

The use of RNAi to analyze gene function in spirotrichous ciliates

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The use of small double-stranded RNA to inhibit expression of genes has proven to be a powerful tool to analyze the biological function of genes in various eukaryotic organisms. We have established the RNAi technology in three genera of spirotrichous ciliates, *Euplotes*, *Oxytricha* and *Stylonychia*. Here we describe this method to silence gene expression in these three genera. Expression of the following genes was inhibited: the catalytic subunit of telomerase (TERT) and the telomerase-associated protein p43 in *Euplotes* and the α -subunit of the telomere-binding protein (α TeBP) of *Stylonychia* and *Oxytricha*. In addition, we report how the biological function of a developmentally regulated gene can be elucidated by this technique in *Stylonychia*.

Key words: Ciliate; MDP1; p43; PIWI; RNAi; Telomerase; Telomere-binding protein.

Introduction

The inhibition of gene expression by RNA interference (RNAi) has been reported for several eukaryotic organisms, from *Saccharomyces pombe* to humans (Fire et al. 1998; Raponi and Arndt 2003; Shi 2003), including the ciliate *Paramecium tetraurelia* (Galvani and Sperling 2002) but so far not in *Saccharomyces cerevisiae*. Double-stranded RNA becomes processed by Dicer, a member of the RNaseIII family of nucleases and part of the endogenous RISC complex (RNA induced silencing complex), into small 21–26 bp RNA fragments. These small RNA fragments pair with homologous regions of the mRNA and as a result of this interaction these regions become also degraded by the RISC complex into 21–26 bp fragments (for review: Hannon (2002)). In addition, small double-stranded RNAs (dsRNAs) seem to target regulato-

ry proteins including enzymes that modify histones at homologous chromosomal loci eventually leading to heterochromatization of these loci (Allshire 2002; Volpe et al. 2002).

Spirotrichous ciliates have proven to be fascinating model systems to study basic biological processes. The organization of macronuclear DNA in small gene-sized DNA-molecules allowed a detailed analysis of telomere structure, function and replication (Jonsson and Lipps 2002; Möllenbeck et al. 2003). Moreover, the macronucleus represents a unique system to study the contribution of specific sequences and associated proteins to global nuclear architecture (Postberg et al. 2001; Jonsson et al. 2002). During macronuclear differentiation extensive DNA rearrangement and elimination processes take place allowing the molecular basis of programmed DNA rearrangements in eukaryotic organisms to be analyzed (Prescott 1994;

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Prescott 2000). Finally, these differentiation processes are accompanied by morphogenetic alterations only rarely observed to the same extent in other differentiating eukaryotic cells (Frankel 1992; Kloetzel et al. 2003). The molecular analysis of all these processes in spirotrichs was greatly limited by the lack of genetic mutants, the lack of efficient transfection systems and unsolved problems in creating knock-out mutants.

In this paper we describe the methodology used for gene silencing by RNAi in three species of spirotrichous ciliates. As examples we show how this technique can be used to identify the biological function of elements involved in replication of telomeres and of genes differentially expressed during macronuclear differentiation in spirotrichs.

Material and methods

Euplotes aediculatus, *Oxytricha nova* and *Stylonychia lemnae* were grown in Pringsheim medium and fed with the algae *Chlorogonium elongatum* as described by Ammermann et al. (1974). During the process of RNAi induction cells were adapted to *E. coli* as a food source (see Results). Conjugation of *Stylonychia* was achieved by mixing two different mating types. Products used for the inhibition of specific genes were obtained by PCR reactions using macronuclear DNA of the various species as a template, these varied in size between 200 bp and 700 bp (inserts: p43: 230 bp; TERT: 570 bp; α TeBP: 600 bp; MDP1: 460 bp out of the coding regions). The PCR products were first cloned into vector pGEM[®]-T Easy (Promega) and subsequently subcloned into vector L4440 and transfected into the RNase III deficient *E. coli* strain HT115 (F⁻, mcrA, mcrB, IN (rrnD-rrnE)1, lambda⁻, rnc14::Tn10 (DE3 lysogen: lacUV5 promoter-T7 polymerase)). A 1:100 dilution of an overnight bacterial culture was grown to an OD₆₀₀ of 0.4. Expression of double-stranded RNA was induced by the addition of 0.4 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and bacteria were grown for an additional 4 hours. Before feeding the bacteria to ciliates they were washed at least twice with Pringsheim medium and resuspended in Pringsheim medium to an OD₆₀₀ = 4.5 (see Results). DAPI-staining as well as *in situ* localization of p43, telomerase and the α -subunit of the telomere-binding protein were done as described earlier (Postberg et al. 2001; Jonsson et al. 2002; Möllenbeck et al. 2003). Macronuclear DNA was labelled by the addition of 1 mM BrdU (5-bromo-2-deoxyuridine). For labelling the old vegetative macronucleus BrdU was added one day prior to conjugation and after washing the cells several times in Pringsheim medium conjugation was induced. For labelling the newly developed

