Expression of S and pre s2+s Hepatitis B surface antigens in mammalian Cos-7 cell line

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Received 4 February 2003; accepted 30 July 2003

Abstract
Hepatitis B virus (HBV) is a serious global health problem. The development of a safe and effective vaccine would help infection prevention. Previous hepatitis B vaccine production involved the isolation of the non-infectious particle from chronic HBV carriers. DNA recombinant technology has been used for vaccine production without having been contaminated with blood-born infectious agents. Vaccine production in mammalian cells has the advantage of being correctly modified and folded in comparison to other lower hosts. The surface protein coding genes, S (Major protein) and pre s2+s (Middle protein) of hepatitis B virus (HBV), were amplified from the mother plasmid containing the adr serotype virus genome. The s and pre s2+s amplicons were separately cloned in pBlueskript IIks(+) vector as pNM-sa2 and pNM-Psa2 intermediates respectively, then released and recloned in pcDNA3 mammalian expression vector. The correct pNM-Sb2 and pNM-Psb2 constructs containing s and pre-s2, respectively, were used to transfect the mammalian Cos-7 cell line. The major and middle proteins were secreted by this cell line and collected from the culture medium. Some features of gene cloning strategy and expression of these proteins are discussed.

Keywords: *HBV, Pre-s antigen, pcDNA3, Cos-7, HBV Vaccine*

INTRODUCTION

Hepatitis B virus (HBV) is a serious global health problem. The 350 million chronic HBV carriers in the world have an increased risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Thoelen *et al*., 1998). Development of a safe and effective vaccine would help against the transmission of HBV (Molnar-Kimber and Jarocki-Witek, 1988). This virus belongs to the family of Hepadnaviridae viruses (Schodel *et al*., 1994). Its partially double-stranded circular DNA genome consists of approximately 3200 base pairs containing various genes which are divided into several overlapping open reading frames (ORF), coding for all viral proteins, including S, C, P, and X9 (Grob *et al*., 1998). The ORF of s region has 3 in-frame AUG codons that divide this ORF into 3 regions, termed pre-s1, pre-s2 and s that encoding the viral envelope proteins (Hepatitis B surface Antigens) (Cabrevizo and Bartolomeh, 2000). Pre-s1, pre-s2 and s regions code for 108-119 (depending on the serotype), 55 and 226 amino acids, respectively (Molnar-Kimber and Jarocki-Witek, 1988). The small protein of HBV carrying the major surface antigen shows property of mobilizing cellular lipids into empty envelope particles collected from both infected liver and transfected cell lines. Such subviral HBsAg particles are non-infectious and are now safely used worldwide for vaccination against Hepatitis B. The surface of HBsAg is antigenically complex, and at least five antigenic determinants have been found on these particles (Robenson *et al*., 1990). The first results of applying HBV vaccination in human was reported after vaccine preparation from plasma of healthy HBV chronic carriers. This vaccine production consisted of purified non-infectious 22nm HBsAg particles (Zmuness *et al*., 1980). However, difficulties such as the risk of contamination with other blood-born diseases, the limit of human serum supplement, and the cost of purification procedure, led to the idea of applying genetic engineering to produce recombinant hepa-

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titis B virus vaccine in different host systems. Although cloning in bacterial system produced massive HBsAg, this antigen did not exhibit its correct modification and/or conformational structure (Thiollais *et al*., 1991).

Since eukaryotic system such as yeast and mammalian cells have shown the ability of synthesizing correct and native HBsAg particles, the application of these hosts has attracted many investigators. Although the expression of eukaryotic proteins in yeast offers certain advantages over bacterial system, the secretion and glycosylation patterns and the product amounts of a given protein are mostly species specific dependent. These matters encouraged other investigators to invest on the application of mammalian cell culture for HBV vaccine production (Pourcel and Dejon, 1985; Thiollais *et al*., 1991). The application of mammalian system offered suitable solution to the profound concern in connection with the matter of the 5-10% non-responding rate among apparently healthy individuals, and a much higher rate of non-responsiveness among immuno-deficient persons who were vaccinated with the currently available yeast-derived recombinant hepatitis B vaccine (Yap and Guan, 1995). The production of recombinant vaccine in mammalian cells induced an active immunization in these subjects. In the present study, we constructed two recombinant plasmids containing the s and pre s2+s regions of HBV genome in order to express them in Cos-7 mammalian cell line following transfection with pcDNA3 vector.

