium bromide staining of the constitutively expressed 28S ribosomal RNA. In comparing transcript levels between different *Tad* containing samples, densitometric analysis of the hybridization signal (minus control background) corresponding to the full-length 7 kb *Tad* transcript was performed using the Instant Imager Analysis software programme (Canberra Packard). The values obtained from this analysis were then expressed as a function of *Tad* copy number to compensate for unequal segregation of *Tad* elements during the crossing procedure and values were compared between strains using Student's *t*-test (two-tailed).

Southern blot analysis

Genomic DNA was isolated from frozen mycelia and ~4 µg were digested and run according to standard protocols. For methylation analysis, the isoschizomer pair of Dpn11 (methylation-insensitive) and Sau3A1 (methylation-sensitive) were used. To analyse copy number, we digested either with Nde1 and used an internal 602 bp Nde1 *Tad* fragment in order to produce a single hybridizing band whose intensity was proportional to the copy number of *Tad* (Figure 1), or we digested with EcoR1 and used a PCR probe comprising nucleotides 54–1569 of *Tad* that hybridizes to a band unique for each *Tad* insertion (not shown). An estimate of copy number was calculated by comparing the total signal in each lane [minus control (non-transformed)

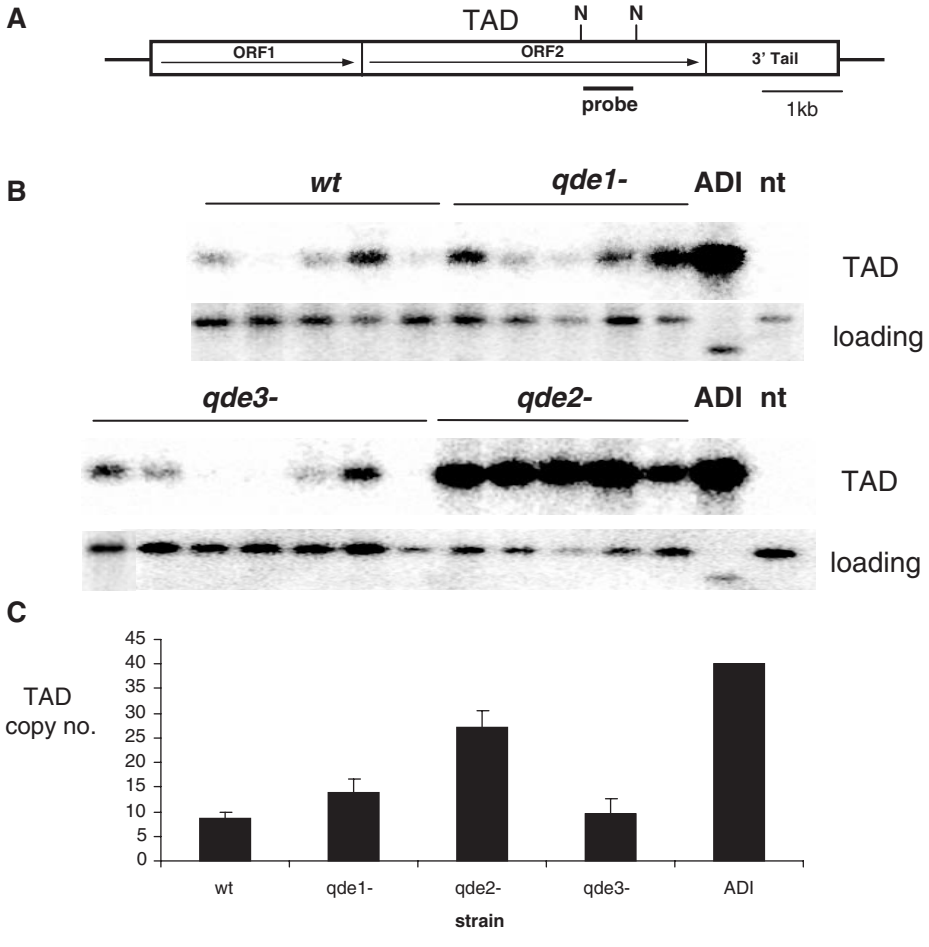

Figure 1. Tad transposition shows a marked increase specifically in a *qde2* mutant background. (**A**) Schematic representation of the *Tad* element. Nde1 (N) sites and the probe used in the Southern-blot analysis in (B) are indicated. (**B**) Representative Southern blots in *Tad*-transformed wild-type (*wt*), *qde-1*$^-$, *qde-3*$^-$ and *qde-2*$^-$ strains showing hybridization to the Tad probe or the single copy al-2 gene used as a loading control (this gene shows a restriction polymorphism in Adiopodouméé). DNA from a non-transformed wild-type strain (nt) and the Adiopodoumé strain (ADI) containing 40 *Tad* copies were included as controls. (**C**) The mean copy number of *Tad* in each strain after 15 asexual generations is shown. A minimum of 10 independent transformants were analysed for each strain. Error bars represent standard error of the mean.

background] to that of Adiopodoumé DNA, which is reported to have 40 copies of the *Tad* element (26). As a loading control, all blots were stripped and re-hybridized with either a probe corresponding to a 1397 bp Sph1 fragment upstream of the single copy *dcl-1* gene or a 1213 bp Nde1–BamH1 fragment of the single copy *al-2* gene. The relative strength of the signal was used to adjust the estimated copy number in each strain.

