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The Homologous Region 3 from *Bombyx mori* Nucleopolyhedrovirus Enhancing the Transcriptional Activity of *Drosophila hsp70* Promoter

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***Drosophila melanogaster* heat shock protein 70 gene promoter (D*hsp70p*) is widely used in transgenic insect to drive exogenous gene, and the homologous region 3 from *Bombyx mori* nucleopolyhedrovirus (BmNPV*hr3*) functions as an enhancer for several promoters. To test whether BmNPV*hr3* can enhance the D*hsp70p*s transcriptional activity, the reporter plasmids, which contain the D*hsp70p*, the reporter β-*galactosidase* gene with SV40 terminator and BmNPV*hr3* fragment, are constructed and transfected into the insect cell lines (Bm-N cells and Sf-21 cells) by lipofectin-mediated method. The results from the transient expression assay show that BmNPV*hr3* significantly increases transcriptional activity of D*hsp70p* both under the normal condition and under the heat-shock treatment, although the effects are significantly different between in Bm-N cells and in sf-21 cells. The enhancing behavior of BmNPV*hr3* on the D*hsp70p* is in an orientation-independent manner. Meanwhile, the effects of heat-shock treatment on D*hsp70p* alone or D*hsp70p*/BmNPV*hr3* combination present no significant difference, indicating that BmNPV*hr3* only enhances the transcriptional activity of D*hsp70p*, but cant alter its characteristic of the response to the heat-shock stress. The above results suggest that the D*hsp70p*/BmNPV*hr3* combination is more effective one to drive exogenous gene for transgene or stable cell expression system in insects.**

Key words: Heat shock protein 70 gene, Promoter, Enhancer, Insect cell line, BmNPV*hr3*, Transient expression

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Introduction

Heat shock protein-70 (Hsp70) belongs to the highly conserved protein class of chaperones (Lindquist *et al.*, 1988; Feder *et al.*, 1999). Heat shock proteins are involved in protecting cells from hyperthermic stress by binding to denatured protein and assisting in exact refolding (Beckmann *et al.*, 1990; Cotto *et al.*, 1999; Feder *et al.*, 1999). Under the normal condition, the expression of *hsp70* is kept at very low level, and increased sharply under the stress conditions, such as heat-shock (Kimura *et al.*, 1999; Uhlirova *et al.*, 2002), hypoxic (Ricchi *et al.*, 2001), chemicals (Hung *et al.*, 1998; Zhao *et al.*, 1999), hormone (Lacoste *et al.*, 2001) and electrical stress (Yanagida *et al.*, 2000) etc. Due to the characteristics of inducible ability and lack of organism- and tissue-specificity, *hsp70* promoter (*hsp70p*) is widely used in the transgene and gene therapy to drive exogenous gene (Uhlirova *et al.*, 2002; Schmidt *et al.*, 2004). In order to elevate its transcriptional activity for transgene and/or make it express specifically for gene therapy, many trials had been carried out *in vivo* and *in vitro* (Huynh *et al.*, 1999; Zhao *et al.*, 1999; Lacoste *et al.*, 2001; Schmidt *et al.*, 2004).

Baculovirus homologous regions (*hr*s) are repeated sequences interspersed in the genomes. Up to date, *hr*s have been identified in almost all of baculovirus, including *Bombyx mori* Nucleopolyhedrovirus (BmNPV). There are seven *hr*s in BmNPV T3 genome, that is *hr1*, *hr2L*, *hr2R*, *hr3*, *hr4 L*, *hr4 R* and *hr5* (Gomi *et al.*, 1999). Almost all of *hr*s plays an important role in viral DNA replication and functions as an enhancer for some viral and nonviral genes transcription (Lu *et al.*, 1997; Lo *et al.*, 2002; Viswanathan *et al.*, 2003). We cloned the *hr3* fragment from BmNPV ZJ8 genome (Genbank accession No

U51328) and find that it can function as an origin of viral DNA replication and increase the transcriptional activity of viral gene promoter, such as *helicase*, *gp64*, *ie-1*, *egt* (Zhang *et al.*, 1995; Xiao *et al.*, 2001; Zhou, 2002; Zhou *et al.*, 2003; Shen *et al.*, 2004).

The aim of the present study was to determine whether the BmNPV*hr3* could also enhance the *Drosophila hsp70ps* transcriptional activity using transient expression system in order to obtain a clue as to its possible application in the transgenic insect or stable cell expression system to increase the product of exogenous gene.

