EXPRESSION OF HEPATITIS B VIRUS SURFACE ANTIGEN IN COS-7 CELLS BY USING A RECOMBINANT pcDNA I VECTOR

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ABSTRACT

A recombinant plasmid containing pre-S_2+S *gene was constructed using pcDNAI plasmid as a vector. The constructed recombinant plasmid was transfected into COS-7 cells. It was shown that the hepatitis B virus surface antigen (HBsAg) was expressed in transfected COS-7 cells. The HBsAg could be rapidly detected within 24 hours and the antigen was accumulated in detectable level for two weeks after the transfection. In addition, the expressed HBsAg was composed of complete polypeptides of pre-S2+S domain at MW. P25, GP28, GP33 and GP36 kDa. The synthesized polypeptides were assembled to form a spherical particle. These spherical particles were shown to have similar structure to those of plasma derived HBsAg under the electron microscope. The antibody specific to HBsAg reacted specifically to the expressed polypeptides. The results suggested that our recombinant HBsAg was morphologically and antigenically close to native HBsAg. Thus, our expression system may be considered as one of the alternative ways to modify the system to produce stable expression of HBsAg and leads to the production of vaccine or diagnostic kits.*

INTRODUCTION

The human hepatitis B virus (HBV) is a member of the family Hepadnaviridae and causes liver diseases ranging from chronic hepatitis to cirrhosis and hepatocarcinoma. Consequently, the life cycle of the virus and its molecular biology are intensively studied. The classic marker for infection by HBV is the hepatitis B surface antigen (HBsAg). The virus particle may occur in two distinctive forms of 42 and 22 nm in size. The larger particles (42 nm) consist of a core containing the viral genome, the core protein, a DNA polymerase and a phospholipid envelope carrying the surface antigen.[1] The smaller particles (22 nm) as spherical structure or filaments are produced in substantial quantities in infected individuals and contain only the elements of the surface envelope.[2] It has been shown that the surface proteins are not of uniform species but a common region is shared by all of them. The proteins are designated as P25, GP28, GP33, GP36, GP43, and GP46.[3] The GP28 protein is the glycosylated form of P25 that contains 226 amino acids and both are the predominant forms among the surface proteins. On the other hand, GP33 and GP36 proteins have 55 more amino acids (pre-S2) while GP43 and GP46 proteins have 162 more amino acids (pre-S1) at the amino terminal ends of P25 and GP28 (S).

The surface protein, HBsAg, is important in clinical application as a vaccine and diagnostic tools. Several approaches have been devised to express HBsAg gene in both prokaryotic and eukaryotic cells[4-6]. Since HBV viruses are very difficult to grow in continuous cell culture systems, the only one source to obtain HBsAg is from plasma of the chronic carriers. However, the disadvantage of using these materials is that it poses an infection risk to users or laboratory workers. Most of the reported HBsAg is expressed in a heterologous system and the expressed proteins are those proteins that located in the S region. Since, the immunologically important epitopes are found in the pre-S2 region. Thus, attempts have been made to obtain the expressed HBsAg[7-9] that consisting of proteins that locate in either pre-S2 region or both pre-S1 and pre-S2 regions. In this study, the constructed recombinant plasmid using pcDNAI as a vector could produce HBsAg containing proteins in the pre-S2 region in COS-7 cells. With this system, the HBsAg polypeptides are synthesized within 48-72 hours of transfection and are released in the culture medium as 22 nm particles.

