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SMALL INTERFERING RNA-MEDIATED TRANSLATION REPRESSION ALTERS RIBOSOME SENSITIVITY TO INHIBITION BY CYCLOHEXIMIDE IN *CHLAMYDOMONAS REINHARDTII*

by

Xinrong Ma

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SMALL INTERFERING RNA-MEDIATED TRANSLATION REPRESSION ALTERS RIBOSOME SENSITIVITY TO INHIBITION BY CYCLOHEXIMIDE IN *CHLAMYDOMONAS REINHARDTII*

Xinrong Ma, Ph.D.

University of Nebraska, 2013

Advisor: Heriberto Cerutti

RNA interference (RNAi) is an evolutionarily conserved gene silencing mechanism in eukaryotes, with regulatory roles in a variety of biological processes, including cell cycle, cell differentiation, physiological and metabolic pathways, and stress responses. RNAi can function by transcriptional silencing, mRNA target cleavage, translation repression and/or DNA elimination. In this study, we used the unicellular green alga *Chlamydomonas reinhardtii* as a model system to study RNAi-mediated translation repression. We demonstrated that small RNAs (sRNAs) generated from exogenously introduced inverted repeat transgenes, with perfect complementarity to the 3'UTR of a target transcript, can inhibit protein synthesis, without or with only minimal mRNA destabilization. In addition, there are no changes in the polyadenylation status of sRNA-repressed transcripts. Moreover, the translationally repressed mRNAs remain associated with polyribosomes, suggesting that sRNA-mediated silencing occurs at a post-initiation step of translation. Intriguingly, we consistently observed reduced sensitivity of the ribosomes associated with these repressed transcripts to inhibition by antibiotics such as cycloheximide, both in ribosome run-off assays and in *in vivo* experiments. Our results suggest that sRNA-mediated repression of protein synthesis in

Chlamydomonas may involve alterations to the function/structural conformation of translating ribosomes. Additionally, since sRNA-mediated translation inhibition is now known to occur in a number of phylogenetically diverse eukaryotes, this mechanism may have been a feature of an ancestral RNAi machinery.

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CHAPTER 1

Literature Review

1. INTRODUCTION

Small RNAs are generally ~20-30 nucleotide-long non-coding RNA species. Despite their small sizes, small RNAs exhibit profound regulatory effects in almost every biological process. More intriguingly, nearly 50% of the transcriptome in humans is subject to small RNA (miRNA)-guided regulation (1, 2). Of note, emerging evidence indicates a strong association of dysfunction of small RNA-directed gene regulation with human diseases like cancer (3-5). Small RNAs are categorized into different classes, predominantly based on their distinct origins and processing. In animals, there are three different types of small RNAs, namely microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting or piwi-associated RNAs (piRNAs) (5, 6). Due to the lack of piwi proteins, plants only have two major classes of small RNAs: miRNAs and siRNAs (6, 7). In *Chlamydomonas reinhardtii*, two recognizable types of small RNAs exist: miRNAs and siRNAs (8, 9). MiRNAs originate from single-stranded (ss) transcripts or introns, which contain stem-loop structures where the miRNAs reside (5, 10-12). On the other hand, the precursors of siRNAs are generally long nearly-perfect complementary double-stranded (ds) RNAs, which are generated from a variety of sources: long inverted-repeat transcripts, transcripts from convergent transcription or RNA-dependent RNA polymerase (RdRP) activity, viral and transposon RNAs or exogenous dsRNAs (5, 11, 13). SiRNAs play roles in gene regulation, defense responses, DNA methylation, and heterochromatin formation (5, 11, 14-16). In general, miRNAs and siRNAs bind to their complementary sequence within target mRNAs, function through multicomponent complexes (e.g., RNA-induced silencing complexes (RISCs) or effector ribonuleoprotein complexes (i.e., miRNPs), a core component of which is

Argonaute (Ago), and regulate gene expression posttranscriptionally through three processes: endonucleolytic cleavage (commonly referred to as RNAi), enhanced mRNA degradation, and/or translational repression (2, 17-20).

The majority of the efforts have focused on understanding the mechanisms employed by small RNAs to control posttranscriptionally gene expression, especially in metazoan systems. However, limited work has been done in plants as well as in algae. Various experimental data could not allow us to come up with a unified model to delineate the mode of action of small RNAs. Particularly, in regard to small RNA-mediated translational repression, several models have been proposed in metazoans, including inhibition at translation initiation, co-translational degradation of nascent polypeptides, a blockage at elongation, and/or premature termination (17, 21-23). Here, representative work that describes mechanisms or models used by small RNAs in the translational repression pathway is reported with a special focus on mechanistic and technical aspects in both metazoans and plants.

2. SMALL RNA-MEDIATED TRANSLATIONAL REPRESSION IN METAZOANS

2.1 Gene regulation at the translational level

Translation of an mRNA consists of three major steps: initiation, elongation, and termination. For efficient protein synthesis, an additional step of ribosome recycling is also necessary. Multiple protein factors are involved to control the translation process,

which guarantees normal functions of organisms. Small RNAs have been shown to repress translation of targets without significant effects on mRNA abundance (24, 25). Current evidence supports an inhibitory effect at both initiation and post-initiation stages of translation by small RNA action.

2.1.1 Translational repression at the post-initiation stage

The first evidence to support a role of miRNAs in posttranscriptional gene silencing comes from the study on heterochronic gene lin-14 and its negative regulator lin-4 in *Caenorhabditis elegans* (25). The lin-4 miRNA binds complementary sites in the 3' untranslated region (3'UTR) of lin-14 mRNA and represses translation of lin-14 without changing its mRNA amounts (25). Later, using sucrose density gradient centrifugation, Olsen *et al*. (24) further proved that the lin-4 miRNA translationally represses lin-14, and this inhibitory effect mainly takes place at the post-initiation stage of translation. Sucrose density gradient assay is one of the standard assays used to probe which stage of the translation process is actually affected (24). For the obtained polysome profiles, if target mRNAs under the repressive state are shifted to the top of the gradients compared to those under normal conditions, translation of the target gene is mainly impeded at the initiation stage; whereas, if profiles from these two conditions remain nearly the same, with mRNAs mainly associated with polysomes, a step after the translation initiation is blocked. Studies by Olsen *et al*. (24) showed that the lin-4 target mRNAs were still associated with polysomes even when repressed, indicating a blockage at the post-initiation stage. Similar observations were obtained from another miRNA target gene lin-28 in *C. elegans* (26) and from human cells (27-29). Consistently, several studies on both

Mus musculus and *Drosophila melanogaster* reached the same conclusion of an inhibitory effect at the post-initiation stage of the translation of mRNA targets by miRNAs, based on the co-sedimentation of miRNAs and target mRNAs with polysomal fractions (30-32).

Another approach frequently exploited in this field is the assay that uses *in vitro*-transcribed mRNAs under the control of an internal ribosome entry site (IRES) at the 5'end to check whether reporter mRNAs are still subject to miRNA-mediated repression. The purpose of putting an IRES upstream of a mRNA is to bypass the requirement of the 5' CAP structure m^7GpppN, more specifically to bypass Cap-dependent translation initiation step. The rationale is that if target mRNAs containing an IRES are still able to be repressed by miRNAs, repression takes place at steps after the initiation stage. Results from mammalian cells showed that IRES-containing reporter mRNAs were still repressed by the corresponding miRNAs (29, 33). Additionally, miRNAs translationally repress target mRNAs regardless of the location of miRNA-binding sites, either in the 5'UTR or in the 3'UTR of target mRNA. However, the use of DNA or RNA transfection methods does yield different outcomes (33).

Several research groups have proposed hypothetical mechanisms of translational repression by miRNAs, aimed at interpreting the seemingly paradoxical observation that target mRNAs are indeed associated with true polysomes, even though no protein products are accumulated (27-29). One of the proposed models is that miRNAs translationally repress target mRNAs at the post-initiation stage through co-translational

degradation of nascent polypeptides (28). Using Hela cells, Nottrott *et al.* (28) found that let-7a miRNA could attenuate the translation of reporter mRNAs with let-7a responsive elements in the 3'UTR by directing the newly synthesized polypeptides for immediate destruction. However, this process was not carried out by the regular cellular proteasome degradation pathway; instead, a specific protease might be involved. Of note, one of the caveats of the model is that the supportive evidence only comes from negative results. Studies by Maroney *et al*.(27) suggest that miRNA-mediated translational repression may result from a reduction in elongation rates. In comparison, Peterson *et al*. (29) used mammalian cell cultures transfected with both a reporter mRNA and synthetic siRNA or a bicistronic (IRES) reporter together with synthetic miRNA to examine the mode of action by small RNAs. Evidence from both sucrose gradient assays and the IRES-containing reporter assay pointed at an inhibitory effect exerted by mi/siRNAs at the post-initiation stage of the translation of their mRNA targets. Furthermore, under the treatment with translation inhibitors, the polysomes dissociated from repressed mRNAs much faster than those from unrepressed mRNAs, which finally prompted the authors to propose a ribosome drop-off model. In this model, mi/siRNAs, upon binding to the target mRNA, lead to ribosome drop-off at multiple sites along the target mRNA, causing a dramatic decrease in the full-length protein yield.

2.1.2 Translational repression at the initiation stage

Evidence to support the post-initiation model cannot exclude the possibility of a blockage effect by mi/siRNAs at the initiation stage. In fact, there is a great deal of experimental data directly pointing at an effect of mi/siRNA-mediated translational repression at the

initiation stage. To address this inhibitory effect at the translation initiation stage, researchers have adopted the sucrose density gradients assay, the tethering assay, as well as the reporter assay. Also, studies were carried out both *in vivo* and *in vitro*. One direct piece of evidence comes from sucrose density gradients assays. Instead of the major association of target mRNAs with polysomal fractions under both normal and repressive conditions, features of a post-initiation effect, a shift towards the lighter gradients fractions of repressed target mRNAs was observed, which makes a case for an inhibitory effect at the translation initiation stage (34). It has been demonstrated that there are two types of inhibitory effects exerted by mi/siRNAs on translation initiation: Cap-dependent inhibition and Cap-independent inhibition.

Type I. Cap-dependent inhibition by mi/siRNAs

Using endogenous Let-7 miRNA and reporter mRNAs containing Let-7 target sites or tethered hAgo2 in human cells, the Filipowicz group (34) found that repressed reporter mRNAs were both shifted to the top of the sucrose gradient. In addition, the downstream reporter mRNA containing an IRES in a dicistronic construct was resistant to miRNA-mediated translational repression. Furthermore, miRNA-targeted mRNAs were relieved from repression when tethered with eukaryotic translation initiation factor 4E (eIF4E) or eukaryotic translation initiation factor 4G (eIF4G) (components of eukaryotic translation initiation complex eIF4F). Thus, this cap-dependent repression involves an inhibitory effect at an early step of translation initiation, probably at the cap-recognition step, i.e., the step of eIF4E binding to the cap structure. Yet, this repression process did not seem to require a poly (A) tail. Subsequently, the translationally repressed mRNAs, together with

miRNAs and Ago proteins, were localized to cytoplasmic foci called processing bodies or P bodies for storage (34). Humphreys *et al*. (35) also used human Hela cells to address the same question. Both the 5'cap structure and the 3' poly (A) tail were found to be involved in the full range of miRNA-mediated translational repression, and eIF4E was also identified as the molecular target of miRISCs. Meanwhile, increasing evidence of Cap-dependent translational repression by mi/siRNAs has been produced by *in vitro* assays. Extracts were prepared from a wide range of resources, including extracts from transfected mammalian HEK-293 cells, *D. melanogaster* embryos, mouse krebs-2 ascites, or rabbit reticulocyte lysate (36-39). The common findings from these different cell-free systems are that translationally repressed target mRNAs shifted to the lighter fractions of sucrose gradients and this repressive process was 5' m^7GpppN Cap-dependent.

More interestingly, some researchers have proposed models to demonstrate how miRNAs inhibit translation initiation of their targets (37, 40-42). Experiments to examine the function of human Ago2 in miRNA-mediated translational repression revealed that human Ago2 contained a 5' cap structure binding motif-MC within its Mid domain region, which exhibited high similarities to the cap-binding motif of traditional eukaryotic 5' cap binding protein-eIF4E. Consequently, a model was proposed to delineate the miRNA-mediated translational repression in humans: miRNA guides Ago2-containing miRISC to load onto target mRNA; Ago2 then uses its MC motif to compete with eIF4E for cap binding and thus inhibits normal translation initiation (42). However, studies on *Drosophila* Ago homologues showed that an Ago1 mutant was not defective in cap binding, but rather its association with miRNAs as well as with GW182 (member of

the GW repeat-containing protein family, as discussed below) was attenuated (41). More recently, one study (40) on allosteric regulation of Argonaute proteins by miRNAs demonstrated that Argonaute protein can bind directly to the 5' cap, but not through the MC motif identified previously (42).

There are increasing data which suggest a role of the poly (A) tail in the repression process as well. Representative work comes from the Hentze group (37), who studied *Drosophila* miR2 and proposed a "two hit model", in which both the 5'end-cap structure and the 3' end-poly (A) tail are targets for functional miRISCs with the 5' cap as the primary target. More importantly, using this experimental system, the authors also demonstrated that the action taken by miRNAs on both ends of a target mRNA is independent from each other. Two studies (38, 39) using an *in vitro* assay also indicated that repression was a poly (A) tail-dependent event, manifested by the enhancement of silencing activity of miRNAs upon extending the length of the poly(A) tail alone.

Type II. Cap-independent inhibition by mi/siRNAs

Eukaryotic initiation factor 6 (eIF6), considered as an anti-association factor, is a ribosomal protein, which could prevent 80S ribosome assembly (43). Mi/siRNAs could inhibit the translation of targets by preventing the 60S ribosomal subunit from joining the 40S subunit via eIF6. Studies on humans and on *C. elegans* using both reporter mRNA and endogenous miRNA targets revealed that eIF6, which prevents the 60S subunit from joining the 40S subunit and thereby impedes 80S monosome assembly, was co-immunoprecipitated with miRISCs. This observation implies a role of eIF6 in miRNA-

mediated repression (43). On the other hand, depletion of eIF6 in human cells leads to a relief of repression of several reporters targeted by different miRNAs (43). The same effect of attenuating miRNA-directed repression was also observed when analyzing two lin-4 miRNA endogenous mRNA targets (lin-14 and lin-28) in *C. elegans* (43). MiRNA-mediated translational inhibition, by preventing the joining of the 60S subunit, is further evidenced by another *in vitro* assay conducted in a rabbit reticulocyte lysate, in which miRNA-targeted mRNAs were associated solely with 40S ribosome components and displayed the 40S subunit characteristic toe print (44). Similarly, in *Drosophila*, miRNAs inhibited the formation of the 48S translation initiation complex before the joining of the 60S ribosomal subunit (37). Lastly, Wang *et al*. (44) proposed a model, by which miRISC complexes use eIF6 to interfere with polysome formation on mRNAs, especially impeding the initial formation of translationally competent monosomes at the start codon of target mRNA.

However, the fact (45-47) that eIF6 is also functional in 60S ribosomal subunit biogenesis weakens the proposed model, since it is possible that eIF6 is indirectly involved in the miRNA-directed gene silencing pathway. This model is still under debate. First, no difference in miRNA-mediated repression was observed before and after knocking down eIF6 from *Drosophila* S2 cells (48), and no difference was observed in knockout mice with a single eIF6 allele deletion (46). Secondly, another investigation of *C. elegans* (49) revealed an opposite effect, that let-7-mediated repression is enhanced by depleting eIF6.

Of note, mi/siRNA-mediated translational repression either in a cap-dependent manner or in a cap-independent manner could be reconciled in one organism. In *Drosophila*, it is known that Ago1-RISC and Ago2-RISC employ different mechanisms to translationally repress their mRNA targets (50, 51). Ago1-RISC inhibits translation after the cap recognition stage, whereas Ago2-RISC represses the cap recognition step via binding to eIF4E, which interrupts the interaction between eIF4E and eIF4G.

