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Distinct RNA-dependent RNA polymerases are required for RNAi triggered by double-stranded RNA versus truncated transgenes in *Paramecium tetraurelia*

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ABSTRACT

In many eukaryotes, RNA-dependent RNA polymerases (RdRPs) play key roles in the RNAi pathway. They have been implicated in the recognition and processing of aberrant transcripts triggering the process, and in amplification of the silencing response. We have tested the functions of RdRP genes from the ciliate *Paramecium tetraurelia* in experimentally induced and endogenous mechanisms of gene silencing. In this organism, RNAi can be triggered either by high-copy, truncated transgenes or by directly feeding cells with double-stranded RNA (dsRNA). Surprisingly, dsRNA-induced silencing depends on the putatively functional *RDR1* and *RDR2* genes, which are required for the accumulation of both primary siRNAs and a distinct class of small RNAs suggestive of secondary siRNAs. In contrast, a third gene with a highly divergent catalytic domain, *RDR3*, is required for siRNA accumulation when RNAi is triggered by truncated transgenes. Our data further implicate *RDR3* in the accumulation of previously described endogenous siRNAs and in the regulation of the surface antigen gene family. While only one of these genes is normally expressed in any clonal cell line, the knockdown of *RDR3* leads to co-expression of multiple antigens. These results provide evidence for a functional specialization of *Paramecium* RdRP genes in distinct RNAi pathways operating during vegetative growth.

INTRODUCTION

RNAi is a conserved eukaryotic mechanism of gene regulation which can be triggered by different forms of double-stranded RNA (dsRNA) (1). Since its discovery, the artificial introduction of dsRNA or the expression of inverted-repeat constructs have become powerful tools to inactivate gene expression. The RNAi machinery typically produces small RNAs (sRNAs), 21–28 nt in length, which have been shown to act in a homology-dependent manner. According to their origin and properties, sRNAs can inhibit gene expression at different levels. Post-transcriptional gene silencing (PTGS) can result from mRNA cleavage targeted by siRNAs, which are processed from long dsRNA precursors, or from translation inhibition. The latter is the most frequent mode of action of miRNAs (microRNAs), which are processed from genome-encoded, stem–loop forming transcripts (2). sRNAs also mediate transcriptional gene silencing (TGS) in organisms as diverse as plants and fungi through mechanisms involving DNA methylation, as seen in *Arabidopsis* (3–5), or histone methylation, as first demonstrated in the case of pericentric heterochromatin formation in budding yeast (6).

Despite the diversity of effector mechanisms, core enzymes involved in RNAi share a high degree of similarity among different organisms (1,7). siRNAs are usually cleaved from dsRNA by RNAse-III-type endonucleases of the Dicer family, yielding duplexes with characteristic 5′-monophosphate ends and 2 nt 3′ overhangs (8). Such is the case of the primary siRNAs that are cleaved from exogenously introduced dsRNA or artificially expressed hairpins in many systems. Stoichiometric considerations revealed that a few dsRNA molecules per cell are

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enough to cause RNAi phenotypes (9), leading to the discovery that the silencing response can be amplified through the action of RNA-dependent RNA polymerases (RdRPs) on the targeted mRNA, which results in the synthesis of secondary siRNAs. cRNA synthesis allows siRNA formation and homology-dependent silencing to spread outside of the initial inducer sequence, a process called transitivity (10). However, the precise mechanisms involved in RdRP-mediated synthesis of secondary siRNAs appear to vary in different organisms.

In *Arabidopsis*, both the 5′ and the 3′ fragments of an mRNA that has been targeted in its central region by primary siRNAs can become substrates for RdRP activity, generating dsRNAs that are then cut into secondary siRNAs by Dicer enzymes (11). While the appearance of secondary siRNAs upstream of the targeted region (3′- to 5′-transitivity) is thought to result from the priming of RdRP activity by the primary siRNAs (12), 5′- to 3′-transitivity appears to involve unprimed RdRP activity initiated at the 3′-end of the 3′ cleavage fragment (11). A different mechanism has been described in *Caenorhabditis elegans*. Targeting an mRNA with primary siRNAs makes it a substrate for an unprimed RdRP activity which directly synthesizes short antisense RNAs, predominantly from the targeted region but also from the 5′ and 3′ regions of the mRNA (13,14). These Dicer-independent secondary siRNAs have a 5′-triphosphate end and are responsible for a potent slicer activity (15). Although their length may be controlled by other factors, *in vitro* studies of a purified RdRP from *Neurospora* revealed two different reactions, one of which synthesizes 9–21-nt RNAs from the entire length of a single-stranded template (16). In view of these mechanistic differences, the common feature that will be used here as a definition of secondary siRNAs is the fact that they are synthesized by an RdRP from the targeted mRNA, rather than processed from the RNA molecule that initially triggers silencing.

Two entirely distinct RNAi pathways have been described in the ciliate *Paramecium tetraurelia* (17). In addition to the meiosis-specific scnRNA pathway, which is involved in epigenetic regulation of genome rearrangements during early development, a constitutively expressed pathway is responsible for homology-dependent gene silencing during the vegetative phase of the life cycle. The latter can be induced experimentally either by feeding cells with an *Escherichia coli* strain producing dsRNA (18), as observed in *C. elegans* (19,20), or by microinjecting 3′-truncated transgenes at high copy numbers into the somatic macronucleus, which leads to the production of aberrantly sized transcripts (21,22). Previous studies showed that both methods result in the accumulation of ~23-nt siRNAs which depend on the Dicer protein Dcr1 (17,23,24). The cloning and sequencing of ~23-nt siRNAs associated with dsRNA feeding suggested the existence of two different subclasses. One appeared to represent primary Dcr1 cleavage products of the dsRNA trigger since it contained sRNAs matching the entire length of that molecule on both strands, including sequences derived from the plasmid vector used for dsRNA production in *E. coli*. A more abundant subclass of sRNAs with a 5′-UTR bias, containing a short untemplated polyA stretch at the 3′-end, was strictly antisense to the targeted mRNA and did not include vector-derived sequences, suggesting that it represents RdRP-dependent secondary siRNAs (17).

Although previous work strongly suggests that ~23-nt siRNAs are responsible for reducing the steady state amount of homologous mRNAs, only limited experimental evidence is available as to whether this occurs through PTGS or TGS in *P. tetraurelia*. One study used run-on assays to examine transgene-induced silencing of the endogenous *T4a* gene and concluded that the effect was post-transcriptional (22). In the case of dsRNA-induced silencing, another study reported northern blot evidence for the cleavage of *Upf1* mRNA in the targeted region (25). Furthermore, the rapid disappearance of silencing phenotypes obtained by this method when dsRNA-producing bacteria are replaced with the normal food bacteria indicates that no lasting modification is set on the gene itself, suggesting that silencing only occurs through PTGS.

The aim of the present study was to investigate the involvement of four *Paramecium* RdRP genes in dsRNA-induced and in transgene-induced RNAi during vegetative growth. By knocking down the function of each gene in cell lines silenced for reporter genes, we show that two putatively functional RdRP genes are required for dsRNA-induced silencing. These genes are not only necessary for the accumulation of putative secondary siRNAs, but also, unexpectedly, for the accumulation of primary siRNAs processed from the dsRNA trigger. Similar tests show that a third RdRP gene, with a highly divergent catalytic domain, is involved in transgene-induced silencing. The same gene is also necessary for the accumulation of a cluster of endogenous siRNAs mapping in an intergenic region, and for the mutual exclusion that characterizes the expression of surface antigen genes.

MATERIALS AND METHODS

Paramecium strains and cultivation

Experiments were carried out with *P. tetraurelia* stock 51. Cells were grown at 27°C in wheat grass powder (Pines International Co., Lawrence, KS, USA) infusion medium bacterized with *Klebsiella pneumoniae* the day before use (unless otherwise stated) and supplemented with 0.8 μg/ml β-sitosterol.

Plasmid constructs

To induce silencing by dsRNA feeding, fragments of the coding region were cloned into the plasmid L4440 and transformed into the RNaseIII-deficient *E. coli* strain HT115DE3. Positions in the coding sequences (cdss) were as follows: *RDR1*: 2423-3162 of accession number GSPATG00024768001 (ParameciumDB, http://paramecium.cgm.cnrs-gif.fr); *RDR2*: 2422-3122 of GSPATG00036857001; *RDR3*: 1789-2462 or 548-1315 of GSPATG00006401001; *RDR4*: 1750-2459 of GSPATG00018564001; *ND169*: 1450-1860 of

GSPATG00008337001; *ICL7a*: 1-580 of GSPATG00021610001 and A^{51}: 380-873 of M65163. To induce RNAi by injection of a *ND169* transgene, a plasmid (pTI+) containing the entire *ND169* cds and 3′-UTR downstream of a bidirectional constitutive promoter was modified by removing the 3′ cds and 3′-UTR downstream from position 1653 of the cds (pTI− construct). *GFP* on the opposite site of the promoter served as a control for the presence in the macronucleus.

Induction of RNAi and analysis of phenotypes

Transgene-induced silencing was carried out by microinjection of the BglII-linearized pTI− (position 1.593). *Paramecium* cell preparation and microinjection was carried out as described (24). Gfp fluorescence level of injected cell lines was observed to estimate transgene copy numbers.

DsRNA-induced silencing was performed by feeding dsRNA-producing *E. coli* as described (26). DsRNA-induced co-silencing of two genes was achieved by providing two types of OD-synchronized feeding bacteria in a 1:1 ratio. Specificity of silencing was verified by semi-quantitative RT–PCRs using four different dilutions of cDNA.

Ability of trichocyst discharge was tested by supplementing small amounts of the culture with saturated picric acid (1:2). Expressed surface antigens were determined by adding specific antisera (anti-A^{51}, B^{51}, C^{51}, D^{51}, E^{51}, G^{51}, H^{51}, I^{51}, J^{51}, N^{51} or Q^{51}, 1:200) to small volumes of the culture (~50 cells). Immobilization of cells expressing the corresponding antigen was completed within 30 min at room temperature. To determine the percentage of cells expressing one or several surface antigens, immobilization reactions were carried out with one antiserum. Then a second antiserum was added to test for immobilization of remaining cells. Additionally, each antiserum was used separately as a cross-check.

