

Isolation, Subtype Determination, Cloning and Expression of HBsAg Gene from an Iranian Carrier in *Saccharomyces cerevisiae*

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ABSTRACT

The Hepatitis B Surface antigen (HBsAg) gene was isolated from an Iranian HBeAg positive carrier by PCR. The gene was cloned in pUC19 for sequencing and pYES2 for expression in *Saccharomyces cerevisiae*, which pNF1 and pDF3 constructs were made respectively. The sequencing data showed that the isolated HBsAg gene shared more than 90% homology with the *ayw* subtype. The pDF3 was transferred into the yeast strain and expression of the HBsAg was induced. Yeast-derived HBsAg was purified by immunoaffinity chromatography and checked by ELISA, Western blot analysis and confirmatory ELISA. The immunogenicity test in mice showed that injection of the yeast-derived recombinant HBsAg could induce the immunologic response against the antigen. 320 fold dilution of the mouse serum was positive for anti-HBsAg. The amount of HBsAg expressed in yeast constitute 1% of the total cell soluble protein.

Keywords: HBV, HBsAg *S. cerevisiae*

INTRODUCTION

The human hepatitis B virus (HBV) belongs to a family of closely related DNA viruses called Hepadna viridae [1]. There are an estimated 300 million HBV carriers in the world and in areas of high prevalence such as south east Asia and Africa up to 20 % of the population are carriers [2]. The studies carried out in Iran have shown that the rate of endemicity in our country is medium [3]. There is a continuing need to assess appropriate immunization strategies which will vary according to the HBV prevalence rates in different parts of the world. The current trend in developing countries in which the rate of endemicity is high or medium is mass immunization of infants [4]. The absence of a cell culture system capable of propagating the virus has greatly impeded the development of a vaccine by classical approach and has led to the production of a vaccine from the serum of HBV chronic carriers [5, 6].

Despite the proven efficacy and safety of the plasma-derived vaccine, the need for an alternative method of subunit vaccine production was felt. Recombinant DNA technology has now developed to the stage where essentially any gene can be manipulated to give at least a reasonable level of expression in a variety of microbial or animal cells and genetic engineering procedures was used to produce subunit vaccines. The immunodominant epitope of 1113sAg, the so called domain a is

sufficient to raise the protective antibody, but since full immunogenicity of HBsAg is highly dependent on its conformation, the approach to produce HBsAg in *E. coli* was not successful and a eukaryotic system such as mammalian cells or yeast seemed essential [7, 8].

Several factors have made the baker's yeast the organism of choice for the production of HBsAg, including its long and successful history of use in baking and brewing industries, its ability to carry out desired posttranslational modifications, its suitability for large scale fermentation and the lower cost of the production in yeast in comparison with other systems. rHBsAg (recombinant I-113sAg) from transformed yeast strains have now been approved for routine use as a human vaccine [9]. However, in spite of the improvements the cost of the vaccine is still too high for infants mass vaccination in the developing countries. Therefore, the research in this field is still continuing to increase the expression level by using different constructs, enhance the purity, increase the thermostability that confer long term immunity after fewer doses, in order to produce a less expensive vaccine. Therefore, this project was undertaken in order to improve expression level of HBsAg in yeast by isolating the gene without non translating region (NTR) from human serum by PCR. These steps are essential in achieving the production of a subunit vaccine in Iran.

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MATERIALS AND METHODS

Strains and vectors. *E. coli* strain Top 10F' {mcrA, (mrr-hsdRMS-mcrBC), F8 lacZDM15, DlacX74, deoR, recA₁, araD139 i (ara, leu), 7697, galU, galK, r, rpsL, endA₁, nupG, F'} and *S. cerevisiae* strains: INVSC1 (MATa, his3-DI, leu2, trp1-289, ura3-52) were used as the recipient of the constructed vectors (Invitrogen Corp.). The *Saccharomyces cerevisiae-E. coli* high copy number shuttle vector pYES2 (Invitrogen Corp.) designed for inducible high level expression of recombinant proteins in *S. cerevisiae* and pUC19 vectors were used in this study. Plasmid digestion and cloning were performed according to standard procedures [10]. All enzymes were from Gibco BRL and were used according to the manufacturer's recommendations.

