Differential structure of the intronic promoter of the *Bombyx mori* A3 actin gene correlated with silkworm sensitivity/resistance to nucleopolyhedrovirus

J. Tavares, J.P. Bravo, F. Gimenes, Q.A.L. Neto, A. Fiorini and M.A. Fernandez

Departamento de Biologia Celular e Genética,
Universidade Estadual de Maringá, Maringá, PR, Brasil

Corresponding author: M.A. Fernandez
E-mail: mafernandez@uem.br / aparecidafernandez@gmail.com

ABSTRACT. Previous reports demonstrated that actin is necessary for nucleocapsid transport and viral gene expression during nucleopolyhedrovirus infection of *Bombyx mori*. The first intron of *B. mori* A3 actin contains a cryptic promoter that drives expression of a rare isoform. We detected differences in the size and nucleotide composition of the first intron of the A3 actin gene from *B. mori* strain C24A, which is more resistant to nucleopolyhedrovirus than the M11A strain (22 and 95% lethality, respectively). We sought to determine if resistance to BmMNPV infection and the A3 actin promoter structure are correlated. Intrinsically bent DNA sites in these sequences, which determine curved structures, were analyzed by electrophoretic mobility assays and the helical parameters ENDS ratio, roll and twist. We found both fragments to have

non-centralized bent DNA sites with distinct ENDS ratio values, nucleotide positions and two-dimensional structures. Additionally, a conformational-sensitive gel electrophoresis assay identified an allelic variation found in strain M11A that is absent in strain C24A. These data suggest that A3 actin intronic sequence variations impair virus propagation and are markers of BmMNPV-resistant populations.

Key words: Actin; Bent DNA; *Bombyx mori*; Nucleopolyhedrovirus; Resistance

INTRODUCTION

The silkworm, *Bombyx mori*, produces a cocoon that is used in the textile industry to make silk tissues with high commercial value. Sericulture, is an important source of income for many families in Brazil, the fifth largest cocoon producer in the world. Unfortunately, sericulture has been damaged by pathogens, primarily *B. mori* multiple nucleopolyhedrovirus (BmMNPV), that kill larvae prior to cocoon production (Brancalhão et al., 2002; Fernandez et al., 2005).

Our previous unpublished results indicated that the M11A *B. mori* strain is highly susceptible to BmMNPV (95% lethality), while the C24A strain is more resistant (22% lethality). Actin is required for BmMNPV nucleocapsid morphogenesis (Kasman and Volkman, 2000) and F-actin inhibitors, such as cytochalasin B, cause formation of irregular capsids and aborted development in a related virus, *Autographa californica* MNPV (Ohkawa and Volkman, 1999). Additionally, Feierbach et al. (2006) reported that neuronal cells infected with α-herpesvirus produce actin filaments physically associated with nascent capsids. Finally, the BmMNPV P95 protein stimulates actin expression levels in *B. mori* cell cultures (Maolong et al., 1998).

Actin functions in several important cellular processes. F- and G-actins exhibit dynamic effects in gene transcription and transcription factor localization (Miralles and Visa, 2006). An alternative transcription start site within the first intron of the cytoplasmic A3 actin gene was described by Fatyol et al. (1998) and it drives an actin isoform transcription. The transcriptional activity of this intronic promoter was tested and found to function as a strong enhancer and can drive actin expression in the absence of the primary 5' UTR promoter (Fatyol et al., 1998). It remains to be determined if the strong promoter activity of the first intron of A3 cytoplasmic actin is dependent on secondary structures in the DNA molecule, which could be bound by viral proteins to increase actin expression and facilitate nucleocapsid development.

Secondary structures like curved DNA are formed by multiple intrinsically bent DNA sites that occur in A/T-rich regions with ~10-bp steps and are often bound by specific proteins that interact with chromatin in promoter regions (Miyano et al., 2001; Wanapirak et al., 2003; Ohyama, 2005; Fiorini et al., 2006; Gimenes et al., 2008). In developmental amplicons, our research group described bent sites in the *Bradysia hygida BhC4-1* gene promoter (Fiorini et al., 2001) and in the *Drosophila melanogaster* DAFC-66D amplicon (Gimenes et al., 2009). Analysis of the promoter structure of insect genes indicates that

those genes coding secreted polypeptides all contain similar bent DNA sites (Gouveia et al., 2008). In this study, we sought to analyze the structure of the first intron of A3 cytoplasmic actin in sensitive and resistant *B. mori* strains, M11A and C24A, respectively.

