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Dominant genetic screen for cofactors that enhance antisense RNA-mediated gene silencing in fission yeast

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ABSTRACT

Specific gene silencing has been demonstrated in a number of organisms by the introduction of antisense RNA. Mutagenesis of host-encoded factors has begun to unravel the mechanism of several forms of RNA-mediated gene silencing and has suggested that it may have been conserved through evolution. This has led to the identification of certain host genes, which, when mutated, abrogate this phenomenon. Conversely, the identification of other factors that, when co-expressed or overexpressed, can enhance gene inhibition is equally important for both elucidating the mechanism of this process and enhancing gene silencing in recalcitrant systems. We have taken such a dominant genetic approach to identify several host-encoded factors that dramatically enhance target gene silencing when co-expressed with antisense RNA in fission yeast. The transcription factor thi1 and, surprisingly, the ATP-dependent RNA helicase ded1 were initially shown to enhance gene silencing in this system. Additionally, screening of a *Schizosaccharomyces pombe* cDNA library identified four novel antisense-enhancing sequences (*aes* factors) all of which are homologous to genes encoding proteins with natural affinities for nucleic acids. These findings demonstrate the utility of this strategy in identifying host-encoded factors that can modulate gene silencing when co-expressed with antisense RNA and possibly other forms of gene-silencing activators.

INTRODUCTION

Gene silencing has been demonstrated in a number of organisms by the intracellular expression of antisense RNA (1). The application of this strategy often results in only partial suppression, depending upon the gene being targeted or the organism in which it is employed (2). This is also true for other forms of post-transcriptional gene silencing (PTGS) such as double-stranded (ds)RNA-mediated gene silencing and RNA interference (RNAi) (3). The reasons for this variability of RNA-mediated gene silencing may include (i) the secondary structure of the complementary RNAs and target site accessibility, (ii) the stability of the antisense RNA molecule, (iii) the ratio of antisense and target RNA, (iv) the metabolic state of the cell, (v) the co-localisation of the complementary RNAs within the cell and (vi) the presence or absence of host factors that are part of the gene-silencing pathway(s) (4,5).

RNA structure, annealing dynamics and stability are dependent on RNA-binding proteins (2,6). Additionally, there are many proteins that bind to duplexed RNA which may affect antisense RNA-mediated gene inhibition, including ADAR, PKR, RNA helicases and RNase III (7). Several cellular proteins have been identified as facilitators of RNA duplex formation (8). These include the ribonucleoproteins (RNPs) (9–11), the tumor suppressor protein p53 (12,13) and the yeast initiation factor TifIII (14). These RNA-binding proteins may act to facilitate RNA hybridisation through various mechanisms. For example, the heterogenous nuclear ribonuceloprotein hnRNP A1 has been shown to strongly enhance RNA:RNA duplex formation through protein–protein interactions (9,11) and has thus been a candidate for modulating antisense RNA efficacy. It is reasonable to expect that alternative proteins would exist in fission yeast that act in a similar fashion to hnRNPs.

RNA helicases have roles in transcription, pre-mRNA splicing, RNA maturation, RNA transport and translation and RNA degradation (15). It is likely that unwinding of RNA duplexes also affects gene silencing. This could result in inhibition of the antisense effect by resolving dsRNA or it is conceivable that such helicases could enhance gene silencing if an RNAi-like mechanism was active where dsRNA was the catalytic molecule (16). Recently, the RNA helicases SDE3 (17), DICER (18) and MUT6 (19) have been shown to be central to dsRNA-mediated gene sil*encing in Arabidopsis thaliana, Drosophila melanogaster* and the alga *Chlamydomonas reinhardtii*, respectively.

Mutagenesis of host-encoded factors has begun to unravel the mechanism of PTGS and has suggested that it may have been conserved through evolution (20). This has led to the identification of certain non-essential factors that, when down-regulated, affect the efficiency of gene silencing. In addition to RNA helicases, these genes include ones encoding an RNA-dependent RNA polymerase [*qde-1* (21), *sde1* (22) and *ego-1* (23)], a RecQ DNA helicase [*qde-3* (24)], an RNase D homologue [*mut-7* (25)], an RNase III homologue (18) and a putative translation initiation factor [*rde-1* (26), *qde-2* (27) and

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ago1 (28)]. Clearly, there may be several additional factors, in particular genes that are essential for cell viability, which are involved in gene silencing that, as yet, have not been identified.

In comparison with mutagenesis strategies, the identification of other factors that, when overexpressed, can enhance gene silencing is equally important for both elucidating the mechanism of these processes and enhancing gene silencing in recalcitrant systems. Here we present a genetic screening approach to identify several host-encoded factors that dramatically enhance target gene silencing when co-expressed with antisense RNA in fission yeast. These factors were named antisense-enhancing sequences (to be referred to as *aes* factors). We discuss the utility of this genetic strategy for identifying cofactors capable of modulating the efficacy of RNA-mediated gene regulation.

MATERIALS AND METHODS

Schizosaccharomyces pombe media and manipulations

All yeast manipulations were performed as previously described (5). Yeast strains were maintained on standard YES or EMM medium (29) and transformed with plasmid DNA by electroporation (30). Repression of *nmt1* transcription was achieved by the addition of thiamine to EMM medium at a final concentration of 4 μM.

