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Double-stranded RNA-mediated gene silencing in fission yeast

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ABSTRACT

Double-stranded RNA (dsRNA) can specifically inhibit gene expression in a variety of organisms by invoking post-transcriptional degradation of homologous mRNA. Here we show that dsRNA-mediated gene regulation also occurs in the fission yeast *Schizosaccharomyces pombe*. We present evidence that: (i) reporter gene silencing is significantly enhanced when additional non-coding sense RNA is co-expressed with antisense RNA; (ii) expression of a panhandle RNA also silences target gene expression; (iii) expression of dsRNA is associated with siRNAs; (iv) a novel host-encoded factor which enhances antisense RNA gene silencing also enhances panhandle RNA-mediated gene inhibition. Both the exogenously introduced *lacZ* and *c-myc* genes are shown to be susceptible to dsRNA-mediated gene silencing in this model. Taken together, these data indicate that RNA-mediated gene silencing can occur through a RNAi-like mechanism in fission yeast.

INTRODUCTION

The utility of double-stranded RNA (dsRNA)-mediated gene interference (known as RNAi in animals) (1) has been demonstrated in a variety of organisms, including *Caenorhabditis elegans* (2), plants (3), *Drosophila* (4), planaria (5), trypanosomes (6), *Hydra* (7), zebrafish (8,9) and, most recently, in mammalian tissue (10–12). Additionally, another form of post-transcriptional gene silencing (PTGS) in which extra copies of a target gene suppress both the endogenous and introduced transgene (co-suppression or quelling) has been observed in *Paramecium* (13), *Neurospora crassa* (14), plants (15), *Dictyostelium* (16) and mammalian cells (17). It has been suggested that in the different categories of RNA-mediated gene silencing, the culprit dsRNA is formed either by (i) the cryptic transcription of antisense RNA in cells where extra copies of the sense gene have been introduced, (ii) the simultaneous expression of antisense and sense sequences, (iii) the formation of RNA hairpins from inverted repeats or (iv) the direct introduction of dsRNA (18). The dsRNA is thought to act as a substrate for dsRNA-dependent RNA polymerase that generates a complementary RNA (cRNA) (19,20). This cRNA may target mRNA for degradation or hybridise to antisense RNA to generate additional dsRNA. It has been shown that the long dsRNA is fragmented into small 21–25 nt dsRNA species (21) and that this is the primary mediator of gene silencing (22). This fragmentation process is mediated by the RNase III-like nuclease DICER (23), however, it has recently been shown that its role may not be essential in all circumstances (24).

In the case of plants and worms it has been shown that RNAi acts in a sub-stoichiometric fashion and has the ability to migrate between cells (2,25). However, the potency of PTGS seems to vary between organisms. For example, dsRNA appears to have an amplification or catalytic component in most of the organisms investigated, but was shown to be less robust in the vertebrate zebrafish where gene suppression was dependent on the concentration of introduced dsRNA (8). This suggests that either the level of dsRNA has not reached the threshold which may be required for activation of a catalytic event or that some of the factors involved in robust forms of RNA-mediated gene silencing are absent and/or inhibitors of the gene silencing phenomenon are present in that organism.

The proteins that are essential for the RNAi pathway are present in *Schizosaccharomyces pombe*, suggesting that this mechanism is also required in this yeast. Indeed, it has recently been shown that this RNAi machinery is required for heterochromatic gene silencing in fission yeast (26) and that this is associated with small interfering RNAs (siRNA) homologous to these centromeric regions (27). In addition, it has been shown that the formation of heterochromatin at the silent mating type region (28) and accurate chromosomal segregation during mitosis requires the RNAi machinery. However, it has not yet been directly demonstrated that dsRNAs are involved in gene regulation in this model yeast system.

The *lacZ* fission yeast model has previously been employed to investigate features of antisense RNA technology *in vivo* (29). It has been shown that gene inhibition is dependent on the dose of antisense RNA while co-localisation of antisense

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and target genes does not affect the level of target gene suppression in this system (30). Additionally, the size of the antisense transcript (31,32) and the region to which it is targeted (33) can affect the efficacy of target gene inhibition. We further demonstrated that overexpression of host-encoded factors can enhance antisense RNA-mediated gene silencing in this system (34). Here we demonstrate that antisense RNA-mediated gene regulation is enhanced by expression of additional sense RNA. Furthermore, it is shown that expression of panhandle RNA also inhibits target gene suppression, suggesting that the generation of dsRNA through either intermolecular or intramolecular hybridisation is central to antisense RNA-mediated gene silencing in *S.pombe*. The usefulness of this model is extended by co-expressing the antisense enhancing sequence *aes2* to (i) further show that antisense RNA-mediated gene regulation is linked to dsRNA-mediated gene silencing and (ii) illustrate that concomitant expression of host-encoded factors can enhance different forms of RNA-mediated gene silencing. In addition, the presence of siRNAs is demonstrated in both antisense RNA- and panhandle RNA-expressing strains, indicating that both function through a common RNAi-like mechanism.