2.1.3 Regulation of small RNA-mediated translational repression and translational activation by mi/siRNAs

Small RNAs are not limited to functioning by repression. mi/siRNAs can directly stimulate expression of their targets under certain conditions. Studies on miR122 and its endogenous target-*CAT-1* mRNA in Huh7 cells revealed a new feature of small RNA-mediated gene regulation. Under amino acid deprivation or other stress conditions, repressed target mRNAs could regain the accessibility to cellular translation machinery (52). This derepression process requires RNA-binding protein HuR, which binds to the AU-rich element within the CAT-1 mRNA 3'UTR region, possibly helping to disengage miRISCs from targeted mRNA (52). Subsequently, another RNA-binding protein Dead end 1 (Dnd1) was also shown to be involved in derepressing miRNA (miR-430)-directed repression in zebrafish and humans through binding to the U-rich elements in the 3'UTR of target mRNA. Since the Dnd 1 binding sites are very close to miRNA binding sites, the derepression could be achieved by the hindrance of accessibility of miRISCs to the target (53).

On the other hand, human Ago2 can directly initiate the activation of their targets when cells are undergoing certain stresses or in cell cycle arrest (54-56). Under serum starvation, several tested miRNAs, such as miR369-3, let-7, and the synthetic miRCXCR4, upregulated the expression of their corresponding target mRNAs. RNA binding protein-fragile X mental retardation-related protein1 (FXR1) was also identified as a requirement in this translational activation process through interacting with Ago protein (57). Again, miR122 has recently been reported to stimulate translation of hepatitis C virus RNA through reinforcing the association of ribosomes with target mRNAs (58). Taken together, miRNAs activate their targets translation when cells are at a quiescence stage, whereas miRNAs inhibit targets translation when cells are proliferating.

Furthermore, not only RNA binding proteins but also modifications on miRNA target sites have been implicated to contribute to the derepression of miRNA-mediated translational inhibition and to translational activation. Several studies (59, 60) showed that modifications on miRNA/target interactive sites, such as shortening or point mutation, can lead to instability or a complete loss of association between miRNA and target, thereby increasing expression of targets. These findings add a new layer of complexity and dynamics to miRNA regulation. Yet, the exact mechanisms of this new emerging aspect of miRNA-mediated gene regulation still remain unclear. Moreover, what is the determinant at the molecular level to control the switch from the repressive mode to the active mode? What are the main protein factors involved? These questions are yet to be answered.

2.2 Deadenylation, a cause, a result or completely independent of mi/siRNA-mediated translational repression

The mRNA poly (A) tail is very important for both mRNA stability and translation initiation. Therefore, researchers have begun to probe the possible connection between miRNA-targeted mRNA deadenylation and miRNA-mediated translational repression. However, the results are rather controversial. On one hand, experimental evidence from *in vitro* assays pointed directly to an essential role of targeted-mRNA deadenylation in miRNA-mediated translational repression. In rabbit reticulocyte lysate containing synthetic miRNA mimics and luciferase mRNAs with multiple binding sites, miRNA-mediated repression requires both a functional cap and a poly (A) tail, and the repression could be further boosted solely by lengthening the poly (A) tail (39). In addition, investigations of let-7 mediated translational repression *in vitro* further confirmed that let-7-directed suppression of reporter mRNA correlated tightly with mRNA deadenylation status, and more intriguingly, deadenylation occurred independently of the 5' cap structure of mRNA as well as mRNA translation activity (38). Finally, Wakiyama *et al.* (61) proposed a model explaining how miRNA-directed deadenylation contributes to translational inhibition: upon binding to targets, miRISC recruits GW182 and then the deadenylase complex, followed by deadenylation, and translation repression as a result of the disruption of the closed-loop formation.

By contrast, other data proved that miRNA-mediated translational repression still takes place normally independently of deadenylation of mRNA targets. mRNAs with 3'end

modifications either by displacing regular poly (A) tail with a histone 3' stem-loop or a 3' end generated by ribozyme, are nonetheless subject to miRNA-mediated translational repression (62, 63). In *Drosophila* S2 cells that lack CCR4-NOT major deadenylase (it is important for deadenylation), target mRNAs are stabilized, yet translational repression proceeds as normal, further suggesting that deadenylation could be completely uncoupled with translational inhibition process (64). A more recent study on zebrafish embryos strongly supports that translation inhibition by miRNAs is independent of deadenylation. These two outputs of miRNA-mediated silencing are actually conducted by different domains of GW182 protein (this will be further discussed in 2.3 of this review) in zebrafish-TNRC6A (65). These observations suggest that miRNA-directed mRNA deadenylation is neither sufficient nor necessary for translational repression. The controversial experimental data could be explained by the potential existence of multiple mechanisms for gene silencing by miRNAs.

2.3 GW182 and its role in mi/siRNA-mediated gene regulation

GW182 belongs to a conserved Glycine/Tryptophan (GW) repeats-containing protein group. GW182 is also the marker protein of subcellular cytoplasmic foci called P-bodies or GW bodies that serve as specific mRNA storage centers as well as mRNA degradation sites. Research in *Drosophila*, *C. elegans*, and human cells all deduced an essential role of GW182 in small RNA-mediated gene silencing (66-68). GW182 has been implicated in at least two miRNA-mediated gene regulatory pathways: non-cleavage decay of target mRNAs and translational repression (41). During mRNA decay, GW182 could act as a scaffold to bring miRISC and the deadenylase CCR4-NOT complex closer to the targets,

followed by deadenylation and decapping of target mRNAs (41). The second pathway is miRNA-mediated translational repression. That GW182 is indeed functional in miRNA-mediated translational repression is supported by evidence from several studies discussed below.

First, the interaction between GW182 and Ago protein is necessary for the miRNA response. The N terminus of the GW182 protein can bind to the C terminus of Ago (63). Studies by Eulalio *et al.* (48) showed that overexpression of the GW182 N terminal domain leads to inhibition of miRNA-mediated silencing because of interruption of GW182-Ago binding. The interaction between those two proteins is so important that tethering only the 3' terminal half of the GW182-binding motif of human Ago2 to the reporter can trigger a similar level suppression as the full-length Ago2 (69).

Secondly, GW182 itself is enough to trigger gene repression. Studies based on the tethering assay suggest that only tethering GW182 protein on the 3'UTR of an mRNA reporter without any miRNA binding sites is enough to trigger a similar level of gene downregulation as by miRNAs. From the tethering assay, it was demonstrated that tethering GW182 alone or tethering Ago alone contributes to comparable levels of repression of reporter expression (64). Genetic analyses in *Drosophila* further confirmed that the C-terminus of GW182 is actually a silencing effector domain, crucial for miRNA-mediated gene silencing (63).

Finally, a model can be proposed on how GW182 functions in miRNA-mediated translational repression. GW182 can directly bind poly (A) tail binding protein (PABP) and Ago and, in turn, interfere with closed-loop formation enhanced by eIF4G and PABP that is required for efficient translation initiation, leading to translation inhibition (36, 70). However, there are certainly additional factors involved in translational inhibition other than those affecting the association of eIF4G-PABP, as evidenced by the susceptibility to suppression of target mRNAs lacking poly (A) tails (41, 67).

2.4 A unified mechanism or multiple mechanisms?

Since the first finding of miRNA-mediated translational repression in *C. elegans* (25) and the subsequent findings about miRNA-directed mRNA degradation (62, 71), the past two decades witnessed a dramatic advance in understanding how mi/siRNAs control gene regulation posttranscriptionally. Yet, it is still unclear as to the exact molecular mechanisms of mi/siRNA-mediated silencing. For instance, do small RNAs direct translational repression through a common and unified mechanism among different species and cell types, or alternatively, are there multiple mechanisms used by small RNAs to regulate their natural targets according to the species, cell types, certain developmental stages, or even to different miRNA/target pairs?

To date, prevailing knowledge suggest that multiple mechanisms co-exist, and that sometimes, distinct mechanisms are functional within a single organism. One example is the case of Ago1-RISC and Ago2-RISC in *Drosophila* (50, 51). If we take a closer look from a mechanistic perspective, the initiation stage of translation is the most likely

primary target, possibly through interruption of the recognition of the 5' cap structure or the translation initiation complex assembly, which requires interaction with the GW182 protein (72). Actively translated mRNAs are in a closed-loop structure maintained by the interaction between PABP and eIF4G. When mRNAs are targeted by miRNAs, miRISCs complexes which include the Ago protein and the GW182 protein (at least in animals) are guided by miRNAs to load onto miRNA-binding sites in the mRNA target. GW182 associates with PABPs, which helps to recruit the deadenylation complex-NOT/CCR4/CAF1 to deadenylate target mRNAs. However, it is not clear whether deadenylation occurs before or after the miRNA-mediated translational repression. In addition, deadenylated mRNAs in animal systems can be stored in a translationally repressed state. Otherwise, deadenylated mRNAs in cell cultures could also undergo further decapping by the decapping complex and finally be degraded by 5'-to-3' exonuclease XRN1 (73). This could be mechanistically separated from the translational inhibition pathway (74).

In terms of miRNA-mediated translational repression, there are currently four types of hypothesized mechanisms in metazoans: inhibition of translational initiation, inhibition of translation elongation, co-translational degradation of nascent polypeptides, and premature termination. An inhibitory effect at the initiation stage of translation could be explained by the interruption of the closed-loop by GW182 proteins, in which, GW182 competes with eIF4G for binding to PABPs. Intriguingly, the investigation of *Drosophila melanogaster* cell-free translation system further extends this model: PABPs and the poly(A) tail initially enhance the binding of miRISCs to mRNA target. Then, the ensuing

PABPs displacement, which is further augmented by mRNA deadenylation, could contribute to miRISC-mediated translation repression (75). The mechanisms of inhibition at the elongation stage and the co-translational degradation of nascent polypeptides have not been investigated in this system (Fig1.) (74).

3. SMALL RNA-MEDIATED TRANSLATIONAL REPRESSION IN PLANTS

3.1 Regulation at the translational level

The nature of full complementarity or nearly-full complementarity between plant mi/siRNAs and their mRNA targets dictates that plant small RNAs act predominantly through RNA cleavage, as opposed to small RNA-guided translational inhibition in metazoans. Insights on mechanisms of mi/siRNAs action in plants have been mainly gained from studies carried out on the land plant *Arabidopsis thaliana*. Interestingly, increasing evidence from the past few years suggested that in addition to performing the canonical endonucleolytic cleavage function, plant mi/siRNAs can also control targeted gene expression by translational repression (76-80). More importantly, this rather newly-discovered pathway directed by plant small RNAs is not unique to *Arabidopsis*. Similar observations have been made from algal species like the marine diatom *Phaeodactylum tricornutum* (81) and the unicellular green alga *Chlamydomonas reinhardtii* (Chapter 2).

In *Arabidopsis*, the interactions among several miRNA/target pairs-miR172/*APETALA2*, miR398/CSD1 or CSD2 (two Cu/Zn superoxide dismutases), miRNA156/157/*SPL3* (a SBP box gene) usually lead to the corresponding target mRNAs to be affected mainly at

the translational level (76, 78, 79, 82). Unlike the extensive work done in metazoan systems, experimental data from plants are largely confined to the phenotypic level. Few studies have been carried out from a mechanistic perspective. Broderson *et al*. (77) took advantage of forward genetic screening for silencing-defective mutants of a constitutively expressed GFP reporter containing a miR171 target site and identified three classes of mutants: Class I-*microRNA biogenesis deficient* (*mbd*) mutants; Class II-*microRNA action deficient* (*mad*) mutants- affected at the mRNA level; and Class III- microRNA action deficient (mad) mutants- affected at the protein level. In terms of class III *mad5* and *mad6* mutants, further investigations of several endogenous miRNA targets showed consistent upregulation of protein expression with no obvious changes at the mRNA level compared to wild type control. Particularly, when researchers chose these tested mRNA targets, they also took into account the location of the miRNA binding sites. These miRNA binding sites are located in the 5'UTR, coding sequence, or the 3'UTR of mRNA targets. Moreover, it was tested whether this mode of action is true for plant siRNA-mediated posttranscriptional silencing by introducing *mad6* and *ago1-27* into a well-established SUC-SUL (SS) RNAi silencing system in *Arabidopsis*. Molecular analyses of the SUL protein, mRNA, and siRNA levels in the SS×ago1-27 plant revealed that there was a clear increased level of SUL protein with no changes at the mRNA and siRNA levels compared to those in the parental SS line. To summarize, these data demonstrated that plant miRNAs and siRNAs are able to operate by translational repression regardless of target site locations.

3.2 Current models of small RNA-mediated translational repression in plants

Above all, in plants, miRNAs or siRNAs bind to perfectly or near-perfectly complementary sites located mainly in the open reading frame (ORF) of target mRNAs, triggering endonucleolytic cleavage and/or translational repression. In the former case, the enzymatic activity responsible for the endonucleolytic cleavage resides in the piwi domain of Ago protein and the resulting 5'- and 3'-cleavage product can be further degraded by the exosome and the exonuclease XRN4 (corresponding to XRN1 in animals), respectively. On the other hand, during translational repression, the endonucleolytic enzymatic activity is somehow prevented, and thus mi/siRNAs can inhibit translation of targets by an unknown mechanism (Fig 2.) (73). Intriguingly, Voinnet's group found mechanistic similarities of small RNA-mediated translational inhibition between plants and animals. Several common functional elements in this pathway have been identified (77): The Argonaute proteins (AGO1 and AGO10), Katanin (KTN-1) which encodes the catalytic subunit of the microtubule-severing enzyme and is involved in microtubule dynamics, and the mRNA decapping factor VCS (the homolog of animal decapping complex component Ge-1). Similar to the requirement for tubulins in miRNA action in *C. elegans*, the identification of KTN-1 in *Arabidopsis* further links cytoskeleton dynamics to miRNA function (83). The discovery of VCS in small RNA-mediated translational repression in *Arabidopsis* suggests that for some miRNA targets, translational inhibition and mRNA decay are possibly coupled (41). A study by Lanet *et al*. (80) provided the first piece of biochemical evidence to demonstrate translational repression directed by *Arabidopsis* miRNAs. The authors found that a portion of several microRNAs tested co-sedimented with actively translated polysomal fractions along with AGO1, the linkage of which was most likely through the target

mRNAs. Furthermore, the association between miRNAs and polysomes is dependent on AGO1 activity as well as the miRNAs themselves. The comparison of the hypomorphic *ago1* mutant with the slicing-inhibited 2b mutant regarding the transcript and protein level of three miRNA targets-*AGO1*, *CIP4*, and *CSD2*, indicates that the small RNA-mediated translational repression pathway might be genetically separable from the RNA cleavage pathway.

The goal of this study:

RNAi has been implicated in a variety of applications, such as in medicine and agriculture (84, 85). To delineate the precise mechanisms acted by RNAi will certainly contribute to its applications. In *Chlamydomonas*, RNAi can be easily achieved by the introduction of inverted repeat (IR) containing transgenes (86). Translational inhibition by RNA interference (RNAi) is a widespread phenomenon in animals, although the molecular mechanism(s) is not well understood. In contrast, there is limited evidence for a role of small RNAs in translational repression in plants and fungi. The major goal of my research project is to examine this mechanism in the single celled organism *Chlamydomonas reinhardtii*.

Figures

Figure 1-1. Model for miRNA-mediated repression in metazoans [modified from (74)]**.** GW182 interacts with one member of the Argonaute family of proteins, upon the interaction between miRNAs and their targets. Downstream of this step, there are different pathways. Which is functional is probably dependent on the composition of the RNA-induced silencing complex (RISC) and interaction with mRNA-or miRNA-ribonucleoprotein (mRNP or miRNP) complex, and/or the specific cell context.

(a) The primary non-cleavage degradation pathway mediated by GW182, followed by de-capping and mRNA decay via NOT/CCR4/CAF1 deadenylation complexes. This is considered independent from the translation repression pathway.

(b) GW182 interaction with eIF4G, preventing it from associating with poly-A binding protein (PABP). This interaction hinders the circularization (i.e., head to tail interaction) of mRNAs required for efficient translation. This represents one type of initiation block.

(c) The 60S ribosome subunit is prevented from joining to the 40S ribosome subunit. The formation of 80S ribosomes is inhibited. This represents a different type of initiation block.

(d) A translation elongation block: slowed or stalled ribosomes along the mRNA.

(e) Premature translation termination.

(f) Co-translation degradation of nascent polypeptides.