Schizosaccharomyces pombe strain and plasmid construction

The construction of the target strain RB3-2 has been previously described (31). The long *lacZ* antisense (pGT2), short 5′ antisense (pGT59) and short 3′ antisense (pGT61) containing episomal plasmids have also been described elsewhere (32). Plasmid pREP4-lacZAs was generated by subcloning the *lacZ* *Bam*HI fragment contained in pGT2 into the plasmid pREP4 (33). pREP4 is identical to pREP1 except that the *Saccharomyces cerevisiae LEU2* gene has been replaced with the *Schizosaccharomyces pombe ura4* selectable marker. The *lacZ* vector encoding sense *lacZ* incapable of producing functional β-galactosidase was generated by end-filling the *Cla*I site of pGT2 and re-ligating (pGT62) (32). This fragment was subcloned into the *Bam*HI site of pREP4 to generate the plasmid pM54-3.

The *thi1* open reading frame (ORF) (accession no. 6523770) was initially PCR amplified from genomic DNA (strain 1913; NCYC) using the 5′ primer 5′-ATG AGA TCT GTG GTT GGT ATT CTA GAG AGA-3′ and the 3′ primer 5′-ATG AGA TCT AAC AAA GAC CTG CAA AAA ACC-3′ to generate an amplicon with *Bgl*II ends. The *ded1* ORF (accession no. AJ237697) was amplified from the same genomic DNA to give it *Bam*HI ends using the forward primer 5′-ATG GGA TCC CAA CCA AAC ACT TCA ACT CAG-3′ and the reverse primer 5′-ATG GGA TCC TCA GAA GCC TGT GCA TAA CAC-3′. The PCR products for *thi1* and *ded1* were each subcloned into the *Bam*HI site of pREP4 in the sense orientation to produce pREP4-*thi1* and pREP4-*ded1*, respectively. The *ded1* ORF was also subcloned into the *Bam*HI site of the pREP2 vector in the sense orientation to generate pREP2-*ded1*.

Northern analysis

Nucleic acid electrophoresis and membrane transfer was performed as described (34). Northern blots were hybridised using ExpressHyb solution according to the manufacturer's instructions (Clontech Laboratories). DNA probes were ^{32}P labelled using the Megaprime labelling kit (Amersham). A 2.2 kb *Pst*I/*Sac*I *nmt1* fragment was used as a probe and radioactive signals were detected by autoradiography and quantitated by phosphorimager analysis (ImageQuant; Molecular Dynamics).

Isolation of *S.pombe* cDNA clones that modulate the efficacy of antisense RNA

The *S.pombe* cDNA library was originally constructed in pREP3Xho by Bruce Edgar and Chris Norbury (35). The vector pREP3Xho is derived from pREP3, which contains the *LEU2* marker and inserts are under control of the conditional *nmt1* promoter (33). A total of 5 μg library DNA was transformed into the strain RB3-2 containing the episomal antisense *lacZ* vector pREP4-lacZAS and grown in EMM liquid medium to mid-logarithmic phase. Transformants were then plated on EMM solid medium and grown at 30°C for 3 days. Colonies were overlayed with medium containing 0.5 M sodium phosphate, 0.5% agarose, 2% dimethylformamide, 0.01% SDS and 500 μg/ml X-gal (Progen, Australia) (31). Plates were incubated at 37°C for 3 h and colonies of interest recovered and assayed for β-galactosidase activity. To remove antisense plasmids from co-transformants, strains were plated on EMM containing limiting uracil and 1 mg/ml 5-fluoroorotic acid (29). Strains were then replica-plated on both selective and non-selective media. Those colonies that did not grow on selective medium were identified as having lost the *ura4*-containing antisense plasmid.

β-Galactosidase assays

Expression of the *lacZ* gene-encoded product, β-galactosidase, was quantitated using a cell permeabilisation protocol as previously described (5). A semi-quantitative overlay assay was employed for rapid screening of yeast transformants (31).

Plasmid segregation

Raw data were normalised to account for plasmid segregation following cell division (36). Strains were plated on both selective and non-selective media. The number of colonies grown on non-selective medium (YES) was taken as the total number of viable cells in the cell population. Cells in the population harbouring either *ura4*- or *LEU2*-containing plasmids were identified by plating onto EMM + leucine and EMM + uracil, respectively. The ratio of the number of colonies grown on selective medium to the total number of viable colonies was used as a quantitative measure of the proportion of plasmid-containing cells. It was found that ~73% of the cell population contained *LEU2*-based plasmids, while ~69% of the cell population contained both *ura4*- and *LEU2*-based plasmids.

RESULTS

Overexpression of a host-encoded factor enhances antisense RNA efficacy

We have recently established a fission yeast model for examining antisense RNA-mediated regulation of the *lacZ* reporter gene (5,31,32). We use the fission yeast strain RB3-2 containing the chromosomally expressed target *lacZ* gene under control of the

