

The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in *Neurospora crassa* interacts with replication protein A

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ABSTRACT

Post-transcriptional gene silencing (PTGS) pathways play a role in genome defence and have been extensively studied, yet how repetitive elements in the genome are identified is still unclear. It has been suggested that they may produce aberrant transcripts (aRNA) that are converted by an RNA-dependent RNA polymerase (RdRP) into double-stranded RNA (dsRNA), the essential intermediate of PTGS. However, how RdRP enzymes recognize aberrant transcripts remains a key question. Here we show that in *Neurospora crassa* the RdRP QDE-1 interacts with Replication Protein A (RPA), part of the DNA replication machinery. We show that both QDE-1 and RPA are nuclear proteins and that QDE-1 is specifically recruited onto the repetitive transgenic loci. We speculate that this localization of QDE-1 could allow the *in situ* production of dsRNA using transgenic nascent transcripts as templates, as in other systems. Supporting a link between the two proteins, we found that the accumulation of short interfering RNAs (siRNAs), the hallmark of silencing, is dependent on an ongoing DNA synthesis. The interaction between QDE-1 and RPA is important since it should guide further studies aimed at understanding the specificity of the RdRP and it provides for the first time a potential link between a PTGS component and the DNA replication machinery.

INTRODUCTION

The initial, paradoxical observation that introducing extra copies of a gene could actually lead to silencing of the same gene has led to the discovery of a wide range of phenomena that all involve post-transcriptional gene silencing (PTGS) of repetitive sequences and that play roles varying from genome defence against viruses and transposons, to development and chromosomal segregation (1,2). In the last few years, the details of how homologous transcripts are either degraded or translationally repressed during PTGS have been largely worked out. A fundamental intermediate, common to all forms of PTGS, is the production of a double-stranded RNA (dsRNA) which is then processed by the RNase III enzyme Dicer into short interfering RNAs (siRNAs) or microRNAs (miRNAs) (3,4). The subsequent processing of siRNAs and miRNAs and their mode of binding through homology to their target transcripts are widely conserved through several organisms and great progress has been made in working out the details of these processes. However, one of the few remaining open questions concerns events upstream of the production of dsRNA—how are repetitive sequences recognized as different from endogenous genes in the first place? This question is particularly important, in light of the fact that PTGS probably first evolved as a defence mechanism against invading sequences such as transposons and viruses (5,6).

In the model fungus *Neurospora crassa* where PTGS (known as quelling) can be induced by transgenes, it has been observed that in order to induce gene silencing,

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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transgenic loci need to be transcribed (2). It has been suggested that an RNA-dependent RNA polymerase (RdRP) called *qde-1* (7), specifically required for quelling, may use the transgenic RNA as its substrate to produce a dsRNA. This idea is supported by the fact that, *in vitro*, QDE-1 has been shown to be able to convert a single-strand RNA template into dsRNA (8).

To gain insight into how the RdRP is able to specifically target only the gene to be silenced, we introduced a tagged version of QDE-1 and used this to immunopurify QDE-1-containing protein complexes. Using this approach, we were able to show that QDE-1 interacts with the *Neurospora* homologue of the largest subunit of Replication Protein A (RPA-1), a single-stranded DNA-binding protein that is important in DNA replication, repair and recombination. Moreover, we were able to show that QDE-1 is enriched at the transgenic locus required to trigger PTGS, and that the accumulation of siRNAs is coupled to DNA synthesis. Taken together, these observations support the view that repeated sequences could be targeted for silencing as they are being replicated, and that it is during replication that these sequences are somehow identified by the cell as qualitatively different from endogenous, non-silenced genes.

MATERIALS AND METHODS

Plasmid construction

The QDE-1 overexpression plasmid pFLAG*qde-1* was created by insertion of the *qde-1* gene (NCU07534.3) immediately downstream of the inducible quinic acid dehydrogenase (*qa-2*) promoter in the pMXY2 vector that contains benomyl resistance as a selectable marker (9).

A 200 bp BstZ171-BglIII fragment surrounding and including the start codon of *qde-1* was removed and replaced with a PCR homologous fragment modified to encode the FLAG epitope (DYKDDDDK) immediately after the ATG (primer sequences available on request).

