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Transgene-mediated post-transcriptional gene silencing is inhibited by 3′ non-coding sequences in *Paramecium*

Angélique Galvani and Linda Sperling*

Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette Cedex, France

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ABSTRACT

Homology-dependent gene silencing is achieved in *Paramecium* by introduction of gene coding regions into the somatic nucleus at high copy number, resulting in reduced expression of all homologous genes. Although a powerful tool for functional analysis, the relationship of this phenomenon to gene silencing mechanisms in other organisms has remained obscure. We report here experiments using the *T4a* gene, a member of the trichoeyst matrix protein (*TMP*) multigene family encoding secretory proteins, and the *ND7* gene, a single copy gene required for exocytotic membrane fusion. Silencing of either gene leads to an exocytosis-deficient phenotype easily scored on individual cells. For each gene we have tested the ability of different combinations of promoter, coding and 3′ non-coding regions to provoke silencing, and analyzed transcription and steady-state RNA in the transformed cells. We provide evidence that homology-dependent gene silencing in *Paramecium* is post-transcriptional and that both sense and antisense RNA are transcribed from the transgenes, consistent with a role for dsRNA in triggering silencing. Constructs with and without promoters induce gene silencing. However, transgenes that contain 3′ non-coding regions do not induce gene silencing, despite antisense RNA production. We present a model according to which different pathways of RNA metabolism compete for transcripts and propose that the relative efficiencies of dsRNA formation and of 3′ RNA processing of sense transgene transcripts determine the outcome of transformation experiments.

INTRODUCTION

In organisms ranging from protozoa to vertebrates, introduction of foreign DNA or RNA can promote unexpected deregulation of normal gene expression at the post-transcriptional level (for recent reviews see 1–6). Expression of all endogenous genes sharing homology with the foreign nucleic acid is reduced or silenced, in what has been likened to a genetic immunity system. Transcription of the targeted endogenous genes is normal, although mRNA levels decrease dramatically. Such post-transcriptional gene silencing (PTGS) offers the opportunity to obtain 'loss-of-function' phenotypes, and for many organisms has opened the door to functional analysis (7,8). Genetic studies have revealed that the same genes are required for different PTGS phenomena: co-suppression or PTGS in *Arabidopsis* (9–12), quelling in *Neurospora* (13–15) and RNAi in *Caenorhabditis elegans* (16–18). This supports mechanistic conservation among phyla, and suggests that PTGS is a universal and ancestral gene regulation mechanism. Mounting evidence indicates that its primary function was to protect organisms against invasive nucleic acids, such as viruses (19) and transposons (16,18).

Biochemical approaches are rapidly uncovering the mechanisms, common to PTGS and RNAi, by which dsRNA activates catalytic, sequence-specific mRNA degradation (20–25). How transgenes produce molecules that activate this mechanism is, however, still an open question. Experiments in plants show that co-transformation with transgenes designed to produce both sense and antisense RNA or hairpin structures are far more efficient in inducing co-suppression than transgenes producing only sense or antisense RNA (26–28), indicating that a dsRNA step is implicated in transgene-mediated PTGS. However, many structurally different transgenes can trigger the phenomenon (29,30) and how these molecules lead to the formation of dsRNA remains obscure. In some cases, dsRNA could be directly produced by the transgenes, by transcription from cryptic promoters or through inverted DNA repeats. In other cases, it has been postulated that the introduced DNA produces aberrant RNAs that would serve as template for an RNA-dependent RNA polymerase (RdRP) to synthesize a complementary RNA (cRNA) that can hybridize with sense RNA to form dsRNA (9,11,14,17,31,32). Alternative models suggest that the formation of aberrant RNA does not require transcription of introduced DNA, but results from ectopic pairing with endogenous DNA that interferes with normal transcription (33,34). In conclusion, it is difficult to reconcile all these models, and the nature of the aberrant triggering RNA is still a matter of debate.

In the ciliate *Paramecium*, exogenous DNA microinjected into the somatic nucleus does not usually integrate into the genome, but undergoes linearization, multimerization and telomerization. Such pseudo-chromosomes are able to replicate autonomously (35) and to express genes if they carry adequate

*To whom correspondence should be addressed. Tel: +33 1 69 82 32 09; Fax: +33 1 69 82 31 50; Email: sperling@cgm.cnrs-gif.fr

regulatory sequences: this allows, for example, functional complementation of mutations (36,37). The transforming DNA is maintained through vegetative growth, but is lost at the next sexual process when the somatic nucleus is replaced by a new one formed from the zygotic germline nucleus (38).

Homology-dependent gene silencing can be obtained in *Paramecium* by microinjection at high copy number (a threshold of 20–30 haploid equivalents for completely silenced phenotypes) of plasmids containing only the coding sequence of a gene into the somatic nucleus, which leads to a dramatic reduction in expression of all endogenous homologs (39). The effect is not obtained with transgenes bearing the flanking regulatory sequences required for expression. Aberrant RNAs, both longer and shorter than the full-length mRNA and easily detectable by northern blot, are systematically present in silenced clones. These aberrantly sized RNA molecules hybridize with a plasmid probe, suggesting that they are synthesized from the microinjected DNA (39). Such abnormal RNAs are also found in some cases of co-suppression in plants (40,41) and are postulated to play a role in initiating gene silencing. In order to determine the relationship that may exist between homology-dependent gene silencing in *Paramecium* and the PTGS phenomena described so far, we have examined the ability of different constructs to trigger gene silencing and analyzed the aberrant RNA molecules produced to try to understand their role.

MATERIALS AND METHODS

Cells and culture conditions

The wild-type reference strain was *Paramecium tetraurelia* d4-2, derived from stock 51. The nd7-1 secretory mutant strain was also used (37,42). Cells were grown at 27°C in grass infusion (Wheat Grass Powder; Pines International, Lawrence, KA), infected with *Klebsiella pneumoniae* the day before use, supplemented with 0.4 µg/ml β-sitosterol, according to Sonneborn (43).

