RNAi in *Dictyostelium*: The Role of RNA-directed RNA Polymerases and Double-stranded RNase

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We show that in *Dictyostelium discoideum* an endogenous gene as well as a transgene can be silenced by introduction of a gene construct that is transcribed into a hairpin RNA. Gene silencing was accompanied by the appearance of sequence-specific RNA ~23mers and seemed to have a limited capacity. The three *Dictyostelium* homologues of the RNA-directed RNA polymerase (RrpA, RrpB, and DosA) all contain an N-terminal helicase domain homologous to the one in the dicer nuclease, suggesting exon shuffling between RNA-directed RNA polymerase and the dicer homologue. Only the knock-out of rrpA resulted in a loss of the hairpin RNA effect and simultaneously in a loss of detectable ~23mers. However, ~23mers were still generated by the *Dictyostelium* dsRNase in vitro with extracts from rrpA−, rrpB−, and DosA− cells. Both RrpA and a target gene were required for production of detectable amounts of ~23mers, suggesting that target sequences are involved in ~23mer amplification.

INTRODUCTION

RNA interference by double-stranded RNA (dsRNA) has been applied efficiently to silence genes in various organisms ranging from fungi and nematodes to plants. dsRNA has been introduced into organisms by microinjection (Fire et al., 1998), by transformation with gene constructs generating complementary RNAs or fold-back RNA (Waterhouse et al., 1998; Wang et al., 2000), or by feeding an organism with *Escherichia coli* -expressing dsRNA (Kamath et al., 2000). There is increasing evidence that the antisense strand in the Escherichia coli-expressing dsRNA (Kamath et al., 2000), or by feeding an organism with *Escherichia coli*-expressing dsRNA (Kamath et al., 2000). There is increasing evidence that the antisense strand in the dsRNA is transcribed into a hairpin RNA. Gene silencing was accompanied by the appearance of sequence-specific RNA ~23mers (Fleenor et al., 2000), but also the initial dsRNA or an amplification product of it apparently serves as a precursor for ~23mers (Fleenor et al., 2000). A nuclease containing a ~23mer guide RNA has been proposed to mediate sequence-specific mRNA degradation (Yang et al., 2000) and appears to be part of a complex termed RNA-induced silencing complex (RISC) (Hammond et al., 2000). However, this nuclease activity is not responsible for the generation of ~23mers from dsRNA. Bernstein et al. (2001) have shown in *Drosophila* that a complex containing the RNase “dicer” and RISC are distinct entities and can be separated by high-speed centrifugation.

Only recently, the small RNAs have been unambiguously characterized as 21 and 22mers (Elbashir et al., 2001b), and it has been demonstrated that synthetic double-stranded 21mers can confer gene silencing in mammalian cells (Elbashir et al., 2001a).

We have previously identified a dsRNase that processes dsRNA to ~23mers in vitro but does not by itself display single-stranded RNA activity (Novotny et al., 2001). This large (~450 kDa) complex may be the *Dictyostelium* equivalent of the *Drosophila* dicer complex.

In a search of genes required for the RNAi mechanism, several RNA helicases have been identified (e.g., Wu-Scharf et al., 2000). In addition, the RNA-directed RNA polymerase (RdRP) has been found to be necessary for RNAi in *Caenorhabditis elegans* (Smardon et al., 2000) and *Arabidopsis* (Dalmay et al., 2000) and for quelling in *Neurospora* (Cogoni and Macino, 1999).

Here we show that RNAi mediates posttranscriptional gene silencing (PTGS) in *Dictyostelium* and that the knock-out of one of three RdRP homologues, RrpA, is sufficient to impair the mechanism. Sequence-specific ~23mers are found in silenced *Dictyostelium* cells in vivo, and these products are similar in size to the ~23mers generated in vitro by the partially purified *Dictyostelium* dsRNase.
MATERIALS AND METHODS

Dictyostelium AX2 cells and transformants were grown in association with Klebsiella aerogenes in suspension culture or on plates or in AX2 medium. Development was done in phosphate buffer suspension (Spudich, 1987). Dictyostelium transformation, was carried out as described previously (Nellen et al., 1987; Howard et al., 1988). Transformation with vectors containing the G418 resistance cassette resulted in multicopy tandem integration into the genome, whereas transformation with the blasticidin resistance cassette gave single- or low-copy integration. Cotransformation was done as described by Nellen and Fertel (1985).