MATERIALS AND METHODS

Schizosaccharomyces pombe strain and plasmid construction

All yeast strains were maintained on standard YES or EMM medium (35). Yeast cells were transformed with plasmid DNA by electroporation (36). Repression of *nmt1* transcription was achieved by the addition of thiamine to EMM medium at a final concentration of 4 μM. The *ura4*-antisense plasmid was generated by subcloning the full-length *lacZ* fragment contained in pGT2 (31) into the plasmid pREP4 (37) in the antisense orientation. The construction of the long *lacZ* antisense and the frameshifted sense *lacZ* containing episomal plasmids have been described (31). This non-coding *lacZ* fragment was generated by introducing a frameshift mutation at the ClaI restriction site (31). The functional *lacZ* sequence was subcloned into the BamHI site of pREP4 to generate pM85-1. The 792 bp BglII *c-myc* antisense fragment (CM-17) (33) was subcloned into pREP4 to generate the sense *c-myc* vector, pN12-1. Construction of the *aes2*-encoding plasmid has been described elsewhere (34). Construction of the antisense *lacZ* integrating vectors and stable antisense *lacZ* strains has also been described (30). A sense *lacZ* version of the integrating vector was made by subcloning the full-length non-coding BamHI *lacZ* fragment (31) into the BamHI site of pRIP2/s in the sense orientation (37). This was transformed into the strain SP41 (*h+*, *ade6-704*, *ura4-D18*), and a single-copy integrant was isolated (M60-3). M60-3 was then mated with RB3-2 to introduce the target *lacZ* gene.

The *lacZ* panhandle integration vector, pM30-8, was generated by first introducing a NotI site into the XmaI site of pRIP1/s (37) using the self-complementary linker 5′-CCG GGC GGC CGC-3′ to generate pL121-14. The 2.5 kb sequence of the 5′ end of the non-coding *lacZ* gene (31) was then PCR amplified using the forward primer 5′-ATG CGG CCG CAA TTC CCG GGG ATC GAA AGA-3′ and the reverse primer 5′-ATG CGG CCG CAA TGC GGG TCG CTT CAC TTA-3′ to produce NotI ends. This product was then subcloned into the NotI site of pL121-14 in the antisense orientation. The full-length frameshifted *lacZ* fragment was then introduced into the BamHI site of this vector in the sense orientation upstream of the 2.5 kb antisense fragment to generate pM30-8. The episomal *lacZ* inverted repeat vector, pM53-1, was generated by subcloning 2.5 kb of the 5′ *lacZ* frameshifted gene in the antisense orientation downstream of the full-length *lacZ* frameshifted sequence in pREP1 (37). The frameshifted sequence was replaced with functional *lacZ* sequence to generate a panhandle capable of expressing β-galactosidase (pM81-2). The control vector pM91-1 was generated by removing the 2.5 kb NotI *lacZ* fragment from pM81-2 and reintroducing it in the sense orientation.

β-Galactosidase assays

The expression of the *lacZ* gene-encoded product, β-galactosidase, was quantitated using a cell permeabilisation protocol as previously described (30). A semi-quantitative overlay assay was also employed for the *in vivo lacZ* panhandle assay (33).

Plasmid segregation

Raw data was normalised to account for plasmid segregation as previously described (34). Briefly, by plating strains on both selective and non-selective media we have found that ~73% of the cell population contained *LEU2*-based plasmids while ~69% of the cell population contained both *ura4*- and *LEU2*-based plasmids. These values were used to normalise the raw data obtained from the quantitative β-galactosidase assays.

Detection of small *lacZ*-specific RNAs

Yeast total RNA was isolated using the glass bead method as previously described (31). This RNA was then separated on a 15% 8 M urea TBE gel and RNAs spanning 20–30 bases in size were excised. The recovered RNA was ethanol precipitated, loaded onto a 15% 8 M urea TBE gel and transferred to nylon membrane. In addition to the RNA samples, *lacZ*-specific DNA oligonucleotides spanning 22–33 bases were included to confirm size and sequence specificity of detected RNAs.

To prepare the *lacZ*-specific RNA probe for detecting small RNAs, a pGem3Zf-based plasmid containing the 3.5 kb *lacZ* gene was linearised with EcoRI and used as a template for *in vitro* transcription. The radiolabelled *lacZ* sense RNA was generated using SP6 RNA polymerase and the MAXIScript kit according to the manufacturer's instructions. The labelled transcripts were sheared to an average size of ~50 nt by adding 300 μl of an alkaline buffer (80 mM sodium bicarbonate and 120 mM sodium carbonate) to the 20 μl reaction and incubating at 60°C for 3 h (38). Pre-hybridisation and hybridisation were performed at 42°C using ExpressHyb solution according to the manufacturer's instructions. The membrane was then washed twice with 2× SSC, 0.2% SDS at 42°C and twice with 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 60 mM sodium chloride and 10 μg/ml RNase A at 37°C for 1 h to remove non-specific background.

RESULTS

Expression of additional sense RNA enhances antisense RNA-mediated target gene suppression

It has been demonstrated that co-expression of antisense RNA with additional sense RNA can enhance gene silencing (3). This is thought to be due to the potential formation of additional dsRNA that may induce an RNAi-like mechanism. To determine whether gene suppression in fission yeast is due to formation of an antisense RNA:target mRNA hybrid or an antisense RNA:sense RNA hybrid, a version of *lacZ* which is unable to be translated into functional β-galactosidase was co-expressed in strains expressing the target and antisense *lacZ* genes. If antisense RNA is required to hybridise to target mRNA for inhibition of the gene expression pathway, then overexpression of the sense RNA should compete with the target mRNA for the available antisense molecules, thereby producing a decrease in *lacZ* gene suppression.

