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RESEARCH

Structural and Functional Characterization of the Actin-1 Gene Promoter From the *Antheraea pernyi* (Lepidoptera: Saturniidae)

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ABSTRACT. The Chinese oak silkworm, *Antheraea pernyi*, is an economically important insect of the Saturniidae family. In this study, genome walking was performed to obtain an *A. pernyi* actin promoter, which can be employed in transgenic or stable cell line expression systems. The putative promoter was analyzed by the online promoter analysis programs at the Berkeley Drosophila Genome Project and the Web Promoter Scan Service, which led to the recognition of several functional elements. With respect to these elements, a series of actin A1 promoter fragments with 5′-deletions were generated that were then used to construct different vectors expressing Green Fluorescent Protein (GFP). The plasmids were transfected into Sf9 cells and GFP expression was determined by observing GFP fluorescence in cells and by measuring GFP mRNA levels with real-time polymerase chain reaction. Sequence comparisons indicated that the sequence cloned from *A. pernyi* was the actin A1 promoter. The basic function of the promoter was verified by constructing expression vectors and observing GFP expression. In addition, real-time polymerase chain reaction revealed a strong inhibitory element may exist upstream of the TATA box, which downregulated gene expression. The actin A1 promoter is an ideal candidate for use in *A. pernyi* transgenic systems.

Key Words: *Antheraea pernyi*, actin, promoter, functional analysis

Actins are the most abundant proteins in eukaryotic cells and are broadly categorized into muscle or cytoplasmic actins according to their N-terminal sequences (Hightower and Meagher 1986, Mounier et al. 1992). Actins participate in more protein–protein interactions than any known proteins and play an important role in many cellular functions, ranging from the maintenance of cell motility, cell shape, and polarity to the regulation of transcription (Dominguez and Holmes 2011). Because of their presence in all cell types, actins are used as reference genes in northern blot analyses and quantitative reverse transcription polymerase chain reaction (PCR) studies (Bunger et al. 2003, Kitade et al. 2008, Yukihiro et al. 2008.). Four actin genes have been previously isolated from *Bombyx mori*, two of which (A1 and A2) are of the muscular type, the other two (A3 and A4) encode cytoskeletal isoforms (Mounier and Prudhomme 1986, Mange and Prudhomme 1999).

A gene promoter is a DNA sequence that directs the positioning of RNA polymerase, and is usually located upstream of the gene's coding sequence. Correct association of an RNA polymerase with a promoter initiates transcription; hence the promoter is a fundamental *cis*-regulatory element for gene expression. Regulation of actin gene expression has been shown to be very complicated and involves several positive and negative *cis*-acting sequences (Parker and Topol 1984, Mangé et al. 1997, Zhu et al. 2012). The functional organization of the *B. mori* A3 promoter has been examined in detail. Two major regulatory *cis*-acting sequences were identified: a positive element containing a serum response element (SRE) sequence that can bind a SRF-related protein present in silk gland extracts and a negative region, R3, which is located just upstream of the SRE. R3 also binds a silk gland factor and is thus a good candidate for downregulating A3 in this tissue (Mange and Prudhomme 1999).

The Chinese oak silkworm, *Antheraea pernyi*, is an economically important insect of the *Saturniidae* family. It is commercially cultivated, mainly in China, India, and Korea. It feeds on the leaves of *Quercus* species and produces coarse silk. It is also an excellent natural bioreactor for the production of recombinant proteins (Huang et al. 2002). Because of its uncultivated, wide distribution, the research and application of *A. pernyi* will provide corroborating supplementation for the model organism, *B. mori* (Liu and Jiang 2008).

In this article, we describe the cloning of an actin promoter from *A. pernyi* using genome walking and sequence analysis with online promoter analysis programs at the Berkeley Drosophila Genome Project and the Web Promoter Scan Service. A series of experiments were performed to assess the functional properties of the sequence.

Materials and Methods

Insects. *A. pernyi* strain 741 was provided by the Liaoning Institute of Sericultural Science.

