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Chicken HS4 insulator significantly improves baculovirus-mediated foreign gene expression in insect cells by modifying the structure of neighbouring chromatin in virus minichromosome

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ABSTRACT

The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is widely used as a eukaryotic expression vector for protein production. In the current study, chicken β-globin 5′-HS4 insulator (HS4) was placed downstream of the polyhedrin promoter-directed foreign gene expression cassette in AcMNPV, and found to markedly increase the expression of target gene. When enhanced green fluorescence protein gene (*egfp*) was used as the reporter gene, cells infected by the recombinant virus with HS4 (AcEGFP-HS4) showed 3.0 and 2.1-fold stronger fluorescence than that by the control virus without HS4 (AcEGFP) at 72 and 96 h post infection, respectively. The level of *egfp* mRNA was also much higher in cells infected by AcEGFP-HS4 than that by AcEGFP. An increase in gene expression was also seen when firefly luciferase gene or secreted alkaline phosphatase gene was used as a reporter. The insertion of HS4 in the polyhedrin locus has no significant effect on virus replication. The effect of HS4 was orientation-dependent, and sensitive to inhibitors of histone acetyltransferase. In DNase I sensitivity assay, HS4 significantly increased the sensitivity of neighbouring DNA to nuclease, but had little effect on DNA of a distal locus. These results suggested that HS4 insulator might affect baculovirus gene expression by modifying the structure of neighbouring chromatin in the virus minichromosome.

1. Introduction

Baculoviruses are enveloped DNA viruses that specifically infect insects (Okano et al., 2006). The prototype baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), is widely used as a eukaryotic expression vector for protein production in insect cells, employing its highly active promoters of polyhedrin or p10. Advantages of the baculovirus expression system include high levels of expression, convenience to generate recombinant virus, ability to incorporate large foreign genes, correct post-translational modifications, and good biosafety profile (Kost et al., 2005; Miller, 1988). However, the expression level of foreign protein was routinely much lower than that of the native viral polyhedrin in wild type AcMNPV (Sano et al., 2002). Efforts have been made to improve foreign gene expression by adding an extra copy of viral homologous region 1 (hr1) (Venkaiah et al., 2004) or 3 (hr3) (Chen et al., 2004), lobster tropomyosin cDNA leader sequence (Sano et al., 2002), the cauliflower mosaic virus 35S promoter (Abe et al., 2005), etc. Increases of 1.5 to 7-fold in target gene expression were reported in these studies.

Insulators are DNA sequences that can isolate the target gene from surrounding regulatory elements to ensure its expression (Wallace and Felsenfeld, 2007). Two classes of insulators have been identified: enhancer-blocking insulators and barrier insulators. Enhancer-blocking insulators can obstruct the effect of distal enhancers and prevent inappropriate activation of gene expression when placed between an enhancer and promoter. Barrier insulators can block the extension of heterochromatin and protect a transcriptionally active region (West et al., 2002). Many insulators have been identified and characterized in the genome of yeast, drosophila, human and other eukaryotes. They frequently locate at or near gene boundaries, and usually contain DNase I-hypersensitive sites. Insulators have been used to ensure transgene expression in retrovirus, adeno-associated virus and adenovirus vectors (Ramezani et al., 2003; Inoue et al., 1999; Steinwaerder and Lieber, 2000).

The 1.2 kb-long chicken 5′-HS4 β-globin insulator (HS4) is the most frequently studied vertebrate insulator. It has both enhancer-blocking and barrier functions (Chung et al., 1993), and is also able to block the effect of silencers (Yao et al., 2003). In the current work, HS4 insulator was placed downstream of the expression cassette of a reporter gene in a baculovirus expression vector, and found to markedly increase the gene expression from the polyhedrin promoter. Further analysis suggested that epigenetic regulations might have contributed to this increase.

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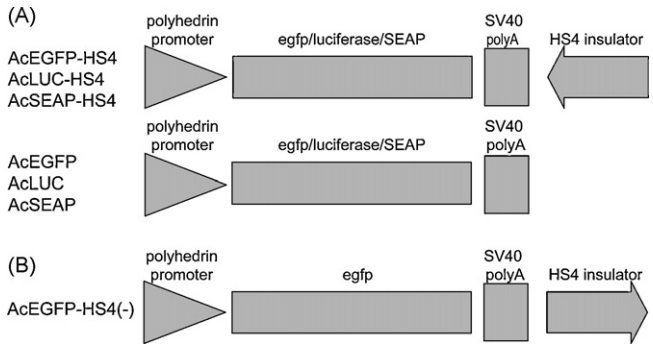

Fig. 1. Schematic diagrams of recombinant AcMNPV *polyhedrin* locus.

