JOURNAL OF VIROLOGY, Jan. 1996, p. 133–140 Vol. 70, No. 1
0022-538X/96/$04.00+0

Intracellular Retention of Surface Protein by a Hepatitis B Virus Mutant That Releases Virion Particles

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Received 2 March 1995/Accepted 12 October 1995

In the course of chronic infection, hepatitis B virus mutants can sometimes be found circulating in the serum as the predominant species. One class of such mutants contains in-frame deletions in the S promoter region. By transfecting hepatoma cells with wild-type or mutant viral genomic DNA, we have shown that one such mutant gives rise to extremely small amounts of S transcripts, as expected, and therefore expresses very little of the middle and small surface (viral envelope) proteins that are translated from these transcripts. In addition, this mutant gives rise to greater-than-wild-type levels of the preS1 transcripts, which are translated into the large surface protein. Because the large surface protein, unlike the other forms of surface protein, is incompetent for secretion, cells transfected with the mutant viral DNA contain large amounts of 20-nm particles within dilated perinuclear vesicles. Therefore, this and similar S promoter mutants may be one contributing factor in the pathogenesis of ground-glass cells, which are hepatocytes containing nonsecretable viral surface proteins within dilated vesicles and are commonly found during chronic hepatitis B. Interestingly, DNA-containing virion particles are secreted into the medium by cells transfected with the mutant DNA, in amounts that are slightly larger than those secreted from wild-type-transfected cells, apparently because the amount of large surface protein is insufficient to block virion secretion. This finding may explain how such mutants can become the predominant circulating species in the serum, especially if there are selection pressures against the wild-type virus.

Hepatitis B virus (HBV) is a DNA virus with a circular genome of approximately 3.2 kb (reviewed in reference 10). Unlike most DNA viruses, it replicates via reverse transcription of an RNA intermediate and is distantly related to the retroviruses. Although most people exposed to HBV recover fully from the infection and become immune to reinfection, approximately 10% are unable to clear the virus from the liver, become chronically infected, and serve as the reservoir for further dissemination of the virus (reviewed in reference 16). Some of these chronic carriers suffer from chronic hepatitis and are at high risk for development of cirrhosis and hepatocellular carcinoma. While the exact mechanism of carcinogenesis is unknown, it is believed that repeated bouts of hepatocellular necrosis and regeneration provide a "fertile field" for endogenous, environmental, and/or viral carcinogens to act (reviewed in reference 33). Most of the cell death during chronic hepatitis B results from immunologically mediated attack by host factors, since HBV is normally noncytopathic (reviewed in reference 16). However, under certain conditions, HBV appears to be capable of directly damaging hepatocytes. With a transgenic mouse model, Chisari et al. (6) have shown that overexpression of the HBV large surface (envelope) protein leads to cell death and regeneration in the liver, eventuating in hepatocellular carcinogenesis. This phenomenon results from the presence of so-called ground-glass cells, which not only spontaneously die but are also exquisitely sensitive to killing by gamma interferon (13). Ground-glass cells contain large amounts of HBV surface protein accumulated within dilated smooth vesicles (11). This accumulation results from the fact that the large surface protein is not competent for secretion, although it is synthesized on the rough endoplasmic reticulum and, hence, directed into the secretory pathway (4, 5, 28, 29). Consequently, the retained proteins are accommodated in greatly dilated smooth vesicles that probably represent the endoplasmic reticulum-Golgi intermediate compartment that has undergone proliferation (17, 18). In contrast, the middle and small surface proteins, which are co-carboxy terminal with the large surface protein (Fig. 1), are efficiently secreted in the form of subviral particles (reviewed in reference 9), although a sufficiently high ratio of large to middle and small surface proteins will cause intracellular retention of all forms of the surface protein via heteromultimer formation. The physiological significance of the lack of secretion of the large surface protein is unknown, but this protein, together with the small surface protein, is necessary for formation of the complete virion, which contains the viral core (nucleocapsid) particle inside the envelope of surface proteins and lipids (reviewed in reference 9).