Genomic DNA and RNA Purification. Total RNA was extracted from fatbodies of silkworm pupa using TRIzol according to the manufacturer's instructions (Invitrogen Shanghai, China). Genomic DNA extraction from fatbodies was performed according to the method described by Kitade et al. (1996), Zhao et al. (2000).

Cloning of the A. pernyi Actin A1 Promoter Region

According to the known sequence of Actin-1 gene of *A. pernyi* (GenBank KC242321.1), specific reverse primers, SP1, SP2, and SP3 were designed. The primer sequences are as followings: SP1: 5′-CGATGGGGTACTTCAGGGT-3′, SP2: 5′-GATACCTCTCCTGC TCTGGGCCTC-3′, SP3:5′- CTGGAGGCGAGGGCGACCTA-3′. Genomic walking procedure was carried out in a thermal cycler with Genome Walking Kit (Takara Co., Dalian, China) according to the manufacture manual with universal primers AP1–AP3, which were degenerate primers provided with the kit, and gene-specific primers SP1–SP3. Simply, the first-step PCR was finished with primers AP1 or SP1 based on template of genomic DNA extracted from fatbodies and extension reaction as following:

94°C	1 min				
98°C	1 min				
94°C	30 s	}			
65°C	1 min	}	5 cycles		
72°C	2 min	}			
94°C	30 s;	25°C	3 min;	72°C	2 min

94°C	30 s;	65°C	1 min;	72°C	2 min	} 15 cycles
94°C	30 s;	65°C	1 min;	72°C	2 min	
94°C	30 s;	44°C	1 min;	72°C	2 min	
72°C	10 min					

Based on template of 1 µl of first-step PCR product, second-step PCR was carried with AP2 or SP2 primers as the following procedure:

94°C	30 s;	65°C	1 min;	72°C	2 min	} 15 cycles
94°C	30 s;	65°C	1 min;	72°C	2 min	
94°C	30 s;	44°C	1 min;	72°C	2 min	
72°C	10 min					

The third-step PCR was finished with AP3 or SP3 primers based on 1 µl of second-step PCR product as template:

94°C	30 s;	65°C	1 min;	72°C	2 min	} 15 Cycles
94°C	30 s;	65°C	1 min;	72°C	2 min	
94°C	30 s;	44°C	1 min;	72°C	2 min	
72°C	10 min					

The PCR products were separated on a 1% agarose gel, and target bands were purified and subcloned to pMD18-T. Methods used for preparation of plasmid DNA, digestion, ligation, and transformation were as described by Sambrook et al. (1989).

Analysis of Promoter Structure and Predication of Transcript Factor-Binding Sites. The DNA fragment was sequenced by using Sanger method in Takara Co. with ABI prism 3730XL DNA analyzer. Putative promoter DNA sequence was submitted to NCBI (http://www.ncbi.nlm.nih.gov/blast) and similar sequences were identified using the CLUSTAL 2.1 program at EMBL-EBI web site (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Bioinformatic analysis was performed using the Berkeley Drosophila Genome Project online Promoter analysis program (http://www.fruitfly.org/seq_tools/promoter.html) and the Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/).

Engineering of A1 Promoter Fragments With 5′-End Deletions. According to the bioinformatic analysis, we designed four PCR primers to generate a series of actin A1 promoter fragments with 5′-end deletions. The primers used were: F1 5′-CCCAAGCTTTCTTTACGTAACTCATGTA-3′; F2 5′-CCCAAGCTTGAATATCGTTCGCCCAACG-3′; F3 5′-CCCAAGCTTTTAAGGGTAGACCTAGGAA-3′; F4 5′-CCCAAGCTTGCCCGACGATATATAAGCC-3′; (the underlined sequence indicates a HindIII site). The reviser primer containing BamHI-site is as following: R 5′-GCGGATCCTTTGTCGTTTGTTTGTGTTGTT-3′ (the underlined sequence indicates a BamHI-site).