Average division rate was determined from single cells cultured in 200 µl silencing medium (*E. coli* producing dsRNA) over 5 days. Cells were counted every 24 h and one cell was transferred to fresh silencing medium.

Total RNA extraction, northern blot analysis and real-time RT–PCR

RNA was extracted using TRIzol® Reagent (Invitrogen, Karlsruhe, Germany), modified by the addition of glass beads. Prior to harvesting, cells were transferred from silencing medium to medium supplemented with *K. pneumoniae* and 0.8 µg/ml β-sitosterol for 30 min to allow for the complete processing of provided dsRNA. Without this procedure, a smear of (partially) degraded dsRNA makes the identification of single RNA species impossible.

For sRNA northern blots, 20 µg of denatured total RNA were separated on 15% polyacrylamide (acrylamide:bis 19:1)-7 M urea gels, transferred to Hybond N^+ membranes (GE/Amersham, Braunschweig, Germany) in 20× SSC by vacuum and UV cross-linked. For standard northern blots, RNA was separated on formaldehyde 1.2% agarose gels (20 µg per lane) and blotted in 10 × SSC. Size markers used for valuation of siRNA migration were 17-, 21- and 25-nt unphosphorylated ssRNA oligonucleotides (microRNA Marker, NEB, Frankfurt am Main, Germany) which were detected by specific probes.

Hybridizations of siRNAs were carried out at 42°C in 1× church buffer (7% SDS, 0.25 M sodium phosphate, 1% SDS, 1 mM EDTA, pH 7.2). Membranes were washed in 2× SSC and 0.1% SDS for 5 and 30 min and subsequently with 0.2× SSC and 0.1% SDS for 5 and 30 min at the same temperature. Riboprobes were hybridized at 60°C in 6× SSC, 2× Denhardt's solution and 0.1% SDS. Membranes were washed at 60°C as described for siRNA northern blots. Labelled membranes were then exposed to phosphor image plates.

Real-time RT–PCR was carried out as described (26). Briefly, total TRIzol®-isolated RNA was additionally purified with the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA (500 ng) was reverse transcribed using an oligo-dT primer. cDNA was amplified with the QuantiTect™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Data from surface antigen expression were set in relation to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was found to be constantly expressed during vegetative growth and *RDR* silencing.

Probes, riboprobes and oligonucleotide probes

Double-stranded probes for siRNA detection in dsRNA-induced silencing spanned the silencing fragments or the polylinker between the T7 promoters. Transgene-siRNAs were hybridized with a double-stranded probe spanning the entire *ND169* cds or the plasmid part adjacent to the shortened *ND169* 3′-end. Probes were generated by random priming with [α-^{32}P] dCTP (3000 Ci/mmol). For strand-specific detection of siRNAs, two adjacent oligonucleotide probes were 5′-end labelled with T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 Ci/mmol) (for siRNAs of *ND169*: position 1580–1629 and 1631–1679; for siRNAs from the L4440 polylinker: position 80–130 and 165–215; for endogenous siRNAs from scaffold 22, ParameciumDB: position 564980–565029 and 565030–565079). Long transcripts of the *ND169* transgene and endogenous mRNA were detected with sense and antisense riboprobes synthesized *in vitro* from the entire coding region of *ND169* cloned into the pGEM-T vector (Promega, Mannheim, Germany). *In vitro* transcription was carried out using the MAXIscript® T7/SP6 Kit (Applied Biosystems/Ambion, Austin, TX, USA) and [α-^{32}P] UTP (800 Ci/mmol).

Biochemical analysis of siRNA

A 22-nt unphosphorylated RNA oligonucleotide and a portion of the same control oligonucleotide, which was 5′-monophosphorylated using T4 polynucleotide kinase were added to total RNA of each individual reaction as a control. Removal of 5′ phosphates was carried out by treating 20 µg of total RNA with 5 U calf intestinal alkaline phosphatase (CIP) (New England

Biolabs, Frankfurt am Main, Germany) for 1 h at 37°C. For verification of 5′-monophosphates, 20 µg of total RNA were incubated with 1 U Terminator 5′-monophosphate-dependent exonuclease (Epicentre, Madison, WI, USA) for 1 h at 30°C. For characterization of 3′-ends of siRNA, periodate treatment and β-elimination were carried out with 20 µg of total RNA as described (27). A 23-nt 5′-triphosphorylated control RNA oligonucleotide [*in vitro* transcribed from the SmaI digested vector pGEM-3Zf (Promega, Mannheim, Germany)] was used for control treatment to check Terminator exonuclease specificity. All samples were precipitated and dissolved in DEPC-treated-water before loading on a 15% (19:1) polyacrylamide gel.

SDS-PAGE, western blot and immunofluorescence staining

Surface antigens were isolated using a standard salt–alcohol extraction procedure (28). Gradient SDS polyacrylamide gels (6–15%) with 3% stacking gels were used (29). Proteins were electro-blotted to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) in 15.6 mM Tris–HCl, 120 mM glycine, 1% SDS and 20% methanol. After staining in 0.1% Ponceau S, membranes were blocked in 5% dried milk in TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.6) and decorated with primary sera and secondary antibodies conjugated with alkaline phosphatase (Promega, Mannheim, Germany). Primary sera were the anti-A^{51}, anti-B^{51}, anti-D^{51} and anti-H^{51} polyclonal sera (kind gift of J. Forney). Indirect immunofluorescence staining was carried out as described (30) using the Y4 monoclonal mouse antibody (31) (kind gift of Y. Capdeville) and polyclonal anti-D^{51} serum.

RESULTS

The *Paramecium* genome reveals four RdRP candidate genes

Four genes encoding putative RdRPs were identified in the *Paramecium* macronuclear genome, two of which (*RDR1* and *RDR4*) are paralogs derived from the intermediate whole genome duplication (32). Figure 1 shows an alignment of the predicted proteins with functional RdRPs from other eukaryotes over a portion of the catalytic domain. Many of the highly conserved amino acids are present in Rdr1 and Rdr2, including the DLDGD motif in which the third aspartatic acid residue was shown to be essential for catalytic activity in other organisms (16,33,34). In contrast, many conserved amino acids are missing in Rdr3 and Rdr4. The latter shows a seven-residue gap in the region of the DLDGD motif, whereas Rdr3 has three aspartic acid residues with different spacings. A phylogenetic analysis based on an alignment of the entire catalytic domains indicates that Rdr3 and Rdr4 have greatly diverged from Rdr1 and Rdr2, which are most closely related to the catalytically active Rdr1 protein from the ciliate *Tetrahymena thermophila* (35) (Supplementary Figure S1). Expression levels of the RdRP genes during vegetative growth were determined by real-time PCR. All four genes appear to be expressed at low levels (Table 1) and no significant upregulation of any of them was observed when cells were submitted to dsRNA feeding (data not shown). All predicted introns were verified by cDNA sequencing. This further revealed two non-predicted and inefficiently spliced introns in the *RDR4* gene, the splicing of which disrupts the integrity of the open reading frame. Thus, Rdr1 and Rdr2 may well be catalytically active, but it is

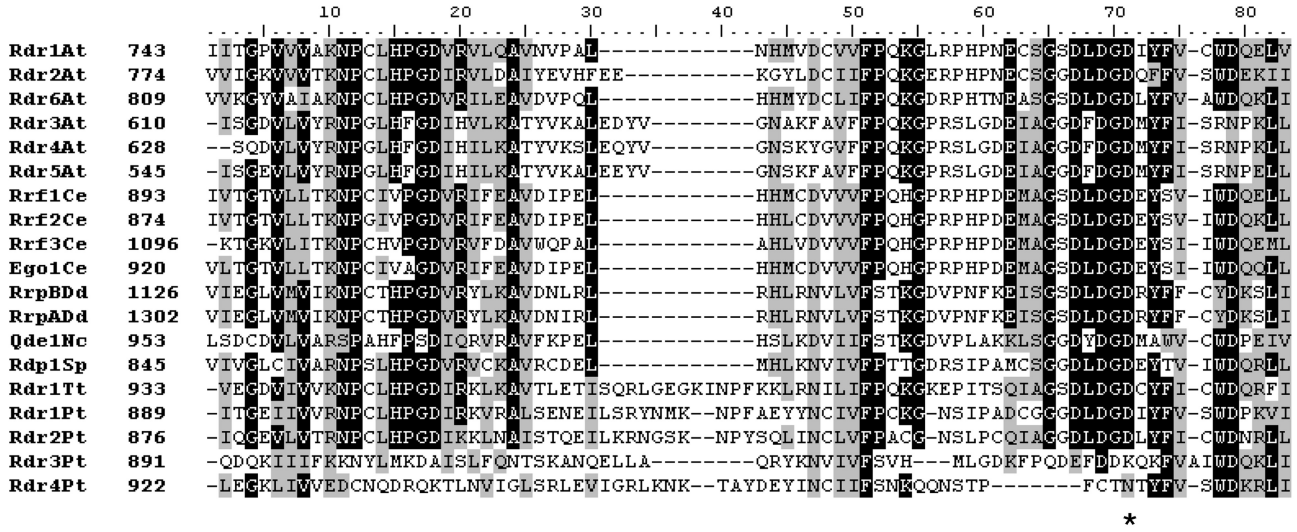

Figure 1. RdRPs in *P. tetraurelia*. Sequences of RdRP catalytic domains from *P. tetraurelia* and other organisms were aligned using the MUSCLE v4 software (67). Conserved residues are highlighted black and grey; the individual position in the protein is given by the position in amino acids. The asterisk indicates the aspartatic acid which was found to be necessary for *in vitro* catalytic activity of Qde1 (*Neurospora crassa*), Rdr6 (*A. thaliana*) and Rdp1 (*S. pombe*) (10,33,34). Accession numbers are as follows: *A. thaliana*, Rdr1-6: Q8H1K9, Q82504, O82190, O82189, O82188, Q9LKP0; *C. elegans*, Rrf1-3: Q9NDH1, Q9BH56, Q19285, Ego1: Q93593; *Dictyostelium discoideum*, RrpA & RrpB: Q95ZG7, Q95ZG6; *N. crassa*, Qde1: Q9NDH1; *S. pombe*, Rdp1: O14227; *T. thermophila*, Rdr1: QOMSN7; *P. tetraurelia*; Rdr1-4: Q3SE67, Q3SE69, Q3SE68, A0DMU3.