MATERIALS AND METHODS

Construction of recombinant pcDNAI (r-pcDNAI) vector

In this study, the *pre-*S_2*+S* gene of HBV was amplified by using polymerase chain reaction(PCR) technique. A pair of primers was designed as a forward fragment (P_1) of 30 nucleotides (5'ACACGGATCCGAGACAGTCATCCTCAGGCC 3') and a reverse fragment (P_2) of 30 nucleotides (5'ACACCTGCAGACGTTTGGTTTTATTAGGGT 3'). The P_1 primer was consisted of 20 nucleotides in the region before *pre-*S_2 gene plus 10 nucleotides of the linker of *Bam*HI restriction site. The P_2 primer was consisted of 20 nucleotides of the reverse sequence located after the stop codon of *S* gene plus 10 nucleotides of the linker of *Pst*I restriction site. To amplify the *pre-S2+S* gene, 1 μL of plasmid DNA of the clone pPM5 that contains whole genome of HBV in the pBS (+ sk) plasmid[10] was used as the DNA template. A 300 ng each of P1 and P2 primers, 5 nanomole each of deoxyribonucleotide triphosphate (dNTPs), 1 unit of Tag polymerase (Promega), 5 μL of 10x polymerase buffer and 3 μL of 25 mM $MgCl_2$ were added to make the total volume of 50 μL for PCR reaction. The reaction was allowed to perform for 30 cycles in a programmable DNA thermal cycle (Perkin-Elmer Cetus). The sample for each cycle was heated at 90°C for 1 minute to denature DNA, and cooled for 2 minutes at 62°C for annealing of the primer. The sample was subsequently incubated for 3 minutes at 72°C for extension of the primer. The PCR product of the *pre-*S_2*+S* gene was observed for the expected size at 906 base pairs (bp) by agarose gel electrophoresis. These fragments were then purified and kept for cloning into pcDNA-I plasmid.

The pcDNA I plasmid bought from In Vitrogen Corporation was used as the expression vector and its organization were shown in figure 1. It consisted of CMV promoter for initiating replication and T7 promoter for the translation of mRNA. These two promoters would enhance the protein synthesis. Also, there were several restriction sites available in the polylinker region and replication origin of SV40 gene that allowed the plasmid to be able to replicate in eukaryotic cells. In addition, there was *SupF* gene that supported the growth of *E. coli* strain MC 1061/P3 on agar containing ampicillin and tetracycline when the plasmid was transferred.

In this study, the recombinant plasmid was constructed by inserting the fragment of *pre-*S_2*+S* gene from PCR reaction into the *Eco*RV digested dT-tailed pcDNA I. The recombinant plasmid was transformed into *E. coli* strain MC 1061/P3. The transformants were selected on LB agar plate containing 7.5 μg per ml of tetracycline and 30 μg per ml of ampicillin. Several

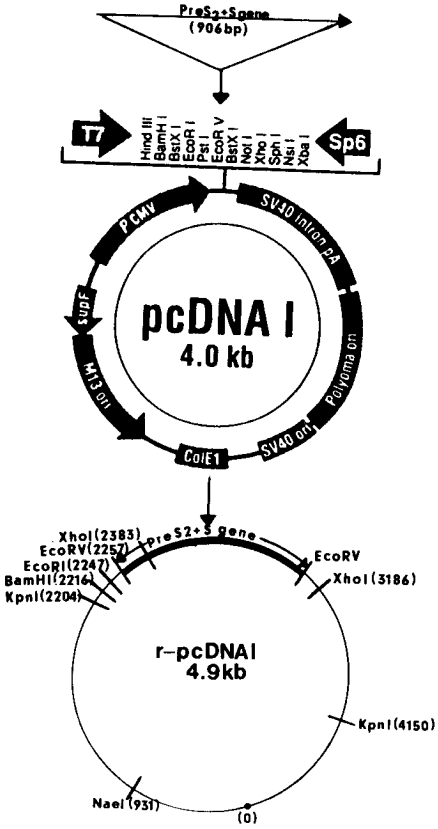

Fig. 1. Schematic diagram of genetic map of pcDNA I (4.0 kb). Amplified $preS_2$ plus S gene (906 bp) was ligated with *Eco*RV digested dT-tailed pcDNAI to obtain recombinant pcDNA I (4.9 kb).