Isolation of HBsAg from Iranian Carriers. The serum from an Iranian HBsAg positive carrier (kindly provided by Hepatitis and Aids department, Pasteur Institute of Iran) was used for isolating the HBsAg gene, using PCR [11]. The following primers were designed using OLIGO and DNASIS (Hitachi software engineering) softwares and synthesized by DNA synthesizer (Applied Biosystem, USA): 5'-CCCTGCGGGATCCATGGAGAACATCACATCAG-3' and 5'-TTTTGTGAATTCTTAAATGTATACCCAAAGAC-3'. BamHI and EcoRI restriction sites (shown in bold face) were incorporated in the primers for further cloning.

PCR was performed for 60 cycles (two sets of 30 cycles) as follows: 1 min in 95°C, 1 min in 40°C, 1 min in 72°C and the last extension was prolonged for 5 min (Gene ATAQ controller, Pharmacia-LKB). Before starting the first run the reaction mixture was put at 95°C for 3 min (hot start). After the first run of 30 cycles, one unit of Taq DNA polymerase and 1 pi 100 mM dNTP mix was again added to the PCR mixture and the amplification procedure was done according to the above mentioned temperature profile. One ill of the PCR product was loaded on 1% agarose gel. The PCR product about 705 by in length was cloned in pUC19 and pYES2. The resulted plasmids were used for *E. coli* Top 10F' transformation. Transformants were selected on media containing 100 mg/ml ampicillin and the isolated constructs were subjected to both restriction endonuclease analysis and PCR using the following primers: 5'-GATTCC TAGGACCCCT

GCTCGTGTTAC-3' and lower 5'- AATTAGAGGACAAACGGGCAACATACC-3'. The temperature profile was: 1 mM at 93°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The last cycle was prolonged for 3 min at 72°C. The obtained PCR product was 310 base pairs in length. Sequencing of the gene was done by dideoxy chain termination technique using Autoread Sequencing kit and automated laser fluorescent DNA sequencer (Pharmacia LKB, Sweden), according to the manufacturer's recommendations.

Yeast transformation. The lithium acetate procedure was used for transformation of the *S. cerevisiae* strain INVSC1 [12]. All glassware and solutions used were sterile and all steps were carried out on ice. The transformants were screened using a synthetic minimal medium containing 0.67% yeast nitrogen base (Difco, USA), 1% glucose 0.1 mM histidine, 20 mg/l tryptophan, 30 mg/l leucine. The plates were incubated at 30°C and checked for a week for transformants.

Yeast Cell Growth and Disruption. Fermentation of yeast cell was carried out in a fed batch culture using above mentioned media at 30°C with aeration and a final volume of 2.5 liters. After glucose exhaustion galactose at a concentration of 1% (w/v) was added as the inducer and cells were incubated for further 24h. The pH of the medium was monitored by 5N sterile NaOH. The culture was then centrifuged (15 min, 10,000 ×g) and cell pellet was washed and dispersed in the disruption buffer containing; 50 mM sodium phosphate buffer, pH 7.2, 1 mM phenylmethylsulfonyl fluoride (Sigma, USA), 1 mM EDTA, 1% Triton X-100. The glass beads (212 to 300 microns, Sigma) were used for disruption and the cells were ground in the presence of liquid nitrogen. The mixture was pelleted at 12,000 ×g, 4° C for 30 min and the supernatant was assayed for total protein concentration [13] and HBsAg by ELISA. The cell extract was applied to the column for further purification, using immunoaffinity chromatography.

ELISA Test. Hepanostika HBsAg and Hepanostika ® HBsAg Uni-Form II Confirmatory Micro ELISA system (Organon Teknika B.V., Holland) were used for HBsAg detection and confirmation according to the manufacturer recommendations. The cut off value was determined in each set of

experiment, using positive and negative controls. During this experiment twice of the positive value given by the kit were considered as positive. Confirmation of the presence of HBsAg was indicated by a reduction of HBsAg immunoreactivity in the presence of specific anti HBsAg as compared to a non-neutralized sample of the same reactive specimen.

HBsAg Purification. HBsAg from yeast cells extract was purified with one step affinity chromatography, using rabbit anti-HBsAg antibody coupled to activate Sepharose CL-4B (Pharmacia, Sweden). Anti-HBsAg was raised in two healthy young New Zealand albino rabbits by injection of recombinant HBV vaccine (Heber Biotech, Cuba) in three doses. The first dose with Freund's complete adjuvant, the second two weeks later with incomplete Freund's adjuvant followed two weeks later with the 3rd dose without adjuvant. Antibody titers measured before each injection. Four weeks after the last injection the animals were bled by transthoracic heart puncture. Sepharose protein A (SPA) column was prepared according to the procedure recommended by the Pharmacia Company (Affinity chromatography, Principles and methods) for purifying the IgG.

