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An OTHBVS Cell Line Expresses the Human HBV Middle S Protein

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An OTHBVS cell line from HepG2 was established. This cell line stably expresses the human hepatitis B virus (HBV) middle S protein that includes the preS2 region which is important for HBV particle entry into the hepatocyte. To establish this cell line, the middle S open reading frame (ORF), with a promoter located in the 5' region and enhancer located in the 3' region, was cloned downstream from the metallothionine (MT) promoter of the OT1529 vector. In this vector, expression of the middle S protein was constructed to be regulated by its own promoter and enhancer. Expression of the large S protein which contains the preS1 region in addition to the middle S protein was designed to be regulated by the MT promoter. When extracts of OTHBVS cells were examined with an S protein detection kit (RPHA, Korea Green Cross Co.), an S protein was detected. Total mRNA of OTHBVS cell examined by northern blot analysis with an S ORF probe revealed small/middle S transcripts (2.1 kb). When the MT promoter was induced by Zn, large S transcripts (2.4 kb) were detected. The GP36 and GP33 middle S proteins were presumably detected, but large S proteins were not detected by immunostain analysis using anti-preS2 antibody.

Key words: HBV, HepG2, surface protein, metallothionine promotor, LTR

Human HBV, the prototype member of a small family of hepadnaviruses, is a major cause of liver disease ranging in severity from chronic infection of hepatocytes to liver cirrhosis and hepatocellular carcinoma (1). The HBV particle is enveloped with three kinds of surface proteins, a small S protein which contains only the S region, a middle S protein which contains both the S region and the preS2 region, and a large S protein which contains a preS1 region in addition to the middle S protein (8). The HBV produces several types of transcripts. The 2.1kb transcript mRNA encodes middle S and small S proteins and the 2.4 kb transcript encodes the large S protein (10). After translation, these proteins are embedded into the endoplasmic reticulum and some of them are glycosylated (7). These glycosylated proteins are important as signals of secretion. These modified or native surface proteins envelop a complex called capsid consisting of core proteins which pack the HBV genome and polymerase. The HBV genome is replicated from pregenomic mRNA inside the capsid. The enveloped particle is secreted by means of glycosylated surface proteins (4). A few of these embedded surface proteins are self-assembled into small particles and secreted. Recent reports indicated that the preS2 region of the middle S protein is important for binding of the HBV particle to the hepatocyte receptor molecule. It is also suggested that the middle S protein plays an important role for entry into the hepatocyte cell (9). The transcription of small/middle S gene is efficient in the hepatocyte cell line because of hepatocyte specific transcription factors which are known as hepatocyte nuclear factor series (HNF) (3,5). This causes efficient expression of the middle S protein which is regulated by its own promoter. A recombinant OTHBVS vector is to be expressed in the hepatocyte cell line because it contains hepatocyte specific nuclear factors. This vector was transfected into the HepG2 cell line and this cell line produced middle S proteins.

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Materials and Methods

Vector construction

The human HBV gene, subtype *adr*, fragment from 2864 to 1300, was cloned into multiple cloning sites downstream from the MT promoter of the OT1529 vector which contains LTR and the neo^r marker. Therefore, these constructs contain preS1 region to enhancer region of the HBV genome (Fig. 1).

Cells and transfection

HepG2 cells were cultured in MEM medium supplemented with 10% FBS. To transfect cells with the OTHBVS construct, 1.6×10^6 cells were seeded

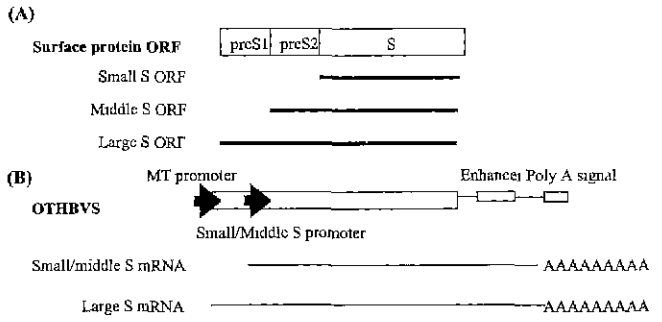

Fig. 1. *Surface protein ORF organization in human HBV* genome and OTHBVS vector construction. (A) ORF region and domain organization of three surface proteins. (B) OTHBVS vector construction for expression of surface proteins. Its own promoter and enhancer regulate transcription of small/middle S mRNA. Thus, small and middle S proteins are constitutively expressed in HepG2 cells, the hepatoblastoma cell line. If the OTHBVS cell line is cultured in MEM medium containing 150 μM Zn, the MT promoter is induced and large S mRNA is transcribed.