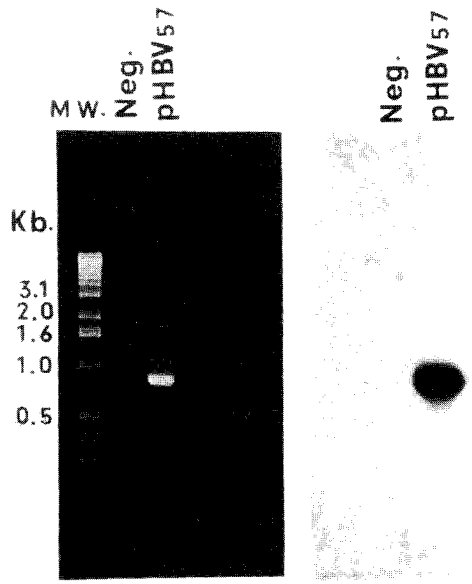

Fig. 2. Agarose gel electrophoresis and southern blot hybridization of DNA fragment of amplified *pre-*S_2*+S* gene from clone pHBV 57.

clones that resist to both of drugs were obtained and used as a source to select the clone with the insertion of *pre-S_2+S* gene. To confirm the insertion, the selected clone was used to extract the recombinant plasmid. The selected clone was subsequently amplified *pre-S_2+S* gene by PCR technique as previously mentioned. The recombinant plasmid was designated as r-pcDNA-I. The size of amplified product was confirmed by agarose gel electrophoresis and southern blot hybridization with oligonucleotide probes specific to HBV[11]. The orientation of the insert was also determined by restriction endonuclease analysis. The clone with the insertion and correct orientation of *pre-S_2+S* gene was propagated and used as a source to extract the recombinant plasmid. The extracted recombinant plasmid was saved for further study of HBsAg gene expression.

COS-7 cells and r-pcDNA I transfection

Since the r-pcDNA-I plasmid was consisting of the replication origin of SV40 gene, therefore it should be able to replicate in COS-7 cells. This cell was derived from African green monkey kidney cells by transformation with the defective SV40 virus resulting in T antigen expression. The T antigen could initiate the replication of plasmid containing the replication origin of SV40 gene in a high copy number. Therefore, transfection was performed with 1-10 μg quantities of the r-pcDNA I carrying *pre-S_2+S* gene per 60 mm dish of cell culture by using calcium phosphate method[12]. The tissue culture dish was seeded with 10^6 COS-7 cells grown overnight in Dulbecco's minimum essential medium. The HBsAg was harvested from culture medium at 24, 48 and 72 hours after transfection. About 30 ml of the supernatant at each time interval was first centrifuged for 16 hr at 34,000 rpm and the pellets were suspended in 2 ml of phosphate buffered saline solution. These solutions were kept for characterization of HBsAg.

Characterization of the secreted HBsAg

The expressed HBsAg in the solutions harvested at 24, 48 and 72 hour's post-transfection was used to determine for the antigen titer by ELISA test. The ELISA test was carried out with ELISA kit bought from Sorin biomedica Diagnostics S.P.A.. The HBsAg titer was expressed in term of average value of optical density (OD). The HBsAg was then concentrated by immuno-precipitation method[13]. The concentrated HBsAg was subsequently characterized. The molecular weight (MW) of its polypeptides was compared with purified HBsAg that derived from plasma of the hepatitis carriers by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The specificity of these polypeptides of HBsAg was determined by using monoclonal antibody specific to **'a'** determinant of HBsAg[14] in immunoblot analysis. In addition, the concentrated HBsAg from immunoprecipitation was also adsorbed on carbon-coated grids and examined the structure of HBsAg by staining with 2% uranyl acetate under electron microscopy.

RESULTS

Construction of expression plasmid

The PCR product of *pre-S_2+S* gene was successfully subcloned into *E. coli* strain MC1061/P3 by using pcDNA-I as the plasmid vector. The recombinant plasmid was designated as pHBV57. This recombinant plasmid was used for the study of HBsAg expression since it could be confirmed as the hybrid clone of pcDNA-I with the insertion of the correct orientation of *pre-S_2+S* gene. The recombinant plasmid pHBV57 was first determined the insertion of *pre-S_2+S* gene by using PCR technique. As shown in Figure 2, the PCR product of the pHBV57