The plasmid-encoding cmycRPA-1 (pMycRepA) was made by first subcloning from cosmid H116G2 (Fungal Genetics Stock Centre, University of Kansas) into pBluescriptSK a 4.6 kb BamHI genomic fragment containing the *RPA-1* coding sequence (NCU03606.3) and 1.6 and 1 kb of upstream and downstream sequence, respectively. To introduce the c-myc epitope at the N-terminus of the protein, a 130-bp BstBI-PmlI fragment was removed and was substituted with a PCR homologous fragment modified to encode the c-myc epitope (EQKLISEEDL) immediately after the ATG (primer sequences available on request).

The construct (pRepAKO) for knocking out the endogenous *RPA-1* gene was prepared by amplifying 1 kb of sequence from both immediately upstream and immediately downstream of the *RPA-1* coding sequence. The upstream and downstream amplified regions were then cloned either side of the hygromycin resistance cassette in the previously described plasmid pCSN44 (10).

Neurospora strains, growth conditions and transformation procedure

The stably silenced *Neurospora* strain, 6XW, and the silencing deficient *qde-1* mutant strain 107 derived from this strain have been previously described (2,11).

Growth conditions for *Neurospora* were essentially as described elsewhere (12). For hydroxyurea (HU) treatments, HU (0.1 M final concentration) was added to overnight-grown cultures (10^6 conidia/ml) for 4 h with shaking at 28°C. Mycelia were then harvested by filtration and washed with an excess of *Neurospora* minimal medium (NMM) and frozen (T_0) or rinsed with fresh pre-warmed NMM and incubated for a further 30 and 100 min with shaking at 28°C and then harvested and frozen (T_{30} and T_{100} , respectively). Quinic acid induction was used to increase the level of production of FLAGQDE-1. This was achieved by placing grown mycelia in 1× Vogel's and 0.3% quinic acid for 4 h with shaking at 28°C. Preparation of *N. crassa* spheroplasts and transformation with recombinant plasmids was performed as described by Vollmer and Yanofsky (13).

Knockout of the *RPA-1* gene was achieved by transforming a KpnI–NotI-linearized version of pRepAKO into the strain FGSC9719 (Δ *mus-52*), which is defective in the non-homologous end-joining pathway, and thus results in a dramatic increase in the frequency of homologous recombination (14). Having verified a heterokaryotic strain containing a knockout event at the endogenous locus, we were unable to purify this strain to homokaryosis despite repeated serial transfers and microconidiation.

Immunoprecipitation (IP)

Large-scale IP was performed by homogenizing 5 g (wet weight) of ground, frozen mycelia in 15 ml lysis buffer (10% glycerol, 150 mM NaCl, 50 mM HEPES, pH 7.4). After centrifugation at 10 000 g at 4°C to remove cellular debris the supernatant was incubated for 3 h at 4°C on a rotating wheel in the presence of 100 µl (packed gel volume) of anti-FLAG M2 agarose resin (Sigma). The resin was then pelleted by gentle centrifugation at 1000 g and washed three times in lysis buffer followed by two washes in tris-buffered saline (TBS). The precipitated proteins were eluted from the resin with FLAG peptide (Sigma F3290) in TBS (250 µg/ml).

A similar procedure was followed in the IP of cmycRPA1 using an anti-cmyc agarose resin (Sigma E6654).

Mass spectrometry of proteins interacting with QDE-1

Immunoprecipitated proteins were resolved on a 7% T–3.3% C SDS-PAGE separating gel (1 + 18 + 18 mm), revealed by Sypro Ruby staining and visualized using a Typhoon 9200 laser scanner (GE Healthcare). Proteins were excised, digested with trypsin and MALDI-TOF/TOF (4700 Proteomics Analyzer; Applied Biosystems) was used to obtain mass spectra, which were analysed using the GPS Explorer software (Version 1.1, Applied Biosystems) against the MASCOT search engine

(Matrix Science). Scores greater than 61 were considered significant ($P < 0.005$).

