HelF, a putative RNA helicase acts as a nuclear suppressor of RNAi but not antisense mediated gene silencing

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DDBJ/EMBL/GenBank accession no. HelF: DDB0168963

ABSTRACT

We have identified a putative RNA helicase from *Dictyostelium* that is closely related to drh-1, the 'dicer-related-helicase' from *Caenorhabditis elegans* and that also has significant similarity to proteins from vertebrates and plants. Green fluorescent protein (GFP)-tagged HelF protein was localized in speckles in the nucleus. Disruption of the helF gene resulted in a mutant morphology in late development. When transformed with RNAi constructs, HelF⁻ cells displayed enhanced RNA interference on four tested genes. One gene that could not be knocked-down in the wild-type background was efficiently silenced in the mutant. Furthermore, the efficiency of silencing in the wild-type was dramatically improved when helF was disrupted in a secondary transformation. Silencing efficiency depended on transcription levels of hairpin RNA and the threshold was dramatically reduced in HelF⁻ cells. However, the amount of siRNA did not depend on hairpin transcription. HelF is thus a natural nuclear suppressor of RNA interference. In contrast, no improvement of gene silencing was observed when mutant cells were challenged with corresponding antisense constructs. This indicates that RNAi and antisense have distinct requirements even though they may share parts of their pathways.

INTRODUCTION

RNAi has been discovered by a stringent evaluation of antisense RNA mediated gene silencing. Fire *et al.* (1) observed that dsRNA was a substantially better trigger for gene knock-downs in *Caenorhabditis elegans* than antisense RNA. This was in contrast to previous models that predicted a direct hybridization between the complementary mRNAs and antisense RNAs. The observation also suggested that antisense mediated gene silencing may be due to contaminating dsRNA. Consequently, antisense RNA experiments could since then be regarded as the same or at least very similar to RNAi. However, a rigorous comparison between antisense RNA and RNAi has not yet been performed.

The standard model for the mechanism of RNA interference involves the RNase III related enzyme Dicer that digests dsRNA into 21 bp fragments (siRNAs) with 2 nt 3′ overhangs (2). According to the 'weak-bond-5′-rule' (3) one of the strands is preferentially transferred to the RNA induced silencing complex (RISC) and thus targeted to a specific mRNA (4). A nuclease activity that is associated with RISC, the Argonaute 2 (5–7) hydrolyses the mRNA in the centre of the hybrid, RISC is then released to target the next mRNA molecule.

Despite the fact that major components like Dicer, Argonaute proteins, different other paz/piwi domain proteins, various putative RNA helicases and others are highly conserved throughout evolution, the ubiquitous RNAi pathway has evolved some specialties in different organisms. RISC for example has been defined in *Drosophila* and mammals but lacks stringent evidence in plants, *C.elegans*, *Neurospora* and *Dictyostelium*. In contrast, all of the latter species require RNA directed RNA polymerases (RdRPs) for RNA interference (8–11) but these enzymes appear not to be encoded in the mammalian and in the *Drosophila* genome.

So far, most proteins that have been identified in the RNAi pathway are required for efficient gene silencing. Except for viral inhibitors of RNAi (12,13), there are only two examples that formally act as endogenous inhibitors of RNAi and result in enhanced silencing when the corresponding genes are knocked out. The *C.elegans* rrf-3 gene, a putative RdRP (14),

provided the first evidence for negative modulation of RNAi. In the rrf-3 loss-of-function mutant, even genes that were refractile to RNAi in the wild-type background could be efficiently downregulated. More recently, the eri-1 gene in *C.elegans* that encodes a siRNA degrading enzyme was defined as a second negative RNAi regulator (15).

Though RNAi is by now a routine tool for gene silencing in many model organisms, important details of the mechanism are still unknown. Further components of the machinery are identified but their functions in transgene induced silencing as well as their endogenous, physiological tasks are insufficiently understood.

In *Dictyostelium*, many of the components of the RNAi machinery have been identified (11). Surprisingly, both Dicer-related genes drnA and drnB do not encode the N-terminal helicase domain found in Dicer proteins of other species. Instead, helicase domains are found in the N-terminus of the three RdRPs rrpA, rrpB and rrpC (formally dosA) suggesting exon swapping between Dicers and RdRPs and implying that both proteins may work in concert (11). By routine searches in the *Dictyostelium* genome (16), we now identified a new gene, helF, with high similarity to the helicase domain usually found in Dicer proteins and to a separate gene from *C.elegans* termed drh-1 for 'Dicer-related helicase' (17). The participation of HelF in RNA mediated gene silencing was highly suggestive. We therefore generated a HelF knock-out strain and investigated the performance of RNAi in the mutant background. In addition, we directly compared antisense mediated gene silencing and RNAi on a set of tester genes in the mutant and the wild-type.

MATERIALS AND METHODS

Cell biological methods

Dictyostelium AX2 cells and transformants were grown on bacterial lawns of *Klebsiella aerogenes*, in suspension culture or on plates with HL5 medium. Cells were developed on filters in a moist chamber, synchronous development was monitored microscopically. After 16 h, cells were washed off the filters and collected by centrifugation.

Dictyostelium transformation was carried out as described previously (18). Transformation with vectors containing the G418 resistance cassette resulted in multi-copy tandem integration into the genome. Co-transformation was done as described in Nellen and Firtel (19). Transformants were sub-cloned on *K.aerogenes* plates and single clones from usually two independent transformations were examined. Disruption of the helF gene was done by homologous recombination. Genomic fragments of 1044 and 896 bp from the HelF region (position −991 to 54 and position 982 to 1877 in the HelF gene) were cloned left and right of the BSr cassette (20,21) *Dictyostelium* cells were transformed with the isolated 3383 kb fragment cut out with BpiI. Successful disruptants were identified by colony PCR and confirmed by Southern blot using the 1037 bp fragment cut out from the left arm with EcoRI and XbaI as a radio-labelled probe.

RNA preparations and northern blotting

Total cellular RNA was prepared as detailed previously (22). For northern blots, 10 µg of total cellular RNA were separated on a 1.8% agarose gel containing 20 mM guanidiniumthiocyanate and blotted to a nylon membrane. Prehybridization and hybridization were carried out as described (23). Radioactively labelled *in vitro* transcripts were used as probes. *In vitro* transcription was carried out with T7 and SP6 RNA polymerase as recommended by the manufacturer (MBI, Fermentas).

For Northern blot analysis of small RNAs, 20 µg total RNA per lane were separated on a 12% polyacrylamide gel containing 7 M urea in 1× TBE and electroblotted to a Hybond N+ membrane (Amersham). Prehybridization and hybridization were carried out at 42°C in a buffer, containing1% BSA, 1 mM EDTA, 0.5 M $NaPO_4$ (pH 7.2), 7% SDS. The oligo probe 5'-GTCTACCCAAGGTTTAGTTC-3' end-labelled with [γ-^{32}P]ATP and T4-PNK (MBI, Fermantas) was used for hybridization to detect discoidin antisense RNA. The membrane was washed two times for 5 min with 2× SSC/ 0.1% SDS and once for 10 min with 1× SSC/0.1% SDS at 42°C and exposed on an imaging plate for analysis in a Fuji Phosphorimager.