macronucleus BrdU was added during macronuclear differentiation after the breakdown of the polytene chromosomes.

Results and discussion

Inhibition of gene expression in *Euplotes aediculatus*

Euplotes is normally fed with algae but grows almost as well, with only slightly reduced growth rate, with *E. coli* as a food source. Therefore no adaption to the new food source is required in this species. Ciliates were fed daily with freshly cultivated induced bacteria but a similar effect was observed when induced bacteria frozen at -20 °C in 2% glycerol were fed. 100 μ l of bacteria (see Material and methods) were added to 5 ml of *Euplotes* culture but to prevent overfeeding due to variations in the number of *Euplotes* cells the feeding regimen was monitored visually. In each case the bacteria have to be taken up completely overnight and if necessary the amount of bacteria has to be adapted since overgrowth of ciliates with bacteria leads to the death of the cells. As a control cells were fed with induced bacteria containing the vector without insert or with induced bacteria without a vector.

Expression of the catalytic subunit of telomerase (TERT, Lingner et al. (1997)) and the telomerase-associated protein p43 (Aigner et al. 2000; Aigner et al. 2003) was inhibited in vegetative cells of *Euplotes aediculatus*. To study the degree of gene silencing by RNAi and its effect on the cells macronuclei were either stained with DAPI after several time intervals of feeding with dsRNA expressing cells, stained with an antibody against p43 or hybridized with a telomerase specific gene probe (Möllenbeck et al. 2003). While in control cells replication bands are visible over the whole period of investigation (Fig. 1a-c), in p43 or telomerase inhibited cells this structure could only be observed up to 3 days of feeding (Fig. 1d). Cells stop dividing after about 3 days of feeding p43 or TERT constructs as implicated by the failure to detect replication bands in these cells. This cannot be due to overfeeding as feeding was monitored and no change in overall morphology and motor behaviour could be seen. Furthermore, control cells fed with induced bacteria containing the vector without insert showed a normal growth rate. After 4-5 days we observed that macronuclei show ab-

normal morphology (Fig. 1d–i), become less well stainable with DAPI and eventually after 2 to 3 weeks these cells die. As shown by *in situ* antibody staining (Fig. 2a, b), FISH analysis (Fig. 2c, d) or Western analysis (Möllenbeck et al. 2003) the level of gene expression can be estimated to be reduced by at least 80%. Analysis of the behaviour of telomeres, telomerase and p43 after silencing these two genes revealed that the telomerase-associated protein p43 is involved in anchoring the telomerase in the replication band. This was the first *in vivo* demonstration of the biological function of a telomerase-associated protein outside yeast (Möllenbeck et al. 2003).

Inhibition of gene expression in vegetative cells of *Stylonychia* and *Oxytricha*

Unlike *Euplotes*, *Stylonychia* and *Oxytricha* have to be adapted to *E. coli* as a food source and feeding living bacteria or overfeeding can easily lead to fungal infection and death of the cells. The following feeding protocol proved to be the most successful leading to unlimited growth of these two species: Transformed bacteria were induced by IPTG, collected 4 hours after induction, and aliquots frozen at -20°C in 2% glycerol. To avoid overgrowth with bacteria and eventual fungal infection, bacteria were washed twice with Pringsheim medium and killed by heating for 5 min. at 65°C . Ciliates were adapted to *E. coli* as food source over a period of 4 days by feeding a mixture of algae ($\text{OD}_{600} = 1$) : bacteria ($\text{OD}_{600} = 4.5$) at a ratio of 4:1 the first day, a ratio of 2:1 the second, 4:3 the third and 1:1 at the fourth and all following days. Even after 14 days of feeding the control cells show no deviations with regard to division rate or motility.