Western blot analysis

Western blots were performed using standard procedures. Both the anti-FLAG antibody (Sigma F3165) and the anti-cmyc antibody (Sigma M4439) were used at a 1:2000 dilution. The secondary antibody was HRP-conjugated anti-mouse produced in goat (Bio-Rad) and used at 1:5000.

Preparation of nuclear extracts

Nuclei were isolated by a modification of the method described by Luo *et al.* (15) using freshly harvested mycelial pads (2–3 g, wet weight) in an initial lysis volume of 30 ml. A more detailed description of this method is provided in the Supplementary Data.

Chromatin immunoprecipitations (ChIP)

ChIP was carried out as described previously (16,17), with modifications which are described in supplementary methods. Essentially, conidia (10^7) were inoculated in 100 ml NMM and grown for 24 h and the mycelia were fixed in 2.5% formaldehyde for 10 min, with shaking. For the immunoprecipitation 15 μ l packed gel volume of anti-FLAG resin was used per ml of lysed, sonicated chromatin (15 μ g in total).

Quantification of immunoprecipitated DNA

Quantification was performed using a real-time PCR machine, LightCycler (Roche), with FastStart DNA Master SYBR green 1 kit (Roche). Data were analysed with built-in LightCycler software, version 3.01, using the second derivative method for determining the crossing point (Cp) value for each sample.

Transgenic DNA was amplified using the primer P2 (5'-GCGCGCAATTAACCCTCAC) derived from the bacterial vector sequence and the primer P1 (5'-AAGA GACCCGGTAGGAGGAG) from the *al-1* transgene to avoid amplification of the endogenous *al-1* gene DNA. The actin primers (5'-CCCAAGTCCAACCGTGAGAA and 5'-GGACGATACCGGTGGTACGA) are derived from the fifth exon sequence of the *actin* gene.

QDE-1 enrichment at the transgenic locus was measured as the relative increase in the amount of transgenic DNA with respect to the actin DNA between the 'IP' and 'input' samples. As a negative control we used the silenced non-FLAG strain, 6XW, from which the FLAGqde-1 strain derives and that thus contains the same array of transgenes. We assayed the enrichment in these two strains in four independent ChIP experiments and compared them using a paired Student's *t*-test.

Small RNA purification and northern analysis

Small RNA purification and northern analysis was performed as described previously (18).

RESULTS

Identification of RPA-1 as an interacting partner of QDE-1

To gain insight into how the RdRP is able to specifically target only the gene to be silenced, we placed *qde-1* under control of the quinic acid inducible promoter (*qa-2*) and tagged the gene with an N-terminal FLAG epitope, with the aim of immunoprecipitating any interacting protein partners. We first reintroduced the tagged, cloned version of QDE-1 (FLAGQDE-1) into a *qde-1* mutant strain 107, identified previously in an insertional mutagenesis screen (11). Strain 107 derives from the reference strain 6XW, which has ~20 copies of the *albino-1* (*al-1*) gene inserted in a head-to-tail tandem repeat fashion (11). Since *al-1* is responsible for carotenoid biosynthesis, strains silenced in this gene are easily identified by visual inspection as white in colour, due to the absence of carotenoid production. Restoration of silencing to FLAGQDE-1-transformed strains showed that our epitope-tagged protein was fully functional (Supplementary Data, Figure S1). We then purified the FLAGQDE-1 protein from large-scale vegetative cultures using anti-FLAG conjugated agarose (Sigma), followed by competitive elution with the FLAG peptide. We analysed the eluted proteins on a 7% 1D PAGE gel and looked for bands that specifically co-purified with FLAGQDE-1, using a silenced non-FLAG strain (6XW) as a negative control (Figure 1A). Despite some non-specific background due to cross-reaction of the FLAG antibody in *Neurospora*, and the presence of several specific bands that turned out to be degraded or truncated versions of FLAGQDE-1, one other protein of ~70 kDa consistently co-purified with FLAGQDE-1. This protein was revealed by mass spectrometry to be the largest subunit (70 kDa) of the heterotrimeric RPA complex (Supplementary Data, Figures S2 and S3). In order to further confirm the interaction between these proteins we cloned the *Neurospora* gene for the large subunit of RPA (*rpa-1*) and tagged this gene with the c-myc epitope (cmycRPA-1). In strains that expressed both FLAGQDE-1 and cmycRPA-1, by immunoprecipitating cmycRPA-1 we were able to co-purify FLAGQDE-1 (Figure 1B).

