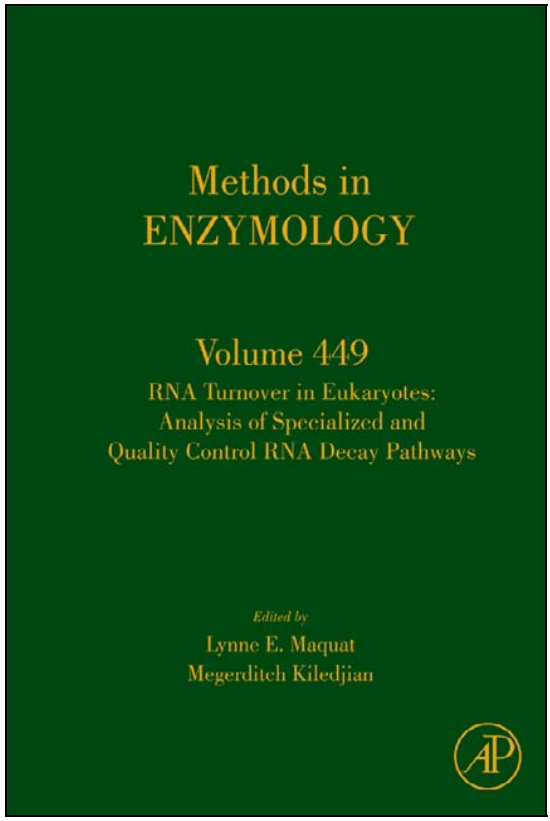

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CHAPTER SIXTEEN

How to Define Targets for Small Guide RNAs in RNA Silencing: A Biochemical Approach

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Abstract

RNA silencing involves various forms of sequence-specific gene silencing triggered by small RNAs. In RNA silencing, Argonautes are crucial protein components that are directed to the target messenger RNAs (mRNAs) through their association with small RNAs by base pairing. Argonautes repress the expression of the target genes at posttranscriptional levels. Full complementarity between a small RNA and its target mRNA results in Argonaute-mediated cleavage ("slicing") of the target mRNA. The D-D-H (asparagine-asparagine-histidine) triad that exists in the PIWI domain of Argonautes is the catalytic center for rendering their target cleavage ("slicer") activity. This chapter describes *in vitro* target RNA cleavage assays using Aubergine in a complex form with PIWI-interacting RNAs. Aubergine is one of the Argonautes expressed primarily in fly germ lines and is immunopurified from fly testes using the specific antibody against it. The method discussed is useful for defining targets for the small RNAs that function in RNA silencing.

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1. Introduction

RNA silencing is an evolutionally conserved cellular process in which small RNAs trigger various forms of sequence-specific gene silencing (Tomari and Zamore, 2005; Zaratiegui *et al.*, 2007). In each case, the small RNAs act as guide molecules that direct effecter complexes to target nucleic acids by base pairing.

RNA interference (RNAi) (Fire *et al.*, 1998) is the prototype of RNA silencing and causes gene silencing by cleaving (slicing) the target mRNAs in a sequence-dependent manner (Tomari and Zamore, 2005). The sequence specificity is rendered by a 21-to-23-nucleotide RNA, termed short interfering RNA (siRNA) (Tomari and Zamore, 2005). Micro-RNAs (miRNAs) are a large subset of endogenous small RNAs (21–23 nucleotides in length) encoded in the genome of a variety of organisms (Ambros, 2004; Kloosterman and Plasterk, 2006; Siomi and Siomi, 2007). miRNAs also function in RNA silencing, where the expression of genes involved in various developmental and metabolic processes is repressed posttranscriptionally. The origin of siRNAs and miRNAs differ from one another but both are processed from a longer double-stranded RNA precursor by an RNase III domain-containing Dicer protein and loaded onto a member of the argonaute family of proteins (Carmell *et al.*, 2002; Parker and Barford, 2006).

