Expression and subcellular localization of cyclin H in *Bombyx mori*

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Abstract: Cyclin H is normally associated with cyclin-dependent kinase 7 (Cdk7). However, cyclin H is also a substrate of protein kinase 2, a ubiquitously expressed serine/threonine protein kinase required for cell viability and cell-cycle progression. Studies of cyclin H have focused mainly on vertebrates, and little is known about its expression in the silkworm *Bombyx mori*. In the present study, the cDNA sequence of cyclin H, which encodes a protein of 250 amino acid residues, was identified in a silkworm pupa cDNA library constructed in our laboratory (GenBank No. AV406047). The open reading frame was 753-bp long and encodes a protein (BmCyc H) with a conserved domain Ccl 1 motif. Recombinant BmCyc H was expressed in *Escherichia coli* BL21(DE3), purified, and used to prepare polyclonal antibodies. Western blotting revealed that the protein was expressed in various tissues in fifth instar larvae, and also in each differentiated growth stage of *B. mori*, with the highest expression in the pupa and spiracle of the fifth instar larvae. The lowest levels were detected in eggs, ovaries, and heart in fifth instar larvae. The results of real-time quantitative polymerase chain reaction revealed that *BmCyc H* mRNA was widespread in different tissues and growth stages of *B. mori*, with the highest expression levels in the moth and pupa and in the spiracles, fatty body, and heart of the fifth instar larvae, with the lowest levels in eggs, and in Malpighian tubules, ovaries, epidermis, gut, and silk gland in fifth instar larvae. The subcellular localization of BmCyc H in *B. mori* BmN cells was examined by immunofluorescence and it was found to be distributed mainly in the nucleus.
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Key words: *Bombyx mori*; *BmCyc H*; Cyclin H; Expression analysis; Real-time PCR; Subcellular localization

Abbreviations

Cdk: cyclin-dependent kinase
CDKI: cyclin-dependent-kinase inhibitor
CAK: Cdk-activating kinase
SKL: sodium N-lauroylsarcosine
ELISA: enzyme-linked immunosorbent assay
IPTG: isopropylthio-β-D-galactoside
TFIIH: RNA polymerase II general transcription factor H

1. Introduction

The main cell-cycle regulatory proteins are the cyclins, cyclin-dependent kinases (Cdks) and cyclin-dependent-kinase inhibitors (CDKIs). The ternary Cdk-activating kinase (CAK) complex composed of Cdk7, cyclin H, and MAT1 of Cdk7-cyclin H-MAT1 catalyzes T-loop phosphorylation of Cdks in most eukaryotic cells and activates many Cdk-cyclin complexes (Lolli G and Johnson,2005; Wu et al.,2011). Cyclin H plays an important role during the cell cycle as a regulatory subunit of CAK, and has been the focus of several in-depth studies(Korsisaari,2002; Liu et al.,2007). After cyclin H binds to Cdk7, Cdk7 phosphorylates Cdk2 and the C-terminal domain YSPTSPS repeat sequence of the largest subunit of RNA polymerase II (Rickert et al.,1999), and the TATA box-binding protein or general transcription factor E (TFIIE) (Andersen et al.,1997; Kaldis,1999). Cyclin H and Cdk7 are commonly found in zebrafish embryo cells. Blastocyst transition during embryonic development is related to Cdk-cyclin complexes, most of which are activated by CAK phosphorylation. Meanwhile, cyclin H is also a basic component of general transcription factor H (TFIIH) (Fribourg et al., 2000). Timmers found the homologs Kin28 and Ccl1 of cyclin H and Cdk7, respectively, in yeast (Timmers, 2002). The Kin28-Ccl1-Tfb3 trimer forms the TFIIH homolog in *Saccharomyces cerevisiae* (Keogh et al., 2000; Liu et al.,2005). Some subunits of TFIIH interact with nuclear hormone receptors, thus accelerating the activation of transcription (Valay et al.,1993; Fukuda et al., 2001). TFIIH plays an important role in cell cycle regulation, DNA repair, and transcription (Timmers, 2002; Egly,2001).