RPA is responsible for binding and stabilizing single-stranded DNA templates and as such is an essential component not just in the replication fork but also in processes such as DNA repair and recombination (19).

The *Neurospora rpa-1* is an essential gene

Having identified RPA-1 as a potential novel component of the PTGS pathway we attempted to knockout this gene in a silenced background to see if silencing was relieved. Although we were able to disrupt the endogenous *rpa-1* locus, these strains could only be maintained in heterokaryosis with wild-type nuclei, suggesting that the homokaryotic state was lethal (Supplementary Data, Figure S4). The lethality of the *rpa-1* knockout has since been confirmed independently by the *Neurospora* Knockout Consortium (http://www.dartmouth.edu/~neurosporagenome/1_s1.html). This observation is in agreement with the crucial role of RPA in replication and

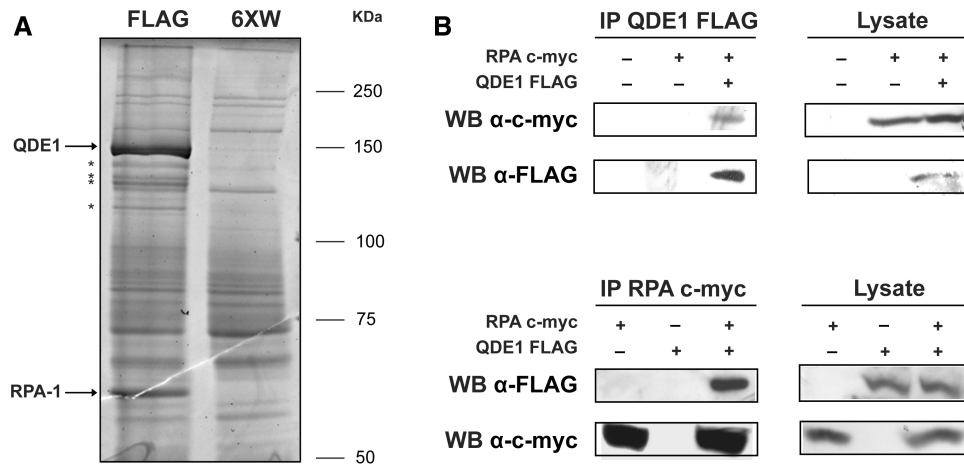


Figure 1. Purification of *Neurospora* QDE-1 and the identification of RPA-1 as an interacting partner. (A) Sypro Ruby staining of immunoprecipitation (IP) of FLAGQDE-1 ('FLAG'). As a negative control a silenced, non-FLAG strain was included ('6XW'). Mass spectrometry analysis of bands specific to the FLAG sample revealed that RPA-1 (13 peptides, 29% sequence coverage) specifically co-purifies with QDE-1. Bands marked with * (asterisk) were identified as degradation products of QDE-1. (B) The interaction between QDE-1 and RPA-1 was confirmed by constructing strains that contained both a cmyc-tagged version of RPA-1 and FLAG QDE-1. In these strains IP of cmycRPA-1 also copurified FLAGQDE-1, and vice versa.

repair and this knockout is lethal in several other organisms (20,21). The inability to knockout *rpa-1* limited our ability to test the functional importance of the RPA-1/QDE-1 interaction. We therefore investigated whether there was additional supporting evidence to suggest that RPA-1 could play a role in silencing, through its role in either DNA replication or repair. In this vein we tested other assumptions that should be true were the interaction between QDE-1 and RPA-1 to be of functional relevance in silencing.