Members of the Argonaute family are defined by the presence of the PAZ and PIWI domains (Carmell *et al.*, 2002). In *Drosophila*, five genes, *AGO1*, *AGO2*, *AGO3*, *Piwi*, and *Aubergine* (*Aub*), are encoded as members of the Argonaute family and, according to sequence, are divided into two subfamilies, namely AGO and PIWI. *AGO1* and *AGO2*, members of the AGO subfamily, are expressed ubiquitously through development. The other three, members of the PIWI subfamily, are only expressed in germ line cells (Williams and Rubin, 2002).

AGO1 and AGO2 in *Drosophila* function in gene silencing through specific binding with miRNA and siRNA, respectively (Miyoshi *et al.*, 2005; Okamura *et al.*, 2004; Tomari *et al.*, 2004). AGO2 functions in RNAi as Slicer and is directly responsible for cleaving a target mRNA that is completely complementary to the siRNA (Miyoshi *et al.*, 2005). Although AGO1 is thought to function in miRNA-mediated translational repression, it also possesses Slicer activity (Miyoshi *et al.*, 2005). However, why and when AGO1 acts as a Slicer remains unelucidated.

Studies have shown that Piwi, Aub, and AGO3, PIWI subfamily members in *Drosophila*, are associated with a subset of endogenous small RNAs, termed piRNAs (PIWI-interacting small RNAs; Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007; Nishida *et al.*, 2007; Saito *et al.*, 2006; Vagin *et al.*, 2006). piRNAs were originally found in the small RNA profiling studies in

Drosophila testis and embryo (Nishida *et al.*, 2007) as a subgroup of small RNAs (24–30 nucleotides in length) that are distinct from miRNAs. (In the initial report, rasiRNA was the original term used for the small RNAs found in germ lines/embryos; however, it was revealed more recently that rasiRNAs are a subset of piRNAs; thus, the term "rasiRNA" is not used in this chapter.) While miRNAs are processed by Dicer from hairpin-formed precursors encoded in the genome, piRNAs are derived from a variety of repetitive intergenic elements such as retrotransposons that are embedded in the genome and produced by a Dicer-independent pathway (Aravin *et al.*, 2003; Brennecke *et al.*, 2007; Saito *et al.*, 2006; Vagin *et al.*, 2006). Proteins of the PIWI subfamily produced in *Escherichia coli* and immunopurified from germ line cells are capable of exhibiting Slicer activity *in vitro* (Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007; Miyoshi *et al.*, 2005). Taken together, it was postulated that the PIWI proteins function, at least in part, as Slicer in silencing repetitive/transposable genes through their association with piRNAs and so protect the genome from invasive elements.

The most abundant (about 46% of the total) class of piRNAs associated with Aub in testes consists of those that derive from *Suppressor of Stellate* [*Su*(*Ste*)] antisense transcripts (Nishida *et al.*, 2007). *Su*(*Ste*) genes are located repeatedly on the Y chromosome, show a significant similarity to *Stellate* genes at the nucleotide level, and are considered paralogs of *Stellate* genes (Livak, 1984). Although *Su*(*Ste*) genes produce nonprotein-coding transcripts, *Stellate* genes encode a protein with striking similarity to the β subunit of protein kinase CK2 (Livak, 1990) and are found repetitively on the X chromosome (Livak, 1984). Deletion of the *Su*(*Ste*) repeats on the Y chromosomes results in overexpression of Stellate in testes and causes an accumulation of aggregates containing Stellate proteins in spermatocytes (Bozzetti *et al.*, 1995). Aggregation then causes male infertility because of severe defects in spermatogenesis. Thus, silencing of *Stellate* genes by *Su*(*Ste*) repeats is essential for the maintenance of male fertility. Genetic approaches later revealed that mutations in the *Aub* gene cause male sterility, which can be attributed directly to a failure to silence the repetitive *Stellate* locus (Schmidt *et al.*, 1999). Biochemical and genetic studies showed that the *Su*(*Ste*) locus produces piRNAs and that the *Aub* gene is required for the accumulation of *Su*(*Ste*) piRNAs (Aravin *et al.*, 2004; Vagin *et al.*, 2006). However, a physical connection between Aub and *Su*(*Ste*) piRNAs has not yet been determined and how Aub is involved in silencing the *Stellate* genes remains unanswered. Through a profiling study for piRNAs associated with Aub in testes, we found that Aub interacts physically with piRNAs derived from *Su*(*Ste*) antisense transcripts in testes (Nishida *et al.*, 2007). Although the nucleotide sequences of *Stellate* and *Su*(*Ste*) genes are not identical, they are strikingly similar to each other. Moreover, we demonstrated that Aub produced in *E. coli* shows Slicer activity *in vitro* (Gunawardane *et al.*, 2007). Considering the aforementioned findings, we speculated that Aub complexes

