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GENOMIC ANALYSIS OF SILKWORM microRNA PROMOTERS AND CLUSTERS

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MicroRNAs (miRNAs) are endogenous single-stranded RNAs of 18~22 nt in length, which can regulate the complementary mRNAs at the post-transcriptional level by cleavage or repression of translation of the target mRNAs. Studies have shown that the majority of animal miRNAs are transcribed from independent transcription units, and some are transcribed together with their host genes. However, the nature of the primary transcript of intergenic miRNAs remains unknown. Silkworm (*Bombyx mori*) miRNAs are representative of those of the *Lepidoptera* insects and many of them are conserved in *Caenorhabditis elegans* and other animal species. To date, little is known about the transcriptional regulation of silkworm miRNA genes. We performed the genomic analysis on the silkworm miRNA transcripts around the promoter region including the transcription start site (TSS) and the TATA-box, and on the organization of the miRNA cluster. In 73 pre-miRNAs from the silkworm 131 promoters were detected via a bioinformatics approach. Among them the portion of non-conserved promoters is greater than that of the conserved ones. The genomic organization of pre-miRNAs of the silkworm was globally analyzed and it was determined that 11 of them were organized into five clusters. Sequence alignment showed that paralogs existed for some of the miRNAs in the cluster. These results may increase the understanding of the specific sequences upstream of the pre-miRNAs and of the functional implications of miRNA clusters in the silkworm.

Keywords: **microRNA, *Bombyx mori*, promoter, miRNA cluster.**

MicroRNAs (miRNAs), encoded by specific genes, are endogenous single-strand RNAs ranging form 19–25 nt in length existing in various organisms from viruses to plants and mammals [1–4]. They play key roles in the regulation of translation or degradation of target mRNAs by inducing post-transcriptional silence through base pairing to the complementary target sites. The precursor miRNAs are transcribed from the genome as pri-miRNA precursors and cleaved by the Drosha endonuclease in the nucleus to form approximately 70 nt pre-miRNAs with a characteristic hairpin structure. Then the pre-miRNAs are transported to the cytoplasm by Exportin-5, and the loop region of the hairpin is removed by the Dicer endonuclease. One strand of the matured double-stranded miRNA is incorporated into a large protein complex, the RNA-induced silencing complex (RISC), in order to guide the RISC to complementary mRNA targets. The RISC either inhibits translation elongation or triggers mRNA degradation, depending upon the degree of complementarity of the miRNA with its target [5, 6]. MiRNAs are involved in numerous cellular processes including development, differentiation, proliferation, apoptosis and response to stress. Dysregulation of miRNA expression also contributes to disease pathology.

The biogenesis of miRNA is complicated and several lines of evidence have shown that most miRNAs are encoded by their own genes located in the intergenic regions or the antisense strands of annotated genes [7–9]. The intergenic miRNA genes are believed to be transcribed independently to form a new gene family. However the intronic miRNAs and the ones interspersed in the human genome with mobile Alu elements are transcribed with their host genes [5, 10]. Studies showed that more than half of all known mammalian miRNAs are located in the introns of either protein-coding or noncoding transcriptional units (TUs), and about 10% of miRNAs are encoded by exons of long nonprotein-coding transcripts, also known as mRNA-like noncoding RNAs [10, 11]. Unlike plant miRNAs, miRNAs in animals are primarily encoded in intronic regions [12], suggesting that transcription of animal pri-miRNA may differ from that of plants [13, 14]. Many pieces of evidence have indirectly suggested that miRNA genes are class-II genes which are transcribed by RNA polymerase II, suggesting that transcription of miRNA may be regulated by a similar mechanism as was established for protein-coding genes and miRNAs may contain promoter elements similar to those of protein-coding genes [15]. It is known that the class-II promoters consist of two parts: the core promoter and the upstream element. The core promoter contains at least two elements: a TATA box beginning at the approximate position of -30 and an initiator centered on the transcription start site (TSS). In *Arabidopsis* 63 miRNA TSSs were identified