QDE-1 is a nuclear protein, which is specifically recruited onto transgenic repetitive loci

In humans and yeast, RPA has been shown to interact physically and functionally with the WRN and BLM RecQ DNA helicases and is essential for their DNA unwinding activity on recombination intermediates at the replication forks (22,23). Strikingly, a direct *Neurospora* homologue of WRN and BLM is QDE-3, another gene encoding a DNA helicase essential for PTGS that, together with QDE-1, is known to be upstream of the production of dsRNA during PTGS (18,24). Together, these previous observations and our demonstration that QDE-1 and RPA-1 interact in a complex led us to investigate the possibility that QDE-1 may be coupled with the DNA replication machinery. For this reason we first investigated whether the interaction between RPA-1 and QDE-1 might occur in the nucleus where replication takes place. The program P-SORT (25) predicts QDE-1 to be a nuclear protein, based on the presence of a bipartite sequence and we confirmed that this was the case by showing that FLAGQDE-1 was enriched in nuclear extracts (Figure 2A). We similarly confirmed that our c-myc-tagged version of RPA-1 was also nuclear (Figure 2A). In yeast it has been shown that the RdRP forms part of a complex (RDRC) that is peripherally associated with the silenced locus (26–28). The RDRC

both interacts with, and is essential for the localization of, the more tightly associated RNAi-induced transcriptional silencing (RITS) complex which is guided by small interfering RNAs (siRNAs) (26,29). We performed ChIP experiments to observe whether QDE-1 was associated with the transgenic locus. We cross-linked the chromatin to fix any DNA–protein associations and immunoprecipitated QDE-1 followed by quantitative PCR to see if the protein was preferentially associated with any specific DNA sequences. After immunoprecipitation using the anti-FLAG antibody, we detected a reproducible (1.7-fold) enrichment ($P < 0.01$) of FLAGQDE-1 at the transgenic *al-1* locus when compared to the unrelated, non-silenced endogenous *actin* gene (Figure 2B). As a negative control we again included the silenced, non-FLAG strain, 6XW. Although low, our level of enrichment is comparable to that found for the RdRP in *Schizosaccharomyces pombe* (RdP1) at the centromeric *dg* and *dh* repeats (26). It has been shown that in *Neurospora* there is a tight correlation between transcription of the transgenic array and the efficiency of silencing, with the RNA, being produced from the transgenes, proposed to be aberrant and converted by the RdRP to a dsRNA (2). Our demonstration that the RdRP QDE-1 is enriched at the transgenic locus suggests that this conversion step happens *in situ* as the aberrant RNA is produced.

siRNA accumulation depends on an ongoing DNA synthesis

In the absence of an *rpa-1* null allele, we tested whether a functional interaction between QDE-1 and RPA-1 might exist through the latter's role in DNA replication. To do this we treated *Neurospora* cultures with HU, a specific inhibitor of DNA synthesis (30). We observed that treatment with HU abolishes the accumulation of siRNAs, (Figure 3) indicating that an ongoing DNA synthesis is required for triggering the silencing machinery. The accumulation of siRNAs was fully restored 100 min after