MATERIAL AND METHODS

DNA sample

Genomic DNA from the C24A and M11A strains was extracted from the silk glands of 5-day-old fifth-instar larvae using the protocol previously described by Mills and Goldsmith (2000) with modifications detailed in Barbosa et al. (2008). The A3 actin first intron from both strains was amplified with primers that anneal to sequences in exon 1 and exon 2 (forward 5'AGG TGG TGC TCG AAC AGT GC3'; reverse 5'AAT TTG CCG CGT GGG TCA GT3') of the *B. mori* actin gene sequence (GenBank U49854). The ~550-bp amplification product was cloned using the pGEM-T Easy vector system (Promega) and transformed into *Escherichia coli* DH5α. Plasmids were extracted and purified using the CTAB protocol (Del Sal et al., 1989).

Electrophoretic mobility assay

*Eco*RI (New England Biolabs) restriction fragments from both clones were analyzed on 6% polyacrylamide (PA) gels, with and without ethidium bromide (EtBr), to determine intrinsic curvature of the DNA molecules. Electrophoresis was performed in 1X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). All gels were stained after running with 1 mg/mL EtBr and photographed under UV light (UVP BioImaging Systems, Upland, CA, USA). As controls, 1% agarose gels were used. The mobility pattern of each fragment was obtained by calculating an R value (ratio of apparent/real fragment molecular size observed in each gel system). Real molecular size was obtained from PA + EtBr and agarose control gels. Apparent molecular size was obtained from the PA gel, where fragments seem to have a different size. R values between 0.9 and 1.10 mean no alteration in the fragment mobility. R values ≥1.11 mean reduced mobility, and R values <0.9 mean increased mobility (de Souza and Ornstein, 1998). Fragments with bent regions on one or both fragment ends display increased mobility (Diekmann and Wang, 1985; Fiorini et al., 2006).

Sequencing and *in silico* analysis

Sequencing was performed using the DYEnamic ET Dye Terminator Kit (Amersham GE) and a MegaBACE 1000 automated DNA sequencer. The helical parameters ENDS ratio, roll, twist, and AT content were calculated using the Map15a software (window 150 bp with a 10-bp step). Two-dimensional (2-D) projections of the 3-D trajectory were constructed with the 3D15m1 program. The Model *it* DNA analysis server from ICGEBnet (International Center for Genetic Engineering and Biotechnology) (Vlahovicek, 2003), available online, was useful for the construction of a 51-bp fragment projection with bent sites centered. The resultant files were visualized in the Jmol software.

Conformation-sensitive gel electrophoresis - CSGE

A pool of first-instar larvae from both strains (M11A and C24A) were macerated with liquid nitrogen, and DNA was extracted as previously described (Barbosa et al., 2008, 2009). DNA fragments were amplified using the primers described in the DNA sample section. As a control, the third intron of the *B. mori* light fibroin chain gene was amplified from C122B and C25B strains, as described elsewhere (Barbosa et al., 2009). The C122B strain is negative for the heteroduplex genotype (Barbosa JB, personal communication), while the C25B strain presents a positive result for the CSGE technique (Barbosa et al., 2009). Screening for polymorphisms using heteroduplex analyses from all strains was performed by denaturation of polymerase chain reaction (PCR) products at 95°C for 10 min followed by hybridization for 1 h at 60°C. The sample was mixed (1:1) with a loading solution consisting of 20% ethylene glycol (Sigma), 30% formamide, and 0.025% (w/v) of both xylene cyanol FF and bromophenol blue. The 10% polyacrylamide CSGE gel was prepared with 10% ethylene glycol, 15% deionized formamide and 40% acrylamide/bis solution (39 g acrylamide and 1 g 1,4-bis(acryloyl)piperazine (Fluka). The gel was pre-run at 110 V for 1 h to remove ionic charges. Electrophoresis was then performed at 300 V for 16 h at room temperature in 0.5X TTE buffer (43.04 g Tris-HCl, pH 9.0, 14.24 g taurine and 0.28 g EDTA in 130 mL). After electrophoresis, gels were stained with 1 mg/mL ethidium bromide (Sigma) in water and documented under UV light using a UVP BioImaging System.