The rabbit serum was diluted by Tris buffer (pH 8.6, 0.05M Tris-HCl, 0.15M NaCl) and loaded on SPA column (equal volume to the SPA column). The column was washed with Tris buffer (pH 8.6) until OD_{280} was zero. IgG was eluted by glycine-HCl buffer (0.05M glycine, 0.15M NaCl, titrated with HCl to pH 3.0). The fractions were collected in the tubes containing neutralization solution (0.5 M sodium phosphate buffer, pH 7.7). The peak fractions were pooled and the IgG thus obtained was examined by SDS-PAGE and used for coupling with Sepharose CL-4B. The collected antibodies were dialyzed overnight against 0.1 M $NaHCO_3$ /0.5 M NaCl. The antibody was centrifuged at $100,000 \times g$ (Beckman TL-100) to remove the aggregates. The supernatant was collected and its absorbance (OD_{280}) was measured to determine the concentration of IgG. The concentration of the IgG was adjusted to 5 mg/ml by 0.1 M $NaHCO_3$ and 0.5M NaCl. A weighed amount of activated Sepharose was transferred to a beaker and equal volume of antibody was added. After gentle stirring for 2 hours at room temperature 50 mM glycine, pH 8.0 was added to saturate the

remaining activated group and slurry was permitted to settle. Aliquot of the supernatant was centrifuged to remove any residual-Sepharose and its A_{280} was measured to compare with the initial value for an estimation of the coupling [14].

HBsAg purification and characterization. The Clarified yeast extract was applied to a column of Sepharose CL-4B (Pharmacia, Sweden) coupled with rabbit antiHBsAg-antibody. The flow rate was adjusted at two column volume per hour. The column was washed with five column volume of TBS (10mM Tris-HCl, 0.15 M NaCl, pH 7.4) buffer (to reach the zero absorbance) and the HBsAg was eluted with 3M KSCN. Fractions containing HBsAg were pooled and dialyzed against TBE buffer to remove KSCN and then concentrated using PEG 20,000. Molecular weight estimation of the purified protein was done, using SDS-PAGE [15]. Samples were boiled in loading buffer (1.6% SDS, 1.5% DTT) for 15 min and loaded onto the 15% gel and the gel was silver stained [16]. The molecular weight markers used were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa) and -galactosidase (116 kDa) which were purchased from Sigma. The HBsAg extracted from sera (kindly provided by Mr. Khabiri, Immunology Dept., Pasteur Institute of Iran) was run alongside the yeast derived one as a control.

For blotting the proteins were transferred onto nitrocellulose membrane (Sigma, 0.2m pore size), using semi-dry transfer system (Novablot Transfer, Pharmacia). The membrane was removed and stained, using rabbit anti-HBsAg antibody and protein A-HRP conjugated (Sigma). Bands were visualized by 4CN(4-chloro-1-naphthol, Sigma) [14].

Immunogenicity testing. Immunogenicity was tested by immunization of five 6 weeks-old Balb/C mice with partially purified S antigen after passing through the 0.2 μ Millipore membrane filter for sterilization. Three doses of about 5 μ g of the antigen were injected with 4 weeks interval. Complete and incomplete Freund's adjuvant were used for the first and second injection and the third one was injected with only S antigen in PBS. Three weeks after the third injection the antibody level was tested, using ELISA.

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pDF3      CCTGCGGGATCCATGGAGAAACATCACAATCCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCCGGGGTTT 70
HBVayw    ATGGAGAACAATCACAATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCCGGGGTTT

pDF3      TTCTTGTTGACAAGAATCCTCACAAATACCGGAGAGTCTAGACTCGTGGTGAGACTTCTCTCAATTTTCTAG 140
HBVayw    TTCTTGTTGACAAGAATCCTCACAAATACCGCAGAGTCTAGACTCGTGGTGAGACTTCTCTCAATTTTCTAG

pDF3      GGGGAACCTACCGTGTGTCTGGCCAAAATTCGCAGTCCCAACCTCCAACTCACTCAACCAACCTCTGTCC 210
HBVayw    GGGGAACCTACCGTGTGTCTGGCCAAAATTCGCAGTCCCAACCTCCAACTCACTCAACCAACCTCTGTCC