MATERIALS AND METHODS

Strains and plasmids: *E. coli* strain, Topl0F´ was from Invitrogen (The Netherlands). SV40 transfected Cos-7 cell line (cv-1 monkey kidney derivative), to express large T antigen (LTAg) purchased from the Iranian cell bank of Pasteur Institute (Teharan, Iran). The pBRHBadr72 plasmid, containing the full genome of hepatitis B virus serotype adr cloned in pBR322, was used as a source for isolating HBsAg genes (Health Research Resources Bank, Osaka, Japan). The pBlueskript IIks(+) was used for further subcloning (Invitrogen, The Netherlands). The pcDNA3 plasmid as an expression vector containing SV40-ori fragment and CMV promoter was used for expression of HBsAg genes into Cos-7 cells (Invitrogen, The Netherlands).

Biochemicals and enzymes: The restriction and modification enzymes used in this study were purchased from Boehringer (Germany) and Cinagen (Iran). PCR primers were from TIBMOLBIOL (Germany). Dulbecco's Modified Eagle's Minimal Essential medium (DMEM), PRMI 1640 and Fetal Calf Serum (FCS) were from GIBCO (USA). Biochemicals of high quality (Molecular Biology Grade) were used in all experiments.

Recombinant DNA techniques: Standard Techniques for recombinant DNA construction were used (Sambrook *et al*., 1989).

Construction of plasmids: The pBRHBadr72 plasmid was used as a template for amplifying the s and pre s2+s genes, using PCR technique. The following primers were designed and used:

Forward S gene primer:

5´-GCGC**GGATCC**ATGGAGAACACAACATCAGGAT-3´

Bam H1

Forward pre s2+s gene primer:

5´-GCGC**GGATCC**ATGCAGTGGAACTCCACAA-3´

*Bam*H1

Reverse primer for both s and pre-s+s genes, since both genes have the same 3´ end:

5´-GGCC**GAATTC**CCTTTGGTTTTATTAGGGTT-3´

*Eco*RI

*Bam*HI, *Eco*RI restriction sites were designed at the beginning of the 5´ of forward and reverse primers and were used when further cloning was planned (shown in bold face).

PCR was performed for 30 cycles as follows: for DNA denaturation, at 93°C for 1 min, for primers annealing, at 69.5°C for 1 min and for strand extension, at 72°C for 1 min. Before starting the first run, the reaction mixture kept at 95°C for 5 min. Also at the end of the last loop, the reaction mixture was left at 72°C for 10 min. The high fidelity *Pwo* DNA polymerase was used for PCR. One PCR program was used for both the genes. The PCR products of pres2+s gene are 846 bp, while s-gene is 681 bp. The blunt-ended amplicons were ligated with the blunt ends of *Hinc*II linearized pBluescript IIks(+) plasmid. This strategy helps us to select the right white colonies faster and without concern about the directionality of the ligated blunt ended PCR fragment. After obtaining the correct construct it is easy to release the cloned fragment from the designed restriction sites and to use it for further cloning. We have also found that it is hard and difficult to recover or to have enough precipitate of the PCR

