Analysis of a Novel Transcription Mode of Fhx/P25 Gene in *Bombyx mori**

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Abstract Fhx/P25 in silkworm, *Bombyx mori*, one of the main components of silk fibroin, is presumed in previous reports to be expressed exclusively in the posterior silk gland (PSG) of the animal with strict territorial and developmental specificities. On the basis of a large-scale analysis of the silkworm EST data, it was found that Fhx/P25 gene is transcribed not only in the posterior silk gland, but in the ovary and in other tissues of the larvae at day 3 of the fifth-instar as well and that this gene has distinct transcription start sites (TSSs) in the posterior silk gland and the ovary. The TSS in the ovary is located about 115 bp upstream sequence of that in the posterior silk gland. Subsequent RT-PCR, FQ-PCR and sequencing have verified the validity of this presumption. In addition, alternative splicing is predicted in pre-mRNA of Fhx/P25 gene and confirmed by RT-PCR. In conclusion, Fhx/P25 gene is not a gene with strictly tissue-specific transcription. Complicated regulation mechanisms may exist for its transcription and expression and it may have other functions to perform.

Key words silkworm (*Bombyx mori*), Fhx/P25 gene, transcription start site, alternative splicing

Silk fibroin has been reported to be specifically synthesized in the posterior silk gland (PSG) cells of silkworm (*Bombyx mori*) and secreted into its lumen in a form of 2.3-MD protein complex, designated as the elementary unit of fibroin, which consists of six sets of heterodimer of disulfide-linked heavy and light chains and one molecule of Fhx/P25 [1~3]. Fhx/P25, a glycoprotein, is associated with $(H\text{-}L)_6$ mainly by hydrophobic interactions and is centrally important in the maintenance of the elementary fibroin unit. It contains three N-linked oligosaccharide chains at *Asn*69, *Asn*113 and *Asn*133 in the form of major molecules (30 ku) or minor molecules (27 ku) for different compositions of oligosaccharide chains[4].

In 1986, Couble *et al*. had sequenced the gene encoding Fhx/P25[5,6]. To understand the regulation of Fhx/P25 expression, they performed an *in vitro* analysis with transgenic *Drosophila* and DNA footprinting and discovered that the determinant *cis*-acting elements were located in the sequence between −254 and −164[7]. Some putative regulatory elements were also identified. The first, BMFA, is a ubiquitous protein thought to be involved in the repression of silk gland-expressed genes at molting, including those encoding silk proteins; the second, SGFB, is a silk gland-specific regulatory protein expressed in both PSG and MSG cells and thus unable, by itself, to specify in PSG expression. Three other proteins (TRIO, Ub2a, and Ub2b) were characterized *in vitro*, but they were all expressed in various larval tissues throughout development[8]. Horard *et al*. found that selective expression of Fhx/P25 in PSG cells was controlled by two main factors, SGFB and PSGF, the latter being a novel regulatory protein. Although present in all silk gland cells, SGFB binds, or has access to, its target sequence only in PSG cells. Not only are SGFB and PSGF involved in PSG-specific *Fhx/P25* activation, they may also be extended to other silk protein-encoding genes, *H-Fib* and *L-Fib*[9].

The foregoing reports suggest that specific expression of Fhx/P25 gene is mainly controlled at the transcription level. However, analysis with our EST (expressed sequence tag) data demonstrates that transcripts of Fhx/P25 gene are present in other tissues than the silk gland, especially in ovary cDNA, and that the transcript start sites (TSSs) of Fhx/P25 gene are different in the silk gland and in the ovary, and that alternative splicing occured in Fhx/P25 pre-mRNA. RT-PCR, FQ-PCR and sequencing performed to verify our analysis showed that Fhx/P25 gene is not so strictly tissue-specific in its transcription and has

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complicated transcription and expression regulation systems. Based on this profile, we postulate that Fhx/P25 may perform functions unknown up to now.