containing *Su*(*Ste*) piRNA silence *Stellate* genes by cleaving the *Stellate* transcripts, analogous to how the AGO2–siRNA complexes function in RNAi (Miyoshi *et al.*, 2005). That is to say, *Stellate* could be one of the "targets of Aub" in testes. To examine if it is indeed the case, the Aub–piRNA complexes were immunopurified from the testis lysate and *in vitro* target RNA cleavage assays were performed. We demonstrated that the Aub complexes immunopurified from testes were able to cleave a target RNA containing part of the *Stellate* transcript (Nishida *et al.*, 2007). Results indicated that *Stellate* genes are a bona fide target of Aub and that *Su*(*Ste*) piRNAs can trigger silencing of *Stellate* in concert with the Slicer activity of Aub in testes.

This chapter describes the details of (1) how Aub–piRNA complexes are immunopurified from *Drosophila* testes as a model system, (2) how target RNAs are prepared for *in vitro* target RNA cleavage assays, and (3) how *in vitro* target RNA cleavage assays are performed. These methods will be useful for identifying small guide RNAs, as well as defining targets for the small RNAs identified in any organisms.

2. Immunopurification of Aub–piRNA Complexes from Fly Testis Lysates

Testes are dissected manually from adult flies in 1× phosphate-buffered saline (PBS). About 1000 testes are collected in a 1.7-ml microcentrifuge tube and washed with 1 ml of 1× PBS three times. Each time, testes were spun briefly at 4 °C. After removing and discarding as much buffer as possible, testis lysates are prepared by grinding them in a cold lysis buffer-containing 30 m*M* HEPES-KOH (pH 7.3), 150 m*M* KOAc, 2 m*M* MgOAc, 5 m*M* dithiothreitol (DTT), 0.1% NP-40, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 0.5% aprotinin. During this step, the microcentrifuge tube should be kept on ice. After spinning at 15,000 rpm for 1 min at 4 °C, the supernatant is transferred to a new 1.7-ml microcentrifuge tube and kept on ice. Lysis buffer is added again to the residual pellets and ground again for reextraction. This step is repeated several times and all the supernatant is then gathered in a microcentrifuge tube. At this point, the final volume should be about 0.5 ml. The supernatant is spun at 15,000 rpm for 20 min at 4 °C to remove all unnecessary debris and is then transferred to a new microcentrifuge tube. This supernatant is a testis lysate that can be used for immunoprecipitation experiments. Immunoprecipitation from the supernatant is performed with the anti-Aub monoclonal antibody (Gunawardane *et al.*, 2007) immobilized on Gamma Bind beads (GE Healthcare) (see Note 1). The amount of anti-Aub antibody used is about 1 ml of the culture supernatant from hybridoma cells generating the anti-Aub antibody. One milliliter should give rise to about 10 μg of anti-Aub antibody. Alternatively,

a purified antibody of the same amount can be used for this step. The lysate–antibody mixture is then rocked at 4 °C for at least 1 h and the beads are washed extensively with 1 ml of the lysis buffer (kept on ice); this is done at least five times. The final wash is carried out with a cleavage reaction buffer (30 m*M* HEPES-KOH, pH 7.4, 40 m*M* KOAc, 5 m*M* MgOAc, and 5 m*M* DTT) (see Note 2).

