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The silk moth *Bombyx mori* U1 and U2 snRNA variants are differentially expressed

Julie M. Sierra-Montes[1], Simone Pereira-Simon[2], Shamayra S. Smail, Rene J. Herrera*

Department of Biological Sciences, OE304, Florida International University, Miami, FL 33199, United States

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Abstract

Five U1 and eight U2 isoforms of the silk moth *Bombyx mori* exhibiting internal nucleotide differences have been previously identified and characterized in various tissues and developmental stages. In this investigation, it is demonstrated that the levels of some snRNA variants differ in egg and silk gland tissue and change during development. Qualitative and quantitative differences in the U1 and U2 variant populations were observed at three developmental points (early, middle and late) of the silk gland (SG) during the fifth instar larval stage of the silk moth. Statistical analyses of the various isoform populations across the fifth instar larval and egg stages show significant differences for some of the U1 and U2 variants. The representation of variant sequences in expressed U1 and U2 sequences (RT-PCR libraries) and in a whole-genome shotgun (WGS) assembly database was confirmed. In addition, conserved elements in the promoter 5′-flanking region of the U1 and U2 variants were identified in the WGS.

Keywords: snRNAs; Differential expression; Spliceosome; *Bombyx mori*; Insect

1. Introduction

Studies on pre-mRNA splicing have provided abundant evidence for the important role of differential or alternative splicing in development and tissue differentiation (for representative publications see Maniatis, 1991; Elliott et al., 1998; Arystarkhova et al., 2002). Invariably, the spliceosomal RNAs and proteins as well as auxiliary proteins involved in the process control differential pre-mRNA splicing. The spliceosome, a nuclear complex comprised of small nuclear RNAs (snRNAs) and their affiliated proteins, performs the function of intron deletion and exon ligation during pre-mRNA splicing. The major small ribonuclear particles (snRNPs), termed U1, U2, U4/U6 and U5, interact in a series of sequential steps to produce the intron-less mature mRNA (Guthrie and Patterson, 1988; Lamond, 1993). Variants of snRNAs have been implicated in the differential expression and/or the alternative splicing of various genes (Kuo et al., 1991; Maniatis, 1991; Rio, 1993; Modrek and Lee, 2002).

U1 and U2 are the two most abundant snRNAs and are the first to initiate the splicing pathway on the pre-mRNA transcript (Guthrie and Patterson, 1988; Will and Luhrmann, 2001). The process begins with the binding of the U1 (which ranges in size from 147 to 1000 nucleotides depending on the species) to the 5′ exon-intron junction. The U2 (which varies in size from 184 to 1175 nucleotides

Abbreviations: snRNA, small nuclear RNA; SG, silk gland; WGS, whole-genome shotgun; snRNP, small nuclear ribonucleoprotein; RT-PCR, reverse transcriptase polymerase chain reaction; PCR, polymerase chain reaction; BLAST, Basic Local Alignment Search Tool; U-snRNA, U-series small nuclear RNA.

* Corresponding author. Tel.: +1 305 348 1258; fax: +1 305 348 1259.
E-mail address: herrerar@fiu.edu (R.J. Herrera).
[1] Present Address: Dept. of Cellular and Molecular Pharmacology, Room 626 Batchelor Children's Institute (R189), 1580 N.W. 10th Avenue, University of Miami Medical Center, Miami, FL 33136, United States.
[2] Present Address: Bascom Palmer Eye Institute, McKnight Vision Research Center, 2nd FL, (D-880), University of Miami Medical Center, 1638 N.W. 10th Avenue, Miami, FL 33136, United States.

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depending on the species) then binds to the branch-site generating the lariat structure with the bulged 2′-OH involved in the subsequent nucleophillic attack. U1 and U2 snRNA's secondary structures are comprised of four (I, II, III and IV) and five (I, IIa, IIb, III and IV) stem/loop domains, respectively. U1, with its associated proteins, is not involved in the catalytic function of the spliceosome while U2, in combination with the U5 and U6 snRNPs, takes part in both transesterfication-splicing reactions.