1 Materials and methods

1.1 Sequence analysis

The original data, which were derived from the silkworm EST data maintained at the Key Sericultural Laboratory of Agricultural Ministry, Southwest Agricultural University (China), contained 81 635 high-quality ESTs, which were collected from 12 cDNA libraries of different developmental stages and tissues of the silkworm strain *Dazao* in 2002. The total ESTs were published on GenBank (ftp://ftp.ncbi.nih.gov/).

BLAST search was carried out, using the local BlastN program; Fhx/P25 CDS sequence (AccessNo: X04226.1) was downloaded from NCBI as query to retrieve the transcripts of Fhx/P25 gene in the silkworm EST data. The sequences were considered identical if the BLAST *E* value was <1.0E-30. Multiple alignments were carried out using the CLUSTALX program package and the promotor of Fhx/P25 gene was predicted with the promotor prediction software of *Drosophila* (http://www.fruitfly.org/seq_tools/promoter.html)

1.2 Silkworm rearing and tissues collection

The silkworm inbred *Dazao*, which was used for EST and genome sequencing, was reared on mulberry leaves under normal conditions. Tissues, i.e. blood, midgut, spermary, ovary, middle silk gland and posterior silk gland, were prepared from the fifth-instar day-3 larvae and kept at −80℃.

1.3 RNA extraction and RT-PCR

Total RNA was extracted with Tripure reagent (Roche) according to its protocol. All RNA was digested DNA with Dnase Ⅰ for 15 min. cDNA was synthesized on 8 μg of total RNA using the reverse transcriptase (Promega) and the oligo (dT) primer (TaKaRa) according to the instruction. The primers were designed from the *B.mori* Fhx/P25 gene (Table 1). The RT-PCR conditions was performed by preheating the samples at 94℃ for 5 min, and 30 cycles of denaturation, annealing, and extension at 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 1 min, respectively. A final extension step was carried out at 72℃ for 10 min. The PCR products were separated on 1.0% agarose gel and sequenced on ABI3100 sequencer.

Table 1 Primers designed from the *B.mori* Fhx/P25 gene (LX04226.1)

Primer names	Primer sequences	Sites
Pf1	5′ CCACGACGCTATTTATTTAACG 3′	1242~1263
Pf2	5′ ATTATTCGCGCAACATGCTGG 3′	1331~1351
Pf3	5′ TTTAACCGTGGACTGTCCGTG 3′	3477~3497
Pf4	5′ AGTTTCCAGGAGTACACGGCACA 3′	3590~3612
Pr1	5′ GGGTTAATGTGGAAGATGGGC 3′	4069~4089
Pr2	5′ GCGAAAAGGCGATATGTCGAA 3′	4362~4382

1.4 Real-time PCR assay

Primers and Taqman probes for Fhx/P25 and Bmactin3 were designed and synthesized by TaKaRa. Primer and probe sequences were as follows: *Fhx/P25* forward, 5′ ACCCTAATGACCAACGCACAG 3′; reverse, 5′ CAACCGAAGTCGCAGAGTGT 3′ (139 bp product). Fhx/P25 probe, 5′ (FAM) TGAAGGACCTCGCCAGCCAGTTCC (Eclipse) 3′. Bmactin3 forward, 5′ CATGAAGATCCTCACCGAGCG 3′; Bmactin3 reverse, 5′ CGTAGCACAGCTTCTCCTTGATA 3′ (89 bp product). Real-time PCR was conducted using an ABI PRISM™ 7000 Sequence Detection System. Each reaction was run in triplicate and included no template and no reverse transcription controls for each gene. 2 μl of 1/50 cDNA template along with 500 mol/L (Fhx/P25) or (Bmactin3) nmol/L primers and 250 nmol/L probe were added to a final reaction volume of 25 μl. Cycling parameters were 95℃ for 5 min, then 40 cycles of 95℃ for 30 s, 58℃ for 30 s and 72℃ for 50 s. The relative amount of each Fhx/P25 and Bmactin3 mRNA was described using the equation $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{(\text{Fhx/P25})X} - Ct_{(\text{atin3})X}) - (Ct_{(\text{Fhx/P25})b} - Ct_{(\text{atin3})b})$, in which, X refered to the target tissues, b (blood) was chosen as calibrator and Bmactin3 as endogenous reference. To detect the speical amplication, the no template and no probe reactions were as controls[10,11].