Using the full-length actin A1 promoter as a template, DNA amplification was performed using the four primer pairs: F1/R, F2/R, F3/R, and F4/R to generate fragments F1, F2, F3, and F4, respectively. PCR conditions were 96°C for 3 min; 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min for 30 cycles; and a final extension at 72°C for 10 min. The amplified products were digested with HindIII and BamHI (Takara) and the large DNA restriction fragment purified by agarose gel electrophoresis. Plasmid pGFP-N2 (Clontech) was similarly digested and the smaller restriction fragment (GFP gene) was gel purified. Purified restriction fragments were ligated to generate expression vectors pGFP-N2-A1.

Cell Culture and Transfection. Recombinant plasmid pGFP-N2-A1 was purified using a Plasmid Mini kit (Qiagen) and transfected into cells (1×10^5 cells per well) using Cellfectin reagent for Sf9, *B. mori* (BmN) and *Trichoplusia ni* (HighFive) cells and FECTIN2000 for HeLa cells (Invitrogen) according to the manufacturer's protocol. Plasmid Mini Kit (25) with 25 QIAGEN-tip 20 columns, reagents, buffers, was from QIAGEN (Germantown, MD). The insect cells were incubated in Grace medium and 1,640 medium was used for HeLa cells supplemented with 10% fetal bovine serum for 24 h and then GFP activity was observed under a fluorescence microscope (Olympus). Transfection efficiency was assayed and a comparable result was obtained when parallel cell samples were measured by flow cytometer (BD FACSCalibur, BD Biosciences).

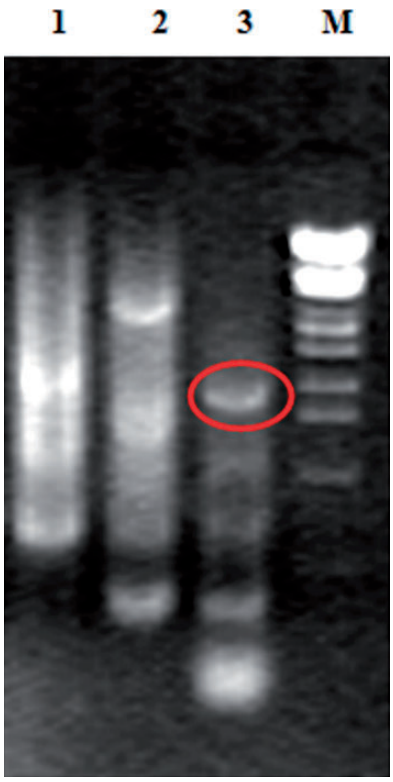

Fig. 1. Results of genome walking. M, DNA marker; 1, product of the first walking step; 2, product of the second walking step; 3, product of the third walking step.

Real-Time PCR Assay of GFP Expression From A1 Promoter Fragments. RNA was isolated from transfected Sf9, *B. mori* (BmN) and *T. ni* (HighFive) cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol and the RNA extracts were treated with DNase before they were reverse transcripted to cDNA. cDNA was generated from total RNA with a Reverse Transcriptase Kit (Biotec, China) using 1–2 µg of total RNA as template.

Two sets of primers were used for real-time PCR experiments designed by webtool of http://www.idtdna.com/PrimerQuest/. The one is for GFP (sense: 5′-TGTCAGTGGAGAGGGTGAA, antisense: 5′-GGCCATGGAACAGGTAGTTT), the other one is for kanamycin resistance gene (sense: 5′-CGTTGGCTACCCGTGATATT, antisense: 5′-CTCGTCAAGAAGGCGATAGAAG).

The resulting cDNA was used for real-time PCR with forward and reverse primers of GFP gene and kanamycin resistance gene designed as above. The real-time PCR was performed according to the protocol of SYBR Premix Ex Taq Kit (Takara). Transcription level of GFP was evaluated and normalized relative to the transcription of kanamycin resistance gene. The data were repeated three times.

Results

Cloning and Characteristics of the Actin A1 Promoter. After genome walking with primers and using *A. pernyi* genomic DNA as template, a PCR product of about 1.7 kb was obtained from third-step PCR (Fig. 1). By nucleotide sequence comparison of the promoter coding region, the sequence of this fragment had high homology (up to 92%) with the actin A1 promoter core region of *B. mori* (GenBank NM_001126252.1), which indicated that the sequence was the A1 promoter of *A. pernyi* (Fig. 2).