pDF3      TCCAACTTGTCCTGGTTATCGCTGGATGTTCTGGGGCGTTTATCAATTTCTCTTCATCCTCTGCTGCTA 280
HBVayw    TCCAACTTGTCCTGGTTATCGCTGGATGTTCTGGGGCGTTTATCAATTTCTCTTCATCCTCTGCTGCTA

pDF3      TGCCTCATCTTCTTGTTGGTCTCTGGACTATCAAGGTATGTTGCCCGTTHGTCTCTAAATTCAGGAT 350
HBVayw    TGCCTCATCTTCTTGTTGGTCTCTGGACTATCAAGGTATGTTGCCCGTTHGTCTCTAAATTCAGGAT

pDF3      CTTCAACTACCAGCACGGGACCATGCGAGAACCTGCACGACTCTCTGCTCAAGGAACCTCTATGTATCCCTCC 420
HBVayw    CTTCAACTACCAGCACGGGACCATGCGAGAACCTGCACGACTCTCTGCTCAAGGAACCTCTATGTATCCCTCC

pDF3      TGTGCTGTACCAAACTTCGGACGGAAAATGGCACCTGTATCCCATCCCATCATCCTGGGCTTTCGGAA 490
HBVayw    TGTGCTGTACCAAACTTCGGACGGAAAATGGCAAATGGCACCTGTATCCCATCCCATCATCCTGGGCTTTCGGAA

pDF3      AATTCCTATGGGAGTGGGCTCAGCCCGTTHCTCTCGGCTCAGTTTACTAGTGCCCAATTTGTTCAGTGGTT 560
HBVayw    AATTCCTATGGGAGTGGGCTCAGCCCGTTHCTCTCGGCTCAGTTTACTAGTGCCCAATTTGTTCAGTGGTT

pDF3      CGTAGGGCTTTCCTCCACTGTTTGGCTTTCAGTTATATGGATGATGGTAATGGGGGCCAAGTCTGTAC 630
HBVayw    CGTAGGGCTTTCCTCCACTGTTTGGCTTTCAGTTATATGGATGATGGTAATGGGGGCCAAGTCTGTAC

pDF3      AGCATCTTGAGTCCCTTTTACCCTGTTACCAATTTCTCTGCTTGGGTATACATTTAAGAATTCACAAAA 700
HBVayw    AGCATCTTGAGTCCCTTTTACCCTGTTACCAATTTCTCTGCTTGGGTATACATTTAAGAATTCACAAAA

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Fig. 1. The sequence of the isolated HBsAg gene and its comparison to the reported sequence of the HBV subtype ayw (Gene bank accession number: X02 H96). The different bases are shown in small letter.

RESULTS

pDF3 and pNF1 constructions. HBsAg gene isolated by PCR, was cloned in pUC19 and the construct was designated pNF1 which was used for sequencing. The other construct which were made by cloning the HBsAg gene into the expression vector pYES2 was designated pDF3.

The sequence of the isolated HBsAg gene and its comparison to the reported sequence of the HBV subtype *ayw* (Gene bank accession number: X0ZH96) is shown in fig 1. The gene isolated in our study shows more than 90% homology with the reported *ayw* subtype. The primers used are shown underlined.



Fig. 2. SDS-PAGE analysis of the purified yeast derived HBsAg. A) total soluble extract from yeast cells expressing HBsAg. B) HBsAg isolated from a human carrier and C) purified recombinant yeast derived HBsAg. D) Molecular weight markers (kDa).

Expression of HBsAg in *S. cerevisiae*. The results of the ELISA tests showed the presence of HBsAg in the samples (OD of yeast extract was 2.41, 1/50 dilution of eluted sample was 2.50 and 1/100 dilution was 1.55). And also the expressed protein had the antigenic determinants of the natural serum HBsAg. The negative control in the case of yeast extract was the cells harboring the pDF3 but were grown in an uninduced condition (glucose), in the case of purified HBsAg the negative control was the buffer used for dialysis. The total volume of the concentrated fraction which

was isolated from the immunoaffinity column was 4.7 ml. The HBsAg concentration obtained from 2.5 liters of primary yeast culture was 495.8 μ g (198.3 μ g/L). By considering the total cell soluble protein which was 10.314 mg/ml, the rate of HBsAg which was isolated to the total cell soluble protein was 0.01 or 1%.

Confirmatory results: The results of the confirmatory test showed that the HBsAg expressed in the *S. cerevisiae* was neutralized by the anti-HBsAg antibody supplied with the kit. It means that the recombinant HBsAg expressed in yeast has a correct tertiary structure which could be recognized properly by the anti-HBsAg antibody.

