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Influence of Nucleotide Sequences Related to the 5'-untranslated Leader Region of Polyhedrin mRNA on the Expression of HBsAg in Insect Cells

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Nai-Yueh Tien, Chu-Fang Lo, Pei-Lain Yang, Ting-Ting Wu, Ming-Der Yang, Chung-Hsiung Wang, Chao-Hung Lee, Malcolm J. Fraser and Guang-Hsiung Kou (1994) Influence of nucleotide sequences related to the 5'-untranslated leader region of polyhedrin mRNA on the expression of HBsAg in insect cells. *Zoological Studies* **33**(2): 140-152. A series of new baculovirus transfer vectors, under the control of the polyhedrin promoter for foreign gene expression, was constructed and utilized to successfully express the HBsAg gene in insect cells. The antigenicity of expressed HBsAg was confirmed by Western blot and immunoprecipitation. Electron microscope was used to examine the morphology of 22-nm lipoprotein particles. Comparison of the levels of nonfused HBsAg proteins expressed by three differently constructed recombinant vectors was made using SDS-PAGE, quantitative analysis of enzyme-immunoassay, and RNA dot blot analysis of total cytoplasmic RNA. The results suggested, the nucleotide sequences related to the 5'-untranslated leader region of polyhedrin mRNA were important to HBsAg expression, and the HBsAg synthesis in insect cells driven by the polyhedrin promoter do not appear to follow the Kozak rule.

Key words: AcMNPV, Baculovirus, Transfer vector.

The baculovirus expression vector system (BEVS), developed by Smith et al. (Smith et al. 1983a,b), has been utilized for the expression of a wide variety of heterologous genes from prokaryotes and eukaryotes (Luckow and Summers 1988a, O'Reilly et al. 1992). In this system, the foreign genes are controlled by the strong polyhedrin promoter of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and expressed in insect, *Spodoptera frugiperda*, cells. The cells appear to provide a suitable environment for proper post-translational modifications including glycosylation, acylation, amidation, phosphorylation, signal-peptide cleavage, and proper folding. It was documented that the recombinant proteins expressed by BEVS have antigenicity and biological activities similar to their authentic counterparts (Luckow and Summers 1988a, Miller 1989).

A variety of baculovirus transfer vectors were constructed for expression of different foreign genes and also for investigation of the expression regulation mechanisms in insect cells (Luckow and Summers 1988a,b 1989, O'Reilly et al. 1992). The factors influencing foreign gene expression may be: codon preference, cell processing pathways, RNA stability, or protein processing, transport, targeting, and stability (related to intrinsic properties of the foreign gene.) Although these factors have not been thoroughly examined, some data have shown that the sequences immediately

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upstream from the polyhedrin initiation codon are important to transcription regulation (Lanford et al. 1988). Also reported is the dramatic influence of sequences near the ATG initiation codon on translation levels (Luckow and Summers 1988b). However, in order to elucidate the importance of these sequences on the gene expression level further research is requisite.

In our study, new AcMNPV transfer vectors, different from known vectors in that they have multiple cloning sites derived from pUC19 for foreign gene insertion, were constructed using exonuclease III and exonuclease VII deletion as well as the polymerase chain reaction (PCR) method. These vectors have been utilized to successfully express several foreign proteins, such as HBsAg, HTLV-1 envelope protein, and fish growth hormone in our laboratory. In this paper, the AcMNPV transfer vectors construction scheme is described. Using these constructed vectors, HBsAg synthesis was studied in order to understand the influence of nucleotide sequences related to the 5'-untranslated leader region of polyhedrin mRNA on the HBsAg expression level in insect cells, and also to determine whether or not the HBsAg synthesis in the baculovirus expression vector system is based on the Kozak rule (Kozak 1986 1989). Our results demonstrated that the nucleotide sequences related to the 5'-untranslated leader region of polyhedrin mRNA were important for HBsAg expression. Furthermore, the HBsAg synthesis in insect cells driven by the polyhedrin promoter did not appear to follow the Kozak rule.

MATERIALS AND METHODS

Cells and viruses

AcMNPV (E2 strain) and recombinant virus stocks were cultured and assayed in confluent *Spodoptera frugiperda* cells monolayers (IPLB-Sf 21-AE) (Vaughn et al. 1977) in TNM-FH medium supplemented with a 10% fetal calf serum (FCS) at 28°C.

Construction of AcMNPV transfer vectors library, pBSdHHd

The p18BSd library was assembled as follows: p18BS was first constructed by digesting *Sph*I/*Bam*HI fragment from AcMNPV *Eco*RI-I fragment and inserting it into pUC18. The p18BS contained a 5'-flanking region, polyhedrin promoter and a portion of the 5'-AcMNPV polyhedrin gene (176 bp). Supercoiled form of p18BS was digested completely by *Bam*HI and *Sst*I, and precipitated with an equal volume of isopropanol for enzyme and reaction buffer removal. Seven eppendorf tubes each containing 2 μg linear DNA (p18/*Bam*HI & *Sst*I), 4 μl 10X exonuclease (*Exo* III) buffer and ddH_2O measuring 40 μl in volume were prepared. They were placed in a 37°C water bath and 8 units of *Exo* III (40U/μl) were added to each tube. *Exo* III reaction time was set at ten second intervals beginning at 60 and continuing to 120 seconds. Following each reaction time, each tube was placed in an ice box to halt the *Exo* III reaction (Guo and Wu, 1983). Immediately following this process, a 40 μl 10X exonuclease VII (*Exo* VII) buffer and 0.2 units of *Exo* VII (2U/μl) were added to the seven tubes, and they were returned to a 37°C water bath for 45 mins. The *Exo* VII was inactivated at 70°C for 15 mins(Yanisch-Perron, 1985). Then the Klenow fragment of DNA polymerase (10U/2μg DNA), 0.2M $MgCl_2$, and free nucleotides were added to the above solution for filling-in. These truncated linear DNAs were precipitated with an equal volume of isopropanol to remove enzymes and buffers, then ligated with T4 DNA ligase and transformed into *E. coli* TB-1 strain completing the p18BSd library.

p19HHd library was constructed as follows: the *Bam*HI fragment (1950 bp) digested from AcMNPV *Eco*RI-I fragment was inserted into pUC19 to construct a recombinant plasmid, p19BB. The p19BB was digested with *Pst*I and *Xba*I and treatedin following the same process used for the p18BSd library. As per Fig. 1, p19BBd was digested with *Hind*III and inserted into pUC19 to establish the p19HHd library.

Using *Nde*I and *Eco*RI digestion, and T4 DNA ligase ligation, the random composition of p18BSd and p19HHd libraries yielded a series of different AcMNPV transfer vectors which were designated as the pBSdHHd transfer vectors library. A dsDNA plasmid sequencing analysis was carried out to determine the polyhedrin promoter sequences in these vectors (USB, Sequenase version 2.0 sequencing kit).