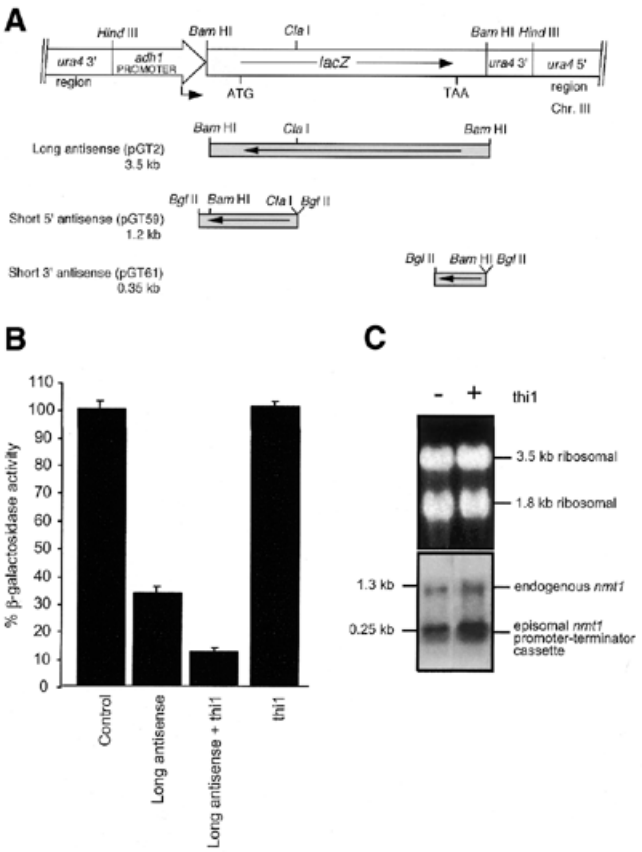

Figure 1. Overexpression of the transcriptional activator *thi1*. (**A**) The long (pGT2), short 5′ (pGT59) and short 3′ (pGT61) *lacZ* antisense constructs are shown in relation to the target *lacZ* cassette. The *adh1*-driven target *lacZ* gene is integrated at the *ura4* locus on chromosome III of the fission yeast strain RB3-2. (**B**) thi1 was co-expressed with the long *lacZ* antisense gene in the strain RB3-2 (long antisense + *thi1*) and co-transformants were assayed for β-galactosidase activity. RB3-2 expressing the antisense *lacZ* (long antisense) or *thi1* (thi1) genes only were also analysed. The control strain was RB3-2 transformed with pREP2 and pREP4 (control). Three independent colonies were assayed in triplicate for each strain. (**C**) Northern blot analysis of RB3-2 containing the pREP2 and pREP4 (–) or pREP2 and pREP4-*thi1* (+) plasmids. RNA was probed with the *nmt1* promoter fragment to detect all transcripts driven by the *nmt1* promoter including the endogenously expressed *nmt1* gene (1.3 kb) and the episomally expressed *nmt1* promoter and terminator cassette (0.25 kb). This latter cassette is transcribed from the control vectors pREP2 and pREP4. The ethidium bromide-stained gel showing the rRNA bands indicates equal RNA loading.

constitutive *adh1* promoter and antisense *lacZ* genes under control of the conditionally regulated *nmt1* promoter (Fig. 1A). In this system, we showed that antisense RNA-mediated gene silencing is dose dependent (5). To demonstrate that a dominant genetic approach could be used to identify factors that enhance gene silencing by antisense RNA, we initially overexpressed the *thi1* gene in this model. This transcriptional activator has previously been shown to up-regulate transcription of the thiamine-repressible promoter *nmt1* (37) and, as such, we hypothesised that activation of the *nmt1*-driven antisense gene would enhance *lacZ* inhibition. The plasmid pREP4-*thi1* was transformed into strain RB3-2 containing the antisense *lacZ* plasmid pGT2 and β-galactosidase assays were performed. Raw data were then normalised to account for plasmid segregation. As predicted, *lacZ* suppression in the *thi1*-expressing strain was enhanced when compared with a strain expressing antisense RNA alone (Fig. 1B). When pREP4-*thi1* was introduced into RB3-2 in the absence of the antisense plasmid, no down-regulation of β-galactosidase activity was observed. As expected, northern analysis demonstrated that overexpression of *thi1* resulted in an increased level of steady-state *nmt1* RNA (Fig. 1C). This was shown for both the endogenous *nmt1* gene and the episomal *nmt1* promoter which is contained in the control vector. These results indicate that the transcriptional activator, *thi1*, could increase the intracellular dose of the antisense RNA that, in turn, enhances gene silencing. Furthermore, it suggests that additional rate-limiting host-encoded factors are present which may affect the robustness of RNA-mediated gene silencing.

Co-expression of the *ded1* helicase enhances antisense RNA-mediated gene silencing

Since RNA duplex formation can directly inhibit translation, RNA helicases were originally proposed to decrease antisense RNA-mediated gene inhibition as overexpression of an RNA helicase would enhance unwinding of the RNA duplex. However, recent evidence has shown that RNA helicases are required for other forms of RNA-mediated gene silencing, such as PTGS and RNAi (17–19). We therefore tested the ability of the *S.pombe* ATP-dependent RNA helicase gene, *ded1* (38), in modulating antisense RNA efficacy by co-expressing it with various antisense genes in fission yeast (Fig. 1A). This particular RNA helicase was chosen because (i) it has been shown to be involved in translation initiation in *S.pombe* (38) and might therefore be a candidate for unwinding a mRNA-containing duplex, and (ii) *ded1* is an essential gene and therefore an ideal candidate RNA helicase to test by the dominant genetic approach.

Surprisingly, co-expression of *ded1* from the pREP4 plasmid and the long antisense *lacZ* (pGT2) from the pREP2 plasmid demonstrated complete inhibition of β-galactosidase activity following normalisation for plasmid segregation (Fig. 2A). When *ded1* was overexpressed in the absence of the antisense *lacZ* vector, β-galactosidase activity was comparable with control strains, indicating that the *ded1* effect was dependent on the presence of antisense RNA. The *ded1* vector was also co-expressed with a short 5′ antisense *lacZ* plasmid (pGT59) which was previously shown to be less effective than the full-length antisense gene (32). Again, overexpression of *ded1* stimulated antisense RNA-mediated *lacZ* inhibition. However, when the *ded1* gene was co-expressed with a short 3′ antisense *lacZ* plasmid (pGT61), which demonstrates negligible suppression (32), no enhancement of gene silencing was observed. When *ded1* was expressed from a *ura4*-based plasmid (pREP4) there was no impact on the phenotype of the transformed strain (Fig. 2B). However, in agreement with previous observations (39), *ded1* expression from the weakly complementing *LEU2*-based plasmid (pREP2) caused aberrant morphology of transformed cells (Fig. 2B). This is most likely due to differences in copy number of *ura4*- and *LEU2*-based plasmids (36,40). As *LEU2*-based plasmids are usually maintained at a higher copy number than *ura4*-based plasmids there would be a consequent increase in the steady-state level of ded1, which would produce a threshold of the protein that impacts on the cell cycle. In addition, we have observed an increased steady-state level of gene expression from *LEU2*-based

