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 duplex determines the sequence-specific degradation of the target mRNA but only in regions of sequence homology to the dsRNA (Hammond *et al.*, 2000; Yang *et al.*, 2000). In silenced *Drosophila* and plants, ~23mers have been identified as products of hairpin RNA (RNAi), which hybridize to both the sense and the antisense strand. It has been shown that in vitro the target mRNA is degraded to ~23mers (Zamore *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 RNase 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 Firtel (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 guanidiniumthiocyanate and blotted 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 (GAAATCACCAATATA-AACCAACTGATC) that binds 3′ in both Rrp genes was used. The final amplification was done with primer pair B (5′- primer: GAA-GACGAGGAAGCAGAGTTCATTATAAC, 3′ primer: GAAAT-CACCAATATAAACCAACTGATC). Amplification products of the two genes could be distinguished by *Cla*I 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 (CCGCTC-GAGATCTATAGCTGAATTGTTGGCTATACG) 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: TAAC-GAGCTCCTGCACTGGATGG, 3′ primer: CCGCTCGAGATCTAT-AGCTGAATTGTTGGCTATACG). As a control we performed RT-PCR on thioredoxin 1 mRNA with oligo (CGCGGATCCTTATTT-GTTTGCTTCTAGAGTACTTC) for first-strand synthesis and primer pair D (5′ primer GAACGAGCTCCATGGCCAATAGAG-TAATTCATG, 3′ primer CGCGGATCCTTATTTGTTTGCTTCTA-GAGTACTTC) 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 BS^R cassette (Sutoh, 1993) when selection for blasticidin resistance was possible.

The pV18gal-i vector was generated by replacing the discoidin promoter in pVEII (Blusch *et al.*, 1992) for the V18 promoter (Ken and Singleton, 1994). The first β-gal fragment of 815 bp was fused in sense orientation to the V18 promoter; the second fragment of 1326 bp was ligated tail to tail to the first fragment. The additional 511 bp constitute the predicted hairpin loop in the fold-back transcript.

The discoidin RNAi construct was introduced into the pDneo2 vector (Witke *et al.*, 1987) with loop and dsRNA sizes of 259 and 509 bp, respectively.

DosA replacement and rrpA and rrpB disruptions were done by homologous recombination in AX3 (for DosA) and AX2 (for rrpA and rrpB), essentially as described by Witke *et al.* (1987). The BS^R selection cassette was flanked by arms of 1.8 and 2.5 kbp for DosA, 798 and 982 bp for rrpA, and 586 and 1191 bp for rrpB. Clones were picked and analyzed by PCR.

PCR was performed on genomic DNA of potential rrpA and rrpB disruption clones using primer pair A (5′-primer: CGCTACTTC-TACTAATTCTAGA, 3′-primer: GAAATCACCAATATAAAC-CAACTGATC) in which one primer binds within the coding sequence of the BS^R 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: GAA-GACGAGGAAGCAGAGTTCATTATAAC, 3′ primer: GAAAT-CACCAATATAAACCAACTGATC), which bind within identical coding sequences of the rrpa and rrpB genes that flank the BS^R cassette. Under the conditions used, PCR did not proceed across the inserted BS^R cassette. Therefore, only products of nondisrupted genes were obtained. Amplification products of rrpA and rrpB could be distinguished by cutting with *Cla*I and *Eco*RV. *Cla*I cleaved the rrpA but not the rrpB PCR product and *Eco*RV cleaved the rrpA PCR product twice and rrpB product once.

β-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 Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, and 7 ml/l mercaptoethanol) by freezing in liquid nitrogen and thawing at 37°C. Cell debris was removed by centrifugation at $10,000 \times g$. The supernatant was incubated with *o*-nitrophenyl-D-galactoside at 37°C. The reaction was stopped with 0.5 volume of 1 M Na$_2$CO$_3$. β-Gal activity was measured photometrically at 420 nm and standardized for 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 nmol o-nitrophenol/min at 37°C. U/mg = (E$_{420}$ × 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 μg of protein cell extract were incubated for 3 h at room temperature with 13 ng of ^{32}P-labeled 260-bp PSV-A dsRNA (131 kBq/μg) or with 40 ng of ^{32}P-labeled 400-bp β-gal dsRNA (13 kBq/μg) in 1× assay buffer (50 mM Tris-HCl, pH 8.0, 25 mM KCl, 5 mM MgCl$_2$, 2 mM dithiothreitol, 250 μg/ml tRNA, 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 ~23mers 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 EUDICT 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 $\pm$ 3.5 U/mg protein. As shown in Figure 1, introduction of a 807-bp-long antisense construct targeted against the first 800 nucleotide-coding region of 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 sense and antisense construct were not successful either (Figure 1, c and d). Similarly, the insertion of a 827-bp β-gal fragment (same sequence as in the sense and antisense constructs) between two promoters (actin 15 and V18) was not effective (Figure 1e). We then tried a construct containing inverted repeats of the same 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

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 T7 promoters and transformed bacteria (strain BL21) were used to feed *Dictyostelium* cells. Only in e was silencing of the transgene observed.