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via 5'-RACE and most of them contain a TATA-box in the core promoter region [16]. However, there are exceptions to this rule, for example, promoters of miR-23a, -27a, -24–2 lack the common promoter elements required for transcription initiation such as the TATA-box and the initiator element, and a large portion of pri-miRNAs does not contain a 5'-cap or a poly (A) tail [14]. Such TATA-less promoters are often found in housekeeping genes [15]. It is therefore necessary to study the upstream sequences including promoters, transcription start sites or specific elements, to understand the location and length of pri-miRNAs, expression patterns of miRNAs, miRNA-mediated regulatory pathways and their network.

However, up to date we have limited knowledge on the transcription of miRNA genes, which is the first and important step of miRNA biogenesis. Understanding the mechanism of miRNA gene expression is fundamentally important. One of the main goals for miRNA research is to elucidate how pri-miRNA genes are transcribed and how is miRNA involved in the complicated gene regulatory network [17]. Recently, five transcription factor (TF) binding motifs have been identified relating to protein-coding gene promoter sequences: AtMYC2, ARF, SORLREP3, LFY and TATA-box [18]. Identification of promoters of intergenic miRNA genes in *Caenorhabditis elegans*, *Homo sapiens*, *Arabidopsis thaliana* and *Oryza sativa* revealed that most known miRNA genes in these four species contain the same type of promoters as the protein-coding genes [19]. As is known, the promoter of a gene is a crucial control region for transcription initiation [20, 21].

Silkworm (*Bombyx mori*) has been domesticated for over 5000 years and is well-known for its industrial importance in sericulture. In addition it is a model system for the *Lepidoptera* insects with a completed genome sequence, which is in great favor for functional genomic research. At present, there are 91 silkworm microRNAs in the miRBase. Although millions of small RNAs from the silkworm were identified via high-throughput sequencing [22] and the number of non-conserved miRNAs identified in the silkworm is growing and is greater than in *Drosophila* and other animal species [23, 24], little is known about the promoters of miRNAs in the silkworm. The method of full-length cDNA sequence alignment may allow to identify some promoters in the silkworm, but most of the cDNA sequence clones do not extend to the transcriptional start site (TSS) [25–27]. Full understanding of miRNA transcription requires a complete description of the location and extent of pri-miRNAs, including transcription start sites and promoters [13, 18, 19, 28].

We are interested in the known miRNA genes that contain their own promoters, and are focused on (1) the identification of specific sequences or motifs adjacent to pre-miRNAs, which relate to the expression of miRNAs, and (2) the pattern of miRNA clusters associated with the upstream specific sequences of pre-miRNA in the silkworm.

EXPERIMENTAL

Upstream sequences of silkworm miRNAs. The silkworm (*B. mori*) pre-miRNA sequences were obtained from the miRBase database (miRBase Sequence Database, http://microrna.sanger.ac.uk; released 14.0 September 2009). The silkworm genome sequences were downloaded from the SilkDB (http://silkworm.genomics.org.cn/) and the Silkworm Genome Research Program (http://sgp.dna.affrc.go.jp/index.html). As there were no reports on silkworm miRNAs in introns, intergenic miRNAs of the silkworm were divided into two groups: non-conserved miRNAs and conserved miRNAs.

The upstream sequences of pre-miRNAs in the intergenic regions were organized according to the method described previously [19, 29]. Briefly, if a pre-miRNA and its upstream gene were in the same direction and the distance between them was more than 2400 bp, the 2000 bp sequence upstream of the pre-miRNA was retrieved by Apollo (a genome annotation tool) on basing on the GFF file ('Gene-Finding Format' or 'General Feature Format') of silkworm miRNAs from the genome sequences [30]. Meanwhile, two hundred random sequences 2000 bp in length were automatically generated by a computer as a control.