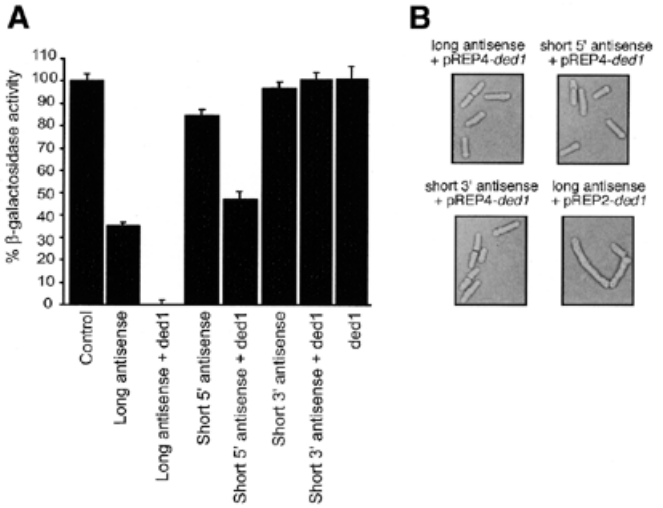

Figure 2. Co-expression of antisense *lacZ* genes and *ded1*. (**A**) β-Galactosidase assay of antisense *lacZ* and *ded1* co-transformants. The pREP4-ded1 plasmid was co-expressed with the pREP2 plasmid containing different antisense *lacZ* constructs. Three colonies were assayed in triplicate for each strain. Strains were co-transformed with the appropriate control plasmid to complement auxotrophy. (**B**) Light microscopic analysis of *ded1* transformants. The strain RB3-2 was co-transformed with the plasmids indicated and grown to mid-logarithmic phase before examination.

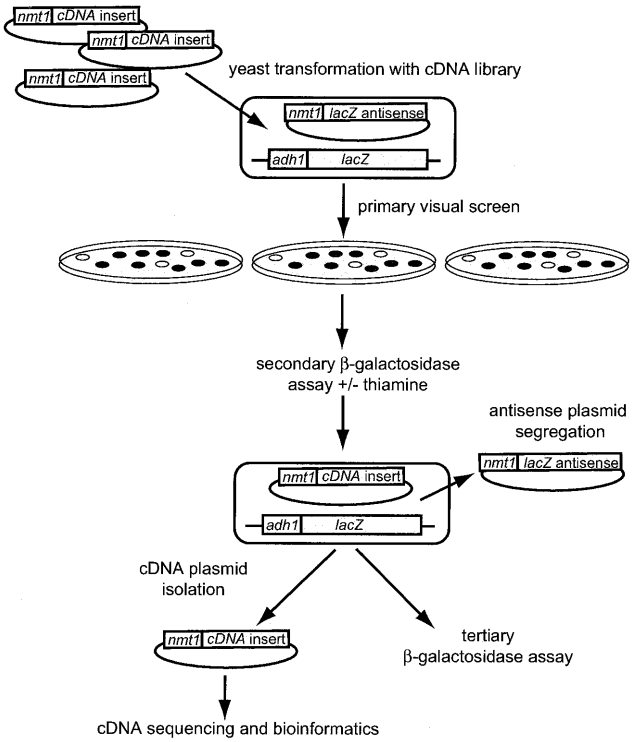

Figure 3. Overexpression screening strategy for antisense RNA-modulating factors. A target strain containing the integrated *lacZ* gene under control of the *adh1* promoter and the episomal vector containing the *nmt1*-driven *lacZ* antisense gene was transformed with a *S.pombe* cDNA library. Library fragments were driven by the *nmt1* promoter. Transformants were individually screened for a change in the *lacZ*-encoded blue-colour colony phenotype and then transformants of interest were further characterised by quantitative β-galactosidase assay and sequence analysis of antisense enhancing sequences.

plasmids compared with *ura4*-based plasmids (data not shown). The above observations confirmed that the *ded1* ORF was generating functional ded1 protein. In conclusion, these data indicate that the host-encoded ATP-dependent RNA helicase, *ded1*, can enhance antisense RNA efficacy when co-expressed with effective antisense RNA molecules.

