HBeAg gene expression with baculovirus vector in silk worm cells

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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 consists 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 N will be a good expression system. For this purpose, we amplified the pre-c signal 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-NPВ transfеr vector pBmo30, the Bm-N cells were co-transfected by p-Bm-HBe and wild-type Bm-NPВ DNA, and at length the recombinant virus with high expression HBeAg were efficiently obtainable after plaque purification.

MATERIALS AND METHODS

Viruses and vectors
Bm NPV transfer vector pBmo30 and silk worm cells were supplied by Virus Research 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 pBm HBE DNA were extracted from E. coli cells by ordinary method. The silk worm cells were infected by BmNPВ DNA, then cultured for 5-7 days at 27°C, 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 7g/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’AGATCTCATGGAACTTTTTACCTCTGCT 3’
Primer 2(-): 5’CCCGGGTTATCTAGAAA-
CAACAGTAGTTTCCGGAA 3’

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 pHm 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 ApIB 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°C. 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°C, 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/HBeAg kit from Medicine
Research Institute of Nanjin was used to perform
ELISA, separately by using the HBeAg-positive
serum of HBV patients and HBeAg generated
from engineered bacteria as positive controls, and
by using ruptured 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 ≥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
sphacryls-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°C, then serum to be detected was added
to it, and after 30 min at 40°C, the sametype of
HBeAg was added to it. At last, TMB H2O2 was
used to colorize it. Those P/N ≥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 as 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
sites at 3’end for cloning.

Figure 1 Partial sequence of HBeAg amplified by PCR.
Construction and identification of the inserted vectors carrying HBeAg gene

The Bm NPV transfer vector pBm 030 was 6.3 kb, containing polycloning site, pBm 030 DNA and the amplified fragment by PCR was digested by Bgl-II/Sma-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, condensation 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 pBm Be DNA and the homol ogous gene of the wt Bm NPV plh gene. The plh gene was exchanged for the plh/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 rBmHBe, 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, 32 000), while the recombinant virus rBm HBe produced HBeAg about M, 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 HBeAg 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 | Positive (n) | Double sandwich method $^a$ | Indirect $^b$ |
|---------------------------|----------|--------------|-----------------------------|--------------|
| Cell culture medium       |          |              |                             |              |
| Culture cell lysate       | +        | +            | +                           | -            |

$^a$The purified HBeAg expressed in karyotic cells was used to coat enzyme-labeled reaction plate (100 ng/well), incubated throughout the night at 4°C, then serum to be detected was added to it. After 30 min at 40°C, HRP-Labeled HBeAg expressed in karyotic cells was added to it, and at last this was colorized by application of TMB-H2O2. Those P/N $\geq 2.1$ were positive.

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

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

| Sample                  | Dilution | Positive (n) | Double sandwich method $^a$ | Indirect $^b$ |
|-------------------------|----------|--------------|-----------------------------|--------------|
| Cell culture medium     |          |              |                             |              |
| Culture cell lysate     | +        | +            | +                           | -            |

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 (mg protein) | Specific activity (μg/mg protein) | Purification factor | Total recovery (%) |
|-------------------------------|-----------------------------|----------------------------------|--------------------|--------------------|
| Cell culture medium           | 1.16 x 10$^4$              | 1.9 x 10$^5$                     | 1.0                | 1.0                |
| (NH4)2SO$_4$                  | 1.32 x 10$^4$              | 1.8 x 10$^5$                     | 2.9                | 2.9                |
| Sephacryl S-200               | 1.12 x 10$^4$              | 1.5 x 10$^5$                     | 6.3                | 18.3               |
| DEAE Sepharose FF             | 8.40 x 10$^4$              | 1.5 x 10$^5$                     | 9.4                | 172.0              |

A: Individual steps; B: Accumulated results.
Characteristics of HBeAg expressed in silk worm cells as compared 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 ≥ 2.1 was used 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 signal peptide, 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 peptide, 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 expressed product inside cells should be HBcAg according to the c gene sequence analysis, 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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