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Independent transcription of miR-281 in the intron of ODA in *Drosophila melanogaster*

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ABSTRACT

MicroRNAs (miRNAs) have recently received much interest for their role in post-transcriptional regulation. However, the study of miRNA transcription lags behind that of gene cloning and functional analysis. The MiR-281 in *Drosophila melanogaster* is located in the first intron of isoform RA of the ornithine decarboxylase antizyme (ODA) gene. ODA has three isoforms because of alternative transcription start sites (TSSs). Expression profile analysis indicated that miR-281 is not co-expressed with any ODA isoform. We amplified the primary transcripts of miR-281 using the RACE technique. The pri-miRNA is 2149 bp with a poly (A) tail and a canonical polyadenylation signal (AATAAA). Chromatin immunoprecipitation analysis confirmed the binding of hypophosphorylated Pol-II and the transcription factor Myc at the core miR-281 promoter region. The abundance of miR-281 does not correlate, either positively or negatively, with the expression of any ODA isoform, indicating that ODA has little influence on the transcription of miR-281.

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MicroRNAs (miRNAs) belong to an abundant class of small (~22 nucleotides), endogenous, non-coding RNAs that function at the post-transcriptional level [1–3]. Thousands of miRNA genes have been reported in diverse organisms, including mammals, insects, plants, viruses, and protistae [4–7]. According to their location in the genome, miRNAs are classified into intergenic miRNAs, exonic miRNAs in non-coding transcription units (TUs), intronic miRNAs in protein coding TUs and intronic miRNAs in non-coding TUs [8,9]. Intronic miRNAs can be co-expressed with host genes [10] and in human, some intronic miRNAs are processed from primary gene transcripts. Intermediate transcripts that include exons and an miRNA-harboring intron, were detected during the biogenesis of intronic miRNA [11]. Another type of intronic miRNA, the mirtron, was discovered to be generated without Drosha-mediated cleavage. These mirtrons have their own special primary nucleotide motifs for recognition and cleavage by Dicer-1 [12,13]. Generally, transcription of intronic miRNAs is more complex than that of their intergenic counterparts. It becomes more complicated if the host gene has alternative transcriptional start sites (TSSs) and undergoes alternative splicing. For example, a miRNA in the intron of one isoform may appear in the exon or 5′UTR of another isoform [8]. We name these miRNAs located in the alternative region of a host gene as AR-miRNAs. It is our current interest to investigate the potential relationships between alternative splicing or alternative TSSs of host genes and the biogenesis of AR-miRNAs.

The ornithine decarboxylase antizyme gene (ODA) in *Drosophila melanogaster* has three isoforms, RA, RB, and RC, as a result of alternative TSSs. The miR-281 is located in the first intron of isoforms RA and RC but is upstream of isoform RB. We found that miR-281 is different from other known intronic miRNAs. It is independently transcribed with its own RNA polymerase II- (Pol-II) like promoter in the ODA intron. We amplified full-length transcripts of the miRNA-281 and demonstrated that RNA Pol-II and the transcription factor Myc bind to the miRNA-281 promoter region.

Materials and methods

Bioinformatics and data collection. Seventy-eight *D. melanogaster* miRNA sequences were downloaded from miRBase (Version 9.0). The gene annotations were retrieved from GenBank. The 5′UTR sequence were downloaded from the UTResource database [14]. Secondary structures of miRNAs were predicted using the Vienna RNA Secondary Structure Prediction server [15]. Target genes of miR-281 were analyzed by both PicTar and MiRanda [16,17]. All miRNA sequences were aligned using ClustalX. The phylogenetic tree was constructed using MrBayes [18] and viewed with TreeView [19]. MiRNA and ODA gene sequences were mapped onto the genome using Blat from the UCSC database (R5.2; August 2007). ESTs that mapped to ODA and miR-281 genes with *E*-values of less than 1.0e−100 were analyzed and displayed with the UCSC genome browser by adding custom EST track settings.

RACE amplification. RACE was carried out with the SMART Rapid amplification of cDNA kit (Takara) using 3 μg of adult fly total RNA,

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according to the manufacturer's instructions. The full-length transcripts were confirmed by PCRs using appropriately designed 3′ and 5′ primers. The sequences of all the primers are given in the supplemental material.

Chromatin immunoprecipitation and RT-PCR. ChIP assays were carried out using the protocol described by Cavalli et al. [20] with some modifications. The detailed procedure is given in the supplemental material. Total RNA was prepared using Trizol (Invitrogen), treated with DNaseI (Takara) and then reverse-transcribed using oligo-dT primers. RT-PCRs were carried out using the M-MLV kit (Promega), according to the manufacturer's protocol. Gene-specific primer pairs were designed from neighboring exons to detect genomic DNA contamination.

Real-time PCR. Total RNA was extracted as described previously and then reverse-transcribed using oligo-dT primers (18). Real-time PCR reactions were conducted using an Applied Biosystems 7300 with a SYBR Premix Ex Taq kit (Takara). *Drosophila* Ribosomal protein L11 (RpL11) was used as the internal control. We calculated the relative quantity of RA, RB, RC and miR-281 by the comparative C_t ($2^{-[\Delta][\Delta]C_t}$) method. The data were normalized against the housekeep gene RpL11.