Library screen for antisense-enhancing plasmids

Next, a *S.pombe* cDNA library was overexpressed in the antisense *lacZ*-expressing fission yeast strain to screen for novel host-encoded factors that would enhance gene silencing in the current system (Fig. 3). A *S.pombe* cDNA library was transformed into RB3-2 containing the episome-based long *lacZ* antisense plasmid (pGT2). The cDNA transformants are under control of the *nmt1* promoter and have the ability to be translated. From 12 000 transformants screened, 48 were initially identified as having a reduced blue phenotype compared with background transformants (Fig. 4A). Each of these transformants was independently isolated from the primary screen. Transformants also displaying an increased blue phenotype were identified, but these were not analysed further. Clearly, this system can also be employed to screen for host-encoded factors that, when overexpressed, inhibit antisense RNA efficacy. Quantitative analysis using the liquid β-galactosidase assay showed that 25 of the 48 transformants displayed a reproducible reduction in β-galactosidase activity compared with the antisense strain alone (Fig. 4B). The control strain, RB3-2, transformed with the *lacZ* antisense construct, consistently demonstrated ~55% of control β-galactosidase activity (prior to normalisation for plasmid segregation). The antisense *lacZ* gene and the host factor cDNA were both driven by the conditional *nmt1* promoter. Repression of the *nmt1* promoter by addition of thiamine to the culture medium resulted in a reversion to control levels of β-galactosidase activity. This indicated that the observed enhancement of suppression in these transformants was dependent on expression of the antisense RNA and/or the host factor cDNA and that this effect was not due to other events such as *lacZ* target gene mutations or modification of protein stability.

Following segregation of the antisense *lacZ* plasmid from these strains, 9 of the 25 transformants returned to the level of β-galactosidase activity observed in the control strain (Fig. 4B). Together with the above data, this indicates that the enhanced gene silencing observed in these nine strains was dependent on expression of antisense *lacZ* RNA. The cDNAs expressed in these transformants were therefore named antisense-enhancing sequences (*aes* factors). The remaining 16 transformants retained a suppressed level of β-galactosidase activity, indicating that the reduced blue phenotype of these transformants was not dependent on the presence of *lacZ* antisense RNA and that the cDNA encoded proteins in these strains were modulating *lacZ* gene expression by an alternative mechanism. It is possible that these factors are affecting the rate of *lacZ* transcription or the stability of the β-galactosidase protein. Although these were not further characterised it will be of interest to determine their nature and mode of action on *lacZ* gene expression.

Characterisation of antisense-enhancing sequences

The library plasmids were recovered from the *aes*-containing strains and their cDNA inserts sequenced using primers

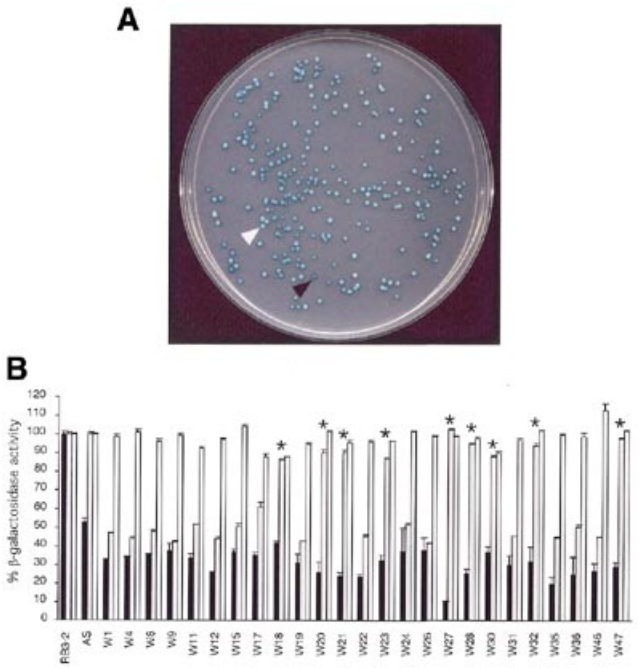

Figure 4. Co-expression screen using a *S.pombe* cDNA library. (**A**) Transformants were grown on minimal medium plates and overlaid with X-gal-containing medium. Those that showed a reduced blue-colour phenotype (white arrow) were analysed further. Transformants demonstrating an enhanced blue-colour phenotype were also identified (black arrow). (**B**) Transformants that showed a visual reduction in the blue phenotype were assayed for β-galactosidase activity in liquid culture in the absence of thiamine (black histograms). Thiamine was added to the medium to repress expression of the antisense and cDNA cassettes (white histograms). Transformants were again assayed for β-galactosidase activity following antisense vector segregation (grey histograms). Asterisks indicate transformants showing an antisense-dependent enhancement of gene silencing. One colony was assayed in triplicate for each transformant.

