A single Hox locus in Drosophila produces functional microRNAs from opposite DNA strands

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MicroRNAs (miRNAs) are ~22-nucleotide RNAs that are processed from characteristic precursor hairpins and pair to sites in messages of protein-coding genes to direct post-transcriptional repression. Here, we report that the miRNA iab-4 locus in the Drosophila Hox cluster is transcribed convergently from both DNA strands, giving rise to two distinct functional miRNAs. Both sense and antisense miRNA products target neighboring Hox genes via highly conserved sites, leading to homeotic transformations when ectopically expressed. We also report sense/antisense miRNAs in mouse and find antisense transcripts close to many miRNAs in both flies and mammals, suggesting that additional sense/antisense pairs exist.

Results and Discussion

Our examination of the antisense transcript that overlaps Drosophila mir-iab-4 revealed that the reverse complement of the mir-iab-4 hairpin folds into a hairpin reminiscent of miRNA precursors [Fig. 1A]. Moreover, 17 sequencing reads from small RNA libraries of Drosophila testes and ovaries mapped uniquely to one arm of the iab-4 antisense hairpin [Fig. 1B]. All reads were aligned at their 5′ end, suggesting that the mir-iab-4 antisense hairpin is processed into a single mature miRNA in vivo, which we refer to as miR-iab-4AS. For comparison, we found six reads consistent with the known mir-iab-4-5p [or mir-iab-4 for short] and one read for its star sequence [miR-iab-4-3p]. Interestingly, the relative abundance of mature miRNAs and star sequences for mir-iab-4AS (17:0) and mir-iab-4 (6:1) reflects the thermodynamic asymmetry of the predicted miRNA/miRNA* duplexes [Khvorova et al. 2003; Schwarz et al. 2003]. Because they derived from complementary near palindromes, miR-iab-4 and miR-iab-4AS had high sequence similarity, only differing in four positions at the 3′ region [Fig. 1B]. However, they differed in their 5′ ends, which largely determine miRNA target spectra [Brennecke et al. 2005; Lewis et al. 2005]: miR-iab-4AS was shifted by 2 nt, suggesting targeting properties distinct from those of mir-iab-4 and other known Drosophila miRNAs. We confirmed robust transcription of mir-iab-4 sense and antisense precursors by in situ hybridization to Drosophila embryos [Fig. 1C]. Both transcripts were detected in abdominal segments in the posterior part of the embryo, but intriguingly in nonoverlapping domains. As described previously [Bae et al. 2002; Ronshaugen et al. 2005], mir-iab-4 sense was expressed highly in abdominal segments A5–A7, showing modulation in levels within the segments: abdominal-A [abd-A]-expressing cells [Fig. 1D]; Karch et al. 1990; Macias et al. 1990] ap-
peared to have more mir-iab-4, whereas Ultrabithorax (Ubx)-positive cells appeared to have little or none [Fig. 1D; Ronshaugen et al. 2005]. In contrast, mir-iab-4AS transcription was detected in the segments A8 and A9, where Abdominal-B (Abd-B) is known to be expressed [Fig. 1C; Yoder and Carroll 2006]. Primary transcripts for mir-iab-4 and mir-iab-4AS were also detected by strand-specific RT–PCR in larvae, pupae, and male and female adult flies (Supplemental Fig. S1), suggesting that both miRNAs are expressed throughout fly development.

To assess the possible biological roles of the two iab-4 miRNAs, we examined fly genes for potential target sites by searching for conserved matches to the seed region of the miRNAs [Lewis et al. 2005]. We found highly conserved target sites for mir-iab-4AS in the 3′ untranslated regions (UTRs) of several Hox genes that are proximal to the iab-4 locus and are expressed in the neighboring more anterior embryonic segments: abd-A, Ubx, and Antennapedia (Antp) have four, five, and two seed sites, respectively, most of which are conserved across 12 Drosophila species that diverged 40 million years ago [Fig. 2A; Supplemental Fig. S2; Drosophila 12 Genomes Consortium 2007; Stark et al. 2007a]. More than two highly conserved sites for one miRNA is exceptional for fly 3′ UTRs, placing these messages among the most confidently predicted miRNA targets and suggesting that they might be particularly responsive to the presence of the miRNA. The strong predicted targeting of proximal Hox genes was reminiscent of previously characterized miR-4 targeting of Ubx in flies and miR-196 targeting of HoxB8 in vertebrates [Mansfield et al. 2004; Yekta et al. 2004; Hornstein et al. 2005; Ronshaugen et al. 2005].