was proved as *pre-S_2+S* gene according to the size and the specificity of the DNA fragment at about 900 bp by agarose gel electrophoresis and southern blot hybridization, respectfully. The recombinant plasmid pHBV57 was also checked for the orientation by restriction endonuclease analysis. The digestion of pHBV57 with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I and *Xho*I endonucleases resulting in a specific size of DNA fragments in agarose gel electrophoresis and results are summarized in Figure 3. The pHBV57 yielded a DNA fragment of 4.9 kilobases (kb) in size after digestion with *Bam*HI, *Eco*RI and *Hind*III endonuclease. It confirmed that there was only one restriction site in pcDNA I whereas no restriction site for these enzymes in *pre-S_2+S* gene as reported by other[10]. It was also shown that there were two DNA fragments appeared at about 3.1 and 1.8 kb when it was digested with *Kpn*I endonuclease. However, there were two DNA fragments appeared at about 4.1 and 0.8 kb after digested with *Xho*I endonuclease. These results were correlated very well to those restriction sites of *Xho*I in pcDNA I and in the *pre-S_2+S* gene. It also agreed with those two restriction sites of *Kpn*I that found in pcDNA I as shown in Figure 1. From the results of restriction analysis, it meant that this pHBV57 had the orientation of the reading frame of *pre-S_2+S* gene concerning the CMV promoter and the SV40 origin fragment.

Characterization of the secreted HBsAg

In the study, pHBV57 was prepared in large quantities in *E. coli* strain MC1061/P3 and used as a source to transfect COS-7 cells. According to the transient expression system, transfected COS-7 cells were analyzed within 72 hours for protein production. It was demonstrated that HBsAg could be detected at 24 hours after transfection. The level of antigen detected was at a low level of average OD = 0.35. The amount of secreted antigen was then increased to the average OD value of 1.8 at 48 hours. The level of antigen declined to the average OD value of 0.60 at 72 hours after transfection. However, large quantities of HBsAg were expected to accumulate if the transfection was allowed to progress for a week. The result of positive HBsAg showed that *pre-S_2+S* gene was functioned in COS-7 cells. The HBsAg product could also interact with antibody specific to the HBsAg.

To find out whether the recombinant antigens contain *pre-S_2* region, SDS-PAGE and immunoblot were used to analyze the secreted HBsAg derived from recombinant plasmid in comparison with plasma derived HBsAg. Results of SDS-PAGE analysis were summarized in Figure 4. The plasma derived HBsAg in lane A showed two predominantly peptides of MW at 25, 28 and 50 kilodaltons (kDa). The 25 and 28 kDa peptides were corresponded to the nonglycosylated (P) and glycosylated (GP) forms of HBsAg, respectively, while the another with MW 50 kDa might be a dimer of 25 kDa protein. The undiluted product of recombinant HBsAg was in lane B and those of serially two fold diluted HBsAgs were in lane C and D. They were consisting of 5 bands of polypeptides at MW of 25, 28, 33, 36 and 50 kDa. The 25 kDa peptide was corresponded to the non glycosylated form while 28, 33 and 36 kDa peptides were corresponded to the glycosylated forms of HBsAg. All of these polypeptides were not immuno-precipitated by normal serum and were not found in supernatants of mock-transfected COS-7 cells. The appearance of higher MW proteins of GP33 and GP36 kDa resulting from the digestion of recombinant HBsAg indicated that the HBsAg produced from *pre-S_2* region was indeed derived from transfected samples. In addition, plasma derived HBsAg lane A showed very faint bands of P25 and 50 kDa (Figure 4 lane A) after reacting with monoclonal antibody specific to HBsAg in immunoblot. On the other hand, the recombinant HBsAg very dense bands of 28, 33, 36 and 50 kDa (Figure 4 lane B)after reacting with monoclonal antibody specific to HBsAg in immunoblot. Results are shown in Figure 5. This result suggested that

