Stable Expression of the Hepatitis B Virus Surface Antigen Containing Pre-S2 Protein in Mouse Cells Using a Bovine Papillomavirus Vector

By TETSUO YONEYAMA,* TOSHITAKA AKATSUKA AND TATSUO MIYAMURA

Department of Enteroviruses, National Institute of Health, 10-35, Kamiosaki, 2-Chome, Shinagawa-ku, Tokyo 141, Japan

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SUMMARY

The large BglII fragment (2-8 kilobases) of hepatitis B virus DNA including the transcription unit for the hepatitis B surface antigen (HBsAg) was inserted into a bovine papillomavirus vector containing the neomycin resistance gene. The recombinant DNA was transfected into mouse C127 cells. A stable transformed cell line (MS128) secreting a large amount of 22 nm HBsAg particles containing pre-S2 protein was established. The secreted HBsAg particles had the receptor for polymerized human serum albumin. Immunoprecipitation and Western blot analyses showed that HBsAg particles consisted of two major proteins of 22K and 26K encoded by the S gene and a minor protein of 35K encoded by the pre-S2 and S genes. Southern blot analysis revealed that the transfected plasmid was integrated into the host chromosomal DNA and that most of the plasmid sequences were present. These results suggest that the stable expression of the HBsAg in MS128 cells is related to the integrated state of the recombinant DNA.

INTRODUCTION

Hepatitis B virus (HBV) is a causative agent of acute and chronic hepatitis in humans and is considered to be implicated in primary liver cancer (Beasley & Hwang, 1984). The hepatitis B surface antigen (HBsAg) is found in sera of infected individuals as 22 nm spherical particles, filamentous structures of similar diameter and 42 nm spherical particles (infectious HBV) (Tiollais et al., 1985). The major HBsAg protein consisting of 226 amino acids is present in non-glycosylated (22K) and glycosylated (26K) forms (Peterson, 1981). Two sets of less abundant larger HBsAg-related proteins encoded by the pre-S region have been identified (Heermann et al., 1984; Stibbe & Gerlich, 1983). One of these larger proteins, called middle S (GP33/GP36), contains 55 additional amino acids (pre-S2) at the amino terminus of the major HBsAg protein. The other, called large S (P39/GP42), contains an extra 108 amino acids (pre-S1) at the amino terminus of middle S. Pre-S proteins are thought to be involved in virus attachment to hepatocytes (Machida et al., 1983; Neurath et al., 1986) and in enhanced immunogenicity (Milich et al., 1985; Neurath et al., 1985). Therefore, much attention has been focused upon the role of pre-S proteins in HBV infection.

Transfection of mammalian cells with a variety of recombinants containing the HBsAg gene has led to success in producing HBsAg particles in cultured cells (Tiollais et al., 1985). Taking advantage of the episomal nature of the viral genome, bovine papillomavirus (BPV) DNA has been used as a vector to express the HBsAg gene in mouse cells (Denniston et al., 1984; Hsiung et al., 1984; Stenlund et al., 1983; Wang et al., 1983). Expression is, however, sometimes unstable in the cell cultures after a long passage. Here we report the introduction of the HBsAg DNA into mouse cells by means of a BPV vector and the establishment of a cell line (MS128) which stably produces HBsAg particles containing pre-S2 protein at a high level. In this cell line, the HBsAg DNA was integrated into the host cell chromosomal DNA.
Methods

Construction of recombinants. The HBV DNA used in our studies was derived from an adr subtype clone (pBRHBVadr72) (Fujiyama et al., 1983). The vector, pDBPV-MMTneo (342-12), which contains whole BPV DNA, pML2 DNA, simian virus 40-derived splicing and poly(A) addition signals (SV), the mouse metallothionein promoter (MMT) and the neomycin phosphotransferase gene (Neo). Four recombinants (at two BamHI sites and in two opposite directions) were constructed. One of the four constructs, which yielded MS128 cells, is illustrated here. Restriction sites: B, BamHI; H, HindIII; G, BglII; X, XhoI; A, AatI; E, BstEII.

Cell culture, transfection and cell cloning. Mouse C127 cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% foetal bovine serum (FBS), penicillin at 100 units/ml and streptomycin at 100 μg/ml. Cells (5 x 10⁵ cells per plate) were transfected with 10 μg of recombinant DNA without carrier DNAs using the calcium phosphate coprecipitation method (Graham & Van der Eb, 1973), and maintained in selection medium containing neomycin (G-418; Gibco) at 1 mg/ml. Individual colonies were isolated and tested for their HBsAg production in culture medium. The selected cell colony was further cloned by limiting dilutions, and the cell clone (MS128) producing the largest amount of HBsAg was selected and used for further experiments.