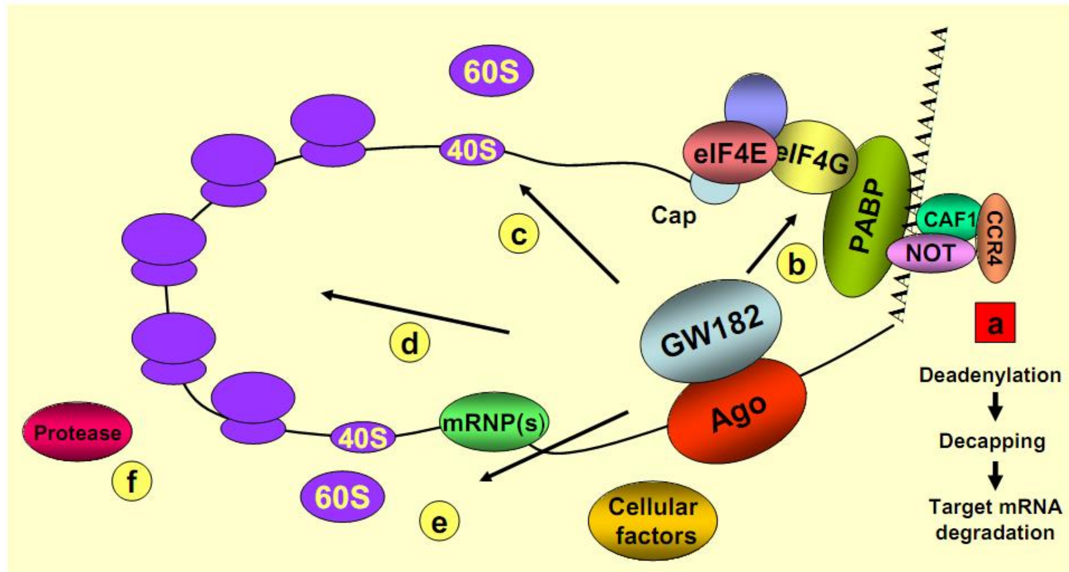
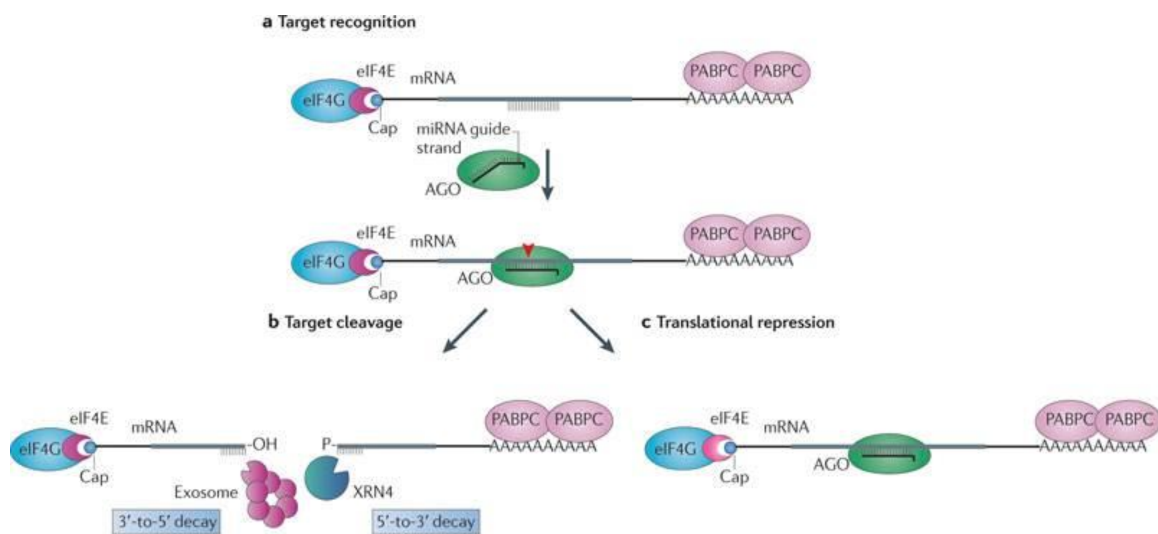

60S
40S
eIF4E
eIF4G
Cap
PABP
CAF1
CCR4
NOT
c
b
a
d
GW182
Ago
Deadenylation
Decapping
Target mRNA
degradation
Protease
f
mRNP(s)
40S
60S
e
Cellular
factors

Figure 1-2. Model for miRNA-mediated repression in plants [modified from (73)].

(a) Plant microRNAs (miRNAs) bind to Argonaute (AGO) and recognize mRNA targets with fully or nearly complementary binding sites located mainly in the ORF.

(b) Plant AGOs can endonucleolytically cleave the mRNA target within the seed region (between nucleotides 10 and 11, opposite the miRNA strand, indicated by the red arrow head). The cleavage products are further degraded by the exosome (3'-5' decay) and the exonuclease XRN4 (5'-3'decay), respectively.

(c) Alternatively, the "slicer" activity of the RISC complex is somehow prevented and the mRNA target is repressed at the translation level by an uncharacterized mechanism.


a Target recognition
eIF4E
mRNA
eIF4G
Cap
PABPC
PABPC
AAAAAAAAAA
miRNA guide strand
AGO
eIF4E
mRNA
eIF4G
Cap
AGO
PABPC
PABPC
AAAAAAAAAA
b Target cleavage
c Translational repression
eIF4E
mRNA
eIF4G
Cap
-OH
P-
PABPC
PABPC
AAAAAAAAAA
Exosome
XRN4
3′-to-5′ decay
5′-to-3′ decay
eIF4E
mRNA
eIF4G
Cap
AGO
PABPC
PABPC
AAAAAAAAAA

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CHAPTER 2

Small Interfering RNA-Mediated Translation Repression Alters Ribosome Sensitivity to Inhibition by Cycloheximide in *Chlamydomonas reinhardtii*

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Abstract

Small RNAs (~20-30 nt in length) play important roles in gene regulation as well as in defense responses against transposons and viruses in eukaryotes. Their biogenesis and modes of action have attracted great attention in recent years. However, many aspects of small RNA (sRNA) function such as the mechanism(s) of translation repression at post-initiation steps remain poorly characterized. In the unicellular green alga *Chlamydomonas reinhardtii*, sRNAs derived from genome integrated inverted repeat transgenes, perfectly complementary to the 3' UTR of a target transcript, can inhibit protein synthesis without or with only minimal mRNA destabilization. The sRNA-repressed transcripts are not altered in their polyadenylation status and they remain associated with polyribosomes, indicating inhibition at a post-initiation step of translation. Interestingly, ribosomes associated with sRNA-repressed transcripts show reduced sensitivity to translation inhibition by some antibiotics such as cycloheximide, both in ribosome run-off assays and in *in vivo* experiments. Our results suggest that sRNA-mediated repression of protein synthesis in *Chlamydomonas* may involve alterations to the function/structural conformation of translating ribosomes. Additionally, sRNA-mediated translation inhibition is now known to occur in a number of phylogenetically diverse eukaryotes suggesting that this mechanism may have been a feature of an ancestral RNAi machinery.

Introduction

RNA-mediated silencing is an evolutionarily conserved process in eukaryotes by which small RNAs induce the inactivation of cognate sequences through a variety of mechanisms, including translation repression, RNA degradation, transcriptional inhibition, and/or, in a few organisms, DNA elimination (1-5). Intriguingly, recent studies indicate that these non-coding RNAs may also participate in transcriptional or translational activation (2, 6, 7). Despite the mechanistic diversity of these processes, in most characterized pathways, sRNAs (~20-30 nucleotides in length) are incorporated into effector complexes containing at their core Argonaute proteins, which include two major subfamilies of polypeptides named after *Arabidopsis thaliana* ARGONAUTE1 (AGO1) and *Drosophila melanogaster* P-element induced wimpy testis (PIWI) (2, 3, 8-10). Some AGO-PIWI proteins function as sRNA-guided endonucleases ("slicers") that cleave complementary transcripts whereas others lack endonucleolytic activity and repress their targets through other mechanisms (3, 4, 10, 11).

Three major classes of sRNAs have been recognized in metazoans: microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs) (3, 5, 12, 13). Land plants and green algae lack PIWI proteins and contain only miRNAs and siRNAs that associate with members of the AGO clade (1, 13, 14). miRNAs commonly originate from endogenous, single-stranded non-coding RNA transcripts or introns that fold into imperfectly paired hairpin structures. They often modulate the expression of genes with roles in development, physiological or metabolic processes, or stress responses (1, 3-5, 12, 13). siRNAs are produced from long, near-perfect complementarity double-stranded RNAs (dsRNAs) of diverse origins (1, 3, 5, 13). In higher plants and

algae, these siRNAs play various roles in suppression of viruses and transposable elements, post-transcriptional regulation of gene expression, DNA methylation, and/or heterochromatin formation (1, 15, 16). Despite considerable advances in our understanding of the biogenesis and function of sRNAs (1-5, 10, 12, 13), key mechanistic aspects of their mode of action remain poorly characterized.

The degree of complementarity between a sRNA and its target site has been considered a main determinant of the post-transcriptional repression mechanism (1, 3, 4, 12). Highly complementary sRNA-mRNA hybrids, with perfect central pairing, activate Argonaute-mediated endonucleolytic cleavage of target transcripts (3, 9, 10, 11). This is the best-characterized mechanism of post-transcriptional silencing mediated by siRNAs and, in land plants, by many miRNAs (1, 3, 4, 17, 18). Conversely, imperfect sRNA-mRNA hybrids, with central bulges or mismatches, enable translational inhibition and/or accelerated exonucleolytic ("slicer" independent) transcript decay; the prevalent mode of repression involving metazoan miRNAs (2, 4, 10, 12). Interestingly, recent evidence indicates that sRNAs perfectly complementary to a target mRNA can also cause translational inhibition without, or with only minimal, transcript destabilization (1, 15, 19-21). This outcome may result from the association of sRNAs with Argonautes that lack endonucleolytic activity (11, 21). However, siRNA-programmed AGO proteins, known to possess the predicted catalytic motif, can also fail to cleave (3, 10, 11), suggesting that our understanding of the determinants of the Argonaute "slicer" activity is insufficient and/or that associated factors may modulate AGO endonucleolytic activity.

Over the past few years, remarkable progress has been made in our understanding of the mechanism(s) of miRNA-mediated post-transcriptional silencing in metazoans, but no consensus has emerged yet unifying all current observations (2-4，22，23). Animal miRNAs have been proposed to repress translation in at least four distinct ways: inhibition of translation initiation, inhibition of translation elongation, co-translational degradation of nascent polypeptides, and premature termination of translation (2, 4, 24-31). miRNAs can also promote sequestration of target mRNAs in discrete cytoplasmic foci, either processing bodies or stress granules (32, 33), but this localization may be a consequence of silencing rather than a requirement for translation repression (4, 34, 35). Additionally, genome wide proteomic and transcriptomic analyses, after the removal or the ectopic expression of miRNAs, have suggested that the "slicer" independent degradation of miRNA targets may account for most of the stable repression mediated by miRNAs in mammalian cell cultures (4, 36-39). One possible explanation for all these disparate and sometimes conflicting observations is that metazoan miRNAs may regulate target transcripts via multiple, interrelated mechanisms that can be modulated by AGO-associated factors and target mRNA effects. Indeed, AGO-binding GW-repeat proteins (TNRC6/GW182-like) have been shown to interact with cytoplasmic poly(A) binding protein and with the CCR4-NOT and PAN2-PAN3 deadenylase complexes leading to mRNA deadenylation as well as translation repression (2, 4, 23, 40-42); although there is also increasing evidence for miRNA-mediated translation inhibition in a deadenylation-independent manner (2, 22, 23, 43-45). Depending on the cell type and/or specific target, mRNAs may be maintained in a translationally repressed state or rapidly degraded (2, 4, 22, 44, 46).

Small RNAs can also cause translation repression in land plants. In *Arabidopsis*, the transcripts of *APETALA2*, a target of miR172, the SBP-box gene *SPL3*, a target of miR156/157, and two copper/zinc superoxide dismutases (*CSD1* and *CSD2*) as well as the copper chaperone for superoxide dismutase (*CCS1*), targets of miR398, were found to be regulated by miRNA-mediated translation inhibition (15, 47-51). Mutations in two genes implicated in sRNA function (encoding the microtubule-severing protein KATANIN and the enhancer of decapping protein VARICOSE) were shown to increase polypeptide levels of several miRNA-regulated genes without causing a corresponding change in the abundance of their mRNAs (1, 20). Moreover, *Arabidopsis* AGO1 and a subset of miRNAs have been demonstrated to associate with polyribosomes, consistent with a role for miRNAs in translation inhibition (52). Indeed, translational regulation may be an important aspect of miRNA function in *Arabidopsis* based on the phenotypes of loss-of-function mutants of *SUO*, coding for a large GW-repeat polypeptide involved in miRNA-mediated repression of protein synthesis (53). However, SUO does not appear to be an ortholog of animal TNRC6/GW182 and the mechanism(s) by which small RNAs inhibit translation in higher plants remains uncharacterized.

Translation inhibition mediated by sRNAs may also operate in unicellular eukaryotes. In the parasitic protozoan *Giardia lamblia*, sRNAs have been shown to repress the expression of reporter genes containing sRNA target sites in their 3'-untranslated regions (UTR) without changes in transcript levels (54, 55). Likewise, in the marine diatom *Phaeodactylum tricornutum*, transformation with an inverted repeat transgene, producing

dsRNA homologous to a phytochrome gene, did not alter target mRNA amounts but significantly reduced cognate protein abundance (56). These observations are consistent with sRNA-mediated translation inhibition, which also occurs in the unicellular green alga *Chlamydomonas reinhardtii*. Here, we show that transgenic siRNAs perfectly complementary to a target transcript can repress protein synthesis at a post-initiation step. Moreover, ribosomes associated with a siRNA-repressed transcript display reduced sensitivity to inhibition by the antibiotic cycloheximide, suggesting that the silencing mechanism(s) alters the function/structural conformation of translating ribosomes.

Results

Inverted Repeat Transgenes Can Trigger Translation Repression of Homologous Endogenous Transcripts

In *C. reinhardtii*, RNA interference (RNAi) has been achieved, among other approaches, by the production of hairpin dsRNA from genome-integrated inverted repeat (IR) transgenes (16). The transcribed dsRNA is processed into siRNAs and, in most cases, reduction in the steady-state levels of target mRNAs is observed (57, 58), implying RNAi-induced transcript degradation. For instance, transformation of *Chlamydomonas* with an IR construct targeting the 3’ UTR of *Amino Acid Carrier 5* (*AOC5*) (Figure 2-8A), encoding a putative basic amino acid permease, results in transgenic lines tolerant to the arginine analog L-canavanine (Figure 2-1A). These strains contain ~22-nt *AOC5* siRNAs and the *AOC5* mRNA amount is significantly reduced (Figure 2-1B).

L-canavanine is a non-proteinogenic α-amino acid structurally related to L-arginine. However, its incorporation in place of arginine during protein translation can generate functionally aberrant polypeptides and eventual cell death (59). Suppression of expression of the AOC5 transporter in the *Chlamydomonas* RNAi strains likely diminishes L-canavanine uptake, allowing cells to survive and grow in the presence of this compound (Figure 2-1A). Intriguingly, ~10% of the transgenic lines showed the expected survival on medium containing L-canavanine (e.g., Figure 2-1C, Aoc5-IR6) but no reduction in the *AOC5* mRNA level (e.g., Figure 2-1D, Aoc5-IR6). These strains were obtained at a frequency much higher than expected for conventional genetic mutation (i.e., natural mutations disrupting the *AOC5* gene) and they displayed no obvious alteration of the endogenous *AOC5* locus, when examined by Southern blotting and hybridization (data not shown). Thus, these observations raised the possibility that IR-mediated suppression of *AOC5* gene expression could occur at the translational level in a subset of *Chlamydomonas* transformants.

To explore whether RNAi was functional in *Chlamydomonas* strains with no significant alteration in target transcript levels we used a tandem IR system, previously demonstrated to suppress simultaneously co-targeted genes (57，60). A hairpin-forming construct homologous to part of the coding sequence of *Cre16.g662000*, encoding a putative RNA helicase, was engineered inside the *AOC5* inverted repeats (Figure 2-8B). Transformation of *Chlamydomonas* with this tandem IR transgene and selection on L-canavanine containing medium allowed the recovery of strains showing reduced transcripts levels for both *AOC5* and *Cre16.g662000* (data not shown). However, as observed before with the

single *AOC5* IR strains, ~5-10% of the tandem IR transformants were able to grow in the presence of L-canavanine (e.g., Figure 2-1C, Aoc5/Helic-IR4) without any obvious change in the *AOC5* mRNA abundance (e.g., Figure 2-1D, Aoc5/Helic-IR4). Interestingly, the *Cre16.g662000* transcript was considerably down-regulated in the same transgenic lines (e.g., Figure 2-1D, Aoc5/Helic-IR4). Since the tandem IR transgene directs production of siRNAs homologous to both *AOC5* and *Cre16.g662000* and the reduction in *Cre16.g662000* mRNA amount is indicative of functional RNAi, these results are consistent with *AOC5* being repressed at the translational level in a subset of transgenic strains. However, we were unable to test this hypothesis directly due to lack of an antibody to assay AOC5 protein abundance.