Initially, the *nmt1*-driven antisense *lacZ* gene was integrated in single copy into a strain (RB3-2) containing the chromosomally expressed *adh1*-driven target *lacZ* gene. The *adh1* promoter is constitutively expressed while the *nmt1* promoter is conditional and only active in the absence of thiamine. A crippled version of the sense *lacZ* gene (driven by *nmt1*) was also integrated in single copy into a separate target strain and then crossed with the antisense and target-containing strain. Strains were then isolated containing (i) the antisense and target *lacZ* genes, (ii) the sense and target *lacZ* genes or (iii) the antisense, sense and target *lacZ* genes. In the strain containing the antisense gene alone, β-galactosidase activity was ~65% of the control strain, while there was no change in the strain expressing sense *lacZ* alone (Fig. 1A). Surprisingly, in the strain expressing both complementary transcripts, the level of gene silencing was not only maintained but moderately enhanced (Fig. 1A). To further increase the potential formation of intracellular dsRNA, an episomal sense *lacZ* plasmid was co-transformed with the episomal antisense *lacZ* plasmid into the target strain RB3-2. Significantly more stimulation of *lacZ* inhibition was observed in the presence of the episomally expressed complementary RNAs (Fig. 1B). When β-galactosidase activity was normalised for plasmid segregation (39) the strain expressing both complementary RNAs exhibited ~100% *lacZ* inhibition, compared with 68% silencing by antisense alone. Expression of the sense *lacZ* RNA had no effect on β-galactosidase activity. Northern analysis demonstrated that the total relative level of episomally expressed *nmt1*-driven *lacZ* RNA in the strain transformed with both sense and antisense genes was approximately equal to the sum of that seen when either plasmid was expressed alone (Fig. 1C). Additionally, there was a concomitant reduction in the steady-state level of target mRNA with a lower signal present in the strain containing both sense and antisense genes compared with the strain containing antisense alone (Fig. 1C). However, since dividing cells unequally segregate episomal plasmids, the total sample population will contain cells that contain *lacZ* mRNA but no antisense RNA or dsRNA specific for this target. As a result, a complete reduction in target mRNA was not seen. These results demonstrate that the presence of additional sense RNA does not titrate the antisense RNA from the target mRNA and therefore indicates that increasing the potential formation of dsRNA, but not necessarily an antisense RNA:target mRNA hybrid, is required for efficient interference of target gene expression in *S.pombe*. Further, this effect seems to be dose-dependent.

A *lacZ* panhandle RNA inhibits *lacZ* gene expression

To confirm that dsRNA is central to efficient antisense RNA-mediated gene silencing in fission yeast a vector was engineered containing the full-length 3.5 kb frameshifted *lacZ* sequence with a 2.5 kb inverted repeat (Fig. 2A). This construct generates a panhandle transcript of ~6.2 kb in length, with a 1 kb loop and 2.5 kb of self-complementarity which, predictably, will form a strong intramolecular RNA duplex. The panhandle construct was driven by the conditional *nmt1* promoter. This gene was initially integrated into a fission yeast strain in single copy and the target *lacZ* gene was then introduced through genetic crossing. These strains were then assayed for target gene silencing. A β-galactosidase assay showed no reduction in target enzyme activity when transcription of the inverted repeat was activated (data not shown). However, RNA analysis indicated that the 6.2 kb panhandle transcript was being generated in this strain (Fig. 2B). Initially, this indicated that the panhandle RNA did not inhibit target gene activity in this system. However, since we have shown that the expression of both antisense RNA and additional sense RNA has a dose-dependent effect on gene silencing, efforts were made here to elevate the steady-state level of the panhandle RNA. To this end, an episomal plasmid containing the *lacZ* panhandle cassette was made and introduced into the *lacZ* expressing strain. β-Galactosidase activity was reduced by ~60% in this strain, while addition of thiamine to the culture medium returned β-galactosidase activity to control levels (Fig. 2C), indicating that *lacZ* inhibition was dependent on expression of the panhandle gene. Northern analysis showed that the episomally based panhandle gene was expressed at a similar level to the antisense *lacZ* gene and was approximately 10-fold higher than the strain containing a single copy of the panhandle gene (Fig. 2B).

To investigate whether expression of the panhandle RNA was impacting on the cellular phenotype of these transformants, cultures were grown to mid-logarithmic phase and then viewed under the light microscope. It was observed that there was no difference in the growth rate of the panhandle-containing strain compared to control cells lacking this construct. Furthermore, there was no difference in the general morphology of the cells (data not shown). We also performed experiments to show that the panhandle *lacZ* was forming dsRNA *in vivo*. To this end we generated a panhandle construct containing a functional *lacZ* gene and a control construct with the 3′ inverted portion of panhandle as a direct repeat. Both were introduced into a yeast strain lacking the *lacZ* target gene. While the control strain showed high levels of β-galactosidase activity, virtually no activity was detectable in the panhandle-expressing strain (data not shown). This indicated that the *lacZ* sequence was unable to be translated, probably due to panhandle formation. Together, these data indicated that a construct capable of forming an intramolecular RNA duplex could inhibit target gene activity in a dose-dependent manner.