Analysis of cell DNA. Total cell DNA was extracted from monolayer cultures of transformed cells by the method of Gross-Bellard et al. (1973). The DNA (10 μg) was digested with appropriate restriction enzymes and separated on a 0.8% agarose gel. As a control, 2 ng of the recombinant plasmid pH2 plus 10 μg of C127 cell DNA were similarly digested and separated on the same gel. The resultant fragments were transferred bidirectionally and analysed by blot hybridization (Southern, 1975; Smith & Summers, 1980). As probes, HBV and BPV-pML DNAs were radiolabelled to a specific activity of >10⁶ c.p.m./μg by nick translation with [α-32P]dCTP (Rigby et al., 1977). Since the mouse metallothionein sequence in the pDBPV-MMTneo vector (342-12) caused a strong background hybridization signal, the BPV-pML vector without the mouse metallothionein sequence (230-08) (Denniston et al., 1984) was used as a probe, instead of the pDBPV-MMTneo vector.
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Purification and analysis of HBsAg particles. Confluent monolayers of MS128 cells were maintained in 5% FBS-DME medium at 37°C. Medium was collected from the cultures and HBsAg was concentrated by precipitation with ammonium sulphate and low speed centrifugation. The pellets were dissolved in 10 mM-Tris-HCl (pH 7.5). After exhaustive dialysis, a portion (0.5 ml) was layered on a 12 ml discontinuous 1.1 to 1.4 g/ml CsCl density gradient and centrifuged at 30000 r.p.m. at 5°C for 24 h in a Beckman SW41 rotor. HBsAg-positive fractions were pooled, layered on a 12 ml 5 to 30% sucrose gradient and centrifuged at 40000 r.p.m. at 5°C for 4 h in an SW41 rotor. HBsAg particles in HBsAg-positive fractions were used for electron microscopy and Western blot analysis.

Labelling and immunoprecipitation of HBsAg. MS128 cells were cultured in methionine-depleted medium, and then labelled for 16 h with [35S]methionine (Amersham; 1 mCi/mmol). For glycosylation analysis, cells were preincubated for 1 h in the presence of tunicamycin (10 µg/ml) and radiolabelled in the same way. Supernatants of the culture medium were incubated with anti-HBsAg rabbit serum or with preimmune rabbit serum at 0°C for 1 h. The immunoglobulins bound to Staphylococcus aureus Cowan I strain were washed and eluted in 2.3% SDS and 5% 2-mercaptoethanol. Samples were electrophoresed on an SDS-13% polyacrylamide gel and autoradiographed.

Western blotting. Purified HBsAg particles were denatured, subjected to SDS-PAGE (13%) and transferred to nitrocellulose paper (Towbin et al., 1979). Antisera used for detection were anti-HBsAg rabbit serum, and mouse monoclonal antibodies against synthetic peptides of pre-S1 and pre-S2, which consisted of 13 amino acid residues from positions 95 to 107 in the pre-S1 region, and 19 amino acid residues from positions 14 to 32 in the pre-S2 region (Okamoto et al., 1985).

RESULTS

HBsAg production by mouse cell transformants

Mouse C127 cells were transfected with recombinant DNAs (Fig. 1) and maintained in selection medium containing 1 mg/ml of neomycin. Cells in neomycin-resistant colonies were picked and amplified. Supernatants from each of these cultures were assayed for HBsAg. Thirteen out of 27 colonies tested were found to be positive in radioimmunoassay tests (Ausria II; Dainabot, Tokyo, Japan). However, most of these colonies were unstable in their expression of HBsAg when passaged (data not shown). One of these cell colonies (BHS21), which showed stable and efficient expression of HBsAg, was selected and further cloned by limiting dilution. From 137 examined the cell clone (MS128) producing the largest amount of HBsAg was selected. In MS128 cells secretion of HBsAg at an average rate of 18 µg/10⁷ cells/day was stable for 51 days (data not shown). Initially, MS128 cells showed a flat non-transformed morphology like that of the original C127 cells, but changed to a fully transformed morphology during further passages (Fig. 2), although HBsAg production was constant in these passages.