Materials and Methods

Bacterial, vectors, insect cells and reagents

E. coli TG1, JM109, vector Bluescript SK(M13-), pSK-BmNPV*hr3*, and pAcDZ1 containing a *LacZ* reporter gene with SV40 terminator driven by the *Drosophila hsp70* promoter (D*hsp70p*) were kindly provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Zuidema *et al.*, 1990; Lei *et al.*, 1994; Zhang *et al.*, 1995). The *B. mori* cell line (Bm-N) and *Spodoptera frugiperda* cell line (Sf-21) were maintained in the Key Laboratory of Silkworm Biotechnology, of the Ministry of Agriculture, China. The normalization plasmid pSK-*ie1-luc* was constructed previously in our laboratory (Zhou, 2002). Enzymes, insect cell culture medium TC-100, fetal bovine serum (FBS) and lipofectin were purchased from Invitrogen. E4030 kit for luciferase assay was obtained from Promega. Other reagents were from Sigma Chemical.

Construction of reporter plasmids

The DNA fragment, consisting of *Dhsp70p* and reporter -*galactosidase* gene with SV40 terminator, was obtained by *Bam*H I/*Xba* I double enzyme digestion from the plasmid pAcDZ1 DNA, then purified and subcloned into the plasmid vector pSK (pD*hsp70p-LacZ*). The BmNPV*hr3* fragment was from the pSK-BmNPV*hr3* plasmid DNA by *Pst* I enzyme digestion and subcloned into the downstream of SV40 terminator of pD*hsp70p-LacZ* with two directions. The insert directions of BmNPV*hr3* were identified by the *Eco*R I enzyme according to their positions in the BmNPV*hr3* fragment. The blank control plasmids (p*LacZ*) was also constructed by subcloning the *Hin*d III/*Bam*H I enzymes digested *LacZ* gene with SV40 terminator fragment from the plasmid pAcDZ1 DNA into the same enzymes digested plasmid vector pSK. The detail was shown in the Fig. 1. Preparation of reporter plasmid DNA, digestion, ligation and transformation were performed as the methods reported by Sambrook (1989).

Transient expression assay and heat-shock treatments

Cell culture was performed as the Summers and Smiths report (1987). The procedure of cell transfection was taken following our previous descriptions (Zhou, *et al.*, 2002; Tang, *et al.*, 2003). Insect cells (Bm-N cells or Sf-21 cells) were seeded into 12 cm^2 flasks with an approximately density of 5×10^5 cell/ml and cultured at 27°C over night. One μg reporter plasmid DNA and 0.5 μg normalization plasmid pSK-*ie1-luc* DNA were mixed with 7.5 μl lipofectin in a 50 μl total volume as transfection solution. After remove of primary medium and being washed twice with serum-free TC-100 medium, cells were transfected

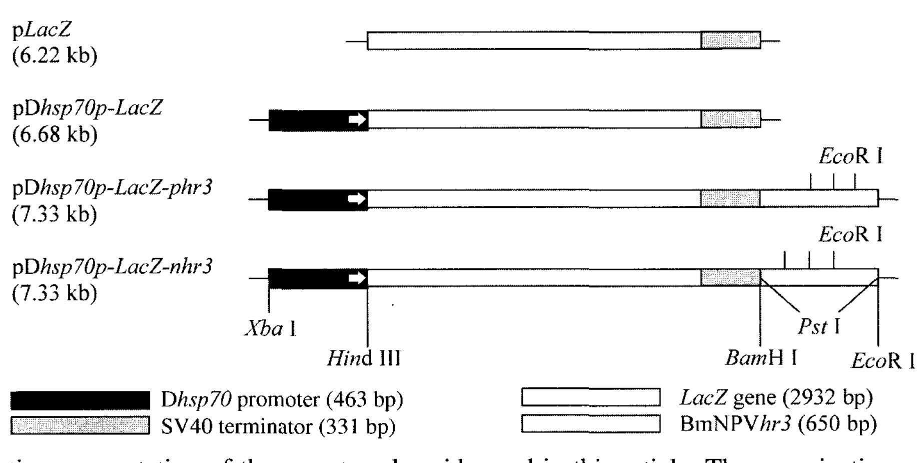

Fig. 1. Diagrammatic representation of the reporter plasmids used in this article. The organization of the reporter plasmids and the enzyme sites used in experiments were shown in the figure. The arms of the Figure were ligated with the pSK plasmid. The BmNPV*hr3* fragment harbors three *Eco*RI enzyme sites as shown in the Figure. The insertion directions of BmNPV*hr3* were identified by *Eco*RI digestion. pD*hsp70p-LacZ-phr3* was designated if the about 270 bp size fragment appeared, and pD*hsp70p-LacZ-nhr3* if this fragment didn't appear.

for 4 – 5 hrs. Then medium was replaced by 3 ml TC-100 medium containing 10% FBS and the hour(s)-post-transfection (hpt) started to be calculated at this moment. Cells transfected with p*LacZ* were taken as the blank control. For the heat-shock treatment experiments, the cells were shifted to 37°C incubator at 40 hpt and maintained for 2 hrs, then recovered under the normal temperature 27°C in certain times. The non-heat-shock treatment transfected cells were taken as the control. The cells were harvested at the required hpt as the text. Three replicates were done for each experiment.