Construction of AcMNPV expression vectors containing the foreign gene HBsAg

An AcMNPV transfer vector, termed pBSd104 HHd113, was chosen from the pBSdHHd transfer vectors library for HBsAg gene insertion. The HBsAg gene (adw type) was subcloned from pSV-

S, kindly provided by Dr. Chao-Hung Lee, and inserted into the AcMNPV transfer vector at the *Eco*RI and *Pst*I cloning sites. The AcMNPV expression vector with HBsAg was named pWYC4. By designing a forward primer (5'-CTATCAATATATAGTTGCTGATATC-3') and a reverse primer (5'-GGAATTCTATTTATAGGTTTTTTTA-3') and using PCR, we modified the sequences related to the 5' untranslated leader of polyhedrin mRNA in the pBSd104HHd113 vector and constructed a new AcMNPV transfer vector, named pYPLT7, which contained the intact sequences related to the polyhedrin mRNA leader. The HBsAg gene was then inserted into pYPLT7 to form pYPLB1. Another HBsAg gene-containing transfer vector, pYPLT11, had 4 extra nucleotides, "AAAT", added in the site between the *Eco*RI cutting site and HBsAg translation start codon by PCR with a forward primer (5'-GAATTCAAATATGGAGAACATCACATCAGGATT-3') and a reverse primer (5'-GTCTAGACTCTGCGGTATTGT-3'). The pYPLB1 contained an intact polyhedrin promoter which was constructed by PCR with a forward primer (5'-CTATCAATATATAGTTGCTGATATC-3'), and a reverse primer (5'-GGAATTCTATTTATAGGTTTTTTTA-3').

Transfection, isolation, and identification of recombinant baculoviruses

Sf 21-AE cells were seeded in a 35-mm petri dish at a density of 2×10^6 cells/dish. After the cells had attached, the media were removed and replaced with a DNA mixture of wild-type (wt) AcMNPV and HBsAg gene containing transfer expression vector, as well as the transfection buffer (1X HEBES and 2.5M $CaCl_2$) and stored at 28°C for 8 hrs. Then, after discarding the transfection buffer, the cells were incubated in TNM-FH media with 10% FCS at 28°C for five days (Summers and Smith 1987). After five days, the media were harvested and the recombinant virus screened by limiting dilution following DNA dot hybridization with a [^{32}P]-labeled HBsAg gene probe synthesized by the random primer method (Fung et al. 1988). The selected recombinant viruses were further identified by PCR (Innis et al. 1990, Goswami and Glazer 1991), using two forward primers, 5'-CTATCAATATATAGTTGCTGATATC-3' (−114 to −90 of polyhedrin gene) and 5'-ATGGAGAACATCACATC-3' (+1 to +17 of HBsAg gene); as well as one reverse primer, 5'-GG(TC)GCGTC(TG)GG(TC)GCAAA(TC)TC(TC)TT(AT)AC(TC)TT(AG)AA-3', which corresponds with a +719 to +687 of polyhedrin gene antisense. Ten μl of virus inoculum was treated with 90 μl of detergent buffer A (50mM KCl,10mM Tris-HCl [pH8.3], 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20) and 10μg proteinase K at 60°C for one hour, and then boiled for 10 mins. A mixture of four nucleotides (dA, dG, dT, dC; final conc. 0.25mM), 0.5 μg of two primers, 2.5 units of Taq polymerase (Promega), 5 μl of detergent buffer B (25mM $MgCl_2$, 10X detergent buffer A) and ddH_2O was added to 50 μl of the above recombinant virus sample in order to carry out PCR. The PCR program was forty cycles of denaturing at 94°C for 1 min, annealing at 42°C for 1 min, and elongation at 72°C for 5 mins. The 10 μl of PCR products was analyzed by electrophoresis (Malitschek and Schartl 1991).

Western blot and immunoprecipitation analysis

Sf 21-AE cells were seeded in 4-well tissue culture plates at a density of 6×10^5 cells/well. After cell attachment the medium was removed. The cell culture was inoculated for one hour with two hundred μl of virus suspension with a multiplicity of infection (m.o.i.) of 10. The viral inocula were then discarded and the cells were incubated in 500 μl of fresh complete TNM-FH medium. Forty-eight hours after infection, the cells were lysed with a 1X SDS sample buffer and heated at 100°C for 5 mins. Proteins were separated by electrophoresis in a 10% SDS polyacrylamide gel and stained with Fast-stain solution (ZOION RESEARCH Inc.). For Western blot analysis, the proteins were semi-dry transferred to nitrocellulose. The HBsAg protein on the nitrocellulose was probed with anti-HBsAg antiserum. The immune complexes were further reacted with peroxidase-conjugated second antibodies. Hydrogen peroxide was used as a peroxidase substrate and 4-chloro-1-naphthol for color development.

For immunoprecipitation, Sf 21-AE cells at a density of 1×10^6 cells/35-mm petri dish were infected with recombinant virus at a m.o.i. of 10. Forty-eight hours after infection, the cells were starved in methionine-free Grace's insect cell culture medium for one hour, then L-[^{35}S]methionine (100μCi/ml) was added. After three hours, the infected cells were disrupted with 1ml RIPA (150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50mm Tris, pH 8.0) and anti-HBsAg antiserum was added for one hour at 4°C. Afterwards, 100 μl of the protein A - Sepharose CL-4B (Pharmacia, 0.1g/ml RIPA) was mixed into the RIPA-Ab mixture for one hour at 4°C. The immune complexes were pelleted to remove the supernatant and washed three times

with RIPA. The complexes were then boiled with 50 μl 1x SDS sample buffer and electrophoresed. The dried gel was autoradiographied with X-ray film.

Radiolabeling of proteins in infected insect cells

At 24-hour intervals over the 6 day postinfection period, the infected cells in the 4-well tissue culture plates were starved in 200 μl of methionine-free Grace's insect cell culture media (GIBCO/BRL Inc.) for one hour. The L-[^{35}S] methionine (1 μCi/10 μl medium) was then added to the media for three hours (Luckow and Summers 1988b). After removing the cell-free medium, the labeled cells were disrupted with 50 μl 1X SDS sample buffer and electrophoresed in 10% SDS/polyacryamide gel. Stained with Fast stain, the gel was dried and exposed at -70°C to X-ray film.