To examine more conclusively whether IR transgenes can suppress gene expression by translation inhibition in *Chlamydomonas* we used an alternative system. Tryptophan synthase β subunit (TSβ, encoded by the *MAA7* gene) is required to convert the indole analog 5-fluoroindole (5-FI) into the toxic tryptophan analog 5-fluorotryptophan. RNAi-mediated suppression of *MAA7* in *Chlamydomonas*, triggered by dsRNA produced from IR transgenes, results in strains resistant to 5-FI which have reduced *MAA7* transcript levels (57). However, ~10% of the *Chlamydomonas* transformants containing an IR transgene designed to produce dsRNA homologous to the *MAA7* 3' UTR showed tolerance to 5-FI (Figure 2-2A) and significantly reduced levels of the TSβ protein, as detected by immunoblotting assays (Figure 2-2B), without any marked change in the *MAA7* mRNA amount (Figure 2-2C; Figure 2-9A). Taken together, our observations strongly suggest that inverted repeat transgenes can induce translation repression of

targeted transcripts in *Chlamydomonas*, although it remains unexplained why the same construct can trigger primarily either mRNA destabilization or inhibition of protein synthesis in different transgenic lines.

siRNAs Are Required for the Translation Repression Mediated by Inverted Repeat Transgenes

The Maa7-IR transgenic lines with marked reduction of the TSβ protein content without changes in *MAA7* transcript levels contain detectable amounts of *MAA7* siRNAs (Figure 2-2D).

To test whether siRNAs are required for the observed suppression of TSβ protein production in *C. reinhardtii*, we identified a deletion mutant of *Exportin 5* (*Cre10.g420400*) (Figure 2-10) by screening a library of insertional mutants generated in the Maa7-IR44s background. In metazoans, Exportin 5 (EXP5), a member of the importin-β/karyopherin family of proteins, mediates the nuclear export of miRNA precursors (pre-miRNAs) and its depletion results in diminished miRNA amounts (62, 63). The *Arabidopsis* ortholog of EXP5, HASTY, also appears to be required for the biogenesis (presumably through the nuclear export of Dicer-processed duplex small RNAs) and/or the stability of some miRNAs since mutant plants show a general reduction in miRNA levels (64). Likewise, in *Chlamydomonas* depletion of the EXP5 ortholog causes a decrease in the abundance of each of four miRNAs selected for analysis (Figure 2-10C, Maa7-IR44s(*exp5*)). The *exp5* mutation likely results in a null phenotype because almost the entire *EXP5* gene is deleted in the *Chlamydomonas* mutant (Figure 2-

10A) and no *EXP5* transcript is detected in Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) assays (Figure 2-10B, Maa7-IR44s(*exp5*)).

The *EXP5* deleted *Chlamydomonas* strain, Maa7-IR44s(*exp5*), becomes sensitive to 5-FI, as expected for a defect in the RNAi-mediated down-regulation of *MAA7* expression (Figure 2-3A). Moreover, it contains TSβ protein amounts quite similar to those in the wild type strain (Figure 2-3B) without appreciable changes in the *MAA7* transcript abundance (Figure 2-3C). Notably, *MAA7* siRNA levels are greatly reduced in the mutant background and barely detectable after prolonged exposure to a phosphorimager screen (Figure 2-3D). To our knowledge, EXP5 plays no direct role in protein translation and, if anything, its depletion might have an overall detrimental effect on protein synthesis due to EXP5 role in the nuclear export of tRNAs (62, 63). Thus, the observed accumulation of TSβ protein in the *Chlamydomonas exp5* mutant is most likely a reflection of the requirement for siRNAs (much diminished in the mutant background) as effectors of the translation repression triggered by inverted repeat transgenes.

siRNA-Mediated Translation Repression of the *MAA7* Transcript Occurs at a Post-Initiation Step

In metazoans mRNA deadenylation is a widespread (although not universal) consequence of miRNA regulation (4, 23, 44-46). Thus, to begin addressing the mechanism of translation repression mediated by inverted repeats in *Chlamydomonas*, we examined first whether the poly(A) tail length is reduced in the siRNA-repressed *MAA7* transcripts. However, we could find no change in the polyadenylation status of this mRNA and of a

control transcript encoding actin (*ACT1*) in Maa7-IR44s in comparison with the wild type and the Maa7-IR44s(*exp5*) strains (Figure 2-3E; Figure 2-11). Thus, siRNA mediated translation inhibition of *MAA7* in *Chlamydomonas* seems to occur in a deadenylation-independent manner.

We next carried out polyribosome profiling to examine whether translation was being repressed at initiation or post-initiation steps. If translation were inhibited at initiation, the *MAA7* transcript would be expected to shift to lighter fractions (with fewer or no ribosomes) when separating Maa7-IR44s cell extracts on sucrose sedimentation gradients. In contrast, if protein synthesis were inhibited after initiation, the *MAA7* mRNA would be expected to associate with heavier polyribosomal fractions in the translationally repressed transgenic line, although the exact distribution would vary depending on the specific translation step being affected. Cells from the wild type, Maa7-IR44s and Maa7-IR44s(*exp5*) strains were treated with a high concentration of cycloheximide (150 μg/ml) to arrest translating ribosomes, resuspended in lysis buffer containing the antibiotic, and broken by one passage through a French press. Lysates were then fractionated by sucrose density gradient ultracentrifugation to separate free mRNAs from those associated with varying numbers of ribosomes. The presence of the *MAA7* transcript, the control *ACT1* mRNA, and the *18S rRNA* in each fraction of the gradient was assayed by slot blot hybridization (Figure 2-4A).

Interestingly, *MAA7* transcripts were found similarly associated with polyribosomal fractions in the three strains examined (Figure 2-4A and 2-4B), regardless of the TSβ

protein accumulation (Figure 2-3B) or the *MAA7* siRNAs content (Figure 2-3D). However, consistent with siRNA-mediated translation repression, part of the *MAA7* siRNAs co-migrated with polyribosomes in the Maa7-IR44s samples whereas these siRNAs were practically undetectable in the heavier gradient fractions of the Maa7-IR(*exp5*) strain (Figure 2-4C, lanes 7-10). In Maa7-IR44s, the proportion of *MAA7* siRNAs associated with polyribosomal fractions (relative to the total *MAA7* siRNA amount) was much larger than that of an endogenous *Chlamydomonas* miRNA such as miR912 (Figure 2-4C). By contrast, in a previously characterized transgenic strain, Maa7-IR5, containing the same *MAA7* IR construct as Maa7-IR44s but inducing target transcript destabilization rather than translation repression (57), the *MAA7* siRNAs were more abundant in the ribosome-free portion of the gradient and virtually absent from the polyribosomal fractions (Figure 2-12).

To determine further whether the fast-sedimenting mRNAs and siRNAs were indeed associated with polyribosomes, we treated lysates with EDTA, known to chelate Mg^{2+} and dissociate translating cytosolic ribosomes into their 40S and 60S subunits (65). This caused, as expected, redistribution of all tested RNAs to the subpolysomal region of the gradient (Figure 2-13). As EDTA may also disrupt some non-ribosomal ribonucleoprotein complexes, we also treated cells with puromycin, prior to cell breakage, in an attempt to induce premature termination of elongating peptide chains and specific disassembly of translating ribosomes (27, 28, 66). However, this treatment caused only a minor reduction in the polyribosomal fractions in *Chlamydomonas* (similarly in the three strain examined), presumably because of poor drug uptake (data not shown). The

sedimentation patterns of the *MAA7* and *ACT1* transcripts in puromycin-treated cells were again indistinguishable among the wild type, Maa7-IR44s, and Maa7-IR44s(*exp5*) (data not shown). Thus, the *MAA7* mRNA appears to associate predominantly with *bona fide* translating ribosomes in the three examined strains (Figure 2-14A). These observations led us to conclude that *MAA7* siRNAs repress translation of the target transcript primarily at a post-initiation stage in the *Chlamydomonas* Maa7-IR44s transgenic line.

Ribosomes Subjected to siRNA-Mediated Translation Repression Show Lower Sensitivity to Inhibition by Cycloheximide

The association of *MAA7* transcripts with polyribosomes in the Maa7-IR44s strain suggested several possible mechanisms of siRNA-mediated protein synthesis inhibition including effects on translation elongation, termination, and/or degradation of nascent polypeptides. We therefore attempted to assess functional differences, between the wild type and Maa7-IR44s strains, in the ribosomes associated with the *MAA7* mRNA. In the absence of cycloheximide, protein translation proceeds for a short time in cell extracts partly depleting ribosomes from mRNA templates (67). This ribosome run-off assay can be used to evaluate the stability of ribosome association with transcripts, which will depend on the elongation rate and susceptibility to premature termination. Cell extracts from the wild type and Maa7-IR44s strains were prepared in lysis buffer lacking cycloheximide and containing 150 mM KCl and 5 mM Mg^{2+}, ionic conditions near the optimum for *in vitro* protein synthesis (68). As expected, upon sucrose density gradient ultracentrifugation, the polyribosomal component in these extracts was much reduced

(Figure 2-5A) in comparison with that observed in high cycloheximide-treated cells (Figure 2-4A). However, the new experimental conditions were uninformative as to the function of siRNA-repressed ribosomes since we found no significant difference in the *MAA7* transcript (and the control *ACT1* mRNA) gradient distribution between the two examined strains (Figure 2-5A and 2-5B; Figure 2-14B).

Cycloheximide is a potent inhibitor of protein synthesis with high specificity for eukaryotic ribosomes. This antibiotic has been reported to inhibit translation elongation by binding to the large ribosomal subunit Exit (E) site, stalling translocation as a consequence of the occupation of the E site by both cycloheximide and a deacylated tRNA (69-71). We next tested low concentrations of cycloheximide (30 μg/ml) to reduce the rate of elongation in the ribosome run-off assay rather than totally inhibit this process. We reasoned that slowing down elongation might increase polyribosomal association if a transcript was being translated by ribosomes already partly repressed at the elongation step whereas a normally translated mRNA might be affected to a lesser degree. Under these low cycloheximide conditions the overall abundance of polyribosomes (Figure 2-6A) was intermediate between the high cycloheximide (Figure 2-4A) and the no cycloheximide (Figure 2-5A) treatments for both tested strains. Intriguingly, in the low cycloheximide ribosome run-off experiments, the *MAA7* transcript was moderately but consistently depleted from the polyribosomal fractions in the translationally repressed Maa7-IR44s strain relative to the wild type (Figure 2-6A and 2-6B; Figure 2-14C). As a control, the distribution of the *ACT1* mRNA in the sucrose density gradients was virtually identical in both examined strains (Figure 2-6A and 2-6B; Figure 2-14C). These results,

although unexpected based on our initial reasoning, provided the first evidence for a functional difference(s) between the ribosomes associated with the *MAA7* transcript in the wild type strain (translationally competent) and the Maa7-IR44s strain (translationally inhibited by a siRNA-dependent mechanism). Similarly, when cells were treated with low concentrations of cycloheximide, the *AOC5* mRNA was also moderately depleted from polyribosomal fractions in the repressed Aoc5/Helic-IR4 strain relative to the CC-124 control (Figure 2-15), suggesting that these observations are indicative of a general feature of siRNA-inhibited ribosomes.

To gain further insight on the function of siRNA-repressed ribosomes we examined the effect of different antibiotics and growing conditions on the accumulation of the TSβ protein *in vivo*. We were particularly interested in testing whether siRNA-mediated translation repression was altered by antibiotics with dissimilar modes of action, inhibiting distinct ribosome functions. A sub-lethal concentration of cycloheximide severely compromised survival of the Maa7-IR44s strain in medium containing 5-FI (Figure 2-7A), consistent with greater *MAA7* expression. In contrast, this treatment had little effect on the phenotype of the previously characterized Maa7-IR5 strain containing the same *MAA7* IR construct integrated into its genome but inducing target transcript destabilization rather than translation repression (57). In cells growing in liquid medium, exposure to low cycloheximide for 18 h (see Methods) had a similar inhibitory effect on overall translation in the three examined strains, wild type, Maa7-IR44s and Maa7-IR5, as reflected by a comparable reduction in histone H3 levels (Figure 2-7B, cf., Ctrl and Chx panels). However, the TSβ protein amount uniquely increased in Maa7-IR44s

subjected to sub-lethal cycloheximide concentrations (Figure 2-7B, cf., Ctrl and Chx panels), whereas the antibiotic did not affect TSβ protein levels (a fairly stable polypeptide) in the wild type or the Maa7-IR5 strains. This suggested that cycloheximide treatment is of no consequence for RNAi-triggered mRNA destabilization in Maa7-IR5. No change in the original *MAA7* mRNA abundance was observed in any of these strains during the 18 h experimental period (data not shown).

Paromomycin, an aminoglycoside antibiotic which influences the decoding center of the ribosome, translation fidelity, and perhaps an early stage of translation after initiation (72, 73), did not alter the survival on 5-FI containing medium or TSβ protein amount of Maa7-IR44s (Figure 2-16A and 2-16B), despite being as effective at inhibiting histone H3 accumulation as cycloheximide (Figure 2-16B). Likewise, anisomycin, an antibiotic that binds to the Aminoacyl (A) site of the large ribosomal subunit and inhibits translation elongation by competing with the binding of aminoacyl-tRNA to the peptidyltransferase center (74, 75), did not affect survival on 5-FI or TSβ protein levels of Maa7-IR44s (Figure 2-16C and 2-16D). Merely reducing growth rate (and overall protein synthesis) by culturing cells in minimal medium also had no apparent consequence on TSβ protein expression in Maa7-IR44s, based on the strain's ability to survive and grow in the presence of 5-FI (Figure 2-16E). Treatment with sub-lethal concentrations of a fourth antibiotic, hygromycin B, slightly increased TSβ protein accumulation in Maa7-IR44s (Figure 2-7B) and reduced to some extent survival of the strain on 5-FI containing medium (Figure 2-7A). Interestingly, hygromycin B seems to have a mode of translation inhibition that differs from other aminoglycoside antibiotics.

In addition to affecting decoding fidelity, it appears to have, like cycloheximide, an inhibitory effect on the translocation of mRNA and tRNAs on the ribosome (72, 73).

The observed effects of cycloheximide, and to a much lower degree of hygromycin B, on TSβ protein accumulation in the Maa7-IR44s strain are unlikely to be indirect, such as destabilization of a short lived protease required for TSβ degradation or of a polypeptide specifically involved in siRNA-mediated translation repression, since, if this were the case, paromomycin and anisomycin would be expected to have a similar consequence on TSβ protein content. Additionally, since *MAA7* suppression by target mRNA destabilization in the Maa7-IR5 strain was not affected by the cycloheximide or hygromycin B treatments, general depletion of core components of the RNAi machinery also appears unlikely. Moreover, exposure to low concentrations of cycloheximide distinctly altered ribosome association with *MAA7* or *AOC5* transcripts, assessed by sucrose density gradient ultracentrifugation, when comparing Maa7-IR44s or Aoc5/Helic-IR4 with the wild type strain (Figure 2-6A and 2-6B; Figure 2-14C; Figure 2-15). These observations, taken together, suggest that ribosomes translationally repressed by siRNAs are differentially (less) sensitive to inhibition by cycloheximide, and to some extent hygromycin B, implying that the siRNA machinery imposes some alteration on normal ribosome function/structure.

Discussion

RNAi has been developed as a practical tool to study gene function in a few algal species (16). In *Chlamydomonas*, hairpin-forming transcripts produced from genome integrated IR transgenes have been successfully used to down-regulate the expression of a number of endogenous genes (16, 58). In most cases, reduction of the steady-state level of targeted mRNAs was observed, implying RNAi-mediated transcript degradation (16, 57, 58). However, in a few instances, discrepancies between protein and mRNA amounts, suggestive of inhibitory effects on translation, have also been reported. For example, a *Chlamydomonas* transgenic line containing an IR transgene designed to suppress expression of *Chlamyopsin*, coding for an opsin related protein, displayed a 50-fold reduction in protein abundance but only a 3-fold decrease in transcript amount, in comparison with the wild type strain (76). In land plants and animals there is convincing evidence that siRNAs perfectly complementary to a target mRNA can mediate translation repression in addition to mRNA degradation (15, 19-21). Our results indicate that this phenomenon also occurs in the unicellular green alga *C. reinhardtii*, triggered by *MAA7* IR transgenes (Figures 2-2 and 2-3) and by *AOC5* IR transgenes (Figure 2-1D; Figure 2-15) designed to produce hairpin dsRNA homologous to the 3' UTR of target transcripts.