In vitro transcription and nuclear run-on transcripts

The discoidin full length cDNA was cloned in pGemT-Easy, the vector was linearized with Bsp120I and SP6 polymerase was used for *in vitro* transcription, resulting in a discoidin antisense transcript of ∼600 nt. Actin15 antisense RNA and Green fluorescent protein (GFP) sense RNA were transcribed with T7 or SP6 polymerase from the appropriate, linearized pGEM vectors. The transcripts were digested with DNase (RNase free) to eliminate the DNA template. The reaction mixtures were extracted with phenol/chloroform, precipitated with ethanol and resuspended in DEPC-treated water. By slot blotting, 1 µg of discoidin antisense RNA, 1 µg of GFP sense RNA and 100 ng of actin antisense RNA were transferred to a nitrocellulose membrane using a vacuum slot blot device. Nuclear run-on transcription was performed as described previously (23). Purified radio-labelled transcripts were hybridized to the slot blots in a volume of 1 ml for 48 h at 50°C in hybridization buffer containing 50% formamide, 50 mM sodium phosphate (pH 6.5) and 5× SSPE, washed in a low salt buffer (0.2× SSC, 0.1% SDS) and exposed on an imaging plate for analysis in a Fuji Phosphorimager.

RT–PCR

Total RNA was isolated from wild-type and the HelF knock-out strain. Reverse transcription was carried out with an oligodT primer on 2 µg of RNA using M-MulV reverse transcriptase (MBI) in a 20 µl reaction. PCR was done on the 2 µl aliquot of the cDNA using the helF specific primers: 5'-CAATAAACTTTTATCAAATGGTG-3' (5' primer) and 5'-CTCTAAATTTTTAATTAAATTATAAATT-3' (3' primer). As a control, primers for the thioredoxin gene were used on an equal aliquot of the same cDNA. Since the trx primers generate a fragment that contains an intron in the gene, DNA contaminations would be readily detected by appearance of a 730 bp genomic product in addition to the spliced trx cDNA fragment of ∼300 bp. Further control PCRs were done with the same primer pairs but without cDNA template.

Western and colony blotting

Western blotting and protein detection was done as described (24) using the monoclonal antibodies 80-52-13 for discoidin and 176-3D6 for coronin and an alkaline phosphatase coupled secondary antibody. For colony blotting (25) the same antibodies were used.

Vector constructs

Fragments of the discoidin, coronin, sp96 and thioredoxin genes were obtained by PCR with primers, containing suitable restriction sites. The RNAi hairpin constructs were introduced into the pDneo2 vector (26). A gene fragment in sense orientation was fused to the Actin6 promoter, followed by a second longer fragment in antisense orientation. Transcription of the constructs formed a fold-back RNA with loop and dsRNA sizes of 259 and 509 bp (discoidin), 261 and 642 bp (coronin), 393 and 321 bp (thioredoxin) and 250 and 405 bp (sp96) (see Supplementary Data).

The discoidin and thioredoxin antisense constructs comprised the whole coding sequence of the gene and were cloned in the pDneo2 vector in antisense orientation to the Act6 promoter. The coronin antisense vector has been described previously (27).

A helF–gfp fusion gene under the control of Actin15 promoter was constructed. The helF coding sequence was amplified by PCR using the primers, Forward: 5′-AAGAATT-CAAAATGACTAAAAATGATTTACAAACT-3′; Reverse: 5′-AAGGATCCCTCTAAATTTTTAATTAAATTATAAA-TT-3′ and cloned via EcoRI and BamHI into the vector pdD-GFP to create a C-terminal GFP fusion.

Fluorescence microscopy

Cell were fixed at −20°C in methanol for 20 min, washed three times with 1× phosphate-buffered saline (PBS) and stained with DAPI (1 mg/ml, diluted 1:15 000 in 1× PBS). The fixed cells were imbedded in Gelvatol and analyzed with a Leica DM IRB inverted fluorescence microscope. For image acquisition a Leica DC 350F digital camera and IM50 software were used. Images were processed in AdobePhotoshop.

Criteria for silencing efficiency

The expression levels of mRNAs in northern blots were normalized to the loading of rRNA and calculated as a percentage of the expression level in wild-type. Clones showing expression levels less then 10% of the wild-type signal were considered as silenced; clones with expression levels between 10 and 50% were considered as partially silenced and clones with less than 50% reduction were considered as non-silenced. By evaluation of western blots, the corresponding bands were referred to the internal loading control (discoidin or coronin), and normalized to the wild-type signal. For colony blots, quantitation was not possible and clones were only categorized as silenced and non-silenced.

Dictyostelium strains

AX2, AX2::HelF⁻, AX2::act15-HelF-GFP

Accession numbers

HelF: DDB0168963, chromosome 2, coordinates 2103998 to 2106511, Crick strand (DictyBase, http://dictybase.org).

RESULTS

HelF is a single copy gene with homology to Dicer-related helicases

The helF gene was identified by BLAST searches in the *Dictyostelium* data base using the Dicer helicase domain as a query sequence. A continuous open reading frame (ORF) of 2511 bp encoding a putative protein of 837 amino acids was found on chromosome 2, region DDB0168963 (28). Domain analysis suggested an N-terminal double stranded RNA binding motif (29) and a C-terminal RNA helicase motif of the DEAH family. The highest similarity within the DEAH domain was found with drh-1 from *C.elegans* (17) (53%), the helicase domain of *C.elegans* Dicer (43%), the helicase domain of DCL1 (51%), a Dicer-like protein from *Arabidopsis thaliana* and the helicase domains of *Dictyostelium* RrpA and RrpB (47%). It should be noted that for all these putative RNA helicases, the enzymatic activity has not yet been demonstrated. Furthermore, some of the motifs that have been shown to be essential for helicase activity (30) are altered or not present in helicases that have been implicated in RNAi. However, other amino acid sequences and additional boxes appear to be conserved in helicase-like genes that are involved in RNAi. For example, in the position of the conserved SAT box, the GLTAS peptide sequence is found in drh-1, dcr-1, DCL1, helF, rrpA and rrpB. Striking similarities are also detected in the C-termini of these proteins in the TSVXEEGXDV sequence that is usually not conserved in DEAH proteins and in QSRGRAR, a typical C-terminal helicase domain, that usually has some small variations (30,31). Figure 1 shows an amino acid alignment of HelF and a selection of related putative RNA helicases.

Southern blot analysis of genomic *Dictyostelium* DNA confirmed the results of the data base search that helF is a single copy gene (Figure 2A). By northern analysis, no mRNA could be detected, but semi-quantitative RT–PCR showed that helF was expressed at approximately the same levels throughout the 24 h developmental cycle of *Dictyostelium* (data not shown).