Cytoplasmic RNA isolation and dot blot analysis

For RNA synthesis in uninfected, wild-type, and recombinant baculovirus-infected insect cells measurement, total cytoplasmic RNA was prepared using the NP-40 lysis method (Luckow and Summers 1988b). Sf 21-AE cells were seeded in 25-cm^2 tissue culture flasks at a density of 6×10^6 cells/flask. After the cells had attached , the medium was removed and replaced with 1 ml (m.o.i.10) of a viral inoculum. The inoculum was removed one hour later and replaced with 5 ml of complete TNM-FH medium which was then incubated at 27°C for 48 hours. The cells were harvested and washed once with cold PBS. The pellet was resuspended in 45 μl of cold 1X TE (10mM Tris, 1.0mM EDTA, pH7.6) and lysed with 5 μl of 5% NP-40. After a 5-min icing, an additional 5 μl of 5% NP-40 was added and the suspension centrifuged for 2.5 min in an Eppendorf microfuge to pellet the nuclei. Exactly 50 μl of the supernatant was transferred to a new microcentrifuge tube containing 30 μl of 20X SCC and 20 μl formaldehyde and then heated at 65°C for 15 min. Denatured cytoplasmic RNA preparations were stored at -70°C for later use. After an initial 50-fold dilution in sterile RNase-free 15X SCC, serial 2-fold dilutions of cytoplasmic RNA were dot blotted onto Hybond-N membranes and hybridized to digoxigenin(DIG)-labeled DNA probes. The nucleotide sequences of polyhedrin promoter (-114 to -1) and HBsAg ($+1$ to $+96$) were used respectively, as DNA probes. Prehybridization and hybridization were performed following the Sambrook et al. methodology (1989). The hybridized filters, were processed with an anti-DIG Fab fragment with AMPPD as a substrate, then exposed to X-ray film at 37°C.

Quantitative analysis of HBsAg using enzyme-immunoassay

Sf 21-AE cells were seeded in 4-well tissue culture plates at a density of 4×10^5 cells/well. After the cells were attached, the cell-free medium was removed and replaced with a viral inoculum (m.o.i. of 10). The inoculum was discarded after one hour and replaced with 500 μl of fresh complete TNM-FH medium. Afterwards it was incubated at 28°C. At 24-hour intervals over six days postinfection, the medium and cells in each well were harvested. Cell-free medium was termed extracellular while infected cells lysed in 500 μl of RIPA were termed intracellular. The two preparations were stored at -20°C for later use. All preparations were done with 10 or 100 folds dilution and assayed for HBsAg concentration using enzyme-immunoassay (SURASE B-96; General Biological Comp.)

Analysis of HBsAg on CsCl gradient ultracentrifugation

Using an insect cell-spinner culture system, large quantities of cultured fluid infected with recombinant virus were harvested at three days postinfection and then centrifuged at 3,000 rpm for 10 mins to remove cell debris. The supernatant was treated with 2% (wt/vol) polyethylene glycol 10,000 (PEG 10,000), stirred for 30 mins. at 4°C and centrifuged at 13,000 rpm (RP-19 rotor, Hitachi) for one hour. The precipitate was discarded, and the PEG 10,000 concentration was raised to 10% (wt/vol) and stirred overnight at 4°C. The solution was again centrifuged as described above to precipitate the HBsAg proteins. The precipitate was then redissolved in NTE buffer (10.15M NaCl, 10mM Tris-HCl [7.5], 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, and 1 μl/ml aprotinin) and density adjusted to 1.2 g/ml by addition of 230 mg CsCl per ml and ultracentrifuged at 50,000 rpm (RPS-55T rotor, Hitachi) at 10°C for 40 hrs. (Price et al. 1988, Price 1989). The 0.5 ml fractions were collected and assayed for HBsAg activity using the HBsAg-EIA kit. The fractions containing maximum HBsAg activity were examined under an electron

microscope following phosphotungstic acid negative-staining.

RESULTS

Construction of AcMNPV transfer vectors: pBSdHHd library

In this investigation, *Exo* III and *Exo* VII were used to hydrolyze various nucleotides of the polyhedrin gene to construct two different plasmid libraries, p18 BSd and p19HHd. All p18BSd plasmids contained the same 5'-sequence flanking the polyhedrin gene. The polyhedrin promoter and 5'-end partial sequence of the polyhedrin gene were modified by deletion to create the p18BSd plasmids. The p19HHd plasmids contained the 3'-sequence flanking the polyhedrin gene and multiple cloning site. The 3' coding region of the polyhedrin gene was modified by deletion in the p19HHd plasmids. A series of AcMNPV transfer vectors, designated the pBSdHHd library, was constructed by random p18BSd and p19HHd composition (Fig. 1).

Fifty-one p18BSd plasmids were selected from TB-1 transformants. The number of nucleotides deleted by *Exo* III and *Exo* VII was estimated to be from 6 to 314. The sequence around the polyhedrin promoter was confirmed in six p18BSd plasmids. The p19HHd plasmids from TB-1 transformants were screened, and the deleted sequences of the polyhedrin gene were confirmed (data not shown).

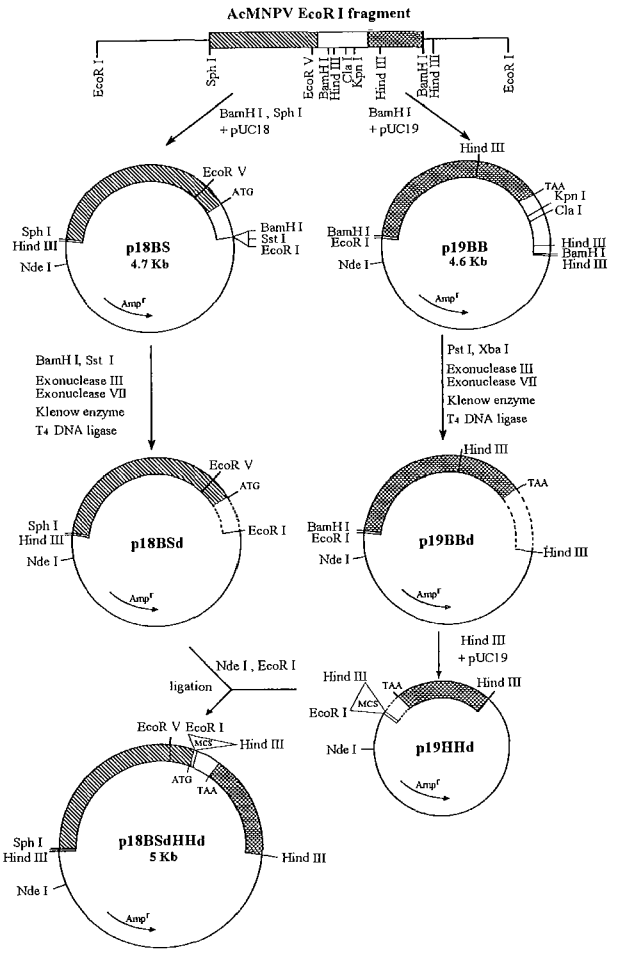

Fig. 1. Construction scheme of AcMNPV transfer vectors, pBSdHHd library. The left section show the procedure scheme of the p18BSd library. The right section presents the construction of the p19HHd library. The hatched and netted areas represent the flanking 5' and 3' sequences, respectively. The polyhedrin gene is represented by the open area.