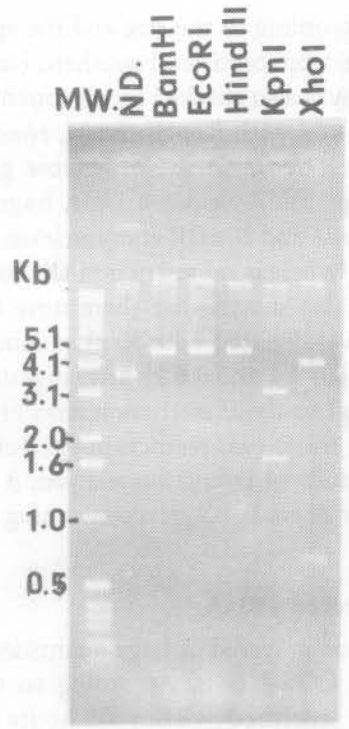

Fig. 3. Agarose gel electrophoresis of DNA fragments of clone pHBV 57 with non digestion (ND.) and digestion with *Bam*HI, *Eco*RI, *Hind*III , *Kpn*I and *Xho*I restriction enzymes.

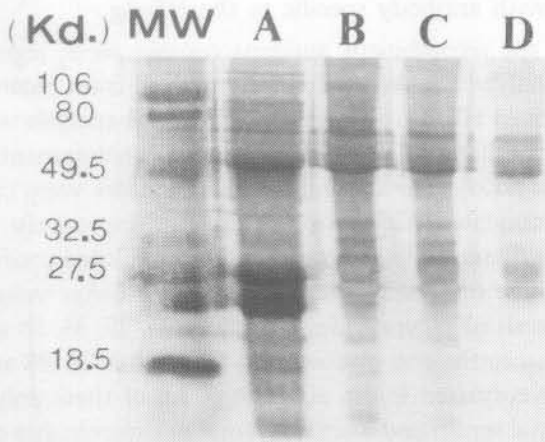

Fig. 4. Demonstration of MW of HBsAg polypeptides by SDS-PAGE and silver staining.
A: plasma derived HBsAg
B, C and D: recombinant HBsAg (serially two fold dilution)

the clone pHBV57 containing *pre-S_2+S* gene could express complete HBsAg consisting of P25, GP28, GP33 and GP36 polypeptides. Moreover, it was demonstrated that the secreted product of COS-7 cells transfected with pHBV 57 contained particles of 22 nm in size under electron microscopy as shown in Figure 6.. These HBsAg particles (Figure 6A) were similar to those particles purified from the serum of hepatitis carriers (Figure 6B). This result indicated that *pre-S_2+S* gene could encode HBsAg polypeptides and the proteins were processed, assembled and finally secreted into the culture medium in the form of 22 nm HBsAg particles.

DISCUSSION

In this study, the pcDNA I was used as the expression vectors to study the role of *pre-S_2+S* gene in the biogenesis of HBsAg. Our findings demonstrated that the recombinant pcDNA-I could be obtained from the insertion of PCR products of *pre-S_2+S* gene instead of using restriction enzyme digested fragments of the foreign genes into plasmids as reported by others.[4-9] The constructed recombinant plasmid derived from pcDNA-I was designated as pHBV57. Results of hybridization and restriction endonuclease analysis of pHBV57 confirmed the correct size and the orientation of *pre-S_2+S* gene. Since COS-7 cells always produce SV40 T antigen, thus, it is appropriated to transfect pHBV57 into COS-7 cells for the study of HBsAg expression. The pHBV57 can replicate in COS-7 cells to produce a high copy number because it contains the replication origin of SV40. The high number of gene copies of pHBV57 that contains CMV promoter can lead to the formation of high cellular recombinant RNA for the synthesis of HBsAg polypeptides. Results suggest that the product of HBsAg protein can be harvested within 72 hours after transfection. The product of HBsAg protein is secreted into the culture medium of the transfected COS-7 cells. Thus, it is quite easy to collected culture medium to use as a source for the purification of HBsAg. It is feasible to obtain enough HBsAg for any analysis eventhough the expression system in COS-7 cells is a transient system. The rate of production of secreted product was estimated to release about 20 to 30 μg per 10^7 cells. The calculation was based on the analysis of SDS-PAGE result using a known amount of purified HBsAg particles in comparison with those particles derived from the expression of pHBV57. This value was found to be about 5 to 7 times higher than those reported by others.[15,16]