2. Materials and methods

2.1. Cell culture and virus

The *Spodoptera frugiperda* cell line Sf9 and *Trichoplusia ni* cell line High5 were cultured in TNM-FH (Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum at 27 °C. The cells were routinely subcultured every three days, with a split ratio of 1:3. Viruses were propagated in Sf9 cells by infecting the overnight culture at a multiplicity of infection (MOI) of 0.1 pfu/cell. Virus inoculum was obtained by centrifuging the infected culture medium for 10 min at 850 × g and stored at 4 °C before use. Virus titres were determined by end-point dilution assay (O'Reilly et al., 1992). All viruses used in the study were within their first two passages.

2.2. Virus construction

The full HS4 insulator (1.2 kb) was obtained from plasmid pSLJCA, a gift from Dr. Andre Lieber, University of Washington, by *Xba*I digestion. It was inserted into the *Xba*I site of pFastBac-1 (Invitrogen, Carlsbad, CA). Recombinant plasmids with HS4 in either orientation were generated and named pFB-HS4 and pFB-HS4(−). Both plasmids had the HS4 fragment downstream of the polyhedrin promoter, with HS4 in the same or opposite orientation as the polyhedrin promoter in pFB-HS(−) and pFB-HS4, respectively (Fig. 1A and B). The plasmid pFB-G was constructed earlier in the laboratory by inserting the cytomegalovirus immediate early promoter (CMV promoter), and the PCR fragment of enhanced green fluorescence protein gene (*egfp*) together with the SV40 polyadenylation signal (polyA) into the sites of *Kpn*I and *Sal*I, respectively, both in the opposite orientation as the polyhedrin promoter. It was digested with *Sal*I to isolate the *egfp*/polyA fragment, which was inserted into the plasmid pFastBac-1, pFB-HS4 or pFB-HS4(−) downstream of the polyhedrin promoter to generate the plasmid pFB-EGFP, pFB-EGFP-HS4 and pFB-GFP-HS4(−), respectively. The plasmids pFB-LUC-HS4, pFB-LUC, pFB-SEAP-HS4 and pFB-SEAP were generated similarly, where the firefly luciferase gene (*luc*), or secreted alkaline phosphatase gene (*seap*) was digested from plasmid pG5-FL (Clontech, Mountain View, CA) or pSEAP2-Basic (Clontech), respectively, and used in the place of *egfp* as above. All constructs were verified by restriction enzyme digestion, PCR and DNA sequencing. Recombinant viruses AcEGFP-HS4, AcEGFP-HS4(−), AcEGFP, AcLUC-HS4, AcLUC, AcSEAP-HS4 and AcSEAP were generated from pFB-EGFP-HS4, pFB-EGFP-HS4(−), pFB-EGFP, pFB-LUC-HS4, pFB-LUC, pFB-SEAP-HS4 and pFB-SEAP, respectively, using the Bac-to-Bac system (Invitrogen) following the manufacturer's protocol.

2.3. Virus growth curve

Actively growing Sf9 cells were counted using a hemocytometer and were seeded at 2×10^5 cells/well in a 24-well plate and infected with AcEGFP and AcEGFP-HS4 at MOI of 5 pfu/cell. The plate was left at 27 °C for 2 h before the supernatant was replaced with fresh medium. The cells were allowed to grow further at 27 °C. The culture