Interestingly, in *Chlamydomonas*, the same inverted repeat construct (homologous to a 3' UTR) can induce predominantly either target mRNA degradation or translation repression in different transgenic lines. One obvious difference among the RNAi strains is the site of integration of the IR transgene in the algal nuclear genome. In mammalian cells it has been recently demonstrated that the promoter driving transcription of an mRNA influences the type of miRNA-mediated translation repression. Transcripts

derived from the SV40 (Simian Virus 40) promoter, containing let-7 target sites in their 3' UTRs, are repressed at the initiation state of translation whereas identical mRNAs derived from the TK (Thymidine Kinase) promoter are repressed at a post-initiation step (27). Bushell and colleagues proposed that a nuclear event linked to the promoter, such as co-transcriptional loading of factors onto the nascent mRNA, might determine the type of miRNA-mediated translation repression (27). Similarly, we speculate that the site of integration of an IR transgene in the *Chlamydomonas* genome may influence its transcriptional activity, site of hairpin dsRNA processing to siRNAs (nuclear vs. cytoplasmic), and the eventual association of a factor(s) with siRNA-loaded AGOs that may modulate the type of repression. Addressing the actual molecular mechanism(s) determining this choice will require further investigation.

In metazoans, the mechanism(s) of miRNA-mediated silencing has been the subject of extensive research (see Introduction). However, because translation repression, deadenylation, and transcript decay are closely linked processes, delineating a unifying model of silencing has been difficult (2-4, 22, 23, 31, 46, 77). Recent studies examining the relative timing of different events suggests that miRNA targets in zebrafish, flies, and HeLa cell lines are first subject to translational inhibition, followed by effects on deadenylation and mRNA degradation (22, 44-46). In this context, deadenylation may consolidate the initial translational inhibition (4), which apparently occurs at the initiation level (22, 44, 77). However, in animal systems, there is also evidence for some repressed mRNAs remaining associated with polyribosomes, a strong argument in support of post-initiation translation inhibition (2, 4, 29, 31). miRNAs have been proposed to slow

translation elongation (24), promote premature termination ("ribosome drop-off") (26), or induce rapid proteolysis of nascent polypeptides (25). In addition, in *D. melanogaster*, which contains two AGO subfamily proteins, both Argonautes can inhibit translation but by different mechanisms. AGO2 specifically represses the cap recognition step whereas AGO1 induces deadenylation of target mRNAs and, secondarily, blocks translation downstream from cap recognition (23, 30). Indeed, alternative sRNA effector complexes, including AGO-PIWI polypeptides and associated factors such as GW-repeat proteins, as well as specific features of the sRNA binding site and the proteins associated with a given target transcript may determine the actual mode of sRNA-mediated repression (2, 4, 12, 29).

Despite these advances, our mechanistic understanding of the sRNA-mediated inhibition of translation at post-initiation steps is still very limited. Several ribosomal proteins have been implicated in sRNA-triggered silencing (78-80) although, in mammalian cells, a relief in miRNA repression of translation by depletion of ribosomal proteins may be caused indirectly by activation of the p53 pathway (81). Nonetheless, certain ribosomal proteins have been demonstrated to co-immunoprecipitate with Argonautes and other components of sRNA effector complexes (79, 80, 82, 83). In nematodes and mammals it was recently demonstrated that the Receptor for activated C-kinase 1 (RACK1), an integral component of the 40S ribosomal subunit, is required for the association of miRNA effector complexes with translating ribosomes and may contribute to silencing at a post-initiation step (84). Similarly, Argonaute proteins can form a complex with PUF (Pumilio/FBF) RNA-binding proteins and with eukaryotic translation elongation factor

1A (eEF1A), reducing its ability to hydrolyze GTP (85). This complex attenuates translation elongation perhaps by interfering with the proper delivery of aminoacylated tRNAs to the ribosome (85). However, in this experimental system target specificity was conferred by the PUF proteins and it remains to be examined whether sRNA-guided AGO proteins can recruit a similar complex to mRNA targets and elicit the same regulatory mechanism. Nevertheless, these studies, taken together, indicate that AGOs or sRNA-guided effector complexes may interact with ribosomes and/or other components of the translation machinery in order to bring about translation inhibition at post-initiation steps in metazoans.

In higher plants and algae, repression of protein synthesis by small RNAs remains poorly characterized (1, 15, 16, 52, 53). Our observations suggest that, in *Chlamydomonas*, siRNA-mediated translation inhibition of the *MAA7* transcript occurs in a deadenylation-independent manner (Figure 2-3E). Sucrose density gradient ultracentrifugation analysis, separating mRNAs according to the number of associated ribosomes, has been the main experimental technique used to support either the initiation or post-initiation modes of sRNA repression (4). In this approach, cells/organisms are commonly treated with cycloheximide to “freeze” translating ribosomes on mRNAs. After the addition of a high molar excess of cycloheximide, the first ribosome initiating on a mRNA becomes locked over the start codon, preventing the loading of additional ribosomes, and elongating ribosomes become blocked on their progression (69， 70). Hence, this experimental condition should ideally reflect the ribosome occupancy *in vivo* and ribosome density on a given transcript (4, 38). Because *MAA7* repressed transcripts were found associated

with *bona fide* translating ribosomes in the high cycloheximide sucrose density gradient assays (Figure 2-4), siRNA-mediated inhibition of protein synthesis in *Chlamydomonas* appears most likely to occur at a post-initiation step. Interestingly, in ribosome run-off experiments in the presence of low concentrations of cycloheximide (unable to cause total elongation block), the *MAA7* and the *AOC5* transcripts were moderately but consistently depleted from the polyribosomal fractions in translationally repressed strains in comparison with the wild type (Figure 2-6; Figure 2-15). The simplest interpretation of these results suggests that ribosome run-off (i.e., elongation and normal termination) and/or "drop-off" (i.e., premature, abnormal termination) can still occur on the siRNA inhibited *MAA7* and *AOC5* mRNAs in the presence of a low dose of the antibiotic.

The ribosome run-off assays imply that siRNA-repressed ribosomes are more active (less inhibited) than normal ones under low concentrations of cycloheximide. Moreover, in 18-h experiments *in vivo* using sub-lethal concentrations of cycloheximide, accumulation of the tryptophan synthase β subunit is blocked in the translationally competent CC-124 strain, without a significant decrease in TSβ abundance since this is a long-lived protein (Figure 2-7B). In contrast, in the translationally repressed Maa7-IR44s strain, TSβ protein levels increase in the presence of low concentrations of cycloheximide, indicating that the ribosomes can still translate the *MAA7* transcript (Figure 2-7B). The loading of the lanes in Figure 2-7B is normalized for equal number of cells and is therefore indicative of average protein content per cell at the end of the 18 h experimental period. Since antibiotic treated cells, as previously reported (86), are arrested in growth and division the observed changes in protein content largely reflect what occurs in the

initially inoculated cells. However, *Chlamydomonas* in control medium undergoes one or two rounds of cell division during the 18 h experimental period and the corresponding increase in culture protein accumulation (as a consequence of an increase in cell numbers) is not displayed in Figure 2-7B. Taking these technical aspects into consideration, the results suggests that siRNA-repressed ribosomes have reduced sensitivity to inhibition by cycloheximide, allowing translation of the TSβ protein from the *MAA7* transcript in the presence of sub-lethal concentrations of the antibiotic. Interestingly, pretreatment of the mammalian ECV-304 cell line with cycloheximide also partly relieved the miRNA-mediated repression of a *Renilla* luciferase reporter (35).

In the recently solved crystal structure of the *Tetrahymena thermophila* 60S ribosomal subunit, cycloheximide was shown to bind in a tight pocket of the E site, previously identified as the binding site for 3'-terminal nucleotides of deacylated tRNAs in the archaeal ribosome (71). This is in agreement with observations in *C. reinhardtii* where substitutions of a proline residue in ribosomal protein L41 (named L36a in higher eukaryotes), which is a conserved component of the *T. thermophila* cycloheximide binding pocket (71), confer resistance to cycloheximide (87). Occupation of the E site by both cycloheximide and a deacylated tRNA, effectively trapping deacylated tRNA at the E site, is thought to block eukaryotic ribosome translocation (69, 70，88). Antibiotics binding to the aminoacyl site, such as anisomycin, or to the decoding center, such as paromomycin, of the ribosome have no effect on siRNA-mediated translation repression in *Chlamydomonas* (Figure 2-16). Thus, our findings are consistent with a fairly specific alteration(s) of the function/structural conformation of translating ribosomes, mediated by

siRNA-effector complexes, which may also affect the binding and/or the action of certain antibiotics such as cycloheximide. However, the exact mechanism of translation repression induced by sRNAs in *Chlamydomonas* remains to be elucidated.

We have previously argued, based on phylogenetic and taxonomic distribution analyses, that a fairly complex RNAi machinery was already present in the last common ancestor of eukaryotes (14). This ancestral RNAi machinery may have been capable of both small RNA-guided transcript degradation as well as transcriptional repression, both widespread sRNA-mediated processes among living eukaryotes (1, 3, 14). By contrast, reports of small RNA-induced translation repression were initially limited to animals and higher plants, suggestive of a more recently evolved mechanism confined to certain lineages (1, 2, 4, 15). However, current evidence indicates that small RNAs can inhibit translation in a much wider range of eukaryotes, including the protozoan parasite *Giardia lamblia*, the diatom *Phaeodactylum tricornutum*, and the green alga *Chlamydomonas reinhardtii* (this work, 54-56). Additionally, there is experimental support for the association of sRNAs and of Argonautes with polyribosomes in the parasites *Trypanosoma brucei* and *Toxoplasma gondii* (89-91). Given the much wider taxonomic distribution of sRNA-mediated translation repression, it is tempting to speculate that a basic process of protein synthesis inhibition may have been another feature of an ancestral RNAi machinery.

Methods

Transgenic Strains, Mutants, and Culture Conditions

Chlamydomonas transgenic strains containing inverted repeat constructs homologous to *AOC5*, *AOC5/Cre16.g662000*, or *MAA7* were generated as previously described (57, 60). DNA fragments for building the IR constructs were generated by RT-PCR amplification with the following primers: for *AOC5*, AA-Per-1 (5'-GCTGACGAGTCTGTGGAGACG-3') and AA-Per-2 (5'-CTTACTCACGCCCAGCAGAGA-3'); and for *Cre16.g662000*, Helic-F1 (5'-GGATGACGTGATCGCCAAG-3') and Helic-R2 (5'-GGCCTGAATCCCATGTCTAGC-3'). The *AOC5* primers amplify a 930-bp fragment that was digested with *Nhe*I to generate a 3' segment of 380-bp used to build the inverted repeat transgene (Figure S2-1). The IR construct targeting the *MAA7* 3' UTR has already been described (57). The *Cre10.g420400* deleted strain, lacking exportin 5, was obtained in an insertional mutagenesis screen designed to isolate mutants defective in RNA interference in *Chlamydomonas* (92, 93). Unless noted otherwise, *C. reinhardtii* cells were grown photoheterotrophically in Tris-Acetate-Phosphate (TAP) medium or photoautotrophically in minimal High Salt (HS) medium (94). For phenotypic analyses, cells grown to logarithmic phase in TAP or HS media were serially diluted, spotted on plates of the appropriate media (see figure legends), and incubated for 7-15 days under dim lights (57). The antibiotic concentrations used in *in vivo* experiments were previously demonstrated to be inhibitory of protein synthesis in *C. reinhardtii* (86).

RNA Analyses

Total cell RNA was purified with TRI Reagent (Molecular Research Center), following the manufacturer's instructions. For northern analyses of mRNAs, the isolated RNA was

separated by agarose/formaldehyde gel electrophoresis, blotted onto nylon membranes, and hybridized with ^{32}P-labeled probes (57, 95). For small RNA analyses, total RNA samples were resolved on 15% polyacrylamide/7 M urea gels, and electroblotted to Hybond-XL membranes (GE Healthcare) (57, 95). Blots were hybridized with ^{32}P-labeled DNA probes at 40°C for 48 h using the High Efficiency Hybridization System (Molecular Research Center). Specific miRNAs were detected by hybridization with complementary DNA oligonucleotides labeled at their 5' termini with γ-^{32}P-ATP and T4 Polynucleotide Kinase (New England Biolabs). The poly(A) tail length of specific transcripts was estimated using a G/I tailing protocol followed by RT-PCR analysis (96), according to a commercially available kit (USB, Affymetrix). The primer sequences for the poly(A) tail analyses were as follows: for *ACT1*, ACT-3'UTR-PF4 (5'-AAGATATGAGGAGCGGGTCA-3') and ACT-3'UTR-PR2 (5'-AAATGGTCCGAGCAGGTTTT-3'); and for *MAA7*, MAA7-3'UTR-PF1 (5'-GTGATTGAAAGGGGAGCGTA-3') and MAA7-3'UTR-PR1 (5'-ACATGCGATTGGTAGCAACA-3').

Immunoblot Analyses

The *Chlamydomonas* TSβ protein was immunodetected, following a standard procedure (Rohr et al., 2004), by overnight incubation at 4°C with a 1:5000 dilution of a rabbit antibody raised against the full length recombinant protein (GenScript). A modification-insensitive polyclonal antibody (Abcam, ab1791) was used to detect histone H3.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analyses

Total RNA was isolated with TRI Reagent and contaminant DNA was removed by DNase-I treatment (Ambion). First-strand cDNA synthesis and PCR reactions were performed as previously described (57, 95). PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining (95). The number of cycles showing a linear relationship between input RNA and the final product was determined in preliminary experiments. Controls included the use as template of reactions without RT and verification of PCR products by hybridization with specific probes (data not shown). The primer sequences were as follows: for *AOC5*, AA-Per-3(F) (5'-CTTCAAAGTGCCGCTGTACCC-3') and AA-Per-4(R) (5'-GTCTCCACAGACTCGTCAGCA-3'); for *EXP5*, Mut3-cod-F1 (5'-ACAGGGACGCAGTCAAGG-3') and Mut3-cod-R2 (5'-CCAGGCTCAGGACCATGTAG-3'); and for *ACT1*, ACT-cod-F (5'-GACATCCGCAAGGACCTCTAC-3') and ACT-cod-R (5'-GATCCACATTTGCTGGAAGGT-3'). The *Cre16.g662000* gene, encoding a putative RNA helicase, has a very close paralog in the *Chlamydomonas* genome (*Cre16.g661900*). Thus, to avoid amplification of the related transcript, reverse transcription was performed with a *Cre16.g662000* specific primer (Helic-R7, 5'-CACATCCGAGCTGAACATGAC-3') and then PCR was carried out with Helic-F2 (5'-CCAAATTTCCAAGATCCTCAGC-3') and Helic-R4 (5'-AGCATGACGTCGCGCTTG-3').

Polyribosome Profile Analyses

Chlamydomonas strains were grown to mid logarithmic phase in liquid TAP medium,

~8x10^{8} cells pelleted by centrifugation, and resuspended in 10 ml of the same medium. Resuspended cells were incubated under dim lights and constant shaking for 15 min in the presence of 150 μg/ml cycloheximide, 30 μg/ml cycloheximide, or 300 μg/ml puromycin. In the no antibiotic experiments, the TAP medium contained an amount of ethanol (solvent) equivalent to that added with the antibiotics. Cells were then pelleted again and resuspended in 10 ml of lysis buffer (25 mM Tris-HCl, pH 7.5; 150 mM KCl; 5 mM $MgCl_2$; and 1 mM DTT) containing the same antibiotics or solvent control and supplemented with an EDTA-free protease inhibitor cocktail (Sigma). From this step on, cells and lysates were always kept on ice. Cells were broken by one passage through a French press at a pressure of ~2,000 psi. To complete cell lysis, 0.1 volume of 5% sodium deoxycholate (pH 8.0) was added to the lysates and mixed gently for ~5 min. Cell extracts were then centrifuged at 10,000x*g* for 10 min at 4°C. Supernatant concentrations were normalized by measuring absorbance at 254 nm and a 0.125 volume of 10% Triton X-100 was added gently. Finally, ~600 μl of the clarified cell extracts were layered on 4.5-45% (w/v) sucrose gradients prepared in 25 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mg/ml heparin, and then centrifuged for 2.5 h at 260,000x*g* in a Beckman SW41 rotor. Gradients were fractionated with an ISCO system while monitoring absorbance at 254 nm. Total RNA was purified from each fraction by two phenol/chloroform extractions and ethanol precipitation. Specific transcripts were detected by slot blot hybridization of RNA treated with DNase-I (Ambion) to remove any contaminating DNA (particularly in sub-polyribosomal fractions). Small RNAs were isolated as previously described (89). For EDTA treatment, antibiotics were omitted and EDTA was added to 50 mM in the lysis buffer and to 10 mM in the sucrose gradients.