Analysis of recombinant DNA in MS128 cell DNA

To examine whether the recombinant DNA (pBH2) was present extrachromosomally we analysed the cell DNA by Southern blot analysis. Total DNA from MS128 cells was treated with restriction endonucleases that recognize a single site (XhoI), or multiple sites (HindIII, BstEII, AvaI) within the recombinant pBH2. As a control, pBH2 itself was cleaved in the same manner. When XhoI was used, a band in the position expected for a linear DNA (17.4 kb) was detected in the digest of pBH2 (Fig. 3 a, b, lane 1), and two bands larger than the linear DNA were detected in MS128 cell DNA (Fig. 3 a, b, lane 5), indicating that the pBH2 sequence was integrated in high Mr cell DNA and was not present in an episomal form. Digestion of the DNAs with HindIII (Fig. 3 a, b, lanes 2 and 6) led to the same conclusion. Digestion of pBH2 DNA with BstEII yielded 8.6 and 4.4 kb bands (Fig. 3 a, b, lane 3). The band of 4.4 kb was considered to contain two fragments with similar sizes. Digestion of the MS128 cell DNA with BstEII yielded 8.6, 4.4 and 4.0 kb bands (Fig. 3 a, b, lane 7). This indicated that sequences of about 400 nucleotides in the original recombinant had been deleted on integration and that the integration occurred near the BstEII site in cellular sequences. Digestion of pBH2 with AvaI produced four fragments, which hybridized with BPV-pML DNA (Fig. 3 a, lane 4) and three fragments which hybridized with HBV DNA (Fig. 3 b, lane 4). The shortest HBV-specific fragment (0.9 kb) and the largest 5.5 kb fragment which hybridized with the BPV-pML DNA probe were missing and
Fig. 2. Morphology of (a) untransformed C127 cells and (b) transformed MS128 cells.

Fig. 3. Blot analysis of DNA from MS128 cells and from the recombinant pBH2. For (a) and (b), 10 μg of cell DNA was digested with XhoI (lanes 1 and 5), HindIII (lanes 2 and 6), BstEII (lanes 3 and 7) and AvaI (lanes 4 and 8). Digested DNA was electrophoresed through a 0.8% agarose gel, and transferred bidirectionally to nitrocellulose papers. Each paper was hybridized with either radiolabelled BPV-pML DNA (a) or HBV DNA (b). The pBH2 DNA sample was a mixture of 2 ng of pure pBH2 DNA and 10 μg of C127 cell DNA, corresponding to 80 copies of pBH2 sequences per cell, was used. DNA from the recombinant (pBH2) is shown in lanes 1 to 4, and cell DNA from MS128 in lanes 5 to 8. Sizes of the marker fragments are indicated. (c) A scheme for the integration state of the recombinant DNA in MS128 cells is shown. Restriction sites A, E, H and X indicate AvaI, BstEII, HindIII and XhoI, respectively. The hatched area indicates the pre-S region; the dotted area, the S gene; and the thick bar, the flanking cellular DNA. Abbreviations as for Fig. 1.
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Fig. 4. Electron micrograph of HBsAg particles released into the culture medium of MS128 cells. HBsAg particles were purified by CsCl density gradient centrifugation followed by sucrose gradient centrifugation. The particles were stained with 2% uranyl acetate. Bar marker represents 100 nm.

high Mr DNA bands appeared in MS128 cell DNA (Fig. 3a, b, lanes 4 and 8). This indicated that the AvaI site which was located in the upstream portion of the pre-S gene was missing and the integration occurred near the missing AvaI site.

A scheme for the integration state of pBH2 DNA in MS128 cells is shown in Fig. 3(c). Southern blot analysis indicated that the transfected plasmid was integrated in the upstream portion of the pre-S region in MS128 DNA, that most of the plasmid sequences were present, and that the pre-S region and the S gene were conserved. From the intensity of the bands in Fig. 3, the copy number of the integrated plasmid was estimated to be 20 to 40 copies per cell. In other cell colonies, which were transformed by our constructs and unstable in HBsAg expression, recombinant DNAs existed as extrachromosomal forms (data not shown).

Characterization of HBsAg released from MS128 cells

The HBsAg from culture medium of MS128 cells consisted of 22 to 27 nm particles with a density of 1.21 to 1.22 g/ml in CsCl (Fig. 4). To determine whether the pre-S gene was expressed in MS128 cells, electrophoretic analyses were carried out. Radiolabelled proteins from MS128 cells were immunoprecipitated by anti-HBsAg antibody and analysed by electrophoresis on a polyacrylamide gel (Fig. 5a). There were three protein bands Mr of 22K, 26K and 35K. After treatment with tunicamycin (10 µg/ml) the 26K and 35K bands disappeared, indicating that both proteins were glycosylated. No non-glycosylated precursor of the middle S protein was detected, but it is known that the non-glycosylated form of some proteins is sensitive to protease digestion (Olden et al., 1982). Western blot analysis showed that the 35K band reacted with anti-pre-S2 antibody (Fig. 5b). The broad band of the 35K polypeptide was due to a difference in the extent of glycosylation (Michel et al., 1984). None of the bands were stained by the anti-pre-S1 antibody (data not shown).