Cyclin H was discovered in the 1990s, since when increasing numbers of studies have focused on this protein, revealing considerable information on its biological characteristics. Cyclin H is highly

conserved in higher eukaryotes and has a variety of biological activities (Barkova et al.,1996). Research in animal models and *in vitro* cell lines has shown that cyclin H and Cdk7 are over-expressed in colorectal cancer (Yin and Wang, 2002), breast cancer (Wang et al.,2002), malignant melanoma, and many other malignant tumors. Cyclin H and its homologs represent an important component of the cell cycle regulatory system, and as such are of significant research value.

Studies of cyclin H have focused mostly on vertebrates, such as humans, mice, *Xenopus*, and zebrafish(Liu et al.,2007). However, cyclin H in *B. mori*, as a model Lepidopteran, has been largely ignored. Research on *B. mori* cell cycle proteins has so far only cloned and expressed cyclin E (cyclin box) from silk glands and examined its localization (Takahashi et al.,1996; Sudhakar et al.,2000), and reported on the amino acid sequence of cyclin B in *B. mori* eggs (Kiuchi,2008).

We constructed a silkworm pupa cDNA library in our laboratory (GenBank No. AV406047) and identified a cDNA showing high homology with the cyclin H gene. The gene was named *B. mori* cyclin H (*BmCyc H*), and was cloned from the *B. mori* pupal cDNA library. In this study, *BmCyc H* was cloned and expressed *in vitro*. We simultaneously analyzed differences in expression and transcription of *BmCyc H* in different tissues and at different developmental stages in *B. mori*, and examined its subcellular localization. This study provides an important basis for further studies of BmCyc H function.

2.Materials and Methods

Bacterial strains and cell line

E. coli TG1 and *E. coli* BL21(DE3), *B. mori* BmN cells (*B. mori* ovary cell line), and plasmid pET-28a(+) were maintained in our laboratory. The *B. mori* pupal cDNA library was constructed in our laboratory. New Zealand male rabbits were purchased from Hanzhou Normal University Experimental Animal Center. The species of *B. mori,* Jingsong×Haoyue, was provided by a Hangzhou silkworm farm.

Main reagents and equipment

The restriction enzymes *Bam*HI and *Eco*RI were purchased from Promega Corporation (Shanghai, China). One Step SYBR® Prime Script™ RT-PCR Kit II was from Takara (Dalian, China), 4',6-diamidino-2-phenylindole (DAPI) was from

Sigma (Shanghai, China), Cy3 label was from Proteintech Group (Wuhan, China), and Taq enzyme and related polymerase chain reaction (PCR) reagents were from Shanghai Boya Biotechnology Co., Ltd (China). The ABI3100A and ABI3010 sequence analysis systems and ABI7300 Quantitative PCR Instrument were from Applied Biosystems (USA), and the ECLIPSE TE2000-E laser scanning confocal microscope was from Nikon (Japan).

BmCyc H cloning and sequence analysis

According to the gene sequence for *BmCyc H*, a pair of primers was designed and synthesized with *Bam*HI and *Eco*RI restriction enzyme sites (underlined): P1: 5'-GGGGGATCCATGAAAGACTTAGTTGAC-3' and P2: 5'-GGGGAATTCTTACAAAGGTTCCGGTT -3'. The BmCyc H gene was amplified by PCR using *B. mori* pupal cDNA (provided by our lab) as a template. PCR amplification was conducted using the following parameters: denaturation at 94°C for 5 min; followed by 32 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; and then a final extension at 72°C for 10 min. The fragment was digested by BamHI/EcoRI (Promega Corporation) and then cloned into the plasmid pET-28a(+). The recombinant plasmid pET-28a(+)-*BmCyc H* was constructed, and the recombinant was identified by PCR, double digestion, and sequence analysis.