Transformants were subcloned on a lawn of K. aerogenes and then grown in plates (Costar, Cambridge, MA). For RNAi analysis, populations of primary transformants and several individual clones were assayed.

Total cellular RNA was prepared as detailed by Maniak et al. (1989); enrichment for small RNA, RNA PAGE, blotting, and hybridization was done according to the method of Hamilton and Baulcombe (1999). For Northern blots and slot blots on total cellular RNA, 10 μg were either separated on a 1.2% agarose gel containing 20 mM guanidinium thiocyanate and botted to a nylon membrane or directly applied to the membrane using a vacuum slot blot device. For slot blotting, RNA was dissolved in 20 mM 3-(N-morpholino)propanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA, 7% (vol/vol) formaldehyde, 50% (vol/vol) deionized formamide, and bromophenol blue. Prehybridization and hybridization were carried out as described by Crowley et al. (1985). Radioactively labeled in vitro transcripts were used as probes. In vitro transcription was carried out with T7 and SP6 RNA polymerase as described by Weber and Gross (1997). Reverse transcription (RT)-PCR on rrpA and rrpB was done with 3 μg of total RNA from wild-type AX2, RrpA-, and RrpB- cells. The RNA was treated with DNase before cDNA synthesis to eliminate genomic DNA contaminations. For first-strand synthesis the oligonucleotide (GAATACCAATTATAAACCAACTGATC) that binds 3’ in both Rrp genes was used. The final amplification was done with primer pair B (5’-primer: GAGCAGGAAGACAGTTCATTATAAC, 3’-primer: GAAATCCATTATAAACCACTGATC). Amplification products of the two genes could be distinguished by Cid cleavage. For semiquantitative RT-PCR on rrpA, equal amounts of total RNA from the RrpB- strain were used either in a multiplex PCR with additional primers for thioredoxin 1 (see below) or in parallel PCRs. 16 RT-PCR on β-galactosidase (β-gal) was done with 1 μg of total RNA. The RNA was treated with DNase to eliminate genomic DNA contaminations. For the first-strand synthesis the oligo (CGGCTGCAAGATCTATAGCCATGCTGTTGGGATGCTACTACG) that binds to a 3’ sequence tag in the β-gal reporter construct was used. The final amplification was done with primer pair C (5’-primer: TACACGAGCTCTGCACTGGATCTG, 3’-primer: CGGCTGCAAGATCTATAGCCATGCTGTTGGGATGCTACTACG). As a control we performed RT-PCR on thioredoxin 1 mRNA with oligo (CGCGAGAATCCATTTTTGTTCCCTTGACCTAGTCCAGTTCCGCAACTGATC) for the PCR reaction.

Western blotting and detection of discoidin was done as described by Wetterauer et al. (1993) using the monoclonal antibody 80–52-13 and a phosphatase-coupled secondary antibody.

Vector Constructs

Fragments of β-gal and discoidin genes for RNAi and antisense constructs were obtained by PCR including suitable restriction sites. β-Gal was expressed from the actin 6 promoter either in the pGem 7z vector for cotransformation experiments or in vectors containing a BSR cassette (Sutoh, 1993) when selection for blasticidin resistance was possible.

The pV18gal-i vector was generated by replacing the discoidin promoter in pVII (Blusch et al., 1992) for the V18 promoter (Ken and Singleton, 1994). The first β-gal fragment of 915 bp was fused in sense orientation to the V18 promoter; the second fragment of 1326 bp was fused in reverse orientation to the V18 promoter. Transformation with the BSR cassette gave single-or low-copy integration. Cotransformation was done as described by Nellen and Fertel (1985).