RNA from the actin 6 promoter directed against the discoidin gene family. Nine clones were randomly chosen and assayed for discoidin expression. Five of them exhibited complete and four exhibited partial discoidin silencing. Similar to antisense experiments (Crowley *et al.*, 1985), it appeared that the entire discoidin gene family was affected. On the RNA level, discoidin transcripts were not detectable in cells exhibiting complete silencing and were strongly reduced in cell lines with partial silencing (Figure 2A). Surprisingly, the RNAi effect appeared to be reduced on the protein level in developing cells (Figure 2B). When these cells were transferred back to axenic medium, the RNAi effect was again observed to the same extent (Figure 2B). This demonstrated that cells were not reprogrammed and that the RNAi machinery as well as the RNAi construct was still functional. Examination of steady-state levels revealed that discoidin mRNA was 35-fold enhanced in developing cells compared with axenic growth, whereas the protein levels were similar (Figure 2B). This suggested that the
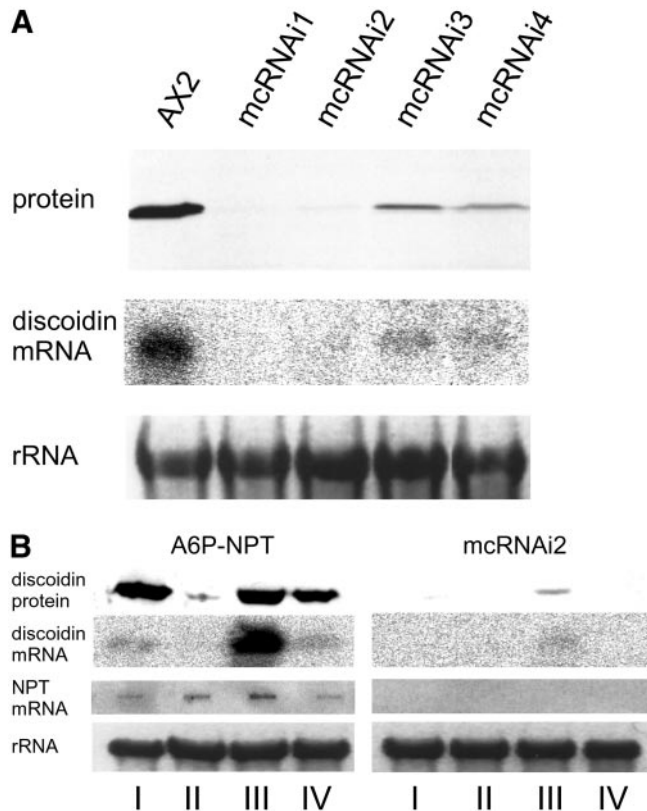
Figure 2. (A) Western blot with a discoidin-specific antibody on protein isolated from wild-type AX2 cells (control) and from four of nine randomly chosen clones silenced by discoidin RNAi (top). mcRNAi1 and 2 display complete silencing, and mcRNAi 3 and 4 display partial silencing. A Northern blot with RNA from the same cell lines, hybridized with a discoidin-specific probe, is shown in the middle. As a control for equal loading, ethidium bromide-stained large rRNA is shown in the bottom. The RNAi construct was introduced into cells via a multicopy vector (mc). (B) Protein and RNA were isolated from an AX2 strain carrying the G418 resistance gene under control of the actin 6 promoter (A6P-NPT) and from the discoidin RNAi strain (mcRNAi2) expressing the RNAi from the same promoter grown in axenic medium to a density of 2×10^6 cells/ml (I), grown in bacterial suspension to a density of 3×10^6 cells/ml (II), grown in bacterial suspension to a density of 3×10^6 cells/ml and then developed in shaking culture for 5 h (III), and grown in axenic culture to a density of 2×10^6 cells/ml after passage through bacterial growth and development for 5 h (IV). First line, cellular protein separated by SDS-PAGE, blotted, and probed for discoidin. Second line, Northern blot probed for discoidin mRNA. Third line, slot blot probed for NPT mRNA. Fourth line, ethidium bromide-stained large rRNA as a loading control.