The sequence was submitted to the Berkeley models (Berkeley Drosophila Genome Project) and to the Web Promoter Scan Service. This analysis showed that it contained typical promoter characteristics. A transcription initiation site was identified, as were a number of

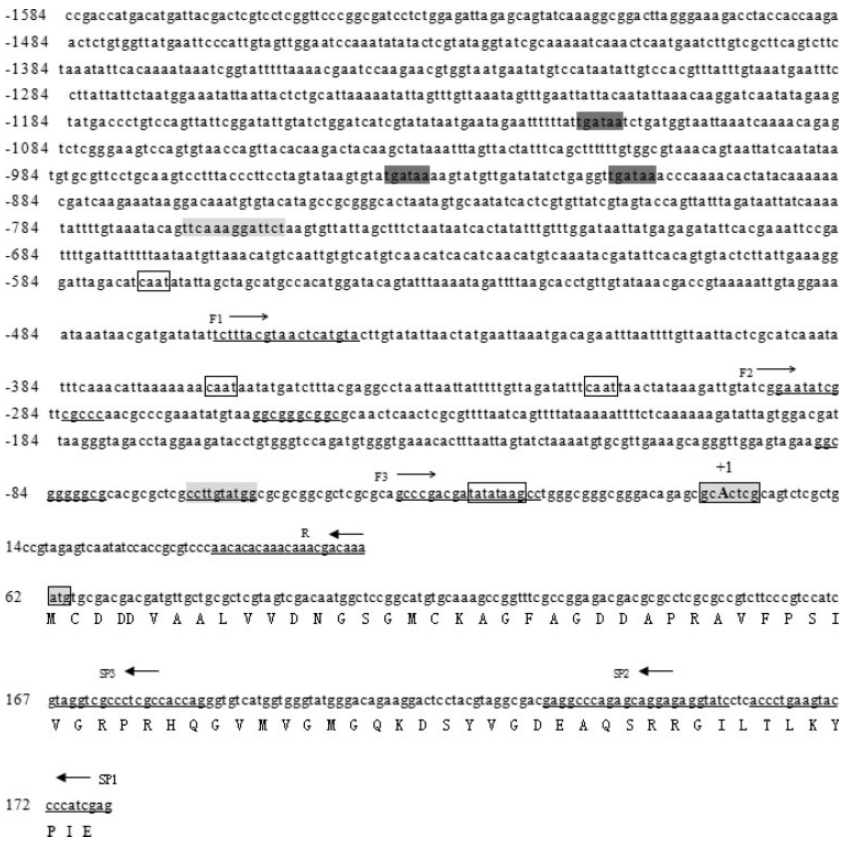

Fig. 2. Nucleotide sequence of actin A1 promoter. CAP site and transcription initiation site were marked. TATA boxes were framed. GATA boxes and CEBP motif were shaded in dark and light gray, respectively. GC-rich boxes were underlined. CArG motif was underlined and shaded in gray. CAAT boxes were framed. F1, F2, F3, and R primers for 5′-end deletions were shown; SP1, SP2, and SP3 primers for genomic walking were shown, too.