PCR was performed on genomic DNA of potential rrpA and rrpB disruption clones using primer pair A (5’-primer: CGCTACTTC-TACTAATTCTAGA, 3’-primer: GAATCCAAATTATAAACCACTGATC) in which one primer binds within the coding sequence of the BSR cassette and one in identical sequence regions of the rrpA and rrpB genes outside the recombinogenic arm. Positive clones were further analyzed with primer pair B (5’-primer: GAGCAGGAAGACAGTTCATTATAAC, 3’-primer: GAAATCCATTATAAACCACTGATC), which bind within identical coding sequences of the rrpA and rrpB genes that flank the BSR cassette. Under the conditions used, PCR did not proceed across the inserted BSR cassette. Therefore, only products of nondisrupted genes were obtained. Amplification products of rrpA and rrpB could be distinguished by cutting with Cid and EcoRV. Cid cleaved the rrpA but not the rrpB PCR product and EcoRV cleaved the rrpA PCR product twice and rrpB product once.

B-Gal Assays

β-Gal assays were done essentially as described by Dingermann et al. (1990). Briefly, cells were harvested, washed with phosphate buffer, and lysed in assay buffer (60 mM NaHPO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4, and 7 ml/1 mercaptoethanol) by freezing in liquid nitrogen and thawing at 37°C. Cell debris was removed by centrifugation at 10,000 × g. The supernatant was incubated with o-nitrophenyl-β-D-galactoside at 37°C. The reaction was stopped with 0.5 volume of 1 M Na2CO3. β-Gal activity was measured photometrically at 420 nm and standardized to the protein concentration of the sample. Activities are given in units per milligram of total protein. One unit is the amount of enzyme that produces 1 mmol o-nitrophenol/min at 37°C. U/mg = (E420 / 1.7 × D) / (0.0045 × t × c), where t is time in minutes, D is dilution of protein sample in the assay, and c is concentration of protein sample in mg/ml.

dsRNase Preparation and In Vitro Assay

dsRNase was prepared as detailed by Novotny et al. (2001). For Figure 5B, a preparation purified by three column steps was used, whereas for Figure 6 crude extracts were used. For the experiment shown in Figure 6, 350 μl of protein cell extract were incubated for 3 h at room temperature with 13 ng of 32P-labeled 260-bp PsaV-A dsRNA (131 kbp/μg) or with 40 ng of 32P-labeled 400-bp β-gal dsRNA (13 kbp/μg) in 1× assay buffer (50 mM Tris-HCl, pH 8.0, 25 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 250 μg/ml RNA, and 15% glycerol). Because of lower specific activity, a higher amount of the β-gal substrate was used. After phenol/chloroform extraction, the assay was precipitated with 3 volumes of 100% ethanol and washed with 70% ethanol. The products were separated on an 8 M urea PAGE and analyzed with a Fuji X BAS 1500 (Raytest, Straubenhardt, Germany) bioimaging analyzer after 4 h of exposure. The amount of 25mers was quantified with the TINA software (Raytest).

Sequence Alignments

Multiple alignments were done with the MultAlign interface (Corpet, 1988) and the LALIGN program (accessed at: http://www.expasy.ch/tools/).
Sequence data for *D. discoideum* chromosome 6 were obtained from The Sanger Center website at http://www.sanger.ac.uk/Projects/D.discoideum/. Sequencing of *D. discoideum* chromosome 6 was accomplished as part of the EU-DICT consortium with support by The European Union.

Further sequence data were obtained from the Genome Sequencing Center, Jena, website at http://genome.imb-jena.de/dictyostelium. The German part of the *D. discoideum* Genome Project is carried out by the Institute of Biochemistry I, Cologne, and the Genome Sequencing Center, Jena, with support by the Deutsche Forschungsgemeinschaft. (grant 113/10-1 and 10-2).