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. NTP mRNA levels varied slightly under the different growth or developmental conditions and were rather higher (2.5-fold) in developing cells
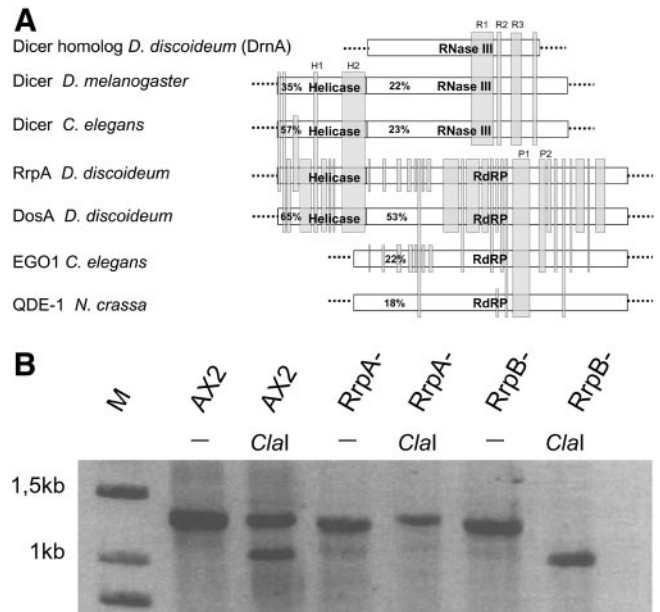

Figure 3. (A) Schematic comparison of the *Dictyostelium* RrpA and DosA genes with RdRP genes from *C. elegans* and *N. crassa* and of a *Dictyostelium* dicer homologue gene with dicer from *Drosophila* and *C. elegans*. Shaded boxes exhibit at least 65% similarity to the *Dictyostelium* genes. Overall similarities to the corresponding *Dictyostelium* genes are given in percentages. The alignments are not exactly drawn to scale. Complete alignments are available as supplementary material on the web. Characteristic homology boxes in the RNaseIII domain (R), the helicase domain (H), and the RdRP domain (P) are marked; amino acid positions refer to the *Dictyostelium* proteins RrpA and dicer homologue: R1, LGDS domain, AA 907–969; R2, EALIG domain, AA 975–994; R3, DAVL domain, AA 1063–1103; H1, PRIL domain, AA 187–197; H2, VLEE domain, AA 441–500; P1, SGSD domain, AA 1306–1370; P2: SDQY domain, AA 1415–1454. (B) RT-PCR reactions with total RNA from AX2 wild type, RrpA⁻, and RrpB⁻ cells. The resulting PCR products were separated on a 0.8% agarose gel before and after *Cla*I cleavage.

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 data base 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

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

Host strain transformation	AX2		RrpA⁻		DosA⁻	
	β-Gal + control	β-Gal + mcRNAi	β-Gal + control	β-Gal + mcRNAi	β-Gal + control	β-Gal + mcRNAi
	Clone 1A 128	Clone 2A 6,1	Clone 3A 378	Clone 4A 10,2	Clone 5A 1257	Clone 6A 4,4
	Clone 1B 383	Clone 2B 8,9	Clone 3B 255	Clone 4B 74	Clone 5B 90	Clone 6B 8,8
	Clone 1C 457	Clone 2C 1,0	Clone 3C 23,2	Clone 4C 24,3	Clone 5C 1024	Clone 6C 14,9
	Clone 1D 155	Clone 2D 0,6	Clone 3D 246	Clone 4D 343	Clone 5D 233	Clone 6D 3,6
	Clone 1E 293		Clone 3E 26	Clone 4E 89	Clone 5E 838	Clone 6E 1,8
			Clone 3F 334	Clone 4F 163	Clone 5F 413	Clone 6F 22,6
			Clone 3G 83	Clone 4G 656	Clone 5G 1184	Clone 6G 5,3
			Clone 3H 319	Clone 4H 16,4	Clone 5H 1271	Clone 6H 1,11
			Clone 3I 24	Clone 4I 140	Clone 5I 1063	Clone 6I 23,0
			Clone 3J 200	Clone 4J 509	Clone 5J 663	Clone 6J 4,6
	Population 316	Population 8,2	Population 210	Population 253	Population 419	Population 15,1
Average β-gal activity (U/mg) clones + pop.	289 ± 128	5 ± 4	191 ± 132	207 ± 214	769 ± 428	9, 6 ± 8
Unpaired *t* test p value	0,0008		0,83		0,0001	
Average reduction by RNAi	98%		0%		99%	