product if enzymatic digestion of the designed restriction sites is directly carried out after the PCR reaction. In other words, further manipulation on PCR product does not only cause the loss of the product, it may also cause a DNA destructing effect. The resulted ligate mixture was used for *E. coli* Topl0F´ transformation according to the producer instruction (Invitrogen, The Netherlands). White transformants were selected on media containing X-gal-ampicillin, and then plasmid extraction was followed. The extracted constructs were subjected to restriction endonuclease R-mapping analysis using the referred restriction endonucleases. The correct oriented fragments were released from the plasmid after digesting with *Bam*HI and *Eco*RI restriction endonucleases. These fragments were ligated to the *Bam*HI and *Eco*RI linearized pcDNA3 plasmid. The overnight incubated ligate mixture was used for *E.coli* Topl0F´ transformation. After excluding the blue colonies that contained the self-ligated pBlurscript IIks(+) on media with X-gal-ampiciline, white transformants were selected. To select white colonies harboring the right size of the recombinat pcDNA3, DNA extraction was carried out and the right constructs were subjected to restriction endonuclease (R-mapping) analysis. The resulting plasmids pNM-sb2 and pNM-PSb2 carrying s and pre-s2, respectively, under transcriptional control of CMV promoter were identified by obtaining the right fragments in length after digestion of plasmids with restriction endonuclease *Xba*I. Both plasmids pNM-sb2 and pNM-Psb2 gave the same length fragment of 606 bp. Plasmids with the correct oriented fragments of both genes were used to transfect Cos-7 cell line.

DNA sequencing: To make sure that the construction and manipulation processes did not affect the base sequences of the pre-s2 and s genes, two correct constructed pcDNA3 that named pNM-Psb2 and pNM-sb2 were sequenced. The resulted recombinant plasmids named pNM-sb2 and pNM-Psb2 containing pre-s2 and s genes, respectively, were prepared for direct sequencing of s and pre-s2+s regions before Cos-7 cell transfection following the use of the standard primers of T7 and SP6 promoters and the application of an automated DNA Sequencer (Sequence laboratories, Gottingen, Germany).

Cos-7 cell transfection: The cells were propagated in DMEM or RPMI-1640, supplemented with 10% FCS and antibiotics (penicillin 50 unit/ml and streptomycin 50 µg/ml) all cultures were maintained at 37°C in a moist atomsphere containing 5% CO_2 and 95% air. Cell subculture and media renewal were done every 3 days and everyday, respectively. Transfection was essentially done as described previously (Conn *et al.*, 1998). Briefly, 1-2×10^5 Cos-7 cells in 100 mm dishes were considered for each trasfection and 15 µg DNA was used. Following incubation at 37°C for 4-5 h and immediate glycerol shock, cells were washed once with DMEM and then re-fed with fresh medium. G-418 (Sigma, USA) at 0.25 mg/ml was added after 72 hours and rich medium containing this antibiotic was changed every 3 days. The formation of colonies resistant to G-418 appeared after further incubation and media renewal. For a large scale of cell growth, good and well shaped colonies were isolated then repeatedly propagated in the same medium in the presence of increasing doses of 0.25-0.5 mg/ml of G-418 for permitting only those cells resistant to neomycin to grow. Those cells were resistant to the added antibiotic were repeatedly reused for HBV surface antigens production.

Analysis of cellular DNA: Cellular DNA was extracted from confluent monolayers of trasfected cells as described (Merante *et al.,* 1998). In order to confirm the presence of HBsAg gene in transfected cells, genomic DNA was applied as a template for PCR by using the primers of each gene. Also, for further confirmation, Southern blot technique was used following DNA extraction from neomycin resistant Cos-7 cells. Briefly, the extracted DNA was digested with the appropriate restriction endonuclease and electrophoresed at 15V for 15 h using 0.8% agarose gels. The gel was transferred to nylon membrane (Nylon$^+$ membrane, Amersham, UK). The blotted membrane was hybridized at 42°C with PCR product of s-gene as a DNA probe that was labeled according to the ECL Labeling Kit (Amersham, UK). The developed film was analyzed for data interpretation.

Protein Analysis

ELISA: Monolisa Ag HBs plus kit (Sanofi, Pasteur-France) was used for HBsAg detection and confirmation according to the manufacturer recommendations. The cut off value was determined in experiment using positive and negative controls.