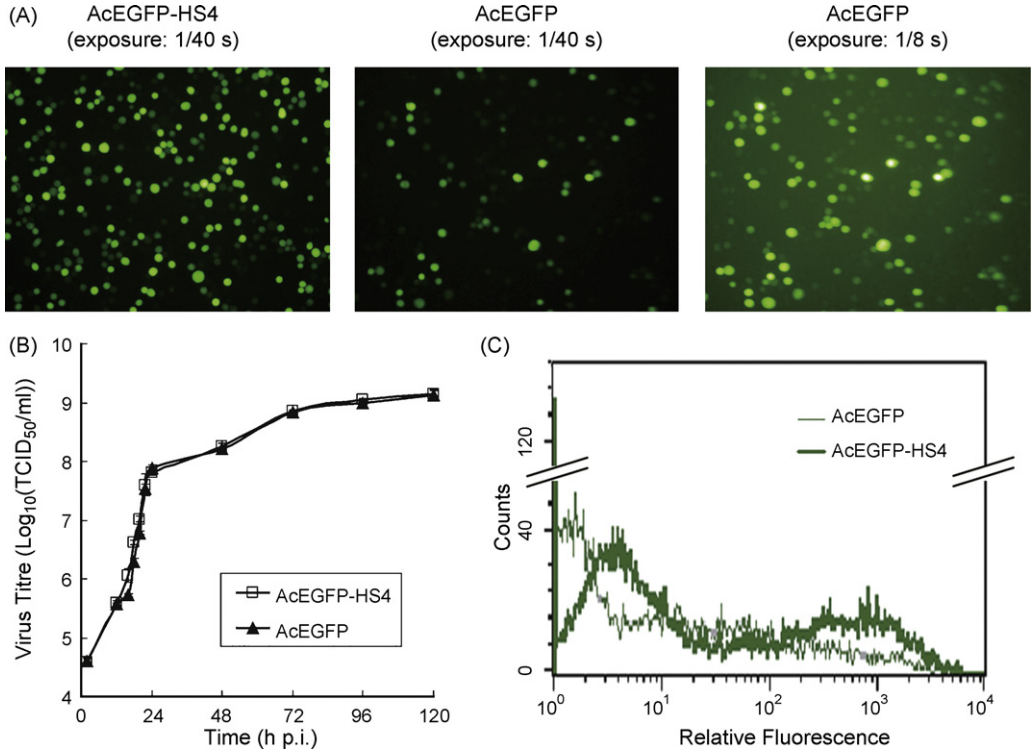

Fig. 2. (A) Fluorescence microscopy of Sf9 cells infected by AcEGFP-HS4 and AcEGFP. Pictures were taken using different exposure time as indicated. (B) The growth kinetics of extracellular virus in AcGFP-HS4 and AcEGFP-infected Sf9 cells. (C) Flow cytometry analysis of Sf9 cells infected by AcEGFP-HS4 and AcEGFP.

supernatant was collected at 0, 24, 48, 72, 96, 120 h post-infection (p.i.) and virus titer was determined.

2.4. Determination of the expression level of EGFP, luciferase and SEAP

Sf9 or High5 cells were seeded into wells of a 24-well cell culture plate at a density of 1.5×10^5 cells/well and then infected with the appropriate virus. To measure the fluorescence intensity of EGFP, AcEGFP-HS4, AcEGFP-HS4(−) and AcEGFP-infected Sf9 cells were collected at specific time points, washed three times with phosphate buffered saline (PBS), and re-suspended in 300 μl PBS. Then, 200 μl cell suspension was added into a well of an opaque 96-well plate and measured for green fluorescence with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA), using 485, 538 and 530 nm as the wavelength of excitation, emanation, and gating cut-off, respectively. Uninfected Sf9 cells were used as the blank control. To determine the luciferase activity, AcLUC-HS4 and AcLUC-infected Sf9 cells were washed with PBS, and 100 μl cell suspension was moved to a well of an opaque 96-well plate. Equal volume (100 μl) of Steady-Glo luciferase assay reagent (Promega, Madison, WI) was then added to each well, and the plate was left at room temperature for 5 min until cell lysis was completed. The luminescence was measured with a SpectraMax M5. To determine SEAP activity, the supernatant of AcSEAP-HS4 and AcSEAP-infected Sf9 cells was collected and clarified by centrifugation at $400 \times g$. Then, 20 μl clarified supernatant was removed to a well in an opaque 96-well plate, and SEAP activity was measured with SpectraMax M5 using a Reporter Assay Kit-SEAP (Toyobo, Osaka, Japan) following the manufacturer's protocol. Alternatively, an equal volume of 1% Triton X-100 in PBS was added to infected cells, and the cell lysate was used to determine the total SEAP activity in infected cells. All experiments were performed in triplicate on at least two independent occasions.

2.5. Immunoblot

AcEGFP-HS4 and AcEGFP-infected Sf9 cells were collected at 96 h p.i. The cellular proteins from approximately 5×10^4 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide) and transferred onto polyvinylidene membrane. Immunoblot was carried out following the standard protocol, using primary antibodies against EGFP (Bioinforbody, Zhuhai, China) and baculovirus envelope protein GP64 (laboratory stock), respectively, followed by alkaline phosphatase-conjugated secondary antibodies (Sigma).

2.6. Flow cytometry

AcEGFP-HS4 and AcEGFP-infected Sf9 cells were harvested at 48 h p.i., and washed twice with PBS. They were then analyzed for green fluorescence on a FACScalibur cytometer (Becton Dickinson, San Jose, CA) following the manufacturer's protocol (filter: 530 nm). Ten thousand cells were counted and examined for each sample.

2.7. Transcriptional analysis

AcEGFP-HS4 and AcEGFP-infected Sf9 cells were collected at 48 and 72 h p.i. Total RNA was extracted from cells using Trizol (Fermentas, Burlington, Ontario) and resuspended in 30 μl RNase-free water (Fermentas). It was digested with RNase-free DNase (TakaRa) for 0.5 h at 37 °C. Reverse transcription was carried out with RevertAid First Strand cDNA synthesis Kit (Fermentas) using 5 μg DNA-free total RNA and 1 μg oligo(dT) primer per sample following the manufacturer's protocol. Quantitative real-time PCR was performed on an Mx3000p system (Stratagene, La Jolla, CA) to determine the transcriptional level of *egfp* gene. Each reaction contained 100 nM Taqman probe (5′-CCCTCGCCCTCGC-3′, Brown et al., 2003), 100 nM each of forward and reverse primer (5′-CGTAAACGGCCACAAGTTC AG-3′; 5′-GGGTCAGCTTGCCGTAGGT-3′, Brown et al., 2003), and 1 μl of the first strand cDNA. In the control reaction, DNase treated-RNA (0.5 μl) was used in the place of cDNA. Polymerase chain reactions were performed using Gold Taq Mix (ABI, Foster City, CA). The level of *egfp* cDNA was normalized with that of viral nucleocapsid protein gene *vp39*, which was quantified using SYBR Green I Real-time PCR Master Mix (Toyobo) with primer pairs of 5′-TTCGACGCGTGCATAACATACA-3′ and 5′-TGCCTAGCGATCGTCATTTTG-3′. The transcription level was calculated from a standard curve using a serial dilution of AcEGFP bacmid DNA as the template.