BmCyc H gene expression and antibody preparation

The recombinant plasmid was transformed into *E. coli* BL21(DE3). Positive clones were selected and incubated in LB medium to an optical density 600 of 0.5 (about 3 h) at 37°C. sopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1 mmol/L to induce protein expression. After induction for 5 h, the bacteria were collected by centrifugation and subjected to ultrasonication. The precipitate after centrifugation for 15 min at 12 000 rpm was dissolved in inclusion-body solubilization buffer containing sodium N-lauroylsarcosine (SKL) (Zhu,1999). The fusion protein was then highly purified with binding buffer using a Ni-NTA chelating column (pH 7.4, 20 mM imidazole, 0.5 M NaCl and 20 mM Tris), wash buffer (120 mM imidazole) and elution buffer (200 mM imidazole). The purified protein BmCyc H was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and used to immunize New Zealand rabbits to prepare polyclonal antibody.

Differential *BmCyc H* gene expression analysis

Total proteins were extracted from different growth stages of *B. mori* and from different tissues of fifth instar larvae. Differential expression of BmCyc H protein in *B. mori* was analyzed by western blotting.

Analysis of transcription of *BmCyc H* gene

Total RNA was extracted using the Trizol method. According to DNAStar's Primerselect software, primers for *BmCyc H* were designed using 18S rRNA as an internal reference. The primer sequences were as follows: Upstream primer: 5'-ATATAGCAGTGGCGT GTTTAACTTT-3', downstream primer: 5'-ATGCAGTTACACATGGCGTCTCCGT-3'; internal reference upstream primer: 5'-CGATCCG

CCGACGTTACTACA-3', internal reference downstream primer: 5'-GTCCGGGCCTGGTGAGATTT-3'. One-step SYBR Green quantitative PCR kit (Invitrogen,USA) was used for quantitative PCR analysis of *BmCyc H* gene transcription at different developmental stages and in different tissues of fifth instar larvae of *B. mori*.

Subcellular localization of BmCyc H protein

BmN cells were cultured overnight in special dishes. The culture medium was discarded, and the cells were washed twice with phosphate-buffered saline (PBS). The stationary liquid (PBS containing 3.7% formaldehyde) was used to fix the cells at room temperature for 10 min. This was followed by incubation in PBST (PBS containing 0.2% Triton X-100) at room temperature for 10 min and blocked in PBS containing 3% bovine albumin fraction V for 2 h at room temperature, with shaking. The first antibody was added at a dilution of 1:100 overnight at 4°C. The second antibody was then added with Cy3-labeled goat anti-rabbit IgG (Proteintech Group) at a dilution of 1:200, together with DAPI (Sigma) at a dilution of 1:2 000, and incubated at 37°C for 2 h. The results were observed by fluorescence and confocal microscopy at the Cy3 emission wavelength of 280 nm and the DAPI wavelength of 353 nm.

3. Results

Molecular cloning and bioinformatic analysis of *BmCyc H*

The *BmCyc H* gene was cloned from a *B. mori* pupal cDNA library. It had an open reading frame of 753 bp, encoding 250 amino acid residues (Figure 1). The recombined plasmid pET-28a(+)-*BmCyc H* was identified by PCR, double digestion, and sequencing. The results demonstrated that the vector had been successfully constructed (Figures 2 and 3).