HelF knock-outs display prolonged slug migration and preferentially develop to stalk cells

To investigate the function of HelF, gene knock-outs were constructed by homologous recombination. DNA from two disruption strains is shown in the Southern blot in Figure 2A. A digestion of genomic DNA with EcoRI and XbaI resulted in an expected 7.6 kb fragment in the wild-type and a 1 kb fragment in the disruption strains. RT–PCR analysis confirmed the lack of HelF expression in the disruption strain (Figure 2B). Mutants were then examined for phenotypic alterations during development. When grown on a lawn of *K.aerogenes*, development proceeded in parallel to the wild-type until ∼10 h, then the mutant lagged behind, displayed a prolonged slug stage and migrating slugs left long tails of stalk-like structure behind. Some slugs were extremely large. A reduced number of culminants was observed after prolonged development on filters. These gave rise to a reduced number of fruiting bodies (10-fold decreased in comparison to the wild-type), some of them with aberrant stalks. Plaques were covered with an
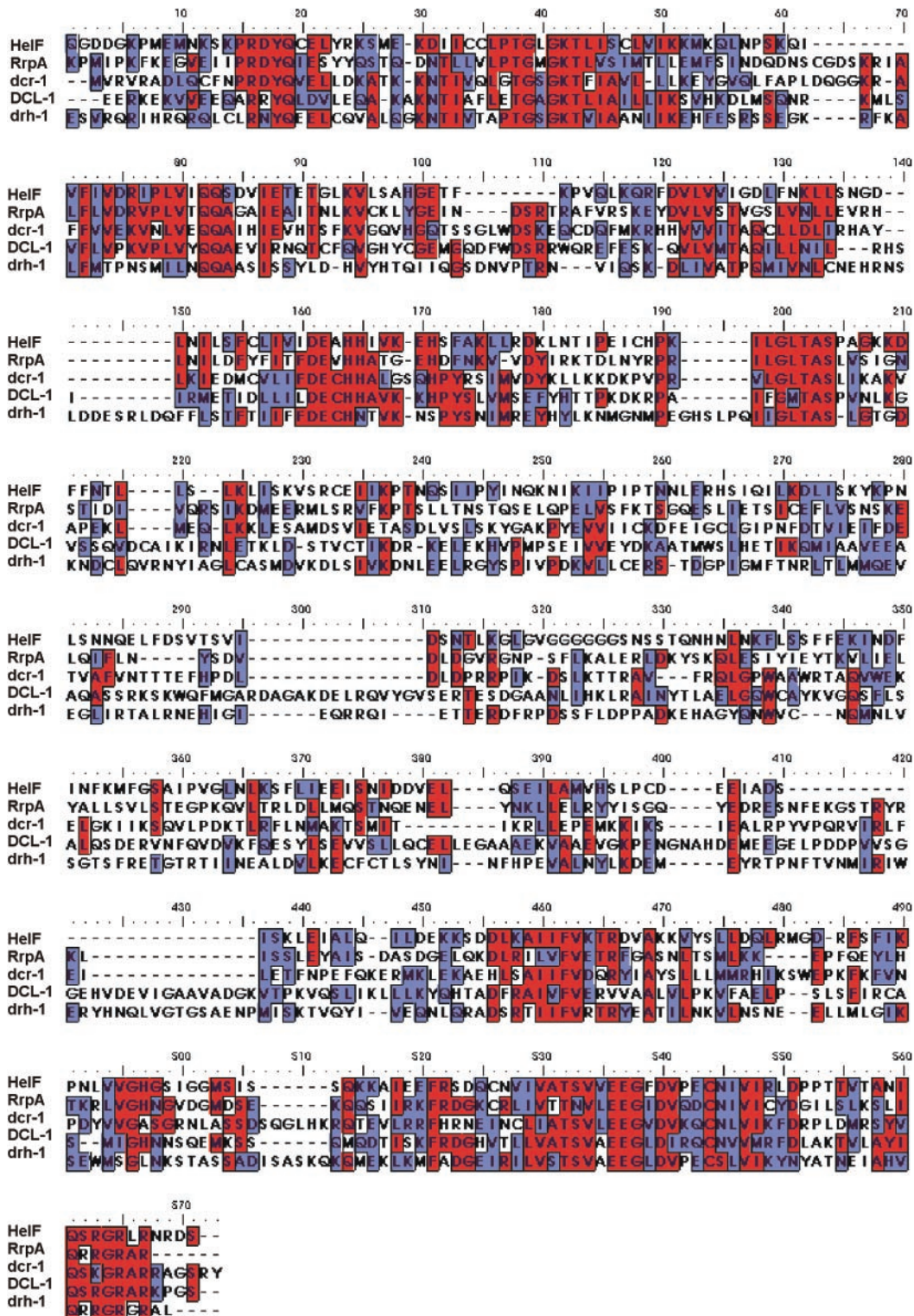
Figure 1. Protein alignment. Alignment of DEAH-box RNA helicase domains from HelF, RrpA (*D.discoideum*, DDB0191515, DictyBase, http://dictybase.org), dcr-1 (*C.elegans*, NP_498761), DCL1 (*A.thaliana*, NP_171612) and drh-1 (*C.elegans*, NP_501018). Identical residues are marked in red, similar residues in blue.

extensive network of dead stalk originating from the long slug tails (Figure 3). As indicated by the appearance of mature fruiting bodies, the penetrance of the phenotype was not complete.

