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HBeAg gene expression with baculovirus vector in silk worm cells

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Subject headings hepatitis B virus; HBeAg BmNPV vector; gene expression; DNA, viral

INTRODUCTION

Miyanohara *et al*[1] first obtained products with the expression of HBeAg activity by constructing a yeast expression system; later researches discovered HBeAg expression in xeropus oocytes[2], COS cells [3], *E. coli* cells[4], Bacillus subtilis[5], which allow us to know more certainly about HBeAg gene. HBeAg has the same 149 amino acid sequence as the amino-terminal of HBcAg which is encoded by c-gene and consist s of 183 amid acids. The c-gene has a 89bp pre-csequence in the upstream of it. The co-expressed product of the two genes (pre-c protein) is cleaved off the amino-terminal signal peptide sequence and the c-terminal alkaline region by hydrolases in the membrane of endoblasmic reticulum, forming the secretable HBeAg[6]. The most important thing is that the signal peptide encoded by pre-c region directs the formation and secretion of HBeAg, suggesting that only by eukaryotic expression systems can we produce HBeAg with high purity and activity. The domestic HBeAg/anti-HBe diagnostic kit is produced in *E. coli* cells, containing a high proportion of HBeAg which affects the quality of the kit. Recently, the technique with baculovirus vector to express foreign gene efficiently in worm cells and body has been applied and popularized[7]. We have replaced the polyhedron protein gene encoding sequence with human INF-α in Bombyx morinuclear poly-hedrosis virus(*Bm* NPV), suggesting that silk worm cells can recognize the signal peptide of human INF-α gene and cut correctly[8]. Ninety-nine percent protein becomes mature only after the secreting stage, the *Bm* NPV-*Bm* N will bea good expression system. For this purpose, we amplified the pre-csignal peptide sequence and the same 149 amino acids sequence homologous with HBcAg at the N-end by PCR, and added appropriate restriction endonuclease sites on both 5' and 3' ends, cloned it into-Bm-NPV transfer vector pBmo30, the Bm-N cells were co-transfected by p-Bm-HBe and wild-type Bm-NPV DNA, and at length the recombinant virus with high expression HBeAg were efficiently obtainable after plaque purification.

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MATERIALS AND METHODS

Viruses and vectors

Bm NPV transfer vector pBmo30 and silk worm cells were supplied by Virus Rese arch Institute of Wuhan University. The cells were cultured in Tc-100 (containing 100 mL/L-fetal calf serum) and then stored frozen. HBeAg gene was generated by PCR from the template DNA obtained from the HBV Library of the Virus Research Institute of Wuhan University[9].

DNA extraction and fragment recollection

The transfer vector pBmo30 and recombinant vector p*Bm* HBe DNA were extracted from *E. coli* cells by ordinary method. The silk worm cells were infected by *Bm*NPV DNA, then cultured for 5-7 days at 27℃, centrifuged at low speed when nuclear polyhedrons emerged. The supernatants containing virus particles were harvested to infect *Bm* N cells again, and the cells were cultured, observed as before, and centrifuged to maintain the cells and supernatants. The *Bm* N DNA was extracted from polyhedrons and viruses according to Summers program. The amplified fragments and enzyme-excised fragments were subjected to 7 g/L-10 g/L agarose gel electrophoresis respectively, and recovered by DE81 membrane method[10].

PCR amplification

At the 3' and 5' ends of the HBV e gene, we took artificially synthesized 30bp sequence as the primers. *Bgl*-II locus was added to the (+) 5' end of the primer, and *xba*-I-TAA-*Sma*-I locus to (-) 5' end of the primer.

Primer 1(+): 5'AGATCTCATGGAACTTT-TTACCTCTGCCT 3'

Primer 2(-): 5'CCCGGGTTATCTAGAAA-CAACAGTAGTTTCCGGAA3'

PCR reaction was performed from the template PHB24 plasmid containing entire gen ic HBV and the above primers. PCR product was subjected to 7g/L-agarosegel electrophoresis.

DNA sequence analysis

DNA sequence was analyzed to identify the amplified fragment through ddNTP/PCR/silver-stained sequence analysis system. PCR amplification was performed under the template DNA of purified 537bp fragment, with the same primers, and under the presence of one type ddNTP according to silver-stained sequence a nalysis protocol. The samples were subjected to the 80 g/L PAG gel electrophoresis, then fixed, stained and colorized and the DNA sequence was read up.

Clone ligation and transformation

The plasmid p*Bm* DNA and PCR fragment were digested respectively by *Bgl* II and *Sma* I, ligated, then transfected into competent *E. coli* cells. The resistance colonies were selected from ApILB plates. *Bgl* II and *Sma* I digested the extracted recombinant DNA, the DNA samples were subjected to 100 g/L-agarose gel electrophoresis to identify the positive recombinant.