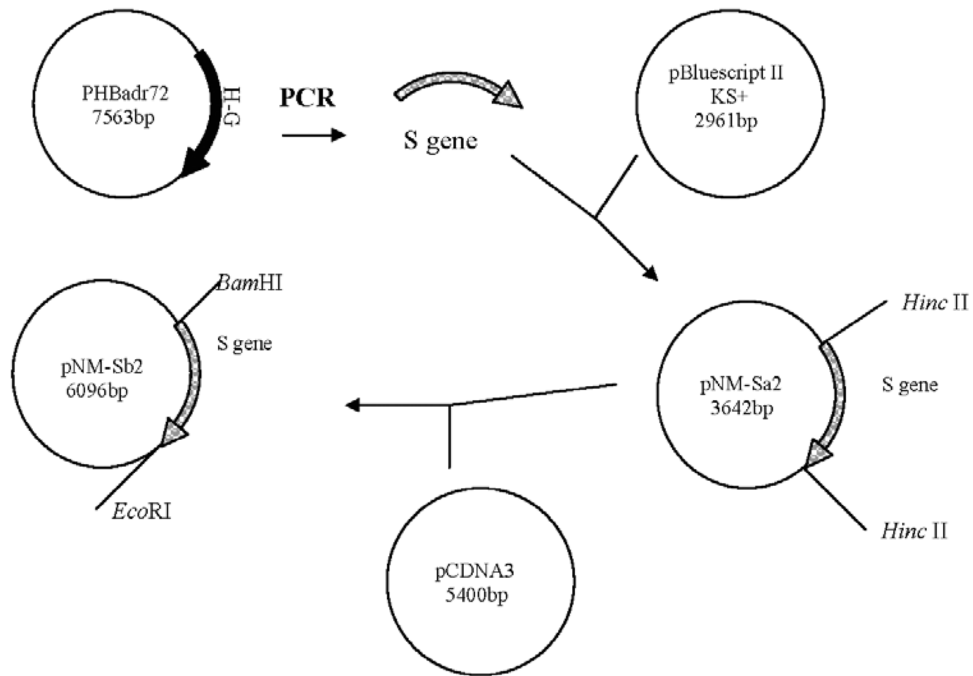

Figure 1: Schematic representation of the pNM-Sa2, pNM-Sb2 plasmid constructions.

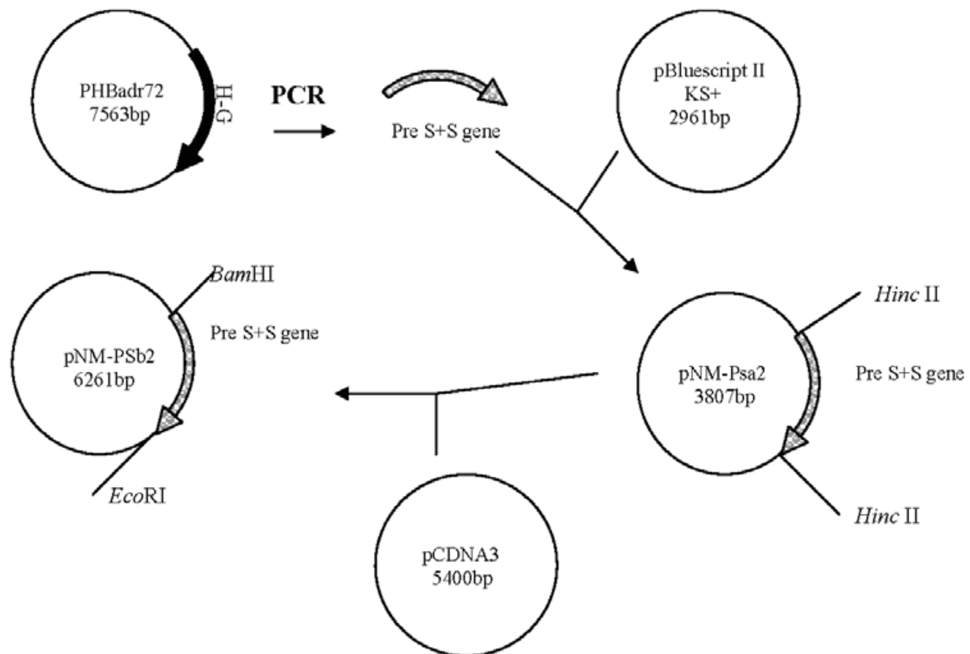

Figure 2: Schematic representation of the pNM-Psa1, pNM-Psb2 plasmids constructions.