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ATGAAAGACTTAGTTGACGTAATGGCTTTGCAAAACAGACGTGAAAAGCGCGTACCCGACTATCGAGGCGCGCCGAACCACAGCTTAGCA  90
 M  K  D  L  V  D  V  M  A  L  Q  N  R  R  E  K  R  V  P  D  Y  R  G  A  P  N  H  S  L  A   30
ACAAATTTTGTTTTTGAATGTGGAATAAAGCTTGGCTTGCAGCCTGCAACTATAGCAACTGCTGCCATATTTTACCACAAATTCTTCAAA 180
 T  N  F  V  F  E  C  G  I  K  L  G  L  Q  P  A  T  I  A  T  A  A  I  F  Y  H  K  F  F  K   60
GAGGCCGACAAGAATGATTACGACTGCTACGTAATCTGCACGCTTGTTTGTGTGCAGCCGGGAAATCTCGAGATGAGCCTGTTAGGCTGC 270
 E  A  D  K  N  D  Y  D  C  Y  V  I  C  T  L  V  C  V  Q  P  G  N  L  E  M  S  L  L  G  C   90
GAGATGCTGTACAATGTAGCACACAATTCGATAAATCGTGGAGCCGGTCCGTTGGAGTTAGGCGACGAGTACTGGTCATGGCGAAGTGCT 360
 E  M  L  Y  N  V  A  H  N  S  I  N  R  G  A  G  P  L  E  L  G  D  E  Y  W  S  W  R  S  A  120
GTAGCTCAAGCCGAGCTTCTTGTGCTGAGACTGCTGGGATTTAATCTGGAAGCGCCGTCGCCACACCGGTATCTATTGCATTATCTCAAA 450
 V  A  Q  A  E  L  L  V  L  R  L  L  G  F  N  L  E  A  P  S  P  H  R  Y  L  L  H  Y  L  K  150
TCGCTGCAAGAATGGTTTCCAGTAGCGCAGTGGCGTTCAGCTCCCATTGCTCGAATGGCAATGGCTTTCCTTCAGGATTTCCATCACTCA 540
 S  L  Q  E  W  F  P  V  A  Q  W  R  S  A  P  I  A  R  M  A  M  A  F  L  Q  D  F  H  H  S  180
CCAGCAGTACTTGATTATCGAGCACCCCATATAGCAGTGGCGTGTTTAACTTTAGCTTTACATGTATTAGGAGTATCAGTGCCATTGGCA 630
 P  A  V  L  D  Y  R  A  P  H  I  A  V  A  C  L  T  L  A  L  H  V  L  G  V  S  V  P  L  A  210
TCCACACTAGATGATGATGCTGCATGGTGTTCAGTTTTCACAAAGGATCTGCAGAAAGAAAAAAATTGGGAAATAATGGAGAAGATAATG 720
 S  T  L  D  D  D  A  A  W  C  S  V  F  T  K  D  L  Q  K  E  K  N  W  E  I  M  E  K  I  M  240
CAAGTTTATGGTCGTGAACCGGAACCTTTGTAA 753
 Q  V  Y  G  R  E  P  E  P  L  *
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Figure 1. cDNA sequence and deduced amino acid sequence of *BmCyc H* gene

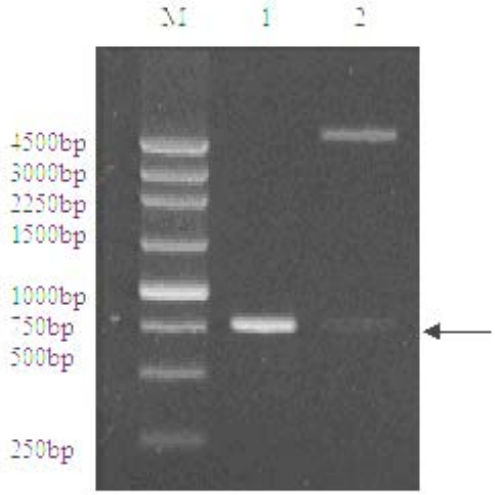

Figure 2. The PCR and double-digestion identification of recombinant plasmid. 1. PCR product; 2. Recombinant plasmid DNA digested with BamHⅠ/EcoRⅠ; M. DNA ladder marker

Figure 3. Sequencing analysis of recombinant plasmid

To identify cyclin H homologs in the silkworm, *cyclin H* DNA sequences from 13 species were screened and then classified (http://www.ncbi.nlm.nih.gov/). MEGA4.1 was used to compare the predicted amino acid sequence of BmCyc H with those of cyclin H from other species (Figure 4). Sequence alignment showed homology of 51.2% between BmCyc H and the *Tribolium castaneum* cyclin H. *BmCyc H* contains a complete Ccl 1 conserved sequence domain. The protein Ccl 1 encoded by the *Ccl 1* gene is involved in DNA replication, transcription, and repair during cell division, as the subtype and homolog of cyclin H (Keogh et al.,2002; Liu et al.,2005).

We constructed phylogenetic trees of cyclin H and BmCyc H for 13 species using the neighbor-joining algorithm of MEGA4.1 (Beta). The results revealed the evolutionary relationships of cyclin H between different species. The closest evolutionary relationship was between BmCyc H and cyclin H from the Valley borer(*Aphomia gularisZeller*), and the next closest was between *Apis mellifera* and *Nasonia vitripennis* (Figure 5). The amino acid sequence of BmCyc H protein was submitted to SWISS-MODEL. Tertiary-structure prediction showed that the BmCyc H protein contains two domains connected by a random coil. Each of the two domains contains five α-helixes, with no β structure (Figure 6). The tertiary structure of BmCyc H is similar to that of cyclin H in other species. Cyclin H also showed significant homology with cyclin C, but only 43% homology with cyclin A(Zhao,et al.,2008; Andersen et al.,1996).