Localization of HelF

Most of the RNAi machinery is localized in the cytoplasm. Although HelF does not contain a conventional nuclear localization signal (NLS), a 76.7% probability for nuclear
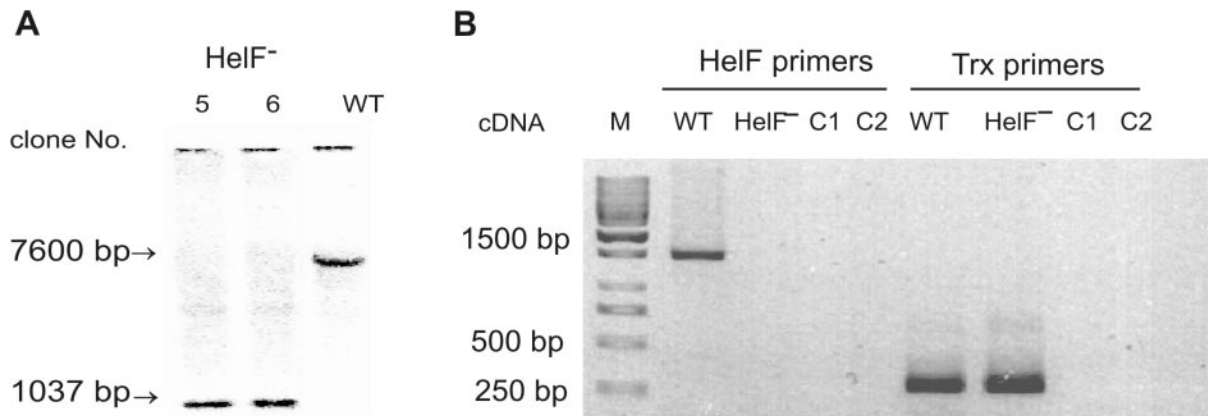
Figure 2. Analysis of HelF⁻ strain. (**A**) Southern blot of genomic DNA, isolated from wild-type cells and from two HelF disruption clones (no. 5 and 6). DNA was digested with EcoRI and XbaI, separated on an agarose gel, blotted and hybridized with an oligo-labelled probe, resulting in labelled fragments of 7600 and 1037 bp in wild-type in the KO strains, respectively (arrows). (**B**) RT–PCR of total RNA isolated from wild-type and the HelF knock-out strain was carried out with an oligodT primer. PCR was done on the cDNA using the helF specific primers. 5′-CAATAAACTTTTATCAAATGGTG-3′ (5′ primer) and 5′-CTCTAAATTTT-TAATTAAATTATAAATT-3′ (3′ primer). The expected product of 1.500 bp was only detected in the wild-type. As a control, primers for the thioredoxin gene were used on the same cDNA. Similar amounts of PCR products were obtained for cDNA of both strains and only the spliced trx fragment of ∼300 bp was detected, thus confirming the efficient removal of DNA contaminants. C1 and C2 are control PCR with helF and trx primers, respectively but without cDNA template.
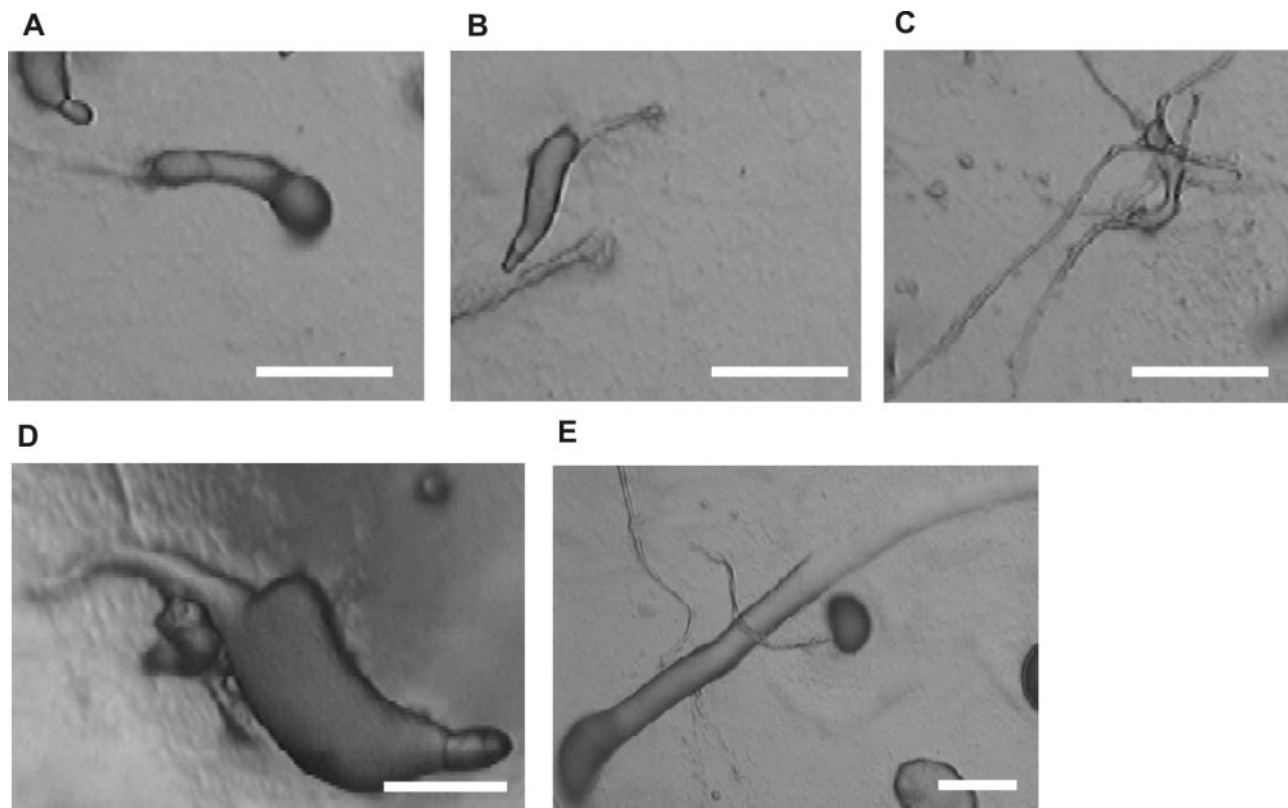

Figure 3. HelF⁻ phenotype. Wild-type cells (**A** and **B**) and HelF⁻ cells (**C–E**) were grown in parallel on a lawn of *K.aerogenes*. Mutant slugs were oversized and aberrant (D and E). After prolonged development, the plaques formed by the mutant strain were covered with a network of dead stalk (C) originating from the long slug tails and a reduced number of mature fruiting bodies. Scale bars represent 0.25 mm.

localization was predicted when analyzed by the PSORT II program with Reinhardt's method for cytoplasmic/nuclear discrimination (32). We constructed a C-terminal HelF–GFP fusion gene under the control of the actin15 promoter and transformed it into wild-type cells. Transformants were cloned and examined by fluorescence microscopy. As shown in Figure 4A, HelF-GFP was predominantly found in nuclear speckles. By confocal microscopy, ∼10 to 20 foci were found per nucleus. Though the nature of these foci is not known, the distinct localization was clearly different from other over-expressed nuclear proteins, e.g. the heterochromatin proteins HcpA and HcpB (33) or DnmA (34). When GFP

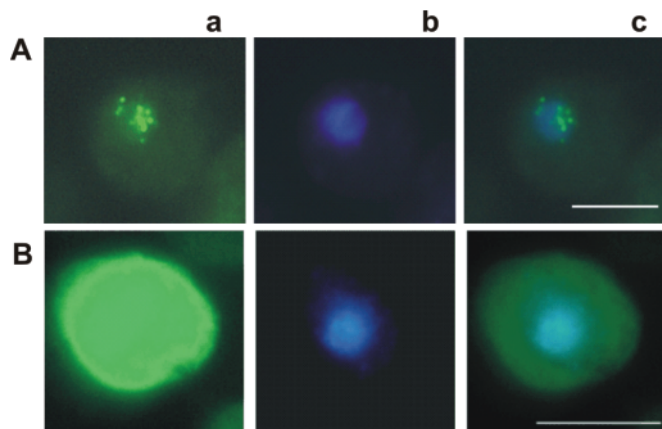**Figure 4.** Localization of HelF. (**A**) Fluorescence microscope image of a HelF-GFP cells; (a) GFP, (b) DNA stained with DAPI, (c) merge of (a) and (b). (**B**) Subcellular distribution of non-fusion GFP as a control. Scale bars represent 5 μm.

alone was expressed from the actin15 promoter, the entire cell was stained (Figure 4B) and GFP coupled to a nuclear localization signal resulted in a diffuse staining of the entire nucleus (35). This argued against a localization artefact of HelF-GFP and we thus concluded that endogenous HelF localized to the nucleus.

HelF knock-outs display increased silencing by RNAi

HelF-KO strains were then transformed in parallel to wild-type strains with RNAi hairpin constructs directed against the endogenous genes discoidin, coronin, thioredoxin and the spore coat protein gene sp96. The inverted repeat sequences were transcribed from the actin6 promoter and constructs were integrated in multi-copy tandem arrays into the genome (19). Gene silencing was monitored by colony blots and western blots for coronin and discoidin and by northern blots for thioredoxin and sp96. Supplementary Figure S1 provides representative examples of the results. The data from at least two independent transformations are summarized in Figure 5.