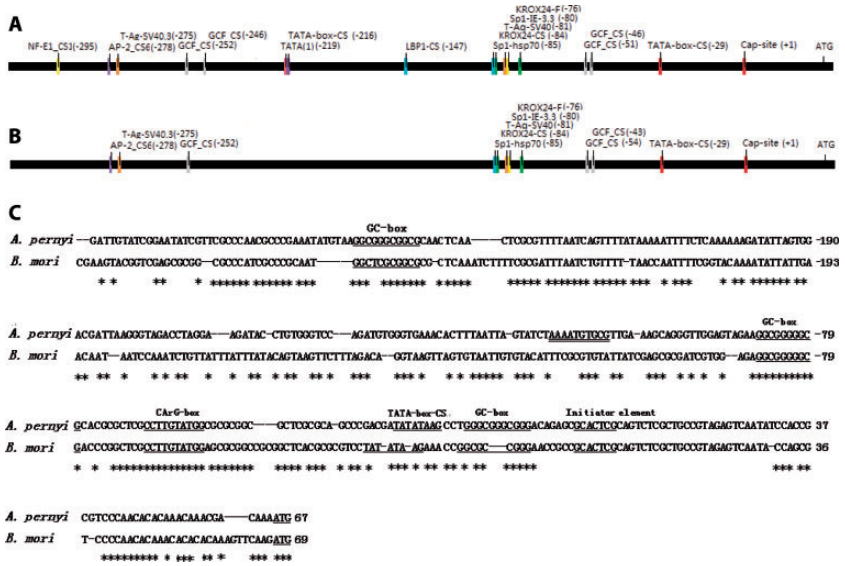

Fig. 3. Comparison of *A. pernyi* actin A1 promoter and *B. mori* actin A1 promoter. (A) Features of *A. pernyi* actin A1 promoter. (B) Features of *B. mori* actin A1 promoter. (C) Sequence alignment between the core region of *A. pernyi* actin A1 promoter and *B. mori* actin A1 promoter. The start code and the transcription factors were indicated by underline, and the putative inhibiting factor sequence was indicated by double-underlined. Missing nucleotides were indicated by - - - -. Identical nucleotides were indicated by ****.

putative transcriptional regulatory elements, including a TATA-like sequence, (TATATAA at −31 to −24 nt), and CAAT (−316 to −313 nt;−366 to −363 nt;−574 to 571 nt), CEBP (−768 to −756 nt) and CArG motifs (−67 to −58 nt). Three purine-rich GAGA boxes and two GC-rich boxes were also identified (Fig. 2). These results revealed that the putative A1 promoter region contains characteristics of eukaryotic promoters (Yutaka et al. 2001).

Predication and Comparison Between A. pernyi Actin A1 Promoter and B. mori Actin A1 Promoter. The actin A1 promoter sequences of *A. pernyi* and *B. mori* were compared using CLUSTAL 2.1 and analyzed using the Berkeley models (Berkeley Drosophila Genome Project) and the Web Promoter Scan Service. The structures were very similar to each other in their core promoter and regulatory element sequences; however, more transcription factor-binding sites were present in the region between -360-ATG in *A. pernyi* than in the same region in *B. mori* (Fig. 3). Several basic transcription factors are the same and some additional factors exhibited different in *A. pernyi*, and the details about binding sites, consensus sequences, function and the references were listed in Table 1.

Analyzing of Core Promoter Sequence. The start site of A1 was determined as the capital A (+1) of initiator element gcActcg. A tataataag sequence is present 30 bases upstream and would represent the

Table 1. Comparison of actin A1 main transcription factors between *A. pernyi* and *B. mori*

Site name	*A. pernyi* A1	*B. mori* A1	Transcription factor	Function	Reference
CAP-site	+1 (+) CANYYY	+1 (+) CANYYY	CAP	Enhancing cAMP complexes affinity to DNA	Lin and Green (1989)
TATA-box-CS	−29 (+) TATAWAW −216(−) TATAWAW	−29 (+) TATAWAW None	TFIID	Activating pol II transcription	Timmers et al. (1992)
TATA(1) GCF_CS	−219 (+) TATAAA −46 (−) SCGSSSC −51 (−) SCGSSSC −246 (−) SCGSSSC −252 (+) SCGSSSC	None −43 (−) SCGSSSC −54 (+) SCGSSSC None −252 (−) SCGSSSC	TMF GCF	TATA element modulator factor Repressor at GC-rich sequences	Garcia et al. (1992) Kageyama and Pastan (1989)
KROX24-F KROX24_CS	−76 (−) CGCCCCCGC −84 (+) GCGSGGGCG	−76 (−) CGCCCCCGC −84 (+) GCGSGGGCG	KROX24	Transcriptional activator	Lemaire et al. (1990)
Sp1-IE-3.3 Sp1-hsp70	−80 (−) CCCGCC −85 (+) GGCGGG	−80 (−) CCCGCC −85 (+) GGCGGG	Sp1	Enhancing gene transcription binding to the GC boxes in the regulatory region	Ding et al. (1999)
T-Ag-SV40 T-Ag-SV40.3	−81 (+) GGGGC −275 (−) TGGGC	−81 (+) GGGGC −275 (−) TGGGC	T-Ag	Transactivation transcription factor	McVey et al. (1989)
LBP1_CS	−147 (−) WCTGG	None	LBP-1	TAR-binding factor	Wu et al. (1988)
AP-2_CS6	−278 (+) CCCMNSSS	−278 (+) CCCMNSSS	AP-2	activating pol II transcription	Moser et al. (1995)
NF-E1_CS1	−295 (−) MYWATCWY	None	GATA-1	High-affinity DNA-binding factor by a single zinc finger motif	Pedone et al. (1996)