Prediction of specific sequences upstream of silkworm miRNA genes. Sequences of the TSS and the TATA-box were predicted using an online web approach (http://www-bimas.cit.nih.gov/molbio/proscan/) and the Eponine method [31].

Clustering of silkworm miRNA genes. For analysis of miRNA clustering, both upstream and downstream sequences with pairwise distance less than 10 kb were considered as clustered miRNAs. When the clustered miRNAs were organized, the 5'-end sequences of the first miRNAs in the upstream regions were fetched following the same rule mentioned above. Some miRNAs that overlap with the transposons were excluded from our data set [29].

RESULTS AND DISCUSSION

Analysis of promoters related to silkworm miRNA genes

Among the 91 silkworm miRNA genes obtained, 7 (e.g. Bmo-miR 1920–1921) could not be located in the chromosome and 11 overlapped with each other, and thus were unsuitable for promoter prediction, so only 73 genes were used for predicting putative promoters.

The 2000 bp long sequences located upstream of pre-miRNAs were analyzed for putative promoters, in total 120 upstream sequences were obtained and used for further analysis. Among the 120 candidates, 27 (22.5%) did not contain a promoter (Fig. 1). And in the other 93 promoter-containing sequences, 28 (30.1%) contained only one promoter, 19 (20.4%) contained two promoters and the rest (49.5%) contained three or more promoters, respectively. Thus a total of 131 putative promoters was predicted.

In plants and animals, miRNA genes are found in diverse genomic locations. In animals, they were either located in the non-coding region of protein-encoding genes, or hosted within the introns [32]. To understand the genomic distribution of silkworm pri-miRNAs in more details, we divided these pri-miRNA sequences into conserved and non-conserved pri-miRNAs. In this case, the proportion of the two pri-miRNAs types varies considerably (Fig. 1). The percentage of conserved pri-miRNAs tended to decrease with the increase of promoter number they contained, whereas the portion of non-conserved pri-miRNA showed an increasing pattern, implying that non-conserved pri-miRNAs of the silkworm contain more than one promoter.

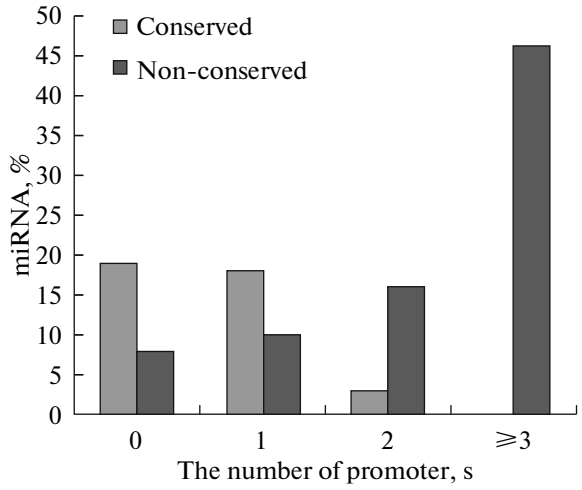

Fig. 1. Promoter predictions in the conserved and non-conserved miRNAs.

Analysis of specific sequences upstream pre-miRNAs of the silkworm

The distribution of putative promoter positions corresponding to pri-miRNA sequences was subsequently analyzed and a total of 76 TSS predictions was obtained within 2000 bp upstream from 120 pre-miRNAs sequences. A clear peak was observed around -30 nt upstream of the TSS (Fig. 2*a*). The majority of predicted TSSs lie in pre-miRNAs from −500 bp to −1100 upstream of the pre-miRNAs (Fig. 2*b*). Distribution of the TSSs of non-conserved miRNA genes can be divided into three distinct regions (Fig. 2*b*). The first peak is close to the pre-miRNA and is within the −200 bp region, the second broad region ranges from −550 to −800 bp, and the third region ranges from −1100 to −1300 bp. TSSs of conserved miRNA genes are located in different regions upstream of the pre-miRNAs too, and reveal three small peaks from −500 to −800 bp, −1000 to −1300 bp and −1500 to −1600 bp upsteam of the pre-miRNAs, respectively.

