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Monitoring miRNA-mediated silencing in *Drosophila melanogaster* S2-cells

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ABSTRACT

MicroRNAs (miRNAs) are small cellular RNAs that participate in post-transcriptional gene regulation. Even though they were only recently discovered, research on the biogenesis, mechanism of repression and biological significance of miRNAs has already received much attention. In this study, we have compared expression strategies for miRNA-activity reporter constructs and have examined the dependence of silencing by a particular *Drosophila* miRNA, *bantam*, on specific argonaute proteins. Consistent with previous biochemical experiments, we found that *bantam* silencing is strongly dependent on Ago1, but in addition we could detect the activity of Ago2-loaded *bantam*. Our experiments suggest that a perfectly complementary design and a transient expression strategy for reporter constructs may – in the case of catalytically active Ago-proteins – lead to a disproportionately strong response mediated by a minor fraction of silencing complexes. We present evidence that *Drosophila* S2-cells of independent sources differ in their RNAi efficiency in response to dsRNA added to the growth medium, and that the selection antibiotic G418 acts as an inhibitor of RNAi induced by soaking.

1. Introduction

MicroRNAs (miRNAs) are small, 21–23 nt RNAs that silence cognate genes. They are encoded in the genome as longer precursors and their biogenesis is characterized by two rounds of nucleolytic processing events – first in the nucleus by an RNase III enzyme called Drosha, then in the cytoplasm by Dicer, another RNase III enzyme. Mature microRNAs are single stranded and reside in a protein complex called RNA induced silencing complex (RISC) where they are bound by a protein of the argonaute family [1]. Target gene repression by microRNAs can occur via translational repression and/or the destabilization of the target mRNA [2]. A similar biogenesis pathway exists for short interfering RNAs (siRNAs), the small RNA guides for an RNA destruction pathway called RNA interference (RNAi). They are also subject to cytoplasmic processing by Dicer and become incorporated into RISC, which in humans and *Drosophila* is characterized by argonaute proteins with efficient nuclease domains (Ago2 in both organisms) [3]. While RNAi probably serves to fend off RNA viruses [4], miRNAs regulate a wide variety of cellular functions during development but also in adult life [5].

Functional specialization of *Drosophila* argonaute proteins has been proposed previously with Ago1 identified as the miRNA effector protein while Ago2 was the siRNA effector protein [6]. The reason for this specialization in biogenesis is not the distinct origin of the small RNAs, but differences in the extent of base-pairing of the miRNA/miRNA* intermediate [7,8]. The two pathways therefore intersect after the dicer-dependent processing step in *Drosophila*. Whether such sorting occurs in other organisms is unclear at the moment.

Drosophila melanogaster has become an important model organism to study the biochemical reactions during RISC-mediated mRNA cleavage [6,9–15] and RISC biogenesis [7,8,15–29]. In addition, reporter-based assays in cell culture and *in vivo* have contributed to our understanding of small RNA biogenesis, sorting and target site selection [7,16,30–37]. In the coming years, the research focus will likely be extended from the mechanism of miRNA biogenesis to the regulation of their activity (both transcriptional and post-transcriptional) [38–40] and reporter assays for specific miRNAs will be an important tool. In this article, we present reporter constructs for the *D. melanogaster* miRNA *bantam* and examine aspects of target site structure and expression strategies to measure *bantam* activity in Schneider-2 (S2) cells. Our results highlight both the strengths and some potential pitfalls of the artificial reporter gene strategy.