in 60-mm plates and cultured overnight. OTHBVS construct DNA was mixed with 2M $CaCl_2$. This mixture was slowly dropped into 2 × Hepes buffer and incubated 30 min at room temperature. This mixture was added dropwise into overnight cultured cells and incubated overnight. Transfected cells were washed with PBS several times and added with 5 ml of MEM medium. After two days, geneticin was added into culture medium to a final concentration of 0.2 mg/ml and the cells were incubated until colonies were visible. The colonies were cultured separately and geneticin concentration in culture medium was increased gradually. In this process, the most resistant cell line was selected.

Detection of surface protein expression with RPHA kit

The screened cells were cultured in MEM medium supplemented with 10% FBS containing 0.5 mg/ml geneticin. At confluence, these cells were lysed with buffer A (30 mM potassium phosphate, 1mM EDTA, 20 mM 2-mercaptoethanol) by sonication and clarified by centrifugation. The volume of buffer A was 1/10 of the culture medium volume. The resulting supernatant was serially diluted with buffer A and tested with a RPHA test kit which can detect small S proteins.

Northern blot analysis

The mRNAs were extracted with PolyATtract® mRNA Isolation Systems (Promega) and were electrophoresed in 1% agarose gel. Separated mRNAs in the agarose gel were transferred to a nylon membrane and hybridized with an S-ORF probe. The hybridized membrane was developed with Dig-High Prime(Roche Molecular Biochemicals).

Western blot analysis

The extracts of cells induced by Zn were separated in 10% polyacrylamide gel and were transferred to a PVDF membrane. The membrane was blocked with 10% skim milk and incubated with anti-preS2 antibody. The membrane was then incubated with secondary anti-rabbit antibody and developed with ECL (Amersham Co.)

Results and Discussion

The OTHBVS construct was transfected into HepG2 cells by the calcium chloride method. To select the cell line containing the OTHBVS construct integrated by the LTR element into the chromosome, geneticin was added into the culture medium to a final concentration of 0.2 mg/ml. In this process, resistant cells formed colonies and they were cultured separately in each culture flask. Each separated colony was cultured in MEM medium with increasing concentrations of geneticin. The most resistant cell line survived at a concentration of 0.5 mg/ml geneticin. It was thought that the most resistant cell line has the most number of integrated OTHBVS vectors. This causes more S proteins to be expressed.

The selected cell line was analyzed with a RPHA detection kit to determine whether the surface protein was expressed or not. The result of the RPHA test indicates that this cell line expresses the surface protein in normal culture medium containing 0.5 mg/ml geneticin. The amount of surface protein

Table 1. Detection of Human HBV surface protein expression with RPHA test kit[*]

Sample	Dilution fold	Relative optical density
Hepavax-B[a]	1/8	+++
	1/160	+++
	1/320	+++
U-vax[b]	1/8	++
	1/160	++
	1/320	+
OTHBVS extract[c]	1/4	++
	1/6	++
	1/8	++

[*] Extracts of OTHBVS cells were tested with RPHA kit that detect the S domain region, thus all kinds of surface proteins could be detected.

[a, b] Concentration of each sample was 20 mg/ml

[c] The OTHBVS cells were lysed with buffer A (30 mM potassium phosphate, 1mM EDTA, 20 mM 2-mercaptoethanol) by sonication and clarified by centrifugation. The volume of buffer A used was 1/10 of the culture medium.