The two subunits of the telomere-binding protein were inhibited by RNAi and led to similar results in both species. As an example inhibition of the α -subunit of the telomere-binding protein in *Stylonychia* is shown in Figure 3. As demonstrated by *in situ* antibody staining (Fig. 3a, c) and Western analysis (data not shown) the level of gene expression is reduced significantly and leads after several days to typical changes in the morphology of macronuclei (Fig. 3b, d) probably due to the breakdown of global nuclear structure. Later, macronuclei become fragmented and eventually resorbed leading to the death of the cells after about 2 to 3 weeks.

Inhibition of gene expression during macronuclear differentiation in *Stylonychia lemnae*

We have recently constructed a subtraction cDNA library of genes expressed during macronuclear development in *Stylonychia lemnae* and characterized three of these genes from which one, MDP1 (Macronuclear Development Protein1), was a homolog of PIWI (Fetzer et al. 2002). To inhibit expression of this gene during macronuclear development cells of different mating types were fed for 4 days with bacteria expressing double-stranded RNA homologous to this gene. Conjugation was then induced and macronuclear differentiation analyzed by DAPI-staining. These cells enter the polytene stage but later stages of macronuclear development (DNA poor stage, macronucleus in the second amplification phase) were morphologically not detectable. In these exconjugants the old macronucleus becomes fragmented but not resorbed and about one week post conjugation typically four macronuclear fragments are visible (Fig. 4c). To analyze whether a new abnormal macronucleus is formed in these cells or whether the observed fragments correspond to the old macronucleus its DNA was tested for IES excision and fragmentation as described (Jonsson et al. 2001). PCR analyses revealed that no IESs were present in these macronuclei and that the DNA was fragmented correctly (not shown) suggesting that these macronuclear fragments correspond to the old macronucleus. To further confirm this assumption labelling experiments with BrdU were performed. BrdU was added prior to conjugation, since the old macronucleus becomes degraded during conjugation the new macronucleus should not be labelled as shown in Figure 4b. In contrast, the macronuclear fragments observed in MDP1 inhibited cells are still labelled with BrdU (Fig. 4d). When BrdU was added after breakdown of polytene chromosomes the new macronucleus of control cells was labelled (Fig. 4f) but no labelling was observed in MDP1 inhibited cells (Fig. 4h). The strong cytoplasmic background is due to variations in incorporation of BrdU and in Figure 4b possibly because of only partially degraded old Mac DNA. These results demonstrate that upon inhibition of MDP1 expression cells can enter macronuclear differentiation but the macronuclear anlage becomes resorbed and no new macronucleus is formed. Instead, the old macronucleus does not become resorbed in these

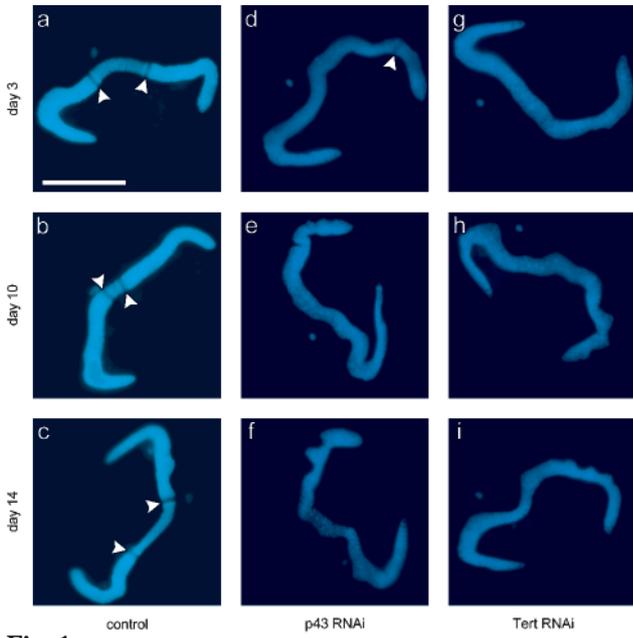


Fig. 1.