2. Materials and methods

2.1. Molecular cloning and sequence of 2′-O-methyl RNA oligonucleotides

Our dsRNA constructs and T7 RNA polymerase mediated transcription were described previously [7]. The *bantam*-responsive GFP expression constructs are based on a P-element vector where the ubiquitin promotor drives expression of GFP (pKF63, [7]). The SV40 poly-A signal is included after the stop codon of GFP. To create plasmid *bantam*_si we annealed the oligonucleotides 5′-GGC CAA TCA GCT TTC

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AAA ATG ATC TCG CAG AAT CAG CTT TCA AAA TGA TCT CA-3′ and 5′-CTA GTG AGA TCA TTT TGA AAG CTG ATT CTG TGA GAT CAT TTT GAA AGC TGA TT-3′ and ligated them into NotI/XbaI cut pKF63. This plasmid harbors two perfectly matched *bantam* target sites. For the bulged match target sites, oligonucleotides 5′-GGC CAA TCA GCT TTC CTC ATG ATC TCA CAG AAT CAG CTT TCC TCA TGA TCT CAC AGA ATC AGC TTT CCT CAT GAT CTC ACA GAA TCA GCT TTC CTC ATG ATC TCA-3′ and 5′-CTA GTG AGA TCA TGA GGA AAG CTG ATT CTG TGA GAT CAT GAG GAA AGC TGA TTC TGT GAG ATC ATG AGG AAA GCT GAT TCT GTG AGA TCA TGA GGA AAG CTG ATT-3′ were annealed and ligated into NotI/XbaI cut pKF63, yielding a construct with four target sites.

The sequence of the cholesteryl-modified antisense *bantam* 2′-*O*-methyl RNA oligonucleotide was 5′-Chol.-UCU UAA AUC AGC UUU CAA AAU GAU CUC AAC CU-3′, for antisense Luciferase it was 5′-Chol.-CAU CAC GUA CGC GGA AUA CUU CGA AAU GUC C-3′ and the seed-mutant form of *bantam* was 5′-Chol.-UCU UAA AUC AGC UUU CAA AAU *ACG AGA* AAC CU-3′ (seed mismatch italicized). All bases were 2′-*O*-methyl modified.

2.2. Cell culture, transfections, FACS analysis and western blotting

Drosophila Schneider 2 (S2) cells were either from the lab's stock or obtained from Invitrogen (order-No. R690–0718064014, Invitrogen, Karlsruhe, Germany) and cultured in Schneider's Medium (Bio&Sell, Nürnberg, Germany) supplemented with 10% fetal bovine serum (Hyclone/Thermo Fisher, Bonn, Germany) at 25 °C. For transfections, cells were seeded at 1×10^6 cells/ml in a 24-well dish (500 µl per well) and 100 µl of transfection mix containing either 1.0 µg of dsRNA or 0.3 µg of plasmid DNA and 4 µl of FugeneHD (Roche, Mannheim, Germany) was added to the cells. The transfection mix was prepared with serum-free medium and incubated 60 minutes at room temperature to allow formation of the complexes between nucleic acids and transfection reagent. When antisense 2′-*O*-methyl RNA oligonucleotides were co-transfected with plasmids, 10 pmol of oligonucleotide was mixed with 0.3 µg of plasmid in 96 µl of medium without serum, then 4 µl of FugeneHD was added and the mix was transfected as described above. For soaking experiments, 0.5 µg of dsRNA (or the amounts indicated) were simply added to the growth medium.

After transfection, the cells were incubated for the indicated times and then analyzed in a Becton-Dickinson FACSCalibur flow cytometer. For quantification, the arithmetic mean of all GFP-positive cells (defined by a window that yields less than 1% GFP-positive cells if untransfected S2 cells are analyzed) was taken. Values represent the mean±SD of three measurements. The data were normalized by dividing all measurement values by the mean of the corresponding control prior to calculating mean and SD, thus yielding a value of 1 for the control with a correspondingly scaled standard deviation.

Western blotting was performed as previously described [7]. The monoclonal Ago1 antibody 1B8 was used in a 1:1000 dilution (TC supernatant) and is a gift of Mikiko Siomi (Keio University Medical School, Japan). As a loading control, monoclonal anti β-tubulin E7 was used at a 1:1000 dilution (TC supernatant) and obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa, USA). Western blot images were acquired on a Fuji LAS-3000 mini imaging system.