BmN cells co-transfected by transfer vector DNA and wild-type BmNPV DNA

The extracted recombinant transfer vector DNA and wt *Bm* NPVDNA were mixed by 5:1 molar ratio, and then co-transfected the fresh growing well wall-adhering-*Bm*-N cells, through the mediation of liperfectin as previously described. Two hours later the medium was removed, and TC-100 (containing 100 mL/L- fetal calf serum) was added and the cells were cultured for 7 -10 days at 27℃. Cells containing recombinant viruses were chosen with plaquepurification on agars plates. Those plaques of 0^- (occlusion) phenotype without polyhedrons, which were the positive recombinant viruses, were selected.

HBeAg expression and determination

Bm N cells were infected by recombinant viruses, cultured for 4 days at 27℃, centrifuged to get cells and supernatants, a 50 g/L SDS-PAG electrophor esis was performed as general method, stained with Cormassie blue, and the prote in expression was observed.

Cells were lysed with guanidine hydrochloride to rupture cell membrane and centr ifuged to get supernatants. Anti-HBe/HBcAg kit from Medicine Research Institute of Nanjin was used to perform ELISA, separately by using the HBeAg-positive serum of HBV patients and HBcAg generated from engineered bacteria as positive controls, and by using reptured -*Bm*-N cell medium containing normal receptor and cultured supernatant as negative controls. P, N values were calculated on the basis of OD value (A), P/N $\geqslant$2 was considered as positive.

Purification of HBeAg expressed in Bm N cells

Cell culture supernatants were collected, precipitated by 27% ammonium sulfate, and then dissolved by PBS (0.02 mol/L-PB, pH 7.0, 0.03 mol/L- NaCl). After separation from pre-balanced sephacryls-200 (1 cm×100 cm) column, electrophoresis and ELISA detection were performed. The peak HBeAg-activity was captured. After gradient elution through DEAE-Sepharose FF ion exchange column, detection of HBeAg activity and SDS-PAG electrophoresis were performed. The general pressured liquid chromatographic system used was from Pharmacia Co.

HBeAg expressed in silk worm cells used in conjunction with anti-Hbe antibody in ELISA kit

The purified HBeAg expressed in silk worm cells was used to coat the enzyme labeled reaction plate (100 ng/well), incubated throughout the night at 4℃, then serum to be detected was added to it, and after 30 min at 40℃, the sametype of HBeAg was added to it. At last, TMB H_2O_2 was used to colorize it. Those P/N $\geqslant$2.1 were positive.

RESULTS

PCR amplification and sequence analysis of HBeAg gene

A series of PCRs were performed with the synthesized primers and plasmid PHB24 a s template DNA; each PCR generated a fragment about 0.5 kb that was homolo gous with the HBeAg gene, within the 361 bp sequence from 5'end analyzed except one site (the 375, T→A), by using ddNTP/PCR/silver staining. The 88 bp sequence from 273-361 of the amplified fragment was identified as HBeAg gene (Figure 1). The amplified HBeAg gene was 537 bp from the 5'signal peptide sequence. We designed *Bgl*-II site at 5'end and *Xba*-I, *Sma*-I si tes at 3'end for cloning.

TGG GGG GAA TTG ATG ACT
CTA GCT ACC TGG GTG GGT
AAT AAT TTG GAA GAT CCA
GCA TCT AGG GAT CTT GTA
GTA AAT TAT GTT AAT ACT

Figure 1 Partial sequence of HBeAg amplified by PCR.

Construction and identification of the inserted vectors carrying HBeAg gene

The *Bm* NPV transfer vector p*Bm* 030 was 6.3 kb, containing polycloning site. p*Bm* 030 DNA and the amplified fragment by PCR was digested by *Bgl*-II/*Sam*-I respectively, ligated by T4 DNA ligase, and allowed the e gene to be inserted into the ploycloning site under the control of plh promoter. The constructive processes was shown in Figure 2. The ligated DNA was transferred into *E. coli* cells, and positive colonies were selected. *Bgl*-II/*Sma*-I were used to digest the recombinant vector, and a fragment of 0.5 kb was obtained on agarose gel electrophoresis, indicating that HBeAg gene cloning was successful. Constructed recombinant viruses carried HBeAg gene.

BmN cells were co-transfected by the transfer vector pBm HBe DNA and wt-BmNPV DNA

Polyhedrosis observed in most cells was the signal of successful co-transfection. Other cells turned to have pathologic characteristics of infection, such asenlargement of cells and their nuclei, condentation of intracellular contents, and irregular granules. Polyhedrosis was not the typical characters of infection in recombinant viruses. So, after co-transfection gene recombination has completed between both plh gene on the 3', 5' ends of p*Bm* Be DNA and the homol ogous gene of the wt *Bm* NPV plh gene. The plh gene was exchanged for the Pplh/HBe gene expression box, as controlled by plh promoter. The recombinant virus by plaque-purification was named r-*Bm*-HBe (Figure 3).