Among the 120 upstream pri-miRNAs, 76 (63%) contained a putative TATA-box, suggesting that the majority of silkworm miRNA genes has the same promoters as the protein-coding genes and are transcribed by RNA polymerase II. And the other 44 (37%) have no predicted TATA-box. The non-TATA box-containing promoters of miRNA, termed as TATA-less promoters have been detected in *Arabidopsis*. Such TATA-less promoters tend to be found in two classes of genes, one is housekeeping genes and the second class is developmental regulation genes such as the homeotic genes that control development of the fruit fly or genes that are active during the development of the immune system in mammals [15].

The location of the TATA-box of pri-miRNAs is similar to that of the TSSs (Fig. 3*a*). The majority of TATA-boxes lie in the −600 to −1200 bp region upstream of pri-miRNAs. In the control (a random intergenic sequence 2000 bp length), distribution of predicted TSSs and TATA-boxes apparently differs from those of the surrounding pre-miRNAs (Fig. 3*b*), suggesting that the distribution of TSSs or TATA-boxes in the indicated regions of pre-miRNAs is specific in the silkworm.

Clustering of silkworm miRNA genes

Previously large-scale surveys of non-conding DNA revealed that miRNA genes tend to form clusters rather than to be randomly distributed across the chromosomes [33–36]. In the human genome many of the known miRNAs are clustered. However, very little is known about miRNA clustering in *Lepidoptera* insects. Some clusters reflect the processing of miRNAs from a single polycistronic transcript, implying that more than one pre-miRNA may be processed from the same primary transcript [32]. Also, this process implies that the co-transcription of functionally different miRNAs in a cluster may simultaneously target several categories of genes [37, 38]. In contrast, some other miRNA clusters demonstrate the independence, transcriptional regulation and relatively long-distant physical location [39]. Evidence suggests that clustered miRNA genes are often, but not always, located in a polycistron and coexpressed with neighboring miRNAs and host genes [40]. The potential clustering of miRNAs in the silkworm genome was examined and the genomic organization of all registered miRNA genes within 10 kb was analyzed.

Almost all of the silkworm miRNA genes lie in the intergenic regions. Our analysis revealed that only five clusters were predicted from 91 silkworm miRNA genes (Table), implying that five families of miRNAs in the silkworm play species-specific roles in development. The reason for such a low number of clusters may be that some of the silkworm miRNAs have not been detected or that the distance between most of silkworm miRNA clusters in the genome is greater than 10 kb. In the five clusters of silkworm miRNAs, one cluster contains three and the others contain two groups of miRNA genes, respectively (Table). Two of such clusters are located in the first chromosome. The length of the miRNA clusters varies from 89 to 2556 bp with an average of 697 bp, suggesting that they may be transcribed from different transcripts and play unique functions in the expression regulation of silkworm