Figures

Figure 2-1. RNA-mediated silencing of the *Amino Acid Carrier 5* (*AOC5*) gene induced by expression of *AOC5* inverted repeat (IR) transgenes in *Chlamydomonas* transformants.

(A) Growth and survival of Aoc5-IR transformants on Tris-Acetate-Phosphate (TAP) medium without (left) or with (right) 400 μg/ml L-canavanine. CC-124, untransformed wild type strain.

(B) Northern blot analyses of mRNAs and small RNAs (siRNAs) in the Aoc5-IR transgenic strains. The left panels correspond to agarose gel separated total RNA samples sequentially hybridized with ^{32}P-labeled PCR products corresponding to the *AOC5* 3' UTR (upper panel), to evaluate the degree of mRNA reduction, or the coding sequence of *Actin* (*ACT1*) (lower panel), as a control for equivalent loading of the lanes. The right panels correspond to total RNA samples separated in a 15% denaturing polyacrylamide gel and probed sequentially with the *AOC5* 3' UTR sequence (upper panel), to detect siRNAs, or the U6 small nuclear RNA sequence (lower panel), to assess the amount of sample loaded per lane.

(C) Growth and survival of the indicated strains on TAP medium alone or containing 400 μg/ml L-canavanine. Maa7-IR44s, strain containing an IR transgene targeting the 3' UTR of the *MAA7* gene (encoding tryptophan synthase β subunit). Aoc5/Helic-IR4, strain containing a tandem IR transgene targeting both *AOC5* and *Cre16.g662000* (encoding a putative RNA helicase).

(D) Semi-quantitative Reverse Transcriptase (RT)-PCR analyses on total RNA samples

from the indicated strains. The panels show reverse images of agarose gel fractionated RT-PCR products corresponding to *AOC5* or *Cre16.g662000*. Amplification of the mRNA corresponding to *ACT1* was used as a control for equal amounts of input RNA and for the efficiency of the RT-PCRs (lower panel). Reactions using RNA not treated with reverse transcriptase as the template were employed as a negative control (data not shown).

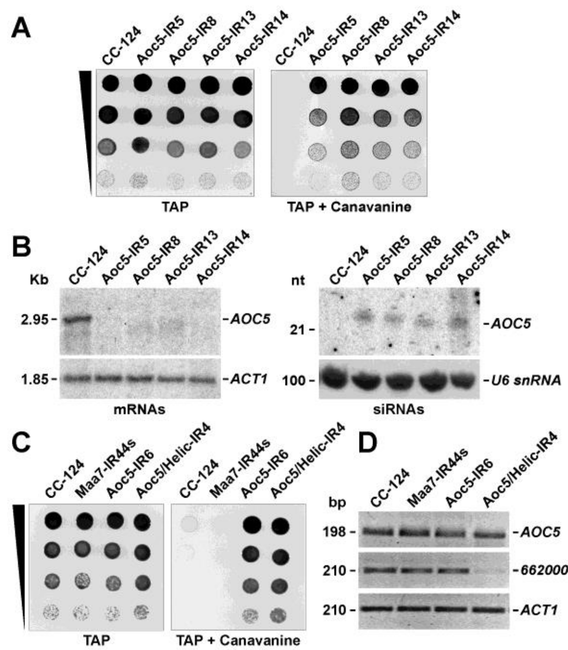


Figure 2-2. RNA-mediated translation repression of the *MAA7* transcript, coding for tryptophan synthase β subunit, induced by expression of *MAA7* IR transgenes in *Chlamydomonas* transformants.

(A) Growth and survival of Maa7-IR transformants on Tris-Acetate-Phosphate medium without (left) or with (right) 7 μM 5-fluoroindole. CC-124, untransformed wild type strain.

(B) Immunoblot analysis of Tryptophan Synthase β subunit (TSβ) levels. The smaller cross-reacting antigen is likely a TSβ degradation product and was not consistently detected in replicate blots. Coomassie-blue staining of an equivalent gel is shown as a control for similar loading of the lanes (lower panel).

(C) Northern blot analysis of agarose gel separated total RNA samples sequentially hybridized with ^{32}P-labeled PCR products corresponding to the coding sequence of *MAA7* (upper panel), to examine the degree of mRNA reduction, or the coding sequence of *Actin* (*ACT1*) (lower panel), to assess the amount of sample loaded per lane.

(D) Detection of siRNAs in transgenic strains undergoing *MAA7* silencing. Total cell RNA was separated in a 15% denaturing polyacrylamide gel, electroblotted onto a nylon membrane, and hybridized with the *MAA7* 3’ UTR sequence (upper panel). The same filter was re-probed with the U6 small nuclear RNA sequence (lower panel) as a control for equivalent loading of the lanes.

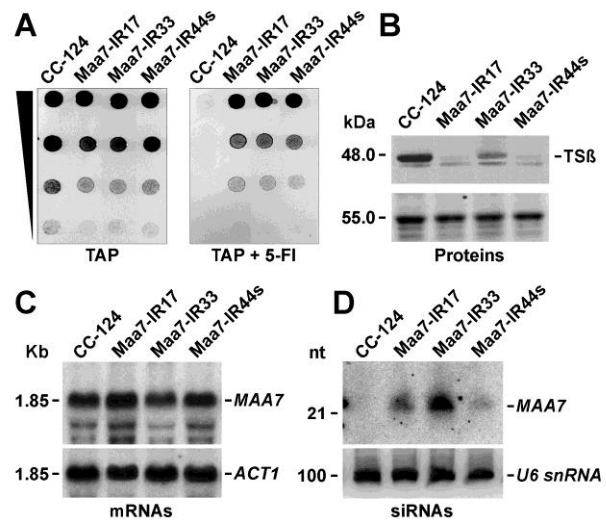

A
CC-124
Maa7-IR17
Maa7-IR33
Maa7-IR44s
TAP
TAP + 5-FI
B
kDa
48.0
55.0
-TSß
Proteins
C
Kb
1.85
-MAA7
-ACT1
mRNAs
D
nt
21
100
-U6 snRNA
siRNAs

Figure 2-3. Translation repression of the *MAA7* gene is greatly diminished in a *Chlamydomonas* mutant deleted for the exportin 5 ortholog (encoded by *Cre10.g420400*) and does not involve transcript deadenylation.

(A) Growth and survival of the indicated strains on Tris-Acetate-Phosphate medium without (left) or with (right) 7 μM 5-fluoroindole. Maa7-IR44s(*exp5*), Maa7-IR44s strain containing a deletion of the *Cre10.g420400* gene.

(B) Immunoblot analysis of tryptophan synthase β subunit abundance. Coomassie-blue staining of an equivalent gel is shown as a control for similar loading of the lanes (lower panel).

(C) Northern blot analysis of agarose gel separated total RNA samples sequentially hybridized with ^{32}P-labeled PCR products corresponding to the coding sequence of *MAA7* (upper panel), to evaluate the degree of mRNA reduction, or the coding sequence of *Actin* (*ACT1*) (lower panel), to assess the amount of sample loaded per lane.

(D) Detection of siRNAs in the Maa7-IR transgenic strains. Total cell RNA was separated in a 15% denaturing polyacrylamide gel, electroblotted onto a nylon membrane, and hybridized with the *MAA7* 3' UTR sequence (upper panel). The same filter was re-probed with the U6 small nuclear RNA sequence (lower panel) as a control for equivalent loading of the lanes.

(E) Analysis of polyadenylated tail lengths of the *MAA7* and *ACT1* transcripts in the indicated strains. Poly(A) tail lengths were examined using a G/I tailing protocol and RT-PCR assays (Figure S2-4). Reactions were performed as described under methods in the presence (+RT) or absence (-RT) of reverse transcriptase.

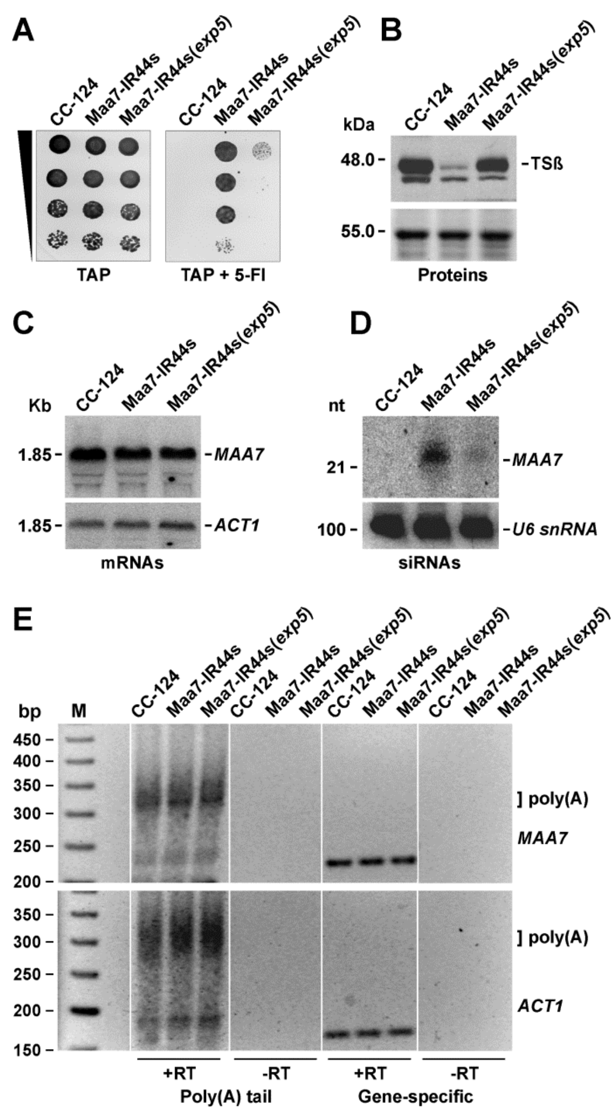

A
CC-124
Maa7-IR44s
Maa7-IR44s(exp5)
TAP
TAP + 5-FI
B
kDa
48.0
55.0
TSß
Proteins
C
Kb
1.85
MAA7
ACT1
mRNAs
D
nt
21
100
U6 snRNA
siRNAs
E
bp
M
450
400
350
300
250
200
150
] poly(A)
+RT
-RT
Poly(A) tail
Gene-specific

Figure 2-4. Translationally repressed *MAA7* transcripts co-migrate with polyribosomes in sucrose density gradients.

(A) Typical polyribosome profiles of the indicated strains treated with 150 μg/ml cycloheximide throughout lysis and ultracentrifugation procedures (upper panels). M, monoribosomes; Poly, polyribosomes. The distribution of the *MAA7*, *ACT1*, and *18S rRNA* transcripts in the gradient fractions was examined by slot blot hybridization (lower panels).

(B) Distribution of *ACT1* and *MAA7* mRNAs across polyribosome profiles of the CC-124, Maa7-IR44s, and Maa7-IR44s(*exp5*) strains. The values represent the average of three independent experiments +/- SEM.

(C) Distribution of *MAA7* siRNAs and of an endogenous microRNA (miR912) in sucrose density gradients of the indicated strains, assessed by northern blot hybridization. Numbers above the lanes indicate pooled gradient fractions.

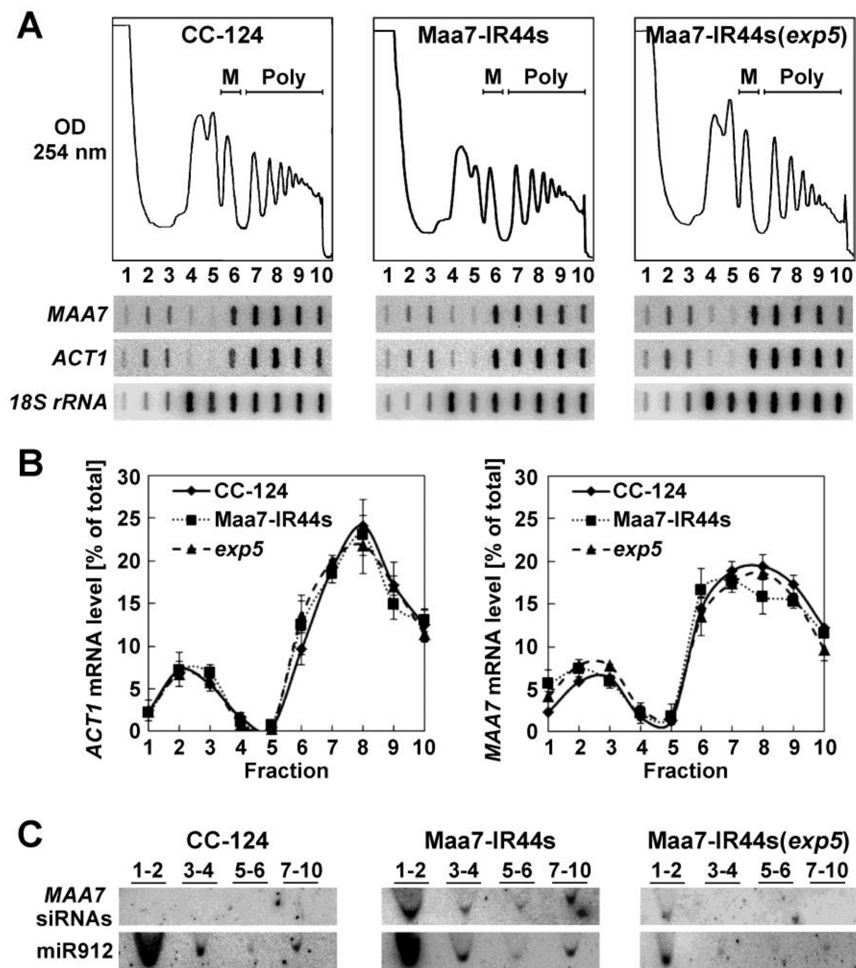

A
CC-124
Maa7-IR44s
Maa7-IR44s(exp5)
M
Poly
OD
254 nm
1 2 3 4 5 6 7 8 9 10
MAA7
ACT1
18S rRNA
B
ACT1 mRNA level [% of total]
MAA7 mRNA level [% of total]
CC-124
Maa7-IR44s
exp5
Fraction
C
1-2
3-4
5-6
7-10
MAA7
siRNAs
miR912

Figure 2-5. The fraction of *MAA7* transcripts co-migrating with polyribosomes is reduced after sucrose density gradient ultracentrifugation of cell extracts subjected to ribosome run-off in the absence of cycloheximide.

(A) Typical polyribosome profiles of the indicated strains (upper panels). M, monoribosomes; Poly, polyribosomes. The distribution of the *MAA7*, *ACT1*, and *18S rRNA* transcripts in the gradient fractions was examined by slot blot hybridization (lower panels).

(B) Distribution of *ACT1* and *MAA7* mRNAs across polyribosome profiles of the CC-124 and Maa7-IR44s strains. The values represent the average of three independent experiments +/- SEM.

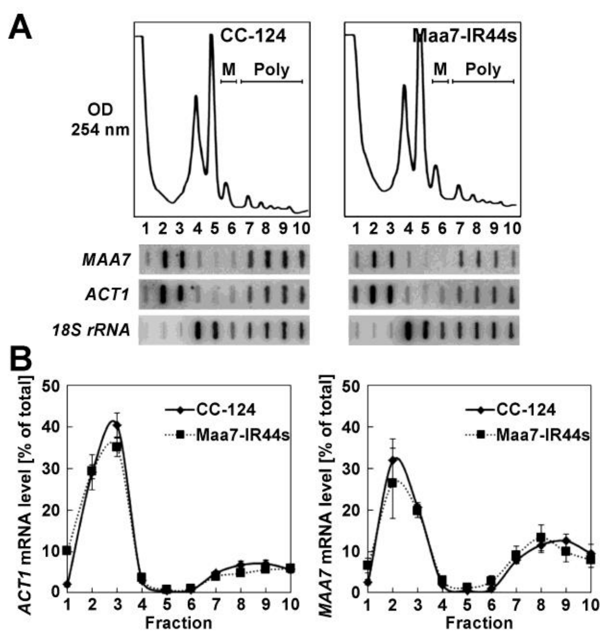


Figure 2-6. siRNA-repressed *MAA7* transcripts are moderately depleted from polyribosomal fractions after ribosome run-off assays in the presence of low concentrations of cycloheximide.