Tad hybridizations were performed at 68°C in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulphate, 100 μg/ml sheared salmon sperm DNA. After hybridization, two very stringent washes of 40 min at 68°C in 0.1× SSC, 1% SDS were included in order to minimize hybridization to RIPed *Tad* relics. In crosses of the mutant strains with either qde2TAD8 or Adiopodoumé, the mutant status of the daughter progeny was checked in each case using probes for *qde-1*, *dcl-1*, *dcl-2*, *qde-2*, as previously described (31,32,35). The probe used to hybridize the ζ–η region when controlling the *dim-2* status was amplified from genomic DNA using the primers zeta-F (5′-CGATTAGCGAATCCTAAGTG-3′) and zeta-R (5′-TTTCTACCATCTATAGCCG-3′).

RESULTS

Adiopodoumé is quelling proficient

To explore the hypothesis that a deficiency in quelling might explain the apparent expansion of *Tad* in Adiopodoumé, we tested for quelling proficiency by using an assay that involves transformation with a segment of the *al-1* gene involved in carotenoid biosynthesis. In quelled strains, silencing of the endogenous gene leads to an albino phenotype. The frequency of quelling in Adiopodoumé was comparable to a wild-type strain (Table 1), ruling out the possibility that *Tad* expansion in Adiopodoumé could be accounted for by a quelling deficiency.

Tad expansion in the *qde* mutants

To ascertain whether the quelling pathway contributed to control of *Tad* transposition, we investigated the expansion of a cloned *Tad* element in various *qde* mutants. Previous work in our lab had identified three genes essential for quelling: *qde-1*, encoding an RNA-dependent RNA polymerase (RdRP) thought to produce a dsRNA template for Dicer (32); *qde-2*, encoding a PAZ-Piwi domain protein constituting part of RISC (31); *qde-3*, encoding a recQ DNA helicase thought to be upstream of *qde-1* (33). We used a cloned full-length version of the *Tad* element, *Tad*1-1 (40), to transform strains that contained insertional disruptions of each of the *qde* genes. As a control, the wild-type reference strain ORS74a was also transformed. We grew single transformants until conidiation, at which point they were germinated on fresh media and allowed to grow again until conidiation and the cycle was repeated. We referred to each of these vegetative cycles as an asexual 'generation'. *Tad*-transformed strains were propagated in this way for up to 15 generations, and the copy number and expression levels of *Tad* were analysed at various timepoints. We performed Southern blots using a probe hybridizing to a single internal Nde1 fragment of the *Tad* element (Figure 1A), the intensity of which is proportional to number of *Tad* elements in the genome. A representative blot is shown in Figure 1B. We found that in the *wt* strain, the mean number of *Tad* copies increased over the course of 15 asexual generations to ∼10 copies per genome (Figure 1C). A similar situation was observed in the *qde-1*$^-$ and *qde-3*$^-$ mutants. However, in a *qde-2*$^-$ mutant background *Tad* showed a very significant increase in transposition rate ($P < 0.01$), approaching 30 copies per genome. These results show that while *Tad* is able to achieve a limited rate of transposition in a *wt* background, this transposition is normally restricted by *qde-2*. Similar results were confirmed when we repeated the experiment using independently created u.v mutants (data not shown). We further analysed the transformed strains by looking at levels of *Tad* transcripts in each. Again, in the *wt* background we found a basal level of *Tad* transcription that was similar in *qde-1*$^-$ and *qde-3*$^-$ backgrounds, while in the *qde-2*$^-$ mutants the level of transcription was markedly increased, as expected from the high copy number in this strain (not shown).

Table 1. Adiopodoumé is quelling proficient

	Wild-type	Adiopodoumé
Orange colonies	222	231
White colonies	99	85
Quelling efficiency (%)	31	28

Specific role for *qde-2* in transposon control in *Neurospora*

Although the above results suggested a role for *qde-2* in the control of *Tad*, we could not rule out that differences in expression and transposition rate were accentuated by varying initial transformation efficiencies with the cloned *Tad* element between the different mutant strains, either in terms of number or arrangement of transgenes, or possible insertions in transposition 'hotspots'. In order to definitively confirm a role for the *qde-2* gene in *Tad* control, we took a *Tad*-transformed *qde-2*$^-$ strain and crossed it with a *wt* strain in order to re-introduce a *qde-2*$^+$ allele. Since RIP inactivates ∼50% of unlinked repeated sequences during a cross, we chose a strain (qde2TAD8) that contained a high *Tad* load (>80 copies) in order to increase the likelihood that intact elements would survive into the daughter progeny.

We noticed an obvious disparity in the levels of *Tad* expression in the first 'generation' growth of daughter progeny that was dependent on the segregation of the *qde-2* allele; in *qde-2*$^-$ progeny *Tad* expression was consistently high (Figure 2A, lanes 1, 4 and 5) whereas in *qde-2*$^+$ progeny, the reverse was true (lanes 2, 3 and 6), confirming that *qde-2* plays an important role in transposon taming in *Neurospora*. In this and subsequent crosses, we have plotted transcription as a function of *Tad* copy number in order to normalize for uneven segregation of elements during the cross (Figure 2B).