Table 1. mRNA levels of the *P. tetraurelia RDR* genes

	RDR1	*RDR2*	*RDR3*	*RDR4*	*GAPDH*
Relative transcript level	2.1 ± 0.8	0.8 ± 0.2	0.6 ± 0.2	0.2 ± 0.2	100 ± 0.0

Steady state levels were determined in vegetatively growing cells using real-time RT–PCR and were quantified in relation to the transcript level of the housekeeping gene *GAPDH*.

difficult to make any prediction about Rdr3; on the other hand, *RDR4* may be a non-functional pseudogene, which would be consistent with its extremely low mRNA level (Table 1) and with the fact that no phenotype was observed upon its silencing, as described below.

Rdr1 and Rdr2 are involved in dsRNA-induced silencing

To test whether *Paramecium* RdRPs are involved in dsRNA-induced silencing, we compared the efficiency of silencing of a reporter gene when each of the *RDR* genes, or an unrelated gene as a control, was co-silenced by dsRNA feeding. Although RNAi-mediated knockdown of genes involved in RNAi is a self-defeating process with variable outcomes, this approach has been used successfully in several genome-wide RNAi screens (36–39). The efficiency of the feeding procedure was verified by the accumulation of *RDR*-specific siRNAs and by the reduction of *RDR* mRNA levels (Supplementary Figure S2).

The chosen reporter genes were *ND169*, whose product is involved in membrane fusion during trichocyst ejection, and the A^{51} gene, encoding a frequently expressed surface antigen. Figure 2 shows the silencing efficiency of the reporter genes, as determined by phenotypic analysis. Feeding cells exclusively with bacteria producing *ND169* or A^{51} dsRNA resulted in close to 100% of cells in the cultures showing the expected phenotypes. When cells were fed a mix of these two bacterial strains in equal amounts, or a mix of each of them with bacteria producing dsRNA homologous to *ICL7a* (encoding a non-essential component of the cytoskeleton), phenotypes were only observed in 60–85% of cells, which is likely due to the lower amount of each ingested dsRNA. Similar results were obtained when cells were fed a mix of each reporter dsRNA with either *RDR3* or *RDR4* dsRNA. In contrast, feeding cells a mix of each reporter dsRNA and either *RDR1* or *RDR2* dsRNA resulted in a significantly lower fraction of cells showing the expected phenotypes (10–15%), which surprisingly indicates that *RDR1* and *RDR2* are involved in dsRNA-induced silencing. To determine whether the reduced silencing efficiency correlates with a lower amount of silencing-associated siRNAs, total RNA was extracted from these cultures and analysed on northern blots.

Two classes of dsRNA-induced siRNAs depend on Rdr1 and Rdr2

Hybridization with gene-specific probes readily revealed silencing-associated siRNAs for both reporters. In these northern blots, some lanes showed a ~1-nt ladder background signal. This likely resulted from ongoing degradation of ingested bacterial dsRNA because it was even more apparent when the cultures were not subjected to a brief starvation period to allow complete digestion of food vacuoles before RNA isolation (see 'Materials and Methods' section). Figure 2C shows that short RNAs associated with A^{51} silencing migrated with an apparent size between 22 and 23 nt. Considering that the RNA oligonucleotides used as markers were 5′-OH (and migrated ~0.5-nt faster than their 5′-monophosphate counterparts in the type of gel used, see below), these A^{51} siRNAs appear to have the same size as previously described *P. tetraurelia* siRNAs (17) and will be referred to as ~23-nt siRNAs. These were also present when the A^{51} surface antigen gene was co-silenced with the other reporter (*ND169*) or with *RDR3* or *RDR4*, but, consistent with the phenotypic analysis, A^{51} siRNAs were clearly less abundant in cultures that were also fed *RDR1* or *RDR2* dsRNA.

SiRNAs associated with *ND169* silencing were also easily detected in double-silencing tests involving *RDR3*, *RDR4* or *ICL7a*, but were almost completely absent in those involving *RDR1* or *RDR2*. However, *ND169* siRNAs migrated as a major band with an apparent size between 21 and 22 nt, i.e. ~1-nt faster than A^{51} siRNAs; taking into account the unphosphorylated state of markers, this band will be referred to as ~22-nt siRNAs. A weaker signal was also observed ~1-nt above the main band (this is more clearly seen with strand-specific probes in Figure 3). To determine whether the ~23-nt signal corresponded to a distinct subpopulation of siRNAs, the same blot was stripped and rehybridized with a probe specific for the plasmid vector sequences present in the dsRNA molecule (polylinker sequences on both sides of the *ND169* insert in the recombinant plasmid, in between the convergent T7 promoters; see Figure 2F). Strikingly, the vector-specific probe only revealed ~23-nt siRNAs (Figure 2E). Since these sequences are absent from the macronuclear genome, the ~23-nt siRNAs must be processed from the ingested dsRNA. Nevertheless, Figure 2E clearly shows that these siRNAs, like the ~22-nt *ND169* siRNAs, were almost completely missing when either *RDR1* or *RDR2* was co-silenced with *ND169*.

The siRNAs associated with *ND169* silencing were further characterized by hybridization with strand-specific oligonucleotide probes. Interestingly, the ~22-nt siRNAs could only be detected with a sense probe of the *ND169* sequence used for dsRNA production, indicating that they were strictly antisense (Figure 3A). In contrast, the ~23-nt siRNAs, although much less abundant, could be detected with *ND169* probes from both strands. This is more clearly seen in RNA samples from cultures that were only fed *ND169* dsRNA, where both the ~22-nt and the ~23-nt siRNAs are more abundant than in double-silencing cultures (Figure 3A, *ND* lane on the right). Similarly, the ~23-nt siRNAs homologous to vector sequences were derived from both strands of the dsRNA (Figure 3B).

The absolute strand bias of the ~22-nt siRNAs, together with the fact that they did not contain any