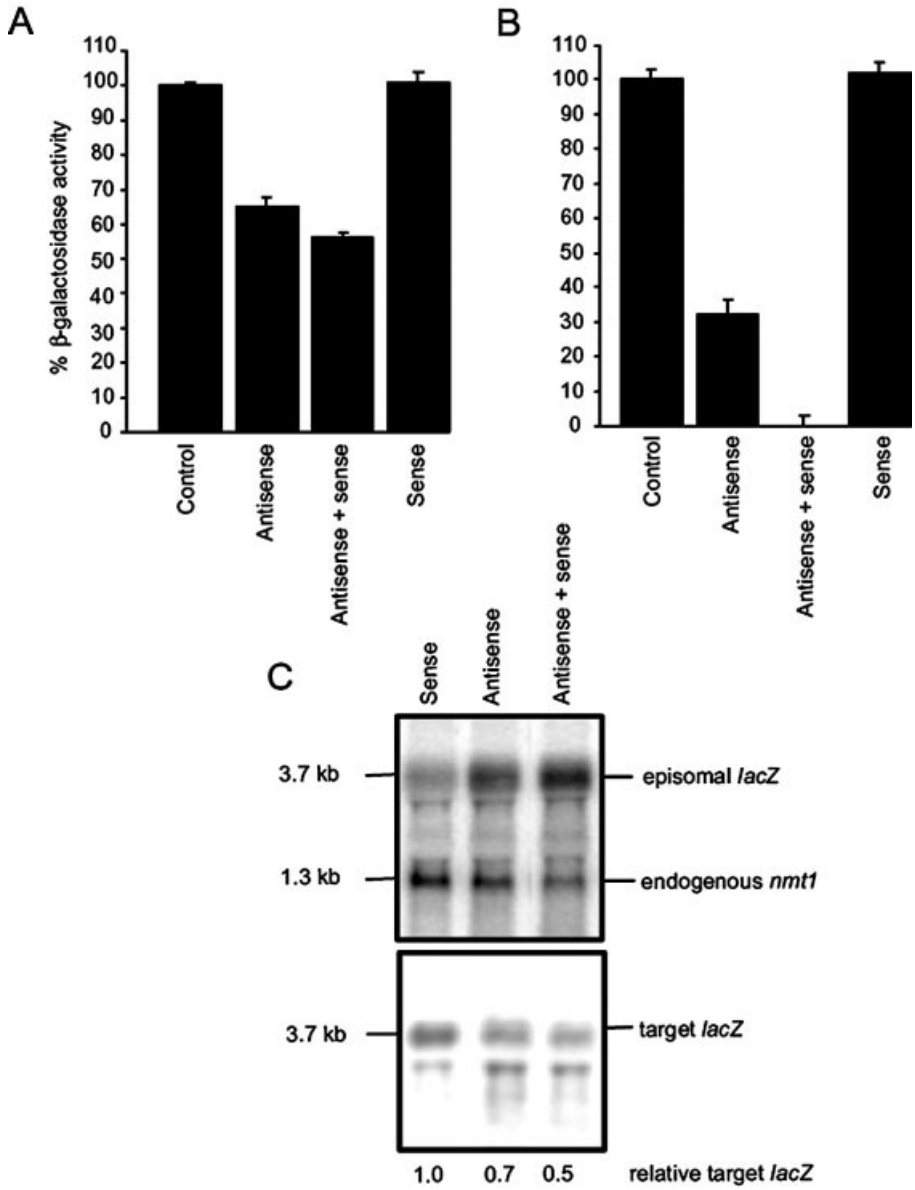

Figure 1. Effect of increasing target sense RNA in antisense RNA-expressing strains. (**A**) Strains containing single integrated copies of the target *lacZ* gene alone (control), the target and the antisense *lacZ* genes (antisense), the target and the sense *lacZ* genes (sense) and the target and both the antisense and sense *lacZ* genes (antisense + sense) were assayed for β-galactosidase activity in the absence of thiamine. (**B**) β-Galactosidase activity was determined in the strain RB3-2 transformed with the episomally expressed antisense *lacZ* plasmid (antisense), the episomally expressed sense *lacZ* plasmid (sense) or both antisense and sense *lacZ* plasmids (antisense + sense). For each strain three independent colonies were assayed in triplicate. (**C**) The 5′ and 3′ UTRs of the antisense and sense *lacZ* transcripts contain *nmt1* sequences. RNA from episomally transformed RB3-2 strains was therefore probed with the *nmt1* fragment to identify episomally expressed transgenes and the endogenous *nmt1* gene (top). Membranes were then stripped and re-probed with the *ura4* 3′ fragment to identify target mRNA (bottom). The relative levels of target and episomally expressed *lacZ* RNA were normalised to endogenous *nmt1* transcript and quantitated by phosphorimager analysis.