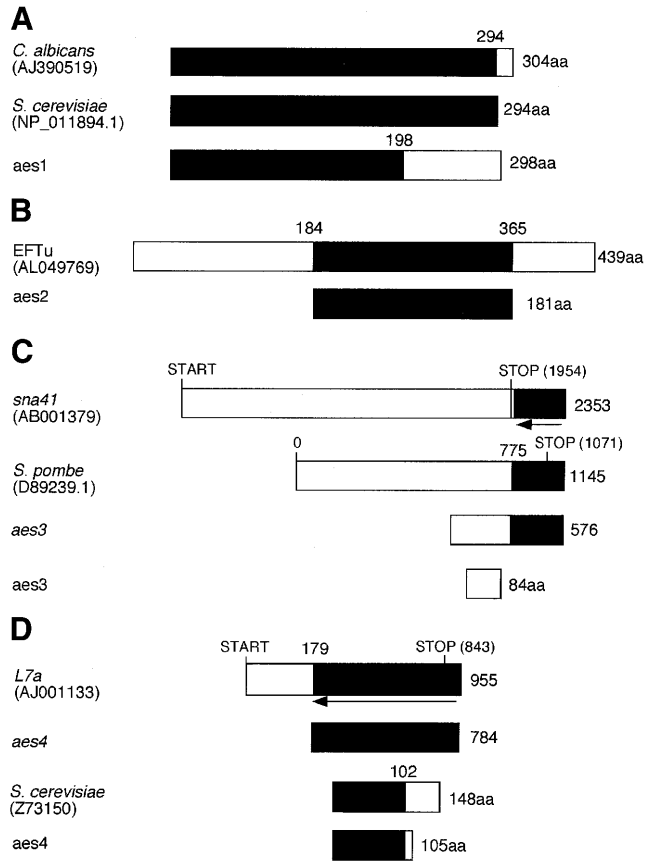

Figure 5. Schematic alignment of *aes* factors with known nucleotide and protein sequences. Regions of identity are shaded black. The length of protein sequences is followed by aa (amino acids). (**A**) Protein alignment of aes1 with *C.albicans* hypothetical protein and *S.cerevisiae* TS protein. (**B**) Region of *S.pombe* EFTu that aligns with the *aes2*-encoded protein. (**C**) Nucleotide sequence of *aes3* aligns with the antisense strand of *S.pombe sna41* (indicated by an arrow) and the sense strand of a *S.pombe* hypothetical protein (partial 3′ sequence shown). The putative *aes3*-encoded protein is indicated. The absence of complete homology to the *sna41* sequence indicates that this factor may be a chimera. (**D**) Nucleotide alignment of *aes4* with the antisense strand of *S.pombe L7a* (arrow). Possible *aes4*-encoded protein alignment with *S.cerevisiae* hypothetical protein. Accession numbers are shown in brackets.

specific for the *nmt1* expression cassette (5; see Supplementary Material). BLASTN and BLASTP analyses were performed on the sequenced cDNA inserts using the NCBI GenBank facility. Four unique *aes* factors were characterised. The extent of homology for each of these is shown in Figure 5.

BLASTN analysis identified the cDNA in the transformants W18, W20 and W30 (named *aes2*) as part of the mitochondrial elongation factor EFTu (accession no. AL049769). EFTu is the mitochondrial analogue of the eukaryotic EF1α, which acts in the cytoplasm transporting tRNA to the A site in the ribosome for peptide elongation (41). The aes2 sequence corresponded to 181 amino acids of the central portion of the encoded protein (Fig. 5A). Interestingly, the absence of the 5′ end of this protein may allow aes2 to act within the cytoplasm as the mitchondrial signal peptides, which allow for transportation from the cytosol to the mitochondria, are found in the N-terminus of mitochondrial proteins (42).

Approximately 50% of the cDNA sequence in transformants W21, W23 and W32 (named *aes3*) had complete identity to the 3′ end of a putative protein (accession no. D89239) that was previously identified in a screen for fission yeast ORFs (43). This portion of *aes3* also had complete homology to the antisense strand of the 3′-untranslated region of the fission yeast gene *sna41* (accession no. AB001379). The protein encoded by *sna41* was previously shown to be involved in DNA replication and has low homology (31% identity) with the *S.cerevisiae* protein CDC45 (44). In addition, analysis of *aes3* showed that its 5′ end was comprised of an ORF which could be translated into an 84 amino acid protein containing a string of 36 arginine–glutamic acid repeats. These types of amino acid repeats are often found in transcription factors, which lends further credence that this factor is acting directly on nucleic acids. Finally, it should be noted that the *aes3* sequence is possibly a chimera generated by library construction since the 5′ portion of this sequence is not homologous to the 5′ end of the *S.pombe sna41* gene.

The cDNA in transformant W47 (named *aes4*) was completely homologous to the antisense strand of the *S.pombe* ribosomal protein L7a (accession no. AJ001133), a component of the 60S ribosomal subunit. Interestingly, several antisense transcripts have been identified in ribosomal loci (45,46). Additionally, a genome-wide screen has found complementarity between many mRNAs and rRNAs (47), while more recently it has been shown that such RNA duplex formation may function

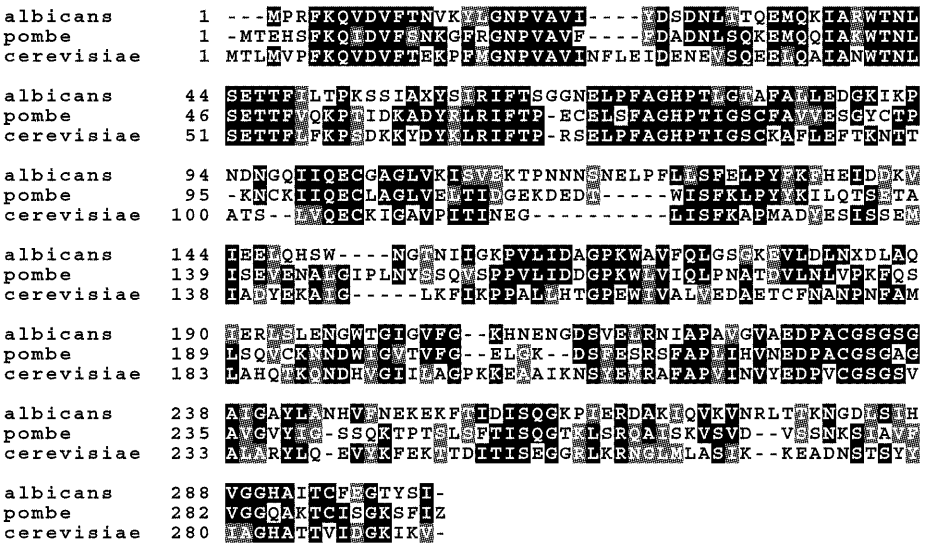

Figure 6. Sequence alignment of the aes1 protein with related proteins. Sequences displayed are *S.pombe* (aes1), *C.albicans* (AJ390519) and *S.cerevisiae* (NP_011894.1). Identical residues are shown in black and conservative substitutions are indicated in grey. The Clusta1W algorithm was used for the alignment and the PrettyBox program (Wisconsin Package v.10.0; Genetics Computer Group, Madison, WI) was used for display.

as a mechanism of translational control (48). *aes4* also contained a small ORF (105 amino acids) of unknown biological function. This protein sequence shared 52% identity with a hypothetical protein in *S.cerevisiae* (accession no. Z73150).