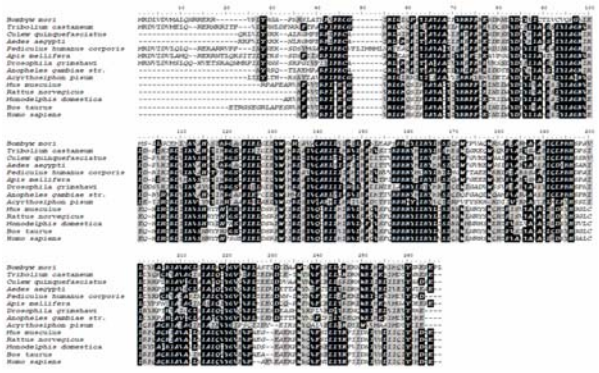

Figure 4. Alignments of cyclin amino acid sequences from multiple species

BmCyc H gene expression and antibody preparation

Recombinant plasmid pET-28a(+)-*BmCyc H* was transformed into *E. coli* BL21(DE3). Positive clones were screened and inoculated into LB medium. Expression of BmCyc H protein was induced by IPTG. The molecular weight of expressed BmCyc H detected by SDS-PAGE was about 32 kD, which conformed to that deduced from the nucleotide sequence (Figure 7). The expressed protein products were purified by Ni-NTA affinity chromatography. New Zealand male rabbits were immunized with the purified proteins to produce polyclonal antibodies. The polyclonal antibody titer detected by ELISA was greater than 1:12 800 (Figure 8).

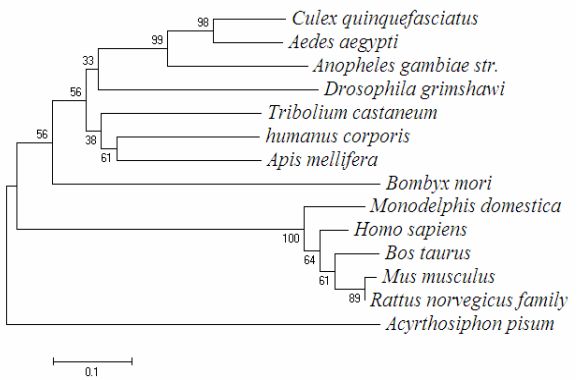

Figure 5. Phylogenetic tree based on cyclin homologs

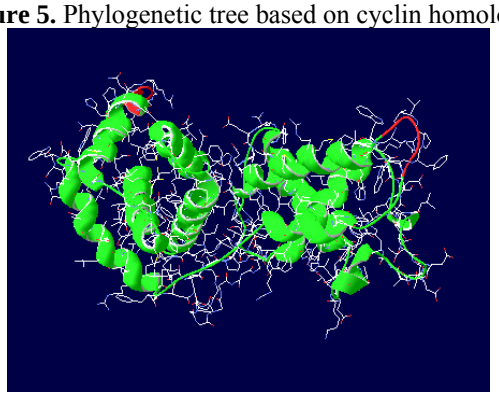

Figure 6. Higher structure of BmCyc H predicted by SWISS-MODEL

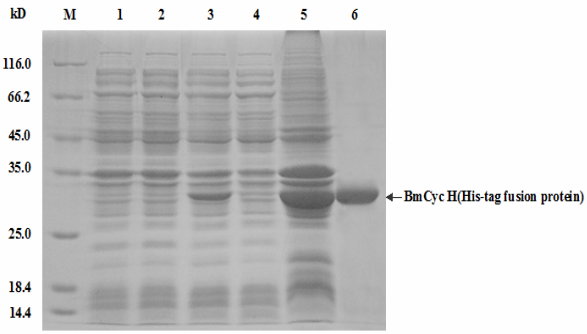

Figure 7. Expression and purification of His-tag fusion protein in BL21(DE3). M; protein molecular weight marker (low). 1. BL21(DE3) (pET-28a(+) after induction with IPTG. 2. BL21(DE3) (pET-28a(+)-BmCycH) without IPTG induction. 3. BL21(DE3) (pET-28a(+)-BmCycH) after IPTG induction. 4. Supernate of supersonic fragmentation. 5. Deposition of supersonic fragmentation. 6. Recombinant protein after His-column purification.