(A) Typical polyribosome profiles of the indicated strains treated with 30 μg/ml cycloheximide throughout lysis and ultracentrifugation procedures (upper panels). M, monoribosomes; Poly, polyribosomes. The distribution of the *MAA7*, *ACT1*, and *18S rRNA* transcripts in the gradient fractions was examined by slot blot hybridization (lower panels).

(B) Distribution of *ACT1* and *MAA7* mRNAs across polyribosome profiles of the CC-124 and Maa7-IR44s strains. The values represent the average of three independent experiments +/- SEM.

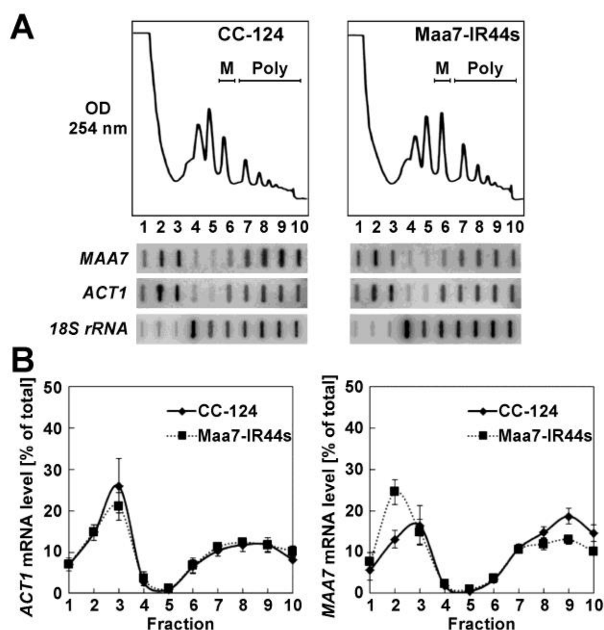


Figure 2-7. TSβ protein synthesis from the *MAA7* transcript, subjected to siRNA-mediated translation repression, shows lower sensitivity to inhibition by cycloheximide.

(A) Growth and survival of the indicated strains on Tris-Acetate-Phosphate medium without (upper panels) or with (lower panels) 7 μM 5-fluoroindole supplemented with solvent (Ctrl) or sub-lethal concentrations of hygromycin B (HygB, 4.0 μg/ml) or cycloheximide (Chx, 2.0 μg/ml). Maa7-IR5, strain expressing a *MAA7* IR transgene that induces target mRNA degradation (57).

(B) Immunoblot analyses of TSβ and histone H3 protein levels. Cells from the indicated strains were cultured for 18 h in liquid TAP medium alone (Ctrl) or containing sub-lethal concentrations of antibiotics (2.0 μg/ml of HygB or 1.0 μg/ml of Chx). Proteins corresponding to equal numbers of cells were loaded per lane.

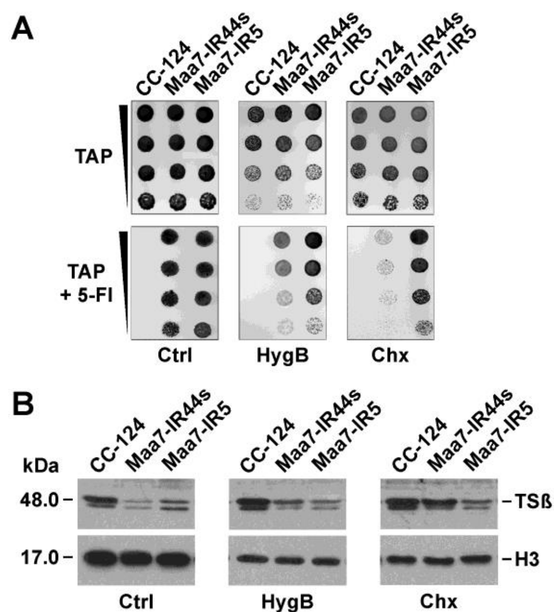
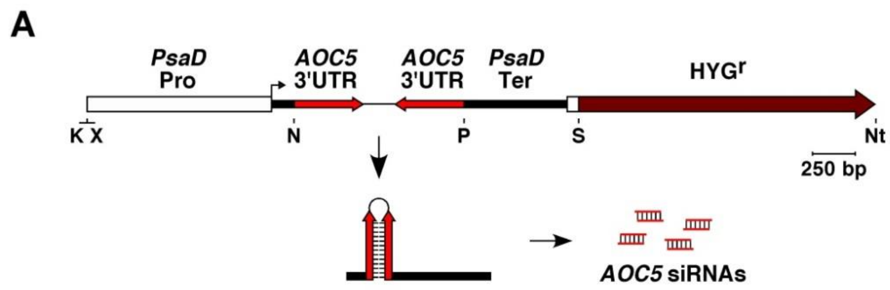


Figure 2-8. Schematic diagrams of inverted repeat (IR) transgenes used to induce RNA interference (RNAi) in Chlamydomonas.

(A) Diagram of the construct triggering *AOC5* silencing. A 380-bp fragment, corresponding to the *AOC5* 3' UTR, was cloned in forward and reverse orientations flanking a DNA spacer and placed under the control of *PsaD* (encoding a Photosystem I subunit) regulatory sequences. This IR transgene was designed to generate, upon transcription, an RNA containing a double-stranded stem loop structure that can be processed into small interfering RNAs (siRNAs). The previously engineered *Aminoglycoside Phosphotransferase* gene (*aph7"*), conferring resistance to hygromycin B (HYG^r) (97), was placed immediately downstream from the *AOC5* IR transgene. Restriction sites: K, *Kpn*I; N, *Nco*I; Nt, *Not*I; P, *Pst*I; S, *Spe*I; X, *Xba*I.

(B) Diagram of the construct triggering *AOC5* and *Cre16.g662000* (encoding a putative RNA helicase) silencing. A 400-bp fragment, corresponding to the *Cre16.g662000* coding sequence (Helic cod sq), was cloned in sense and antisense orientations, flanking a DNA spacer, in between the arms of the *AOC5* 3' UTR inverted repeat. This tandem IR transgene was designed to generate a double-stranded RNA transcript that can be processed into both *AOC5* and *Cre16.g662000* (Helic) siRNAs.


A
PsaD
Pro
AOC5
3'UTR
AOC5
3'UTR
PsaD
Ter
HYGr
K X
N
P
S
Nt
250 bp
AOC5 siRNAs

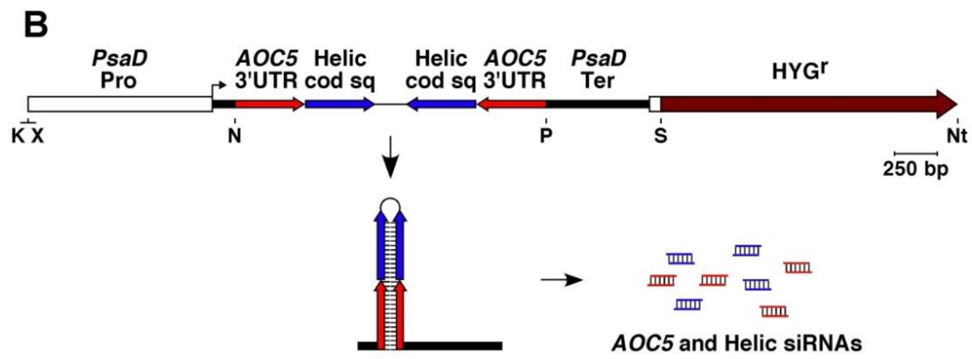

B
PsaD
Pro
AOC5
3'UTR
Helic
cod sq
Helic
cod sq
AOC5
3'UTR
PsaD
Ter
HYGr
K X
N
P
S
Nt
250 bp
AOC5 and Helic siRNAs

Figure 2-9. Abundance of the MAA7 (encoding tryptophan synthase subunit) mRNAs and siRNAs in transgenic strains undergoing RNA-mediated silencing. (A) *MAA7* transcript levels in Maa7-IR transgenic strains and the CC-124 untransformed wild type strain. Total cell RNA was separated in agarose gels under denaturing conditions and hybridized to the *MAA7* coding sequence. The same blot was re-probed with the *ACT1* (encoding actin) coding sequence. Signal intensities from phosphorimager images were quantified with Quantity One software and the *MAA7* transcript levels normalized to those of the *ACT1* mRNA for each sample. For illustration purposes, the *MAA7* normalized amount in CC-124 was set to 1.0 and the remaining samples adjusted accordingly in the bar graph. The values represent the average of four independent experiments +/- SEM.

A

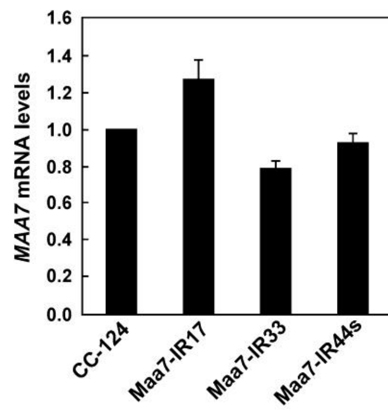
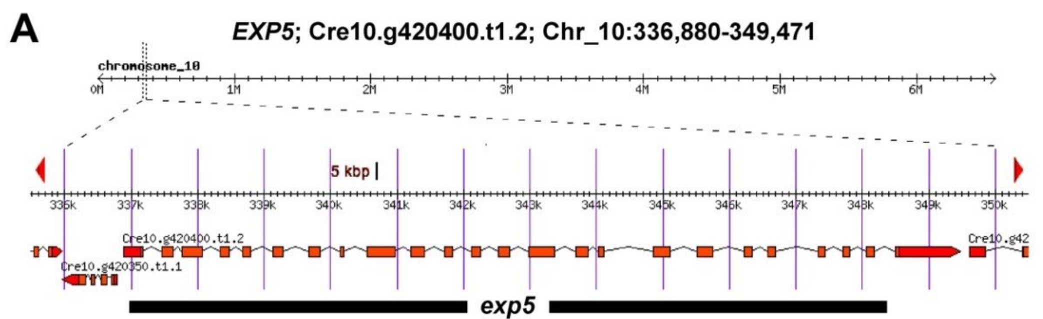


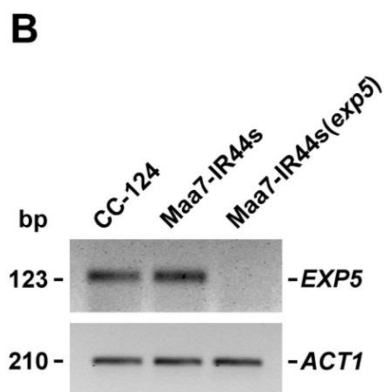
Figure 2-10. A Chlamydomonas Exportin 5 deletion mutant shows reduced steady-state levels of several endogenous microRNAs.

(A) Diagram of the *Cre10.g420400* gene, encoding the *C. reinhardtii* exportin 5 ortholog. The dark horizontal bar indicates the extent of the deletion in the Maa7-IR44s(*exp5*) mutant.

(B) Semi-quantitative Reverse Transcriptase (RT)-PCR analysis of the *Exportin 5* steady-state mRNA levels in the indicated strains. Amplification of *ACT1* transcripts is shown as an input control. The panels show reverse images of agarose gel fractionated RT-PCR products corresponding to representative results out of three independent experiments. Reactions using RNA not treated with reverse transcriptase as the template were employed as a negative control (data not shown). CC-124, wild type strain; Maa7-IR44s, CC-124 transformed with an IR transgene targeting the 3' UTR of *MAA7* for silencing; Maa7-IR44s(*exp5*), Maa7-IR44s strain containing the *Cre10.g420400* deletion.

(C) Northern blot analyses of small RNAs isolated from the indicated strains and detected with probes specific for several *Chlamydomonas* miRNAs. Cad112, candidate miRNA 112. The same filters were re-probed with the U6 small nuclear RNA sequence as a control for equivalent loading of the lanes.


A
EXP5; Cre10.g420400.t1.2; Chr_10:336,880-349,471
chromosome_10
5 kbp
Cre10.g420400.t1.2
Cre10.g420350.t1.1
exp5


B
bp
CC-124
Maa7-IR44s
Maa7-IR44s(exp5)
123
EXP5
210
ACT1

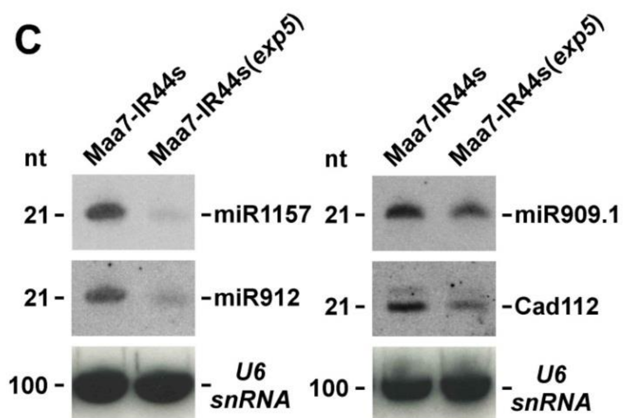
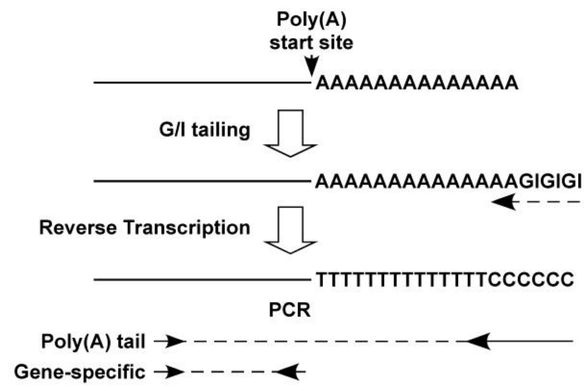

C
nt
Maa7-IR44s
Maa7-IR44s(exp5)
21
miR1157
21
miR912
100
U6 snRNA
nt
Maa7-IR44s
Maa7-IR44s(exp5)
21
miR909.1
21
Cad112
100
U6 snRNA

Figure 2-11. Schematic diagram of the G/I tailing protocol used to examine mRNA poly(A) tail length. A limited number of guanosine and inosine residues were first added to the 3' ends of transcripts by poly(A) polymerase. Tailed RNAs were then converted to DNA by reverse transcription using the newly added G/I tails and 2 nucleotides of the endogenous poly(A) tail as the priming sites. Finally, PCR amplification products were generated using two primer sets. A gene-specific forward and reverse primer set, designed to anneal upstream of the polyadenylation site, was used to produce a specific fragment that serves as a control for the gene of interest. The gene-specific forward primer and a universal reverse primer were used to generate another PCR product that includes the poly(A) tail of the gene of interest. After separating the PCR products on an agarose gel, the poly(A) tail length of the gene of interest can be determined by subtracting from the length of the poly(A) PCR product the length of the universal reverse primer and the distance of the gene-specific forward primer to the known polyadenylation start site.



Poly(A)
start site
AAAAAAAAAAAAAA
G/I tailing
AAAAAAAAAAAAAAGIGIGI
Reverse Transcription
TTTTTTTTTTTTTTCCCCCC
PCR
Poly(A) tail
Gene-specific

Figure 2-12. Distribution of *MAA7* siRNAs in sucrose density gradients of the Maa7-IR44s and Maa7-IR5 strains, assessed by northern blot hybridization. Numbers above the blots indicate pooled gradient fractions. The upper panels show typical polyribosome profiles of the indicated strains treated with 150 μg/ml cycloheximide throughout lysis and ultracentrifugation procedures. M, monoribosomes; Poly, polyribosomes. Note that in order to improve isolation of small RNAs, cell extracts were separated through low salt sucrose gradients, as previously described (89).