Preparation of cell extracts and assay of β-galactosidase specific activity

Cells were harvested by centrifuging at 9,000 g for 5 min at 4°C. Cell extracts were prepared with a kit (E4030, Promega), and lysates were processed with a freeze-thaw cycle at 20°C and room temperature followed by centrifugation at 4°C to remove cell debris and supernatants were ready for β-galactosidase assay. The specific activity of the β-galactosidase was determined as described previously (Jeffrey, 1992), using a spectrophotometric assay with *o*-nitrophenol-β-D-galactopyranoside (ONPG) as substrate. The measurement of the luciferase activity from the normalization plasmid was undertaken upon a liquid scintillation spectrometer (Beckman LS600 series, USA) (Idahl *et al.*, 1986). The amount of total protein in the lysate was estimated using the Bradford method as described (Moos, 1995). The data were analyzed using Statistical Analysis System (SAS).

Results

The transcriptional activity of D*hsp70p* enhanced by BmNPV*hr3*

The insect cells (Bm-N and Sf-21) were transfected with the reporter plasmids DNA pD*hsp70p-LacZ*, pD*hsp70p-LacZ-phr3*, and pD*hsp70p-LacZ-nhr3*, respectively. The cells were harvested at 48 hrs post transfection (hpt). Their specific activities of the β-galactosidase were 105.91, 633.16 and 624.55 in Bm-N cells, and 49.68, 859.46 and 874.27 (unit/mg protein) in Sf-21 cells, respectively (Table 1). The results showed that the D*hsp70ps* transcriptional activity presented higher in Bm-N cells than that in Sf-21 cells, although both of them were at detectable level under the normal condition. But they were enhanced significantly by about 6 times in Bm-N cells and about 17 times in Sf-21 cells from the insertion of BmNPV*hr3*. The enhancement effect of BmNPV*hr3* on D*hsp70ps* in Sf-21 cells was higher than that in Bm-N cells. This maybe resulted from the basal transcriptional activity was lower in Sf-21 cells. The similar results were also found in our previous reports (Xiao *et al.*, 2001; Zhou *et al.*, 2003).

The enhancement effects of two insertion directions of BmNPV*hr3* on the transcriptional activity of D*hsp70p* showed no significant differences in Bm-N cells (F value = 0.05, Pr > F = 0.8271 >> 0.05) and in Sf-21 cells (F value = 0.29, Pr > F = 0.6180 >> 0.05). This indicated that the enhancing behavior of BmNPV*hr3* on the transcriptional activity of D*hsp70p* was in an orientation - independent manner.

The effects of heat-shock treatment on the transcriptional activity of D*hsp70p* and D*hsp70p*/BmNPV*hr3* combination

The lipofectin-embedded reporter plasmids DNA, pD*hsp70p-LacZ* or pD*hsp70p-LacZ-phr3*, transfected into insect cells lines. At 40 hpt, the transfected cells were moved to 37°C incubator and maintained for 2 hrs, then recovered in the normal temperature 27°C for 6 hrs. The cells were harvested at 48 hpt. The non-heat-shock treatment cells were taken as the control. Under the heat-shock treatment, D*hsp70p* transcriptional activity was increased by 2.17 ± 0.07 times in Bm-N cells and 3.45 ± 0.81 times in Sf-21 cells, while D*hsp70p*/BmNPV*hr3* combinations transcriptional activity was increased by 2.14 ± 0.17 times and 3.23 ± 0.19 times, respectively (Fig. 2). It showed that the heat-shock treatment had significant enhancement effects on the transcriptional activities of both D*hsp70p* (F value = 20.13, Pr > F = 0.0012 < 0.01) and D*hsp70p*/BmNPV*hr3* combination (F value = 18.08, Pr > F = 0.0017 < 0.01). Moreover, the enhancements for them are

Table 1. Effects of BmNPV*hr3* and its insertion direction on the transcriptional activity of D*hsp70* promoter

Insect cell lines	Bm-N			Sf-21		
Repoter plasmids	1	2	3	1	2	3
Specific activity of the β-galactosidase (unit/mg)	105.91 ± 17.53	633.16 ± 60.21	624.55 ± 23.94	49.68 ± 20.68	859.46 ± 45.28	874.27 ± 17.16

Note: The reporter plasmids 1, 2 and 3 represented as pD*hsp70p-LacZ*, pD*hsp70p-LacZ-phr3* and pD*hsp70p-LacZ-nhr3*, respectively. The luciferase normalizing system was introduced into each transfection. The p*LacZ* transfected cells were taken as the blank. The data represented the mean ± S.D. from triplicate samples in three separate transfections.