Western blot analysis and Immunogenicity test. The purified yeast derived recombinant HBsAg was analysed in SDS-PAGE (Fig. 2). The size of the HBsAg monomer expressed in yeast was estimated to be about 25 kDa. The size of it was smaller than the monomers of the antigen which was purified from the sera. Besides the monomer the trimer and dimer forms of the antigen could also be observed. The protein bands were transferred to the nitrocellulose membrane for Western blot analysis and antibodies raised against rHBsAg were used for detection (Fig. 3). It can be seen that the HBsAg which was expressed in yeast was smaller than the antigen isolated from the sera. The trimer, dimer and monomer of the antigen protein were also detected.

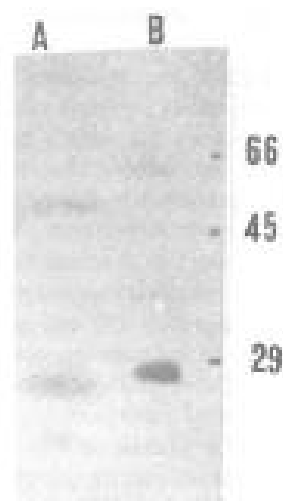


Fig. 3. Western blot analysis of the rHBsAg. A) recombinant yeast-derived Ag. B) serum-derived HBsAg. Numbers indicate the sites of MW markers (kDa).

In order to check the immunogenicity of the isolated yeast-derived HBsAg group of mice were injected with the purified antigen (Fig. 4.). It was shown that the HBsAg which was expressed in yeast could raise the anti-HBsAg response in mouse. ELISA test showed that 1/320 fourth dilution of the mouse sera was still positive for anti-HBsAg.

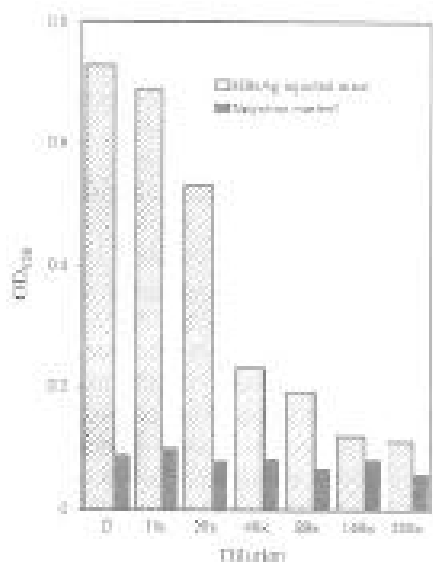


Fig. 4. Immunogenicity test result of the rHBsAg in mice. The negative control was mice injected with PBS.

DISCUSSION

The envelope of the infectious Dane particle of HBV contains all three forms of proteins (large, middle and major), but with a predominance of major protein and variable amounts of middle (M) and large (L) proteins which are encoded by pre-S₂ and pre-S₁ regions respectively. The sera of infected individuals also contains large numbers of empty viral envelopes composed predominantly of major envelope protein (S) and sometimes small amounts of the M and trace amounts of L [17]. The first vaccine against HBV to be used in human involved injection of such particles, which had been purified from the plasma of chronic carriers [6]. This plasma-derived vaccine was effective, but was not appropriate for mass immunization because of limited supply of chronically infected human plasma.

In the preliminary studies to produce the vaccine, HBsAg (subtype *adw*) was expressed in *S. cerevisiae*, using an expression vector containing yeast alcohol dehydrogenase-I as a promoter. In this

study they showed that HBsAg can be isolated in a highly purified form by a single application of immune affinity chromatography [18]. Our results also showed that this procedure can yield a product pure enough for further analysis. It was previously shown [19] that the long non-translated regions in mRNA could reduce the transcriptional efficiency and/or alter its stability. Comparing the rate of the expression of the HBsAg in this study with the results obtained in our previous work (Farhoudi-Moghaddam et al., submitted for publication) it was found that removing the NTR from both 3' and 5' sides of the gene, increased the product yield about 100 times (from 0.01% to 1% of the total cell soluble protein). It was also supposed that the yield of the HBsAg particle from yeast depends largely on the condition of cell disruption, in other word changing these conditions and further processing may help to increase the yield of HBsAg particles. It means that further works in these areas are justifiable.