Expression and detection of HBeAg in silk worm cells

The silk worm cells were infected by recombinant virus r*Bm*HBe, cultured for 72 hours. And the cells and supernatants were harvested for SDS-PAG electrophoresis (Figure 4). The wt *Bm* NPV could produce polyhedron protein (M_r 32 000), while the recombinant virus r*Bm* HBe produced HBeAg about M_r 18 000 instead of polyhedrosis because of the exchange of plh gene. The expression of HBeAg was also observed in the cultured medium, but with smaller molecular weight. So most of the expressed HBeAg was secreted out of cells induced by the signal peptide at N-end. ELISA was performed on culture cell lysate and cell culture supernatant to detect the activity of the expressed HBeAg (Table 1). A positive reaction can be found when the culture cell lysate was diluted 1 : 2 000. The antigenic activity of the culture supernatant was much higher, reaching a dilution of 1 : 32 000. Also no HBcAg was detected in the cell culture supernatant, and HBcAg in culture cell lysate was detectable only at a dilution lower than 1 : 160. The above results definitly proved that HBeAg antige nicity was expressed in silk worm cells.

Table 1 HBeAg antigenicity detection with ELISA

Sample	Dilution						
	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
Cell culture medium	+	+	+	+	+	+	-
Culture cell lysate	+	+	-	-	-	-	-

Detection of anti-HBe antibody by double antibody sandwich method

The HBeAg expressed in silk worm cells was used to coat the enzymelabelled plate, then anti-HBe antibodies in samples were detected. The results were showed in Table 2.

Table 2 Comparison of two methods in anti-HBe antibody detection

Conventional method	*n*	Positive (*n*)	
		Double sandwich method[a]	Indirect[b]
Anti-HBe(+) and anti-HBc(-)	35	35	35
Anti-HBe(-) and anti-HBc(+)	52	4	52
Anti-HBe(+) and anti-HBc(+)	37	37	37
Anti-HBe(-) and anti-HBc(-)	42	2	0

[a]The purified HBeAg expressed in karyotic cells was used to coat enzyme-labeled reaction plate (100 ng / well), incubated throughout the night at 4℃, and then serum to be detected was added to it. After 30 min at 40℃, HRP-Labeled HBeAg expressed in karyotic cells was added to it, and at last this was colorized by application of TMB-H_2O_2. Those P / N ⩾2.1 were positive.

[b]The anti-HBe antibody detection kit for sale in market employed the competition inhibition method.

Purification of HBeAg

Sephacryls-200 chromatography was performed firstly, and showed five protein peaks. ELISA detection indicated that most HBeAg existed in the fourth peak fraction. After concentration, DEAE-Sepharose FF chromatography was performed to get purified HBeAg. The recovery rate was about 52% (Table 3).

Table 3 Purification of HBeAg Produced by Bm N cells

Method	Total activity (by ELISA)	Specific activity (per mg protein)	Purification factor		Total recovery (%)
			A	B	
Cell culture medium	$1:1.60\times10^6$	$1:2.9\times10^5$	1.0	/	/
$(NH4)_2SO_4$	$1:1.32\times10^6$	$1:8.5\times10^5$	2.9	2.9	80
Sephacryl S-200	$1:1.12\times10^6$	$1:5.3\times10^4$	6.3	18.3	70
DEAE Sepharose FF	$1:8.40\times10^5$	$1:5.0\times10^5$	9.4	172.0	52

A: Individual steps; B: Accumulated results.

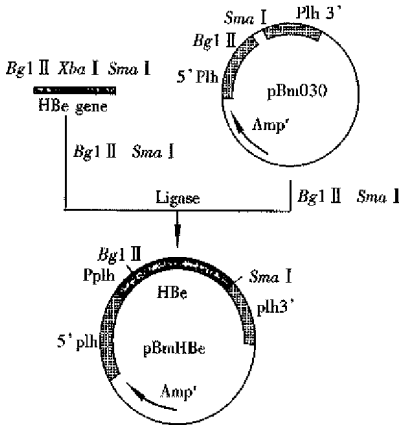

Figure 2 Construction of recombinant vector p*Bm* HBe.