The gene sequences are derived from unfinished contigs. They have been mapped by restriction analysis but may still contain sequencing errors.

**Accession Numbers**


**RESULTS**

**PTGS by RNAi**

To test whether PTGS by RNA interference is functional in *Dictyostelium*, we examined gene silencing by dsRNA using different methods and constructs, starting with a transgenic strain expressing β-gal from the actin 6 promoter with an average reporter activity of 26.6 ± 3.5 U/mg protein. As shown in Figure 1, introduction of a 807-bp-long antisense construct targeted against the β-gal mRNA did not result in efficient silencing (Figure 1b), even though antisense RNA is functional in many cases (e.g., Crowley *et al.* 1985). Transformation of the same fragment in sense orientation and cotransformation of the two promoters (actin 15 and V18) was not successful (Figure 1e), even though antisense RNA is functional in many cases. Similarly, the insertion of a 827-bp fragment (same sequence as in the sense and antisense constructs) between two opposing promoters (V18 and actin15), (f) an 800-bp inverted repeat (2bXG0nt), 500-bp linker, corresponding to the β-gal sequences that we used in the two-promoter construct separated by an ~500 bp spacer (also consisting of β-gal sequences) under the control of the V18 promoter (Figure 1f). The transcript was expected to fold into a stem-loop structure consisting of 802 bp of dsRNA and a 538-base hairpin loop. With this, expression of β-gal could be reduced to undetectable levels. RT-PCR on β-gal mRNA in the silenced strain yielded no detectable product, whereas the unsilenced strain showed the expected PCR product (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results). Using a 100-bp inverted repeat—hairpin loop—construct resulted in some reduction (~50%) of total β-gal activity (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results). Feeding *Dictyostelium* cells with *E. coli* cells expressing β-gal dsRNA from a two-promoter construct (both T7) also targeted against the first 800 nucleotides of the β-gal mRNA was not successful (Figure 1g).

**RNAi-mediated Silencing of Endogenous Genes**

To demonstrate that endogenous genes can be silenced by RNAi, we made a similar construct expressing a stem-loop RNAi activity of 26.6 ± 3.5 U/mg clones showing silencing clones tested

<table>
<thead>
<tr>
<th>Construct</th>
<th>Expressing regulatory RNA</th>
<th>Average 8-gal activity of tested clones in U/mg</th>
<th>Clones showing silencing clones tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) no RNA (empty vector)</td>
<td>↑</td>
<td>27.5 ± 1.8</td>
<td>0/4</td>
</tr>
<tr>
<td>b) antisense 600nts</td>
<td>↑</td>
<td>28.4 ± 3.99</td>
<td>0/12</td>
</tr>
<tr>
<td>c) sense 800nts</td>
<td>↑</td>
<td>27.5 ± 3.13</td>
<td>0/6</td>
</tr>
<tr>
<td>d) sense + antisense 800nts (different loci)</td>
<td>↑</td>
<td>28.5 ± 3.01</td>
<td>0/24</td>
</tr>
<tr>
<td>e) sense + antisense 600nts (sense locus)</td>
<td>↑</td>
<td>28.1 ± 3.14</td>
<td>0/24</td>
</tr>
<tr>
<td>f) inverted repeat (2680Gnt, 500nts spacer)</td>
<td>↑</td>
<td>not detectable</td>
<td>5/5</td>
</tr>
<tr>
<td>g) sense + antisense 800nts (in E. coli for feeding)</td>
<td>↑</td>
<td>27.8</td>
<td>n.a</td>
</tr>
</tbody>
</table>