Results

AR-miRNA: microRNAs in alternative regions

Due to alternative splicing or alternative TSSs, some genomic regions can be both intronic or exonic, relative to different gene isoforms. The miRNAs within these alternative regions we have named AR-miRNAs. There are 21 intronic miRNAs located in 13 host genes in *D. melanogaster*. Three AR-miRNAs, including the miR-281, miR-7, and miR-31b are located in the alternative regions of three host genes (Table S1 and Fig. S1).

We selected the miR-281 to investigate transcription relations between the host gene and AR-miRNAs. The miR-281 is a member of the mir-46 family and is located in the intron of the ornithine decarboxylase antizyme gene (ODA, CG16747). The ODA gene is on the negative strand of chromosome 2 and has three isoforms (RA, RB, and RC) because of alternative TSSs. The predicted precursor of miR-281, according to miRBase, is upstream of isoform RB but in the first intron of RA and RC. Another protein coding gene, SmD3, appears in the positive strand of this region (Fig. 1).

Duplication of miR-281 in Drosophila

The miR-281 includes two tandem repeating miRNAs, pre-miR-281-1 and pre-miR-281-2. According to the annotation in miRBase, the distance between pre-miR-281-1 and pre-miR-281-2 is 128 bp. This cluster produces three types of mature miRNAs, miR-281, miR-281-1*, and miR-281-2* (Fig. S2). Among these three, there is only a one nucleotide difference between the arms, whereas there are 13 nucleotides differences in the loop region, implying that the mature miRNAs are under high evolutionary constraint. Interestingly, though homologs are found in diverse organisms, duplication of miR-281 is only seen in *Drosophila* species. In other insects such as *Apis mellifera* and *Anopheles gambiae*, genome-level scanning only found one copy of pre-miR-281 (Fig. S3). The phylogenetic tree of the miR-46 family, inferred by a Bayesian algorithm, shows that pre-miR-281-1 and pre-miR-281-2 are clustered into two different clades (Fig. S4).

Expression of the miR-281 and the ODA gene

Because the ODA gene has three TSSs, we designed isoform-specific, as well as gene-specific primers to study the relationships between miR-281, SmD3 and the ODA gene isoforms. Isoforms RA, RB, and RC are expressed at all stages of *D. melanogaster* development, whereas miR-281 expression was detected only in the third instar, pupa, and adult stages (Fig. 2), suggesting that the biogenesis of miR-281 is not related to activity of the host ODA gene. The SmD3 gene is expressed at the egg and the first and second instar stages, which is opposite to that of miR-281. It is possible that SmD3 inhibits the accumulation of miR-281 transcripts by an antisense gene silencing mechanism because SmD3 overlaps with miR-281 on the opposite strand, however, we will not discuss this further. In all, these results suggest that miR-281 expression might be independent of the host ODA gene, which is in contrast with the known mechanism of intronic miRNA expression.

Full-length transcript of intronic miR-281

The expression profile analysis indicated that miR-281 might be transcribed independently of ODA. To exclude the possibility of co-transcription of ODA and miR-281, we designed primers to detect the intermediate transcript which was suggested by Kim and Kim

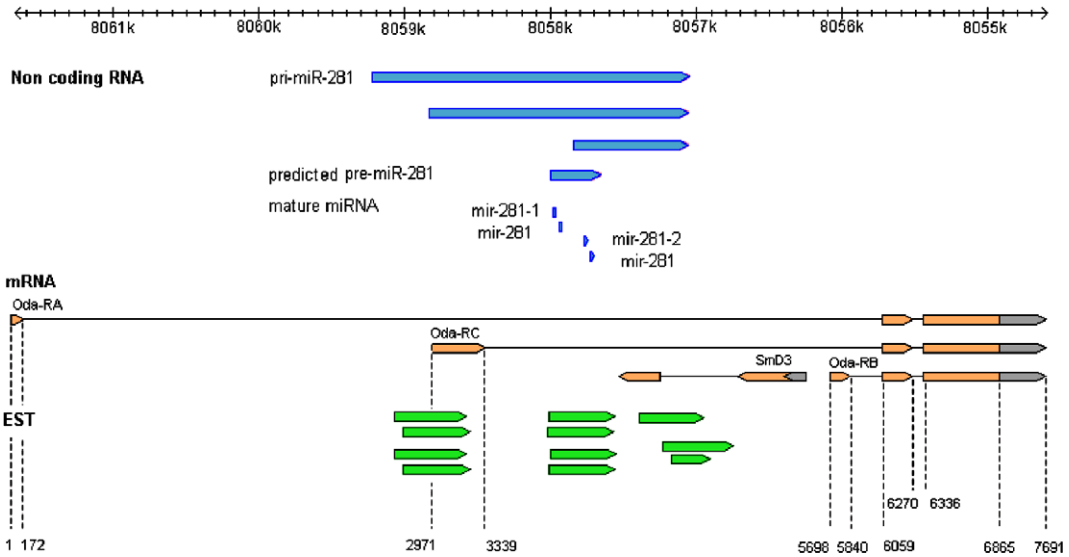

Fig. 1. Schematic depiction of the ODA, SmD3, and miR-281 genes.

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