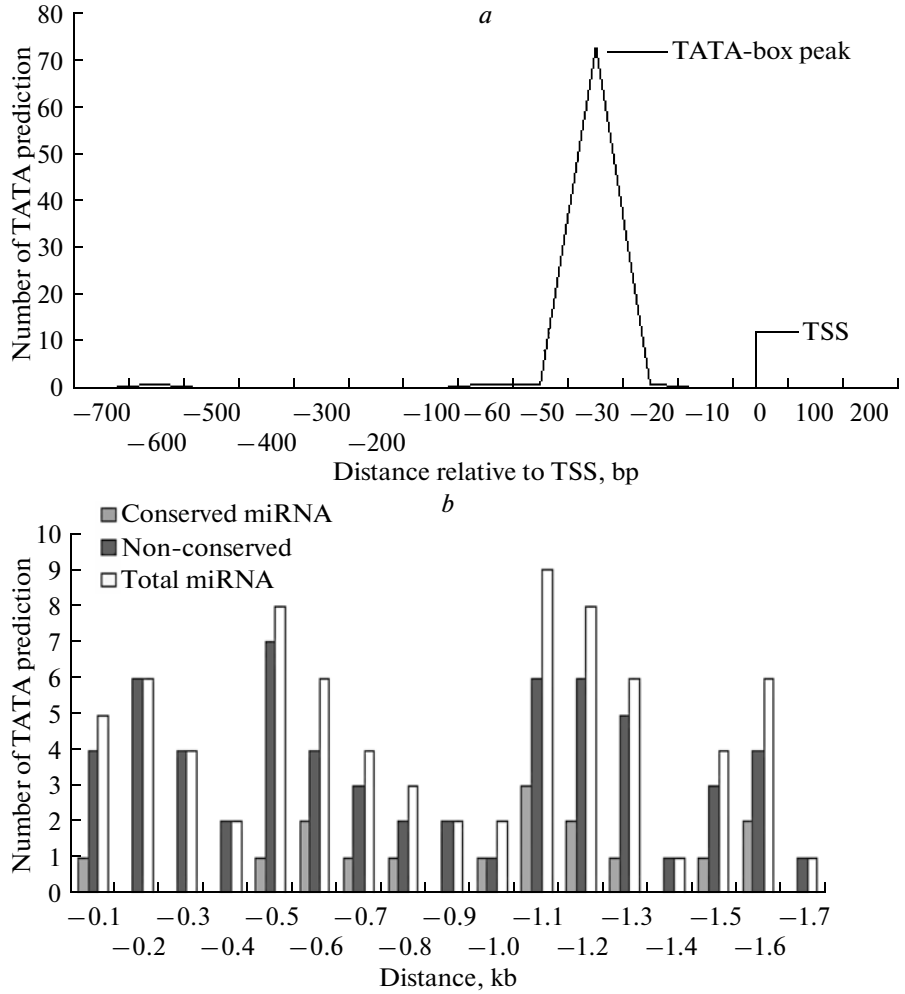

Fig. 2. Locations of the TSS with relation to the TATA-box/promoter on the genome of silkworm miRNAs (*a*) and TSS predictions in the conserved and non-conserved miRNAs (*b*).

genes. In addition, four of the five clusters including miR2a-1-2-2b, miR13a-b, miR2731a-b and miR9b-9c, pre-miRNAs contain a single promoter, suggesting that such miRNAs may be transcribed under the control of a single promoter (Table). The pre-miRNAs of these four clusters are derived from the same family. However, miR927–1926 is an exception, its pre-miRNA belongs to a different family and no promoters were predicted for it, even though it shares the same precursors and can generate two kinds of different mature miRNAs. In the human genome most miRNA clusters contain miRNAs from different families and may consist of several different groups of non-homologous miRNAs [41]. The studies indicated that about 50% of all miRNA genes throughout the *Drosophila* genome were clustered, whereas only a few of miRNA genes of the human genome might be clustered [33, 42]. However, the increasing number of identified miRNAs suggests that miRNAs are more likely to cluster together than previously estimated. Additionally, the human miRNA clusters are frequently observed to have super sized clusters. For example, human hsamiR-127 (also hsa-miR134) that resides on chromosome 14q32, is the largest miRNA cluster to date, and contains more than 50 members [43].