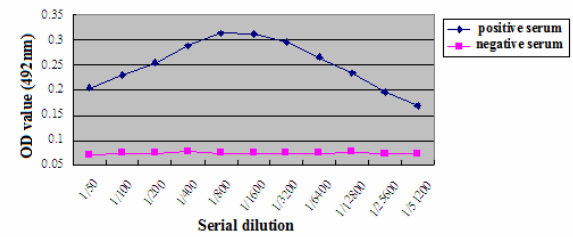

Figure 8. Determination of polyclonal antibody titer

Developmental specificity of transcription and expression of *BmCyc H* gene

Total RNA was extracted from *B. mori* eggs, fifth instar larvae, pupae and moths. Detection of *BmCyc H* by fluorescence quantitative PCR showed that the transcription levels in four developmental stages of *B. mori*, ranking from high to low, were pupa, moth, fifth instar larvae, eggs (Figure 9A).

Total proteins were extracted from *B. mori* eggs, fifth instar larvae, pupae, and moths. Western blotting was used to detect the expression of the *BmCyc H* gene in different developmental stages of *B. mori*. The results showed that BmCyc H protein levels were highest in moths, followed by pupae and fifth instar larvae, and were lowest in eggs (Figure 9B).

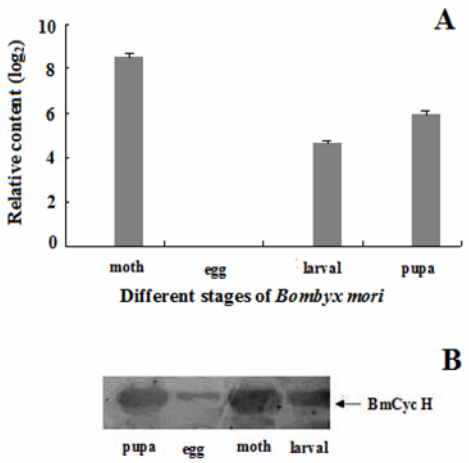

Figure 9. Transcription(A) and expression (B) levels of BmCycH in different developmental stages of *B. mori*

Tissue specificity of transcription and expression of *BmCyc H* gene

Total RNA and proteins were extracted from the head, Malpighian tubules, ovaries, epidermis, spiracle, silk gland, midgut, fatty body, and other tissues of *B. mori* fifth instar larvae, and analyzed by fluorescence quantitative PCR and western blotting, respectively. According to fluorescence quantitative PCR, the transcription levels in different tissues of *B. mori* fifth instar larvae were highest in spiracle, followed by fatty body, head, Malpighian tubules, ovaries, epidermis, midgut, and silk gland (Figure 10A). Western blotting analysis showed that BmCyc H protein was also expressed in various tissues of *B. mori* fifth instar larvae, and was widely distributed in the head, Malpighian tubules, ovaries, epidermis, spiracle, silk gland, midgut, fatty body, and other tissues, with the highest levels in the spiracle and lowest in the head and ovaries (Figure 10B).

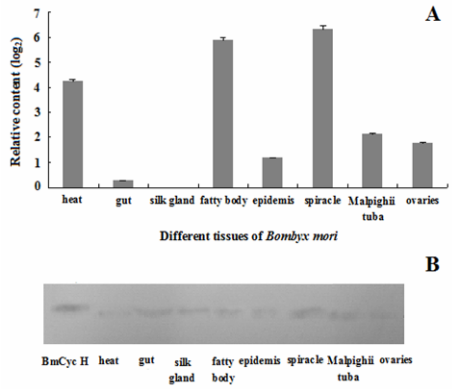

Figure 10. Transcription (A) and expression (B) levels of *BmCycH* in different tissues of fifth instar larva of *B. mori*