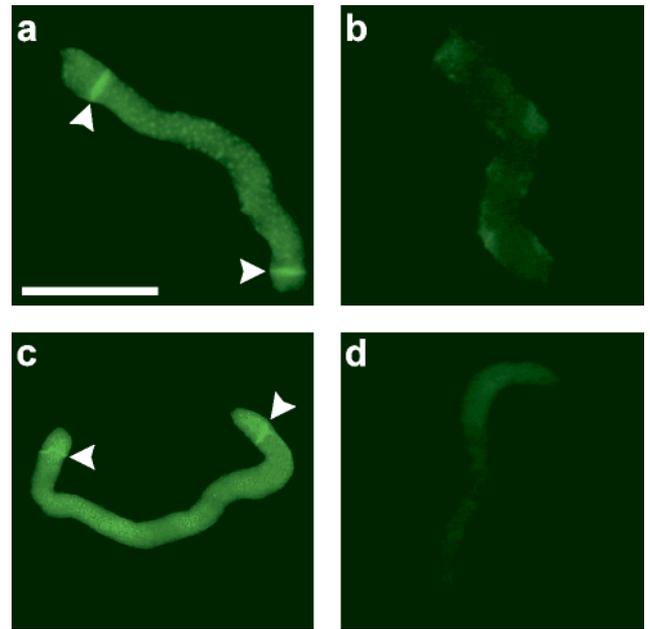


Fig. 2.

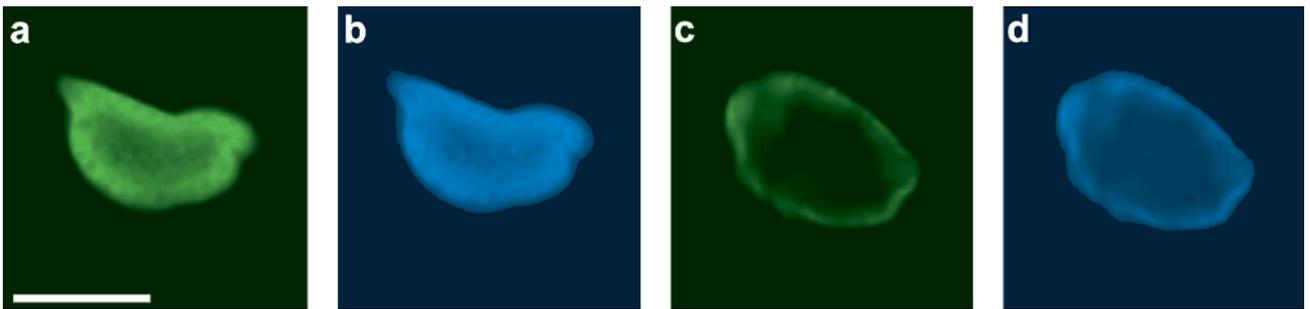


Fig. 3.