Variants of snRNAs have been detected in a multitude of species. U1 RNA isoforms have been reported in *Tetrahymena* (Eugen-Olsen et al., 1997), mice (Lund et al., 1985), *Drosophila melanogaster* (Lo and Mount, 1990) as well as in wheat and peas (Musci et al., 1992) to name a few examples. The expression of some of these plant and animal U-snRNA variants are developmentally and tissue-specifically regulated (Lund and Dahlberg, 1987; Lo and Mount, 1990; Morales et al., 1997). Our laboratory has also reported isoforms for U1, U2, U3, U4, U5 and U6 in the silk moth *Bombyx mori* (Adams et al., 1985; Adams and Herrera, 1987; Sierra-Montes et al., 2002, 2003; Pereira-Simon et al., 2004; Smail et al., submitted for publication). Variant forms of U1 (Sierra-Montes et al., 2003; Pereira-Simon et al., 2004) and U2 (Sierra-Montes et al., 2002) snRNAs have been specifically identified in fifth instar larval silk gland (SG) of *B. mori*. In these studies, five U1 and eight U2 isoforms exhibiting internal nucleotide differences were identified. In this study, the differential expression of these *B. mori* U1 and U2 variants is reported. U1 and U2 RT-PCR libraries from egg and silk gland tissues and developmental stages were generated and recombinant clones containing full-length sequences were identified. Sequencing and statistical analysis revealed that several of the U1 and U2 isoforms are differentially expressed during the early, middle and late fifth instar larval stages of the SG. In addition, U1 and U2 sequences from the egg stage were examined as a basis of comparison to another tissue and from genomic DNA to verify the existence of the variants in the polyploid SG genome. The presence of specific U1 and U2 variants in the recently released whole-genome shotgun (WGS) assembly database was confirmed as well as the existence of variant-specific 5′-flanking promoter regions exhibiting conserved blocks of nucleotides.

2. Materials and methods

2.1. Growth of larvae and isolation of total RNA

Eggs and larvae from the European 703 *B. mori* silk moth strain were obtained courtesy of Dr. Thomas H. Eickbush, Dept. of Biology, University of Rochester. Larvae were grown and the SGs acquired as previously described (Sierra-Montes et al., 2002; Pereira-Simon et al., 2004). Total RNA was isolated, DNAsed and stored at −90 °C as reported in prior publications (Sierra-Montes et al., 2002).

2.2. U1 and U2 RT-PCR libraries

U1 and U2 RT-PCR libraries from the fifth instar larval SG of the European 703 strain of *B. mori* were created as formerly described in Sierra-Montes et al., 2002. The U1 primer sequences (20mers) were designed based on *B. mori* (Adams et al., 1985) and *D. melanogaster* (Mount and Steitz, 1981) terminal internal sequences (Sierra-Montes et al., 2003). The sequences of the U1 primers are as follows: upstream primer 5′-ATACTTACCTGGCGTGGAGG-3′ and downstream primer 5′-CGGGGACAGCGCGTACGCAG-3′. The U2 primers (33mers) were designed against the 5′ and 3′ termini of the *D. melanogaster* U2 snRNA sequence (accession number: X04256). The 5′-ends of each primer were designed to include specific overlapping restriction-enzyme sites (*Bam*HI/*Pst*I, upstream primer; *Eco*RI/*Pst*I, downstream primer) for cloning purposes. The primer sequences are as follows (restriction sites underlined): upstream primer 5′-GCGGATCCTGCAGATCGCTTCTC-GGCCTTATGG-3′ and downstream primer 5′-GAATTCC-CTGCAGAGTTGGGCCAAAATCCCGGC-3′.

A total of four independent RT-PCR libraries for each U1 and U2 snRNAs were created for each tissue/developmental stage [eggs, and early (days 1 and 2), middle (days 3 and 4) and late (days 5 and 6) fifth instar larval stages]. Genomic PCR libraries of U1 and U2 genes from *B. mori* were also created to assess variant representation in SG DNA. PCR products were cloned into pCR 2.1-TOPO plasmids and sequencing of recombinant clones was performed as previously described (Sierra-Montes et al., 2002, 2003).

2.3. Statistical analysis

Chi-square (χ^2) and significance (p) values were obtained using the Crosstabs and Chi Square Tests (programs courtesy of the Statistics Department at FIU). Values were significant at $\alpha \leq 0.05$ and $\chi^2 \geq 5.99147$, and were used to ascertain whether individual U1 and U2 variants, across the three SG developmental stages, exhibit significant differences from one larval stage to another. Data files comparing the individual U1 and U2 isoforms from the early, middle and late fifth instar larval (E=Early, M=Middle and L=Late) stages were set up in a tabular, matrix format. Matrixes consisting of N (number of U1 or U2 clones per isoform per stage) rows and x (developmental stage) columns were tabulated and processed. Chi-square (χ^2), significance (P) and standard error values were generated. Follow-up Pair Wise Comparisons were conducted using Holm's Sequential Bonferroni method $p<0.05$ to determine where the significant differences reside for the individual variants when comparing the number of variants in each larval stage.

2.4. Comparative genomics

BLAST searches against the *B. mori* WGS sequences were performed using U1A snRNA and U2A snRNA *B.*