2.8. DNase I-sensitivity studies

Real-time PCR was used to quantify DNase I-sensitivity of target DNA (McArthur et al., 2001; Peng et al., 2007). AcGFP and AcGFP-HS4-infected Sf9 cells were collected at 24 h p.i., washed in PBS and resuspended in 300 μl of supplemented RSB buffer (20 mM Tris–HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$,

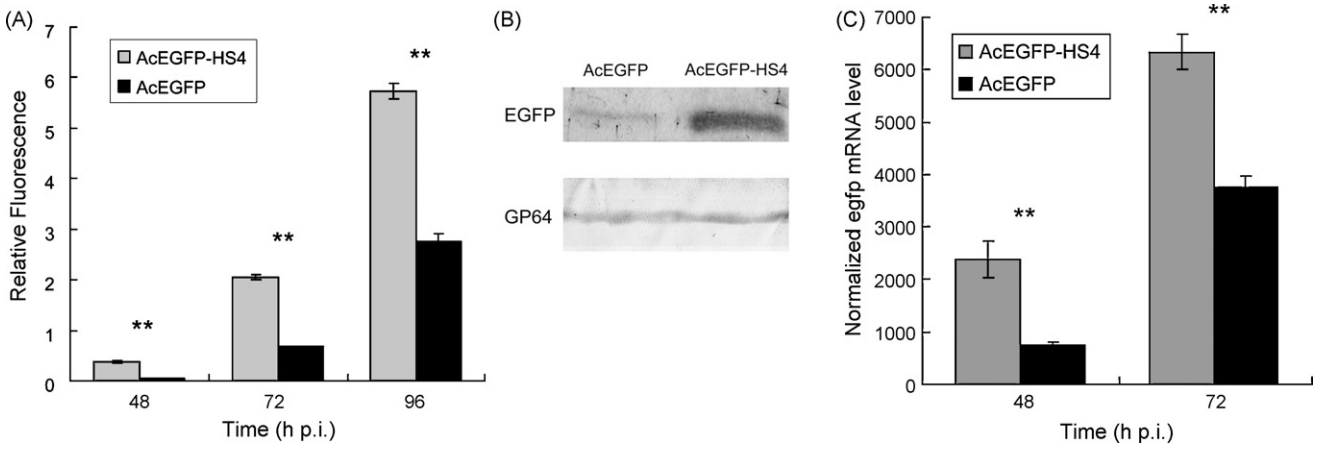

Fig. 3. The effect of HS4 on EGFP expression. (A). Relative fluorescence intensity in Sf9 cells infected by AcEGFP-HS4 and AcEGFP at different times post infection (p.i.). (B). Immunoblot analysis of EGFP expression in Sf9 cells infected by AcEGFP-HS4 and AcEGFP at 96 h p.i. Viral protein GP64 was used as an internal control. (C). Real-time quantitative RT-PCR of *egfp* mRNA in Sf9 cells infected by AcEGFP-HS4 and AcEGFP. The level of *egfp* mRNA was normalized by baculovirus *vp39* mRNA in infected cells, which was determined in parallel. The double asterisks indicate a statistically significant difference ($p < 0.01$) between viruses.