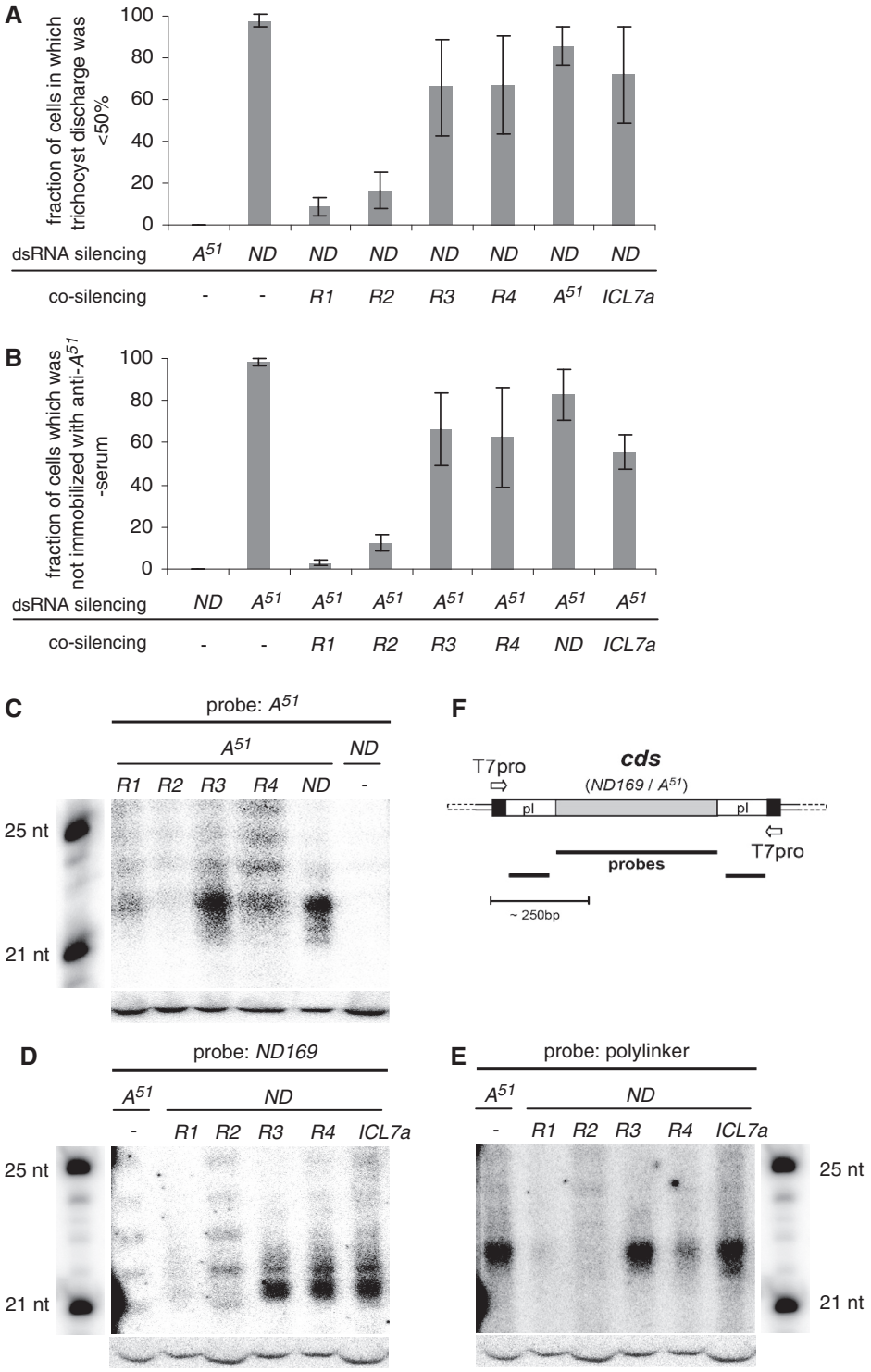

Figure 2. *Rdr1* and *Rdr2* are involved in dsRNA-mediated silencing. (**A** and **B**) Phenotypic analysis. Silencing induced by dsRNA feeding of the two reporter genes *ND169* (*ND*) (A) and A^{51} (B) was compared when they were co-silenced either with *RDR1-RDR4* (*R1-4*) or control genes (*ICL7a*; A^{51} or *ND169*). Silencing efficiency is indicated as percentage of cells which (A) discharged <50 % of trichocysts compared with negative control cells or which (B) were not immobilized by anti-A^{51} serum. Values result from 13 (A) and 9 (B) independent experiments (±standard deviation). Phenotypes were determined 48 h after first dsRNA feeding. Significance levels (*P*-values) between *RDR1* or *RDR2* samples and controls ranged between 0.04 and 0.0001 (one-way ANOVA). (**C**–**F**) Northern blot analysis of silencing associated sRNAs. Total RNA was isolated 48 h after first dsRNA feeding and run on 15% polyacrylamide–urea gels. Above each blot, the individual probes as well as the dsRNA feeding combinations are indicated: (C) A^{51} was co-silenced with *RDR1-RDR4* (*R1-4*) or the control gene *ND169*. (D and E) *ND169* (*ND*) was co-silenced with *RDR1-RDR4* (*R1-4*) or the control gene *ICL7a*. (F) Plasmid map (L4440) for T7 polymerase-mediated dsRNA synthesis in *E. coli*. Polylinker sequences (white) were located at both ends of the gene-specific fragment (grey) between the T7 promotors. Probes used in (C–E) are indicated as black bars. The lower panels show hybridization to glutamine tRNA as a loading control. *RDR4* silencing revealed lower amounts of polylinker siRNAs in Figure 2E, whereas the same blot showed no reduction of secondary ~22-nt siRNAs (D).

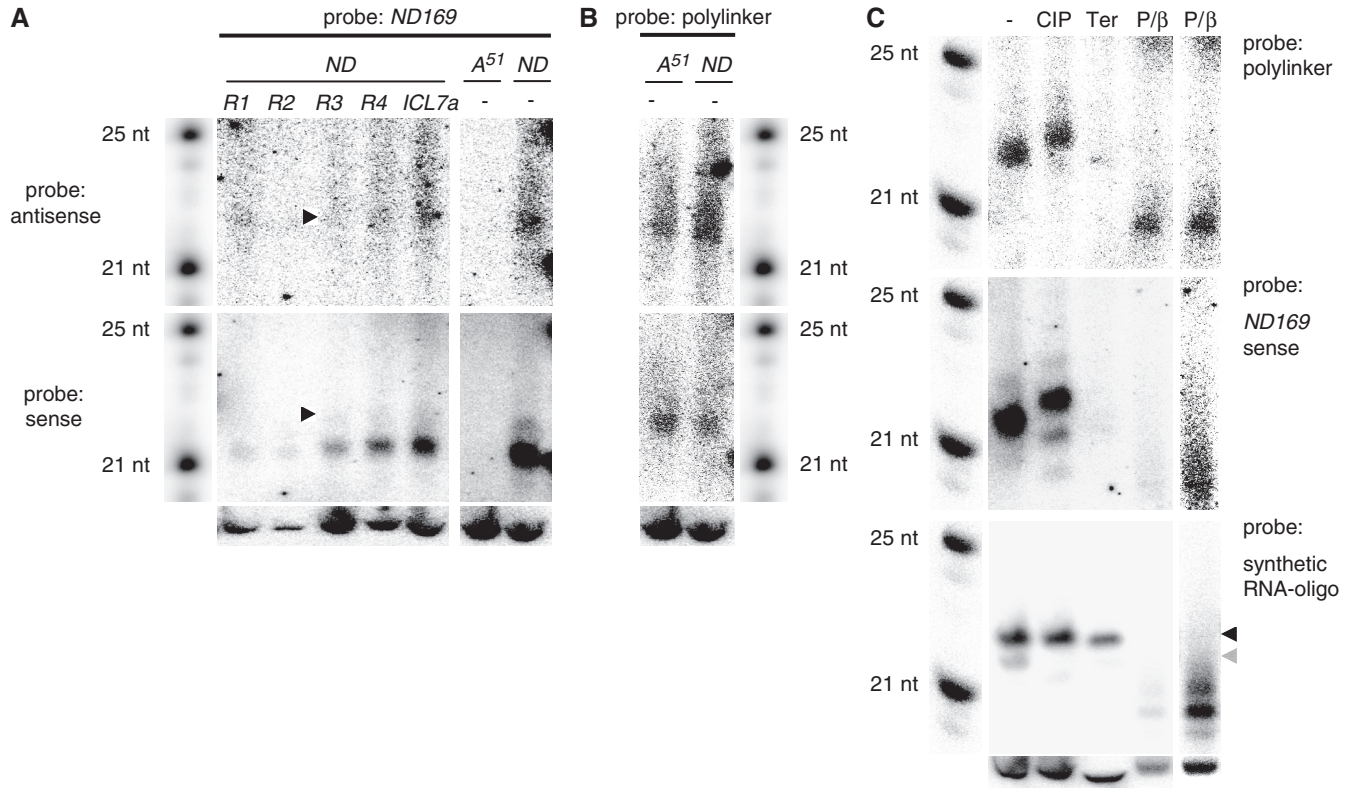

Figure 3. Characteristics of dsRNA-induced primary and secondary siRNAs. (**A** and **B**) Strand bias of siRNA. Blots described in Figure 2D and E were hybridized with two adjacent 50-nt strand-specific oligonucleotide probes located in the centre of the *ND169* dsRNA fragment (A) or a single oligo in the polylinker sequence (B) (upper blot: antisense-orientated probe, lower blot: sense-orientated probe). Arrowheads indicate small amounts of ~23-nt *ND169* siRNAs from both strands. (**C**) Properties of 5′- and 3′-ends of dsRNA-induced siRNA were analysed by treatment with CIP, Terminator (Ter) and periodate followed by β-elimination (P/β). Treatment of total RNA with CIP alkaline phosphatase, removing all 5′ phosphates, resulted in a ~0.5-nt slower migration of siRNA in comparison to untreated samples. This was found for polylinker-specific ~23-nt siRNA (upper blot) and *ND169*-specific ~22-nt siRNA (middle blot). Treatment of total RNA with Terminator 5′-monophosphate-specific exonuclease (Ter) degraded both classes of siRNA. Periodate treatment and subsequent β-elimination (P/β) resulted in ~1.5-nt faster migration of both classes of siRNAs as it was also observed for the 3′-unmodified control oligo. The second P/β-lane (right) represents the latter one with increased contrast. A 5′-monophosphorylated (grey arrowhead) and a 5′-unphosphorylated (black arrowhead) 22-nt RNA oligonucleotide, both lacking a 3′ modification, were added to each reaction as a control (lower blot). The lower panels show hybridization to glutamine tRNA as a loading control.

vector sequence, strongly suggests that they are not processed from the symmetrical dsRNA ingested, but represent secondary siRNAs synthesized by an RdRP activity from the endogenous *ND169* mRNA. Conversely, the much less abundant ~23-nt siRNAs, which can be found on both strands of the entire dsRNA including vector sequences, likely represent primary Dcr1 cleavage products. This further suggests that dsRNA-induced silencing of the A^{51} gene only generates primary siRNAs, a difference with *ND169* silencing which may be explained by the special regulation of surface antigen genes (see 'Discussion' section). The results of our functional tests of *RDR* genes thus unexpectedly indicate that both primary and secondary siRNAs depend on the *RDR1* and *RDR2* genes.

Different biochemical tests were then carried out to characterize the 5′- and 3′-ends of both classes of siRNAs (Figure 3C). As controls for the efficiency of reactions, small amounts of 5′-monophosphate and 5′-OH versions of a synthetic RNA oligonucleotide, both with a 3′-OH end, were added to total RNA from a culture that was fed *ND169* dsRNA. The sample was first treated with phosphatase (CIP), and the same northern blot was then successively hybridized with probes specific for the polylinker part of the dsRNA, for the antisense strand of the *ND169* sequence and for the synthetic oligonucleotide. CIP treatment resulted in an upward shift of the 5′-monophosphate control to its 5′-OH position (~0.5-nt slower). The same shift was observed for the ~23-nt siRNAs with both the polylinker and the *ND169* probes, and for the ~22-nt siRNAs with the *ND169* probe. Both classes of siRNAs were also completely degraded after treatment with the 5′-monophosphate-specific exonuclease Terminator, as was the 5′-monophosphate version, but not the 5′-OH version, of the control. Thus, both types of siRNAs appear to have 5′-monophosphate ends.

To test for a possible 2′-*O*-methylation on the 3′-terminal ribose, which is mediated by the Hen1 methyltransferase in diverse classes of short RNAs (40), we used periodate treatment and subsequent β-elimination, which will cleave the last nucleotide only if it is both 2′-OH and 3′-OH. As can be seen with the control, this results in a downward shift of ~1.5 nt because

of the retention of the 3′ phosphate. The same shift was observed for both classes of siRNAs and no resistant fraction could be detected, indicating that the 3′-terminal ribose was unmethylated.