For discoidin, RNAi mediated gene silencing was increased from 47% of the examined clonal isolates in the wild-type background to almost 100% in the HelF⁻ background when cells were grown in suspension culture. When cells were grown on a lawn of *K.aerogenes*, RNAi mediated silencing was usually not observed when clones were processed for colony blots (11). However, in the HelF-KO background, a significant number of clones displayed no detectable discoidin expression (Supplementary Figure S1 and Figure 5). For coronin, RNAi mediated gene silencing was never observed in the wild-type background. In helF disruptions, ∼60% of the clonal isolates expressed no coronin (Supplementary Figure S1 and Figure 5).

For thioredoxin, complete silencing was found in 15% and partial silencing by 45% of RNAi transformants in the wild-type background, while 94% of the clones displayed complete silencing in the mutant strain (Supplementary Figure S1 and Figure 5).

The spore coat protein gene sp96 is expressed in late development around the time when the phenotype of the HelF-KO

strain was detectable. To address the question if the HelF knock-out had an influence on late gene expression and, more importantly, to see if the RNAi enhancing effect was also observed with late developmental genes, we transformed wild-type and HelF⁻ cells with a sp96-RNAi hairpin construct. RNA isolated from cells developed for 16 h on filters showed complete silencing in 9% and partial silencing in 47% of the transformed clones in wild-type background. In the mutant background, the number of completely silenced clones (77%) was increased to 8-fold (Supplementary Figure S1 and Figure 5).

Even though HelF is mostly nuclear and the RNAi machinery mostly cytoplasmic, it was possible that the enhancement of silencing in the mutant was due to an inhibition of Dicer activity by HelF in the wild-type strain. To test this, we performed *in vitro* Dicer assays (36) and found that the activity was not enhanced in the mutant (Figure 6). It was therefore unlikely that HelF directly interacted with Dicer and decreased its nucleolytic activity or processivity in wild-type cells.

Disruption of HelF does not enhance antisense RNA mediated gene silencing

Since antisense mediated gene silencing is mostly considered to employ the same pathway as RNAi, we were interested to see if a HelF disruption had a similar enhancing effect in antisense experiments. Discoidin, thioredoxin and coronin were used as target genes and wild-type as well as the HelF⁻ strain was transformed with antisense constructs directed by the actin15 promoter. As shown in Supplementary Figure S2 and Figure 7, about 50% of the discoidin antisense transformants were silenced in colony blots and western blots in the wild-type as well as in the HelF disruption strain. For coronin, minimal and partial silencing (<5%, n > 50) was observed in colony blots of either strain (Supplementary Figure S2 and Figure 7). Similarly, no silencing of the thioredoxin gene family could be found on the RNA level in both cell lines (Supplementary Figure S2 and Figure 7) even though efficient antisense transcription could be seen (Supplementary Figure S2). In contrast to RNA interference experiments, antisense mediated gene silencing also was not enhanced in colony blot assays for discoidin and coronin. The data provided further evidence that even though antisense and RNAi mediated gene silencing share common components, there are specific factors that influence one but not the other pathway (Figure 7).

Retroactive enhancement of silencing

In all model organisms, RNAi usually does not result in complete silencing in all cells or clonal isolates. For unknown reasons, the physiological status of a cell, the integration site of the construct or other parameters appear to influence the efficiency of the knock-down. Since HelF was considered to be a regulator of silencing efficiency, it was of interest to see if silencing could be retroactively improved by disrupting the helF gene in an inefficiently silenced strain.

Two non-silenced clones transformed with a discoidin RNAi vector were selected. One of them (Disci9/WT) showed partial silencing, the other one (Disci10/WT) appeared completely unaffected in discoidin expression.
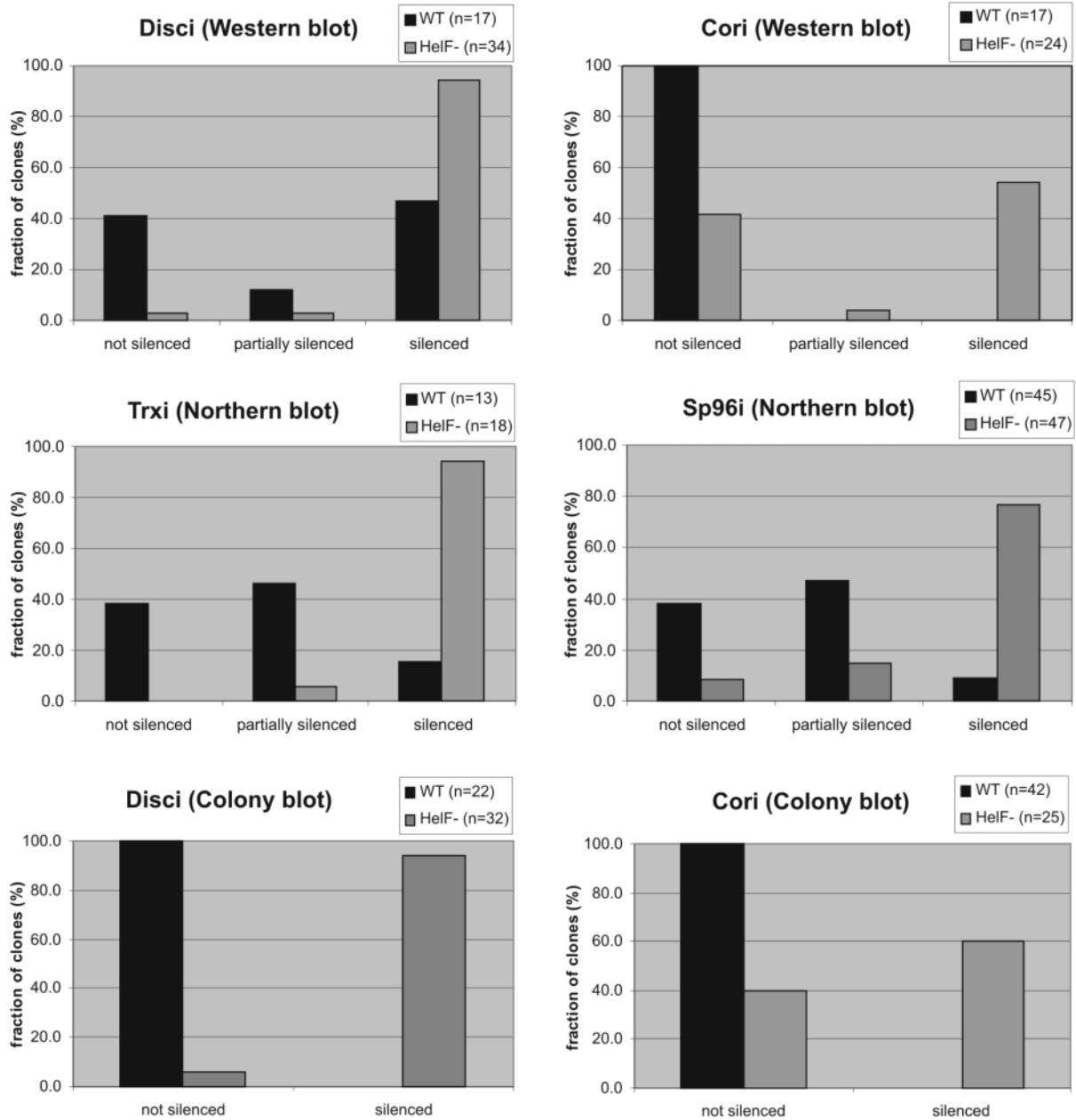
Figure 5. Silencing by RNAi. RNAi mediated silencing of discoidin (disci), coronin (cori), thioredoxin (trxi) and sp96 (sp96i) is summarized. Bars represent the fraction of clones (in %), showing different silencing efficiencies in wild-type and HelF⁻ background on the level of northern, western and colony blots. 'Silenced' was defined as 0 to 10%, 'partially silenced' as 10 to 50% and 'non-silenced' as more than 50% of the wild-type signal. For colony blots, partially silenced clones were not defined. n = number of clones examined.