Figure 1. Constructs used for β-gal gene silencing and their efficiency. In the first two lanes, the construct introduced into the cells is described. In the third lane, the average β-gal activity and SD of randomly chosen tested clones are given in units per milligram of total protein. The last lane shows how many clones have been tested and how many of them exhibited gene silencing. As (a) a control an empty vector was introduced; (b) an antisense copy (800-bp fragment) of the transgene was introduced (V18 promoter), (c) an additional copy (800-bp fragment) of the transgene controlled by the V18 promoter was introduced (equivalent to cosuppression approaches in plants), (d) sense and antisense RNA both under the control of the V18 promoter were expressed from different gene constructs in the same vector, (e) the 800-bp gene fragment was inserted between two opposing promoters (V18 and actin15), (f) an 800-bp inverted repeat with a 500-bp linker, corresponding to β-gal gene sequences, was fused to the V18 promoter, and (g) an 800-bp fragment was inserted between two 17 promoters and transformed bacteria (strain BL21) were used to feed *Dictyostelium* cells. Only in e was silencing of the transgene observed.
RNAi machinery was saturated and possibly not capable of eliminating the high amounts of discoidin RNA in development. Alternatively, the RNAi mechanism could be developmentally regulated. To rule out that incomplete silencing was due to different expression levels of dsRNA, we tested for expression of a transgene (neomycine phosphotransferase, NPT) by the actin 6 promoter. NPT mRNA levels varied slightly under the different growth or developmental conditions and were rather higher (2.5-fold) in developing cells compared with axenic growth (Figure 2B). In colony blots, discoidin expression levels in developing cells were almost indistinguishable from the wild type (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results).

**RrpA Is Required for RNAi in Dictyostelium**

It has been shown that the RdRP is required for PTGS in *Neurospora* (Cogoni and Macino, 1999), *Arabidopsis* (Dalmay et al., 2000), and *C. elegans* (Tabara et al., 1999; Catalanotto et al., 2000; Smardon et al., 2000). A search in the *Dictyostelium* genome database revealed three RdRP-related genes. RrpA and RrpB are closely related and differ by only 49 amino acids (3%) in the available sequence, whereas DosA is less conserved. Nevertheless, the gene product is clearly identified as an RdRP homologue and shows similarity to all RdRPs from other organisms (Figure 3A). Interestingly, all three RdRPs have an N-terminal extension that shows good homology to the helicase domains in the *Drosophila* and *C. elegans* dicer nucleases and the plant CAF protein (see DISCUSSION). This N-terminal part is separated by an intron
from the RdRP homology domain. The intron in rrpA and/or rrpB has been identified by cDNA sequencing (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results).

rrpA, rrpB, and DosA were disrupted by homologous recombination, and the knock-outs were confirmed by PCR and restriction analysis. Transcription of rrpA and rrpB was demonstrated by RT-PCR in the wild-type, the RrpA−, and the RrpB− strains. Figure 3B shows that both genes were transcribed in the wild-type strain, whereas in the knock-out strains only the undisrupted gene was expressed. RT-PCR products from rrpA and rrpB transcripts were distinguished by ClaI cleavage. Pretreatment of RNA with DNase confirmed that the RT-PCR products were derived from RNA and not from contaminating DNA (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results). Expression levels of both genes were too low to be detected by Northern blots.

The disruption strains and the parent AX2 strain were examined for RNai function using the β-gal gene (Table 1) and the discoidin gene family as targets (Figure 4). β-Gal activity was measured by o-nitrophenyl-D-galactoside hydrolysis (Dingermann et al., 1990). Discoidin expression was quantified by Western blotting.

Gene silencing by RNai was assayed in the following ways: The β-gal vector was cotransformed together with the RNAi construct into the knock-out strains (RrpA−, DosA−) and, for comparison, also into the AX2 wild type. As shown in Table 1, an average of 98–99% RNAi mediated gene silencing of total β-gal activity was observed in the DosA−, and the wild-type strain, whereas no silencing was found for the RrpA− strain. The high variability in β-gal expression is due to the cotransformation method, which may result in different copy number integrations of both vectors at different sites in the genome of the tested clones. However the p values calculated from an unpaired t test show the significance of the data (p values: 0.0008 for AX2, 0.0001 for DosA−, and 0.83 for RrpA−; see also Table 1).