RESULTS

pNM-sa2 and pNM-Psa2 construction: Coding sequence for the major part of the HBsAg was amplified following the use of pBRHBadr72 plasmid (s-gene) as a template then subcloned in *Hinc*II cleaved pBlueskript IIks(+) and referred as pNM-sa2 clone (Fig. 1). Coding sequence of s plus pre-s2 of the mother plasmid pBRHBadr72 was PCR amplified. The amplicon was ligated with the *Hinc*II cleaved pBlueskript IIks(+) and named as pNM-Psa2 clone (Fig. 2).

Construction of plasmids pNM-sb2 and pNM-Psb2 and transfection of Cos-7 cells: The pre-s and s genes of the HB virus were released from pNM-sa2 and pNM-Psa2, respectively, then placed under transcriptional control of CMV promoter in plasmid pcDNA3, resulting in plasmids pNM-Psb2 and pNM-sb2, respectively as described in materials and methods (Figures1 and 2). Two recombinant plasmids were used for transfection: I) pNM-Sb2 and II) pNM-Psb2. Transfectants were selected by neomycin-resistant phenotype after 26 days, and the G-418 resistant colonies appeared with an efficiency of 1.25 per 10cm^2 per 1 μg DNA (Table 1). The average number of foci in a 35 mm dish was calculated with respect to the rate of transected DNA and the efficiency of focus appearance was determined.

DNA Sequencing: The pNM-sb2 and pNM-Psb2 clones were sequenced by standard primers of T7 and Sp6 promoters. In pNM-Sb2 clone, the sequence of s-gene was corresponding to the sequence of s-gene in pBRHBadr72 and in pNM-Psb2 clone, the sequence of the pre-s 2+s gene differs from sequence of the same gene in pBRHBadr72 in two positions 63 and 134. In position 61 (codon 21), amino acid was the same of the wild type, while in position 134 (codon 45), Ser altered to Phe (data not shown). On the other hand, the presence of s-gene and pre s2+s gene in transformed cells was confirmed following PCR amplification using forward and reverse primers of these two genes.

State of recombinant DNA in transformed cells: In order to examine whether the pNM-Sb2 and pNM-Psb2 plasmids are replicating extrachromosomally in the transformed cells or were integrated into the host genome, we analyzed total DNA by blot analysis. DNA was extracted from each clone then treated with *Hind*III and electrophorsed on 0.8% agarose for 15 hr at 15V. Hybridization was done with the labeled PCR product of s-gene as a probe. No signal was observed in negative control, while a 1.9 kb band was seen in pNM-sb2 transfectant with pattern of complete *Hind*III digestion. Also three bands as 1.9, 3.7, 15.5 kb were observed in pNM-Sb2 clone. The appearance of such three bands may be due to the *Hind*III partial digestion of the Cos-7 genomic DNA. This may be an indication of pNM-sb2 integration at a single locus in the host chromosome. It is also possible to suggest that these 3 bands can be considered as an integration of 3 separate plasmids in different chromosomal loci.

ELISA Test: ELISA showed that both major and middle proteins of hepatits B surface antigens were expressed in Cos-7 cells after neutralization with the anti-HBsAg antibody. It confirms that the expressed recombinant HBsAg in Cos-7 cells has the correct tertiary structure and was recognized properly by the anti-HBsAg antibody (Fig. 3, and Table 2). According to the kit protocol, and with respect to OD, the concentration of the extracted protein was calculated (Table 3). The samples of A, B, D, E samples are negative and positive controls, serum of normal individual, and serum of HBsAg positive individual, respectively. All of these samples were considered as controls in ELISA test. On the other hand, samples of C, G are medium culture and cell lysate of Cos-7 cells, respectively, and were regarded as negative controls of DNA transfected Cos-7 cells.

In respect to the total volume of culture media and

Table 1: Efficiency of colony formation per μg DNA.

No. of Foci per 10 cm^2 per 1 μg DNA (no/10 cm^2/μg DNA)	Treatment of cells with
1.2	pNM-Sb2
1.3	pNM-Psb2

Table 2. Results of ELISA test.