Immunofluorescence also showed that MS128 cells produced HBsAg containing the pre-S2 protein but no pre-S1 protein (Fig. 6). Polymerized human serum albumin (pHSA) was prepared from the monomeric form of human serum albumin by glutaraldehyde coupling (Machida et al., 1983). An aliquot of culture medium was applied to plastic wells precoated with pHSA, and bound HBsAg was detected with anti-HBsAg serum by ELISA. Binding to pHSA was detected in the medium from MS128 cells but not in the medium from C127 cells or BP11 cells transformed with the vector DNA alone (data not shown).
DISCUSSION

We have established a stable transformed cell line (MS128) producing HBsAg containing pre-S2 protein. The HBsAg particles released into the culture medium had the same density and morphology as those found in the blood of carriers. The amount of HBsAg produced and released into the medium was estimated to be 1 to 4 μg/ml/day (18 μg/10⁷ cells/day). The production rate of HBsAg particles from MS128 cells was 20 to 30 times higher than that of the well-known human hepatoma cell line PLC/PRF/5 (Macnab et al., 1976). Efficient production of HBsAg in mammalian cells (6 to 12 μg/10⁶ cells/day) has been reported previously (Hsiung et al., 1984; Michel et al., 1984; Wang et al., 1983); the level of HBsAg production for MS128 cells was equivalent to, or higher than, those reported. The stable expression of HBsAg particles was maintained in MS128 cells passaged for at least 1 year (more than 400 generations).

Plasma-derived HBsAg particles have been used as both a diagnostic antigen and a protective vaccine. However, the shortage of supplies of carrier plasma has prompted us to prepare HBsAg particles using recombinant DNAs. Yeast cells have been successfully used for this purpose, but have some drawbacks (Imamura et al., 1987; Miyano-hara et al., 1983; Valenzuela et al., 1982); first, HBsAg in yeast cells is not secreted into the medium, second, the middle S product is readily degraded by proteases, and third, HBsAg is not glycosylated in yeast cells. It is, therefore, preferable to purify HBsAg particles from stably expressing mammalian cells. The majority of such cell constructs use recombinants containing a part of the HBV genome coding for the HBsAg only. Since the pre-S2 region may protect from HBV infection in chimpanzees (Itoh et al., 1986), and have enhanced immunogenicity in animals (Milich et al., 1985; Neurath...
et al., 1985), the inclusion of pre-S2 protein seems preferable. Electrophoretic and pHSA binding analyses showed that the pre-S2 protein was indeed included in the HBsAg particles released from MS128 cells. Southern blot analysis revealed that the pre-S region was conserved in MS128 cells. It is known that the pre-S1 protein inhibits the secretion of HBsAg from mammalian cells (Persing et al., 1986). The absence of the pre-S1 protein in MS128 cells was consistent with the efficient release of HBsAg into the medium.

One of the features of MS128 cells is their stability in production of HBsAg particles. BPV DNA has been reported to replicate extrachromosomally in mouse cells (Dimaio et al., 1982; Law et al., 1981, 1983; Sarver et al., 1982). However, it is becoming clear that there are cases in which BPV DNA is integrated into cell DNA (Stephens & Hentschel, 1987). In MS128 cells the recombinant DNA was present in an integrated form and not in an extrachromosomal form. In other cell colonies, which were transformed by our constructs and unstable in HBsAg expression, recombinant DNAs were present in extrachromosomal forms (data not shown). Although the mechanism or the reason for integration of pBH2 DNA in MS128 cells is unknown, it is suggested that stable expression of HBsAg in MS128 cells is due to the integrated state of pBH2 DNA in cell DNA.

Extrachromosomally replicating BPV plasmids exist in a high copy number (10 to 200 copies) per cell (Stephens & Hentschel, 1987); in MS128 cells the copy number of the integrated plasmid was estimated to be 20 to 40 copies per cell. It has been shown that amplified sequences in mammalian cells can be of variable length (Schimke, 1984) and so in the present case it is conceivable that gene amplification has followed integration so that all of the integrated plasmids have the structure shown in Fig. 3(c), although the precise structure of the integrated sequences remains to be elucidated.
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