Plasmid constructs

The different constructs were obtained by cloning PCR fragments into pGEM-T vector (Promega). The PCR fragments correspond to the *T4a* (GenBank accession no. U47117) or *ND7* (GenBank accession no. Y07803) gene with or without regulatory sequences (see Fig. 1 for nomenclature). Each PCR reaction (100 µl) contained 50 ng DNA matrix, 50 pmol each primer, 0.2 mM each dNTP and 2.5 U *Taq* DNA polymerase (Boehringer Mannheim). The primers used for amplification were: 5′-AAGGAATTTTAAGATATACA-3′ (pT4+ and pT4pro sense); 5′-ATGGCTAGATCATTACAAATATTGGC-3′ (pT4– and pT4ter sense); 5′-AATACAATTTAGGTCCC-3′ (pT4+ and pT4ter antisense); 5′-TCAAAATACTTCTTCTCTGACTTGGAGG-3′ (pT4– and pT4pro antisense); 5′-AATGGAAATATAATTCATC-3′ (pND7+ and pND7pro sense); 5′-ATGAGAAAAATAATATAATTATTG-3′ (pND7– and pND7ter sense); 5′-TCACTTTTCTTCCTATTTTTC-3′ (pND7– and pND7pro antisense); 5′-CTAAATACAATTATTAGGG-3′ (pND7+ and pND7ter antisense). PCR reactions were carried out as described (39).

Plasmid preparation and microinjection

Large amounts of plasmids were obtained using the QiaFilter Plasmid Midi kit (Qiagen). All of one preparation was linearized at a unique site in the vector and extracted with phenol. After precipitation with ethanol, DNA was resuspended in water at 10–15 mg/ml. Microinjection of DNA into the macronucleus of young cells (five divisions after autogamy) was carried out as previously described (39).

Evaluation of exocytotic capacity

The genes chosen for this study encode proteins implicated in the regulated secretory pathway (for a review see 44). The phenotype associated with silencing of these genes mimics secretory mutants: the cells are unable to release their secretory granules (trichocysts). Exocytotic capacity was monitored using picric acid (45). This lethal fixative induces complete release of all exocytosis-competent trichocysts. The discharged material can be visualized under dark field light microscopy at low magnification as a halo surrounding the cell. In exocytosis-deficient cells, no secreted trichocysts are visible. Shape and intracellular localization of the trichocysts in exocytosis-deficient cells were observed by immunofluorescence as described (39).

Dot blot analysis

The amount of transforming DNA maintained in different clones was determined by dot blot analysis as described (39). For all experiments with constructs that did not produce gene silencing or functional complementation when introduced into wild-type or mutant cells, respectively, clones maintaining at least 30 haploid equivalents (the average of three measurements for each clone) of the injected sequence were chosen for molecular characterization. This ensures that copy number is above the previously established threshold for gene silencing (39).

RNA extraction and northern blot analysis

Total RNA was prepared from log phase cultures using the Trizol reagent (Gibco-BRL) as described (46). Total RNA (10 µg/lane) was fractionated on formaldehyde–1.25% agarose gels and transferred to positively charged nylon membranes (Ambion) by capillary action. Membranes were UV crosslinked and washed in 2× SSC for 5 min. Filters were prehybridized for 2 h at 60°C in 6× SSC, 2× Denhardt's solution, 0.1% SDS. Hybridizations were carried out at the same temperature for 16–24 h in the same buffer, with 10^6 c.p.m. of ^{32}P-labeled probe per ml hybridization buffer. Filters were washed for 5 min at room temperature and 30 min at 60°C, first in 2× SSC, 0.1% SDS and then in 0.2× SSC, 0.1% SDS.

Preparation of radioactive DNA probes

DNA for ^{32}P-labeling reactions consisted of PCR amplification products of plasmids containing the coding sequence of the gene *T4a*, *ND7* or *ICL1a* (GenBank accession no. U35344). PCR reactions and primers used for the amplification of pT4– and pND7– are described in the section 'Plasmid constructs'. The primers used for amplification of *ICL1a* were: 5′-GGCACGAAGAGGATAGT-3′ (ICL1a– sense); 5′-GCAAAGGTCTTTTTTGTCATAATG-3′ (ICL1a– antisense). Probes were synthesized by [α-^{32}P]CTP (3000 Ci/mmol) incorporation using a Random

Primers Labeling System (Gibco-BRL) according to the supplier's protocol.

Riboprobe synthesis

The coding region of the *T4a* or *ND7* gene was cloned into vector pGEM-T, which contains the T7 and SP6 promoters separated by a polylinker. Sense and antisense riboprobes were synthesized *in vitro* using the T7/SP6 MaxiScript kit (Ambion). Unincorporated nucleotides were removed with Sephadex columns (MicroBio Spin 30; Bio-Rad).

In vitro nuclear run-on experiments

The protocol used has been previously described (46). DNA probes were fixed on nylon membranes prepared by PCR amplification of the coding region of the *T1b* (GenBank accession no. U47115), *T2c* (GenBank accession no. U47116), *T4a* and β-tubulin (GenBank accession no. X67237) genes, the *T4a* 3′ non-coding region and plasmid pGEM-T. The primers used for *T4a* amplification have been described. For the other genes, the primers used were as follows: 5′-ATGTATAAATTAGCAGTCTGCACAT-TGC-3′ (*T1b* sense); 5′-CAGCTCTTTGGAATTCAGC-3′ (*T1b* antisense); 5′-ATGAAGACAATAATCCTTGCCTTAGCAC-3′ (*T2c* sense); 5′-TCAGATTTCTTCTCCAGCTGATTATCTTA-3′ (*T2c* antisense); 5′-ATGAGAGAAATCGTTCATATTCAAG-3′ (β-tubulin sense); 5′-GTTGTGATAAAAATCACTTAGATTATC-3′ (β-tubulin antisense). An aliquot of 500 ng each DNA was mixed with 200 µl of 5× SSC, 0.4 N NaOH, and incubated for 30 min at 65°C. The denatured DNA was then loaded on positively charged nylon membranes (Ambion) using a home-made dot blot apparatus and hybridized as described (46).