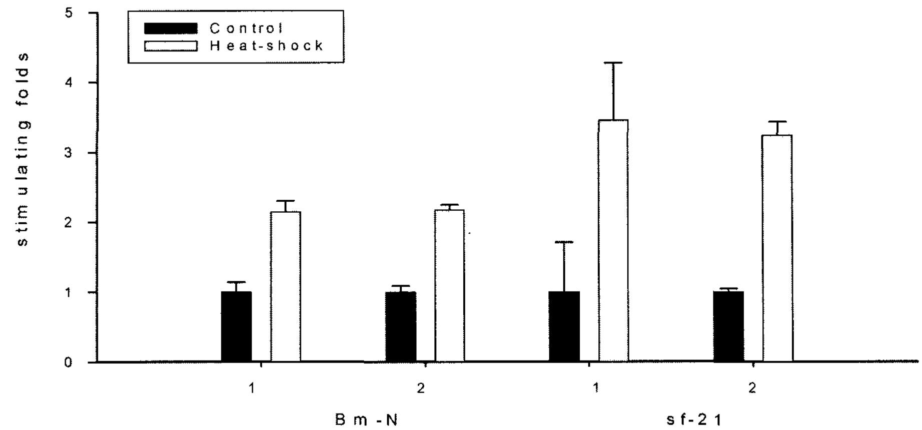

Fig. 2. The effects of heat-shock treament on the transcriptional activities of D*hsp70* promoter and D*hsp70*p/BmNPV*hr3* combination. 1, 2 on X-axis represented as pD*hsp70p-LacZ*, pD*hsp70p-LacZ-phr3*, respectively. The methods of heat-shock treatment were described in Materials and Methods. The increments of specific activity of the β-galactosidase is indicated on Y axis as stimulating folds over the cells without heat-shock treatment which was arbitrarily set at 1.00. The luciferase normalizing system was introduced into each transfection. The p*LacZ* transfected cells served as the blank control. The results represented the mean ± S. D. (error bars) from triplicate samples in three separate transfections.

almost identical (F value = 0.05, Pr > F = 0.8359 >> 0.05), suggesting that the BmNPV*hr3* has no effect on the characteristics of D*hsp70p* on the response to heat-shock stress.

Discussions

In the baculorvirus-infected Sf cells, the *LacZ* gene driven by the D*hsp70p* expressed constitutively (Zuidema *et al.*, 1990). At present study, the D*hsp70p* showed a certain level transcriptional activity under the normal conditions. This maybe resulted from general stresses from transfection and harvesting, such as the treatments of refreshment of medium, the chemicals (including lipofectin, antibacterial materials in the medium etc.) during transfection and chilling PBS and lysate buffer during harvesting. The identical results were also observed in cultured mosquito cells (Zhao *et al.*, 1999) and in *Aedes albopictus* C6/36 cells (Huynh *et al.*, 1999). Thus, the effects of heat-shock treatment on the D*hsp70p*s transcriptional activity presented quite weaker in transient expression *in vitro* here than that of *in vivo* (Kimura *et al.*, 1999; Uhlirova *et al.*, 2002).

We previously reported that the BmNPV*hr3* could increase the transcriptional activity of viral gene promoters, including *helicase*, *gp64*, *ie-1*, *egt* (Xiao *et al.*, 2001; Zhou, 2002; Zhou *et al.*, 2003; Shen *et al.*, 2004). The enhancement effects varied from 100- to 7740-fold in insect cells or in silkworms. At the present study, it was only about 6-fold in Bm-N cells and 17-fold in Sf-21 cells. It maybe due to the BmNPV*hr3* has higher enhancement ability for NPV-derived promoters than that of D*hsp70p*. As for the enhancement effects of BmNPV*hr3* on D*hsp70*s were better in Sf-21 cells than in Bm-N cells, it might result from the fact that the basal transcriptional activity in Sf-21 cells is much lower and the enhancing behavior of BmNPV*hr3* is related with different cell lines factors (Zhou *et al.*, 2003).

When combined with heat-shock stress, the enhancement effects of BmNPV*hr3* on the transcriptional activity of D*hsp70p* were about 10 ~ 50 folds in insect cells, compared with that of D*hsp70p* alone under the normal conditions. The above results showed that the BmNPV*hr3* gave significant increment on the D*hsp70p*s transcriptional activity in insect cells and couldnt change its characteristic of the response to the heat-shock stress, suggesting that D*hsp70p*/BmNPV*hr3* combination has a promising future to drive exogenous gene for transgene or stable cell expression system in insect. Recently, Viswanathan *et al.* (2003) reported that AcMNPV*hr1* can enhance the transcriptional activity of D*hsp70p* in mammalian cells. We are underway to test whether BmNPV*hr3* also has this characteristics. If so, the application fields for the combination of D*hsp70p*/BmNPV*hr3* will be widen.

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