Construction of two AcMNPV expression vectors containing the HBsAg gene

The pBSd104HHd113 vector was chosen from the pBSdHHd library for HBsAg gene insertion. Six nucleotides "ATAAAT" (−1 to −6) in the polyhedrin mRNA 5'-untranslated leader sequences were deleted from this vector (+1 is the A of translation start codon, ATG). An extra "C" was unexpectedly added at position −6 during cloning. In addition, the vector contained part of the 3'-polyhedrin coding region and its downstream region (+642 to +1242) including the polyadenylation signal. The pWYC4 vector was constructed by HBsAg insertion. Additionally, another two expression vectors, pYPLT11 and pYPLB1, were constructed using PCR. In pYPLT11, the 4 nucleotides "AAAT", were added before the translation start site of HBsAg. The pYPLB1 contained an intact polyhedrin promoter and an extra "A" nucleotide between the polyhedrin promoter and the *Eco*RI cloning site in order to avoid new "ATG" codon formation before the translation start site of the HBsAg gene (Fig. 2).

Isolation and identification of three recombinant viruses

After 3-4 days of co-transfection, polyhedra inclusion bodies (PIB) were produced in the insect cells. The HBsAg antigenicity was detected in co-transfected cell surrounding media. These results suggest that in addition to the wt AcMNPV virus, recombinant viruses were also co-transfected cell produced. The recombinant viruses WYC4, YPLT11, and YPLB1 were isolated by limiting dilu-

tion and dot hybridization without polyhedral body formation. Using PCR in combination with three specific primers, two DNA fragments (ca. 1200 bp and 1090 bp) were amplified within these three recombinant viruses. However, only one 930-bp fragment was synthesized within wt AcNPV (Fig. 3). This outcome confirmed that the three recombinant viruses contained the HBsAg gene located at correct positions and lack of wt virus contamination. Insect cells morphology, infected with either recombinant virus or wt virus, could be distinguished by microscopy (Fig. 4). The insect cells infected with wt virus produced many PIBs at 2 days postinfection. However, no PIBs were found in the recombinant virus-infected cells. The morphology of both infected cells were gradually expanded and became larger than normal. Large vacuoles were observed in infected cells.

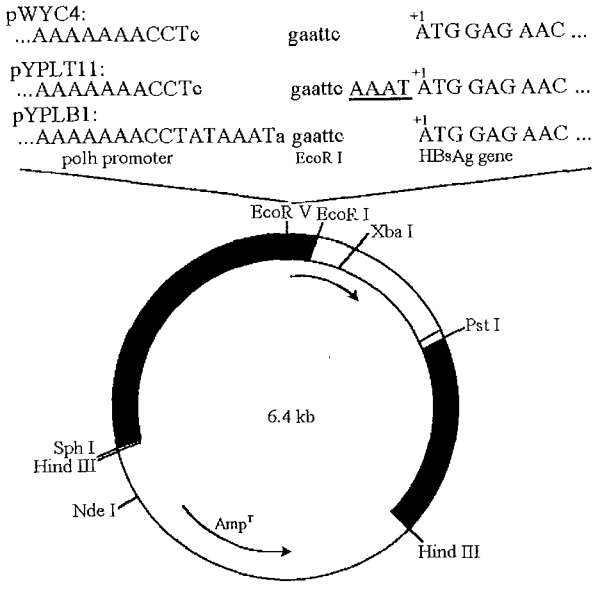

Fig. 2. Circular map and partial nucleotide sequences of two AcMNPV expression vectors containing the HBsAg gene, pWYC4, pYPLT11, and pYPLB1. The structures of both vectors are almost similar, the only differences are the polyhedrin promoter 3'-ends and HBsAg translation initiation codon. The solid areas represent the 5' and 3' sequences flanking the polyhedrin gene, which have been replaced. The HBsAg gene is represented by the open area. The thin line stands for the partial pUC19 plasmid sequences.

Expression of HBsAg by recombinant viruses

In HBsAg proteins expression examination, total lysate of uninfected and infected Sf 21-AE cell monolayers were analyzed by SDS-PAGE, Western blot, and immunoprecipitation (Fig. 5). Uninfected and recombinant virus-infected cell protein profiles were hardly distinguishable on SDS-PAGE with Fast staining. However, wt AcNPV-infected cells synthesized the obvious 29K polyhedrin protein. Western blotting showed that the three recombinant virus-infected cells produced a major 22K protein and a minor 24K protein bearing a weakness on the nitrocellulose. A 20K protein, possibly a degraded form of the 22K protein, was also detected (Fig. 5A). Additionally, similar results with 22K and 20K proteins were obtained by immunoprecipitation. Furthermore, an obvious but smaller amount of 24K glycosylated HBsAg protein was precipitated with anti-HBsAg

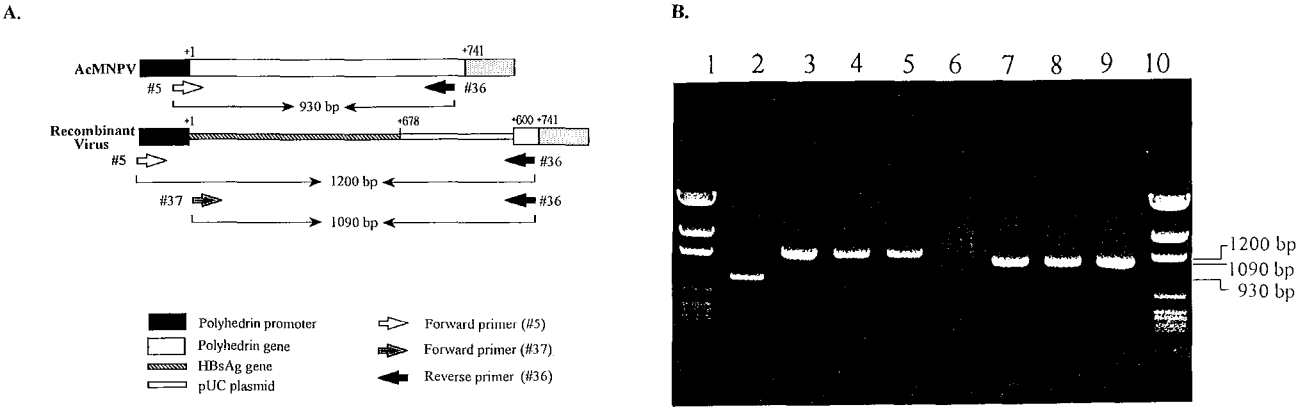

Fig. 3. (A) Schematic drawing of the primer positions and the lengths of DNA fragments amplified with PCR. (B) Analysis of the PCR products of wide-type (lane 2, 6) and the three recombinant AcMNPV types (WYC4: lane 3,7; YPLT11: lane 4,8; YPLB1: lane 5,9). Lanes 1 and 10 represent the pGEM DNA marker. The DNA fragment was synthesized by using primer #5 and #36 in lane 2-5, as well as by primer #37 and #36 in lane 6-9.

polyclonal antibody (Fig. 5B). The Western blot of HBsAg suggested that the amount of HBsAg secreted to the medium was either nonexistent or inconsequential (data not shown).