Transgene-induced RNAi depends on Rdr3

To test the involvement of *RDR* genes in transgene-induced silencing, we injected an *ND169* truncated construct (pTI−) lacking the last 229 bp of the cds and the 3′-UTR into the macronucleus (Figure 4A). This resulted in a trichocyst non-discharge phenotype in ~100% of the cells, indicating that the endogenous *ND169* gene was silenced. Wild-type cultures and cells injected with the pTI+ transgene, containing the untruncated *ND169* gene, showed wild-type trichocyst discharge. The silencing of each *RDR* gene was then induced by dsRNA feeding as in the above experiments. As shown in Figure 4B, the pTI− phenotype was neither altered in the *ICL7a* and A^{51} dsRNA feeding controls nor by the feeding of *RDR1*, *RDR2* and *RDR4* dsRNAs. In contrast, the feeding of *RDR3* dsRNA had severe effects, suppressing the silenced phenotype in ~80% of cells. Northern blots of total RNA samples revealed *ND169* transgene-induced siRNAs migrating at ~23-nt in cultures without dsRNA feeding and with A^{51} dsRNA feeding as a control (Figure 4C). These siRNAs were not detectable in wild-type cells or pTI+ injected cells and were clearly reduced in pTI− cultures when *RDR3* was silenced, suggesting that their accumulation is responsible for silencing of the endogenous *ND169* gene. Hybridization of the same blot with a probe specific for the plasmid sequences located between the truncated *ND169* gene and the end of the injected linear molecule revealed siRNAs migrating slightly slower (~0.5 nt) than those detected with the *ND169*-specific probe; these were also clearly reduced in *RDR3* silencing samples (Figure 4D). All attempts to test the strand specificity of transgene-induced *ND169* siRNAs with oligonucleotide probes failed. We were only able to detect them by hybridization with probes of the entire *ND169* gene, which may be due to their low density or uneven distribution over the length of the construct.

Biochemical analysis of the 5′- and 3′-ends was carried out as described above. The bulk of transgene-induced siRNAs showed a ~0.5-nt slower migration after CIP treatment and disappeared after Terminator treatment, indicating that the vast majority (if not all) of them have a 5′-monophosphate end (Figure 4E). The residual signal seen at ~23 nt on the blot may simply be part of the 1-nt ladder background. In contrast to dsRNA-induced siRNAs, the migration of transgene-induced siRNA was unaffected after periodate treatment and β-elimination. Successful β-elimination was shown by the shift of the control oligonucleotide in the same sample. Although the treatment resulted in a reduced signal for both transgene-induced siRNAs and the control oligonucleotide, no shortened version of the siRNAs migrating ~1.5-nt faster was detectable even after a longer exposure. We therefore conclude that transgene-induced siRNAs carry a modification on either the 2′ or the 3′ of the 3′-terminal ribose.

Although the same reporter gene (*ND169*) was used here to study dsRNA- and transgene-induced silencing, ~22-nt siRNAs were clearly not present among transgene-induced siRNAs. Our data thus indicate differences between the two methods used to induce RNAi in *Paramecium*, both in terms of RdRP requirements and in the associated siRNAs, suggesting that the mechanisms involved are at least partially distinct. This conclusion is further supported by an experiment in which efficient silencing of the A^{51} gene was induced by dsRNA feeding in a pTI− injected culture in which transgene-induced silencing of the endogenous *ND169* gene was inhibited by *RDR3* silencing (Figure 4F). Simultaneous *RDR3* and A^{51} dsRNA feeding resulted in a loss of the *ND169* silencing phenotype comparable with single *RDR3* dsRNA feeding and, in contrast to *RDR1* or *RDR2* silencing, still led to efficient silencing of A^{51}.

Transgene-induced siRNAs are likely derived from aberrant transcripts of the truncated transgene. To test whether Rdr3 is involved in the synthesis of long antisense cRNA, a northern blot of total RNA samples from a pTI− injected cell line, with or without dsRNA-induced silencing of *RDR3*, was hybridized with sense and antisense *ND169* probes (Figure 4G). While the antisense probe only detected the ~1.8-kb *ND169* mRNA in wild-type cells and, in much greater amounts, in pTI+ injected cells, pTI− samples contained a ~3.2-kb sense transcript, which corresponds to the length between the promoter and the end of the linear transgene. The sense probe revealed a similar-sized antisense transcript specifically in pTI− samples. However, neither the sense nor the antisense aberrant transcripts were decreased in abundance upon dsRNA-induced silencing of *RDR3* or A^{51}. The antisense transcript of the transgene may arise from promiscuous transcription of the transgene as such full-length RNA was previously also observed for promoter and terminatorless sequences injected at high copy numbers into the *Paramecium* macronucleus (22). We conclude that the role of Rdr3 in the accumulation of transgene-induced siRNAs is likely downstream of the synthesis of sense and antisense aberrant transcripts.

Rdr3-dependent endogenous siRNAs

During RdRP dsRNA feeding experiments, we noticed reduced growth rates of *RDR3*-silenced cultures compared with others. Precise measurements after long-term dsRNA feeding revealed a very significantly reduced division rate of 1.4 ± 0.3 division/day, compared with 3.6 ± 0.6 division/day for cells that were fed *RDR1*, *RDR2*, *RDR4*, *ND169*, A^{51}, *ICL7a* or *GFP* dsRNAs (Figure 5A). However, this effect became obvious only after 4 days of continuous silencing, raising the possibility that *RDR3* silencing results in the progressive deregulation of endogenous genes important for vegetative growth. Although RNAi-mediated regulation of endogenous genes has not been reported in *Paramecium* so far, a small-scale sRNA sequencing study identified a cluster of endogenous siRNAs in a region between two