Medium culture of cells expressing-middle protein	Cell lysis of normal Cos-7	Medium culture of cells expressing major protein	Serum HBsAg (+)	Serum HBsAg (-)	Medium culture of normal Cos-7	Positive control	Negative control	
H	G	F	E	D	C	B	A	**OD at 450 nm**
0.883	0.014	1.249	1.731	0.020	0.018	1.692	0.012	

Table 3. Concentration of protein expressing in culture medium.

	Culture medium of cells expressing major protein	Culture medium of cells expressing middle protein	Positive control
Concentration (ng/ml)	10	14	20

Figure 3. ELISA Results: A. Negative control of kit, B. Positive control of kit, C. Culture medium of normal Cos-7 cells, D. Serum of HBsAg (-) individual, E. Serum of HBsAg (+) individual, F. Culture medium of pNM-Psb2 transfected Cos-7 cells, G. Cell lysate of normal Cos-7 cells, H. Culture medium of pNM-Sb2 transfected Cos-7 cells

number of cells, the production rate of cells expressing major protein was calculated as; 1.5 mg/day/10^5 cell and 2.1 mg/day/10^5 cell for cells expressing the middle protein.

DISCUSSION

To eliminate blood-born infectious agents contaminated HBV vaccines, investigators tried virus and their propagation in cultured cells. However, the dependence of the virus on liver cell receptor and the internal condition of gene expression of the infected cell, made the application of cell culture technique unsuccessful (Yee *et al*., 1989; Hertogs *et al*., 1994). On the other hand, cloning of HBV vaccine in bacterial system gave not only a low yield, the vaccine also showed structural conformation deficiency (MacKay *et al*., 1981; Murray *et al*., 1988). Although, the application of yeast as a host overcame this problem somehow, the unresponsiveness of a fraction of HBV vaccinated subjects suggested that the recombinant antigen could not be properly recognized by the immune system. This defective and inefficient recognition was subsequently attributed to the lack of glycosylation modification that the vaccine needs and this may be the cause of the difficulty or inability of *Saccharomyces cervesiae* and other yeast strains to secret HBsAg (Cregg *et al*., 1987; Lanford *et al*., 1988). Therefore observation of a small percentage of non-responders among those healthy subjects to the yeast derived recombinant HBV vaccines and their relative ineffectiveness among immuno-deficient recipients suggested the need for further improvement of HBV vaccine industry.

The application of mammalian cells as a host for recombinant HBV vaccine has been found promising and circumvented the complexities that non-responsive HBV vaccinated individuals previously faced. Injection of CHO cell produced recombinant HBV

vaccine in mice confirmed the responsiveness in comparison to mice received the same amounts of yeast-drived HBVsAg vaccine (Shouval *et al*., 1994). Also a high immunogenicity and 100% seroconversion were reported in a study concerned a group of young adults injected with CHO cell produced pre-S-s recombinant vaccine (Hourvitz *et al*., 1996). Due to these outlined encouraging achievement in recombinant HBV vaccine technology, we preferred to extend such studies by applying Cos-7 cell line as a host. These cells are transfected with the SV40 large T antigen and are able to produce this antigen dominantly. Such continuous production can be used properly for DNA replication of pcDNA3 vector which contains the SV40 origin site. Therefore, although previous studies have mostly applied CHO cell line, the application of Cos-7 cells together with the use of pcDNA3 vector may offer superior performance for both vector continuous presence and gene expression.