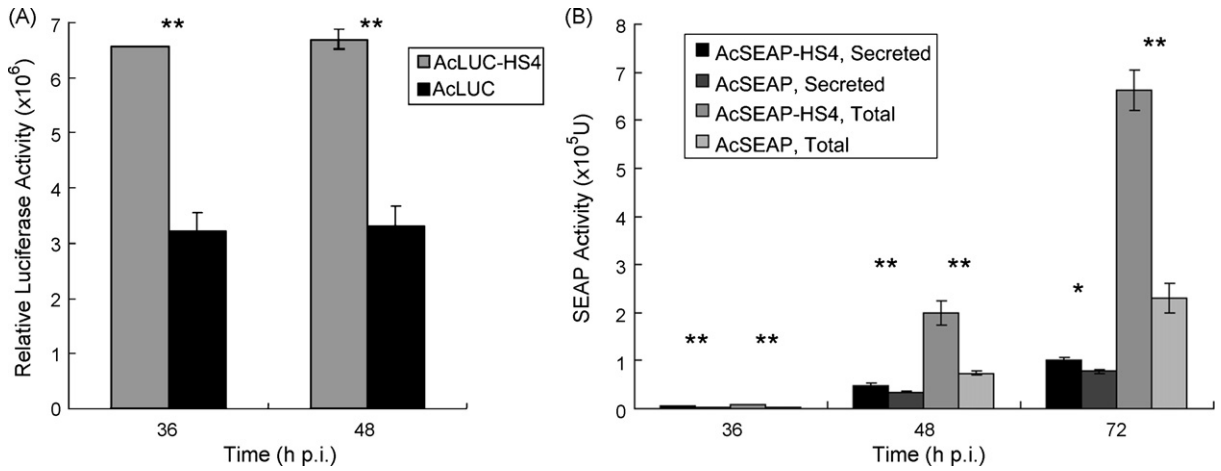

Fig. 4. The effect of HS4 on the expression of firefly luciferase (A) and SEAP (B) in infected Sf9 cells. The luciferase activity in AcLUC-HS4 and AcLUC-infected cells, and the SEAP activity in the culture medium and total cell lysate of AcSEAP-HS4 and AcSEAP-infected cells were determined at different times post infection. The asterisks indicate a statistical difference between viruses (*$p < 0.05$; **$p < 0.01$).

0.5% NP-40, 100 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml RNase A). The sample was divided into 50 μl aliquots, which were either left untreated or treated with various amount of DNase I (TaKaRa). Digestion was carried out at 37°C for 5 min and stopped by adding an equal volume of stop buffer (0.1 M NaCl, 0.1% (w/v) SDS, 50 mM Tris–HCl, pH 8.0 and 100 mM EDTA). Samples were treated with proteinase K for 5 h and the DNA were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl H_2O. Real-time PCR was performed using SYBR Green I Real-time PCR Master Mix (Toyobo) with the primer pairs of the *egfp* gene (see above). As a control, the primer pair of the *lef8* gene, which was located approximately 35 kb from HS4, was also used: 5′-AATGGCTACTCTGTGGCGGTAA-3′, 5′-ATGTGCGGAATGTACGGATC-3′. The expected size of PCR products were 68 and 99 bp in length for *egfp* and *lef8*, respectively. Serially diluted AcEGFP bacmid DNA samples were used to prepare the standard curve.

2.9. Treatment with histone acetyltransferase inhibitors (HATis)

The medium of the overnight culture of Sf9 cells was replaced with fresh medium containing 25 μM curcumin (Sigma) or 5 μM garcinol (BioMol, Plymouth Meeting, PA). The cells were then incubated for 2 h, before the virus inoculum was added. After infection, the cells were further cultured in the presence of drugs at 27 °C. Cell viability was measured by MTT assay as described previously (Wang et al., 2006).

2.10. Statistical analysis

Representative results from two or more independent experiments were shown. Mean and standard deviation was calculated from triplicate experiments and shown in the text and figures. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. HS4 insulator improved baculovirus-mediated EGFP expression in Sf9 cells

A recombinant baculovirus with *egfp* gene driven by the polyhedrin promoter and HS4 insulator downstream of the *egfp* expression cassette was constructed and designated AcEGFP-HS4. A similar virus without the insulator, AcEGFP, was used as the control (Fig. 1A). When examined by fluorescence microscopy, it was found that AcEGFP-HS4 resulted in much stronger EGFP expression than AcEGFP in virus-infected Sf9 cells (Fig. 2A). At a short exposure time (1/40 s), many more cells with green fluorescence were observed in the infection with AcEGFP-HS4 than with AcEGFP. This was not due to the difference in infectivity between the two viruses, since more than 90% of infected cells were seen to express EGFP for both viruses when longer exposure time (1/8 s) was used (Fig. 2A). In fact, the presence of HS4 in the polyhedrin locus had no major effect on virus replication, as two the two viruses had identical growth curves (Fig. 2B). In the flow cytometry analysis, compared to AcEGFP-infected cells, a significant proportion of AcEGFP-HS4-infected cells had a high level of fluorescence (Fig. 2C).

The green fluorescence in infected cells was quantified with a fluorescence plate reader (Fig. 3A). The mean fluorescence intensity (MFI) of AcEGFP-HS4-infected cells was nearly six times as high as that of AcEGFP-infected cells (3899 ± 276 vs. 585 ± 15) at 48 h p.i. The difference decreased at the later time points, though still highly significant ($20{,}484 \pm 574$ vs. 6790 ± 114 at 72 h p.i., and $57{,}238 \pm 1504$ vs. 27673 ± 1509 at 96 h p.i.).

In the immunoblot analysis, the EGFP band was much stronger in AcEGFP-HS4-infected cells than in AcEGFP-infected cells, while the bands of viral surface protein GP64 were similar to each other (Fig. 3B). These results implied that HS4 increased the expression of the nearby *egfp* gene, but had little effect on viral *gp64* gene, which was about 30 kb away from HS4.