RESULTS

Electrophoretic mobility of the first intron of A3 cytoplasmic actin from M11A and C24A strains

Curved DNA molecules display different mobility patterns on polyacrylamide gels with and without EtBr (PA + EtBr and PA, respectively). If curvature is centered in the DNA molecule, fragments have a reduced mobility on PA gels. In contrast, fragments with non-centered curvatures migrate more rapidly (Du et al., 1995; Fiorini et al., 2006; Gimenes et al., 2008). Although *Eco*RI restriction fragments from both strains are of the same size, they had different degrees of accelerated mobility on PA gel as compared with PA + EtBr gel (Figure 1). The R value of both samples was calculated by determining the ratio of apparent/real fragment molecular size observed on each gel system. The R values were 0.82 (C24A) and 0.86 (M11A).

In silico analysis of restriction fragments showed two bent sites

The sequences from both strains were analyzed in the Map15a software to determine the AT% and the helical parameters ENDS ratio, roll and twist (Figure 2). The BmMNPV-resistant strain, C24A, showed one bent DNA site (ENDS ratio = 1.25) near one end of the DNA fragment (475-bp position). The BmMNPV-susceptible strain, M11A, showed one bent site (ENDS ratio, 1.11) near the other end of the DNA fragment (175-bp position). These data were supported by a negative roll angle and a twist angle >34°. The location of these bent DNA sites explains the anomalous mobility pattern on the PA gel. Fragments from C24A and
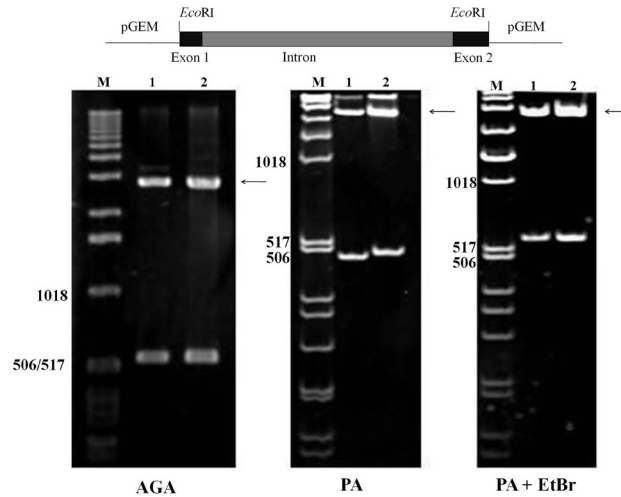
Figure 1. Electrophoretic mobility assay of *Bombyx mori* A3 actin first intron gene fragments. A PCR product containing the entire first intron (gray bar) and sequences from exons 1 and 2 (black bars) was cloned in pGEM plasmid (black lines and black arrows) and was digested with *Eco*RI. Both samples migrate faster on the PA gel than on the PA + EtBr gel, although there is a discrete difference between them. This result indicates that both fragments contain a non-centered bent DNA site. *Lane 1* = C24A ($R = 0.82$); *lane 2* = M11A ($R = 0.86$); M = 1-kb molecular weight marker (Invitrogen). AGA = 1% agarose gel; PA and PA + EtBr = 6% polyacrylamide gel without and with ethidium bromide, respectively.
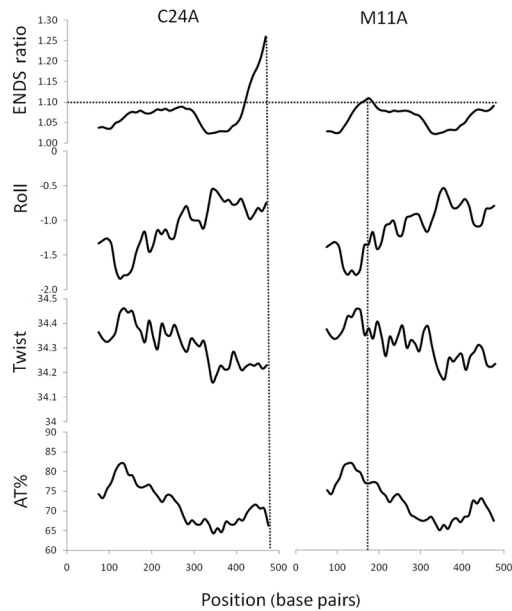