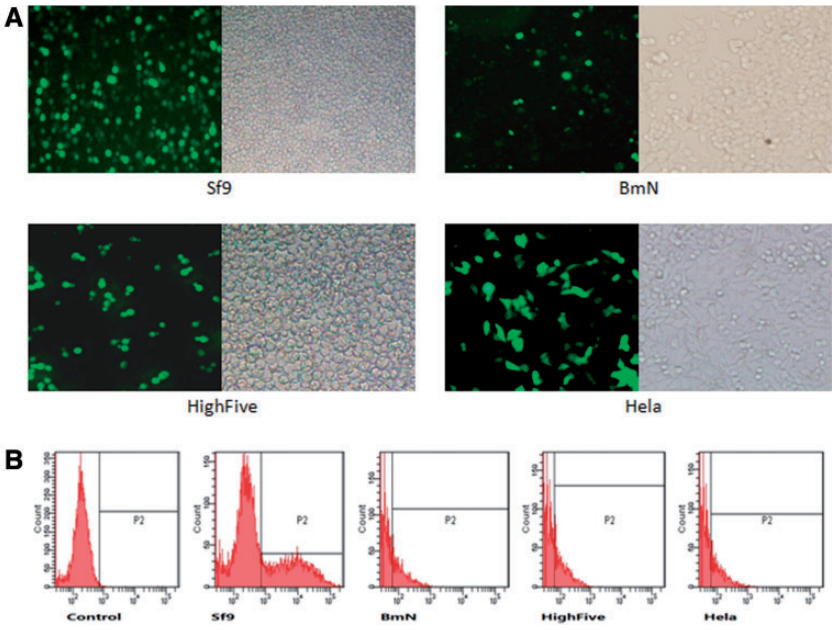

Fig. 4. Expression analysis of GFP under A1 promoter in different cell lines and transfection efficiency analysis. (A) Left showed GFP expression of Sf9 cells, BmN cells, HighFive cells, and HeLa cells, right-showed light images. (B) Transfection efficiency analysis of Sf9 cells, BmN cells, HighFive cells, and HeLa cells measured by flow cytometer.