BLASTP analysis showed that the inserts in transformants W27 and W28 (named *aes1*) shared 43% identity with amino acids 4–202 of a *Candida albicans* hypothetical protein (accession no. AJ390519) which was identified in a screen for genes essential for cell growth (M. De Backer, personal communication). It also shared weak homology (39% identity) with the *S.cerevisiae* thymidylate synthase (TS) protein (accession no. NP_011894.1). TS is required for the *de novo* synthesis of thymidine 5′-monophosphate (dTMP) and also has RNA-binding properties (49). The degree of homology between the *S.pombe* aes1 protein and the *S.cerevisiae* and *C.albicans* proteins is illustrated in Figure 6.

A tertiary β-galactosidase assay showed that these cofactors enhanced antisense suppression by up to 50% when co-expressed with antisense RNA in the *lacZ* strain (Fig. 7A). In this assay three individual colonies of transformants W27 (*aes1*), W30 (*aes2*), W21 (*aes3*) and W47 (*aes4*) were assayed in triplicate and normalised for plasmid segregation. All of the *aes*-expressing strains displayed normal growth rates and cellular morphologies, indicating that overexpression of the exogenous cDNAs did not affect general metabolism (Fig. 7B). Northern analysis also confirmed RNA expression of selected *aes* factors in these strains, while transcripts of predicted sizes were observed (Fig. 7C). These results demonstrated that over-expression of a cDNA library was an effective way of identifying novel cofactors that magnify the suppressive effect mediated by antisense RNA.

DISCUSSION

A common genetic strategy for analysing the cellular function of a gene is to examine phenotypes associated with reduced gene expression levels. Previous studies have used mutagenesis approaches to identify proteins involved in different categories of RNA-mediated gene silencing. For example, a screen of *Neurospora* mutants that were defective in quelling of an endogenous gene (50) identified several proteins involved in PTGS, including an RNA-dependent RNA polymerase (21) and a RecQ DNA helicase (24). A similar screen in *Arabidopsis* also identified mutants impaired in co-suppression (51), while mutagenesis of *C.elegans* strains have identified genes that are involved in RNAi (25,26). A common outcome of these approaches was that all of the genes identified were non-essential for cell viability. Clearly, one reason for this is that genes essential for cell growth or proper development will be selected against if their expression is perturbed.

'Dominant genetics' is an alternative approach to elucidate gene function based on increasing the intracellular concentration of an endogenous gene's encoded product and examining the resulting phenotype (52). This may result in either supplementation of the protein or in its inhibition via a transdominant negative effect. Here a unique overexpression strategy was developed for the identification of novel host-encoded factors that enhance antisense RNA-mediated gene silencing in fission yeast. It overcomes the major limitation of the mutagenesis approaches enabling genes that are both essential and non-essential for cell growth to be selected. It must be considered, however, that overexpression of certain genes can also be deleterious to the cell and, as a result, this screening strategy may also fail to identify a subset of host genes involved in gene silencing. It is therefore suggested that this approach should be used to complement mutagenesis strategies.

The first gene that was tested in modulating antisense RNA-mediated gene regulation in the present model was the *S.pombe* transcription factor *thi1*. As expected, overexpression of this gene up-regulated *nmt1*-driven antisense *lacZ* transcription. Since antisense RNA-mediated gene silencing is dose-dependent in this system (5), co-expression of *thi1* with the *nmt1*-driven antisense *lacZ* gene resulted in enhanced *lacZ* gene silencing. This result validated our approach of co-expressing