2 Results

2.1 The novel transcription profile of Fhx/P25 gene

By carrying out the local BLAST search, we obtained a total of 617 EST sequences of Fhx/P25 mRNA with an *E*-value of <1E-30. Fhx/P25 mRNA targets were derived from different cDNA libraries. Unexpectedly, only 298 ESTs homologous with Fhx/P25 mRNA were derived from the silk gland

library. Of the others, 279 ESTs homologous with Fhx/P25 mRNA were detected from the ovary, 31 from the testis, 6 from the blood and 5 from the fat body (Figure 1).

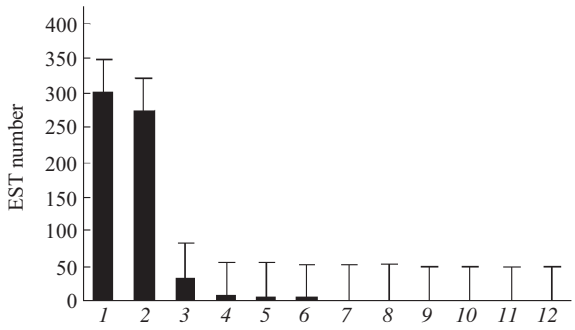

Fig.1 Distribution of Fhx/P25 cDNA in different tissues in EST data

1: silk gland; *2*: ovary; *3*: spermnary; *4*: fat(female); *5*: fat(male); *6*: blood(female); *7*: dissapause egg; *8*: unfertile; *9*: midgut; *10*: blood (male); *11*: fat(pupal); *12*: culture cell (ovary).

Since it was previously reported by many researchers that the Fhx/P25 gene was exclusively expressed in the post silk gland, the novel expression profile of Fhx/P25 in other tissues through EST data needed further confirmation. Thus, primers Pf2 and Pr2 were designed to carry out the RT-PCR reaction. As is shown in Figure 2, the corresponding bands of cDNA were detected in all the tissues studied except in

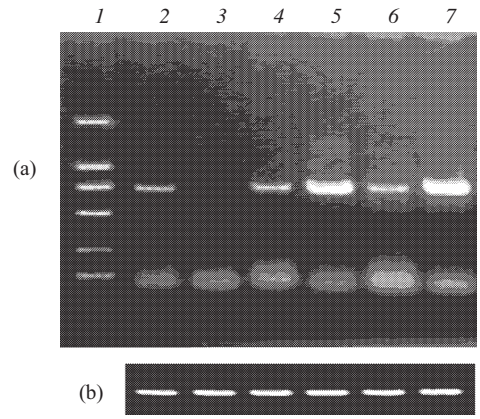

Fig.2 RT-PCR verification of the novel transcription mode of Fhx/P25 gene in different tissues

1: marker DL2000; *2*: blood; *3*: midgut; *4*: testsis; *5*:ovary; *6*: MSG; *7*: PSG. (a) the PCR products with the primer Pf2 and Pr2; (b) the control of BmBmactin3.

the midgut, which was consistent with the analysis result. The products were analyzed on ABI3100 sequencer and the result indicated that the sequences were all identical with the recorded Fhx/P25 mRNA. For the RT-PCR result could not compare the expression quantity of Fhx/P25 in different tissues, the FQ-PCR was carried on. The relative quantification method $2^{-\Delta\Delta\Omega}$ was adapted to count the relative expression of Fhx/P25 in four tissues including PSG, MSG, ovary and blood of the larvae at day 3 of the fifth-instar. The results showed (Figure 3) that the