RESULTS

Homology-dependent gene silencing in *Paramecium* acts at the post-transcriptional level

Although homology-dependent gene silencing in *Paramecium* seems to be related to PTGS described in other organisms, a rigorous demonstration that the phenomenon is post-transcriptional is lacking. We performed *in vitro* nuclear run-on assays using cells silenced for the *T4a* gene. This gene belongs to the *TMP* (trichocyst matrix protein) multigene family, encoding proteins stored in secretory granules. *TMP* genes are transcribed at a rate easily detected by nuclear run-on experiments and the resulting mRNA is abundant and stable (46). The *TMP* gene family consists of several subfamilies, designated *T1*, *T2*, *T4*, etc. Genes belonging to different subfamilies do not share significant sequence identity; however, within each subfamily there are between four and eight co-expressed genes, sharing more than 85% sequence identity (44,47). Thus, *T4a* belongs to the *T4* gene subfamily. As previously shown (39), injection of a plasmid with only the *T4a* coding region, pT4– (see Fig. 1 for plasmids and nomenclature), induces a strong reduction in *T4* subfamily mRNA and generates exocytotic mutants. As illustrated in Figure 2A, secretory granule biogenesis is perturbed and the aberrantly shaped trichocysts cannot attach to the cortex to be secreted (39,48).

Nuclear frameworks from uninjected and pT4– injected wild-type cells were prepared and the transcription rate for three *TMP* gene subfamilies (*T1*, *T2* and *T4*) was measured. To differentiate exogenous from endogenous *T4* genes, PCR fragments corresponding to the 3′ non-coding region of the *T4a* gene (exclusively present in the endogenous gene) and pGEM-T plasmids (specific for the exogenous DNA) were fixed on a membrane. The β-tubulin gene was used as an invariant control. As shown in Figure 2B, we observed a strong pGEM-T signal specific for the injected cells. This demonstrates that the transgenes are highly transcribed, despite the absence of any known *Paramecium* regulatory sequences. For the *T1* and *T2* genes, the transcription rate is 5-fold higher in injected than in control cells. This is not surprising because the *TMP* genes are co-regulated at the transcriptional level and their transcription rate is inversely correlated with the number of trichocysts docked at the plasma membrane (46). Silencing of one of the *TMP* gene subfamilies creates secretory mutants without any docked trichocysts, a situation that promotes the transcriptional activation of all *TMP* genes. The *T4* signal, representing the sum of pT4– transgene transcription and of endogenous *T4* gene transcription, is 35-fold higher in the injected cells. However, the *T4a* 3′ non-coding signal, specific for the endogenous gene, follows the 5-fold activation of the other *TMP* genes. This indicates that the endogenous *T4a* gene is normally transcribed and regulated. Northern blot analysis performed on the same population confirmed a reduction of 75% in the *T4* steady-state mRNA level (not shown), as previously established (39). Taken together, these data provide direct evidence that gene silencing in *Paramecium* does not affect transcription of endogenous genes but acts at the post-transcriptional level.

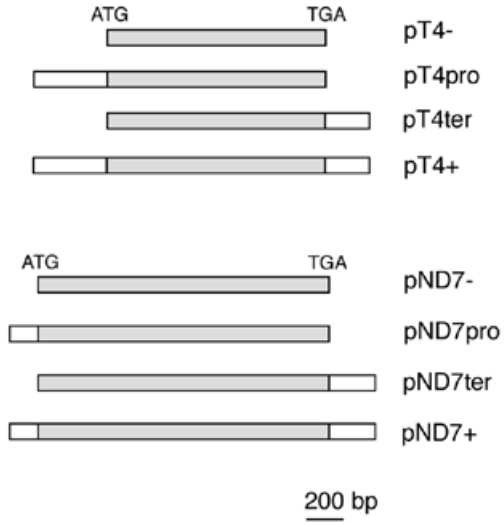

Figure 1. Plasmids used to transform *Paramecium*. Gray boxes represent the coding regions of the *T4a* and *ND7* genes, of 1143 and 1521 bp, respectively. White boxes represent the 5′ (392 and 157 bp) and 3′ (241 and 249 bp) regulatory sequences of the *T4a* and *ND7* genes, respectively. The regions indicated were cloned in the pGEM-T vector. The name of each of the resulting plasmids is given to the right.

Sense and antisense RNA molecules are present in aberrant RNA formed during PTGS

The next step was to better characterize the aberrantly sized RNA molecules that appear in silenced populations. Wild-type

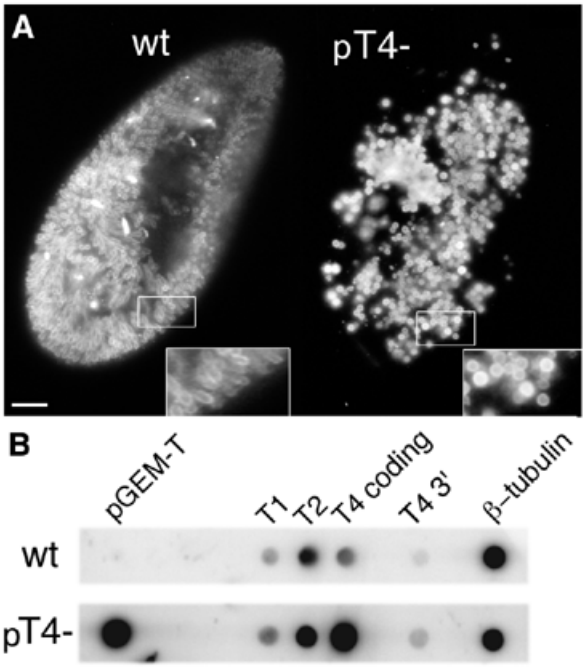

Figure 2. Run-on transcription indicates that the silencing mechanism is post-transcriptional. (**A**) Immunofluorescence images of trichocysts in control and pT4– silenced cells. The control wild-type cell on the left has spindle-shaped trichocysts aligned at the plasma membrane. Only the trichocyst body is stained by an antibody that recognizes the whole family of TMPs; the tip by which the trichocyst is attached to the plasma membrane is not visible. The trichocysts in the silenced cell appear spherical and cannot attach to the plasma membrane or be secreted. Bar: 10 µm; 5 µm for the enlarged insets. (**B**) Run-on transcription of control and pT4– silenced cells. The probes fixed on the membrane are, from left to right, plasmid pGEM-T, the *T1b*, *T2c* and *T4a* coding regions, *T4a* 3′ sequences not present in the pT4– transgene construct and the β-tubulin PTBPT1 gene coding region as an invariant control. After hybridization of the membranes to nascent transcripts elongated *in vitro* in the presence of [α-^{32}P]UTP, the signals were quantified by phosphorimaging.