Subcellular localization of BmCyc H

To determine the localization of BmCycH protein in the B. mori ovarian epithelial cell line BmN, we used polyclonal antibody purified by protein A column as the primary antibody, normal rabbit serum as the negative control, and Cy3-labeled goat anti-rabbit IgG as the secondary antibody (emitting red fluorescence with wavelength of 550 nm). BmCyc H protein in BmN cells was located in the nucleus, as determined by DAPI staining (emitting blue fluorescence with wavelength of 353 nm). A Nikon ECLIPSE TE2000-E confocal microscope was used to observe the results of western blotting. BmCyc H protein was located almost exclusively in the nucleus and was evenly distributed. Few localization signals were detected in the cytoplasm (Figure 11).

4. Discussion

Separation and refolding of prokaryotic expression products of *BmCyc H*

The recombinant strain BL21(DE3)-pET-28a(+)-*BmCyc H* was induced with IPTG and ultrasonicated, and the precipitate and supernatant were detected by SDS-PAGE. BmCyc H was located in the precipitate. High levels of expression often cause foreign proteins to accumulate in the cytoplasm or periplasmic space in the form of inclusion bodies. These inclusion bodies can usually be dissolved by 6–8 mol/L urea or 6–7 mol/L lysate of guanidine HCl(Yang et al.,2008). Recombinant BmCyc H protein cannot be readily dissolved in this solution, but can be dissolved in SKL, which is also widely used to dissolve inclusion bodies and for protein renaturation (Yang et al.,2008).