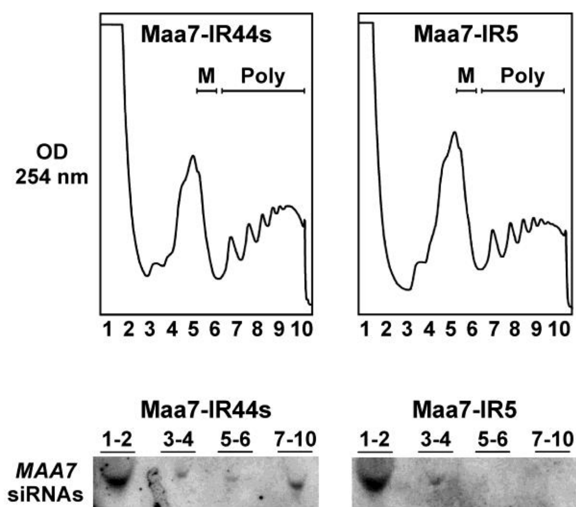
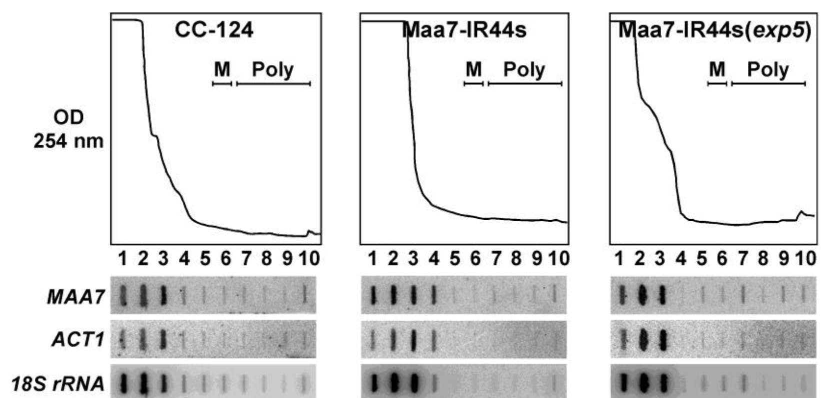
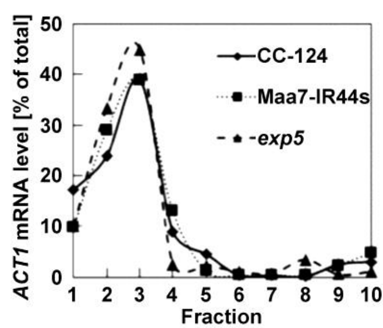

Maa7-IR44s
Maa7-IR5
M
Poly
OD
254 nm
1 2 3 4 5 6 7 8 9 10
Maa7-IR44s
Maa7-IR5
1-2
3-4
5-6
7-10
MAA7
siRNAs

Figure 2-13. Migration of MAA7 and ACT1 transcripts in sucrose density gradients when examining cell extracts in the presence of 50 mM EDTA.

(A) Typical polyribosome profiles of the indicated strains. EDTA disrupts ribosome subunit association and the expected location of monosomes (M) and polyribosomes (Poly) is indicated (upper panels). The distribution of the *MAA7*, *ACT1*, and *18S rRNA* transcripts in the gradient fractions was examined by slot blot hybridization (lower panels).

(B) Distribution of *ACT1* and *MAA7* mRNAs across the EDTA sucrose density gradients for the CC-124, Maa7-IR44s, and Maa7-IR44s(*exp5*) [*exp5*] strains. The values represent the average of two independent experiments.


CC-124
Maa7-IR44s
Maa7-IR44s(exp5)
M
Poly
OD
254 nm
1 2 3 4 5 6 7 8 9 10
MAA7
ACT1
18S rRNA


ACT1 mRNA level [% of total]
CC-124
Maa7-IR44s
exp5
Fraction

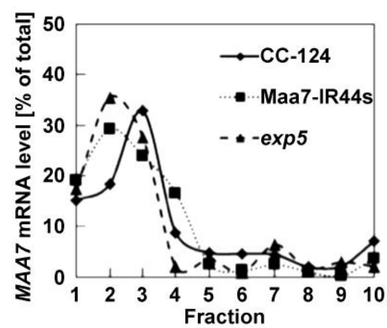
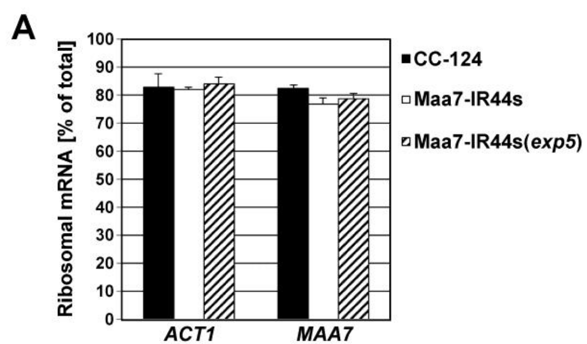

MAA7 mRNA level [% of total]
CC-124
Maa7-IR44s
exp5
Fraction

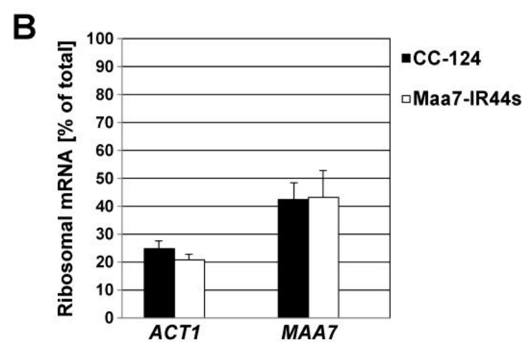
Figure 2-14. Ribosome occupancy, the fraction of a specific mRNA associated with ribosomes, for the MAA7 and ACT1 transcripts after separation on sucrose density gradients.

(A) Ribosome occupancy in the indicated strains treated with 150 μg/ml cycloheximide throughout lysis and ultracentrifugation procedures. The values represent the average of three independent experiments +/- SEM.

(B) Ribosome occupancy in the indicated strains after sucrose density gradient ultracentrifugation of cell extracts subjected to ribosome run-off in the absence of cycloheximide. The values represent the average of three independent experiments +/- SEM.

(C) Ribosome occupancy in the indicated strains after sucrose density gradient ultracentrifugation of cells extracts subjected to ribosome run-off in the presence of 30 μg/ml cycloheximide. The values represent the average of three independent experiments +/- SEM. Samples marked with an asterisk are significantly different ($P<0.05$) in a two tailed Student's *t*-test.


A
Ribosomal mRNA [% of total]
100
90
80
70
60
50
40
30
20
10
0
ACT1
MAA7
CC-124
Maa7-IR44s
Maa7-IR44s(exp5)


B
Ribosomal mRNA [% of total]
100
90
80
70
60
50
40
30
20
10
0
ACT1
MAA7
CC-124
Maa7-IR44s

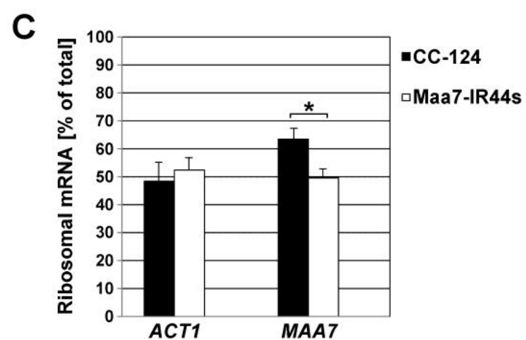
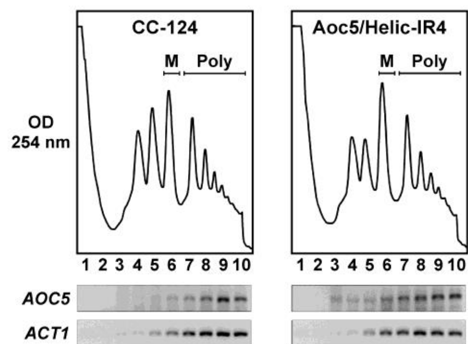

C
Ribosomal mRNA [% of total]
100
90
80
70
60
50
40
30
20
10
0
*
ACT1
MAA7
CC-124
Maa7-IR44s

Figure 2-15. IR-repressed *AOC5* transcripts are moderately depleted from polyribosomal fractions after ribosome run-off assays in the presence of low concentrations of cycloheximide.

(A) Typical polyribosome profiles of the indicated strains treated with 30 μg/ml cycloheximide throughout lysis and ultracentrifugation procedures (upper panels). M, monoribosomes; Poly, polyribosomes. The distribution of the *AOC5* and *ACT1* transcripts in the gradient fractions was examined by RT-PCR (lower panels).

(B) Distribution of *ACT1* and *AOC5* mRNAs across polyribosome profiles of the CC-124 and Aoc5/Helic-IR4 strains.


A
CC-124
Aoc5/Helic-IR4
M
Poly
OD
254 nm
1 2 3 4 5 6 7 8 9 10
AOC5
ACT1

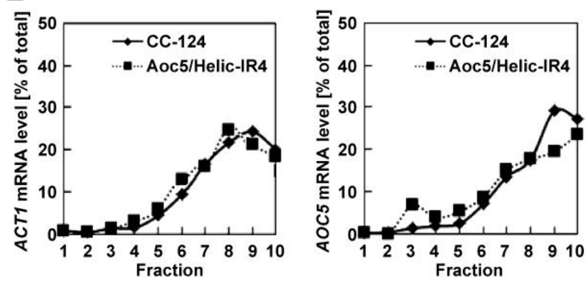

B
CC-124
Aoc5/Helic-IR4
ACT1 mRNA level [% of total]
AOC5 mRNA level [% of total]
Fraction

Figure 2-16. TSβ protein synthesis from the *MAA7* transcript, subjected to siRNA-mediated translation repression, is not affected by treatment with paromomycin, anisomycin, or by slow growth on minimal medium.

(A) Growth and survival of the indicated strains on Tris-Acetate-Phosphate medium without (upper panels) or with (lower panels) 7 μM 5-fluoroindole supplemented with solvent (Ctrl) or with a sub-lethal concentration of paromomycin (Paro, 1.5 μg/ml). Cells grown to logarithmic phase in TAP medium were serially diluted, 5 μl-aliquots spotted on plates, and incubated for 7 to 15 days under dim lights. Maa7-IR5, strain expressing a *MAA7* IR transgene that induces target mRNA degradation (57). Please note that this strain also carries an aminoglycoside 3'-phosphotransferase transgene and is therefore insensitive to paromomycin.

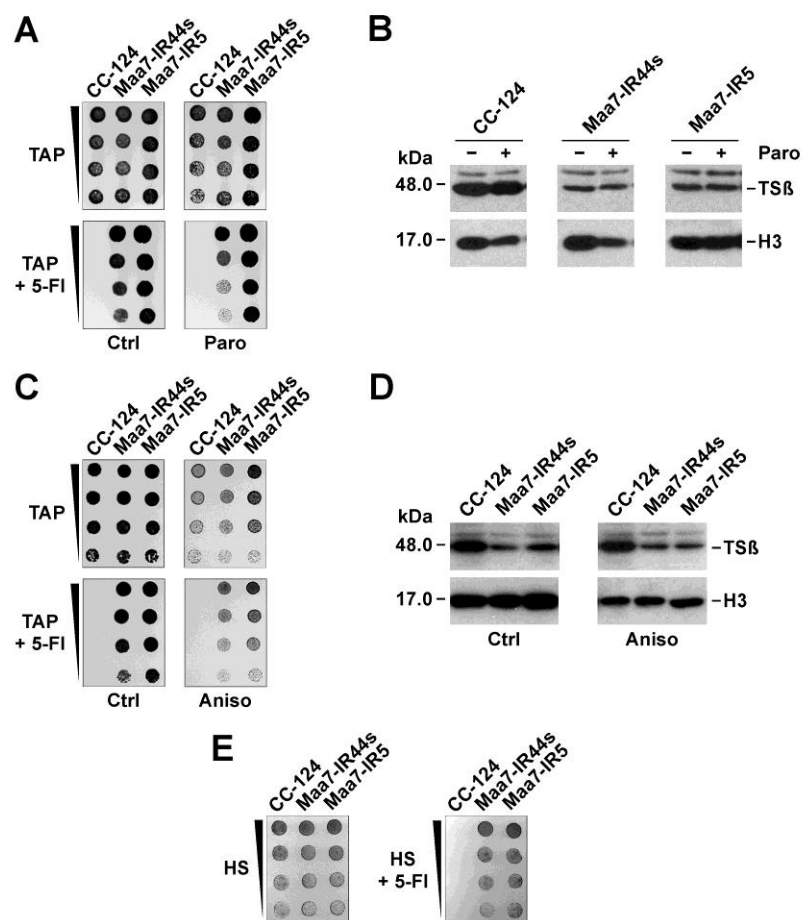
(B) Immunoblot analyses of TSβ and histone H3 protein levels. Cells from the indicated strains were cultured for 18 h in liquid TAP medium alone or containing 0.8 μg/ml of paromomycin. Proteins corresponding to equal numbers of cells were loaded per lane. Since aminoglycoside 3'-phosphotransferase inactivates paromomycin in Maa7-IR5, the antibiotic has no effect on histone H3 accumulation (and on overall protein synthesis) in this strain.

(C) Growth and survival of the indicated strains on Tris-Acetate-Phosphate medium without (upper panels) or with (lower panels) 7 μM 5-fluoroindole supplemented with solvent (Ctrl) or with a sub-lethal concentration of anisomycin (Aniso, 1.0 μg/ml).

(D) Immunoblot analyses of TSβ and histone H3 protein levels. Cells from the indicated strains were cultured for 18 h in liquid TAP medium supplemented with solvent (Ctrl) or containing 0.6 μg/ml of anisomycin (Aniso). Proteins corresponding to equal numbers of

cells were loaded per lane.

(E) Growth and survival of the indicated strains on High Salt (HS) minimal medium without (left panel) or with (right panel) 7 μM 5-fluoroindole. Cells grown to logarithmic phase in HS medium were serially diluted, 5 μl-aliquots spotted on plates, and incubated for 7 to 15 days under dim lights.


A
CC-124
Maa7-IR44s
Maa7-IR5
TAP
TAP
+ 5-FI
Ctrl
Paro
B
kDa
Paro
48.0
17.0
TSß
H3
C
Aniso
D
E
HS
HS
+ 5-FI

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CHAPTER3

CONCLUSIONS

RNAi can function by three main mechanisms: transcriptional silencing, mRNA destabilization, and/or translation repression. Due to the widespread recognition of RNAi as a potent experimental tool in a variety of fields, the molecular basis of RNA interference has attracted increasing attention. In particular, translation inhibition by RNAi is now known to be a widespread phenomenon in animals. However, the mechanisms involved are still not well understood. In addition, there is very limited information regarding a role of small RNAs in translation repression in other eukaryotes such as plants and fungi.

In the work described here, we have adopted the unicellular green alga *Chlamydomonas reinhardtii*, as a model system to examine small RNA-mediated translation repression. Initially, we were able to demonstrate that RNAi could function by translation inhibition, besides targeting mRNAs for degradation, in studies of two independent inverted repeat (IR) systems, namely a single IR system targeting the *MAA7* gene and a tandem IR system targeting both the *AOC5* and *Cre16.g662000* genes. We found that in approximately 10% of the transformed strains, sRNAs derived from genome-integrated inverted repeat transgenes, perfectly complementary to the 3'UTR of a target transcript, can inhibit protein synthesis without or with only minimal mRNA destabilization. Furthermore, when we examined the poly (A) tail length of sRNA-repressed transcripts, there was no appreciable change in their polyadenylation status. Sucrose density gradient assays revealed that sRNA-repressed transcripts were still associated with polyribosomes. Together, these results suggest that siRNA-mediated translation repression occurs at a post-initiation step, in a deadenylation-independent manner. To gain further mechanistic

insights into sRNA-mediated translation inhibition in *Chlamydomonas*, we probed for functional differences in the ribosomes associated with the *MAA7* mRNA in the wild type and Maa7-IR44s strains. Intriguingly, we observed that ribosomes associated with sRNA-repressed *MAA7* mRNAs showed reduced sensitivity to translation inhibition by low concentrations of cycloheximide, both in *in vitro* ribosome run-off assays as well as in *in vivo* experiments. Together, our results suggest that sRNA-mediated repression of protein synthesis in *Chlamydomonas* may involve alterations to the function/structural conformation of translating ribosomes. In addition, sRNA-mediated translation repression is now known to occur in a number of phylogenetically diverse eukaryotes suggesting that this mechanism may have been a feature of an ancestral RNAi machinery.

Our findings contribute to the elucidation of the molecular basis of translation repression by small RNAs. As RNAi becomes a more powerful experimental and therapeutic tool, a better mechanistic understanding of the RNAi process will certainly facilitate progress in its use for practical purposes.