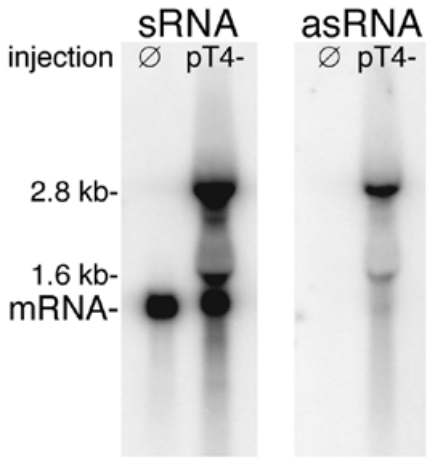

Figure 3. Cells silenced by transgenes with coding sequences contain both sense and antisense aberrant RNA. Duplicate northern blots of total RNA from control and pT4– injected wild-type cells were hybridized with antisense and sense riboprobes corresponding to the coding region of the *T4a* gene. The large and small subunit RNAs migrate at 2.8 and 1.6 kb, respectively, as indicated. T4 mRNA is ~1.2 kb.

cells were injected with plasmids carrying the coding region of the *T4a* gene. Steady-state mRNA was analyzed by northern blot, using riboprobes consisting of *T4a* sense or antisense coding sequences. As shown in Figure 3, aberrantly sized RNA produced in injected cells hybridized with each of the probes, showing that these molecules are in both orientations, sense and antisense. We verified that these are indeed RNA molecules by RNase A digestion of the samples (data not shown).

To understand the functional significance of the sense and antisense aberrant RNA molecules produced from transgenes, we decided to test the ability of constructs with and without different regulatory regions to lead to aberrant RNA formation and to provoke gene silencing.

Transgenes containing promoter and coding sequences can induce gene silencing

The different transgenes tested are presented in Figure 1 and the phenotypes obtained in transformation experiments are summarized in Table 1. In addition to the *T4a* gene presented above, the *ND7* gene was also used. *ND7* is a unique gene required for exocytotic membrane fusion, the final step of regulated secretion in *Paramecium* (37). This gene is transcribed at a rate too low for analysis by nuclear run-on experiments; however, the steady-state mRNA level can be detected on northern blots. Transformation of wild-type cells with the pND7– transgene (containing the coding sequence) produces exocytotic mutants unable to secrete their trichocysts (39).

A first set of experiments was carried out with constructs containing the promoter region and the coding sequences of

Table 1. Exocytotic phenotypes of clones transformed with different transgenes

Transgene	Host strain	
	Wild-type	nd7-1
Uninjected control	+	–
pT4+	+	ND
pT4–	–	ND
pT4pro	–	ND
pT4ter	+	ND
pND7+	+	+
pND7–	–	–
pND7pro	–	+ → –[a]
pND7ter	+	+

This table summarizes the effects of injection of different transgenes on the exocytotic phenotype of wild-type and nd7-1 mutant cells. The transgene constructs are given in Figure 1. Results were scored for 5–12 independent clones. In all cases where injection of transgenes did not affect the exocytotic phenotype of the host strain, clones maintaining >30 copies/haploid genome of the transgene, a value above the threshold necessary to obtain gene silencing, were selected for molecular analyses. ND, not done.
[a]Functional complementation is observed 24 h after injection but is then superceded by a silenced phenotype starting 48 h after injection.

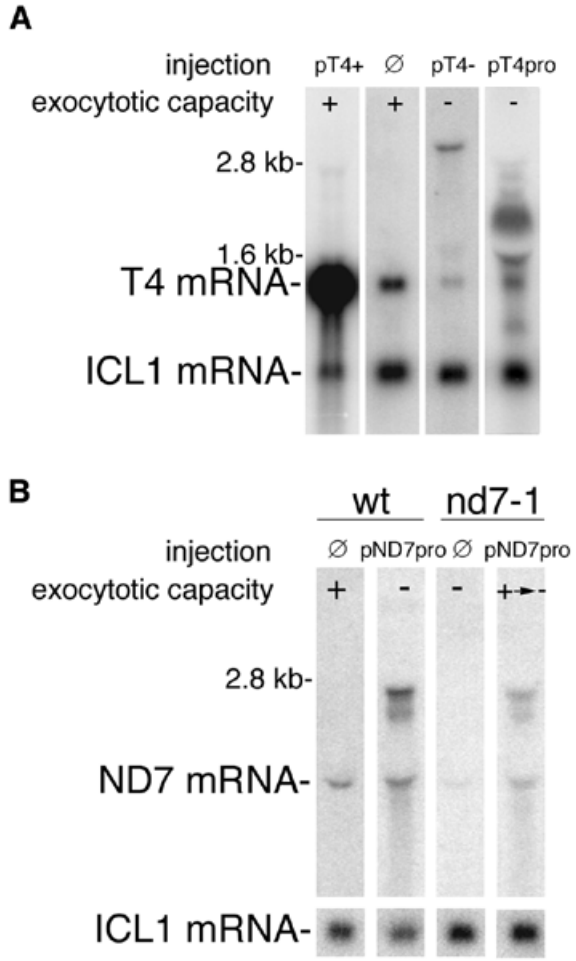

Figure 4. Transgenes with promoters and coding sequences produce silenced phenotypes and aberrant RNAs. Northern blots of total RNA from populations injected with different constructs, hybridized with a DNA probe specific for the *T4a* (**A**) or *ND7* (**B**) gene and with an *ICL1* gene probe to normalize the mRNA levels, are shown. In (B), the injected strain (wild-type or nd7-1) is indicated. Phenotypes, i.e. exocytotic capacity (+ or –), of the clones used for RNA extraction are given above the blots and refer to 100% of the 50–100 cells tested. T4 mRNA is ~1.2 kb, ND7 mRNA is ~1.5 kb and ICL1 mRNA is ~600 nt.