Both clones had been propagated for several weeks and in multiple western blots expression levels were stable. Cells were supertransformed with the helF disruption construct and, after selection for the disruption vector, subcloned on a bacterial lawn. Colony blots (data not shown) showed complete silencing in 20 randomly selected colonies for Disci9 (Figure 8A). Ten of these were examined by PCR for successful disruption of the helF gene and all proved to have the vector integrated into the target gene (Figure 8B). Western blots with an anti-discoidin antibody were performed on all 20 clones (Table 1), four representative examples are shown in Figure 8A. For strain Disci10, 12 out of 24 colonies

displayed silencing in colony blots and were confirmed in western blots (7 completely, 5 partially silenced, see Table 1). Subsequent analysis of helF disruption showed that all silenced clones had the HelF gene disrupted. One clone had a gene disruption but did not show improved silencing. Though helF disruption did not result in 100% silencing in all clones it still improved RNAi efficiency significantly.

The data suggested that the Disci9 and Disci10 strains contained dsRNA that was either expressed below a required threshold level or had structural features that did not allow for efficient silencing. With the disruption of helF, the
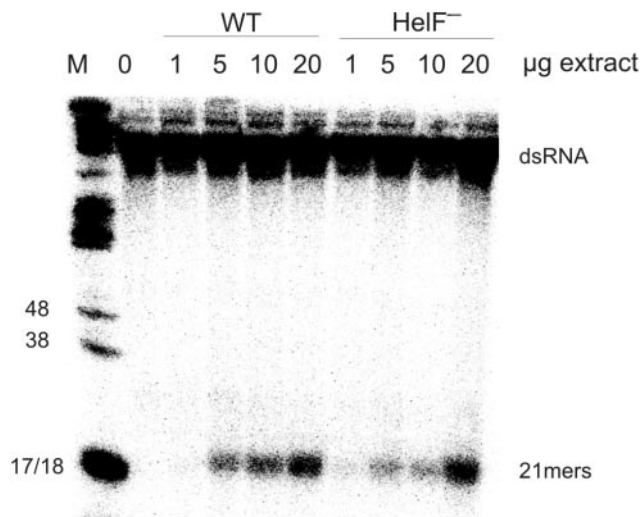
Figure 6. Dicer activity is not enhanced in the helF knock-out strain. Dicer activity in cell free extracts of wild-type and helF knock-out strains was examined as described previously (36). A 600 bp dsRNA generated from *in vitro* transcripts of the discoidin gene was used as a substrate and increasing amounts of extract (1, 5, 10 and 20 μg of protein as determined by Bradford assay) were added. Incubation was for 60 min at room temperature. The dsRNA substrate and the 21mer products are indicated. The marker represents end-labelled DNA fragments (pGEM-3Z digested with Sau3A).

requirements for silencing were less stringent and the sub-optimal dsRNA was sufficient for PTGS.

HelF enhanced gene silencing is post-transcriptional

As shown in Figure 9A, Disci9 and Disci10 in wild-type (/WT) and mutant (/helF⁻) background displayed different levels of steady-state mRNA in northern blots. Since the two silenced clones Disci9/HelF⁻ and Disci10/HelF⁻ originated from their partially silenced parent Disci9/WT and non-silenced parent Disci10/WT, respectively, they both had the same integration site and copy number of the RNAi construct. Therefore a direct comparison of the two strains was possible.

In order to confirm that the enhancement of silencing by HelF disruption was a post-transcriptional event, we performed run-on assays. Actin15 was used as an internal control to determine the relative amount of nascent discoidin transcripts; GFP sense transcripts served as a negative control (see Supplementary Figure S3).

Transcription levels were measured in nuclei from wild-type cells and Disci clones, showing different levels of silencing. Disci1/WT and Disci2/WT showed complete silencing, Disci9/WT was partially silenced and Disci10/WT did not show any silencing at all (see above). Clones Disci9/HelF- and Disci10 /HelF⁻ came from the retrosilencing experiment described above and were completely silenced.
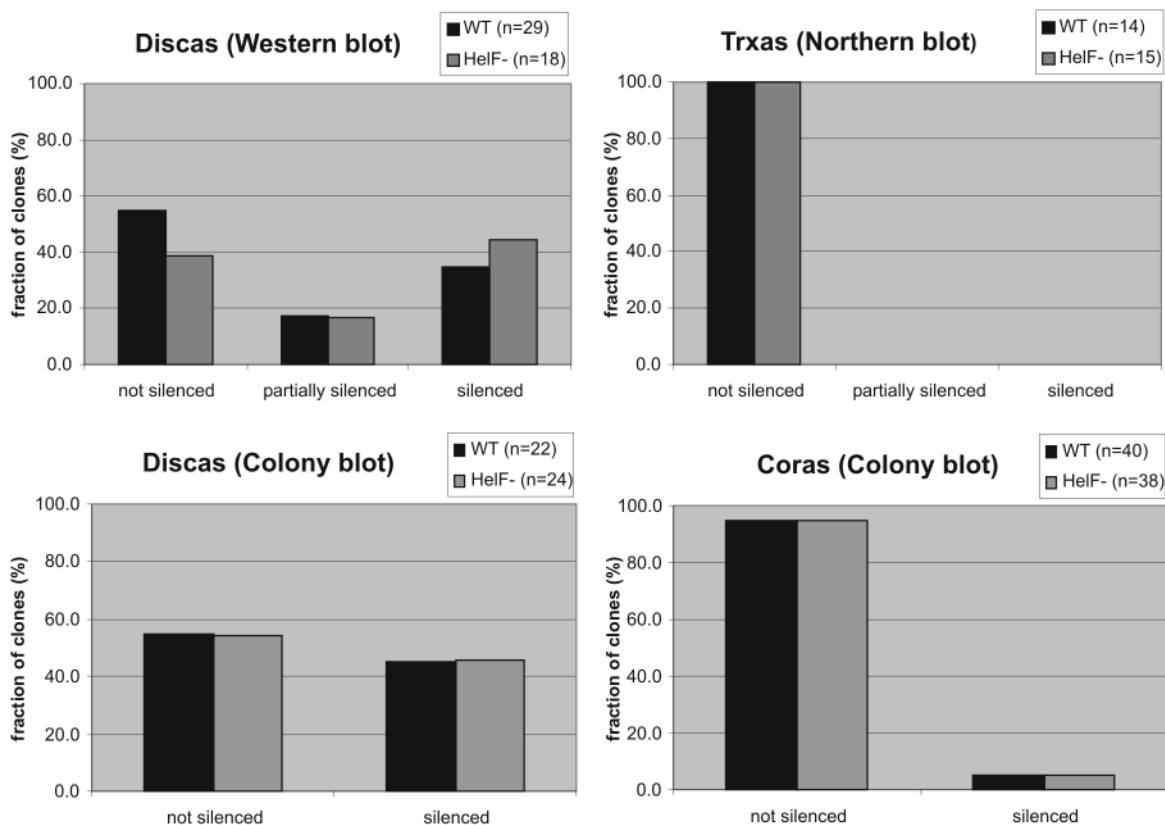