Figure 2. *In silico* analysis of the first intron of the A3 actin gene from strains C24A and M11A. The Map15a software with 150-bp window width and a 10-bp step was used to calculate the helical parameters ENDS ratio, roll, twist angle, and AT%. ENDS ratios >1.1, twist angles >34.0° and negative rolls indicate intrinsically bent segments with left-handed superhelical writhe. The horizontal dotted line indicates the cut off value (1.1) for ENDS ratio analysis. Curved DNA structures often occur in AT-rich regions, but require dA·dT tracts of 10 bp or multiples of 10 bp. Bent DNA sites are identified by vertical dotted lines - in the C24A strain at position 475 bp (site B) and in the M11A strain at position 175 bp (site A).

M11A both have non-centered bent sites, located 93 and 175 bp away from the fragment end, respectively. The curvatures of both fragments cause increased mobility; however, C24A accelerated more than M11A because it is closer to the fragment end. The sequences analyzed contain high AT%, characteristic of bent DNA segments. The 3D15m1 software was used to build a 2-D projection of the 3-D trajectory (Figure 3). The fragment from the resistant strain, C24A, showed a strong curvature in the fragment end while the fragment from the sensitive strain, M11A, showed a discrete non-centered curvature further from the fragment end. The circles indicate the ΔG variation, which is higher in bigger circles. The inset in Figure 3 shows a 51-bp fragment around bent DNA sites from both samples.
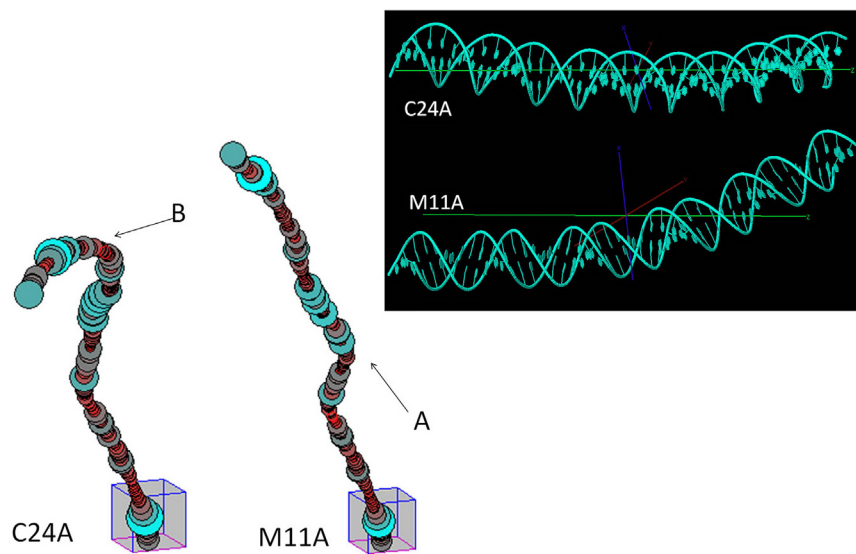

Figure 3. Two-dimensional (2-D) projection of the 3-D trajectories of the intron of the *Bombyx mori* actin A3 gene from strains C24A and M11A. Arrows indicate the intrinsically bent DNA sites **A.** 175-bp position. **B.** 475-bp position. The gray to black circles indicate the ΔG variation (higher in clear gray and big circles), which predicts double-helix stability. The gray box identifies the 5' end of each fragment. The inset shows a 51-bp fragment around of bent sites from both samples.