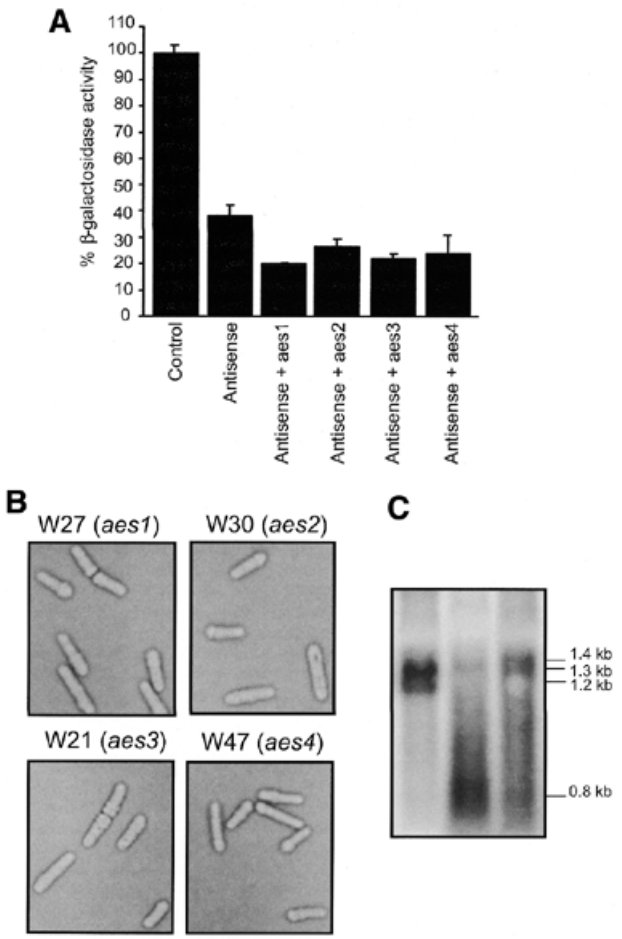

Figure 7. Expression of *aes* factors. (**A**) Co-expression of the unique *aes* factors with the long antisense *lacZ* plasmid in RB3-2. Three colonies were assayed in triplicate for each transformant. In this tertiary assay data were normalized to account for plasmid segregation. (**B**) Microscopic analysis of transformants expressing *aes* factors. Transformants were examined at mid-logarithmic phase. (**C**) Northern analysis of *aes*-containing strains. RNA was fractionated on a 1% MOPS/formaldehyde agarose gel and transferred to a nylon membrane. RNA was then probed with the *nmt1* fragment. The endogenous *nmt1* fragment fractionates at 1.3 kb. W30 contains *aes2* (~1.2 kb), W21 contains *aes3* (~0.8 kb) and W27 contains *aes1* (~1.4 kb).

host-encoded genes with antisense RNA for identifying factors that enhance gene silencing. To further validate this system we wished to co-express a gene that might inhibit gene silencing. To this end we chose the ATP-dependent RNA helicase gene *ded1* (38). *ded1* is an essential gene which has previously been characterised as a suppressor of sterility (39), a suppressor of checkpoint and stress responses (53) and a general translation initiation factor (38). If antisense RNA mediates gene silencing by forming an RNA duplex with mRNA, thereby sterically hindering translation, it might be a reasonable assumption that overexpression of an RNA helicase that is involved in translation initiation would interfere with antisense action, thereby increasing the level of *lacZ* gene activity.

Surprisingly, co-expression of the *ded1* gene from the *ura4* plasmid with the long antisense *lacZ* gene significantly enhanced antisense RNA-mediated *lacZ* inhibition by a further 50% compared with control strains. When *ded1* was co-expressed with an ineffective antisense plasmid no enhancement was observed. These results suggest that *ded1*-mediated augmentation of gene silencing was dependent on an antisense RNA that was capable of some partial gene inhibition. This could be due to the absence of RNA duplex formation with the ineffective antisense RNA and the consequent lack of a substrate for the RNA helicase. This would be consistent with the role of an RNA helicase in dsRNA-mediated gene silencing. Interestingly, DICER in *C.elegans* (18), MUT6 from the unicellular green alga *C.reinhardtii* (19) and SDE3 in *Arabidopsis* (17), all of which contain RNA helicase motifs, have recently been shown to be involved in dsRNA-mediated gene silencing. It will be interesting to examine whether homologues of these helicases also increase the efficacy of antisense RNA in fission yeast. We further address the possibility that antisense RNA is acting through a dsRNA-mediated gene silencing pathway in another study (M.Raponi and G.M.Arndt, manuscript submitted).

The enhanced gene silencing seen when antisense RNA was co-expressed with *thi1* or *ded1* established this system for the screening of novel factors that increase antisense RNA efficacy. Arndt *et al.* (31) previously described an *in vivo* screening strategy for identifying the most effective antisense constructs against any gene of interest using the *lacZ* fission yeast model. In that study, conditions were identified that established a link between the blue colour colony phenotype and the degree of *lacZ*-encoded β-galactosidase activity within fission yeast transformants. In the work presented here similar conditions were utilised to screen the impact of a fission yeast cDNA library on the degree of antisense RNA-mediated *lacZ* gene silencing. From 12 000 transformants 48 were initially found to have a reduced blue phenotype compared with background colonies. Approximately half of these showed a reproducible phenotype when analysed in a secondary assay. Notably, isolation of transformants that contained the same cDNA sequence verified the power of this screening strategy in that it demonstrated the reproducible nature of the visual screen. Two classes of gene-silencing modulators were observed. The first acted independently of antisense RNA and may function either at the transcriptional level or by modifying the stability of the *lacZ* encoded protein. The second class only functioned in the presence of antisense RNA and were therefore named antisense-enhancing sequences (*aes* factors).

Four novel *aes* factors were identified using this screen (named *aes1–aes4*); co-expression of these factors enhanced antisense RNA-mediated gene silencing by up to an additional 50%. Sequence analysis suggested that each of the *aes* factors has the potential to interact with nucleic acids. Although each of these factors has the ability to be translated, further studies will be required to demonstrate whether their proteins are being expressed. Interestingly, evidence exists showing that L7a and EFTu interact during biosynthesis (54). This suggests that aes2 and aes4 may act through a similar mechanism to enhance gene silencing. However, while it can be speculated as to how these factors might be functioning in the gene-silencing phenomenon, further work will be required to understand their precise mechanism of action.

Overall, by using a co-expression strategy in a *lacZ* fission yeast model, five novel genes that enhance antisense RNA-mediated gene silencing have been identified. The overexpression strategy described herein overcomes some limitations associated

with mutagenesis by identifying several genes that are essential for cell viability and with a potential role in gene silencing. In addition, this strategy complements other systems by allowing the isolation of cellular factors that modify the efficacy of RNA-mediated gene inhibition *in vivo*. Furthermore, the co-expression of these factors with different forms of gene silencing activators, including sense RNA, antisense RNA or dsRNA, could be one way of enhancing the efficacy of these methods. This may be especially important for application of antisense RNA and dsRNA to mammalian cells and tissues or to genes that have been recalcitrant to these forms of regulation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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