Notwithstanding the role demonstrated above for *qde-2* in controlling *Tad* elements introduced initially by transformation, there remained the possibility that this effect might not be representative of a natural invasion of *Tad*. To answer this point, we crossed a *qde-2*$^-$ strain with a naturally infected strain (Adiopodoumé) in order to segregate *wt* and mutant alleles of *qde-2* into the progeny. Again, we observed a

significant de-repression of *Tad* only in *qde-2*$^{-}$ progeny ($P < 0.05$) (Figure 3C and D). This demonstrates that our observations on *Tad* control are representative of a natural scenario of *Tad* invasion. Furthermore, introducing a mutant *qde-1*$^{-}$ allele into an Adiopodoumé-derived background mirrored the situation observed with introduced cloned *Tad* elements, in that no derepression was observed in the absence of *qde-1*$^{+}$ (data not shown). This finding is consistent with our previous failure to detect a significant expansion of a cloned TAD element in the *qde-1*$^{-}$ strain (Figure 1B) indicating that the RdRP required for transgene silencing has no obvious role in transposon control in *Neurospora*.

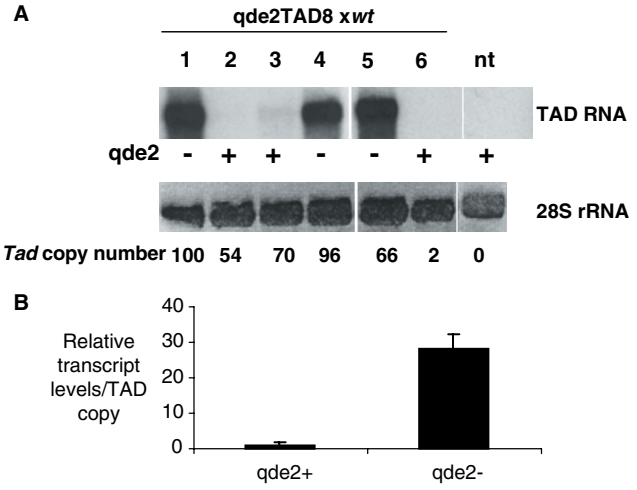

Figure 2. Release of *Tad* repression segregates with the *qde-2*$^{-}$ allele. (**A**) A Tad-transformed *qde2*$^{-}$ strain (qde2TAD8) was crossed with a wild-type strain in order to re-introduce a *qde-2*$^{+}$ allele into a *Tad* background. Expression of the full-length TAD transcript was monitored by northern blot in cultures deriving from six different ascospores (1–6). RNA form a non-transformed *qde-2*$^{-}$ strain (nt) was used as a negative control. The *qde-2* status of each ascospore is indicated as '+' (wild-type allele) or '−' (mutant allele). Ethidium bromide staining of the ribosomal 28S RNA was used as a loading control. The estimated copy number of *Tad* in each ascospore is indicated under each lane. (**B**) Levels of *Tad* transcripts were expressed as a function of copy number in order to normalize for differences due to uneven segregation of elements during the cross and the mean calculated for each type of allele. Error bars display standard error of the mean.

The two *Neurospora* Dicer genes (*dcl1* and *dcl2*) are mutually redundant in *Tad* control

The Dicer enzymes DICER-LIKE1 and DICER-LIKE2 are mutually redundant in the quelling pathway during the silencing of transgenes (35). However, recent reports have demonstrated that in organisms with multiple Dicers, different enzymes are each responsible for processing of different substrates such as microRNA precursors, hairpin dsRNA structures and viral RNA templates (43,44). To explore further similarities between quelling and the *Tad* control pathway, and also to investigate the possibility of a transposon-specific role for either of the Dicers in *Neurospora*, we performed a cross between a double-*dcl* mutant and Adiopodoumé, and analysed *Tad* expression in the progeny that contained either wild-type, single- or double-*dcl* mutant genotypes (Figure 3A and B). As a control comparison, we also crossed a *qde-2*$^{-}$ strain with Adiopodoumé (Figure 3C). Similar to the situation in quelling of transgenes (35), we only noticed an obvious increase in *Tad* expression in the double-*dcl* mutant (Figure 3A, lanes 3 and 8; Figure 3B), whereas no effect was observed in either of the single mutants. This elevation was similar in magnitude