3. Analyzing Protein Components Present in Immunoprecipitates by Silver Staining and Western Blot Analysis

After extensive washing of the beads, immunoprecipitated proteins are eluted with SDS sample buffer without DTT (see Note 3). After elution, DTT is added to a final volume of 100 m*M*. Samples are then boiled and loaded onto SDS-acrylamide gels. After electrophoresis, protein bands are visualized by silver staining using SilverQuest (Invitrogen; Fig. 16.1A). To ascertain if Aub is indeed contained in the immunoprecipitates, Western blot analysis of the immunoprecipitates should be carried out using the anti-Aub antibody (Fig. 16.1B). To do so, after electrophoresis, protein components are transferred to a Protran nitrocellulose transfer membrane (Whatman) and probed with the anti-Aub antibody for at least 1 h at room temperature. Prior to this, the membrane should be incubated in blocking buffer to minimize nonspecific protein–antibody interactions with 1× PBS buffer containing 5% nonfat milk for a half an hour at room temperature. After incubation with the primary antibody, the membrane is washed three times with PBST buffer (0.1% Tween-20 containing 1× PBS) and incubated in PBST buffer containing the secondary antibody conjugated with peroxidase (peroxidate-conjugated sheep IgG fraction to mouse IgG; MP Biomedicals). After extensive washing with PBST buffer, the protein band corresponding to Aub can be visualized using detection reagent 1.2 (GE Healthcare) according to the manufacturer's instructions.

4. Analyzing Small RNAs Present in Immunoprecipitates by Northern Blot Analysis

RNAs that are bound by, and immunoprecipitated with, the Aub protein from testes extract can be isolated using the ISOGEN RNA extraction procedure according to the manufacturer (Nippon Gene). The resultant RNAs are resolved in a 6 *M* urea-containing 12% acrylamide denaturing gel and transferred to Hybond-N^+ (GE Healthcare) by electrophoresis.

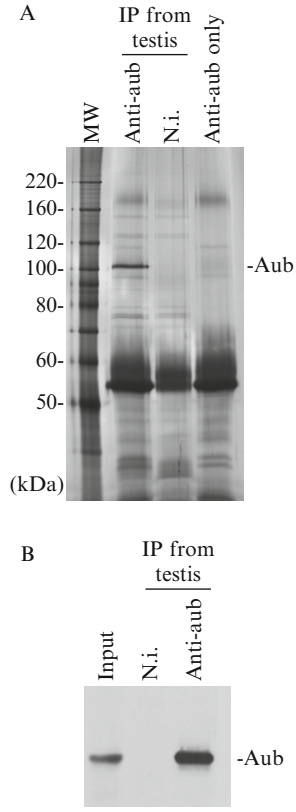

Figure 16.1 **Analyzing the protein components in Aub immunoprecipitates. (A) Silver-stained proteins immunoprecipitated from *Drosophila* testes using anti-Aub antibody. N.i., nonimmune IgG (negative control). In anti-Aub immunoprecipitates, a band corresponding to Aub itself is clearly observed. (B) Western blot analysis of the immunoprecipitates shown in (A). Anti-Aub antibody was used as the primary antibody. N.i., nonimmune IgG (negative control).**

After transferring, the membrane is UV cross-linked and baked at 80 °C for 1 h to immobilize the RNAs. An oligonucleotide probe (a DNA oligonucleotide containing a sequence fully complementary to the small RNA to be detected) is labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP and hybridized to the membrane at 42 °C in 7% SDS containing 0.2 *M* Na_2PO_4 (pH 7.9) overnight. The membrane is then washed twice at 42 °C with 2× SSPE containing 0.1% SDS. The blot is exposed to an imaging plate, and signals are analyzed on BAS-2500 (Fuji) or equivalent.