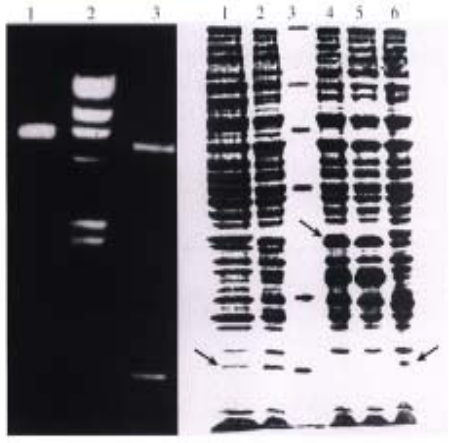

Figure 3 Restriction endonuclease analysis plasmid p*Bm* HBe (Left).
1. p *Bm* HBe / *Bgl* II, linerization; 2. λ DNA / Hind III marker; 3. p Bm Hbe / *Bg*-II + *Sma* 1 I, released 0.54 kb HBeAg gene.
Figure 4 PAG Electrophoresis analysis of HBeAg prote in expressed in *Bm* N cells(Right). 1 and 2: *Bm* N cells infected withr *Bm*-HBe, the arrow shows HBeAg protein, M_r18 000; 3: Standard protein molecular weight marker, M_r94 000,17 000,43 000,20 000, 17 500; 4 and 5: *Bm*- N cells infected with wt *Bm* NPV, the arrow shows M_r32 000 polyhe dea protein; 6: Culture supernatant, the arrow shows HBeAg protein.

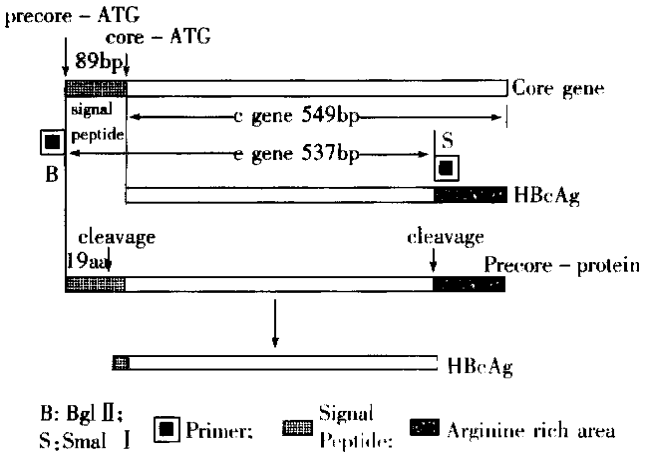

Figure 5 Structure of HBeAg gene and PCR design.

Characteristics of HBeAg expressed in silk worm cells as compa red with those in E. coli cells

The purified HBeAg expressed in silk worm cells and E. coli-cells was adjusted to a protein concentration of 2 g/L-each, then detected by HBeAg detection kit and detection anti-HBe antibody kit sallied in market (Table 4). Those values indicated in Table 4 were dilution magnitude; the P/N $\geqslant$ 2.1 wasused to determine the end point.

Table 4 Characteristics of HBeAg expressed in *E. coli* cells as compared with those in karyotic cells with the double-antibody Sandwich

Antigen	Cell	HBeAg kit	Anti-HBc kit
HBeAg 1	karyotic	10-7	10-1
HBeAg 2	*E. coli*	10-5	10-3

DISCUSSION

We constructed a transfer vector pBm HBe with HBeAg gene fragment from PCR. This vector was used together with wt Bm NPV to co-transfect the silk worm cells, and the recombinant viruses carrying HBeAg gene was obtained. The amplified HBeAg gene was 537 bp long, including an 89 bp sequence of signal peptide from 5'-end to 3'-end of e gene. The relation between c gene and it was shown in Figure 5. While HBeAg was expressed in silk worm cells, the signal peptide was clipped off in granular endoplasmic reticulum, and 19 amino acids were lost with the HBeAg secreting into cell cultured medium. And some antigens in non-secretary form also existed in cells. On the basis of gene sequence analysis, an ATG was found to locate at both 5'-and 3'-ends of the signal peptide, and the second ATG obeyed the Kozak rule completely. When 40s subgroup of ribosome was scanned to the first AUG codon, some of the 40s submits and 60s submits would fit into ribosomes, begin to transcribe and produce protein carrying signalpeptide, which was processed and secreted out of the cells as soluble HBeAg. The HBeAg reaction rate was 100-fold higher, and the anti-HBc cross reaction rate was 100-fold lower, compared with the reaction using expression by prokaryotic cell in the same concentration of protein, because prokaryotic cell system did not differentiate and cut message peptide sequence. The products expressed were cellular c antigen (Table 4).

The other 40s subgroup continued to scan until the second AUG, combined with the 60s subgroup and began to transcribe, and produce protein without signal peptid e, which remained inside of

the cells. Such a result was consistent with the the orctic basis of the initial regulation of mRNA transcription[2]. The exp ressed product inside cells should be HBcAg according to the c gene sequence ana lysis, but ELISA results proved it was HBeAg (1:2 000) mostly, with little HBcAg (<1:160). So the phenomenon suggested that the arginine abundant region at the carboxyl end should be very important for HBcAg expression; it took part in the self-fitting into core particle of HBcAg protein. The amplified e gene fragment did not contain the arginine abundant region, so its expressed product contained little HBcAg.

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