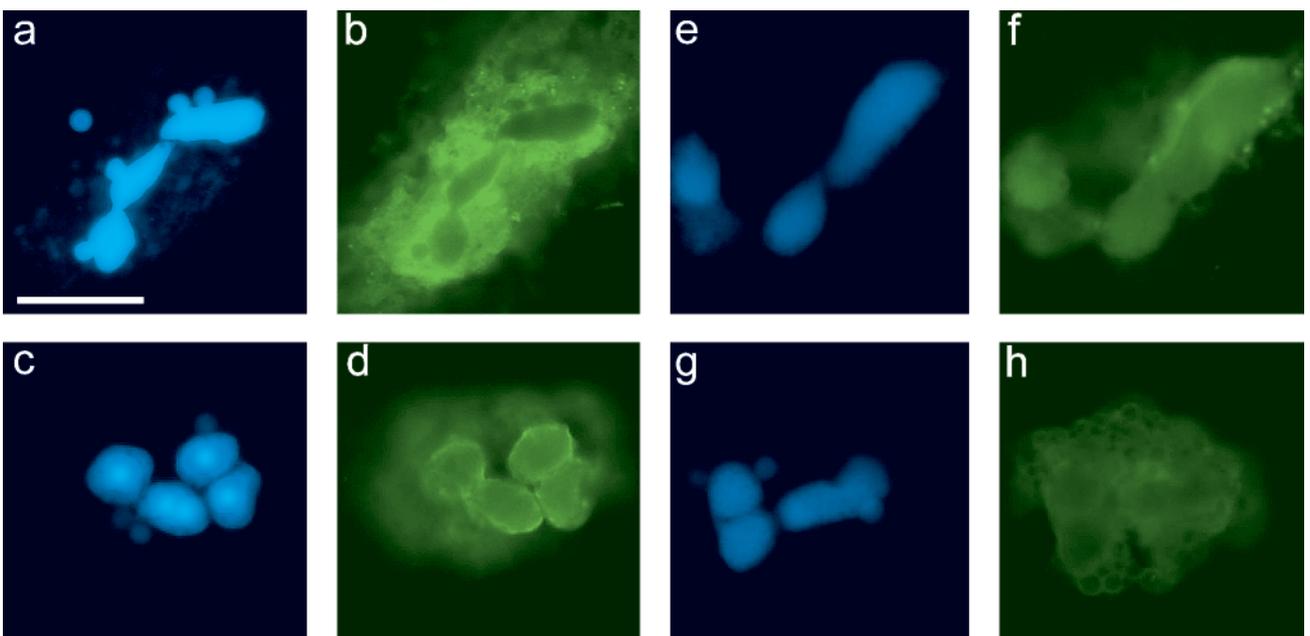


Fig. 4.

cells. Since MDP1 is homologous to PIWI, a well characterized part of the RISC complex (Hannon 2002) these results strongly suggest that the RNAi machinery is involved in macronuclear development of spirotrichous ciliates.

Conclusions

Molecular analysis of basic biological phenomena in spirotrichous ciliates has been hindered by the lack of genetically tractable systems. We report how expression of specific genes can be inhibited by RNAi in three species of spirotrichous ciliates. These experiments have already led to the identification of the biological function of gene products. However, it should be mentioned that probably the expression of some genes can not be inhibited using this technique. Optimal conditions for these experiments differ between the three species and have to be established for each gene and for each species individually.

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◀ **Fig. 1.** Morphology of *Euplotes* macronuclei before and after inhibiting either expression of p43 or the catalytic subunit of telomerase for different periods of time. Cells were fed daily with freshly cultivated induced bacteria. a–c. DAPI staining of macronuclei after feeding bacteria containing the vector L4440 without insert for 3 (a), for 10 (b) and for 14 (c) days. d–f. DAPI staining of macronuclei after silencing p43 expression by RNAi for 3 (d), for 10 (e) and for 14 (f) days. g–i. DAPI staining of macronuclei after silencing TERT expression by RNAi for 3 (g), for 10 (h) and for 14 (i) days. Arrowheads point to the replication band. Bar, 10 μ m.

Fig. 2. Inhibition of either p43 or the catalytic subunit of telomerase expression in *Euplotes* by RNAi by feeding for 14 days. Control cells were fed with induced bacteria without vector. a, b. *In situ* staining of macronuclei with an anti-p43 antibody before (a) and after (b) silencing p43 expression. c, d. FISH analysis of the RNA subunit of telomerase of macronuclei before (c) and after (d) inhibition of TERT expression. Arrowheads point to the replication band. Bar, 10 μ m.

Fig. 3. Inhibition of the expression of the α -subunit of the telomere-binding protein in *Stylonychia* by RNAi by feeding for 10 days. Control cells were fed with induced bacteria without vector. a. *In situ* staining of a macronucleus with an anti- α -telomere-binding protein antibody, b. same macronucleus as in (a) stained with DAPI. c. Macronucleus after inhibition of the α -subunit of the telomere-binding protein stained with an anti- α -telomere-binding protein antibody, d) same macronucleus as in (c) stained with DAPI. Bar, 10 μ m.

Fig. 4. Inhibition of MDP1 expression during macronuclear differentiation in *Stylonychia lemnae*. Control cells were fed with induced bacteria without a vector in a mixture with algae.

All figures show cells one week after conjugation.

a. Control cell stained with DAPI, b. same cell as in (a) labelled with BrdU one day before conjugation. c. DAPI staining of an exconjugant cell after inhibition of MDP1 for 4 days prior to conjugation, d. same cell as in (c) labelled with BrdU one day before conjugation. e. As in (a) control cell, f. same cell as in (e) labelled with BrdU during macronuclear differentiation after the breakdown of polytene chromosomes. g. As in (c) DAPI staining of an exconjugant cell after inhibition of MDP1 for 4 days prior to conjugation, h. same cell as in (g) labelled with BrdU during macronuclear differentiation after the breakdown of polytene chromosomes. BrdU labelling was detected by *in situ* staining with an anti BrdU antibody (Sigma). Bar, 10 μ m.

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