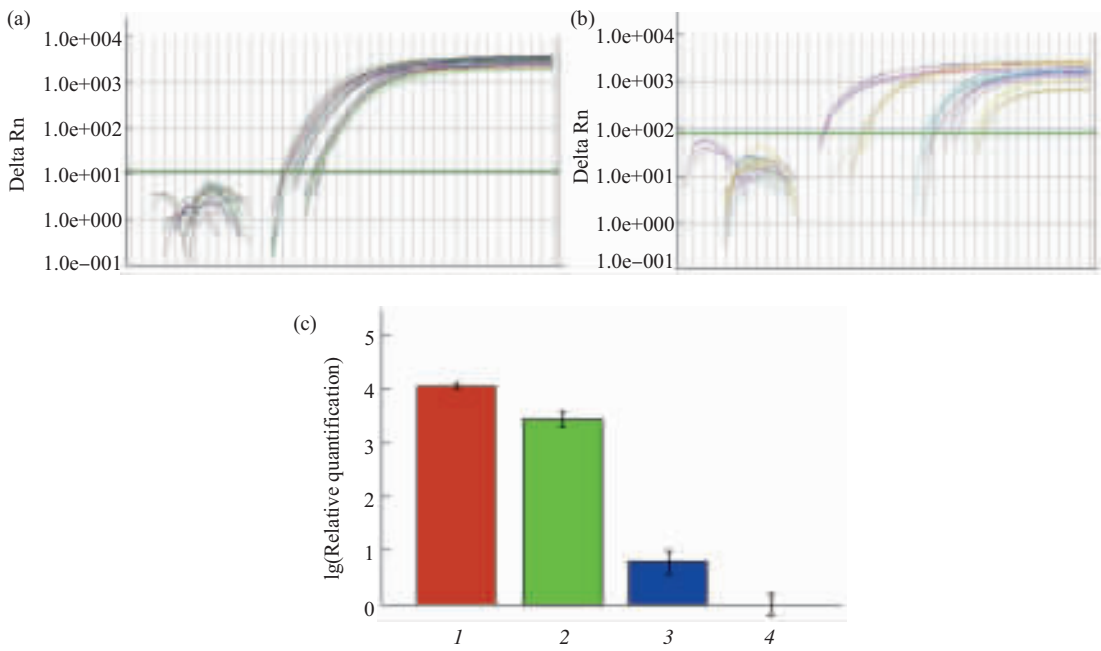

Fig.3 FQ-PCR results of expression of Fhx/P25 transcripts in different tissues with the equation $2^{-\Delta\Delta\Omega}$

(a,b) The amplication plots of the endogenous reference gene of Bmactin3 and the target gene of Fhx/P25 respectively. (c) The relative expression quantifiaction of Fhx/P25 gene in different tissues. *1*:PSG; *2*: MSG; *3*,*4*: Ovary and blood with the the equation $2^{-\Delta\Delta\Omega}$.

expression of Fhx/P25 in PSG was the highest, the second was the MSG, in ovary and blood, the Fhx/P25 transcripts were also detected, in controls, no any fluorescence signals.

2.2 Different transcript start sites of Fhx/P25 gene

It was found that one EST sequence (rswhb_0010002.y1) from the ovary cDNA contained 105 bp upstream sequence of the recorded transcript start site (TSS) of Fhx/P25 gene[6] and, furthermore, some other ESTs from the silk gland and the ovary also contained a partial upstream sequence of the TSS (Figure 4), thus suggesting that the Fhx/P25 gene had more than one TSS. To validate this postulation, RT-PCR reaction was carried out in different cDNA with Pf1 and Pr2. The result showed that the corresponding product was detected in the ovary cDNA only (Figure 5), indicating that Fhx/P25 gene in the ovary had a TSS distinct from that in the silk gland. Therefore, the upstream sequence of Fhx/P25 gene was analyzed in the Promoter Predictions Software of *Drosophila* (http://www.fruitfly.org/seq_tools/ promoter.html) and a predicted core promoter was obtained from −146 to −96 (score=0.96) (Figure 4).