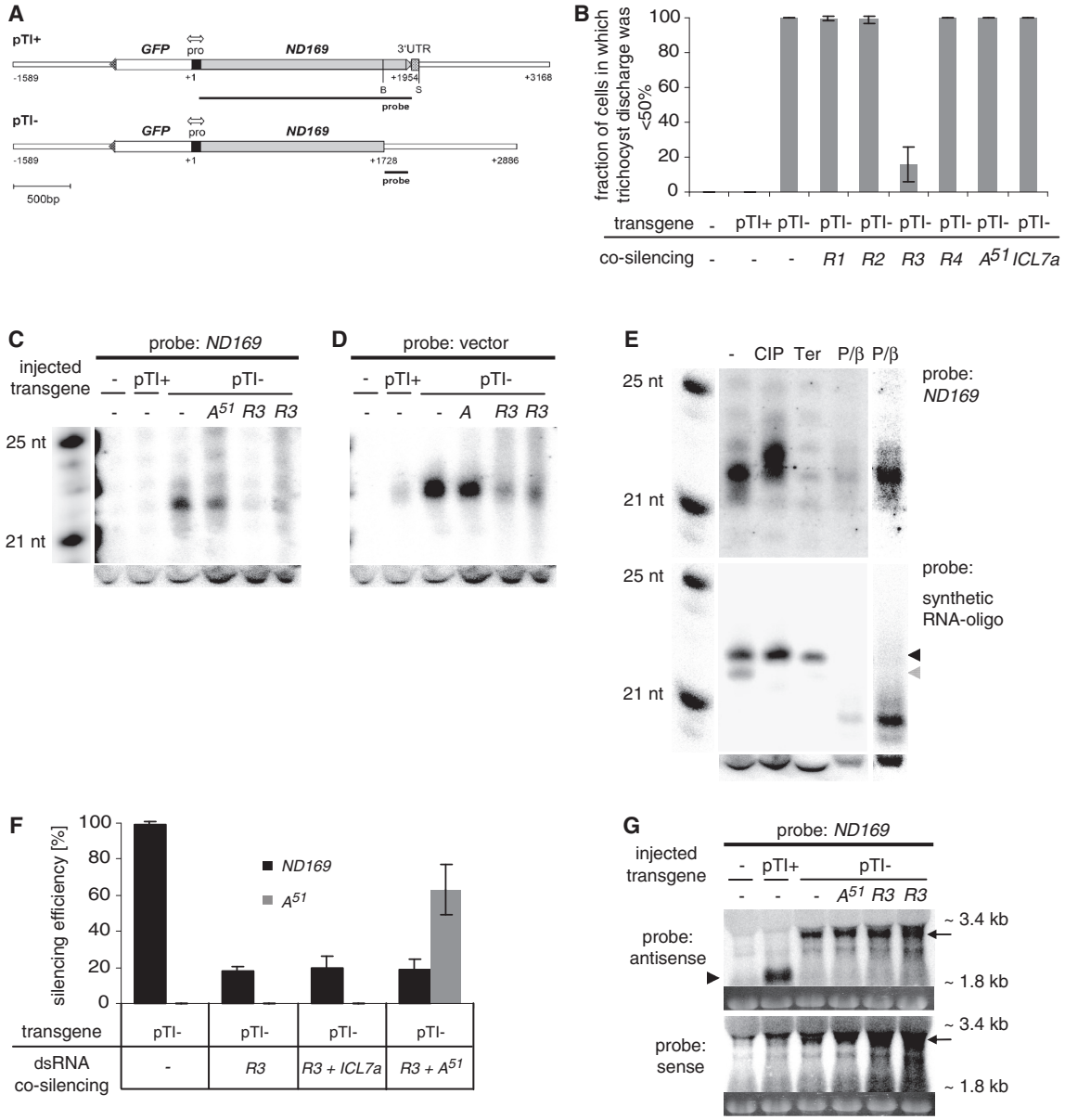

Figure 4. Transgene-mediated silencing involves Rdr3 and is independent from the dsRNA-induced pathway. (**A**) Linearized transgene plasmid construct to induce *ND169* silencing. The pTI− construct contains a 3′-truncated version of the *ND169* gene. *GFP* on the opposite site of a bidirectional promoter served as a control for presence of the construct in the macronucleus. The pTI+ construct containing the entire *ND169* gene served as a negative control in the following experiments. Probes for detection of siRNA and long transgenic transcripts are indicated as black bars (B = BmgBI; S = SmaI; +1 = first base of bidirectional promoter). Experiments were carried out with three injected cell clones; all displayed results from northern blots originate from the same clone. (**B**) Phenotypic analysis. Each of the *RDR* genes (*R1-4*) and two control genes (*ICL7a*; A^{51}) were co-silenced by dsRNA feeding. Silencing efficiency was measured as percentage of cells which discharged <50% of trichocysts compared with non-injected wild-type cells or the pTI+ control. Phenotypes were determined 96 h after first dsRNA feeding. Values are generated from 11 independent experiments (±standard deviation); values did not differ significantly between injected clones. (**C** and **D**) Northern blot analysis of silencing-associated siRNAs. Total RNA was isolated 96 h after the first dsRNA feeding and loaded on 15% polyacrylamide–urea gels. Probes were corresponding to the entire *ND169* cds (1700 bp) (C) and to the 3′ plasmid part of the transgene construct (D) (for probes see A). The lower panels show hybridization to glutamine tRNA as a loading control. (**E**) Properties of 5′- and 3′-ends of transgene-induced siRNA (upper blot). Removal of 5′ phosphates with CIP alkaline phosphatase resulted in a ~0.5-nt slower migration of *ND169*-siRNA in comparison to the untreated sample. sRNAs showed sensitivity to Terminator 5′-monophosphate-specific exonuclease (Ter) but were resistant to periodate treatment and subsequent β-elimination (P/β). The second P/β-lane (right) represents the latter one with increased contrast. Controls (lower blot) were added in the same way as described for Figure 3C. The lower panels show hybridization to glutamine tRNA as a loading control. (**F**) Independency of dsRNA- and transgene-induced silencing. pTI− injected cells were cultivated to develop a trichocyst non-discharge phenotype (100%). Transgene-induced silencing was then inhibited by feeding *RDR3* (*R3*) dsRNA and *R3* + *ICL7a* dsRNA as a double feeding control. A^{51} silencing by dsRNA was induced to determine dsRNA-silencing efficiency when transgene-induced silencing was inhibited. Phenotypes were observed after 48 hof dsRNA feeding. (**G**) Long sense and antisense transcripts from the transgene. RNA samples shown in (C and D) were separated on a denaturing 1.2% agarose gel. Northern analysis was carried out with strand-specific riboprobes corresponding to the 1700 nt *ND169* cds (above: antisense-orientated probe, below: sense-orientated probe). *ND169* mRNA occurs in non-injected and pTI+ controls (overexpressed) and in *RDR3*-silenced pTI− injected cells (indicated by the arrowhead). In each of the pTI− samples aberrantly sized *ND169* RNA of both strands was detected (arrows). The lower panels show ethidium bromide staining of total RNA as a loading control.

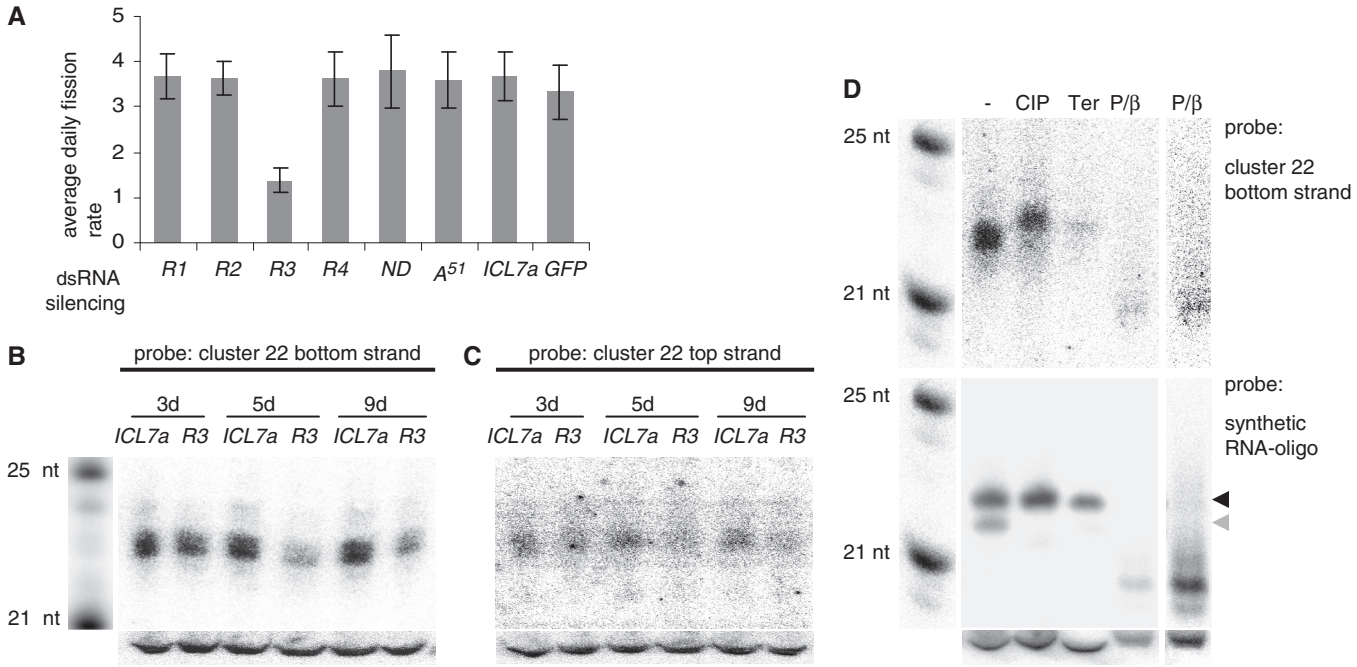

Figure 5. Rdr3 is involved in accumulation of endogenous siRNAs. (**A**) Average division rate of *RDR1-RDR4* (*R1-4*) and control (*ND169*; *ICL7a*; A^{51}; *GFP*) silenced cells (±standard deviation). Single cells were grown in silencing medium and individualized every day. Reduction of division rate of *RDR3* silenced cells started on the fourth day of the experiment. (**B** and **C**) Rdr3 dependency of endogenous siRNAs. Total RNA was isolated on days 3, 5 and 9 of *RDR3* (*R3*) and *ICL7a* (control) silencing. Northern blots were probed with two adjacent 50-nt oligonucleotides corresponding to endogenous siRNAs produced from an intergenic region of scaffold 22. Probes were orientated top (B) and bottom (C), relative to transcription of the 5′-marginal ORF. The lower panels show hybridization to glutamine tRNA as a loading control. (**D**) Properties of 5′- and 3′-ends of endogenous siRNAs. Removal of 5′ phosphates with CIP alkaline phosphatase resulted in a ~0.5-nt slower migration in comparison the untreated sample. Endogenous siRNAs showed sensitivity to Terminator exonuclease (Ter) and were sensitive to periodate treatment and subsequent β-elimination (P/β), indicated by ~1.5-nt faster migration (upper blot). The second P/β-lane (right) represents the latter one with increased contrast. Controls (lower blot) were added in the same way as described for Figure 3C. The lower panels show hybridization to glutamine tRNA as a loading control.

convergent genes on scaffold 22 (17). We therefore tested whether their accumulation depends on Rdr3. Figure 5B and C show that silencing of *RDR3* resulted in a decreased abundance of these siRNAs, in comparison with an *ICL7a* silencing control, after 5 days of continuous silencing, i.e. at about the same time as the growth rate defect became clearly apparent. The endogenous siRNAs migrated at ~23 nt, showing two distinct bands separated by <0.5 nt. Although sequencing had only revealed siRNAs from the top strand (Figure 5B), a weaker signal was also detected on the complementary strand, which likewise decreased after *RDR3* silencing (Figure 5C).

Biochemical analysis of endogenous siRNAs was carried out as described above. After treatment with CIP, the blot indicated a ~0.5-nt slower migration of the top strand siRNAs (Figure 5D); the bottom strand siRNAs were too difficult to detect in these experiments. After treatment with Terminator, the signal was greatly reduced; however, some residual amount of sRNAs could be seen. Since the control oligo was completely digested, this suggests that a minor fraction of cluster 22 siRNAs may not have 5′-monophosphorylated ends. Moreover, the signal remaining after Terminator treatment corresponded to the upper band of the doublet seen with native siRNAs (Figure 5B), suggesting that the two bands may represent 5′ modification isoforms. These endogenous siRNAs were sensitive to periodate treatment and β-elimination, as indicated by the appearance of a ~1.5-nt faster migrating band (Figure 5D). Because the treatment resulted in a decrease of the total signal, we cannot rule out the existence of a minor fraction of insensitive molecules. Nevertheless, these endogenous siRNAs clearly differ from Rdr3-dependent transgene-induced siRNAs, for which no evidence for sensitive molecules was obtained.

Exclusive expression of variable surface antigens is controlled by Rdr3

In addition to the loss of endogenous siRNAs, we found that the expression of surface antigen genes was altered during silencing of *RDR3*. This multigene family encodes alternative surface proteins whose expression is mutually exclusive: only one gene is normally expressed at a time. The expressed gene defines the serotype of the cell, which is stably maintained during vegetative divisions; this can easily be identified by the immobilization reaction observed upon addition of specific antiserum (see 'Materials and Methods' section). *RDR3* dsRNA feeding resulted in a destabilization of the serotype in vegetative clones. Figure 6A shows the percentage of cells reacting to specific sera during *RDR3* silencing, starting with a pure A^{51}-expressing culture. After 48 h, >80% of