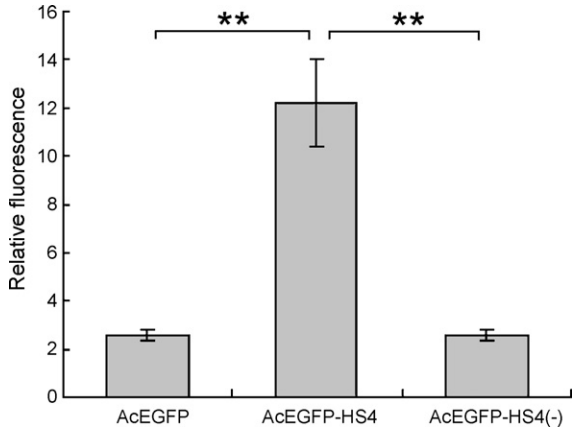

Fig. 5. The orientation effect of HS4. Relative fluorescence intensity in Sf9 cells infected by AcEGFP, AcEGFP-HS4 and AcEGFP-HS4(−) at 48 h p.i. The double asterisks indicate a statistically significant difference ($p < 0.01$) between viruses.

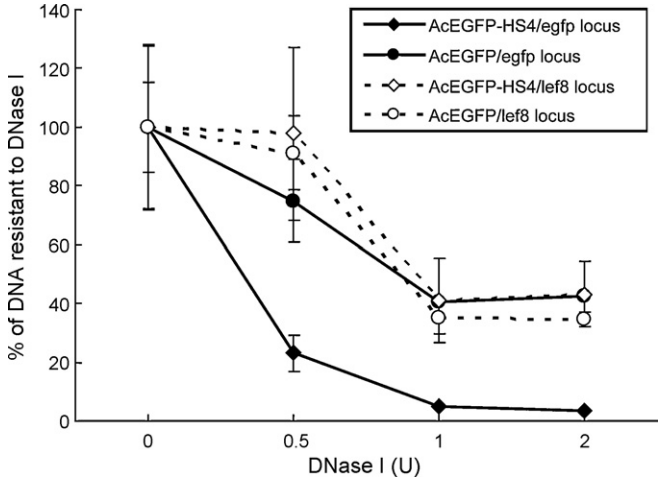

Fig. 6. The effect of HS4 on the sensitivity of viral DNA to DNase I. Viral DNA isolated from AcEGFP-HS4 and AcEGFP-infected cells were digested with DNase I. The percentage of DNA resistant to the nuclease was determined with real-time PCR using primer pairs for *egfp* and *lef8*.

A similar effect of HS4 was also seen in another insect cell line, *T. ni* High5 cells, where the MFI at 48 h p.i. was determined to be 63,500 ± 2517 and 21,500 ± 2517 for AcEGFP-HS4 and AcEGFP, respectively.

3.2. HS4 insulator increased egfp transcription in infected cells

The transcriptional level of *egfp* was quantified by real-time PCR, using the viral nucleocapsid protein gene *vp39*, which was located approximately 24 kb from HS4 in AcEGFP-HS4, as an internal control. When the same amounts of cDNA templates were used, the levels of *vp39* mRNA were similar for both viruses (AcEGFP-HS4: 7.4×10^4 copies, AcEGFP: 7.8×10^4 copies) at 48 h p.i. However, the levels of *egfp* mRNA differed greatly, being about 3.2 and 1.7-fold higher in AcEGFP-HS4-infected cells than in AcEGFP-infected cells at 48 h and 72 h p.i., respectively (Fig. 3C). The melting curve showed that the primers were specific. A no-reverse transcription control was performed to exclude the possibility of DNA contamination in the sample (data not shown). These results implied that HS4 improved *egfp* expression by increasing its transcription.

3.3. HS4 also increased the expression of firefly luciferase and SEAP

Recombinant viruses expressing firefly luciferase and secreted alkaline phosphatase (SEAP) were constructed (Fig. 1A). Unlike EGFP, luciferase has a short half-life in cells, which could be advantageous to reflect the difference in gene expression at a particular time point. As shown in Fig. 4A, the mean luminescence of AcLUC-HS4-infected Sf9 cells was $(6.57 \pm 0.01) \times 10^6$ at 36 h p.i., about 2-fold higher than that of AcLUC-infected cells, which was $(3.21 \pm 0.35) \times 10^6$. A similar trend was seen at 48 h p.i. (Fig. 4A).

AcSEAP-HS4-infected cells also produced a significantly higher level of SEAP than AcSEAP-infected cells (Fig. 4B). The difference in SEAP activity was 1.4 and 1.3-fold in the culture medium, and 2.7 and 2.9-fold in the total culture at 48 and 72 h, respectively.