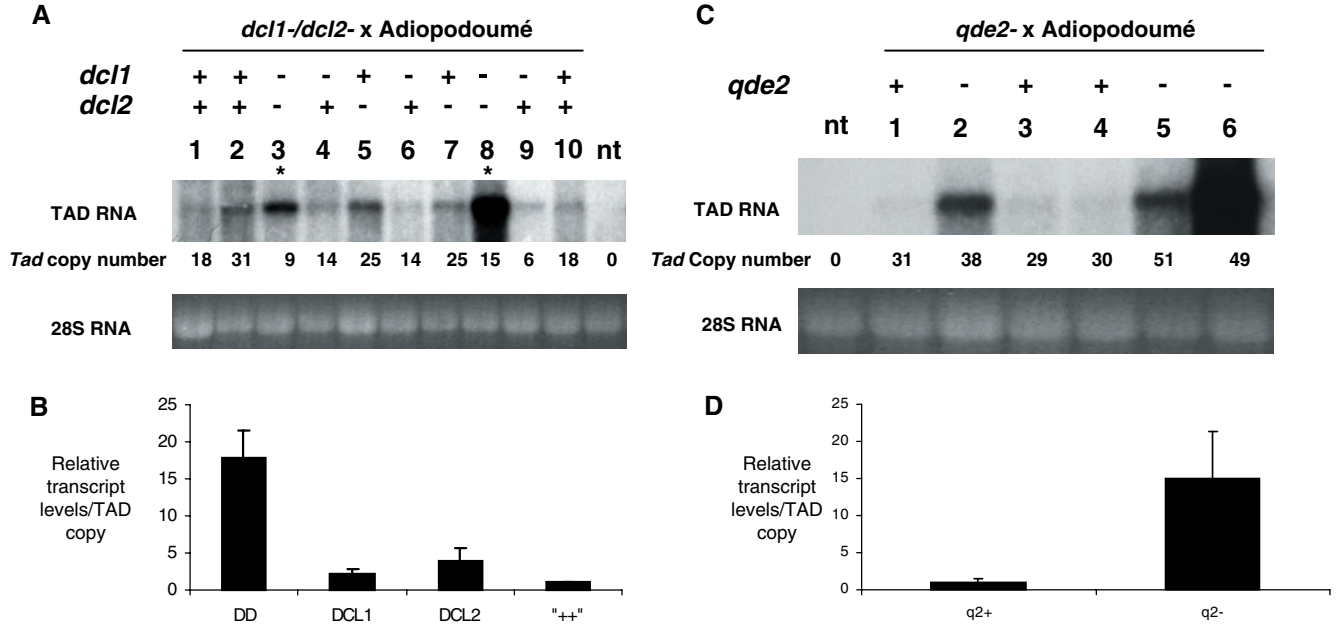

Figure 3. The two *Neurospora dcl* genes are mutually redundant in *Tad* control. Expression of the full-length *Tad* transcript was monitored by northern blot in cultures grown from individual ascospores deriving from the cross of (**A**) a double *dcl* mutant or (**C**) a *qde-2*$^{-}$ strain with Adiopodoumé. Double *dcl*$^{-}$ progeny are indicated with asterisks. Mutant strains not tran sformed with *Tad* (nt) were included as negative controls. (**B**) The mean relative TAD transcript levels in each of the segregating genotypes from (C) the double *dcl*$^{-}$ cross or (**D**) the *qde-2*$^{-}$ cross. Representative blots are shown, a total of 15 ascospores were analysed in the double *dcl*$^{-}$ cross, and 16 ascospores in the *qde-2*$^{-}$ cross. Error bars display standard error of the mean. DD, double *dcl*$^{-}$.

(~15-fold) to that observed in *qde-2*$^{-}$ progeny (cf. Figure 3B and D) from a similar cross, in agreement with the role of DICER-LIKE and QDE2 in the same pathway that controls *Tad*.

siRNAs against *Tad* suggest a quelling-related silencing mechanism

The involvement of *qde-2* in reducing *Tad* expression suggested similarities with a quelling-like mechanism. Another feature of quelling is the production of siRNAs of 21–25 nt that correspond to the targeted sequence (41). We isolated total RNA and enriched for the small RNA fraction in order to look for *Tad* siRNAs in our strains. We found that siRNAs accumulated in both the *qde-2*$^{-}$ strains transformed with *Tad* (Figure 4A), and all of the *Tad qde2*$^{-}$ progeny resulting from the cross of qde2TAD8 with *wt* (Figure 4B, lanes 1, 4 and 5). siRNAs were detected using either probes homologous to the ORF1 (proximal end) or ORF2 (central region) of *Tad*, suggesting that the siRNAs were distributed along most of the element (Figure 4A and data not shown). This observation is consistent with the fact that QDE2 is downstream of the production of siRNAs in the quelling pathway (41). However, our inability to detect siRNAs in either the *Tad*-transformed *wt* strain, the Adiopodoumé isolate or the *qde-2*$^{+}$ progeny from the *wt* cross (Figure 4A and B, compare lanes 2, 3 and 6 with 1, 4 and 5) are not consistent with the usual features of quelling, in which siRNAs accumulate in a quelling-proficient background.

To explain the apparent lack of siRNAs in the presence of QDE2, we considered the idea that QDE2 might function in a complex with siRNAs to direct a form of TGS, in a manner similar to *S.pombe*, where a negative feedback loop exists in the production of siRNAs (2,20,21).