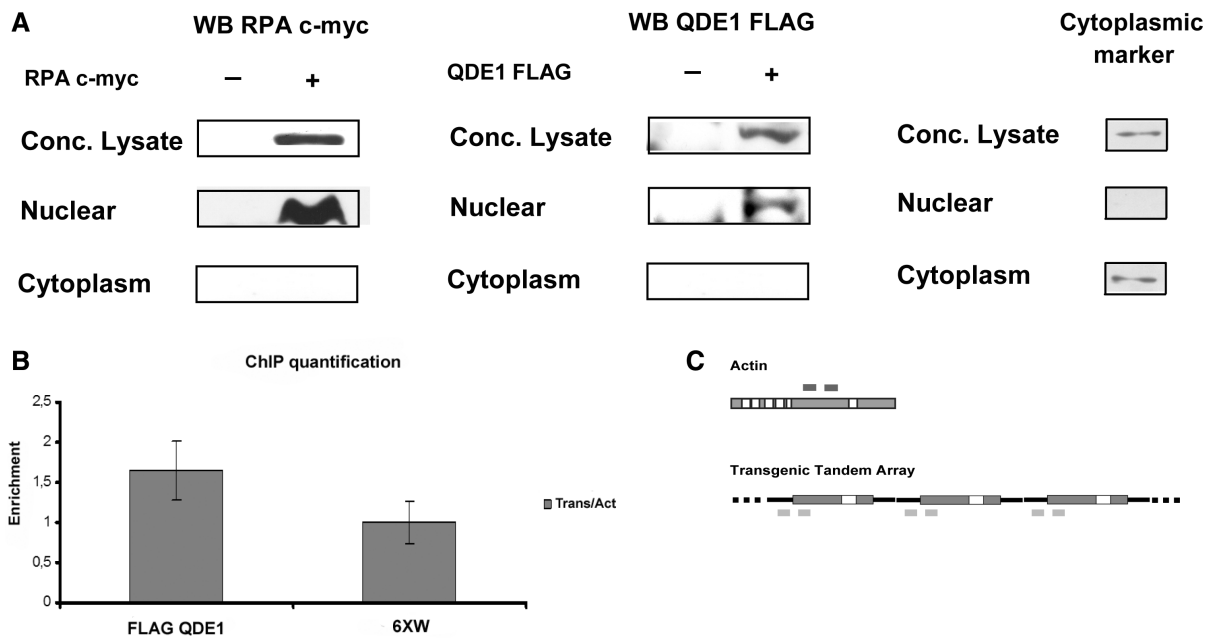


Figure 2. QDE-1 localizes to the nucleus and is enriched at the transgenic repeats that trigger silencing. (A) Western blots showed that cmycRPA-1 and FLAGQDE-1 were greatly enriched in nuclear extracts. Equal amounts of protein were loaded for each nuclear and cytoplasmic extract fraction. A more concentrated form of total lysate was also loaded in order to visualize bands that were otherwise too weak to be visualized in this fraction ('conc. lysate'). (B) ChIP analysis using an anti-FLAG antibody revealed that QDE-1 is enriched at the transgenic locus compared to the non-silenced actin locus. The silenced non-FLAG strain 6XW was used as a negative control (four independent experiments; error bars denote standard deviation; $P < 0.01$). (C) A schematic diagram showing where the primers bind (bars) on the transgenic array and on the actin control gene.

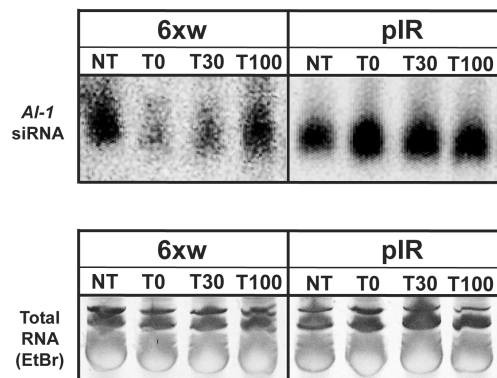


Figure 3. The accumulation of transgene-specific siRNAs correlates with DNA replication. After HU treatment of mycelia to inhibit DNA replication siRNAs were extracted at various timepoints (from 0–100 min, T_0 – T_{100}) following removal of HU. The accumulation of *al-1* siRNAs is abolished in a strain where silencing is induced by a transgenic array (6XW) but not in a strain where silencing is induced in a *qde-1/qde-3*-independent fashion by direct expression of a hairpin dsRNA with homology to the *al-1* gene (pIR). By 100 min after HU release siRNAs in 6XW were restored to the normal level found in non-treated mycelia (NT). As a loading control an ethidium bromide stain of total low-molecular-weight RNA (RNA) is shown.

removing the HU from the cultures, suggesting a direct and reversible effect of HU. Previous results showed that the direct expression of a hairpin dsRNA from an inverted repeat efficiently elicits silencing, bypassing the requirement of both *qde-1* and *qde-3* (24,31). Strikingly, the accumulation of siRNAs produced in a strain similarly expressing a hairpin dsRNA is not affected by HU, indicating a qualitative difference between the formation

of siRNAs that result from a tandem transgene and those that result from an inverted repeat (Figure 3). Although we cannot exclude an indirect link between replication arrest and the disappearance of siRNAs, our data would suggest that it is only the phases of RNA silencing concerned with recognition of the repeated sequence and production of a dsRNA, mediated by QDE-3 and QDE-1 that are linked to DNA replication.