5. Target RNAs for Small RNA-Guided Cleavage

To make a target harboring a partial sequence of Stellate sense mRNA (Stellate target), a short DNA fragment produced by annealing a set of DNA oligonuclotides (5′-AATTCTCTGGCTTGTTGTACGGCGATGAAAG-3′ and 5′-GATCCTTTCATCGCCGTACAACAAGCCAGAG-3′) is inserted into a pBS SK+ vector digested with *Bam*HI and *Eco*RI. This yields a plasmid containing the Stellate target sequence. The sequence of Stellate mRNA was selected as the target sequence because it shows striking complementarity to Su(Ste)-4 piRNA, the most abundant piRNA found in Aub–piRNA complexes in testes (Nishida *et al.*, 2007). The Stellate target sequence is polymerase chain reaction (PCR) amplified from the Stellate target-containing plasmid using primers for the T7 and T3 promoter sequences. The resulting PCR products are subsequently used as templates for *in vitro* transcription using a MEGAscript T7 Kit (Ambion). Transcription is performed according to the manufacturer's instructions. After the reaction, the transcribed RNA is electrophoresed in a 6 *M* urea-containing 6% acrylamide denaturing gel, which is then stained with toluidine blue O solution (0.016% toluidine blue O) (Waldeck), 2% methanol, and 0.04% acetic acid), and the target RNA band with the expected size is excised from the gel using a clean razor blade. The gel piece is put in a 1.7-ml microcentrifuge tube and crushed into small pieces by grinding with a disposable pipette tip. Two gel volumes of RNA elution buffer (0.5 *M* ammonium acetate, 1 m*M* EDTA, 0.2% SDS) are added to the tube and rotated at 4 °C overnight to elute the RNA fragments. The gel pieces are sedimented by centrifugation (at 15,000 rpm for 1 min), and the supernatant is transferred to a new microcentrifuge tube. The target RNA is extracted using phenol and is subsequently precipitated with isopropanol in the presence of Pellet-paint Co-precipitant (Novagen). The resulting RNAs are then radiolabeled at their 5′ ends by the addition of a 5′-m^7G cap using guanylyltransferase (Ambion) (see Note 4). To do so, combine the following capping reaction mixture in a total volume of 15 μl: 1.5 μl of 10× capping reaction buffer (Ambion), 4.5 μl of [α-^{32}P]-labeled GTP (3000 Ci/mmol) (Perkin Elmer), 1 μl of *S*-adenosyl methionine (Ambion), 0.5 μg of gel-purified target RNA, and 1 μl of guanylyltransferase enzyme. The reaction mixture is incubated at 37 °C for 60 min and filtered through a P-30 column, according to the manufacturer's instructions (Bio-Rad), to remove unincorporated [α-^{32}P]GTP. The target RNA labeled is again gel-purified from a 6% acrylamide denaturing gel as indicated earlier. The resultant RNA is dissolved in nuclease-free water to a working concentration of 3000–5000 cpm/μl.

6. *In Vitro* Target RNA Cleavage (Slicer) Assay

Gamma Bind beads containing the immunopurified Aub protein from testes (see the earlier discussion) are drained and the following materials are added to the beads.

- 6 μl of 5× cleavage buffer (150 m*M* HEPES-KOH, pH 7.4, 500 m*M* KOAc, 25 m*M* MgOAc, 25 m*M* DTT, 50 m*M* creatine phosphate, and 2.5 m*M* ATP)
- 1 μl of RNasin plus (3 U/ml) (Promega)
- 1 μl of yeast RNA (0.5 mg/ml) (Ambion)
- 1 μl of creatine kinase (900 μg/ml) (Roche)
- 1 μl of target RNA (3000–5000 cpm/μl)

The final volume is adjusted to 30 μl by adding H_2O. The reaction mixture is incubated at 26 °C for 180–270 min. The resultant RNA is then purified using ISOGEN-LS (NipponGene) and separated in a 6% acrylamide denaturing gel. The gel is exposed to an imaging plate and the gel image is analyzed using a BAS-2500 (Fuji; Fig. 16.2) or the equivalent.