In parallel to the development and the matter of looking for better host for expressing the HBV vaccine, the surface antigens of this virus have also been considered for obtaining effective and reproducible immunogenicity. The important role of HBV surface pre-s1 and pre-s2 proteins in immunological recognition and reaction T and B cell receptors is well documented (Neurath *et al*., 1987). The presence of pre-s2 on the surface of HBV has been believed to overcome the observed difficulty in non-responsive status to s protein through pre-s2 specific T-helper cell functions (Neurath *et al.*, 1986; Neurath *et al*., 1987). Other studies also indicated the immunogenic superiority of pre-s2 over s at the humoral level under normal immunization and time of onset primary response. On the other hand, studies on yeast even showed that s region was not glycosylated while both pre-s1 and pre-s2, each received N-glycosylation on one of their amino acid. These evidences and criteria, have justified the inclusion of pre-s2 region in most vaccine design together with the application of mammalian cells as hosts for gaining better protection against HBV transmission. Recent data also suggest that the pre-s1 and pre-s2 proteins induced protective antibodies and their inclusion with protein s could enhance the vaccine's immugenicity. Although the inclusion of pre-s2 region to s protein was efficiently secreted by transfected cells, the fusion of pre-s1 sequence to these two sequences caused the block of secretion of such three sequences combination. This blockage of protein secretion was suggested to be due to some modification in the plasma membrane (Prange *et al*., 1995; Yap and Guan, 1995). Due to the above documented observations, we concentrated on the use of pre-s2 and s regions of env gene to produce effective Hepatitis B vaccine in mammalian cells. Both s and pres2+s genes were cloned separately in Cos-7 cells in order to study the immunogenicity of the expressed proteins for the purpose of developing more effective vaccine. Having such aims in mind, the frames of both genes coding for surface antigens, pres2+s and s were cloned in pBlueskript IIks(+) plasmid with proper restriction sites. The choice of using this plasmid as an intermediate is due to the easy and fast selection of white colonies picking the right amplified fragment. The correct fragment of this intermediate construct were released from the plasmid, then cloned into pcDNA3 expression vector and used for Cos-7 cell line transfection.

The SV40 large T antigen (LTAg) transfected Cos-7 cells, has the ability of producing LTAg dominantly and this in turn cause pcDNA3 plasmid (containing SV40-ori) to replicate efficiently and with a high copy number. Foci formation of neomycin resistant Cos-7 cells transfected with the circular form of recombinant pcDNA3 (containing either s or pre-s2-s sequences), took about 26 days. These foci were found to be positive for major and middle proteins. Southern blot analysis suggests that pNM-Sb2 and pNM-Psb2 clones were predominately integrated within the genome of the transformed cells, and that always led to a stable expression of the major and middle proteins whenever they were grown in complete media. It is also possible that episomal form of this plasmid can be actively expressed. However, studies have declared that the continuous supplement of high copy number of episomes in the nucleous of propagating cells after transfection will finally lead to their efficient integration in the host genome, and this in turn would result in their stable expression as integrated sequences

Direct sequence of pNM-Sb2 clone showed no alteration in DNA sequence in comparison to the sequence of the mother s-gene in pBRHBadr72 during the plasmid construction and manipulation. However, the sequence of pNM-Psb2 differs from the sequence of the mother pre s2+s gene in pBRHBadr72 in two base positions, inside codons 21 and 46. The alteration in the codon 21 caused no change in the amino acid sequence, while change in codon 45 caused the alteration of Serine to Phenylalanine. It seems that this change in the amino acid sequence did not cause the

loss of the cloned protein immunogenecity.

Expression of both genes in transfected cells proved to be in a proper rate and comparable to other reports concerning HBsAg production in other hosts. Also, we should not ignore the possible secretion and the contamination of the cell culture with proteases that cause degradation of a fraction from the secreted vaccine product and during storage, particularly the pre-s2 part of the vaccine that is sensitive to proteases (Kitano *et al*., 1987; Chen *et al*., 1988; Langley *et al*., 1988). Therefore, what we measured may not be the real secreted amounts of the antigenic proteins. To verify this, more attention to be paid on cell culture condition optimization and extraction measures. Finally, although the expression of such HBV surface antigens in mammalian cells was previously reported, application of pcDNA3 vector and Cos-7 host cell line is this study may offer the advantage of having stable and maintained integration or episomal states of the vector in addition to a good quality of proper human protein folding and modification.

Acknowledgments

The authors are deeply indented to Dr. Khabiri, A.R., Immunology Department, Pasteur Institute, Tehran, Iran, for performing ELISA technique. We thank the Research Council of Tehran University for the full financial support of the presented work under project number of 521/1/105.

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