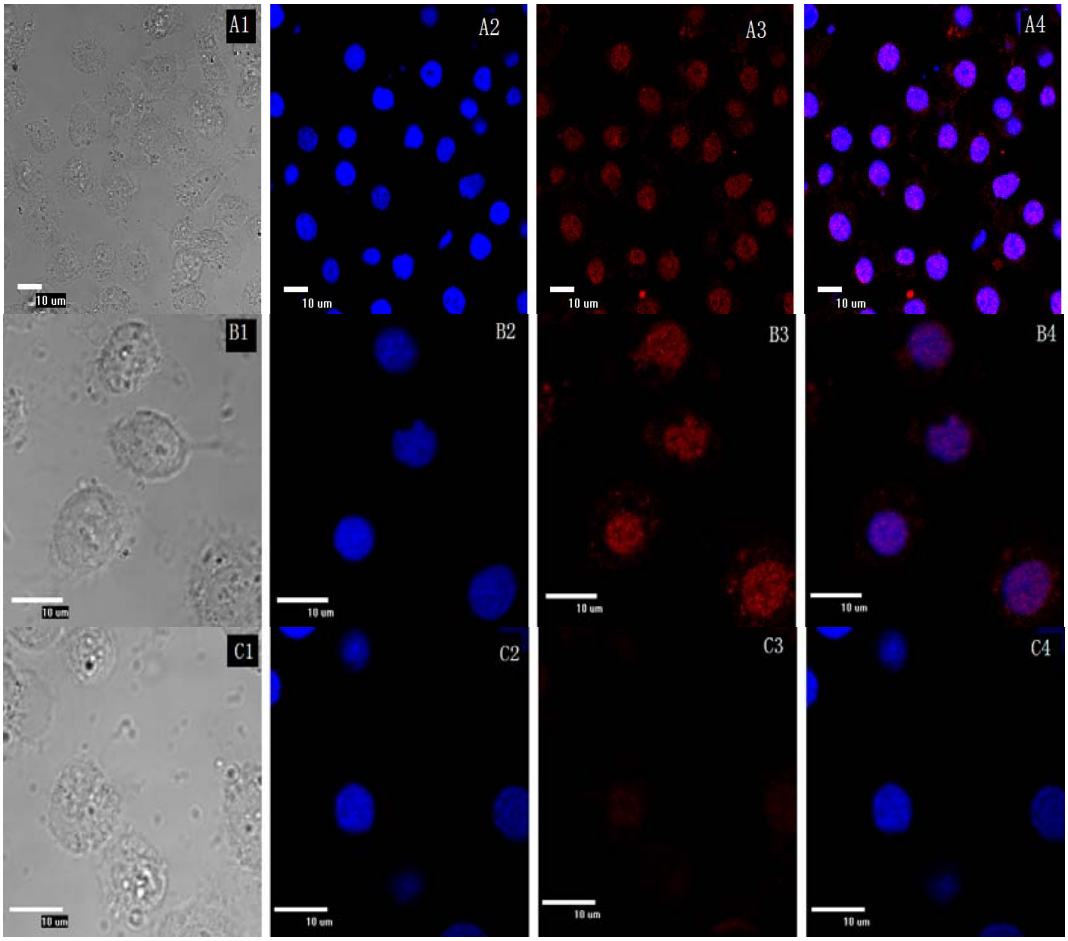

Figure 11. Subcellular localization of BmCyc H
A1, B1, C1: Image of cell under transmitted light. A2, B2, C2: Images of nuclei stained with DAPI. A3, B3, C3: Images of intracellular BmCyc H stained by Cy3. A4, B4, C4: Overlapped images of A2 and A3, B2 and B3, C2 and C3, respectively. C1, C2,C3, C4: Negative controls. Scale indicates 10 μm.

Recombinant BmCyc H was therefore dissolved in SKL solution, refolded, and then purified in a Ni-NTA affinity column. Using a buffer system of 0.5 mol/L NaCl and 20 mmol/L Tris (pH 7.4), the upper concentration limit of imidazole eluent is 100 mmol/L and the lower limit is 150 mmol/L. Imidazole 20 mmol/L was therefore set as the starting concentration, with 120 mmol/L imidazole as the washing concentration, and 200 mmol/L imidazole was finally used to elute the recombinant protein. The recombinant protein purified by this method had a high concentration, purity, and natural activity and was used to prepare polyclonal serum.

Differential expression of BmCyc H protein

Transcriptional (detected by fluorescence quantitative PCR) and translational (analyzed by western blotting) levels of the *BmCyc H* gene were similar in all four developmental stages of *B. mori*. Transcription levels of *BmCyc H* were highest in *B. mori* moths, followed by pupae, fifth instar larvae, and eggs. Western blotting showed that BmCyc H protein levels were very low or absent in eggs, but high in moths, fifth instar larvae, and pupae. This indicates that BmCyc H expression was stable during growth and development, reaching its highest level in the adult moth stage, and suggesting that it plays a specific role in the adult moth. Liu discovered that expression of a dominant-negative form of cyclin H delayed the onset of zygotic transcription in the early zebrafish embryo, leading to sever defects in tissues normally exhibiting high levels of Cyclin H expression(Liu et al.,2007). This suggest that Cyclin H is an essential gene in early larval development, which may be closely linked to silkworm metamorphosis.

The transcription levels of the *BmCyc H* gene in the tissues of fifth instar larvae of *B. mori* were highest in the trachea, followed by the fat body, head, Malpighian tubules, ovary, epidermis, midgut, and silk gland. Western blotting showed BmCyc H proteins were also expressed in various tissues in fifth instar larvae, but the expression levels differed, and were slightly different from the transcription levels.

Distribution of cyclin H in zebrafish transcripts was ubiquitous during early stages embryo development, but became to restricted to the anterior neural tube, brain, eyes, reproductive tissues, liver and heart by 5 days post-fertilization(Liu et al.,2007). This suggests a potential role of maternal inheritance.

Subcellular localization of BmCyc H protein

Cyclin H in humans has been reported to be predominantly located in the nucleus throughout the whole cell cycle. Krempler et al. discovered that cyclin H was targeted to the nucleus by C-terminal nuclear localization sequences (Krempler et al., 2005), while Kayaselcuk et al. observed cyclin H expression in the nuclei of endometrial cells (Kayaselcuk et al., 2006). Subcellular localization of BmCyc H protein in *B. mori* BmN cells also showed that the protein was present in the nucleus. These observations are therefore in agreement with previous studies. Bioinformatics predicts that BmCyc H has a conserved cyclin H domain, indicating that BmCyc H is a regulatory protein related to the cell cycle.

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