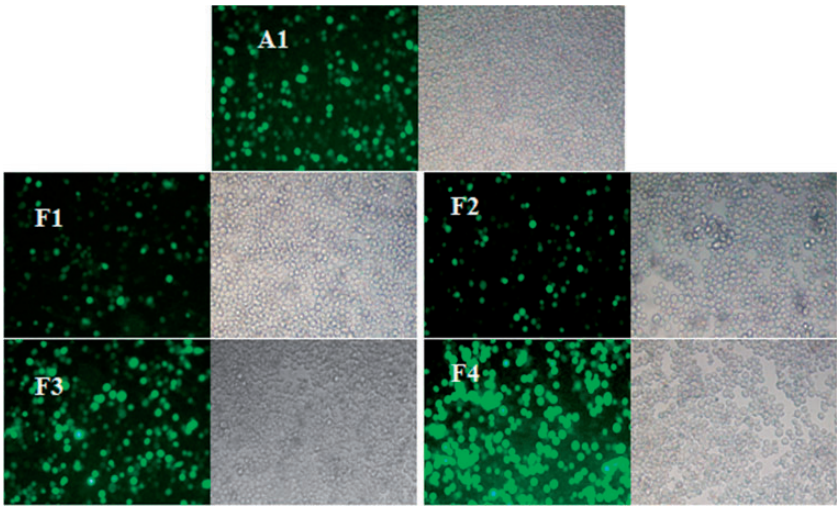

Fig. 5. GFP expression under a series of 5′-end deletions of A1 core promoter region in Sf9 cells. Excitation wavelength 460–490 nm; emission wavelength 510–550 nm; A1: transfected with pA1, including all functional elements; F1–F4: transfected with pF1, pF2, pF3, pF4. Light images were shown.

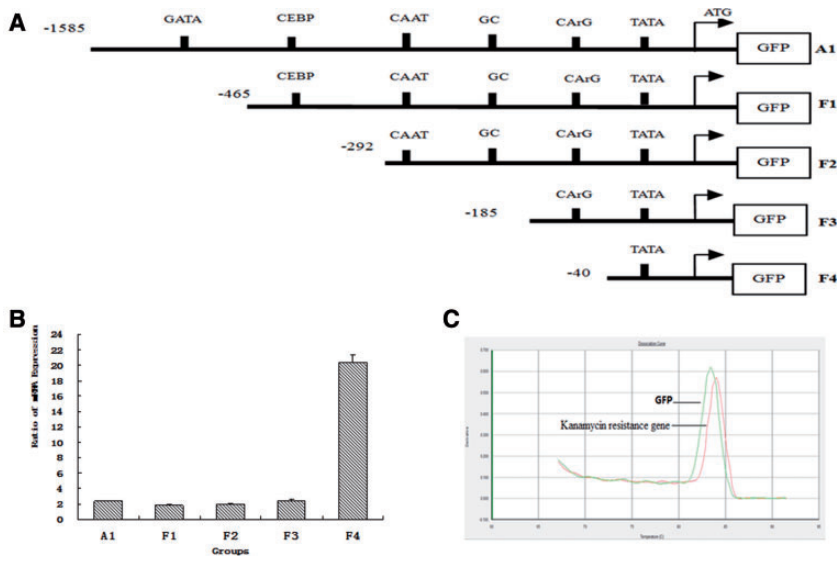

Fig. 6. Reverse transcription PCR analysis of GFP expression after a series of deletions of A1 core promoter region in Sf9 cells. (A) A series of actin A1 promoter fragments with 5′-end deletions were amplified using PCR method. The amplified products were digested with HindIII and BamHI and inserted into pGFP-N2 to generate expression vectors F1, F2, F3, and F4. The coordinates of the relative deletion fragments are indicated. The transcription start sites (ATG) are shown by arrows. (B) Ratio (GFP/kanamycin) of mRNA expression level in Sf9 cells determined by reverse transcription PCR and normalized to kanamycin. Averages of three independent experiments ± SD are shown. (C) Melting curve of qPCR of GFP and kanamycin resistance gene.

TATA element of the promoter. The proximal promoters carry the motif, ccttgtatgg, which almost fits the CArG consensus CC(A/T)$_6$GG of the vertebrate SRE. A 10-nucleotide element, AAAATGTGCG (−120 to −110 bp), was identified by comparison to the sequence of A3 in *B. mori* (Fig. 3).

To test how broad A1 promoter can be applied to different hosts, an expression vector pGFP-N2-A1 was constructed and Sf9, *B. mori* (BmN), *T. ni* (HighFive), and HeLa cells were transfected. The transfection efficiency was assayed by flow cytometer, and the ratios of transfection efficiency of Sf9, *B. mori* (BmN), *T. ni* (HighFive), and HeLa cells are 39.2, 31.7, 30.2, and 29.9%, respectively. Our results showed that upon transfection of plasmids pGFP-N2-A1, strong GFP expression was observed in Sf9, lower in HighFive and BmN cells, and the lowest expression in HeLa cells (Fig. 4).