To test whether mir-iab-4AS is functional and can directly target abd-A and Ubx, we constructed Luciferase reporters carrying the corresponding wild-type 3′ UTRs and control 3′ UTRs in which each seed site was disrupted by point substitutions. mir-iab-4AS potently repressed reporter activity for abd-A and Ubx (Fig. 2B). This repression was specific to the mir-iab-4AS seed sites, as expression of the control reporters with mutated sites was not affected. We also tested whether mir-iab-4AS reduced expression of a Luciferase reporter with the Abd-B 3′ UTR, which has no seed sites. As expected, mir-iab-4AS expression did not affect reporter activity, consistent with a model where miRNAs do not target genes that are coexpressed at high levels [Farh et al. 2005; Stark et al. 2005]. In addition to demonstrating specific repression dependent on the predicted target sites, these assays confirmed the processing of the mir-iab-4AS hairpin into a functional mature miRNA.

If miR-iab-4AS were able to potently down-regulate...
Ubx in the fly, its misexpression should result in a Ubx loss-of-function phenotype, a line of reasoning that has often been used to study the functions and regulatory relationships of Hox genes. Ubx is expressed throughout the haltere imaginal disc, where it represses wing-specific genes and specifies haltere identity (Weatherbee et al. 1998). When we expressed mir-iab-4AS in the haltere imaginal disc under bx-Gal4 control, a clear homeotic transformation of halters to wings was observed (Fig. 3). The halters developed sense organs characteristic of the wing margin and their size increased severalfold, features typical of transformation to wing (Weatherbee et al. 1998). Consistent with the increased number of mir-iab4AS target sites, the transformation was stronger than that reported for expression of iab-4 (Ronshaugen et al. 2005), for which we confirmed changes in morphology but did not find wing-like growth (Fig. 3D).

We conclude that both strands of the iab-4 locus are expressed in nonoverlapping embryonic domains and that each transcript produces a functional miRNA in vivo. In particular, the novel mir-iab-4AS is able to strongly down-regulate neighboring Hox genes. Interestingly, vertebrate Hox clusters, which lies at an analogous position in the vertebrate Hox clusters, is transcribed in the same direction as mir-iab-4AS and most other Hox genes, and targets homologs of both abd-A and Ub x (Mansfield et al. 2004; Yekta et al. 2004; Hornstein et al. 2005). With its shared transcriptional orientation and homologous targets, mir-iab-4AS appears to be the functional equivalent of mir-196.

The expression patterns and regulatory connections between Hox genes and the two iab-4 miRNAs show an intriguing pattern in which the miRNAs appear to reinforce Hox gene-mediated transcriptional regulation (Fig. 3A). In particular, miR-iab-4AS would reinforce the posterior expression boundary of abd-A, Ub x, and Antp, supporting their transcriptional repression by Abd-B. mir-iab-4 appears to support abd-A- and Abd-B-mediated repression of Ub x, reinforcing the abd-A/Ubx expression domains and the posterior boundary of Ub x expression. Furthermore, both iab-4 miRNAs have conserved target sites in Antp, which is also repressed by Abd-B, abd-A, and Ub x. The iab-4 miRNAs thus appear to regulate the established regulatory hierarchy among Hox transcription factors, which exhibits “posterior prevalence,” in that more posterior Hox genes repress more anterior ones and are dominant in specifying segment identity (for reviews, see McGinnis and Krumlauf 1992, Pearson et al. 2005). Interestingly, Abd-B and mir-iab-4AS are expressed in the same segments, and the majority of cis-regulatory elements controlling Abd-B expression are located 3′ of Abd-B (Boulet et al. 1991). This places them near the inferred transcription start of mir-iab-4AS, where they potentially direct the coexpression of these genes. Similarly, abd-A and mir-iab-4 may be coregulated as both are transcribed divergently, potentially under the control of shared upstream elements.

Our data demonstrate the transcription and processing of sense and antisense mir-iab-4 into functional miRNAs with highly conserved functional target sites in neighboring Hox genes. In an accompanying study (Bender 2008), genetic and molecular analyses in mir-iab-4 mutant Drosophila revealed that the proposed regulation of Ub x by both sense and antisense miRNAs occurs under physiological conditions and, in particular, the regulation by miR-iab-4AS is required for normal development. These lines of evidence establish mir-iab-4AS as a novel Hox gene, being expressed from within the Hox cluster and regulating Hox genes during development.