T4a and *ND7*, using the pT4pro and pND7pro plasmids. For comparison, plasmids containing the *T4a* coding sequence alone (pT4–) or the gene with its complete regulatory sequences (pT4+) were injected into wild-type cells. As previously shown, injection of pT4– generates an exocytotic mutant phenotype and northern blot analysis confirmed a reduction in *T4* mRNA and the presence of aberrant RNA (Fig. 4A, lane 3). Injection of pT4+ causes overexpression of the gene, manifested as an increase in *T4* mRNA level, and no detectable phenotype (Fig. 4A, lane 1). Injection of pT4pro produced exocytosis-deficient phenotypes. Northern blot analysis revealed a reduction in mRNA levels and the appearance of aberrantly sized RNA (Fig. 4A, lane 4). Injection of pND7pro into wild-type cells similarly resulted in an exocytosis-deficient phenotype and the appearance of aberrant RNA (Fig. 4B, lane 2). We note that *ND7* transgenes lead to production of aberrant RNA of the same size as the endogenous mRNA (A.Galvani and M.Froissard, unpublished observations), so that any reduction in the latter is masked. These experiments show that constructs containing a functional promoter can efficiently induce gene silencing.

The *nd7-1* mutation is a frameshift that moves the stop codon 300 bp downstream from its normal position (37). Northern blot analysis of total RNA from nd7-1 mutant cells revealed that the ND7 mRNA level is dramatically reduced, leading to the conclusion that the mutation disturbs the stability of the mRNA (Fig. 4B, lanes 1 and 3). The pND7pro construct was injected into nd7-1 mutant cells. The day after injection (after four or five divisions), cells were able to secrete numerous trichocysts, indicating that the mutation is complemented by pND7pro. Strikingly, 48 h after injection, i.e. after the time required to establish a silenced state, recovery of the wild-type phenotype disappears and the cells become mutant again, although they contain over 30 haploid equivalents of the transgene, i.e. values well over the threshold for silencing. mRNA analysis was performed on several clones (Fig. 4B, lane 4). Aberrant RNAs were detected, similar to those formed after injection of pND7pro into wild-type cells, indicating that the transgenes are transcriptionally active. Our interpretation of these results is that enough functional ND7p is produced by the pND7pro transgene to complement the mutation; however, phenotypic rescue is transitory since gene silencing is then established, as in wild-type cells injected with the same construct. This is the first example of silencing of a transgene in *Paramecium*.

Transgenes containing coding sequences followed by the 3′ non-coding region are unable to induce gene silencing

We next tested transgenes containing the coding sequence of a gene with its 3′ regulatory region, but lacking a promoter. This was performed for the *T4a* (pT4ter) and *ND7* (pND7ter) genes. No silencing phenotypes were observed after microinjection of wild-type cells with either construct, although transgene copy numbers were largely above the threshold necessary to induce the phenomenon in *Paramecium*. Analysis of RNA did not reveal any significant reduction in endogenous mRNA levels (Fig. 5 and data not shown).

Complementation experiments were performed with the pND7ter construct: as for pND7pro, injection of the pND7ter transgene into the nd7-1 mutant restores the wild-type phenotype within 24 h (Table 1). Unlike cells injected with the pND7pro transgene, rescue is stable: we never observed the appearance of a silencing phenotype, even several days after microinjection. Taken together, the data obtained for the *T4a* and *ND7* constructs clearly show that transgenes containing a coding sequence followed by its 3′ non-coding region are unable to induce genetic extinction of the corresponding genes.

Transgenes containing the 3′ non-coding region produce antisense RNA

In order to see whether aberrant RNA is produced in the presence of 3′ non-coding sequences, we probed total RNA from uninjected wild-type cells and wild-type cells injected with the pT4+, pT4pro and pT4ter constructs with sense and antisense *T4a* riboprobes. As shown in Figure 5, each of these constructs (like the pT4– construct, Fig. 3) leads to antisense RNA

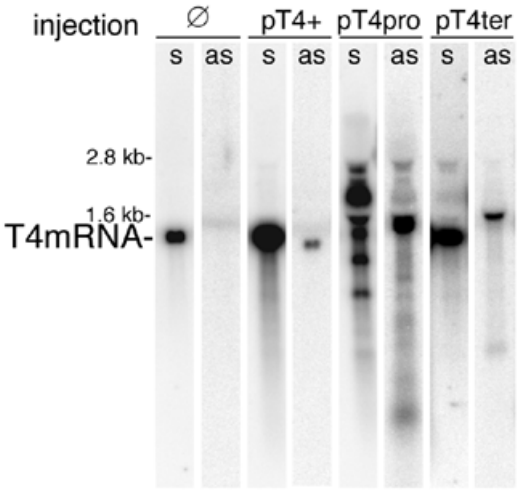

Figure 5. Antisense RNA is produced by transgenes containing 3′ sequences that do not provoke gene silencing. Northern blots of total RNA from wild-type cells injected with pT4+, pT4pro and pT4ter constructs were hybridized with sense and antisense riboprobes specific for the *T4a* coding sequence. The orientation of the hybridizing RNA (s for sense and as for antisense) is indicated beneath each blot.

production. In particular, pT4ter-transformed cells present an antisense band migrating more slowly than the mRNA, superimposed on a smear with some weak, fast migrating bands. Surprisingly, even the pT4+ construct leads to production of a small amount of antisense RNA migrating as a discrete band just ahead of the mRNA. Similar results were obtained for the pND7pro and pND7ter constructs (not shown). Thus transgenes containing the 3′ non-coding region still lead to production of antisense RNA molecules, even though they do not induce gene silencing.

DISCUSSION

PTGS in *Paramecium*

Homology-dependent gene silencing in *Paramecium*, which has proven to be an efficient tool for functional analysis (39,48–51), is induced by microinjection into the somatic nucleus of transgenes carrying only the coding sequence of the target gene. In order to clarify the nature of *Paramecium* gene silencing and to understand the sequence requirements of this transgene-induced phenomenon, we have analyzed the ability of different combinations of promoter, coding and 3′ non-coding regions to provoke silencing and characterized the RNA produced in the transformed cells, by run-on transcription and northern blot analysis.