Temporal expression of protein synthesis in infected cells was analyzed by pulse-labeling with [^{35}S]-methionine at various times after infection, followed by 10% SDS-PAGE. Results indicated that in wt AcNPV-infected cells, polyhedrin protein was obviously detectable at 48 hr p.i., with gradual accumulation to high levels up to 120 hr p.i. (Fig. 6A). Figures 6B, 6C and 6D demonstrate that the 22K HBsAg protein was also obviously expressed at 48 hr p.i. in Sf 21-AE cells infected with WYC4, YPLT11, and YPLB1, respectively. The amounts of 22K HBsAg protein gradually decreased after 72 hr p.i., while the smaller 20K protein also appeared after 72 hr p.i.

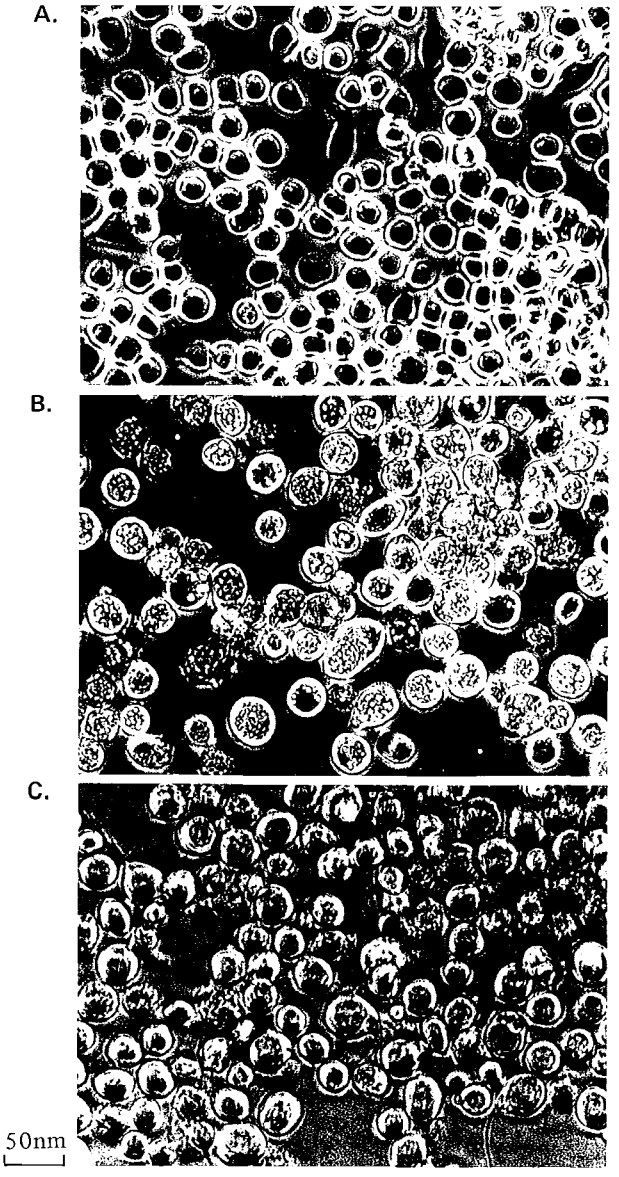

Fig. 4. The morphologies of Sf 21-AE cells. (A) shows the normal Sf 21-AE cells. (B) reveals that the insect cells were infected with wild-type AcMNPV. (C) shows the infected-recombinant virus cells.

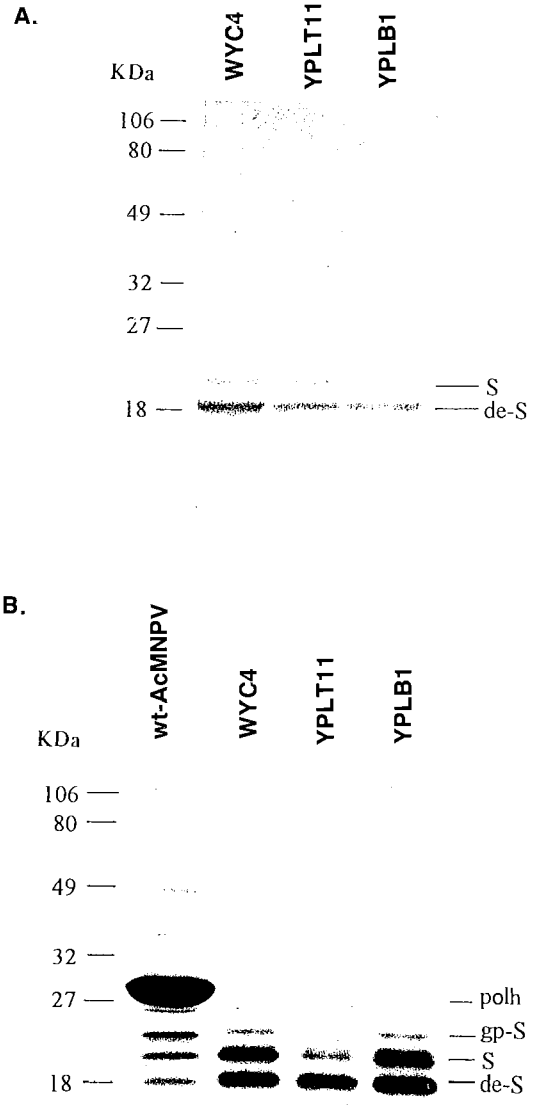

Fig. 5. SDS-PAGE of HBsAg produced from Sf 21-AE cells infected with WYC4, YPLT11, and YPLB1, respectively. (A) Analysis of the infected cells by Western blot with anti-HBsAg polyclonal antibody. (B) Analysis of the [^{35}S]-methionine labeled proteins from the cells infected with recombinant virus or wild-type AcMNPV and noninfected cells. S: HBsAg, gp-S: glycosylated HBsAg, de-S: degraded HBsAg, polh: polyhedrin.

Kinetics of production and secretion of HBsAg

The level of HBsAg synthesis for WYC4, YPLT11, and YPLB1 was determined by comparing medium and cellular extract to an HBsAg standard using an HBsAg enzyme-immunoassay kit. The results revealed that the largest amounts of HBsAg proteins were synthesized and stored in the infected Sf 21-AE cells, and these amounts reached their peak at 3 to 4 days after infection, then gradually decreasing through to day 6. Only small amounts of HBsAg were detected in the medium; the amounts gradually increased from the first to the sixth day after infection (Fig. 7). The highest level of HBsAg synthesis was calculated respectively at 1.16 μg/ml for YPLB1, 1.07 μg/ml for WYC4 and 0.94 μg/ml for YPLT11 (4×10^5 Sf 21-AE cells), respectively. Through medium and cell comparison it was found that the HBsAg from the cells was not secreted to the medium.