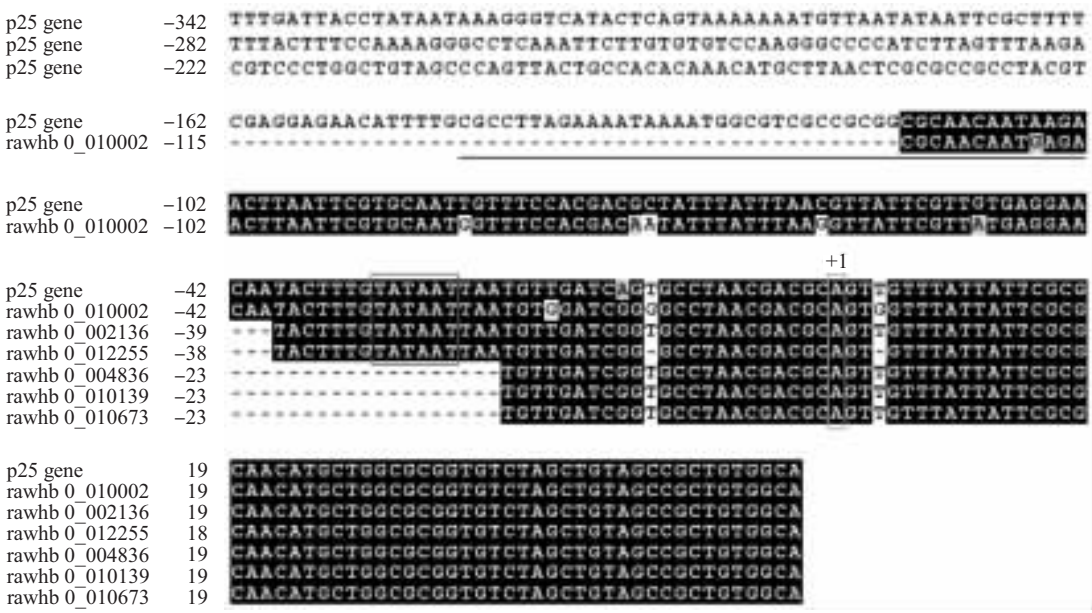

Fig.4 Different transcription start sites in ovary and silk gland

In this figure, rswhb refers to the EST derived from ovary cDNA, rswab corresponds to silk gland cDNA, the underline sequence is the core promotor predicted in http://www.fruitfly.org/seq_tools/promoter.html, the sequence in pane is the predicted TATA box, and +1 is the TSS predicted previously in silk gland.

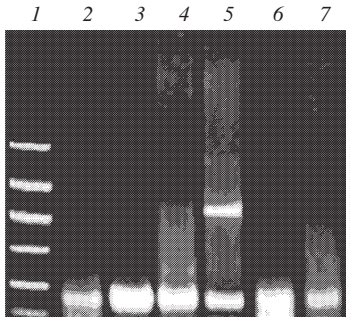

Fig.5 RT-PCR identification of the special transcription start site in ovary

1: marker DL2000; *2*: blood; *3*: midgut; *4*: testsis; *5*:ovary; *6*: MSG; *7*: PSG.

2.3 The probable alternative splicing of Fhx/P25 pre-mRNA

Many genes produce different mRNAs through alternative splicing of pre-mRNA and encode different proteins that function in different tissues and at different times. In this study, three isoforms of Fhx/P25 mRNA were detected in the ovary and silk gland cDNAs in EST data (Figure 6). Most mRNAs contained five extrons as reported, but there were 13 EST sequences containing the third intron or the third and fourth introns. The abnormal mRNAs were predicted to code truncated protein compared with the normal, for many TAAs were present in the third intron. But no base mutant was found at the boundaries

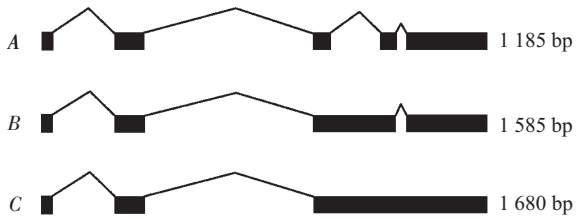

Fig.6 Possible isoforms and the length of Fhx/P25 mRNA in EST data

A: the normal mRNA; *B*: The abnormal mRNA containing the third intron; *C*: The abnormal mRNA containing the third and the fourth introns.

between the extrons and introns. It seems that alternative splicing might have happened to Fhx/P25 gene. Therefore, RT-PCR reaction was carried out to test the analysis result. But only one band of about 250 bp was obtained with Pf3 and Pr1 primers (Figure 7) and it was just the same length as the sum of the third extron and the fourth extron, however, two bands were detected with the primer Pf4 and Pr2, the length about 750 bp and 650 bp respectivly, the two bands subjected to sequence, it was just the two isforms B and C, the large contained the third and forth intron, the small only the third intron. So it was confirmed that the isforms exsited in the the cytoplasm.