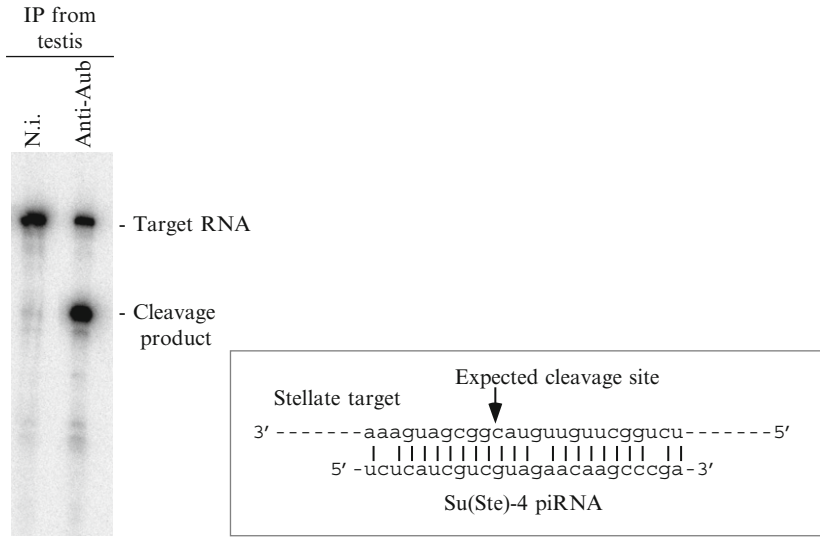

Figure 16.2 Aub–piRNA complexes show Slicer activity *in vitro*. An *in vitro* target RNA cleavage assay was carried out using Aub complexes immunopurified from testes (Fig. 16.1). An RNA target containing part of the *Stellate* transcript was used. The expected cleavage product is observed with the Aub complexes, but not in the negative control lane (N.i.), indicating that Aub complexes from testes have the ability to cleave the target RNA. N.i., nonimmune IgG (negative control). Sequences of Su(Ste)–4 piRNA and part of the *Stellate* transcript are shown in a box on the right. The expected cleavage site on the target is indicated by an arrow.

Notes

Note 1. From our experience, the volume of the Gamma Bind beads should be about 20 μl. When the volume is more than 20 μl, the background is often undesirably high.

Note 2. We previously immunopurified Ago1–miRNA complexes from S2 cells under stringent conditions with a salt concentration of 1 *M* NaCl to strip away associated proteins (in particular, other argonautes) (Miyoshi *et al.*, 2007). Under the same salt concentration, most of the proteins that are associated with human Ago2 are also stripped away from the complexes when immunopurified with specific monoclonal antibodies from human cells (HeLa; Miyoshi *et al.*, 2008). However, we noticed that such stringent conditions are not necessary in the case of immunopurifying Aub–piRNA complexes. Indeed, we confirmed by Western blot analyses that even under nearly physiological conditions (see earlier discussion) the Aub complexes did not contain other argonaute proteins (Nishida *et al.*, 2007). However, if desired, the argonaute–small RNA complexes should be purified under more stringent conditions. Indeed, it has been noted that the potassium chloride concentration in the wash steps of the affinity column purification could be increased up to 2.5 *M* without loss of RISC activity (Martinez and Tuschl, 2004). It is critical to adjust immunoprecipitation conditions (including salt and/or detergent concentrations and binding times) empirically according to the focus of the particular experiment.

Note 3. At this stage, it is quite important to use SDS sample buffer without DTT, as DTT elutes most antibodies from the beads and, as a result, the background of the silver-stained gel becomes much higher.

Note 4. The particular RNA used in the *in vitro* target RNA cleavage assays can be radiolabeled at the 5′ end by guanylyltransferase in the presence of [α-^{32}P]GTP or by T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP. Alternatively, the target RNA can be radiolabeled at the 3′ ends by RNA ligase in the presence of [^{32}P]-pCp (Nishida *et al.*, 2007).

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