DISCUSSION

In our attempts to better understand how the silencing of transgenes is initiated in *Neurospora*, we identified RPA-1, a DNA-binding protein essential for both DNA repair and replication, as an interacting partner of QDE-1, the RdRP responsible for producing the dsRNA intermediate required in PTGS. This finding is significant since it has implications for the possible mode of action of QDE-1. Since RPA-1 is a nuclear protein we might also expect QDE-1 to be so, given their interaction. When we investigated the localization of QDE-1 we found that not only was it nuclear, but that it was also enriched at the transgenic tandem repeats that are required to trigger silencing. Thus the most likely scenario is that QDE-1 acts on the transgenic locus *in situ* to produce a dsRNA. A similar situation exists in the fission yeast, where the RdRP is also enriched at repeated loci that trigger silencing, yet in this case the localization of the RdRP to the locus is dependent on another complex, RITS, which is guided by siRNAs homologous to the target locus (26). There is currently no evidence in *Neurospora*

for a siRNA-directed complex similar to RITS that targets repeat sequences. Indeed assembly of heterochromatin seems to be siRNA-independent in this organism (16,32). We therefore considered the possibility that the enrichment of QDE-1 at repeat sequences could be mediated through its interaction with RPA-1, rather than through siRNAs. Our ability to test this hypothesis was limited by the lethality of the *rpa-1* null allele, which prevented us from directly assaying the functional role of this gene. Faced with this problem we decided to test our hypothesis in an indirect fashion by blocking one of the processes for which *rpa-1* is essential replication—and monitoring the effect on silencing. We showed that in the case of the silenced transgenic tandem repeats, where the accumulation of siRNAs, the hallmarks of silencing, is dependent on both QDE-1 and the RecQ DNA helicase QDE-3, this accumulation was also blocked in the absence of replication. On the other hand, and importantly, in a strain producing a dsRNA directly as a hairpin, where accumulation of siRNAs is both QDE-1- and QDE-3-independent (33), blocking replication had no effect on the levels of siRNAs. This finding suggests that DNA synthesis is only coupled to RNA silencing through nuclear events mediated by QDE-1 and QDE-3, which are concerned with the recognition and transcription of repetitive DNA elements.

In summary, we have shown that QDE-1 interacts with RPA, that QDE-1 can reside at the transgenic locus, and that the accumulation of siRNAs is correlated with DNA synthesis. These findings, coupled with the previous observation that in other organisms RPA interacts with, and is necessary for the function of, the direct homologue of the QDE-3 DNA helicase required for transgene silencing in *Neurospora* (23), can provide several insights into the possible nature of how the cell recognizes in the first instance a transgenic repeat which is to be silenced. Based on the above, we propose that it is during the act of replication that transgenic sequences are recognized by QDE-1, through its interaction with RPA-1, as different from endogenous genes and are thus targeted for silencing by the *in situ* production of a dsRNA. However, since RPA-1 must bind to the entire genome at some point during DNA replication the question remains of what distinguishes transgenic sequences as different from the rest of the genome during replication? Here a clue could lie in the requirement in *Neurospora* for sequences to be inserted in tandem in order to trigger silencing (2,34). We speculate that repeated sequences in close proximity could be recognized at the time of replication through their ability to pair with each other, perhaps forming unfavourable intermediates. It is known that tandem repeats, during DNA replication, frequently may form a slippage intermediate or 'slipped' misalignments during recombination at stalled replication forks, both of which are cruciform-like structures that have to be resolved in order to allow progression of the fork (35–38). In different organisms RPA, together with homologues of QDE-3 the RecQ DNA helicase, has been found to be required in promoting the progression of the replication fork by resolving such cruciform structures and preventing genome instability (23,39,40).

While several questions remain unanswered in our speculative model, including the source of initial RNA which QDE-1 converts into a dsRNA, it is nonetheless attractive in that it goes some way to answering longstanding questions related to the triggering of transgene-induced gene silencing, offering a silencing mechanism that relies on the detection of one of the intrinsic characteristics of these sequences: their repetitiveness. Such a system could potentially function to silence other repetitive elements including also non-IR transposons, viruses and certain chromosomal duplications. Interestingly, and supporting our model of RPA in marking repeated sequences is the recent identification of RPA mutants in *Arabidopsis* which are defective in the transcriptional repression of a subset of transposons, suggesting that our model could be applicable across a range of species (41,42).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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