Together with a previous characterization of gene silencing in *Paramecium* (39), which established that the phenomenon is vegetative, homology-dependent and has a threshold in terms of transgene copy number, the results reported here confirm that the phenomenon is closely related to transgene-mediated PTGS. Run-on transcription experiments not only showed that the silenced endogenous gene is normally transcribed, but also that the transgene is transcribed, even in the absence of defined *Paramecium* promoter elements. The analysis unequivocally establishes that *Paramecium* gene silencing is post-transcriptional. In addition, we have obtained the first evidence that a transgene can be silenced in *Paramecium* as in co-suppression in other organisms. Injection of pND7pro, consisting of the coding sequence of the *ND7* gene preceded by its promoter, in the nd7-1 loss-of-function mutant leads to complementation of the mutation within 24 h of injection. However, by 48 h, the time required to establish stable silenced phenotypes for the *Paramecium* genes studied to date, a silencing phenotype appears. Finally, we have shown by northern blot analysis using riboprobes that both sense and antisense RNA is transcribed from transgenes. This is consistent with the formation of large dsRNA molecules, supposed to be the agent that initiates cognate mRNA degradation in *Paramecium*, as is now established *in vivo* (22,52) and *in vitro* (21,23) for RNAi and is also experimentally supported for PTGS in plants (27,28,53).

Inhibitory effects of 3′ non-coding sequences

Our examination of transgene sequence requirements using two different genes that affect independent steps of the regulated secretory pathway (cf. 44) shows that the presence in the transgene construct of the 3′ non-coding region of a gene prevents gene silencing, whether or not a promoter is also present. This inhibitory effect of 3′ sequences is probably general in *Paramecium*, since somatic transformation experiments with many different genes bearing homologous or heterologous flanking sequences required for expression have been carried out in different laboratories for over a decade and no gene silencing was ever reported. Instead, complementation or overexpression are obtained with complete gene constructs, as shown here for *ND7* and *T4a*, respectively. One possible explanation is that the presence of the 3′ non-coding region limits read-through transcription so that not enough efficient triggering molecules (antisense RNA than can form dsRNA for example) are produced. However, aberrantly sized RNA molecules are detected in cells injected with constructs unable to induce gene silencing. These molecules are probably produced by transcription from cryptic initiation sites on either strand of the injected plasmids, a postulate consistent with the abundance of transcripts that hybridize with vector probe in nuclear run-on experiments.

To explain the inhibitory effect of the 3′ non-coding sequences, we suggest that RNA processing signals present in these sequences may be responsible for sequestering RNA molecules away from the gene silencing pathway. It is important to keep in mind that in *Paramecium*, transforming DNA is maintained in an episomal state and does not integrate into the genome, so that transgene expression is not affected by position effects or host *cis* regulatory sequences at insertion sites. As schematized in Figure 6, we interpret our results as follows. Constructs lacking 3′ non-coding sequences are transcribed on both strands from cryptic promoters and the aberrant sense transcripts either hybridize with the antisense transcripts or serve as template for an RdRP (9,11,14,17), leading to dsRNA formation. The dsRNA then triggers the catalytic, sequence-specific RNA degradation pathway defined by recent *in vitro* studies (reviewed in 54). Transgenes with 3′ non-coding sequences are also transcribed on both strands, as shown by the presence of transgenic antisense RNA in the transformed cells. However, the sense transcripts now carry 3′ processing signals important for mRNA formation. Not enough sense RNA can enter the silencing pathway since it is processed and sequestered.

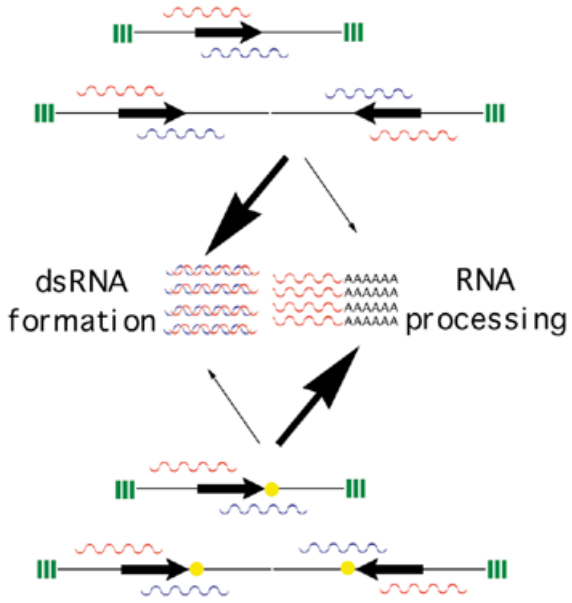

Figure 6. The quality of transgenic sense transcripts determines the outcome of somatic transformation. Transgenes without (top) or with (bottom) 3′ non-coding sequences (yellow circles) are shown. Thin line, plasmid; thick arrow, *Paramecium* gene coding sequences; vertical green lines, telomeric repeats. In both cases, the transgenes are transcribed to produce sense (red) and antisense (blue) RNA. In the absence of 3′ non-coding sequences (top), the sense RNA transcripts either hybridize with the antisense transcripts to form dsRNA or serve as template for an RdRP, in either case leading to dsRNA formation which triggers gene silencing. In the presence of 3′ non-coding sequences (bottom), the 3′ processing signals make RNA processing efficient and/or sequester the sense transcripts before they can enter the gene silencing pathway or lead to dsRNA formation.

Processed sense RNA would be translated or, if not all signals for translation are present or correctly positioned, it would be degraded without triggering the sequence-specific pathway implicated in gene silencing. The outcome of a transgenic experiment in *Paramecium* would thus depend on the quality of the transgenic sense transcripts, since different metabolic pathways are in competition for these molecules.

The idea that different pathways of RNA metabolism may coexist and interact in the cell is supported by recent studies in different organisms. Experiments in *C.elegans*, *Drosophila* and human cell lines provide evidence that the RNAi and stRNA (small temporal RNA; 55) pathways of gene regulation via tiny RNA molecules are linked (56,57). The conserved RNA processing gene *Dicer* (25) is involved both in processing dsRNA to 21–23 nt siRNA molecules and in processing *let-7* and *lin-4* precursors to the 22 nt mature stRNA forms that regulate stage-specific development.