Alignment analysis highlights differences between M11A and C24A strains

An alignment of sequences from M11A, C24A, and the *B. mori* reference sequence (GenBank accession No. U49854) was constructed using the online ClustalW platform (EMBL-EBI) (Figure 4). Consistent with previous study by Fatyol et al. (1998), the actin isoform transcriptional start site (bold a) and some structural motifs (TATA box and CAAT box) were found in all sequences analyzed. Interestingly, we found a deletion of two thymines near the bent region in strain M11A. This deletion modifies the AT periodicity and may explain why the bent DNA present in strain M11A is absent in strain C24A. The bent DNA site of strain C24A is localized in the first exon of the rare A3 actin isoform. The different structure in the C24A strain may impair expression of this isoform and may explain why this strain is more resistant to BmMNPV.

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C24A    GAATTCGATTAAGGTGTGCTCGAACAGTGCGCATTCGCATCTACGTACTTGTCACTTATT 60
M11A    GAATTCGATTAAGGTGTGCTCGAACAGTGCGCATTCGCATCTACGTACTTGTCACTTATT 60
B.mori  GAATTCGATTAAGGTGTGCTCGAACAGTGCGCATTCGCATCTACGTACTTGTCACTTATT 60
        ************************************************************

C24A    TAATAATACTATgtaagttttaattttaaaatttcgaaagaaaaaaaaaacatatttatt 120
M11A    TAATAATACTATgtaagttttaattttaaaatttcgaaagaaaaaaaaaacatatttatt 120
B.mori  TAATAATACTATgtaagttttaattttaaaattgcgaaagaaaaaaaa-catatttatt 119
        *************************************** ************* *********

C24A    tattggtaaaattttaatttcgaagattcttcgtcc------tttaagtattacatatg 173
M11A    tatttgtaaaattttaatttcgaagattcttcgtcc------tttaagtattacatatg 173
B.mori  tatttgtaaaatttgaatttcgaaggttctccgtccctttaccttttaagtattacatatg 179
        **** ********* ********** **** *****        ***************

C24A    tttgagtgtttttttttttt-aataatacgctaatgataacgtgttacgttacataatcgt 232
M11A    tttgagtgtttttttttt◯-aataatacgctaatgataacgtgttacgttacataatcgt 230
B.mori  tttgagtgttttttttttttttaataatacgctaatgataacgtgttacgttacataattgt 239
        ****************    *******************************  **

C24A    tgcataactagtgaagtgaaattttttataaaaaaaaaaacagttttcggaatt-agtgta 291
M11A    tgcataactagtgaagtgaaattttttataaaaaaaaaaacagttttcggaatt-agtgta 289
B.mori  tgcataactagtgaagtgaaattttttataaaaaaaaa-cattttcggaatttagtgta 298
        ************************************** ** ********** ******

C24A    atgccgttg----------ctactaaataagaaataagttitatgggijacgcacatttcaaa 341
M11A    atgccgttg----------ctactaaataagaaataagttitattggacgcacatttcaaa 339
B.mori  ctgcagatgttaataaacactactaaataagaaataagttitattggacgcacatttcaaa 358
         *** * **  *************************iV'V''*********

C24A    gtgtccactcgcatcgatcaattcggaaacagaaattgggaacagtgaattatgaatctt 401
M11A    gtgtccactcgcatcgatcaattcggaaacagaaattgcgaacagtgaattatgaatctt 399
B.mori  gtgtccactcgcatcgatcaattcggaaacagaaattgggaacagtgaattatgaatctt 418
        ***************************************** *******************

C24A    agatagttttctttaacgtcactaaattgatgaacgcaaataaatttgtcgtttact---458
M11A    agatagttttctttaacgtcactaaattgatgaacgcaaataaatttgtcgtttact---456
B.mori  atacagttttctttaacgtcactaaatagatggacgcaaataaatttgtcgtttacttag 478
        * * ********************* **** *********************

C24A    -ataatttatggaatgagaatgtcagtttgaatcagtt-tttactttttcttgcagACTAA 516
M11A    -ataatttatggaatgagaatgt-agtttgaattgtttattttctttttcttgcagACTAA 514
B.mori  tataatgtatggaatgagaatgt-agtttgaattgtttttttttcttttcttgcagACTAA 537
         **** *************** ********  ** ** **************