A QDE2/Dicer-dependent silencing pathway, rather than DNA methylation, is the major contributor to *Tad* control

To investigate the possibility of a form of TGS that might be directed by a QDE2/siRNA complex, we considered DNA methylation; evidence from *Arabidopsis* has shown that DNA methylation can be targeted to transposon loci via an siRNA/Argonaute complex. Moreover, a role for DNA methylation in the control of *Tad* elements has previously been demonstrated; an artificially introduced *Tad* element became *de novo* methylated and showed an increased transposition rate in the absence of methylation (30). Therefore, both to evaluate the relative contributions of *qde-2* and methylation to transposon taming in *Neurospora*, and to see if indeed the two might be linked via a TGS mechanism, we introduced *qde-2*$^{-}$ and *dim-2*$^{-}$ mutations into similar *Tad* backgrounds by crossing qde2TAD8 with a *dim-2*$^{-}$ strain; the *dim-2* gene is responsible for all known methylation in *Neurospora* (45). To confirm the *dim-2* status of the segregants, we performed Southern blots digested with either the methylation-sensitive enzyme Sau3A1, or its methylation-insensitive isoschizomer DpnII and probed for the control ζ–η region, a RIP'd locus that is constitutively methylated in a *dim-2*$^{+}$ background (Figure 5B) (23). In the progeny of the cross, all possible combinations of the *qde2* and *dim2* alleles segregated. Interestingly, when we used a probe covering the first 1.5 kb of *Tad* (Figure 5B) to check the methylation status of the elements in the progeny, we noticed

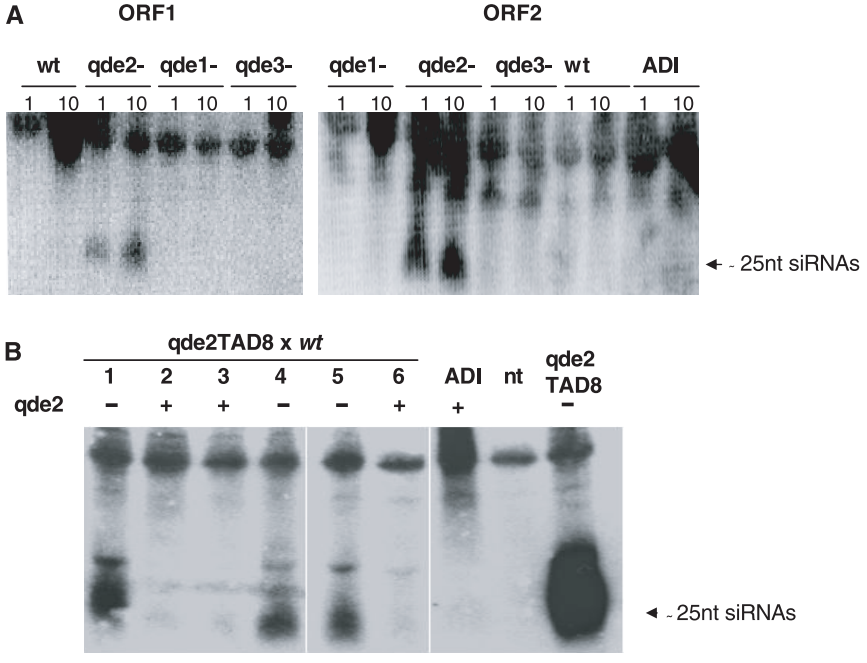

Figure 4. siRNAs against *Tad* accumulate only in a qde2 mutant background. (**A**) In the *Tad*-transformed wild-type (*wt*), *qde-1*$^{-}$, *qde-3*$^{-}$ and *qde-2*$^{-}$ strains, Tad siRNAs could only be detected in the *qde-2*$^{-}$ strain. siRNAs were detected in both the first (1) and tenth (10) asexual generation after transformation with the cloned Tad element. No siRNAs were detected in the Adiopodoumé strain (ADI). Probes recognizing sequence from both ORF1 of *Tad* (left panel) and ORF2 of *Tad* (right panel). (**B**) In a cross of qde2TAD8 with a wild-type strain, the presence of Tad siRNAs segregates strictly with the absence of QDE2. The cultures used in this analysis derive from the same ascospores analysed in Figure 2A. A non-transformed *qde-2*$^{-}$ strain (nt) was used as a negative control.