Co-expression of antisense and sense RNA enhances inhibition of a *c-myc* target

To test the ability of dsRNA to specifically interfere with other target sequences in fission yeast, complementary *c-myc* sequences were co-expressed in a strain containing an integrated *c-myc–lacZ* fusion cassette (Fig. 3A) (33). The target sequence is capable of generating β-galactosidase, and as the target sequence is fused to the 5′ end of the *lacZ* gene, gene silencing of the *c-myc* sequence will also result in down-regulation of *lacZ* expression. A 792 bp antisense *c-myc* fragment from exon 2 of the human *c-myc* gene (named CM-17) was previously found to suppress β-galactosidase activity within the *c-myc–lacZ* fusion target strain (AML1) by 47% (33). Both the sense and antisense *c-myc* genes were under control of the conditional *nmt1* promoters. The region of the *c-myc–lacZ* target to which this fragment is homologous is shown in Figure 3B. The CM-17 fragment was subcloned into the BamHI site of pREP4 in the sense orientation to generate pN12-1. The antisense *c-myc* vector (pCM-17) and the sense *c-myc* vector were then transformed into AML1 both independently and together. When β-galactosidase activity

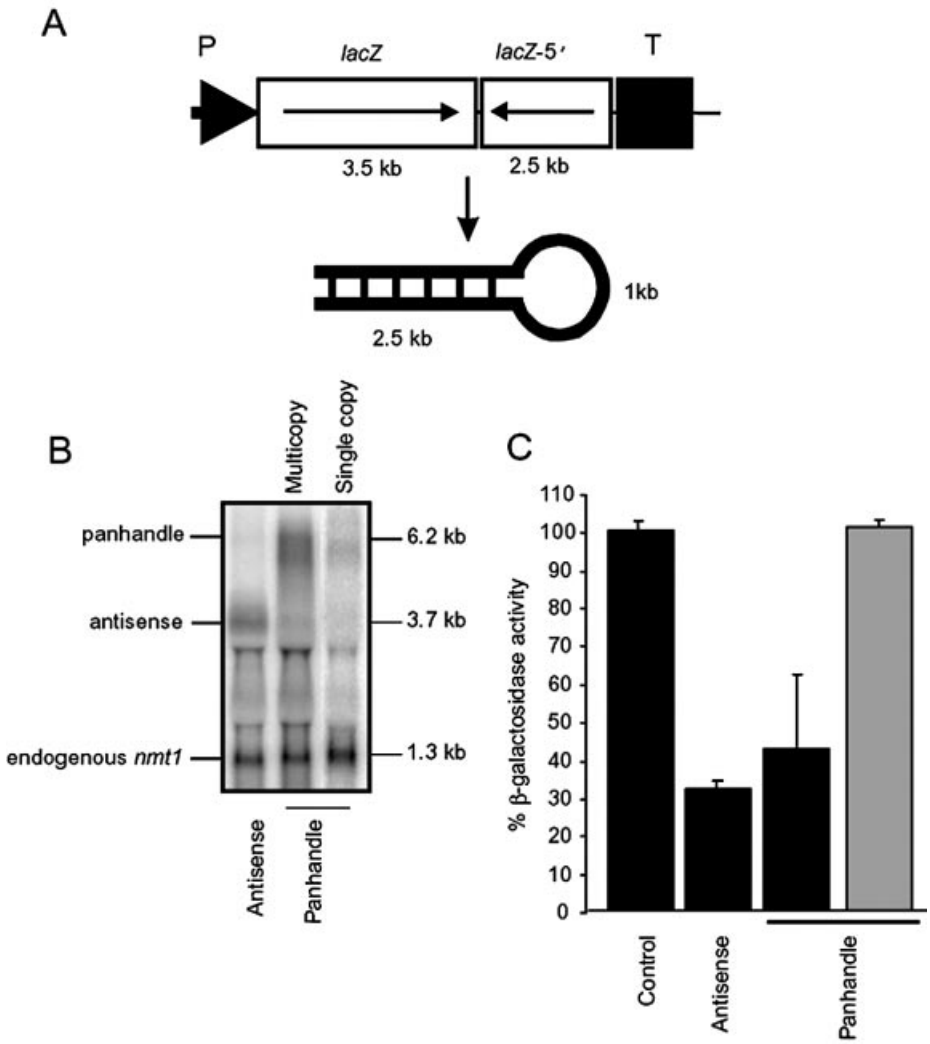

Figure 2. *lacZ* panhandle-mediated gene silencing. (**A**) The *lacZ* panhandle construct contains the full-length *lacZ* gene (with an internal frameshift mutation) which is followed by the inverted 5′ 2.5 kb *lacZ* fragment. Intramolecular hybridisation generates an RNA with 2.5 kb RNA duplex and a 1 kb loop. The *nmt1* promoter and terminator sequences are indicated by P and T, respectively. (**B**) The relative steady-state level of the panhandle *lacZ* RNA (6.2 kb) expressed from single copy and multi-copy genes is shown in comparison to episomally expressed antisense *lacZ* RNA (3.7 kb). The northern blot was probed with the *nmt1* fragment to identify the *nmt1*-driven episomal transcripts in addition to the endogenous *nmt1* transcript. The *lacZ* signals were normalised to the endogenous *nmt1* transcript (1.3 kb) and quantitated by phosphorimager analysis. (**C**) The target strain was transformed with the episomally expressed *lacZ* panhandle and analysed for β-galactosidase activity. The appropriate plasmids were co-introduced to complement auxotrophy. The panhandle strain was assayed in the presence of thiamine (grey). At least three independent colonies were assayed in triplicate for each strain.