3.4. The effect of HS4 was orientation-dependent

When HS4 was inserted in the same position as in AcGFP-HS4, but in the opposite orientation (AcEGFP-HS4(−), Fig. 1B), the effect of HS4 to increase *egfp* expression was no longer observed (Fig. 5). Sf9 cells infected by AcGFP-HS4(−) produced a similar level of EGFP as that infected by AcEGFP. The result indicated that HS4 worked in an orientation- specific manner, which was different from transcriptional enhancers.

3.5. HS4 increased the sensitivity of neighbouring DNA to DNase I

DNase I-sensitivity assay is commonly used to analyze the condensation state of chromatin (Felsenfeld, 1992). Here, it was utilized to compare the chromatin structure of AcEGFP-HS4 and AcEGFP DNA isolated from infected cells at loci either close or distant to HS4. As shown in Fig. 6, the *egfp* locus in AcEGFP-HS4 was more sensitive to DNase I treatment than the same locus in AcEGFP. When 0.5 u DNase I was added, 80% of *egfp* DNA in AcEGFP-HS4 was degraded, while about 80% of *egfp* DNA in AcEGFP remained intact. With the increase of DNase I dosage, nearly all *egfp* DNA in AcEGFP-HS4 was lost, while 40% of that in AcEGFP was preserved (Fig. 6). In contrast, the *lef8* locus in AcEGFP-HS4, which was 35 kb from HS4, had a similar sensitivity to DNase I treatment as the same locus in AcEGFP (Fig. 6). Similar degradation kinetics was seen between *egfp* DNA of AcEGFP, and *lef8* DNA of both viruses (Fig. 6). These results indicated that HS4 caused the relaxation of local chromatin, while had little effect on distant locus.

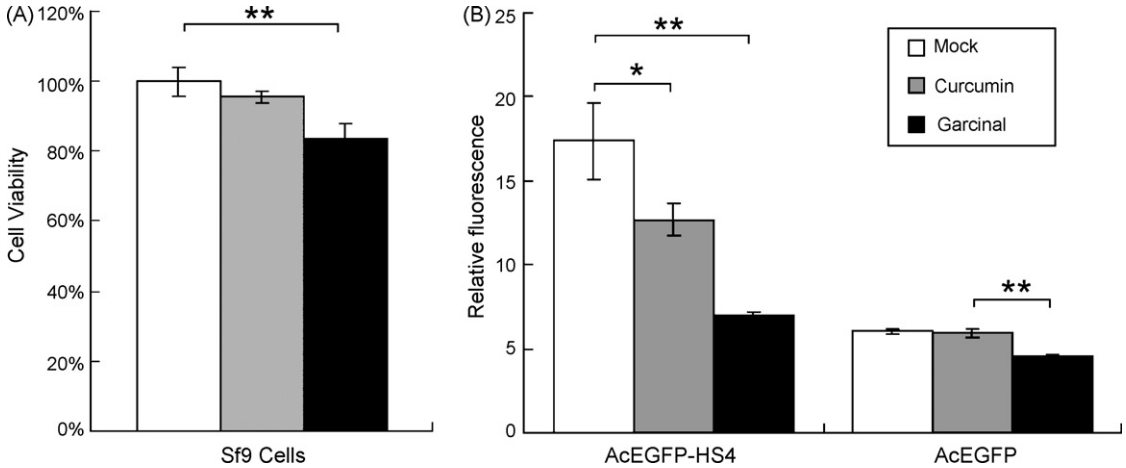

Fig. 7. The Effect of histone acetyltransferase inhibitors on HS4-mediated enhancement of gene expression. (A) The viability of cells cultured in the presence of curcumin (25 μM) or garcinol (5 μM) for 18 h. (B) The fluorescence intensity of AcEGFP-HS4 and AcEGFP-infected cells in the presence of histone acetyltransferase inhibitors curcumin or garcinol at 48 h p.i. The asterisks indicate a statistical difference between viruses ($^*p < 0.05$; $^{**}p < 0.01$).

3.6. The effect of HS4 was inhibited by histone acetyltransferase inhibitors

Histone acetyltransferase inhibitors (HATis) curcumin and garcinol were applied to virus-infected cells to determine that the effect of HS4 was associated with chromatin acetylation. The concentrations of curcumin and garcinol were chosen to be 25 μM and 5 μM, respectively, at which both the viability of Sf9 cells and the expression level of *egfp* by AcEGFP were only slightly affected (Fig. 7A and B). However, the *egfp* expression of AcEGFP-HS4 was significantly inhibited by 27% and 60% in the presence of curcumin and garcinol, respectively (Fig. 7B). This result, together with the results of DNase I sensitivity assay, strongly suggested that HS4 improved the expression of reporter genes by increasing the chromatin acetylation, which in turn caused the relaxation of local chromatin.