In other study [20] the vector constructed for expression of HBsAg contained the glyceraldehyde-3-phosphate dehydrogenase gene promoter and a chemically synthesized DNA segment that optimized the translation initiation in yeast. With this cassette they received the level of 1-2% of total cell protein which that constructing new expression vectors containing sequences like upstream activating one can augment the production of the gene.

Results obtained from other studies showed that the mature form of HBsAg, held together by disulfide bonds, was not present in the initial yeast cell extract but rather must be made outside the cells by some chemical process. After cell lysis, the clarified extract contained HBsAg particles that have the same sedimentation coefficient as mature particles but are not held together by covalent bonds. This initial particle was called form I. It migrates as a 25 kDa monomer in SDS-PAGE under non-reducing conditions. During early phases of purification, some interchain disulfide bonds form so that the antigen migrates as a mixture of monomer and dimer under non-reducing conditions. This disulfide-linked dimer recognized in non-reducing SDS-PAGE was called form II. After treatment with concentrated ammonium or potassium thiocyanate, additional interchain disulfide bounds formed between dimmers and the fully cross-linked particles was obtained. This mature protein was called form III which could not enter the running gel of the SDS-

PAGE system, unless a reducing agent was used. These three forms do not represent differences in aggregation state (particle size). The monomer was co-migrated with major band, the second one apparent molecular weight of 45 kDa.

Immunoblotting detects all of the HBsAg polypeptide, regardless of formation or aggregation state [20, 21]. In our study even by increasing the concentration of the reducing agents the rate of dimer and trimer forms in SDS-PAGE was more than that reported by others, which could be resulted by promotion of thiocyanate in formation of interchain disulfide bonds.

In SmithKline Biological Company which is one of the firms producing HBV vaccine in the market a study was carried out on the expression of HBsAg in *S. cerevisiae* [22]. The vector which was used directed the synthesis of about 250 µg HBsAg per 400 mg total cell protein extracted from one liter of bradytroph culture. During our work the synthesis of HBsAg was about 198.3 µg/l. It seems that this initial result justify the further work for optimizing the growth culture, extraction, production and purification conditions. We have used the Gall promoter which was different from that, which was used by them and this is the first report of the efficiency of Gall promoter in expressing the HBsAg gene. It must be mentioned that during our experiment, switching on the promoter from the first (cultivating the yeast transformant in media containing only galactose as carbon source) was deleterious to the survival of the cells. In another work the yeast 3-phosphoglycerate kinase promoter and a plasmid capable of autonomous replication in yeast was used for expression of HBsAg [22]. Cells containing this plasmid produce non-glycosylated form of the HBsAg at a level of about 1-2 percent of total cellular protein, which this estimation was based on band intensity (on SDS-PAGE) with respect to other yeast proteins.

In all of the reported expression of HBsAg in east, it seems that this system could not excrete the product into the medium. This was also seen in expression of HBsAg in insect cells using Baculovirus expression vector. Insect cells were not capable of efficiently secreting HBsAg particles, which was the difference observed between them and mammalian cells. However, efficient secretion of mammalian proteins in insect cells was observed. The requirements for HBsAg secretion may be significantly different than for most

proteins, since HBsAg secreted as a very large lipoprotein particle [24, 25].

S. cerevisiae offered certain advantages over bacteria as a host for the production of eukaryotic proteins. However, features as secretion, glycosylation pattern and expression level of a given protein are highly species specific. For extending the spectrum of proteins which can be faithfully and efficiently expressed for industrial purposes, the additional yeast expression systems, other than *S. cerevisiae* was being used. Besides, the expression of HBsAg in *S. cerevisiae*, its production was so verified in *Hansenula polymorpha* and *Pichia pastoris*. In these two systems the integrative vectors were used and both of them were methylotrophic yeasts [26, 27]. In both of them the expressed S protein was un-glycosylated and also was not secreted, but the yield of the production because of using a powerful alcohol oxidase promoter was different. As can be seen in Figs 2 and 3, the HBsAg produced in the system which was used by us was also un-glycosylated. Expressing the HBsAg under the control of alcohol oxidase promoter and expressing it in other yeast system to compare the yield of production is under study.

ACKNOWLEDGMENTS

We gratefully acknowledge the help of Dr. S. Amini from Hepatitis and AIDS Dept. for performing ELISA tests, Mr. A.R. Khabiri from Immunology Dept. for helping with purification of HBsAg, Dr. M. Azizi from Biotechnology Dept. for sequencing and my colleagues in Molecular Biology Unit for stimulating discussions.

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