As another example, the wild-type strain AX2 and the knock-out strains (RrpA−, RrpB−, DosA−) were transformed with the discoidin RNAi construct and assayed for discoidin expression. RNAi-mediated silencing of the discoidin gene family is shown in Figure 4. In the RrpA− strain, none of six independent clones exhibited any gene silencing, whereas in both the RrpB− and the DosA− strain three of six clones each showed complete and the others partial gene silencing. Thus, RrpB− and DosA− cells were susceptible to RNai to the same extent as wild-type cells (see also Figure 2A).

To address the question whether differential expression of RrpA was responsible for incomplete silencing of discoidin

Table 1. Silencing efficiency in wild type and RdRP mutant cells

<table>
<thead>
<tr>
<th>Host strain transformation</th>
<th>β-Gal + control</th>
<th>β-Gal + mcRNAi</th>
<th>β-Gal + control</th>
<th>β-Gal + mcRNAi</th>
<th>β-Gal + control</th>
<th>β-Gal + mcRNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1A 128</td>
<td>Clone 2A 6,1</td>
<td>Clone 3A 378</td>
<td>Clone 4A 10,2</td>
<td>Clone 5A 1257</td>
<td>Clone 6A 4,4</td>
<td></td>
</tr>
<tr>
<td>Clone 1B 383</td>
<td>Clone 2B 8,9</td>
<td>Clone 3B 255</td>
<td>Clone 4B 74</td>
<td>Clone 5B 90</td>
<td>Clone 6B 8,8</td>
<td></td>
</tr>
<tr>
<td>Clone 1C 457</td>
<td>Clone 2C 1,0</td>
<td>Clone 3C 23,2</td>
<td>Clone 4C 24,3</td>
<td>Clone 5C 1024</td>
<td>Clone 6C 14,9</td>
<td></td>
</tr>
<tr>
<td>Clone 1D 155</td>
<td>Clone 2D 0,6</td>
<td>Clone 3D 246</td>
<td>Clone 4D 343</td>
<td>Clone 5D 233</td>
<td>Clone 6D 3,6</td>
<td></td>
</tr>
<tr>
<td>Clone 1E 293</td>
<td></td>
<td>Clone 3E 26</td>
<td>Clone 4E 89</td>
<td>Clone 5E 838</td>
<td>Clone 6E 1,8</td>
<td></td>
</tr>
<tr>
<td>Clone 1F 334</td>
<td>Clone 2F 163</td>
<td>Clone 3F 334</td>
<td>Clone 4F 163</td>
<td>Clone 5F 413</td>
<td>Clone 6F 22,6</td>
<td></td>
</tr>
<tr>
<td>Clone 1G 319</td>
<td>Clone 2G 65,6</td>
<td>Clone 3G 83</td>
<td>Clone 4G 140</td>
<td>Clone 5G 1184</td>
<td>Clone 6G 5,3</td>
<td></td>
</tr>
<tr>
<td>Clone 1H 24</td>
<td>Clone 2H 16,4</td>
<td>Clone 3H 319</td>
<td>Clone 4H 140</td>
<td>Clone 5H 1271</td>
<td>Clone 6H 1,11</td>
<td></td>
</tr>
<tr>
<td>Clone 1I 24</td>
<td>Clone 2I 1063</td>
<td>Clone 3I 24</td>
<td>Clone 4I 509</td>
<td>Clone 5I 1063</td>
<td>Clone 6I 23,0</td>
<td></td>
</tr>
<tr>
<td>Clone 1J 200</td>
<td>Clone 2J 4,6</td>
<td>Clone 3J 200</td>
<td>Clone 4J 509</td>
<td>Clone 5J 663</td>
<td>Clone 6J 4,6</td>
<td></td>
</tr>
<tr>
<td>Average β-gal activity</td>
<td>289 ± 128</td>
<td>5 ± 4</td>
<td>191 ± 132</td>
<td>207 ± 214</td>
<td>769 ± 428</td>
<td></td>
</tr>
</tbody>
</table>

Average reduction by RNai

Unpaired t test p value

0.0008 0% 0.0001 99%
in development, the RrpB− strain was assayed for transcription of rrPA by semiquantitative RT-PCR. No significant changes were detectable under the different growth and developmental conditions, which were used for the experiment in Figure 2B (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results).