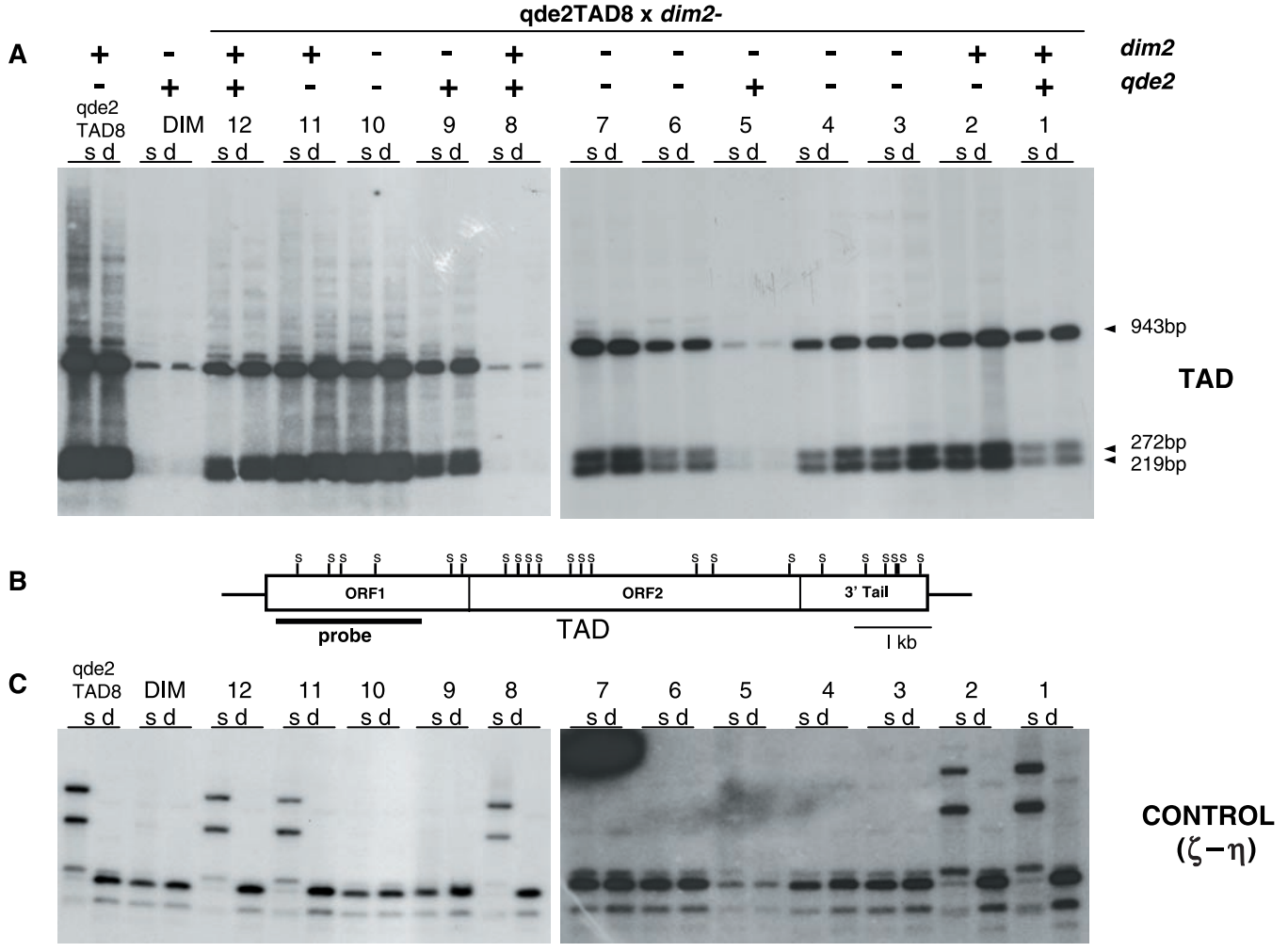

Figure 5. The major bulk of *Tad* elements are not significantly methylated. (**A**) The methylation status of *Tad* elements in the progeny of a qde2TAD8 × *dim-2*$^{-}$ cross was determined by digesting genomic DNA with either the methylation-insensitive enzyme DpnII (d) or its methylation-sensitive isoschizomer Sau3AI (s). The digested DNA was hybridized with the probe shown in (**B**), covering the first four Sau3AI (s) sites of the *Tad* element. The sizes of fully digested hybridizing bands are indicated at the right of (A). (**C**) The blot shown in (A) was stripped and re-probed for the constitutively methylated control region ζ–η to check the *dim-2* status of the progeny. The *dim-2* and *qde-2* status of each ascospore is indicated by a '+' (wild-type allele) or '−' (mutant allele) above each lane (DIM, non-transformed *dim-2*$^{-}$ parent strain).

that there was very little, if any, methylation, judging by the near identity of Sau3A1 and DpnII digests in all of the *dim-2*$^{+}$ segregants, regardless of *qde2* status (Figure 5A, lanes 1, 2, 8, 11 and 12). The absence of methylation was similarly observed when we controlled a 0.6 kb region comprising the proximal portion of ORF2 of *Tad* (not shown). Furthermore, DIM2 had no obvious effect on the expression levels of *Tad*. In contrast, *Tad* expression correlated only with the *qde-2*$^{-}$ allele, similar to the situation in the qde2TAD8 × *wt* cross (Figure 6A, lanes 2–4, 6, 7, 10 and 11), indicating that it is the QDE2-based pathway, rather than methylation, that plays the major role in the control of this transposon. We similarly failed to detect methylation of *Tad* elements that were introduced into a wild-type background, ruling out the possibility that the *qde-2* gene might be required only in the initiation of *Tad* methylation at the time of transformation (data not shown).

Our failure to detect significant *Tad* methylation also rules out the possibility of any QDE2-directed TGS mechanism functioning via the DIM2 methyltransferase.

DISCUSSION

Transposition can represent a threat to genome stability by causing gene disruptions, illegitimate recombination and unregulated transcription. Indeed, in most species the major bulk of transposons are transcriptionally silent due to either DNA methylation and/or histone modifications (13,15,17,19). In addition to these TGS mechanisms, PTGS pathways similar to RNAi are also involved in the silencing of transposons. Moreover, it has been shown recently that DNA methylation of repeated sequences can be directed by the PTGS machinery, providing a direct link between the two mechanisms (16,19,46).