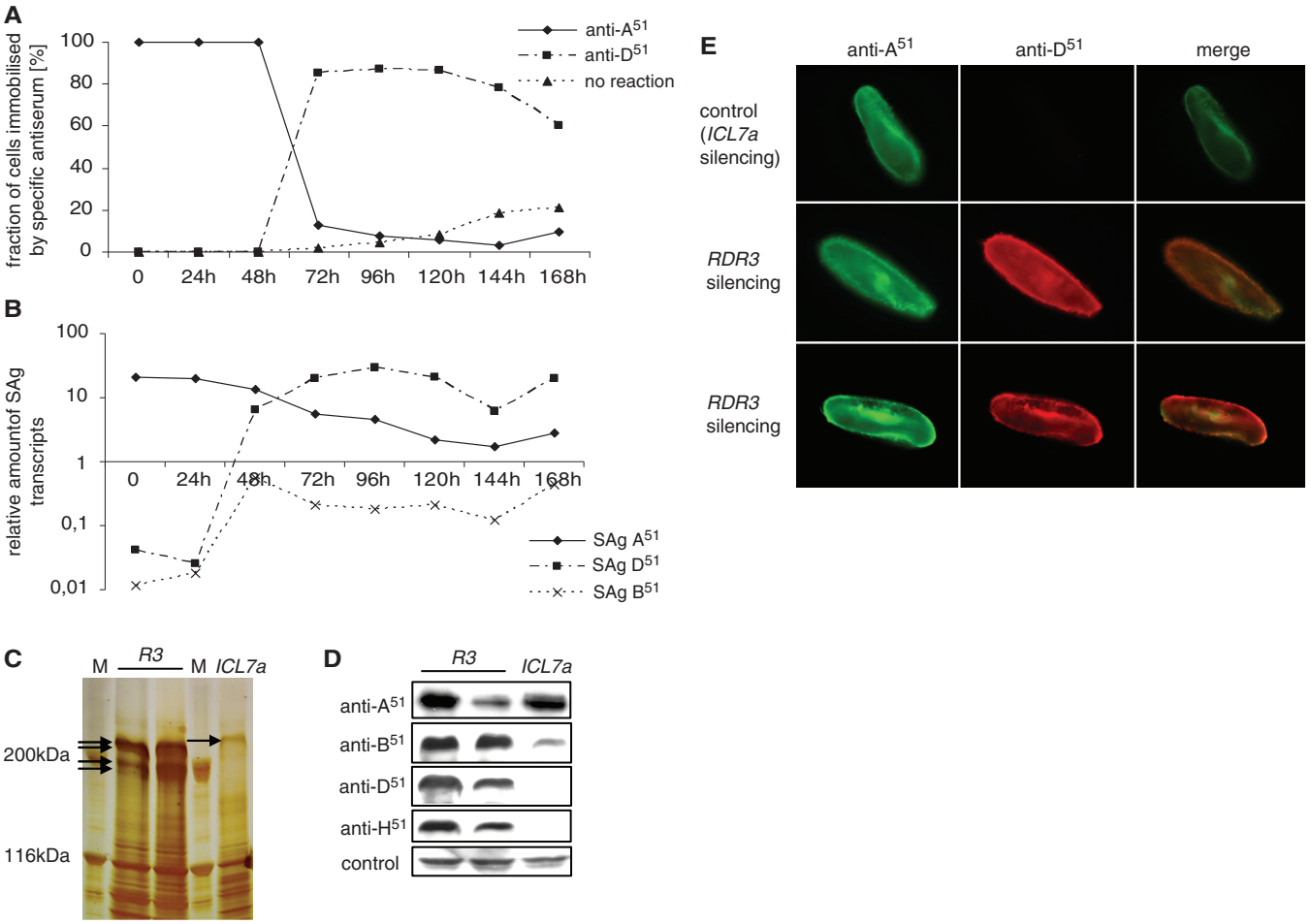

Figure 6. Rdr3 is involved in exclusive expression of surface antigens. Long-term silencing of *RDR3* leads to a destabilization of the currently expressed surface antigen (SAg). (**A**) Protein expression was monitored in immobilization reactions using anti-sera raised against surface antigen A^{51}, B^{51}, C^{51}, D^{51}, E^{51}, G^{51}, H^{51}, I^{51}, J^{51}, N^{51} or Q^{51}. Starting with a SAg A^{51}-expressing culture, cells began to immobilize in response to anti-D^{51} serum after 48 h. In the following, reaction to anti-D^{51} serum was more and more reduced. After 8 days (data not shown) the cultures reacted to none of the available antisera. (**B**) Monitoring of the transcriptional level by real-time PCR revealed that other antigens, B^{51} and D^{51}, are strongly upregulated during silencing of *RDR3*. Expression data were normalized to *GAPDH*-mRNA level, which was found to be constant during *RDR3* knockdown (noRT-control not shown). (**C**) After 9 days of *RDR3* (*R3*) silencing surface proteins were analysed on silver-stained SDS–polyacrylamide gels showing several large proteins present on the surface (~200 kDa, indicated by arrows). *ICL7a* silenced cells expressing surface antigen A^{51} were used as a control. M = size marker (**D**) Western blots identify the surface proteins expressed in *RDR3* (*R3*) silenced cells to be A^{51}, B^{51}, D^{51} and H^{51}. A smaller 80 kDa protein, which was constitutively expressed (bottom blot) and cross-reacted with anti-A^{51} serum, served as a loading control. (**E**) Indirect immunofluorescence staining of surface proteins A^{51} and D^{51} revealed their co-expression on individual cells. Antibodies used were Y4 mouse monoclonal (primary) and Alexa anti-mouse (secondary) for SAg A^{51}; anti-serum (primary) and TexasRed anti-rabbit for SAg D^{51}.

cells did not react to anti-A^{51} serum any more, but an increasing percentage of cells were immobilized by anti-D^{51} serum. In the following days, a growing fraction of cells did not react to any sera tested. This fraction reached 100% after 8 days of *RDR3* silencing, which would seem to indicate that no antigen was expressed.

We therefore directly examined surface antigen mRNAs and proteins. Besides A^{51}, the most frequently expressed antigens are B^{51} and D^{51}. Real-time RT–PCR revealed a strong upregulation of steady state mRNA levels for the B^{51} and D^{51} genes after 24 h of *RDR3* silencing (Figure 6B). Surprisingly, the A^{51} mRNA was still present at relatively high levels during the entire experiment, even when cells were no longer immobilized by anti-A^{51} serum. Thus, different genes seemed to be co-transcribed. To test whether the corresponding proteins were produced, surface antigens were isolated from *RDR3*-silenced and control cultures and run on SDS–polyacrylamide gels. Silver staining revealed different proteins in the size range of surface antigens, ~200 kDa (Figure 6C). In contrast to *RDR3*-silenced cultures, the control culture fed with *ICL7a* dsRNA showed a single band. Western blots confirmed that this was A^{51}, and further showed that A^{51}, B^{51}, D^{51} and H^{51} were all produced in *RDR3*-silenced cultures (Figure 6D). The presence of multiple antigens at the surface of individual cells was revealed by indirect immunofluorescence staining, which showed an even distribution of the A^{51} and D^{51} antigens on the entire cell surface (Figure 6E). This differs strikingly from reports of transient co-occurrence of two surface antigens at the surface during serotype switching, which showed distinct distributions of the old

and new antigens on the cilia and cell cortex (41,42). The simultaneous presence of many antigens at the surface could possibly explain the lack of immobilization by corresponding antisera. Indeed, this reaction requires cross-linking of antigens by antibody molecules (43), which could become inefficient if the concentration of each antigen on the ciliary membrane is too low. This is the first report of a disruption of the mutual exclusion that characterizes the regulation of surface antigen gene expression in *Paramecium*.

DISCUSSION

RdRPs are not as widely distributed among eukaryotes as other RNAi components. They are found neither in mammals nor in the unicellular organisms *Trypanosoma* and *Chlamydomonas* (7). Other organisms, such as *Arabidopsis thaliana* and *C. elegans*, have several RdRP homologs with specialized functions in different silencing mechanisms (11,44). In contrast, the genomes of *Schizosaccharomyces pombe* and *T. thermophila* only contain a single RdRP gene (35,45). Surprisingly, we identified four putative RdRPs in the genome of the ciliate *P. tetraurelia*. The closest homolog of Rdr1 and Rdr2 in other organisms is *T. thermophila's* Rdr1 (35). Together with the close relationship of *Paramecium*'s Dcr1 and *Tetrahymena*'s Dcr2 (17), this suggests that RNAi mechanisms are at least partially conserved in these two ciliates. The highly divergent catalytic domain of the Rdr3 protein raises the question of its possible function. In *Paramecium*, homology-dependent gene silencing can be triggered by dsRNA feeding or by injection of 3′-truncated transgenes (18,21,22). In this study, we used northern blots to characterize sRNAs associated with both pathways and also analysed their RdRP requirements. We used dsRNA feeding to knock down each RdRP gene, and the results appear to indicate that different RdRPs are required for these two pathways. Formally, we cannot exclude the involvement of more RdRPs in each pathway because of the unpredictable efficiency of recursive RNAi. However, other results do indicate that dsRNA- and transgene-induced RNAi are different mechanisms. This is based on the appearance of different classes of sRNAs with different biochemical properties (Table 2). Similarly, it has recently been shown that different sets of Piwi proteins are involved in these two mechanisms (K. Bouhouche *et al.*, submitted for publication). However, the genetic requirements of the two mechanisms are not entirely different since Dcr1 was found to be involved in both pathways (17, S.M. and M.S., unpublished results).

Table 2. Summary of the characteristics and genetic requirements of different siRNAs

	dsRNA primary	dsRNA secondary	Transgene induced	endogeneous
Migration (nt)	~23	~22	~23	~23
Standard specifity	s/as	as	n.d.[a]	s/as
5′ phosphorylation	Mono-P	Mono-P	Mono-P	Mono-P
3′ modification	No	No	Yes	No
RdRP dependency	Rdr1/Rdr2	Rdr1/Rdr2	Rdr3	Rdr3

[a]Experimental attempts to show strand specificity of transgene-induced siRNAs failed, see text for details. s, sense; as, antisense.

DsRNA feeding triggers Rdr1- and Rdr2-dependent accumulation of primary and secondary siRNAs

We found that two distinct classes of siRNAs with apparent sizes of ~23 and ~22 nt are associated with dsRNA-induced silencing and that both classes depend on Rdr1 and Rdr2. The ~22-nt class appears to represent secondary siRNAs, because these molecules are strictly antisense to the targeted mRNA and are not detectable in the vector part of the ingested dsRNA, suggesting that they are synthesized using the mRNA as a template. The absence of this class when dsRNA feeding targeted the A^{51} surface antigen gene is consistent with this hypothesis and may be due to discontinued production of the mRNA: silencing of a member of the antigen family triggers a serotype shift in *Paramecium* (26), which is normally accompanied by transcriptional or co-transcriptional silencing of the previously expressed gene (see below). This would perfectly explain why only the ~23-nt siRNAs can be detected after dsRNA-induced silencing of A^{51}. Our results further indicate that secondary siRNAs are more abundant than primary ones, consistent with the results of a small-scale sequencing study in *Paramecium* (17) and with studies of secondary siRNAs in *C. elegans* (46). However, *Paramecium* secondary siRNAs are monophosphorylated, which stands in contrast to the triphosphorylated 5′-end of sRNAs resulting from unprimed RdRP activity in *C. elegans* (13,14). It is still possible that *Paramecium* secondary siRNAs are initially synthesized with 5′-triphosphate ends, followed by the removal of the γ and β phosphates by a Pir-1-like RNA phosphatase (47,48). Alternatively, the 5′-monophosphate of *Paramecium* secondary siRNAs may indicate that they are cut by Dicer from long dsRNA as demonstrated for plant secondary siRNAs (11). In *Paramecium*, a polarized Dicer activity, which could be due to its interaction with the RdRP as suggested in *Tetrahymena* (35), would allow selective stabilization of the antisense strand after cleavage of the dsRNA.