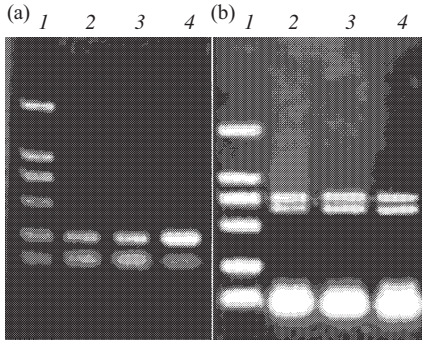

Fig.7 The PCR results with the primers Pf2 and Pr2 (a), Pf3 and Pr1 (b) to test the alternative splicing of Fhx/P25 mRNA

1: marker DL2000; *2*: ovary; *3*: testis; *4*: PSG.

3 Disscusion

In the study reported, the transcripts of Fhx/P25 gene were found in other tissues than the silk gland from the EST analysis results and it was verified by RT-PCR and FQ-PCR. Both procedures revealed that Fhx/P25 gene was transcribed not only in the silk gland but in other tissues as well, strongly suggesting that Fhx/P25 transcription is not strictly restricted to the silk gland spatially. Such results are in some disparity with the reports of other researchers, which may be attributed to two causes: (1) the expression level of Fhx/P25 in other tissues is too low to be detected by Northern blotting; or (2) some tissues were not tested in the researches of other researchers for the presence of transcripts of Fhx/P25 gene.

What is the most interesting in our results may be the transcripts and the different TSSs of Fhx/P25 gene present in the ovary cDNA based on the RT-PCR and FQ-PCR. Because, according to other researchers, the main regulatory elements SGFB and PSGF, which can bind to the 5′ flanking sequences CTATTTATTT-AACG and GGAACAATACTTT of Fhx/P25 gene, respectively, and control the selective expression of Fhx/P25 in PSG cells, are absent in the ovary [9]. Furthermore, the binding sites of the two proteins are presumed to be located at the downstream sequence of the TSS of Fhx/P25 gene in the ovary. It seems that other regulators, such as TRIO, Ub2a, and Ub2b, but not SGFB and PSGF, drive the transcription of Fhx/P25 gene in the ovary (Figure 8). Through the large-scale analysis of silkworm EST data, many other special silk transcripts than Fhx/P25 were also detected in ovary cDNA. Moreover, the expression profiles of the silk gland were very similar to those of the ovary, implying that the fibroin genes in silk gland may have some relation with the ovary. Of course, many structural genes perform not only one function, but also others in different tissues in many organisms and

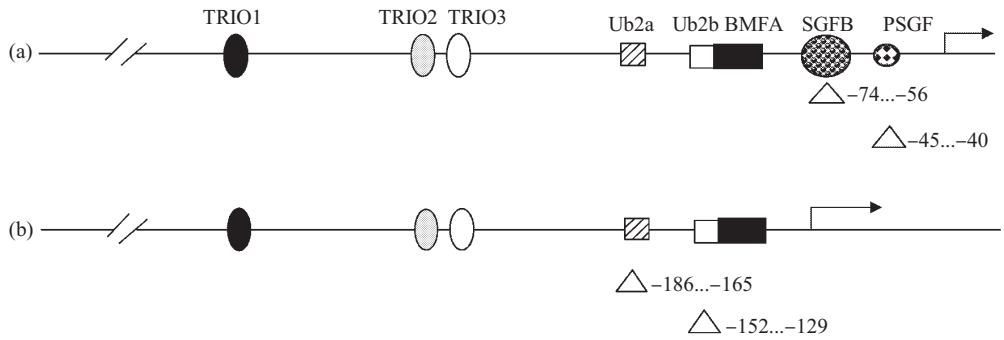

Fig.8 The transcription regulation model of Fhx/P25 gene in the silk gland (a) [12] and the ovary (b)