In our analysis, we evaluated the contribution of both DNA methylation and components of the PTGS pathway in the control of *Tad* expression. The principle determinant of *Tad* expression and expansion in our strains was *qde-2*, and the presence of *Tad* siRNAs hints at a quelling-related mechanism. In mutants lacking QDE2, an introduced *Tad* element

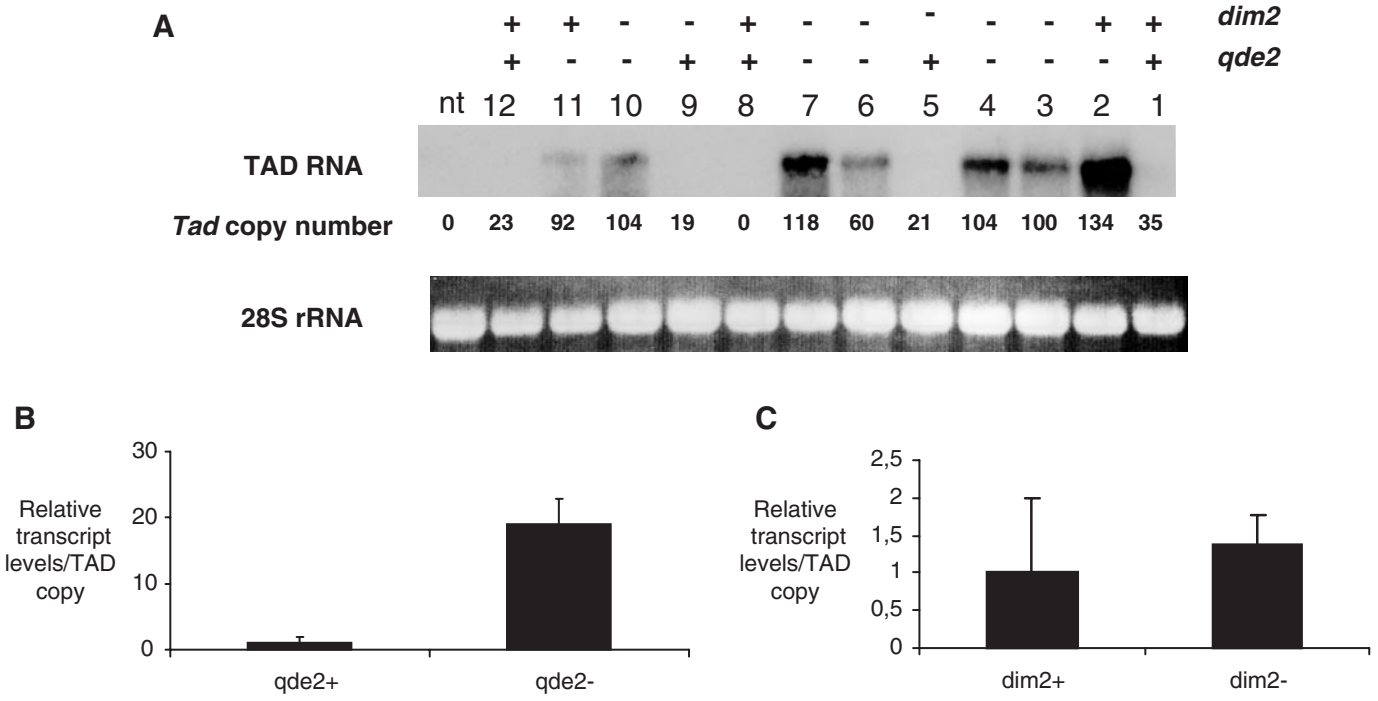

Figure 6. QDE2, not DNA methylation, is the major determinant in limiting *Tad* expression. (**A**) Expression of the full-length *Tad* transcript was monitored by northern blot in cultures grown from individual ascospores deriving from the cross of qde2TAD8 × *dim-2*$^{-}$. The cultures used in this analysis derive from the same ascospores analysed in Figure 5A. Mean relative transcript levels were plotted based on segregation of either (**B**) the *qde-2*$^{-}$ allele or (**C**) the *dim-2*$^{-}$ allele.

showed a rapid expansion over the course of 15 asexual generations and, in outcrosses, the mutant allele segregated strictly with a release of *Tad* repression. Moreover, this dependence on the *qde-2* gene was also observed in a naturally infected, Adiopodoumé-derived strain. On the contrary, we did not observe a significant level of methylation of the *Tad* element in our strains. Strengthening this finding, *Tad* expression levels were unaffected by the absence of DIM2, the DNA methyltransferase responsible for all known methylation in *Neurospora*. This highlights a major difference between *Neurospora* and other DNA methylation-proficient species in which blocking methylation leads to a large increase in transcription of diverse transposon families (15–17). Our data suggest that in the vegetative phase of *Neurospora*, a QDE2/siRNA-based mechanism, rather than DNA methylation, is mainly responsible for *Tad* control. The fact that this QDE2-based mechanism is able to limit *Tad* expression by itself, and without recourse to methylation, is again in contrast to other methyltransferase-containing species where siRNAs and methylation of several classes of transposon DNA are inexorably linked. Our data are in agreement with the recent observation that heterochromatin formation can occur in *Neurospora* in the absence of the RNAi machinery, suggesting that in this species the two silencing pathways are self-contained mechanisms (47,48).

Previous reports have shown that a *Tad* element at the *am* locus could be methylated and, in the absence of methylation, there is an increased mobilization of the element (30). However, methylation was rare and sporadic and indeed the major bulk of *Tad* elements are similarly not methylated in Adiopodoumé (28), suggesting that any role for methylation should be minor in comparison to the *qde-2*-silencing pathway.