4. Discussion

In the current study, 5′-HS4 chicken β-globin insulator was found to significantly improve baculovirus-mediated foreign gene expression in insect cells. The effect of HS4 was independent of the target gene and host cells, since improvement in gene expression was seen for EGFP, Luciferase and SEAP, and in both Sf9 cells and High5 cells. Transcriptional analysis showed that HS4 increased mRNA of the target gene.

HS4 has a more dramatic effect on gene expression in the early phase of infection. The enhancement of EGFP expression was 6.7, 3.0 and 2.1-fold at 48, 72 and 96 h p.i., respectively. The enhancement of *egfp* mRNA was also higher at 48 h p.i. (3.2-fold) than at 72 h p.i. (1.7-fold). A similar phenomenon was seen for the expression of luciferase, where the enzyme activity was detected at 16 h p.i. in AcLUC-HS4-infected cells, while remaining undetectable until 24 h p.i. in AcLUC-infected cells (data not shown). Since the promoter used to drive the expression of these genes, polyhedrin promoter, is only active in the very late stage of infection when most viral proteins have been synthesized, the observation may imply that some viral proteins or virus-induced host factors that are required for the activity of polyhedrin promoter appear earlier or are no longer essential when HS4 was present.

Many regulatory sequences may up-regulate gene expression. These sequences include enhancer, anti-silencer, insulator, etc. It was shown that HS4 improved reporter gene expression in an orientation-specific manner in baculovirus, which was consistent with the finding of Hanawa et al. (2009) in lentivirus vectors. This indicated that HS4 was not an enhancer in baculovirus. It was also observed that *p78/83*, a viral gene downstream of *egfp*, was expressed at a slightly lower level in AcEGFP-HS4-infected cells than in AcEGFP-infected cells (data not shown), which further implied that HS4 was not an enhancer.

This study suggested that HS4 works as an insulator in the baculovirus genome as it does in vertebrate genomes. In vertebrate cells, histones adjacent to HS4 insulator were found to be highly acetylated (Litt et al., 2001). Generally, histone acetylation would lead to chromatin relaxation and high transcriptional activity (Davie and Chadee, 1998). The DNase I-sensitivity assay indicated that HS4 caused the relaxation of local viral DNA, while having little effect on distant DNA. This was consistent with the results using inhibitors of histone acetyltransferase, curcumin and garcinol. Curcumin is an inhibitor of histone acetyltransferase p300/CBP with little cell toxicity, while garcinol inhibits histone acetyltransferase p300 (IC_{50} = 7 μM) and PCAF (IC_{50} = 5 μM) both in vitro and in vivo (Balasubramanyam et al., 2004a,b; Chen et al., 2007). Both inhibitors strongly reduced *egfp* expression of AcEGFP-HS4, while having little effect on *egfp* expression of AcEGFP. These results strongly indicated that the improved gene expression by HS4 was associated with histone acetylation and chromatin relaxation.

The effect of sodium butyrate (NaBu) on baculovirus-mediated foreign gene expression in Sf9 cells was also studied (data not shown). NaBu is a histone deacetylase inhibitor that can increased gene expression in a wide range of cells. It was found to increase baculovirus-mediated gene expression by CMV promoter in both mammalian and insect cells (Sarkis et al., 2000; Peng et al., 2007; Wang et al., 2006). However, we observed that NaBu significantly inhibited *egfp* expression from polyhedrin promoter in AcMNPV-infected Sf9 cells (data not shown), which was consistent with the finding of Peng et al. (2007) using another very late promoter of baculovirus, p10. This might be due to the broad activation of viral and cellular genes by NaBu, which could interfere with the regulation of viral gene expression. Nevertheless, HS4 had a stronger effect on elevating *egfp* expression without NaBu than with it (data not shown). This might imply that HS4 has a similar effect as NaBu, which is to keep the viral genome surrounding the polyhedrin cassette in a high histone-acetylation and relaxation state, giving transcription factors access to viral promoters.

The genomes of many DNA viruses are known to be organized into chromatin in the nucleus, and subjected to various host epigenetic regulations (see review Lieberman, 2008). For example, Hepatitis B virus replication was regulated by the modification of histones binding to virus covalently closed circular DNA (Pollicino et al., 2006). There are only limited studies on the role of insulators in the replication and gene expression of viruses. An insulator-like sequence has been identified in herpes simplex virus 1 (HSV1), and found to act as an enhancer-blocker to maintain virus latency (Amelio et al., 2006). Heterogeneous insulators have been employed in the integrative lentivirus or adeno-associated virus (Ramezani et al., 2003; Inoue et al., 1999) and adenovirus vector (Steinwaerder and Lieber, 2000; Danielsson et al., 2008) to ensure the stable and efficient expression of transduced genes in gene therapy. However, little is known about the effect of insulators on replication and gene expression in a lytic virus, such as baculovirus. The finding that HS4 insulator significantly increased baculovirus-mediated foreign gene expression might be useful to further improve the baculovirus expression vector for protein production. It may also provide important information about the epigenetic regulation of baculovirus gene expression during virus replication.

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