it is an effective method to utilize their genome. Fhx/P25, one of the main components of silk fibroin, is of primary importance in the maintenance of the structure of the fibroin and it possibly performs other functions in the ovary or other tissues. Many transcripts of Fhx/P25 gene were also detected in middle silk gland in our RT-PCR and FQ-PCR, but they cannot translate into Fhx/P25 protein[13]. Thus the remaining question is whether these mRNAs in the ovary were translated into corresponding proteins or degraded. A Western blotting will be carried out to test the fate of the Fhx/P25 mRNAs in the ovary and other tissues in the next step.

Alternative pre-mRNA splicing is an important mechanism in gene regulation [14] and has been confirmed to exist in the genes *Sericin*, *Bmdsx*, *Annexin* Ⅸ and *Bmchtinase* in silkworm[15~18]. In some previous reports, Fhx/P25 gene produced only one mRNA form [6,10], but in our analysis results, three mRNA species were present in the silk gland and the ovary cDNA, one of which was the same as the reported mRNA sequence and the other two contained the third (and the fourth) intron(s). RT-PCR results revealed that the isoforms exsited not only in PSG and ovary certainly, but also in testis. Therefore, alternative splicing seems to exist in Fhx/P25 gene. Deduced from its amino acid sequence, the predicted mature form of Fhx/P25 contains eight Cys residues and three potential N-glycosylation sites [4]. The deduced truncated protein (152aa) from the abnormal mRNA contains three potently N-linked oligosaccharide chain sites but lacks three Cys residues. Whether these mRNAs will translate into protein and perform functions needs more research. What should be noted is that the intron-containing mRNAs normally were prevented from being transported to the cytoplasm due to the formation of the spliceosome complexes [19]. Probably, some suboptimal splice sites may prevent complex formation, thereby allowing an intron to be retained and the intron-containing mRNAs to be transported to the cytoplasm. This often happens to the large EST project[20]. So an error in splicing may give rise to the isoforms of Fhx/P25 mRNA. We have also found that some other fibroin genes are similar to the Fhx/P25 gene, which are transcribed in different tissues and have alternative splicing. Most structural genes, such as fibroin genes (*fib-H*, *fib-L* and *fib-Fhx/P25*), have been specialized in the evolutionary history. As a result, they are expressed with strict territorial and developmental specificities and involved with the synthesis of protein. It is, therefore, comprehensible that the highly specific genes are not expressed in other tissues and have no regulation function. But on the other hand, the highly specific genes in evolution may be captured by some pathway and perform different biological functions. If these structural genes do perform other biological functions, they should certainly be expressed in different times and tissues or in distinct protein forms. A recent study of 30 ku protein also indicates that the storage protein may have some enzyme activity in specific tissues or periods. Likewise, the result reported in this paper merits further study in the regulation of silkworm or other insect genes.

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家蚕 Fhx/P25 基因的一种新的转录模式分析研究 *

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摘要 家蚕 Fhx/P25 蛋白是丝素蛋白的主要成分之一，过去报道只在家蚕后部丝腺特异的转录表达. 通过对大规模的家蚕 EST 序列分析发现，Fhx/P25 基因不仅在家蚕后部丝腺高效转录，而且在家蚕幼虫五龄第三天的卵巢组织及其他组织也有转录；分析还发现 Fhx/P25 基因在丝腺和卵巢组织中有不同的转录起始位点，在卵巢组织中的转录起始位点比在丝腺中的至少要提前 115 bp 左右. 用 RT-PCR 和 FQ-PCR 进一步验证，以上分析结果均正确. 分析还发现 Fhx/P25mRNA 存在选择性拼接. 以上结果表明 Fhx/P25 基因并不是组织特异转录基因，它的转录表达存在复杂的调控机制，可能还有其他功能.

关键词 家蚕，Fhx/P25 蛋白，转录起始位点，选择性拼接

学科分类号 Q522，S881.2

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