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 *Cla*I 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
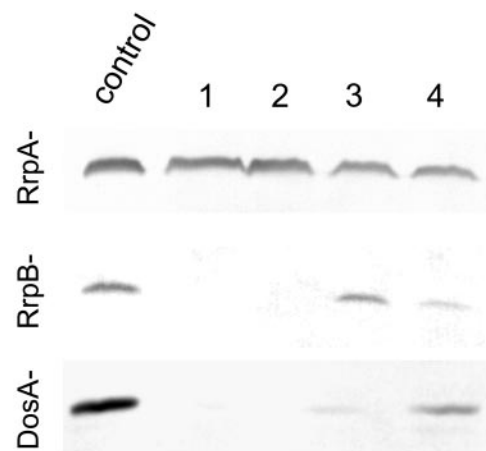

Figure 4. A discoidin Western blot is shown from four of six independent clones, each of the RrpA⁻, RrpB⁻, and DosA⁻ transformants carrying the discoidin RNAi construct. Clones in lane 1 and 2 display complete silencing in RrpB⁻ and DosA⁻, whereas clones in lanes 3 and 4 show only partial silencing. No significant reduction of discoidin levels was observed in RrpA⁻ transformants.
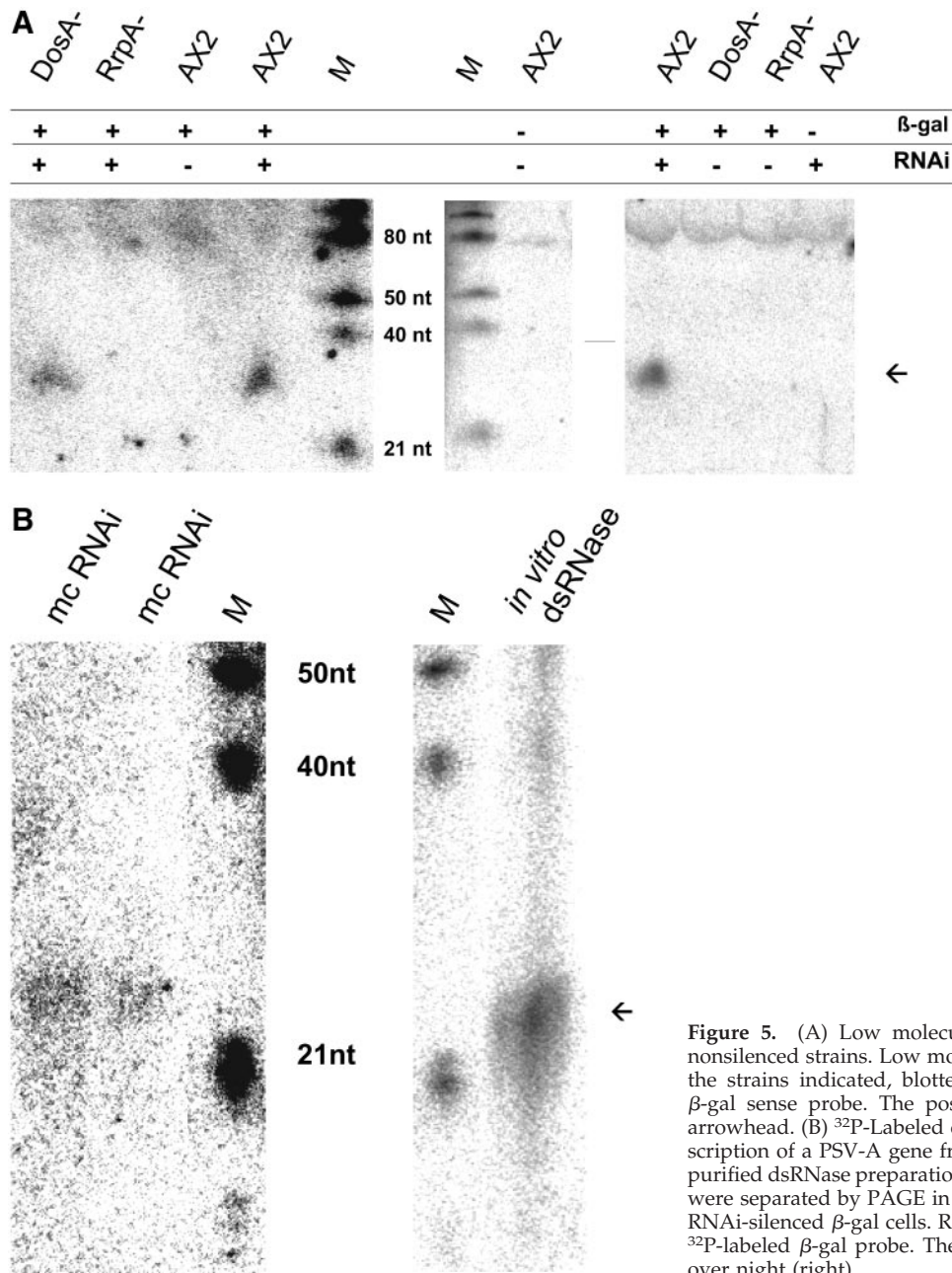
Figure 5. (A) Low molecular weight RNAs from silenced and nonsilenced strains. Low molecular weight RNA was isolated from the strains indicated, blotted, and hybridized with a ^{32}P-labeled β-gal sense probe. The position of ~23mers is indicated by an arrowhead. (B) ^{32}P-Labeled dsRNA was generated 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 ^{32}P-labeled β-gal probe. The blot was exposed for 1 wk (left) and over night (right).