Gene Silencing Is Accompanied by the Production of ~23mers

In several organisms, it has been shown that RNA interference is accompanied by the production of ~23mers of the RNAi and/or the target gene. To test this, we hybridized a β-gal sense probe to enriched small RNA isolated from various cell lines. As shown in Figure 5A, β-gal-specific antisense ~23mers were found in strains with, but not in the strains without, RNAi. Hybridization with a β-gal antisense probe yielded similar amounts of sense ~23mers, whereas no ~23mers were detectable with a β-gal probe that was not covered by the RNAi construct (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results). In the rrpA knock-out mutant, ~23mers were not found.

Figure 5. (A) Low molecular weight RNAs from silenced and nonsilenced strain. Low molecular weight RNA was isolated from the strains indicated, blotted, and hybridized with a 32P-labeled β-gal sense probe. The position of ~23mers is indicated by an arrowhead. (B) 32P-Labeled dsRNA was generated by in vitro transcription of a PSV-A gene fragment and incubated with a partially purified dsRNase preparation from Dictyostelium. Reaction products were separated by PAGE in parallel with small RNA isolated from RNAi-silenced β-gal cells. RNA was blotted and hybridized with a 32P-labeled β-gal probe. The blot was exposed for 1 wk (left) and overnight (right).
DISCUSSION

RNA interference proved to be functional in *Dictyostelium* when constructs transcribing inverted repeats separated by an unpaired loop were stably transformed into the cells. Double promoters and feeding of bacteria expressing sense and antisense RNA from a target gene did not result in silencing in the experiments performed here.

The *rrpA* gene, one of three homologues to RdRP, was strictly required for RNAi, whereas knock-outs of *rrpB* and *dosA* had no obvious effect on the mechanism. This was surprising because *rrpB* differed from *rrpA* in only 49 of 1780 amino acids within the known sequence. The possibility that *rrpB* was a nontranscribed pseudogene could be ruled out since RT-PCR products were detected. Both genes are transcribed at very low levels and could not be shown by Northern blotting.

As in other organisms, RNA interference resulted in the production of sequence-specific siRNAs (small interfering RNAs). The RNAi construct alone (without the target gene) did not show any detectable ∼23mers in the wild type in vivo and the same was true for RNAi plus target in *RrpA*− cells. In both cases, however, ∼23mers were found in the in vitro assay. Because we assume that the dsRNase generates RNAi ∼23mers in vivo, we have to conclude that detection of ∼23mers requires amplification by *RrpA*. The RNAi supplied in vivo is therefore not sufficient to generate detectable ∼23mers and most likely not a target for RdRP. We propose that interaction between RNAi and the target mRNA is necessary to initiate the amplification process by *RrpA* and that amplification is required for efficient gene silencing. This hypothesis is supported by the following observation: Large quantities of antisense ∼23mers detected in silencing strains can obviously not be degradation products of mRNA, and they can also not be (exclusively) derived from the RNAi because they are not seen in strains with only the RNAi construct. Small amounts of ∼23mers produced by the dsRNase may serve as “primers” for *RrpA*, which synthesizes the antisense strand using the mRNA as a template. The resulting dsRNA could then again be degraded by the dsRNase into ∼23mers. These could reintegrate the amplification cycle or mediate mRNA degradation by a putative RISC homolog. This finding appears to contrast with experiments done in *Drosophila*, in which degradation of mRNA and coinjected dsRNA were readily observed. In these experiments, the appearance and persistence of ∼23mers correlated precisely with gene silencing (Yang *et al*., 2000). However, the authors could not exclude an amplification process in which the products mediated gene silencing. ∼23mers generated directly or indirectly (e.g., by RdRP) would not be labeled and would have thus escaped detection in their assay. In the wild-type background without β-gal reporter gene, the “silent” β-gal RNAi construct becomes an active interference agent when a target gene is subsequently introduced. It is therefore likely that ∼23mers are produced from the inverted repeat but are amplified to detectable levels only when the target is present.