was normalised for plasmid segregation it was found that co-expression of the antisense and sense constructs enhanced *c-myc* suppression by an additional 20% compared with the antisense *c-myc* vector alone, while expression of pN12-1 alone showed no inhibition of enzyme activity (Fig. 3C). This enhancement of gene silencing was consistently observed with this human gene target. Transformation of RB3-2 (the strain expressing only the *lacZ* target) with the antisense and sense *c-myc* constructs resulted in no down-regulation of β-galactosidase activity, indicating that the action of dsRNA is sequence-specific in the fission yeast model (Fig. 3C). Northern analysis demonstrated that the *c-myc* constructs were being expressed in all strains analysed (Fig. 3D). The RNA was probed with the *nmt1* fragment which hybridises to both the endogenous *nmt1* sequence (1.3 kb) and the episomally expressed *c-myc* sequences. Normalisation of the *c-myc* RNA to the endogenous *nmt1* transcript indicated that both antisense and sense constructs were being expressed in the co-transformed strains as the steady-state level was approximately equivalent to the sum of that seen in the strains transformed with the sense or antisense constructs only (Fig. 3D).

Detection of small interfering *lacZ* RNA

Given our observations that antisense RNA and panhandle RNA suppressed expression of the homologous *lacZ* gene, we decided to examine the relevant transformants for the presence or absence of *lacZ*-specific siRNA, a hallmark of gene regulation by RNAi (22). Using a hydrolysed *lacZ*-specific sense RNA probe, small RNAs were clearly identified in the

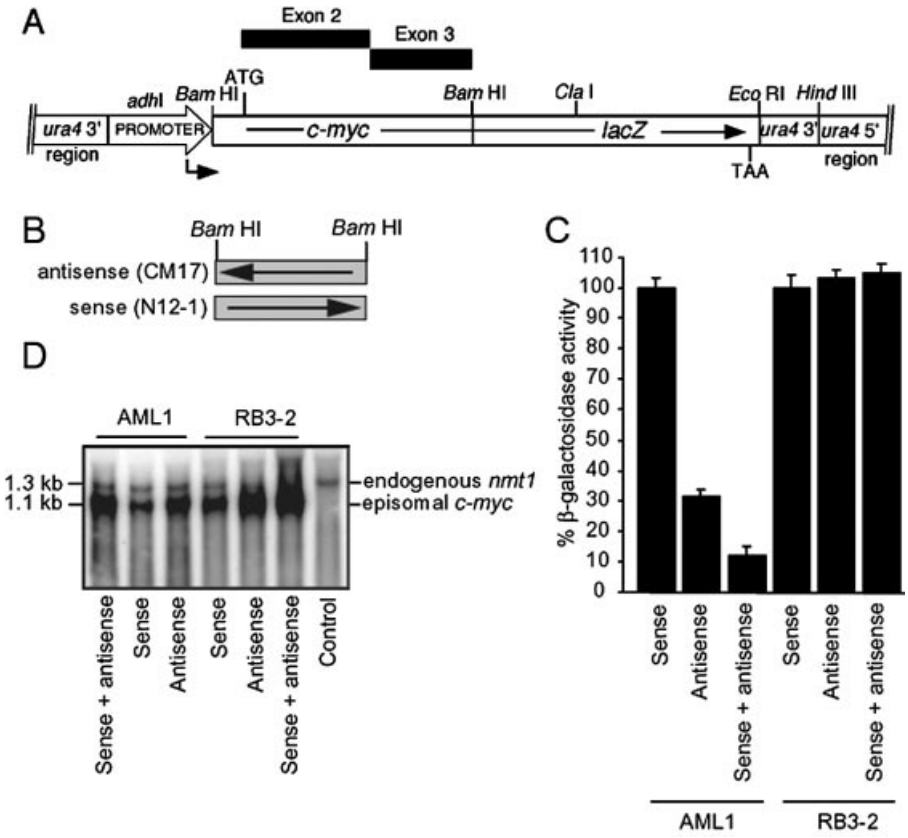

Figure 3. dsRNA-mediated suppression of a *c-myc* target. (**A**) The AML1 strain contains a *c-myc–lacZ* fusion cassette integrated at the *ura4* locus. Exons 2 and 3 of the human *c-myc* gene were employed as a target. The relative position of the *c-myc* antisense fragment is shown. The transcription initiation site is indicated by the bent arrow. The straight arrow represents the normal direction of transcription for a particular DNA fragment. (**B**) The region of the *c-myc* target from which the antisense fragment was derived is shown. The fragments are aligned to the illustration in (A). (**C**) The target strains AML1 and RB3-2 were transformed with the sense construct pN12-1 (sense), the antisense construct pCM-17 (antisense) or both (antisense + sense). Transformants were grown in the absence of thiamine and assayed for β-galactosidase activity. Strains were transformed with appropriate control plasmids to complement auxotrophy. (**D**) RNA was probed with the *nmt1* fragment. The control strain did not contain the episomally expressed *c-myc* sequence (1.1 kb) but shows the endogenous *nmt1* transcript at 1.3 kb.

pandhandle-expressing yeast transformants (Fig. 4). Small RNAs were also observed in the antisense RNA-expressing strain, albeit to a much lesser extent. These same RNAs were not detected in transformants containing vector controls or expressing only *lacZ* sense RNA. These results indicate that antisense RNA- and panhandle RNA-mediated regulation of *lacZ* gene expression in *S.pombe* involves the production of small RNAs of the size range expected for a mechanism involving RNAi. However, no correlation between the level of siRNAs present in the transformants and the degree of gene silencing was found in this study. Nevertheless, this is the first demonstration of single gene regulation through RNAi in any yeast species and further supports the utility of fission yeast as a model for better understanding the biological role(s) of this evolutionarily conserved mechanism of controlling gene expression (28).