C24A    TTCAAGATGTGCGACGAGGAAATTTGCCGCGTGGGTCAGT 556
M11A    TTCAAGATGTGCGACGAGGAAGTT-GCCGCGTTGGT-AGT 552
B.mori  TTCAAGATGTGCGACGAAGAAGTT-GCCGCGTTGGT-AGT 575
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Figure 4. Sequence analysis of the first intron of the *Bombyx mori* A3 actin gene from strains C24A and M11A. Uppercase and lowercase letters indicate exon and intron sequences, respectively. The bold (**t**) marks curvature centers according to ENDS ratio results. The bold (**a**) identifies the transcriptional start site for the A3 actin isoform described by Fatyol et al. (1998). Regions containing structural motifs described by Fatyol et al. (1998) are marked with boxes. This alignment identifies some differences between the strains analyzed that could alter the DNA molecular structure. Interestingly, there is a transversion in the second box (CAT-BOX) that can be an important domain in DNA-protein interaction (within the dotted rectangle). Circle indicates a double-T deletion that could be responsible for M11A bent site existence.

The first intron of A3 actin in strain M11A displays a heteroduplex pattern

CSGE detects conformational differences between homo- and heteroduplexes and can be used to verify if both alleles have the same DNA sequence. The analysis was carried out together with the DNA sequences of two additional strains, C122B and C25B, which are considered as negative and positive controls in the experiment, respectively. DNA from the C122B strain does not show any heteroduplex bands for fibroin light chain gene third intron sequences, which indicates that the two alleles in the strain larvae pool have similar sequences. In contrast, DNA from the C25B strain shows two extra bands for the fibroin light chain gene produced by heteroduplex, which indicates variation among the alleles. The M11A strain showed allelic polymorphism of A3 actin first intron, which was absent in the C24A strain (Figure 5). This result indicates that the susceptible strain contains two different alleles for the same sequence. By contrast, in the resistant strain, all individuals have the same sequence and the same tridimensional structure of the A3 first intron.
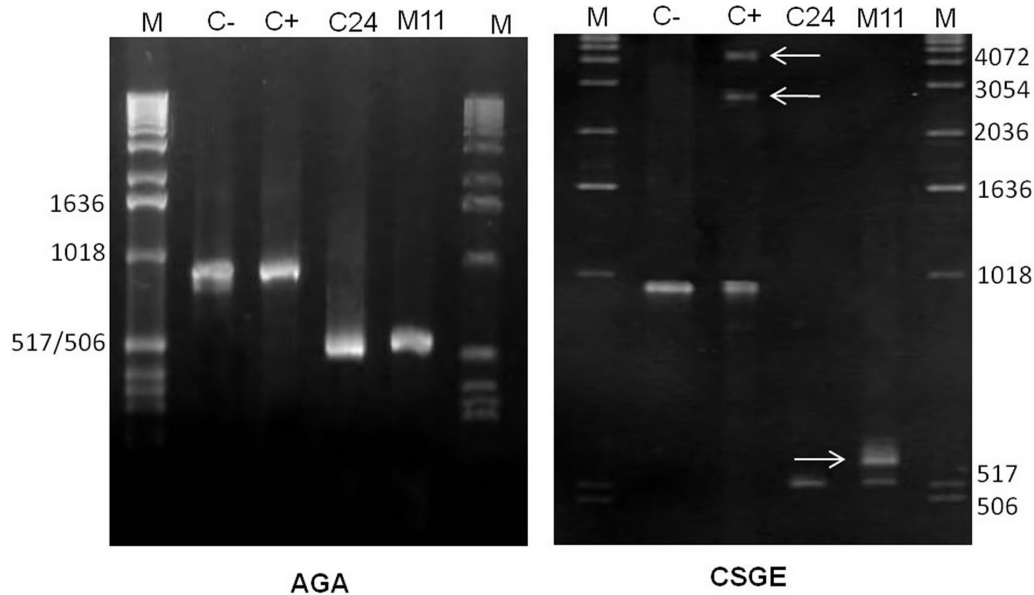