Because *RrpA*− cells still generate ∼23mers in vitro, it is likely that the dsRNA is also degraded in the knock-out strain but that the products are below the level of detection. dsRNA should be rather stable and others have shown that only a fraction of it is processed in vivo (Yang *et al*., 2000)

![Figure 6](image-url)
and in vitro (Zamore et al., 2000). However, we did not see any residual dsRNA in Northern blots (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results).

The substrate specificity of the partially purified Dictyostelium dsRNase and the size of the products strongly suggested that this enzyme complex generates the RNAi ~23mers. Although the sequence similarity between Dictyostelium RpA and other RdRPs is significant (22% similarity to EG01 from C. elegans) the Dictyostelium enzyme contains an N-terminal extension not found in other RdRPs. Surprisingly, this domain, which is separated by an intron from the rest of the coding sequence, shows similarity to various RNA helicases and gives the best match to the helicase domain of K12H4.8 from C. elegans, a member of the dicer gene family (see Figure 3A; Bass, 2000). Dicer is the recently identified bidentate RNase that cleaves dsRNA to ~23mers (Bernstein et al., 2001). Furthermore, it is a homologue of the Arabidopsis CAF gene (Jacobsen et al., 1999) mutations of which cause a floral phenotype. Members of this family consist of an N-terminal helicase domain, a C-terminal RNase III homology domain and a dsRNA binding domain. We have recently identified two dicer/CAF homologues in Dictyostelium that do not contain the helicase motif but show high similarity to the RNaseIII domain of dicer and CAF (Martens, Novotny, Oberstrass, Postlethwait, and Nellen, unpublished results). Assuming that RdRP and dicer/CAF are both components of the same RNAi machinery, it is intriguing to speculate that domain swapping has occurred between the nuclease and the polymerase. If the Dictyostelium dicer homologue and RdRP are really components of the same complex, this may suggest that cleavage of dsRNA and amplification of the signal/guide RNA are spatially linked.

DosA displays 63% similarity to RpA and 22% similarity to EG01 from C. elegans. Good matches in highly conserved regions support our conclusion that the gene encodes a genuine RdRP. The observation that DosA is not required for RNAi makes this an “orphan RdRP” with no known function. This is similar to the situation in plants in which several RdRP-related genes were found but only specific ones appear to be involved in gene silencing. More surprising is the fact that RrpB cannot compensate for a knock out of the closely related RpA gene. A detailed analysis of these two genes may help to specify the features of an RNAi RdRP.

The feasibility of gene silencing was shown with the endogenous Dictyostelium discoidin gene family. Both mRNA and protein were clearly reduced, in many cases to nondetectable levels. The observation that more discoidin expression was found in developing cells and that almost no RNAi effect was observed in cells grown on a bacterial lawn indicated that RNAi mechanisms are either under developmental control or that the limited capacity of the RNAi machinery cannot completely abolish the high amounts of discoidin mRNA transcribed during development. Experiments described previously (Novotny et al., 2001), and here, rule out that reduced activity of dsRNase or reduced transcription of rrpA during development caused the residual expression levels of discoidin in silenced developing cells. A similar reduced silencing effect of the mybB gene in Dictyostelium has also been observed by others (H. Otsuka, R. Dottin, and J. Gross, personal communication). This is reminiscent of the situation in C. elegans in which silencing in specific cell types was found to work poorly (Tavernarakis et al., 2000).

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**Note added in proof.** While this paper was under revision, Lipardi et al. (Cell 2001; 107, 297–307) and Sijen et al. (Cell 2001; 107, 465–479) submitted, revised, and published data that confirmed our conclusions, that siRNAs serve as primers for RdRP to amplify the RNAi effect.

**REFERENCES**