Figure 7. Silencing by asRNA. Antisense RNA mediated silencing of discoidin (discas), coronin (coras) and thioredoxin (trxas) is summarized. Bars represent the fraction of clones (in %), showing different silencing efficiencies in wild-type and HelF⁻ background on the level of northern, western and colony blots. 'Silenced' was defined as 0 to 10%, 'partially silenced' as 10 to 50% and 'non-silenced' as more than 50% of the wild-type signal. For colony blots, partially silenced clones were not defined. *n* = number of clones examined.
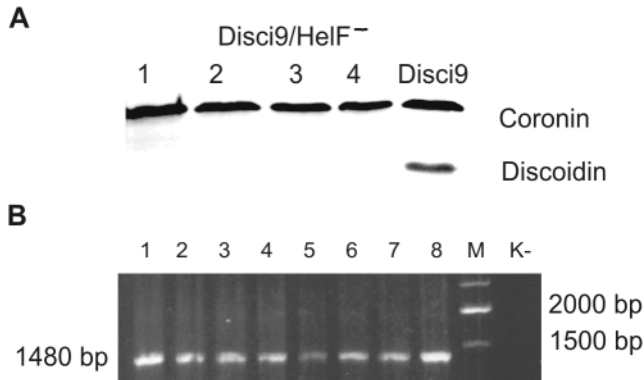
Figure 8. Retroactive silencing. A partially silenced clone (Disci9), transformed with the discoidin RNAi vector was supertransformed with the helF KO construct. After selection on blasticidin, the transformants (Disci9/HelF⁻) were assayed for discoidin expression on western blots (**A**). Expression in the Disci9 parent clone is shown as a control. Disruption of the helF gene was examined by PCR (**B**), the band of 1480 bp is indicative for a successful disruption. K- indicates the PCR control without template.

Table 1. Retroactive silencing

Strain	No. of clones tested	No. of clones silenced	HelF disrupted	Silenced and disrupted	Silenced and undisrupted	Non-silenced and disrupted
Disci9	20	20	20	20	0	0
Disci10	24	12	13	12	0	1

Total number of clones and their genotypes examined for retroactive silencing.

Relative transcription levels of discoidin were determined by calculating the ratio of discoidin and actin transcripts hybridized to the blot. A full length *in vitro* antisense transcript of discoidin that recognized both the mRNA and the hairpin transcripts was used as a target on the filter (Supplementary Figure S3). This was done in order to obtain comparable signals with the same specific radioactivity for both transcripts. Probing on antisense was also done for a selected set of clones to prove the integrity of the hairpin in run-on's.

Different levels of hairpin expression were detected in individual clones (Figure 9B and Supplementary Figure S3). These levels correlated to the level of discoidin silencing in the wild-type background. In two independent clones, that were completely silenced (Disci1 and Disci2), the level of hairpin expression was ∼16-fold higher than the level of endogenous discoidin mRNA. A partially silenced clone (Disci9) revealed an intermediate level of hairpin expression (∼7-fold higher) and a non-silenced clone (Disci10) showed a low, only 2-fold higher level of hairpin expression than that of mRNA. Thus, low expression levels of hairpin constructs could not initiate silencing in the wild-type.

Both pairs, Disci10/WT and Disci10/HelF⁻ as well as Disci9/WT and Disci9/HelF⁻, displayed the same relative levels of run-on transcripts thus demonstrating that transcription was not impaired by disruption of helF and that silencing was truly on the post-transcriptional level.

Most importantly, northern blots on small RNAs further supported the notion that gene silencing was post-transcriptional: only in efficiently knocked-down strains but not in the wild-type or in non-silenced strains, discoidin specific siRNAs could be detected. Surprisingly, the quantity of siRNAs was the same in all silenced strains and did not depend on the expression level of hairpins (Figure 9C).

Hairpin transcripts are completely degraded

Since the levels of siRNA did not correlate with the amounts of hairpin run-on transcripts, we examined the accumulation of dsRNA in northern blots. RNA from clones displaying different expression levels was hybridized with a discoidin sense probe (Supplementary Figure S4) in order to specifically detect the hairpin transcript. Surprisingly, no dsRNA was detected at all, even in strains that synthesized 16-fold more hairpin than the endogenous discoidin mRNA. This confirmed the northern blot results obtained with an antisense probe that also did not detect the hairpin transcript (data not shown). We conclude that most of the original dsRNA was degraded but not diced to siRNAs.

DISCUSSION

HelF is a new component involved in the RNAi pathway. Similar to the putative RNA directed RNA polymerase rrf-3 (14) and eri-1 (15) in *C.elegans*, HelF is a negative regulator and thus implies a control of endogenous RNAi mechanisms. Functional components of the RNAi machinery like Dicer and RISC have been isolated from the cytoplasm (36–38). Related nuclear proteins like DCL4 (39,40), Drosha (41), the RITS complex (42) and possibly some RdRPs are apparently not involved in post-transcriptional gene silencing but rather in processing of miRNAs, transacting siRNAs and in RNA mediated DNA methylation. Unexpectedly, HelF-GFP is mostly nuclear and accumulates in distinct speckles. We propose that HelF plays a regulatory role in very early steps where a cell has to distinguish between genuine transcripts that may have fortuitous double stranded fold-back regions (43) and truly aberrant RNAs that should induce the RNAi pathway.

Our experiments on retroactive silencing support the notion that recognition of RNA as a target for RNA interference is important. To date it is not known, why RNA interference fails in some cases. In *C.elegans*, specific cell types (mostly neuronal cells) are refractive to RNAi (44), probably due to different biochemical properties like e.g. high expression of eri-1 (15). However, in *Dictyostelium* vegetative cells are of a single type. Differences in silencing efficiency should therefore be due to intrinsic features of transcripts from the RNAi constructs. These may either be synthesized in insufficient amounts, not provide good Dicer substrates or they may not be exported from the nucleus. The two non-silenced strains Disci9 and Disci10 apparently expressed potentially functional dsRNA but this was not recognized as such in the presence of HelF.

Run-on experiments with these retroactively silenced strains clearly showed that HelF acted post-transcriptionally. Silenced and non-silenced cells displayed the same transcription of the target gene but only in the helF disruption strain the levels of steady-state mRNA were dramatically reduced.

Run-on experiments showed that silencing efficiency depended on transcription levels of the dsRNA trigger. Apparently, dsRNA had to be transcribed at higher levels

as the endogenous mRNA and that a threshold level had to be exceeded to achieve silencing. In addition, we observed partial silencing at intermediate dsRNA transcription levels. In contrast, HelF⁻ cells displayed efficient silencing below the threshold that was required in wild-type cells.