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.
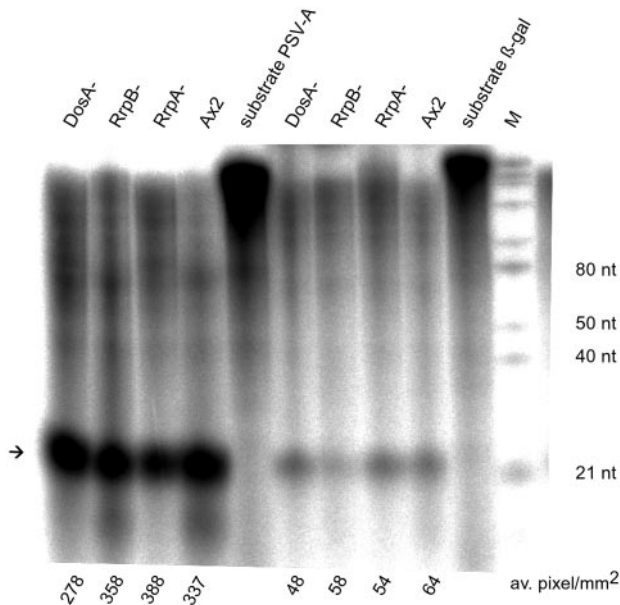
Figure 6. In vitro assays with dsRNase preparations from various strains on a ^{32}P-labeled in vitro prepared PSV-A dsRNA gene fragment and a ^{32}P-labeled β-gal dsRNA fragment. Crude extract (350 μg) was incubated with 13 ng of PSV-A or 40 ng of β-gal dsRNA. ~23mers are indicated by an arrow. ~23mers were quantified from four independent assays by the TINA program (Raytest,). Averages are given in pixels per square millimeter below the lanes.

No ~23mers were observed in a strain containing only the RNAi construct but not the β-gal target gene. When this cell line was subsequently transformed with the β-gal reporter construct, no reporter activity was detected in the transformed population or in six randomly chosen clones (background activity below 2 U/mg). The strain was thus predisposed to silence the newly introduced transgene.

Extracts from the rrpA, the rrpB, and the DosA Knock-Out Strains Generate ~23mers In Vitro

We have previously described a dsRNase from *Dictyostelium* that specifically digests any dsRNA to fragments of ~23 nucleotides or base pairs (Novotny *et al.* 2001). It was of interest to examine whether these products could be related to the ~23mers observed in RNAi silenced strains. Figure 5B shows an in vitro assay of partially purified dsRNase on a 260-bp dsRNA substrate from the PSV-A gene (Sadiq *et al.,* 1994). The digestion products of the dsRNase are very similar in size to the ~23mers found in vivo in RNAi silenced cells, which are shown for comparison in the adjacent lane of the gel. We therefore suggest that the *Dictyostelium* dsRNase is involved in the generation of RNAi-mediated ~23mers. The lack of in vivo ~23mers in the RrpA⁻ strain raised the question whether extracts from these cells were able to produce ~23mers from dsRNA in vitro. Figure 6 shows that crude dsRNase extracts from all mutant cell lines had similar activities on both PSV-A and β-gal dsRNA, thus demonstrating that none of the three RdRP homologues per se was required for the production of ~23mers in vitro.

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 reinitiate 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)

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 suggest that this enzyme complex generates the RNAi ~23mers

Although the sequence similarity between *Dictyostelium* RrpA and other RdRPs is significant (22% similarity to EGO1 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 RrpA and 22% similarity to EGO1 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 RrpA 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).

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.

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