To analyze elements within the A1 promoter, a series of A1 promoter fragments with 5′-end deletions were amplified and cloned into the pGFP-N2 expression vector to replace its Cucumber Mosaic Virus (CMV) promoter. The resulting plasmids contained the defined promoter sequences F1 through F4. The plasmids were transfected into Sf9 cells and GFP expression was assayed under fluorescence microscopy (Fig. 5). The results showed that plasmid containing the F4 fragment produced the highest level of GFP expression.

GFP gene transcription level was normalized relative to the transcription of kanamycin resistance gene by using of real-time PCR after cDNA was synthesized based on total RNA extracted from infected cells with vectors, which containing a series of A1 promoter fragments with 5′-end deletions. The results indicated that the level of GFP

transcription was significantly upregulated when expression was driven by F4, and was ~10-fold higher than when expression was driven by A1 or F1–F3 (Fig. 6).

Discussion

Expression vectors that express high levels of heterologous proteins are important for numerous transgenic applications, and many factors must be considered in the construction of a successful expression vector. The promoter has a significant effect on the expression of an exogenous gene and endogenous promoters have been shown to drive higher levels of expression (Fatyol et al. 1999). We isolated an actin A1 promoter from the Chinese oak silkworm, *A. pernyi*, to construct an expression plasmid that is efficiently recognized by the endogenous *A. pernyi* transcription machinery. Assays demonstrated that this new promoter is functional not only in insect cells but also in mammalian cells using plasmid transfection. Our results showed that the core region of this promoter had a certain universal adaptability to several difference species, so it may be an ideal candidate for use in *A. pernyi* transgenic systems in the future.

Sequence analysis revealed that the *A. pernyi* A1 promoter had a high level of identity with the A1 promoter of *B. mori*, which was a muscular-type actin promoter. The initiator element gcActcg and the TATA element tatataag were identical to that of A1 of *B. mori*. The proximal promoters carry the motif, ccttgtatgg, which almost fit the CArG consensus $CC(A/T)_6GG$ of the vertebrate SRE. This element, required for serum induction of c-fos transcription, has been proved to be essential for muscle-specific transcription in mammals (Mohun et al. 1989). But further study should be carried out on whether the role of this regulatory factor in insects has the same function.

Comparison of the core regions indicated that the *A. pernyi* A1 promoter had more transcription factor-binding elements than the *B. mori* A1 promoter, which may show a stronger ability to regulate gene expression for its better adaption to the more complicated wild surroundings (Cases and de Lorenzo 2005). Unlike domestic silkworms, the Chinese oak silkworms are wild silkworms, and hard to be raised indoor (Liu et al. 2010). Various transcription regulation elements are necessary to response to diverse habitats and conditions, and adapt to changes of the external environment.

Assays of promoter deletion fragments indicated the presence of silencer sequences upstream of the TATA box, which dramatically downregulated gene expression. To our knowledge, no functional tests of *Bombyx* Actin-1 promoter have been performed, only for the Actin-3 promoter. So the sequence of this negative region was compared with that of *B. mori* A3 sequence and a 10-nucleotide element, AAAATGT GCG (−120 to −110 bp), was identified. This sequence is similar to the RA3 region, AAAAGATGCG, which is required for the transient expression of pA3-LacZ in *B. mori* (Mange and Prudhomme 1999). However, we did not identify a positive SRE sequence, which might interact with a SRF-like protein, as occurs in A3 of *B. mori*, indicating that *A. pernyi* A1 may be regulated in another way. In-depth research would be carried out on the function of these elements.

Conclusion

In conclusion, we cloned and characterized the actin A1 gene promoter from the Chinese oak silkworm, *A. pernyi.* The isolated sequence has the highest identity with the *B. mori* actin A1 promoter. Analysis using the Berkeley models Berkeley Drosophila Genome Project and the Web Promoter Scan Service revealed typical eukaryotic promoter characteristics. Sequential 5′-deletions identified basic functional regions of the promoter. These results indicate that a strong inhibitory component may exist upstream of the TATA box, which downregulates gene expression.

Acknowledgments

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