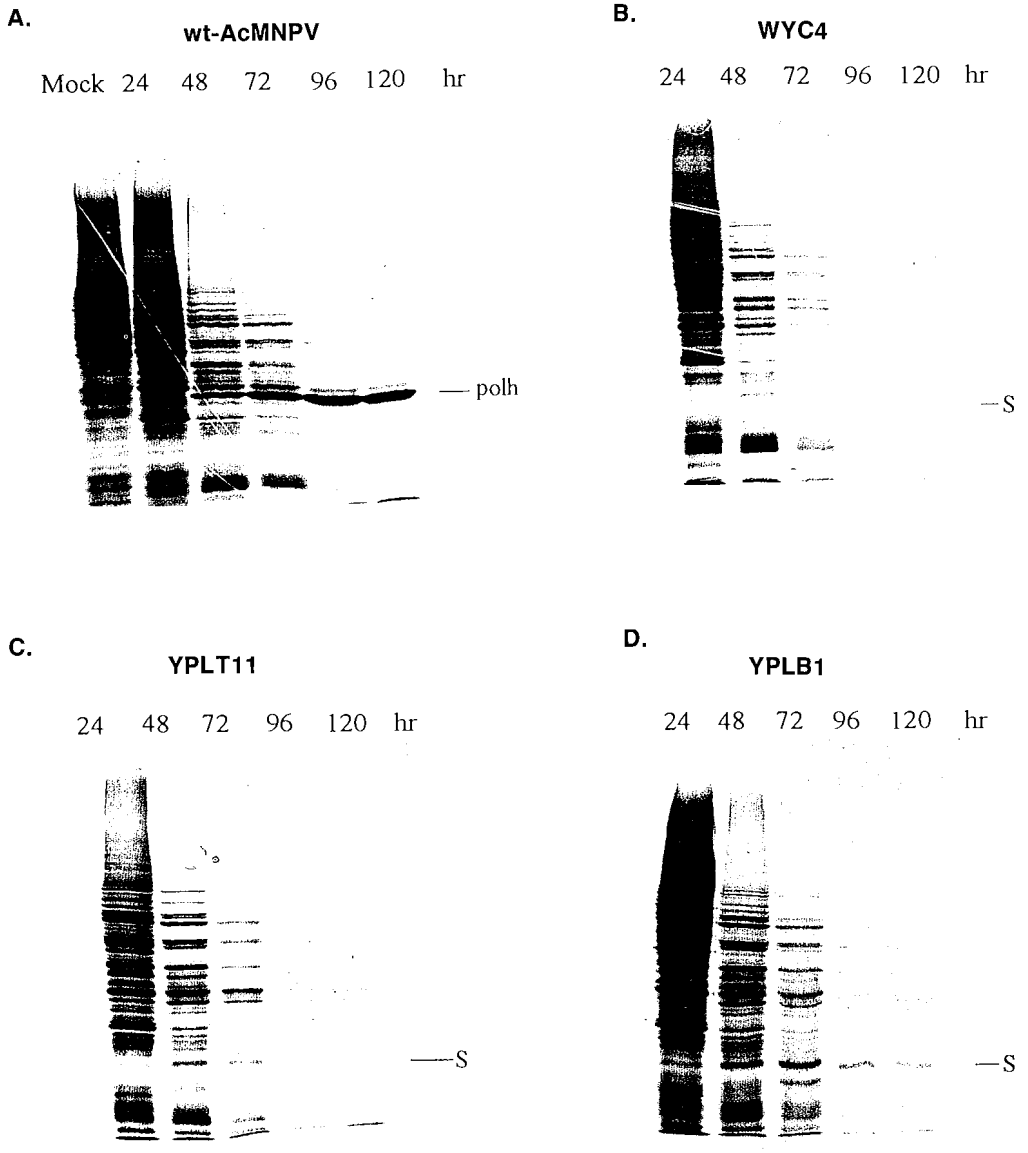

Fig. 6. Temporal expression of proteins in three recombinant viruses and wt AcMNPV-infected cells. Sf 21-AE cells were infected with either one of experimental viruses and pulse-labeled with [^{35}S]-methionine at various postinfection times (24 to 120 hr) for 3 hr. Extracts were analyzed in 10% SDS-PAGE, dried and exposed for autoradiography. (A) wild-type AcMNPV, (B) WYC4, (C) YPLT11, (D) YPLB1; (S: HBsAg, polh: polyhedrin).

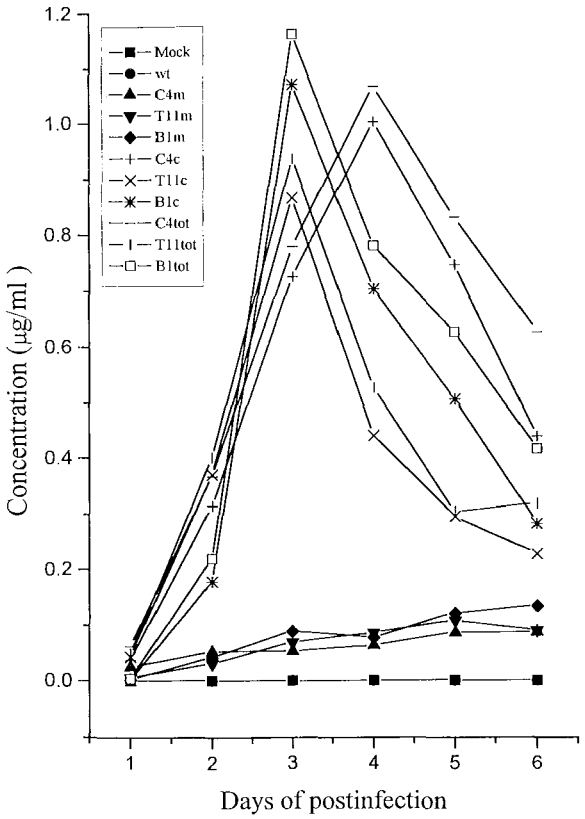

Fig. 7. Kinetics of HBsAg synthesis from Sf 21-AE cells infected with the recombinant WYC4, YPLT11 and YPLB1 from postinfection day 1 to 6. The amounts of HBsAg harvested from infected cells (intracellular), medium (extracellular) and total amount (both intracellular and extracellular) were analyzed respectively.

Analysis of steady-state levels of mRNA in infected cells

In order to determine whether or not protein synthesis from three different recombinant and wt AcNPV infected cells was related to differences in the levels of steady-state RNA affecting translation of the corresponding mRNAs encoding the HBsAg and polyhedrin polypeptides, cytoplasmic RNA was isolated from culture cells as described above. A 50-fold dilution and serial two-fold dilutions of cytoplasmic RNAs were blotted onto Hybond-N membranes which were then probed with DIG-labeled DNA specific for each mRNA. The 114-bp DNA fragment of polyhedrin promoter from −114 to −1 (+1 represents the polyhedrin translation start codon) was used as the template for preparing the polyhedrin promoter DNA probe to hybridize all of the polyhedrin promoter-driven transcripts. The HBsAg DNA probe was synthesized according to the 5'-end nucleotides of HBsAg gene from +1 to +96 (96 bp).