In addition to QDE2, the requirement for DICER-LIKE activity in the repression of *Tad* highlights further similarities to quelling. On the other hand, the superfluity of the RdRP QDE1 and the RecQ DNA helicase QDE3 in this repression, and the accumulation of siRNAs only in the absence of the QDE2 protein are uncharacteristic of quelling. These features could point to a transposon-specific control pathway in addition to quelling. Recent evidence from *Drosophila* and *Arabidopsis* has demonstrated that different *Dicer* homologues can provide distinct substrate specificity (43,44). However, this does not seem to be the case in *Neurospora* as we have demonstrated that each of the two DICER-LIKE enzymes are able to repress *Tad*, and this mutual redundancy is also seen in the silencing of transgenes during quelling (35). It is possible that other RdRP homologues of the *qde-1* gene, such as *sad-1* (involved in MSUD) and *RdRP-3* (function unknown), neither of which is necessary for quelling, might be involved in a transposon-specific pathway (24). Similarly, the *qde-3* homologue, *recQ-2*, might have a role in transposon silencing (49).

An explanation for our failure to detect siRNAs in a *qde-2*$^{+}$ background might rely on the degradation by siRNA-loaded RISC of *Tad* precursors upstream of the formation of dsRNA transcripts, thereby creating a negative feedback loop in which the siRNA pool could be reduced to levels below our detection limits. However, this does not appear to be the case in quelling of transgenes where siRNAs accumulate in both the presence and absence of QDE2, perhaps suggesting either greater abundance of the precursors upstream of dsRNA or lower susceptibility to degradation by RISC.

Again, a transcriptional silencing mechanism, other than DNA methylation, guided by an siRNA/QDE2 complex

similar to the RNA-induced initiation of transcriptional silencing complex (RITS) in yeast or AGO4-containing complexes in plants would have a similar effect in terms of siRNA prevalence. While our data do not exclude this possibility, recent evidence indicates that both maintenance and establishment of heterochromatin (both histone methylation and DNA methylation) at silenced transgenes and endogenous sequences can occur independently of qde-2/RNAi in *Neurospora*, meaning that, as yet, there is no precedent for siRNA-based transcriptional silencing in this species (16,19,20,48).

Models of transposon silencing need to provide a mechanism by which these elements are recognized. In the control of certain transposable elements for example, one such mechanism might rely on the presence of inverted repeat (IR) sequences contained at their termini. Sijen and Plasterk found that in *C.elegans* read-through transcription of these IRs is able to trigger silencing of the *Tc1* family of transposons by forming a hairpin dsRNA structure that can enter the RNAi pathway (50). However, *Tad* and many other transposons, such as non-LTR retrotransposons that include the human and mouse LINE elements, do not contain IR sequences. Thus, an alternative explanation is needed in these cases. One possible reason why *Tad* triggers a QDE2-dependent silencing pathway may rely on the very complex transcriptional profile of the element that, in addition to a full-length transcript includes at least two shorter anti-sense transcripts that originate from its 3′ end (51). Inter-molecular base pairing of sense and anti-sense transcripts would lead to a dsRNA intermediate, providing a template for Dicer cleavage and subsequent RISC-mediated degradation of *Tad* templates. In support of this, the direct production of dsRNA has been shown to bypass the requirement of both QDE1 and QDE3, but is upstream of QDE2 in the silencing of transgenes, a situation similar to that which we observed (52). However, an explanation based on intermolecular pairing of sense transcripts and 3′-originating anti-sense transcripts needs to explain the presence of siRNAs from ORF1, into which the main anti-sense transcripts do not extend (51). An auxiliary role for one of the three *Neurospora* RdRP genes in extending a paired dsRNA molecule in a 3′ direction could speculatively explain this observation. Alternatively, other uncharacterized more extensive anti-sense transcripts might be responsible.

The fact that transposon control via QDE2 is not linked to DNA methylation would appear to set apart *Neurospora* from other methylation-proficient species. In addition to the QDE2-based mechanism that we have identified for the silencing of *Tad*, *Neurospora* also possesses RIP that mutates repeated sequences during the pre-meiotic sexual phase of the life cycle. Strikingly, the methylated component of the genome largely represents RIP-inactivated sequences (53). Indeed, almost any RIP-inactivated sequence is able to function as a *de novo* methylation signal when re-transformed into *Neurospora* (54). It is therefore possible that, in *Neurospora*, methylation of repeated sequences is largely signalled by previous RIP inactivation, obviating the requirement for an RNAi-directed methylation mechanism in this species.

Since Adiopodoumé appears to be proficient for both mechanisms, we prefer the previously proposed hypothesis that the presence of Tad in Adiopodoumé represents a recent invasion (29) that should eventually be cleared by a combination of the two silencing pathways. It would appear likely that *Neurospora*, during the process of a *Tad* invasion, uses a *qde-2*/quelling-related mechanism to limit the expansion of *Tad* during vegetative growth whilst the resulting restricted set of *Tad* elements is gradually eliminated by RIP in successive cycles. It seems reasonable to expect that many of the transposon relics that litter the *Neurospora* genome will have at one time been subject to a similar concert of silencing mechanisms, possibly also involving MSUD, that function to ensure their deactivation and maintain genome integrity.

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Conflict of interest statement. None declared.

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