It remains unclear why the ~22-nt siRNAs were not observed in previous analyses of dsRNA-induced silencing of the *ND7* gene. A small-scale sequencing study of associated siRNAs only examined gel-purified fractions between 23 and 26 nt (17), which may be part of the reason. This study also provided evidence for a distinct subclass of siRNAs with features suggestive of secondary siRNAs (i.e. strictly antisense to mRNA and not observed for the vector part of ingested dsRNA; one was found to match the mRNA upstream of the region targeted by the dsRNA). However, these *ND7* secondary siRNAs migrated at 23–24 nt and were found to carry a short untemplated polyA tail. Whether polyadenylation of secondary siRNAs is only observed at the specific stage of the life cycle when these were analysed (early conjugation) will need to be addressed by further sequencing studies.

It is surprising that any RdRP should be necessary for dsRNA-induced RNAi. One possible explanation may be that amplification of the trigger dsRNA by RdRPs may be necessary to generate dsRNA ends that are efficiently recognized by the Dicer enzyme. Indeed, studies in the related *Tetrahymena* showed that Dcr2 requires a 5′-triphosphate to generate discrete siRNA products (35). In our experiments, the dsRNA resulting from T7 Pol transcription in *E. coli* is 5′-triphosphorylated, which would allow only one siRNA duplex to be cleaved by Dcr1 if this had the same specificity as *Tetrahymena*'s Dcr2. Because the dsRNA produced in *E. coli* contains vector-derived sequences on both sides of the insert, cleaving of a single siRNA duplex from each end would not elicit silencing of the target gene. After cleavage, the remaining portion of the dsRNA would be 5′-monophosphorylated, and an RdRP activity might be required to provide new 5′-triphosphate ends by copying each strand. In this hypothesis, the generation of primary siRNAs would be a cyclic process of RdRP activity and Dicer cleavage, each cycle moving the cleavage sites closer to the centre of the initial dsRNA.

Alternatively, it is conceivable that the dsRNA present in the food vacuoles during digestion of *E. coli* is not imported as such into the cytoplasm, but in the form of ssRNA. RdRP activity would then be required to provide dsRNA substrates for Dcr1. This hypothesis is not exclusive of the first one, since the remaining portion of the dsRNA after the first round of Dcr1 cleavage would still be 5′-monophosphorylated. So far, the mechanism of RNA import from food vacuoles to cytoplasm is unknown and genes similar to *C. elegans SID1*, encoding a transmembrane protein necessary for dsRNA uptake (49,50), cannot be found in the *Paramecium* genome.

Whatever the reason may be, our results indicate that both Rdr1 and Rdr2 are required for the accumulation of primary siRNAs and for dsRNA-induced silencing. Because the effects of each knockdown are the same, there is no evidence that the two proteins have distinct functions. The requirement of these proteins for primary siRNAs also precludes any conclusion as to their possible involvement in the synthesis of secondary siRNAs, since this also depends on the targeting of the mRNA by primary siRNAs.

Transgene-induced silencing differs from dsRNA-silencing

Several lines of evidence indicate that the mechanism underlying transgene-induced silencing are at least partially distinct from those of dsRNA-induced silencing. First, Rdr3 is required for transgene-induced silencing and no evidence was obtained for the involvement of Rdr1 or Rdr2, even in experiments where both *RDR1* and *RDR2* were co-silenced (data not shown). A high degree of functional specialization of RdRPs in the recognition of different types of RNAs triggering RNAi may help to avoid widespread and unspecific silencing. Secondly, although transgene-induced siRNAs have the same apparent size as dsRNA-induced primary siRNAs (~23 nt), they differ from the latter in that they do not trigger the synthesis of ~22-nt secondary siRNAs homologous to the *ND169* gene which was used to study both silencing methods. Thirdly, of all the sRNAs analysed in this study, only transgene-induced siRNAs carry a modification of the 3′ ribose. In the related *Tetrahymena*, the meiosis-specific scnRNAs are 2′-*O*-methylated on the 3′-ribose by the RNA methyltransferase Hen1 (51), which is also present in the *Paramecium* genome. This modification was shown in other systems to protect sRNAs from addition of untemplated nucleotides and degradation (40,52). Finally, distinct mechanisms are also supported by the finding that different (though overlapping) sets of Piwi proteins are involved in the two silencing methods (K. Bouhouche *et al.*, submitted for publication).

Interestingly, an analysis of *Tetrahymena* endogenous ~23–24-nt siRNAs bound by five different Piwi proteins showed that only those bound by the Twi8 protein were modified on the 3′ ribose (53). The same study found that a Twi8–GFP fusion localized to the macronucleus, while two other Twi proteins binding ~23–24-nt siRNAs localized in the cytoplasm. Furthermore, the sequencing of Twi8-bound siRNAs showed them to be derived from both strands of mRNA-producing loci, leading the authors to propose that Twi8 may be mediating co-transcriptional, rather than post-transcriptional, regulation (53). The modification of the 3′ ribose of transgene-induced siRNAs in *Paramecium* thus raises the possibility that this process affects transcription of the endogenous gene in the macronucleus. The absence of secondary siRNAs could then be due to impairment of mRNA production. Conflicting with this hypothesis, one study concluded that transgene-induced silencing of the *T4a* gene was post-transcriptional on the basis of run-on assays indicating that its 3′-UTR was continuously transcribed (22). However, it is now known that another gene (GSPATG00004256001) is present very close downstream of *T4a* in a convergent orientation, with overlapping 3′-UTRs and only 26 bp between the two stop codons. Since the probe used for the run-on assay of the *T4a* 3′-UTR was double stranded, it may well have been measuring the ongoing transcription of the downstream gene.

Rdr3 is also involved in the accumulation of endogenous siRNAs

In our study, northern blots revealed endogenous siRNAs previously identified by cloning and sequencing (17), which map to an intergenic region between convergent genes on scaffold 22. We found that these endogenous siRNAs, whose apparent size is similar to that of transgene-derived siRNAs, also depend on Rdr3. Strand-specific probes indicated that they derive from both strands, although the bottom strand was more difficult to detect by hybridization. A partial strand bias may explain why small-scale sequencing only revealed siRNAs from the top strand (17). In contrast to transgene-derived siRNAs, however, these endogenous siRNAs were not modified on the 3′ ribose, at least for the majority of top-strand siRNAs. In view of the apparent diversity of endogenous siRNAs in *Tetrahymena*, further

sequencing, biochemical and functional characterization of *Paramecium* endogenous siRNAs will be required to ascertain their relationship with the transgene-induced silencing pathway.

RNAi controls antigenic variation in *Paramecium*

As mentioned above, the surface antigen gene family is controlled by unknown mechanisms ensuring that only one gene is expressed at a time. Several studies concluded that surface antigen genes are regulated at the level of transcription (54–57), although cases of post-transcriptional regulation have also been described (26,58). It is not known whether transcriptional and post-transcriptional control mechanisms interact. Importantly, studies of chimeric constructs indicated that the promoters of these genes are not sufficient to mediate mutual exclusion of expression, and that this also involves at least the beginning of the cdss (54,55).

Our experiments showed that the silencing of *RDR3* results in the simultaneous expression of multiple surface antigens at the cell surface. Rdr3 is therefore somehow involved in maintaining most surface antigen genes silent. Similarly, antigenic variation in *Giardia lamblia* was shown to be controlled by RNAi (59). In wild-type cells, several surface antigen genes are transcribed simultaneously, and all but one are silenced post-transcriptionally. Silencing of *Giardia's* RdRP and Dicer leads to a disruption of this system and to expression of multiple surface antigens (59). Although this is superficially similar to our results, it is clear from previous run-on assays that the regulation of *Paramecium* surface antigen genes is not entirely post-transcriptional (54–57), suggesting that Rdr3-mediated silencing may affect transcription itself.

One possibility is that surface antigen genes are co-transcriptionally silenced. The probes used in previous run-on assays were usually not directly located in 5′ part of the long (7–8 kb) cdss, so that they do not formally demonstrate the absence of transcription of the 5′ parts of the genes which may also be below the detection limit (54,56,57). In this hypothesis, Rdr3-dependent siRNAs could affect the elongation of transcription, rather than its initiation. There is increasing evidence from plants and fungi that sRNA-mediated heterochromatin formation actually requires transcription of the targeted loci, and that nascent transcripts are cleaved co-transcriptionally (6,60). In many organisms, the process results in the targeting of H3K9 methylation. In *Tetrahymena*, however, this histone modification only occurs in developing macronuclei, and results in the elimination of modified chromatin (61–63). In contrast, H3K27me3 is present in the macronucleus of vegetative cells, and this modification is also thought to be required in the developing macronucleus as an intermediate in the process of scnRNA-directed H3K9 methylation (62).

Whether or not Rdr3-mediated silencing of *Paramecium* surface antigen genes involves histone modifications, an RNAi-based regulation mechanism may explain the long-known maternal inheritance of serotypes during sexual reproduction, which implies that the particular expression pattern observed in the maternal macronucleus is transferred through the cytoplasm to the developing zygotic macronuclei (64–66). The identification of surface antigen-related siRNAs in different serotypes would certainly provide new insight into this question. Biochemical studies will also be required to determine whether Rdr3, despite its highly divergent catalytic domain, has RdRP activity or whether it is only required as part of a complex for the dicing of dsRNA formed by the pairing of sense and antisense RNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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