Of possible direct relevance to our own experiments, gene silencing is also linked to general mRNA surveillance. In *C.elegans*, some of the *smg* genes required for nonsense-mediated decay are also necessary for RNAi-mediated mRNA degradation (58). In *Chlamydomonas*, the *Mut6* gene required for PTGS is also involved in transposon silencing and in mRNA surveillance via degradation of aberrant nuclear RNAs (59).

The study of *Mut6* (59), along with recent work in plants (28,60; reviewed in 61), suggests that aberrantly processed RNA specifically retained in the nucleus can lead to dsRNA formation and efficient gene silencing. This may well be the explanation of our observations in *Paramecium.* Transcripts that lack signals for 3′-end formation are probably not efficiently exported to the cytoplasm, as is well established in yeast and metazoa (for a review see 62). Retained in the nucleus, they lead to dsRNA formation and gene silencing. Transcripts with signals for 3′-end formation would be efficiently exported to the cytoplasm and hence would be unable to participate in dsRNA formation, explaining why constructs with 3′ non-coding sequences do not provoke gene silencing. *Paramecium* may provide a useful system to test the hypothesis that nuclear localization of aberrant sense transcripts is critical in promoting dsRNA formation and initiation of homology-dependent gene silencing.

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REFERENCES

1. Bastin,P., Galvani,A. and Sperling,L. (2001) Genetic interference in protozoa. *Res. Microbiol.*, **152**, 123–129.
2. Bosher,J.M. and Labouesse,M. (2000) RNA interference: genetic wand and genetic watchdog. *Nature Cell Biol.*, **2**, E31–E36.
3. Cogoni,C. and Macino,G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Dev.*, **10**, 638–643.
4. Plasterk,R.H. and Ketting,R.F. (2000) The silence of the genes. *Curr. Opin. Genet. Dev.*, **10**, 562–567.
5. Sijen,T. and Kooter,J.M. (2000) Post-transcriptional gene-silencing: RNAs on the attack or on the defense? *Bioessays*, **22**, 520–531.
6. Matzke,M.A., Matzke,A.J., Pruss,G.J. and Vance,V.B. (2001) RNA-based silencing strategies in plants. *Curr. Opin. Genet. Dev.*, **11**, 221–227.
7. Gonczy,P., Echeverri,G., Oegema,K., Coulson,A., Jones,S.J., Copley,R.R., Duperon,J., Oegema,J., Brehm,M., Cassin,E., Hannak,E., Kirkham,M., Pichler,S., Flohrs,K., Goessen,A., Leidel,S., Alleaume,A.M., Martin,C., Ozlu,N., Bork,P. and Hyman,A.A. (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature*, **408**, 331–336.
8. Fraser,A.G., Kamath,R.S., Zipperlen,P., Martinez-Campos,M., Sohrmann,M. and Ahringer,J. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature*, **408**, 325–330.
9. Dalmay,T., Hamilton,A., Rudd,S., Angell,S. and Baulcombe,D.C. (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell*, **101**, 543–553.
10. Fagard,M., Boutet,S., Morel,J.B., Bellini,C. and Vaucheret,H. (2000) AGO1, QDE-2 and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi and RNA interference in animals. *Proc. Natl Acad. Sci. USA*, **97**, 11650–11654.
11. Mourrain,P., Beclin,C., Elmayan,T., Feuerbach,F., Godon,C., Morel,J.B., Jouette,D., Lacombe,A.M., Nikic,S., Picault,N., Remoue,K., Sanial,M., Vo,T.A. and Vaucheret,H. (2000) *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101**, 533–542.
12. Dalmay,T., Horsefield,R., Braunstein,T.H. and Baulcombe,D.C. (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO J.*, **20**, 2069–2078.
13. Cogoni,C. and Macino,G. (1999) Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science*, **286**, 2342–2344.