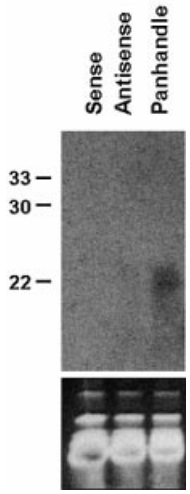

Figure 4. Detection of siRNA in dsRNA-expressing strains. Total RNAs spanning 20–30 bases in size were fractionated on a 15% 8 M urea TBE gel, transferred to nylon membrane and probed with a radiolabelled *lacZ* sense RNA that had been sheared to an approximate length of 50 nt. DNA oligos were also run as size controls. The ethidium bromide stained gel, which shows the total RNA spanning 20–30 bases, indicates equal RNA loading.

Co-expression of *aes2* with panhandle RNA enhances gene silencing

Recent studies on RNA-mediated gene silencing, including our current work, suggest that antisense RNA, co-suppression and dsRNA-mediated interference may share similar mechanisms (40). We therefore explored whether overexpression of

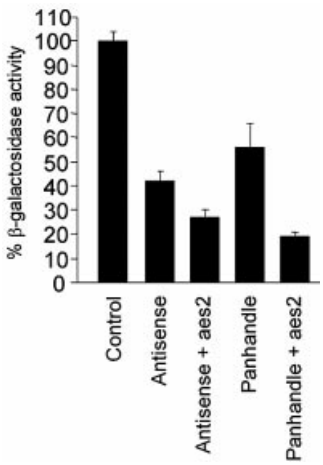

Figure 5. Co-expression of *lacZ* panhandle construct and *aes2* factor. β-Galactosidase activity was determined in co-transformants. The control strain was RB3-2 co-transformed with pREP2 and pREP4 to complement auxotrophy. The panhandle strain was RB3-2 transformed with pM53-1 and pREP2. Five independent colonies were assayed in triplicate for each strain.

a host-encoded factor previously shown to enhance antisense RNA-mediated gene silencing (34) could also enhance dsRNA-mediated regulation. To this end, the effect of co-expressing the antisense enhancing sequence, *aes2*, with a *lacZ* panhandle construct (pM53-1) in a yeast strain containing the *lacZ* target gene (RB3-2) was tested. The *aes2* factor was isolated from a *S.pombe* cDNA library and encodes part of the elongation factor EF-Tu. It was previously shown to enhance antisense RNA-mediated inhibition of the *lacZ* reporter gene by an additional 15% (34) (Fig 5). The panhandle vector pM53-1 was either co-transformed with the control plasmid pREP2 or with the *aes2* plasmid into the target strain RB3-2. Under these conditions, the *aes2* transformant displayed an additional 35% suppression of β-galactosidase activity when compared to the transformant expressing only the panhandle *lacZ* RNA (Fig. 5). This result indicated that expression of the *aes2* gene could specifically enhance both antisense RNA- and, to a greater degree, panhandle RNA-mediated gene inhibition. In addition to the presence of siRNA in both the antisense- and panhandle-expressing strains this data suggests that both antisense- and dsRNA-mediated gene silencing may share a common mechanism.

DISCUSSION

RNA-mediated gene silencing encompasses a variety of phenomena including antisense-mediated gene suppression, co-suppression and dsRNA-mediated gene interference (RNAi in animals), all of which have been implicated in the cellular control of gene expression and defence against viral infection (40). Recent evidence suggests that all of these gene silencing categories may operate through similar mechanisms, with the generation of dsRNA being the key factor (18). We have presented evidence to suggest that dsRNA (i) is a key mediator in antisense RNA-mediated gene silencing in fission yeast, (ii) acts in a dose-dependent manner and (iii) acts through an RNAi-like mechanism.

If antisense RNA was acting by hybridising to its target mRNA and directly inhibiting translation, it would follow that excess non-coding sense RNA would act as a competitor of the antisense RNA:target mRNA interaction. This competitive reaction would titrate out the available antisense RNA allowing for coding sense mRNA to be translated, with a consequent reduction in target inhibition. However, additional expression of both genomic and episomal non-encoding *lacZ* sense RNA failed to increase the level of β-galactosidase activity, but instead stimulated gene suppression. We have previously shown that the level of suppression within a given yeast strain was dependent upon the absolute level of antisense RNA (30). Similarly, results presented here suggest that gene silencing is also dependent on the dose of sense RNA. The concentration of sense RNA was increased by first expressing the sense transgene in single copy (stably integrated) and then in multiple copies (episomally maintained) in *lacZ*-expressing strains. Episomal co-expression of the complementary *lacZ* genes resulted in a further enhancement of *lacZ* gene silencing compared with their co-expression in single copy. This suggested that dsRNA was functioning in a dose-dependent manner. In addition, we have observed that increasing the steady-state level of target *lacZ* mRNA enhances the level of antisense RNA-mediated gene silencing (G.M. Arndt and D. Atkins, unpublished data). Consistent with gene silencing in other organisms, the increase in gene inhibition was associated with a concomitant reduction in *lacZ* mRNA, although a complete reduction was not seen. The specificity of dsRNA-mediated gene interference was also demonstrated by targeting a *c-myc–lacZ* fusion gene with co-expressed sense and antisense *c-myc* constructs. In this case, enhanced gene silencing was observed when additional *c-myc* RNA was generated while there was no effect of *c-myc* dsRNA on the *lacZ* target gene alone.