The genomic arrangement of two miRNAs that are expressed from the same locus but on different strands

Figure 3. Misexpression of miR-iab-4AS transforms halters to wings. [A,B] Overview of an adult wild-type Drosophila (B) and an adult expressing mir-iab-4AS using bx-Gal4 [A]. The halters, balancing organs of the third thoracic segment, are indicated by arrows. [C] Wild-type haltere. [D] Expression of mir-iab-4 using bx-Gal4 induces a mild haltere-to-wing transformation. Sensory bristles characteristic of wild-type wing margins [shown in B′] are indicated by an arrow. [E] Expression of mir-iab-4AS using bx-Gal4 induces a strong haltere-to-wing transformation, displaying the triple row of sensory bristles (inset) normally seen in wild-type wings (shown in B′). Note that C–E are at the same magnification.
miRNAs are located in introns of host genes transcribed on the opposite strand or are within 50 nt of antisense ESTs or cDNAs [Supplemental Table S1]. These include an antisense transcript overlapping human mir-196 (see also Mainguy et al. 2007). However, because of the contribution of noncanonical base pairs, particularly G-U pairs, that become less favorable A-C in the antisense strand, many miRNA antisense transcripts will not fold into hairpin structures suitable for miRNA biogenesis, which explains the propensity of miRNA gene predictions to identify the correct strand [Lim et al. 2003]. Nonetheless, in a recent prediction effort, 22 sequences reverse-complementary to known Drosophila miRNAs showed scores seemingly compatible with miRNA processing [Stark et al. 2007b]. Deep sequencing of small RNA libraries from Drosophila confirmed the processing of small RNAs from four of these high-scoring antisense candidates [Ruby et al. 2007], and the ovary/testes libraries used here showed antisense reads for an additional Drosophila miRNA (mir-312) [see Supplemental Tables S2, S3]. In addition, using high-throughput sequencing of small RNA libraries from mice, we found sequencing reads that uniquely matched the mouse genome in loci antisense to 10 annotated mouse miRNAs. Eight of the inferred antisense miRNAs were supported by multiple independent reads, and two of them had reads from both the mature miRNA and the star sequence [Supplemental Table S2]. These results suggest that sense/antisense miRNAs could be more generally employed in diverse contexts and in species as divergent as flies and mammals.

Functional sense/antisense microRNAs

Figure 4. Regulation of gene expression by antisense miRNAs. (A) miRNA-mediated control in the Drosophila Hox cluster. Schematic representation of the Drosophila Hox cluster (Antennapedia and Bithorax complex) with miRNA target interactions [check marks represent experimentally validated targets]. miR-iab-4 [blue] and miR-iab-4AS [red] target anterior neighboring Hox genes and miR-10 [black] targets posterior Sex-combs-reduced Scr) [Brennecke et al. 2005]. Abd-A and miR-iab-4 and Abd-B and miR-iab-4AS might be coregulated from shared control elements (cis). Note that mir-iab-4AS is expressed in the same direction as most other Hox genes and its mammalian equivalent, mir-196. (B) General model for defining different expression domains with pairs of antisense miRNAs [black]. Different transcription factor[s] activate the transcription of miRNAs and genes in each of the two domains separately [green lines]. Both miRNAs might inhibit each other by transcriptional interference or post-transcriptionally [vertical red lines], leading to essentially nonoverlapping expression and activity of both miRNAs. Further, both miRNAs likely target distinct sets of genes [diagonal red lines], potentially re-enforcing the difference between the two expression domains.

This might provide a simple and efficient means to create nonoverlapping miRNA expression domains [Fig. 4B]. Such sense/antisense miRNAs could restrict each other’s transcription, either by direct transcriptional interference, as shown for overlapping convergently transcribed genes (Shearwin et al. 2005; Hongay et al. 2006), or post-transcriptionally, possibly via RNA–RNA duplexes formed by the complementary transcripts. Sense/antisense miRNAs would usually differ at their 5′ ends and thereby target distinct sets of genes, which might help define and establish sharp boundaries between expression domains. Coupled with feedback loops or coregulation of miRNAs and genes in cis or trans, this arrangement could provide a powerful regulatory switch. The iab-4 miRNAs might be a special case of tight regulatory integration in which miRNAs and proximal genes appear coregulated transcriptionally in cis and repress each other both transcriptionally and post-transcriptionally.