Figure 5. Conformation-sensitive gel electrophoresis (CSGE) analysis of the first intron of the *Bombyx mori* A3 actin gene from strains C24A and M11A. Genomic DNA from third-instar larvae of each strain was purified, and the first intron of the A3 actin gene was amplified and analyzed by CSGE. C- and C+ indicate negative and positive controls to amplified segments of the third intron of the fibroin light chain gene from strains C122B and C25B, as described by Barbosa et al. (2009). A 1% agarose gel (AGA) was useful as a control because allele differences are not identified in this system. On the CSGE gel, the white arrows indicate heteroduplex DNA molecules. The results show that C24A strain is a homozygote and M11A strain is a heterozygote for the sequence analyzed. M = 1-kb molecular weight marker (Invitrogen).

DISCUSSION

Several studies have been conducted to explore possible mechanism of intron mediated enhancement of gene expression. Functional studies using transient transfection of LMTK mammalian cells in culture with a LacZ reporter system allowed the characterization

of the *B. mori* A3 actin first intron promoter (Fatyol et al., 1998). The results showed that the first intron functions as a strong enhancer and can drive reporter gene expression in the absence of upstream promoter sequences.

Enhancers often contain binding sites for regulatory proteins that can increase transcription rates (Carey and Smale, 1999) and DNA-protein interaction suggests the existence of secondary structures in DNA molecule like cruciform or intrinsically bent DNA sites (Calladine et al., 2004). DNA can become curved by a protein binding force or can be intrinsically curved. The intrinsically curved DNA structures often occur in or around origins of DNA replication and regions that regulate transcription, such as enhancers (Ohyama, 2005). When A or T tracts occur with a periodicity near or equal to the DNA helical repeat length, around 10.5 bp, DNA forms a planar curve (Calladine et al., 2004). Most of the curved DNA structures that have been identified are upstream of core promoters and transcription factor binding sites are often located in this curved DNA (Bash et al., 2001). Curved DNA is also found downstream from core promoters as in the *Escherichia coli* CAA gene (colicin A) (Ohyama, 2005). These data indicate a clear relationship between DNA structure and transcriptional regulation. In our study, a bent DNA site was discovered in the A3 actin intronic promoter. The curvature of the A3 actin intronic promoter may modulate promoter strength.

Curved DNA may also modulate the geometry of promoters in collaboration with structure-specific transcriptional regulators (Ohyama, 2005). We suggest that different curvatures of the *B. mori* A3 actin first intron promoter may differentially alter gene expression. This regulation could be affected by viral infection. When infected, susceptible strains increase actin levels more than resistant strains do, probably as a result of different tridimensional DNA structure.

Maolong et al. (1998) found a BmMNPV protein (P95) that causes increased actin expression in *B. mori* cells, proving that actin functions in the infection process. Different responses to P95 could be the result of different DNA structures. If actin is necessary for viral development, alterations of DNA-protein interaction in the actin promoter may be highly related to resistance and susceptibility to BmMNPV infection.

The silkworm is considered a good candidate for recombinant protein production due to its large size and high protein synthesis ability. Baculovirus-based systems have been developed as expression systems to produce some recombinant proteins (Guo et al., 2005). In light of this, it is important to understand the influence of virus infection on host gene expression. Zhang et al. (2008) used injection of recombinant viruses with reporter genes driven by *B. mori* cytoplasmic actin gene promoter to demonstrate that this promoter displays tissue-specific expression variations, highest in the silk gland followed by fat body and middle gut of the infected larvae. The time curves of A3 promoter activity are parallel to the time curves of viral copy number (Zhang et al., 2008). Actin is involved in many cellular events and is greatly synthesized during the fifth-instar larvae. Understanding A3 actin gene regulation and responses during viral infection is a crucial step to the development of baculovirus-based expression systems to produce recombinant proteins for human interests.

This paper described intrinsically bent sites in the first intron of A3 actin. Further experiments with expression cassettes should be conducted to show the functionality of the bent sites described in differential expression.

ACKNOWLEDGMENTS

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