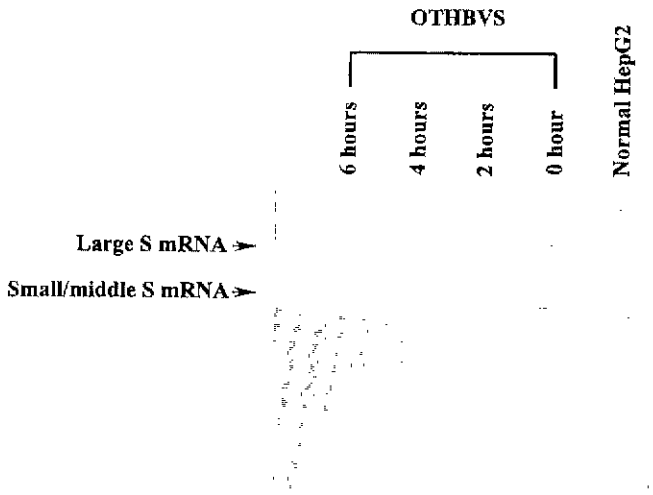

Fig. 2. Northern blot analysis with mRNAs from OTHBVS cells induced with a Zn. The small/middle S mRNA band(lower band) was detected in cells which were not induced by Zn. After inducing for two and four hours, not only the small/middle S mRNA but also large S mRNA was detected. These results indicate that small/middle S mRNA was constitutively transcribed. When the MT promoter was induced by Zn, large S mRNA was transcribed from the integrated OTHBVS vector DNA.

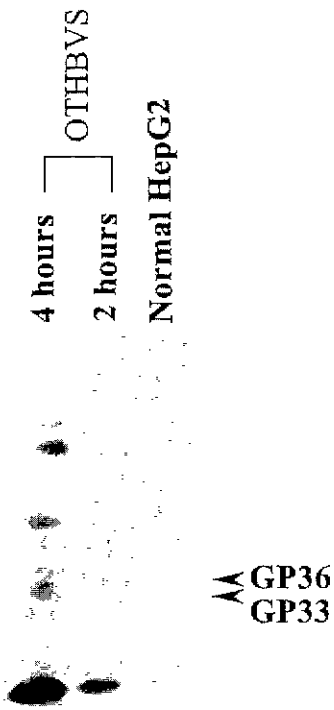

Fig. 3. The extracts of OTHBVS were immunostained with anti-preS2. After 2 and 4 hours of induction, GP33 and GP36 band were presumably detected. However, when the MT promoter was induced by Zn, the large S protein was not detected.

was compared with those of existing vaccinces such as Hepavax B (Korea Green Cross Co.) or U-vax (LG Chemical Ltd.), which were developed for prevention of HBV infection and composed of small S proteins. The concentration of the expressed surface protein was roughly equal to the 1/25 of the U-vax's concentration (Table 1).

In the recombinant OTHBVS vector, large S protein expression was designed to be regulated by the MT promoter. The cell line which expressed the surface protein was named OTHBVS. After this cell line was cultured in MEM medium containing 150 μM Zn to induce the MT promoter, cells were harvested at 2 h intervals. The mRNAs from each harvested cell were analyzed by northern blot analysis with an S-ORF probe. In the analysis of mRNAs of cells not induced by Zn, only the small/middle S mRNA band was detected. Two or four hours after induction, not only a small/middle S mRNA band, but a large S mRNA band was also detected. However, after 6 hours, neither bands were detected (Fig. 2). Cells may have died due to the presence of Zn. The above data indicates that small/middle S mRNA was transcribed constitutively and large S mRNA was transcribed through induction by the MT promoter.

These induced cells were analyzed by Western blot analysis with anti-preS2 antibody. Only two bands were detected and those were presumably two middle S proteins, GP33 and GP36. Although large S mRNA was detected, large S proteins were not detected in this analysis (Fig. 3).

The above data conclusively indicates that the HBV middle S protein was expressed in HepG2 cells by its own promoter and enhancer. Thus, transcriptional regulation of middle S protein expression and modification may occur similarly *in vivo*. In a recent report, it was stated that the preS2 region activates the tumor gene through a PKC-dependent cascade pathway (2). Furthermore, this region is important for HBV infection as a receptor binding protein.

Although many cell-lines expressing surface proteins have been established, this is the first cell line expressing only the small/middle S protein and the expression was regulated by its own regulatory element. The expression of middle S protein was confirmed in this report. The OTHBVS cell line will be useful for future studies on the regulation of middle S protein expression.

Acknowledgments

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