It is perhaps surprising that no antisense miRNA had been found previously, even though, for example, the intriguing expression pattern of the iab-4 transcripts had been reported nearly two decades ago (Cumberledge et al. 1990; Bae et al. 2002), and iab-4 lies in one of the most extensively studied regions of the Drosophila genome. The frequent occurrence of antisense transcripts [Yelin et al. 2003; Katayama et al. 2005] suggests that more antisense miRNAs might exist. Indeed, up to 13% of known Drosophila, 20% of mouse, and 31% of human miRNAs are located in introns of host genes transcribed on the opposite strand or are within 50 nt of antisense ESTs or cDNAs [Supplemental Table S1]. These include an antisense transcript overlapping human mir-196 (see also Mainguy et al. 2007). However, because of the contribution of noncanonical base pairs, particularly G-U pairs, that become less favorable A-C in the antisense strand, many miRNA antisense transcripts will not fold into hairpin structures suitable for miRNA biogenesis, which explains the propensity of miRNA gene predictions to identify the correct strand [Lim et al. 2003]. Nonetheless, in a recent prediction effort, 22 sequences reverse-complementary to known Drosophila miRNAs showed scores seemingly compatible with miRNA processing [Stark et al. 2007b]. Deep sequencing of small RNA libraries from Drosophila confirmed the processing of small RNAs from four of these high-scoring antisense candidates [Ruby et al. 2007], and the ovary/testes libraries used here showed antisense reads for an additional Drosophila miRNA (mir-312) [see Supplemental Tables S2, S3]. In addition, using high-throughput sequencing of small RNA libraries from mice, we found sequencing reads that uniquely matched the mouse genome in loci antisense to 10 annotated mouse miRNAs. Eight of the inferred antisense miRNAs were supported by multiple independent reads, and two of them had reads from both the mature miRNA and the star sequence [Supplemental Table S2]. These results suggest that sense/antisense miRNAs could be more generally employed in diverse contexts and in species as divergent as flies and mammals.

Materials and methods

Plasmids

3′ UTRs were amplified from Drosophila melanogaster genomic DNA and cloned in pcR2.1 for site-directed mutagenesis. The following primer pairs were used to amplify the indicated 3′ UTRs: Abd-A (tc...ttgcag), Ubx (tctagaATCCTTAGATCCTTAGATCCT; ctcgagATGGATGGGTTCTCGTTGCAG), Ubx [tctagaATCCTTAGATCCTTAGATCCTTAGTTGCAG], Abd-B [tctagaGGGTCAGCAAAGTCAACTCAACTCAGTGAC], and Abd-D [tctagaGGGTCAGCAAAGTCAACTCAGTGAC]. Quick-Change multisite-directed mutagenesis was used to mutate all miR-iab-4AX seed sites from ATACGT to ATAGGT, per the manufacturer’s directions (Stratagene). Wild-type and mutated 3′ UTRs were subcloned into pC[40] between Sacl and Notl sites to make Renilla luciferase reporters. Plasmid pC[71] contains the Abd-A wild-type 3′ UTR, pC[72] contains the Ubx wild-type 3′ UTR, pC[74] contains the Abd-B wild-type 3′ UTR, pC[75] contains the Abd-A mutated 3′ UTR, and pC[76] contains the Ubx mutated 3′ UTR fused to Renilla luciferase. The control let-7 expression vector was obtained by amplifying let-7 from genomic DNA with primers 474 base pairs (bp) upstream of and 310 bp downstream from the let-7 hairpin and cloning it into pMPT-puro. To express miR-iab-4 and miR-iab-4AS, a 430-bp genomic fragment containing the miR-iab-4 hairpin was cloned, in either direction, downstream from the tubulin promoter as described in Stark et al. (2005). For the UAS-miR-iab-4 and UAS-miR-iab-4AS constructs, the same 430-bp genomic fragment containing the miR-iab-4 hairpin was cloned downstream from pUAST-DsRed2 [Stark et al. 2003] in either direction.

Reporter assays

For the luciferase assays, 2 ng of p2129 (firefly luciferase), 4 ng of Renilla reporter, 48 ng of miRNA expression plasmid, and 48 ng of pCD2 (GFP) were cotransfected with 0.5 µl Fugene HD per well of a 96-well plate. Twenty-four hours after transfection, expression of Renilla luciferase was induced by addition of 500 µM CuSO4 to the culture media. Twenty-four hours after induction, reporter activity was measured with the Dual-Glo luciferase kit (Promega), per the manufacturer’s instructions on a Tecan Safire II plate reader.

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The ratio of Renilla:firefly luciferase activity was measured for each well. To calculate fold repression, the ratio of Renilla:firefly for reporters compared with mutant reporters. Geometric means from 16 measurements are shown. Error bars represent the fourth highest and lowest values of each set.

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**References**


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