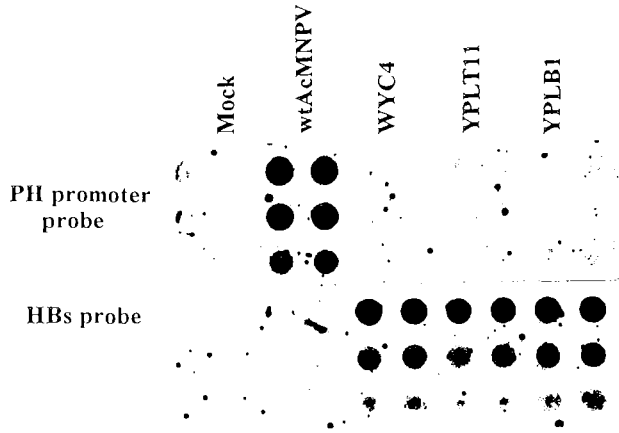

Fig. 8. mRNA steady-state levels from uninfected and infected cells at 48 hr p.i.. Serial two-fold dilutions of cytoplasmic RNA prepared from cells infected with wt AcMNPV, WYC4, YPLT11, YPLB1 and uninfected cells were blotted onto nitrocellulose and hybridized with DIG-labeled DNA probes specific for each mRNA. (A) was probed by polyhedrin promoter nucleotides (114 bp), and (B) was probed by 5'-end nucleotides of HBsAg (96 bp).

As illustrated by Fig.8, the polyhedrin promoter and HBsAg probes were specific. Neither probe hybridized with RNA from mock-infected cells, nor did the HBsAg probe hybridize with RNA from wt AcNPV-infected cells. The signal strength for cytoplasmic RNA diluted dot was measured using a densitometer (data not shown). The results revealed that the signals did lose their strength through gradual dilution. Both probes hybridized to the RNA from the wt and recombinant viruses in a parallel pattern (Fig. 8), indicating that the probes were specific for RNA transcripts driven by the polyhedrin promoter. The probes reflected the different levels of polyhedrin and HBVsAg transcripts. Probed with HBsAg DNA fragment, and examined by a scanning densitometer RNA levels of three recombinant viruses fell in the following order: YPLB1, WYC4, and YPLT11. However, when hybridized with a PH probe, a high level of steady-state RNA was observed in wt AcMNPV-infected cells, higher than the steady-state RNA level of the three recombinant viruses. The amount of wt-RNA was almost ten times that of recombinant RNA (data not shown).

Analysis of 22-nm particles on CsCl gradient centrifugation

The assembly of HBsAg into 22-nm particles

in insect cells infected with YPLB1 was investigated by equilibrium sedimentation in CsCl gradients and electron microscopy. The infected cell cultured fluid was harvested at 3 days postinfection. The HBsAg was concentrated and analyzed on a CsCl gradient. The results showed that the HBsAg bands in the CsCl gradient fractions at a density of 1.14-1.19 g/ml (Fig. 9A). In three fractions, lipoprotein particles with a diameter of 20-22 nm were readily seen under an electron microscope (Fig. 9B). This result suggested that HBsAg did form lipoprotein particles in insect cells.

DISCUSSION

To date a variety of transfer vectors suitable for production of fused or nonfused proteins have been constructed (Luckow and Summers 1988a, O'Reilly et al. 1992). These vectors can be utilized according to expressed foreign genes necessity. Vectors pAcYM-1 (Miller 1989), pAcDZ-1 (Zuidema et al. 1990), pVL941 (Luckow and Summers 1989), and pVL1393 (Luckow and Summers, unpublished data) were designed to express nonfused proteins under normal and intact AcMNPV polyhedrin promoter transcription control. Among these, only pVL1393 contained linker sites (*Bam*HI, *Sma*I, *Kpn*I, *Xba*I, *Eco*RI, *Not*I, *Eag*I, *Pst*I, *Bgl*II). In this investigation, we constructed available AcMNPV transfer vectors which contained linker sites derived from pUC19 (*Eco*RI, *Sst*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I); these are commonly used in prokaryotic expression systems for cloning foreign genes. The polyhedrin coding region from +1 to +600 was deleted to increase the transfer plasmid capacity. In addition, we demonstrated that HBsAg can be expressed in insect cells, under polyhedrin promoter control using both pBSd104HHd113 and pYPLT7 vectors. The antigenicity and morphology of 22-nm particles resembles those particles found in the sera of chronic HBV carriers. Moreover, both HBsAg forms, unglycosylated and glycosylated, were found in *S. frugiperda* cells infected with the three recombinant viruses WYC4, YPLT11 and YPLB1. These results reveal that our AcMNPV transfer vector construction scheme was exact and feasible for expression of the inserted foreign genes; it also offered more transfer vectors for foreign gene expression in insect baculovirus expression vector systems.

The question of HBsAg secretion in *S. frugiperda* cells has not yet been clarified. Kang et al. (Kang et al. 1987, Scully and Kang 1988) indicated