14. Cogoni,C. and Macino,G. (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature*, **399**, 166–169.
15. Catalanotto,C., Azzalin,G., Macino,G. and Cogoni,C. (2000) Gene silencing in worms and fungi. *Nature*, **404**, 245.
16. Ketting,R.F., Haverkamp,T.H., van Luenen,H.G. and Plasterk,R.H. (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell*, **99**, 133–141.
17. Smardon,A., Spoerke,J.M., Stacey,S.C., Klein,M.E., Mackin,N. and Maine,E.M. (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.*, **10**, 169–178.
18. Tabara,H., Sarkissian,M., Kelly,W.G., Fleenor,J., Grishok,A., Timmons,L., Fire,A. and Mello,C.C. (1999) The rde-1 gene, RNA interference and transposon silencing in *C. elegans*. *Cell*, **99**, 123–132.
19. Marathe,R., Anandalakshmi,R., Smith,T.H., Pruss,G.J. and Vance,V.B. (2000) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. *Plant Mol. Biol.*, **43**, 295–306.
20. Tuschl,T., Zamore,P.D., Lehmann,R., Bartel,D.P. and Sharp,P.A. (1999) Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.*, **13**, 3191–3197.
21. Zamore,P.D., Tuschl,T., Sharp,P.A. and Bartel,D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25–33.
22. Yang,D., Lu,H. and Erickson,J.W. (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.*, **10**, 1191–1200.
23. Hammond,S.M., Bernstein,E., Beach,D. and Hannon,G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, **404**, 293–296.
24. Elbashir,S.M., Lendeckel,W. and Tuschl,T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.*, **15**, 188–200.
25. Bernstein,E., Caudy,A.A., Hammond,S.M. and Hannon,G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, **409**, 363–366.
26. Chuang,C.F. and Meyerowitz,E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **97**, 4985–4990.
27. Waterhouse,P.M., Graham,M.W. and Wang,M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA*, **95**, 13959–13964.
28. Smith,N.A., Singh,S.P., Wang,M.B., Stoutjesdijk,P.A., Green,A.G. and Waterhouse,P.M. (2000) Total silencing by intron-spliced hairpin RNAs. *Nature*, **407**, 319–320.
29. Muskens,M.W., Vissers,A.P., Mol,J.N. and Kooter,J.M. (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Mol. Biol.*, **43**, 243–260.
30. Kooter,J.M., Matzke,M.A. and Meyer,P. (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.*, **4**, 340–347.
31. Schiebel,W., Pelissier,T., Riedel,L., Thalmeir,S., Schiebel,R., Kempe,D., Lottspeich,F., Sanger,H.L. and Wassenegger,M. (1998) Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell*, **10**, 2087–2102.
32. Wassenegger,M. and Pelissier,T. (1998) A model for RNA-mediated gene silencing in higher plants. *Plant Mol. Biol.*, **37**, 349–362.
33. Voinnet,O., Vain,P., Angell,S. and Baulcombe,D.C. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell*, **95**, 177–187.
34. Palauqui,J.C. and Balzergue,S. (1999) Activation of systemic acquired silencing by localised introduction of DNA. *Curr. Biol.*, **9**, 59–66.
35. Bourgain,F.M. and Katinka,M.D. (1991) Telomeres inhibit end to end fusion and enhance maintenance of linear DNA molecules injected into the *Paramecium primaurelia* macronucleus. *Nucleic Acids Res.*, **19**, 1541–1547.
36. Haynes,W.J., Ling,K.Y., Saimi,Y. and Kung,C. (1996) Toward cloning genes by complementation in *Paramecium*. *J. Neurogenet.*, **11**, 81–98.
37. Skouri,F. and Cohen,J. (1997) Genetic approach to regulated exocytosis using functional complementation in *Paramecium*: identification of the ND7 gene required for membrane fusion. *Mol. Biol. Cell*, **8**, 1063–1071.
38. Prescott,D.M. (1994) The DNA of ciliated protozoa. *Microbiol. Rev.*, **58**, 233–267.
39. Ruiz,F., Vayssie,L., Klotz,C., Sperling,L. and Madeddu,L. (1998) Homology-dependent gene silencing in *Paramecium*. *Mol. Biol. Cell*, **9**, 931–943.
40. Metzlaff,M., O'Dell,M., Cluster,P.D. and Flavell,R.B. (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. *Cell*, **88**, 845–854.
41. van Eldik,G.J., Litiere,K., Jacobs,J.J., Van Montagu,M. and Cornelissen,M. (1998) Silencing of beta-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates. *Nucleic Acids Res.*, **26**, 5176–5181.
42. Lefort-Tran,M., Aufderheide,K., Pouphile,M., Rossignol,M. and Beisson,J. (1981) Control of exocytotic processes: cytological and physiological studies of trichocyst mutants in *Paramecium tetraurelia*. *J. Cell Biol.*, **88**, 301–311.
43. Sonneborn,T.M. (1970) Methods in *Paramecium* research. *Methods Cell Physiol.*, **4**, 241–339.
44. Vayssié,L., Skouri,F., Sperling,L. and Cohen,J. (2000) Molecular genetics of regulated secretion in paramecium. *Biochimie*, **82**, 269–288.
45. Pollack,S. (1974) Mutations affecting the trichocysts in *Paramecium aurelia*. I. Morphology and description of the mutants. *J. Protozool.*, **21**, 352–362.
46. Galvani,A. and Sperling,L. (2000) Regulation of secretory protein gene expression in paramecium: role of the cortical exocytotic sites. *Eur. J. Biochem.*, **267**, 3226–3234.
47. Madeddu,L., Gautier,M.C., Vayssié,L., Houari,A. and Sperling,L. (1995) A large multigene family codes for the polypeptides of the crystalline trichocyst matrix in *Paramecium*. *Mol. Biol. Cell*, **6**, 649–659.
48. Vayssié,L., Garreau de Loubresse,N. and Sperling,L. (2001) Growth and form of secretory granules involves stepwise assembly but not differential sorting of a family of secretory proteins in *Paramecium*. *J. Cell Sci.*, **114**, 875–886.
49. Froissard,M., Keller,A.M. and Cohen,J. (2001) ND9P, a novel protein with armadillo-like repeats involved in exocytosis. Physiological studies using allelic mutants in paramecium. *Genetics*, **157**, 611–620.
50. Garreau de Loubresse,N., Ruiz,F., Beisson,J. and Klotz,C. (2001) Role of delta-tubulin and the C-tubule in assembly of *Paramecium* basal bodies. *BMC Cell Biol.*, **2**, online.
51. Ruiz,F., Beisson,J., Rossier,J. and Dupuis-Williams,P. (1999) Basal body duplication in *Paramecium* requires gamma-tubulin. *Curr. Biol.*, **9**, 43–46.
52. Fire,A., Xu,S., Montgomery,M.K., Kostas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.
53. Hamilton,A.J. and Baulcombe,D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**, 950–952.
54. Sharp,P.A. (2001) RNA interference-2001. *Genes Dev.*, **15**, 485–490.
55. Reinhart,B.J., Slack,F.J., Basson,M., Pasquinelli,A.E., Bettinger,J.C., Rougvie,A.E., Horvitz,H.R. and Ruvkun,G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, **403**, 901–906.
56. Grishok,A., Pasquinelli,A.E., Conte,D., Li,N., Parrish,S., Ha,I., Baillie,D.L., Fire,A., Ruvkun,G. and Mello,C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell*, **106**, 23–34.
57. Hutvagner,G., McLachlan,J., Pasquinelli,A.E., Balint,E., Tuschl,T. and Zamore,P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*, **293**, 834–838.
58. Domeier,M.E., Morse,D.P., Knight,S.W., Portereiko,M., Bass,B.L. and Mango,S.E. (2000) A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. *Science*, **289**, 1928–1931.
59. Wu-Scharf,D., Jeong,B., Zhang,C. and Cerutti,H. (2000) Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. *Science*, **290**, 1159–1162.
60. Mette,M.F., Matzke,A.J. and Matzke,M.A. (2001) Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. *Curr. Biol.*, **11**, 1119–1123.
61. Matzke,M., Matzke,A.J. and Kooter,J.M. (2001) RNA: guiding gene silencing. *Science*, **293**, 1080–1083.
62. Zhao,J., Hyman,L. and Moore,C. (1999) Formation of mRNA 3′ ends in eukaryotes: mechanism, regulation and interrelationships with other steps in mRNA synthesis. *Microbiol. Mol. Biol. Rev.*, **63**, 405–445.