3. Results

Our aim was to provide functional data for the argonaute-dependence of *bantam* silencing in *Drosophila melanogaster* S2 cells and compare transient and stable expression strategies for miRNA reporter genes. We used GFP as the reporter gene in an expression system analogous to previous work with miRNA-277 [7]. Two different types of *bantam* target sequences were used, one with perfect complementarity that allows for RISC-mediated mRNA cleavage and a partially complementary target sequence that can only be repressed translationally or via mRNA destabilization (Fig. 1A). First, we verified that our reporter constructs were robustly and specifically repressed by *bantam* in *Drosophila* S2 cells. To this end, we co-transfected the reporter plasmids with 2′-*O*-methyl RNA oligonucleotides antisense to *bantam*, a seed-mutant version of the *bantam* inhibitor and a completely unrelated sequence derived from luciferase. As shown in Fig. 1B, GFP expression increased 7.1-fold (perfect match) and 43-fold (bulged match) in the *bantam* reporters while the control construct without any target sequence showed no change in GFP expression. Furthermore, this de-repression was completely abrogated (perfect match) or strongly reduced (bulged match) when the antisense inhibitor lacked seed-complementarity. Similar results – but with a reduced amplitude – were obtained when cell lines with stable expression of the reporter constructs were used (Fig. 1C). In this case, transfection of the antisense inhibitor for *bantam* leads to a 2.6-fold (perfect match) and 6.7-fold (bulged match) de-repression, respectively. Thus, our reporter constructs are robustly and specifically silenced by the *bantam* miRNA.

3.1. Transfection of dsRNA results in more efficient RNAi than soaking

To dissect the mechanism of reporter silencing it is often necessary to deplete specific factors via RNA interference (RNAi) prior to quantification of reporter expression. Besides the exact order of events (see below), an important question is how the dsRNA that triggers RNAi should be introduced into the cells. Previously, both soaking (i.e. addition to the growth medium without transfection reagent) and transfection approaches were successful in *Drosophila* cells [41–43]. We directly compared the efficiency of RNAi in a time-course following soaking and transfection of the same amount of dsRNA directed against GFP (final conc.: 2 µg/ml) in a clonal cell line expressing high levels of GFP (Fig. 2A). At all time points (3, 5 and 7 days), the transfected dsRNA induced more robust RNAi. In both cases, the knock-down reached maximal efficiency after 5 days and was stable on day 7. Therefore, we chose to induce RNAi in our reporter assays by transfecting the corresponding dsRNA.

3.2. Drosophila bantam can silence via both, Ago1 and Ago2

Biochemical assays have established that *bantam* is predominantly loaded into Ago1 [6,7]. *Drosophila* Ago1 and Ago2 can be distinguished by their preference for particular target site architectures: While Ago2 is an efficient, multiple-turnover nuclease on perfectly complementary target sites, it is inefficient at repressing bulged match target sites that cannot be cleaved as directed by the small RNA. Essentially the inverse is true for *Drosophila* Ago1 [7], so that a bulged match reporter will be predominantly silenced via Ago1 while a perfect match reporter is silenced preferentially through Ago2. Since our perfect match reporter was silenced specifically by *bantam* (Fig. 1B and C), we asked whether in this case Ago1 could repress both types of reporters, or whether we were detecting the activity of Ago2-loaded *bantam*.

When transient transfections were used to introduce the reporter constructs, depletion of Ago2, but not Ago1, could de-repress the perfectly matched reporter (Fig. 2B, central panel). On the other hand, depletion of Ago1, but not Ago2, lead to moderately increased GFP expression from the bulged match reporter (Fig. 2B, right panel). The simplest interpretation of these results is that Ago2-loaded *bantam* is repressing the perfect match reporter. However, this reporter did not respond to depletion of Ago2 in stably expressing cell lines (Fig. 2C, central panel), even though in these cells the reporter is still detectably repressed (see Fig. 1C). In contrast, the bulged match reporter responded much better to Ago1-depletion in stably expressing cells than in transient transfections (Fig. 2C, right panel, 7.7-fold vs. 2.2-fold de-repression).

3.3. Different populations of S2 cells have distinct properties

Certain properties are known to vary between different batches of *Drosophila* S2-cells. As an attempt to work with cells of traceable