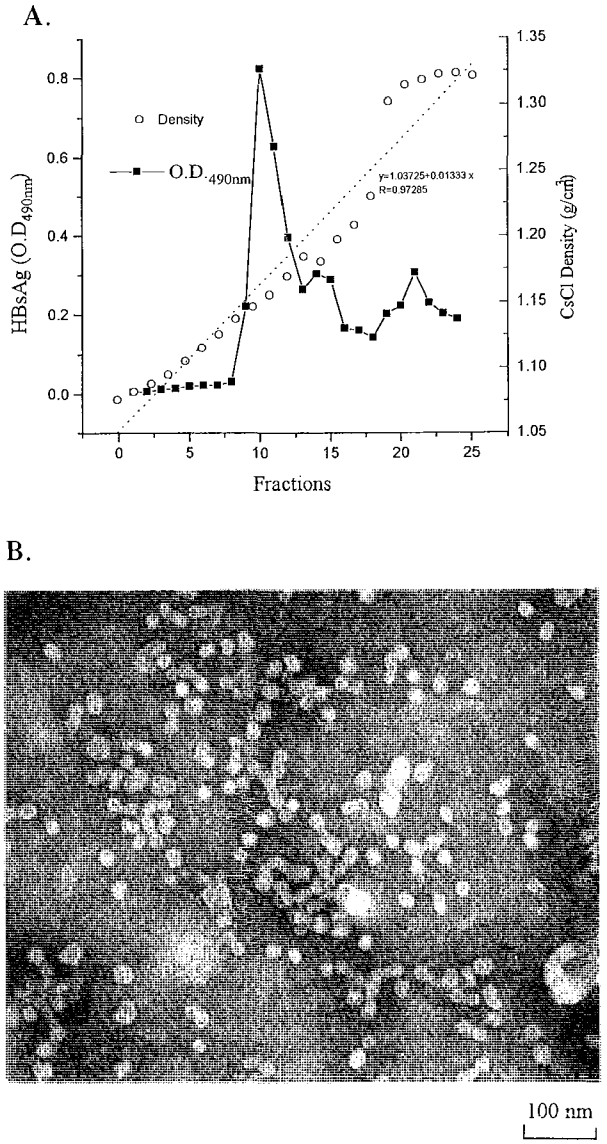

Fig. 9. CsCl centrifugation detection of HBsAg particles from the cell culture media infected with recombinant viruses. (A) Analysis of the HBsAg activity and density of each CsCl-fraction (0.5 ml per fraction). (B) Electron micrographs of HBsAg particles. The particles were observed at the HBsAg activity peak exhibiting a density of 1.14-1.19 g/cm^3.

that HBsAg could be secreted, at significant levels, from *S. frugiperda* cells under AcMNPV polyhedrin promoter control. However, Price et al. (1988) reported that although some HBsAg protein was secreted, most of it was remained within the cell cytoplasm. Moreover, Lanford et al. (1989) suggested that significant HBsAg levels were not detected in the culture media, and that HBsAg polypeptides were membrane associated (presumably in the ER). In this study, the media, which cultured the cells infected with recombinant viruses,

contained very low concentrations of HBsAg protein (under 0.2 μg/ml) from postinfection day 1 to 6 (Fig. 7). But, ten times the amount of HBsAg proteins found in culture media was retained in the cell cytoplasm. Therefore, the HBsAg protein seems to be unsecreted or secreted in small amounts. The HBsAg production peak was reached at 3 to 4 days postinfection, gradually decreasing thereafter. It is suggested here that after the HBsAg synthesis peak, the HBsAg proteins in the cells may be digested by the lytic enzyme released during cell death.

Lanford et al. (1989) reported that the level of HBsAg synthesis was estimated at approximately 6 mg/liter for non-fusion HBsAg. Their estimates were extrapolated from the amount of antigen derived from culture grown in 25-cm^2 flasks (4×10^6 cells) to a 1-liter spinner culture (2×10^9 cells). In our results (shown in Fig. 7), the level of HBsAg was 1.16 μg/ml for YPLB1, 1.07 μg/ml for WYC4, and 0.94 μg/ml for YPLT11 from cultures grown in 4-well culture plates (4×10^5 cells/well). Similarly in this study, using the methodology of Lanford et al., we estimated the level of HBsAg synthesis at approximately 4.7-5.8 mg/liter for the three recombinant viruses. The importance of the sequences related to the 5' untranslated leader of polyhedrin mRNA next to polyhedrin ATG is apparent for foreign gene production (Luckow and Summers 1988a, Matsuura et al. 1987). In addition, the absence of an AT-rich section from nucleotides −8 to −1 (upstream from the polyhedrin ATG and approximately 40 bases downstream from the polyhedrin transcriptional initiation site) would decrease the expressed amount of foreign gene product (Lanford et al. 1989). The results of this study suggest that an intact sequence related to the 5'-untranslated leader of polyhedrin mRNA is important, and the level of HBsAg synthesis under the control of the intact sequences (YPLB1) is higher than that the truncated sequences (WYC4).

Kozak has confirmed that ACCATGG is the optimal sequence for eukaryotic ribosomes initiation. A purine in position −3 has a dominant effect. When a pyrimidine replaces the purine in position −3, translation becomes more sensitive and lower than to changes made in position −1, −2, and +4 (Kozak 1986 1989). In order to examine foreign gene expression corresponded in *S. frugiperda* cells to the Kozak rule (Kozak 1986 1989), we designed three recombinant viruses which expressed the same foreign gene. We found that the level of HBsAg produced in WYC4 infected cells was higher than those in the cells infected with YPLT11. The position −3 was a pyrimidine — "T" from the *Eco*RI cloning site (gaattc) in WYC4. However, the same position was a purine — "A" in the YPLT11, due to the addition of the "AAAT" sequences between the *Eco*RI cloning site and the HBsAg translational initiation codon. By comparing the level of HBsAg synthesized in *S. frugiperda* cells infected with WYC4 or YPLB1 it was revealed that the intact sequences related to the 5'-untranslated leader of polyhedrin mRNA (YPLB1) was more efficient in HBsAg synthesis than truncated sequences. Additionally, analyzing the condition of HBsAg synthesis among three recombinant viruses, we found that the characteristic of the intact sequences related to the 5'-untranslated leader of polyhedrin mRNA was more effective than the Kozak rule for HBsAg synthesis in *S. frugiperda* cells; this is because the levels of HBsAg produced by YPLB1 and WYC4 were higher than those produced by YPLT11. According to the results of Luckow and Summers (1989), the foreign gene expression driven by a polyhedrin promoter in constructed AcMNPV expression vectors does not correspond with the Kozak rule (Kozak 1986 1987). Moreover, the fact that the action of the "AAAT" sequences preceding the HBsAg translation initiation codon seems not to be beneficial to HBsAg synthesis. By determining the relationship between mRNA and protein levels in each of these recombinant viruses, we also analyzed the steady-state mRNA of infected Sf 21-AE cells. As our data, in recombinant viruses, RNA and protein levels of the HBsAg gene are much lower than those of the polyhedrin gene in wt AcMNPV. Therefore, factors other than the Kozak rule could have significant effects on the low level of recombinant gene expression of baculovirus in *S. frugiperda* cells.

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多角體蛋白mRNA 5′端不轉譯引導序列相關之DNA片段對昆蟲細胞中B型肝炎病毒表面抗原表現之影響

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構築一系列的昆蟲桿狀病毒傳送載體，並成功地在昆蟲細胞中表現B型肝炎病毒表面抗原蛋白質。利用西方轉印法及免疫沈澱法，證實所生產之B型肝炎病毒表面抗原具有正確之抗原性；亦於電子顯微鏡下觀察到有22-nm的表面抗原之蛋白顆粒的形成。另分別以蛋白質電泳法，酵素免疫分析法及RNA點墨轉印法等，比較B型肝炎病毒表面抗原在不同的傳送載體(pBSd104HHd113及pYPLT7)控制下，其表現情形之差異。結果顯示多角體蛋白基因啟動子的完整性，對B型肝炎病毒表面抗原基因之表現很重要；且在Sf昆蟲細胞中，以多角體蛋白基因啟動子驅動B型肝炎病毒表面抗原基因時，該基因之表現並不依隨Kozak rule。

關鍵詞：苜蓿尺蠖核多角體病毒, 桿狀病毒, 傳送載體。