One of the important feature of the HBsAg secreted into the medium is the presence of the pre-S_2 region in the polypeptide. This characteristic has been analyzed by SDS-PAGE and immunoblot analysis. The presence of P25 and GP28 could be seen clearly in plasma derived HBsAg whereas other high molecular weight proteins from preS_2 region, GP33 and GP36, could be detected in recombinant HBsAg. Moreover, the pre-S_2 band was seen clearly on immunoblot analysis. This finding is probably reflected the fact that the pre-S_2 region is the most immunogenic epitope which can stimulate the synthesis of an effective antibody to neutralize HBV infection[17]. Results of analysis by electron microscopy showed that the secreted HBsAg particles coded by the expression of *pre-S_2+S* gene formed a similar structure to those of the 22 nm paticles detected in the serum of infected individuals. This result indicated that the coding region of *pre-S_2+S* gene was enough in the processing of assembly of mature HBsAg into spherical structures. These spherical particles have been reported to be more immunogenic antigen than the unassembled ones[18,19]. Therefore our expression system that could produce spherical particles containing *pre-S_2+S* polypeptides gives an advantage over those pre-S_1+pre-S_2+S polypeptides that expressed in other systems in which they could not be secreted to form spherical particles[20,21].

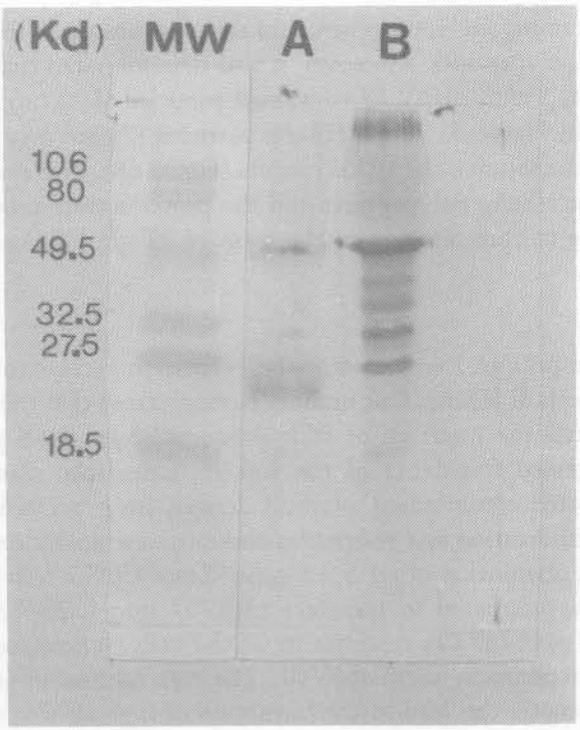

Fig. 5. Immunoblot analysis of recombinant HBsAg (B) in comparison with plasma derived HBsAg (A).