Expression of a *lacZ* panhandle construct also inhibited target gene expression, consistent with the prediction that dsRNA was the integral factor in antisense RNA-mediated gene silencing. A construct which generated a transcript with a 1 kb loop and 2.5 kb of intramolecular complementarity inhibited the *lacZ* gene by ~60%. When one-tenth of the level of panhandle RNA was expressed no gene silencing was observed, again demonstrating that dsRNA-mediated gene silencing was dose-dependent in this system. The ability of a sequence with the potential to form an intramolecular RNA duplex to inhibit target gene expression is consistent with the use of similar constructs in plants (3), *C.elegans* (41) and trypanosomes (42). Furthermore, short *lacZ*-specific RNAs (~21–23 nt long) were identified in strains expressing the panhandle construct, and to a lesser extent in the antisense RNA-expressing strain. The presence of siRNA is one of the hallmarks of the RNAi mechanism of gene silencing and therefore demonstrates for the first time that fission yeast employs the RNAi pathway for gene regulation.

The inability of the panhandle construct to inhibit gene expression to a greater extent than the antisense construct in this study may be due to the following reasons. First, it has recently been demonstrated that panhandle constructs are less effective at inhibiting target genes than hairpin RNA produced from direct inverted repeats (43). Secondly, it is well known that long inverted repeats can lead to genomic instability in prokaryotes and lower eukaryotes (44). It has also been shown

that, even in systems where dsRNA acts in sub-stoichiometric amounts, the gene silencing effect is more robust when higher doses of dsRNA are introduced (1). However, we found that the panhandle strain had the highest levels of the siRNA species while the level of target gene suppression was not as robust as that seen in the antisense-expressing strain. Therefore, although increasing the potential formation of dsRNA correlated with the level of gene silencing, there is no correlation between the level of siRNA and gene inhibition in this system. This suggests that the nature of the siRNA population impacts on the observed level of gene silencing. For example, the antisense strains have the ability to form dsRNA of the entire length of the *lacZ* gene (3.5kb), however, the panhandle transcript only forms dsRNA of the 5′ 2.5 kb *lacZ* sequence. Therefore, while the panhandle generates more siRNA it could be that the short dsRNAs from the 3′ end of the *lacZ* gene are more efficient at silencing gene expression.

We have shown that overexpression of a cofactor of antisense RNA-mediated gene silencing (*aes2*) can also enhance dsRNA-mediated gene silencing. If antisense RNA acts through a dsRNA intermediate in this system then a factor that enhances antisense RNA-mediated gene silencing should also stimulate dsRNA-mediated gene inhibition. To test this hypothesis we co-expressed the previously identified *aes2* factor (elongation factor EF-Tu lacking the mitochondrial localization domain) with the panhandle *lacZ* construct to investigate if it could also stimulate dsRNA-mediated gene silencing. Surprisingly, it reduced β-galactosidase activity by an additional 35% in the presence of the panhandle RNA. These data demonstrate that both antisense RNA- and dsRNA-mediated gene silencing pathways can be modulated by overexpression of the same host-encoded factor. The notion that dsRNA and antisense RNA pathways may share a common mechanism is further supported by our previous observation that the ATP-dependent RNA helicase, ded1, also enhances antisense RNA-mediated gene silencing (34). Both ded1 and EF-Tu act on dsRNA. The first acts in translation initiation by unwinding RNA duplexes while EF-Tu transports tRNA to the ribosome by first recognising the RNA duplex in tRNA stems. Interestingly, the level of gene silencing was enhanced by *aes2* to a much greater extent in the panhandle strain than in the antisense strain. This coincided with the presence of a higher level of short *lacZ*-specific RNAs in the panhandle strain, again suggesting that the nature of the dsRNA may influence the level of gene silencing. The precise molecular role of *aes2*, and other *aes* factors (34), is unknown at present and will require further analysis using the fission yeast system described.

In conclusion, we have shown here that co-expression of complementary transcripts can enhance antisense RNA-mediated gene silencing, that panhandle RNA can inhibit target gene expression and that the antisense RNA- and dsRNA-gene silencing mechanisms may be linked through a common RNAi-like mechanism. Finally, the genetic tractability of fission yeast, including the ease of targeted integration, amenability to antisense RNA- and dsRNA-mediated gene silencing and knowledge of its genomic sequence, should allow for its continued use as an experimental model for the elucidation of gene silencing mechanisms, including investigation of components of the RNAi machinery that are essential for cell survival.

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