The hairpin transcripts appeared to be efficiently degraded but surprisingly, siRNAs did not reflect the quantity of dsRNA transcripts and could therefore not represent primary siRNAs. Furthermore, siRNAs were only detected when the mRNA was

efficiently degraded. Consequently, the small RNAs seen on the northern blot have to be secondary siRNAs derived from dicing of RdRP dependent conversion of the mRNA to a double stranded RNA. This is in agreement with the previous report that RNAi in *Dictyostelium* depends on RdRP activity and that the vast majority of detected Dicer products is derived from the target (11). This can also explain the low and equal amounts of siRNAs in all strains that may correspond to the amount of mRNA. A helF disruption would thus not be expected to increase the amount of siRNAs in comparison to silenced wild-type cells.

In *C.elegans*, the HelF related drh-1 gene is required for RNAi and a loss-of-function mutant (17) impairs gene silencing. The authors imply a function of drh-1 in the Dicer complex since the protein is associated with rde-1 (argonaute), dcr-1 (Dicer) and rde-4 (dsRNA binding protein).

In contrast, HelF acts as a suppressor of RNAi in wild-type cells and a gene disruption enhances silencing. If HelF acted by directly binding to Dicer, an increased Dicer activity would be expected in the helF knock-out strain. This was clearly not the case and also unlikely due to the different localization of both proteins in the cell.

We assume that HelF may act as a surveillance system for intramolecular dsRNA formation of partially complementary RNAs. If HelF functions as a helicase it could unwind short range double strands with low efficiency. Only when complementary regions are longer and/or dsRNA levels rise beyond a certain threshold, the capacity of HelF could be exhausted and RNAi mediated silencing would be initiated. A knock-out of HelF would abolish the surveillance system and thus improve RNA interference by transgene expressed dsRNA. HelF could also be involved in nuclear miRNA processing. It was intriguing that the morphological phenotype was only detectable in later stages of development. This may provide a first indication for developmental control by RNAi or miRNA pathways in *Dictyostelium*.

Antisense RNA most likely does not act via direct hybrid formation with the corresponding mRNA but rather by providing a target for an RdRP that synthesizes the complementary strand (11,45). This assumption was supported by the observation that in some cases of inefficient silencing,
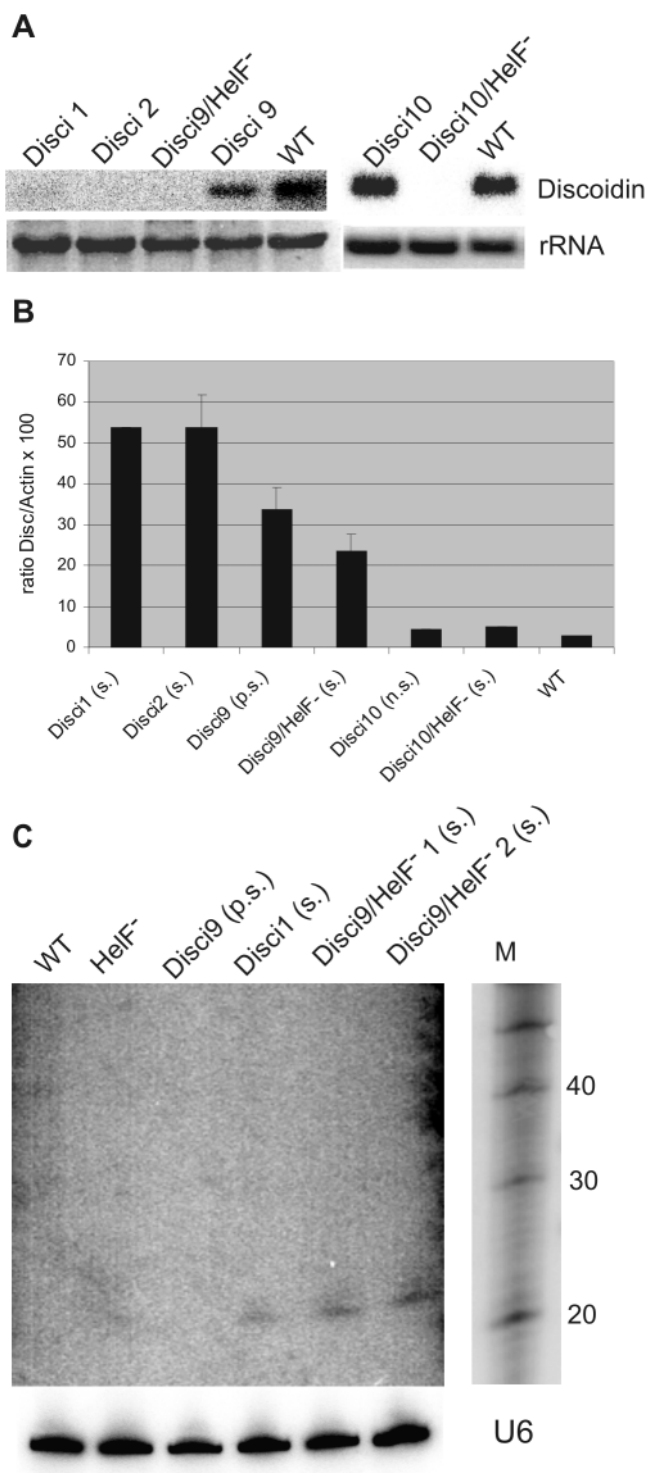
Figure 9. Transcriptional analysis of discoidin. (**A**) Northern blot analysis of discoidin steady-state expression in silenced (Disci1, Disci2, Disci9/HelF⁻ and Disci10/HelF⁻), partially silenced (Disci9) and non-silenced (Disci10) clones, used for run-on assays. Ethidium bromide staining of the large rRNA was used as a loading control. (**B**) Nuclear run-on assays. The relative ratios of discoidin/actin transcription levels (×100) were calculated for each individual filter, hybridized to labelled run-on transcripts from the different clones. The filters were stripped and re-hybridized to wild-type run-on transcripts. The relative ratio of discoidin/actin15 was subtracted from the overall level of run-on transcripts in the mutant strains. The difference comprised the expression level of the discoidin hairpin construct. This calculation was required since the discoidin *in vitro* transcripts, applied on the filter, hybridized to both endogenous and hairpin run-on transcripts. Relative transcription levels of discoidin in wild-type cells (WT) are shown for a comparison. Silencing levels are indicated (s = silenced, p.s. = partially silenced, n.s. = non- silenced). (**C**) Northern blot analysis for discoidin siRNAs. Detection of siRNAs in silenced (s), partially silenced (p.s.) and non-silenced (n.s.) clones carrying the discoidin RNAi construct in the wild-type or HelF⁻ background. M: RNA decade marker (Ambion), shown after short exposure for better resolution. U6 spliceosomal RNA (A. Hinas, P. Larsson, L. Avesson, L.A. Kirsebom, A. Virtanen, F. Söderbom, manuscript submitted) is shown as a loading control.

sense and antisense RNAs can both be detected as full length transcripts in the same cell (46). These have obviously not hybridized since a double strand should be immediately diced. In agreement with the putative functions of HelF discussed above, antisense mediated gene silencing would not be affected by a helF gene disruption if the helicase interfered with intramolecular dsRNA formation but not with the synthesis of dsRNA by an RdRP. Furthermore, antisense RNA acts in the cytoplasm while HelF is at least predominantly nuclear.

The putative target genes that may be regulated or influenced by HelF are still elusive but their definition may cast a new light on the mechanisms to control gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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