Features of the predicted silkworm clusters in the genome

Cluster	Cluster length, bp	Distance between miRNAs, bp	Location	Number of promoters	Chromosomal location
miR2a-1-2-2b	545	121	Intergenic	Single	Chr1
miR9b-9c	2556	2466	Intergenic	Single	Chr12
miR13a-b	206	127	»	Single	Chr1
miR927-1926	89	11	»	None	Chr10
miR2731a-b	89	6	»	Single	Chr15

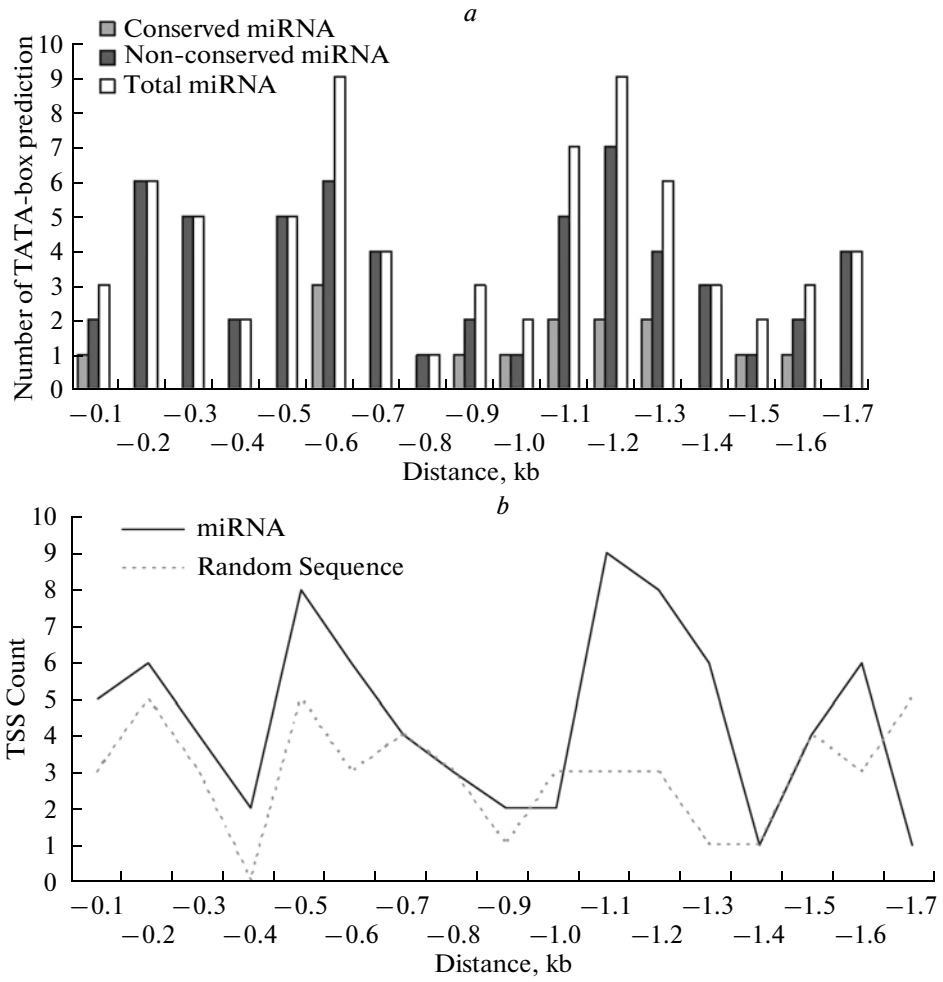

Fig. 3. TATA-box predictions in the conserved and non-conserved miRNAs (*a*) and genomic distribution of TSS and a random sequence form of silkworm pre-miRNA (*b*).

In summary, upstream specific regulatory sequences (TSS, TATA-box) of silkworm pre-miRNAs were extensively analyzed and 131 promoter was detected from 73 silkworm miRNAs. The results showed that non-conserved silkworm miRNA genes contain more promoters than the conserved ones. Global analysis on the genomic organization of the registered miRNA genes from the silkworm determined only five clusters containing 11 miRNA genes. These results will be beneficial to understand the miRNA promoters and regulation of miRNA expression in the silkworm in detail, and provide a foundation for further investigations of transcription factors in the regulation of silkworm miRNAs.

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