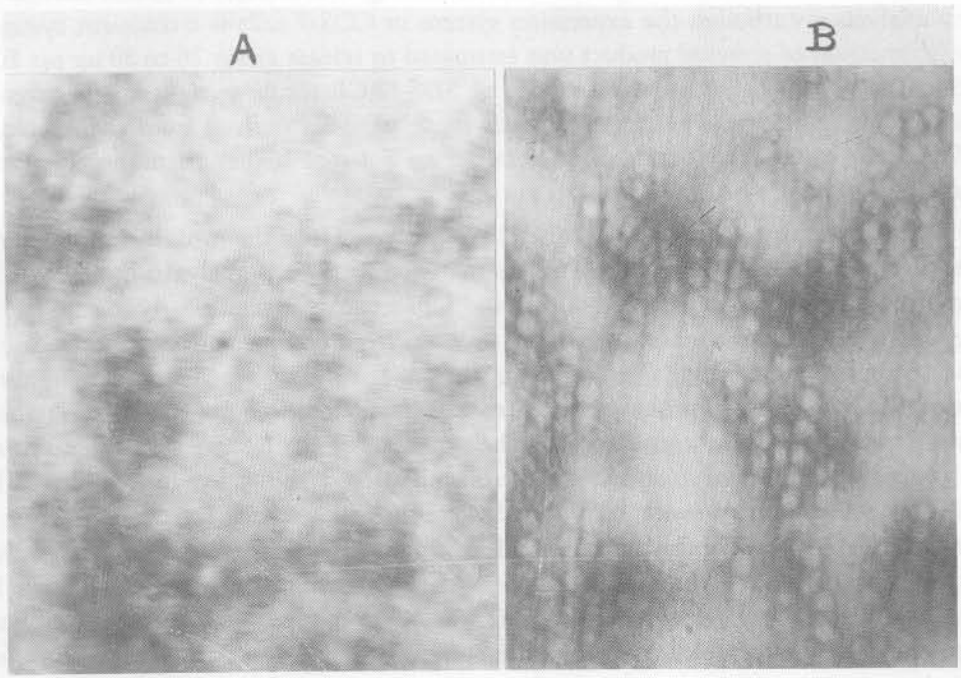

Fig. 6. Comparison of HBsAg particles purified from pHBV 57 transfected COS-7 cells (A) and plasma of hepatitis carriers (B) revealed under electron microscopy.

The findings in this report give an another example of how recombinant DNA technology could be used to produce HBsAg containing pre-S_2 polypeptide in cultured medium of COS-7 cells. This system is allowed the secreted HBsAg protein to assemble into spherical particles. These particles were shown to have morphological and antigenic characteristics similar to native HBsAg. Although the system itself was a transient expression, it could be potentially modified into stable expression of HBsAg for using as vaccine or diagnostic purposes.

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บทคัดย่อ

เซลล์ Cos-7 ที่ถูก transfect ด้วยพาหะ recombinant pcDNA I ซึ่งสร้างขึ้นให้มียีน *pre-S_2+S* ของไวรัสตับอักเสบบีอยู่ด้วย สามารถผลิตแอนติเจนชนิดผิวของไวรัสตับอักเสบบี (HBsAg) ได้ แต่การผลิตแอนติเจนนี้จะเป็นแบบชั่วคราว (transient expression) ซึ่งพบว่า เซลล์ดังกล่าวผลิตแอนติเจนออกมาประมาณ 2 สัปดาห์ การผลิตแอนติเจนนี้เกิดขึ้นอย่างรวดเร็ว โดยสามารถตรวจพบแอนติเจนได้ในเวลา 24 ชั่วโมงหลังจากทำ transfect นอกจากนี้ยังพบว่า HBsAg ที่เซลล์ผลิตขึ้นนี้จะประกอบด้วย polypeptide จำนวน 4 ชนิด ซึ่งมี glycosylation ด้วย โดยมีน้ำหนักโมเลกุลเป็น 25, 28, 33 และ 36 กิโลดาลตัน โปรตีนดังกล่าวนี้รวมตัวกันเป็นอนุภาครูปทรงกลมเหมือนกับ HBsAg ที่แยกได้จากพลาสมา จากการศึกษาสรุปได้ว่า HBsAg ที่เซลล์ Cos-7 ผลิตขึ้นมาหลังจากถูก transfect ด้วยพาหะพลาสมิดลูกผสมนี้ มีคุณสมบัติของแอนติเจนและมีรูปร่างเหมือนกันกับ HBsAg ที่พบในผู้ป่วยไวรัสตับอักเสบบี ดังนั้นการนำระบบนี้มาใช้ในการผลิต HBsAg สามารถใช้เป็นพื้นฐานในการพัฒนาให้สามารถผลิต HBsAg ได้อย่างถาวร (stable expression) ต่อไปนี้ เพื่อนำเอาแอนติเจนที่ได้นี้ไปพัฒนาในการผลิตวัคซีนหรือผลิตชุดน้ำยาสำเร็จรูปต่อไป