While the ground-glass cells in transgenic mice result from artificial overexpression of the large surface protein from heterologous promoters (6), ground-glass cells containing accumulated intravesicular surface proteins are also commonly seen in the livers of people with chronic hepatitis B (reviewed in reference 11). The cause of the apparent overexpression of the large surface protein during natural infections is unknown. The large surface protein is expressed from the preS1 promoter, which is upstream of the entire S open reading frame (ORF) (Fig. 1). The middle and small surface proteins, on the other hand, are translated from internal AUG codons present within 5′-heterogeneous transcripts derived from the S promoter, which is embedded within the S ORF (Fig. 1). Transcriptional initiation from these two promoters is regulated by distinct *cis*-acting DNA elements (reviewed in reference 39), although certain sequences within the S promoter also downregulate the amount of preS1 transcripts by a posttranscrip-

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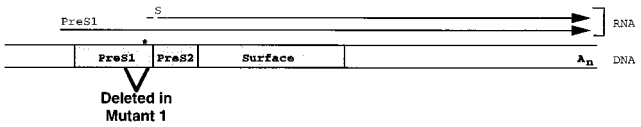

FIG. 1. Map of the surface gene region in the HBV genome. The large surface protein includes the preS1, preS2, and surface regions; the middle surface protein includes the preS2 and surface regions; while the small surface protein includes only the surface region. *, S promoter CCAAT element; A_n, HBV polyadenylation signal.

tional mechanism (22, 23). Recently, the appearance of viral mutants during the course of chronic hepatitis B has been repeatedly observed. One class of such mutants contains in-frame deletions of the S ORF in the preS1 region that is unique to the large surface protein and that overlaps the S promoter (Fig. 1) (8, 12, 26, 35, 38). Therefore, these mutants should give rise to decreased amounts of S transcripts and increased amounts of preS1 transcripts. In turn, there should be both absolute and relative overexpression of the large surface protein, compared with the middle and small surface proteins. This scenario suggests that hepatocytes containing these mutants may then accumulate the large surface protein within the cytoplasm and, hence, turn into ground-glass cells. We have used one such mutant to transfect cultured hepatoma cells and confirmed that there is, indeed, sufficient dysregulation of surface protein expression in these cells to lead to accumulation of HBV surface protein particles within dilated cytoplasmic vesicles. Interestingly, DNA-containing virion particles appear to be secreted from these cells at a slightly higher rate than from cells transfected with the wild-type virus. Therefore, our data suggest that naturally occurring HBV S promoter mutants are one contributing cause of ground-glass cells during chronic infection. They also suggest that these mutants have a slight advantage over the wild-type virus. This advantage, together with possible host immune system-mediated selection against the wild-type virus, can account for the appearance of these mutants as the major viral species circulating in the serum of chronically infected people.

MATERIALS AND METHODS

Plasmids. Plasmid pHBV1.2, also called adwR9, was obtained from T. J. Liang, Harvard Medical School, and contains slightly more than one complete copy (with terminal redundancy of nucleotides 1420 to 2187) of the HBV strain adw2 genome, which is competent for replication (1). A deletion of 129 bp was introduced into the S promoter region of pHBV 1.2 by PCR with the following primers: 1, GCGGGTCACCATATTCTTGG; 2, TGCCTTCCTGACTGCTTG GCTGCTGGCCAGTCGTC; 3, CTGGCCAGCAGCCAAGCAGTCAGGAA GGCAGCCTA; 4, GCCTAGCAGCCATGGAAATG. Specifically, primers 1 and 2 were used to amplify a fragment extending upstream of the deletion, while primers 3 and 4 were used to amplify a fragment extending downstream of the deletion. The two products were purified, mixed, and used in a second round of amplification with primers 1 and 4. This long PCR product, which comprises nucleotides 2820 to 1309 of the HBV DNA, was digested with restriction endonucleases *Bst*EII and *Xba*I and ligated to the large *Xba*I-to-*Bst*EII fragment of pHBV1.2. Two separately generated clones (E5 and E6) of this mutant, confirmed to have the expected deletion and no other mutations by restriction enzyme digestion and sequencing of a 185-nucleotide region surrounding the deletion, were used in further experiments. Plasmid pSAg contains the large *Bgl*II fragment of HBV strain adw2 DNA (36) inserted in vector pTZ19U (40) and, hence, gives rise to and secretes subviral particles containing all three forms of the surface protein. Plasmid pSVLM-S- (3) is similar to pSAg, with two differences. The ATG start codons for middle and small surface proteins have been mutated to ACG, and the preS1 promoter has been replaced with the simian virus 40 early promoter. As a consequence, there is expression of only the large surface protein. Plasmid pSVE5M-S- was derived from pSVLM-S- by using PCR to delete the same 129 bp deleted in mutant 1; therefore, it expresses an internally deleted form of the large surface protein, similar to mutant 1. Plasmid pCMV-β (24) contains the *Escherichia coli* β-galactosidase gene under the control of a cytomegalovirus immediate-early promoter and was obtained from J. Chan, University of California, San Francisco.

Cell culture and transfection. HuH-7 well-differentiated human hepatoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C under an atmosphere of 7% CO_2 and 93% air. Plasmids were transfected by the calcium phosphate coprecipitation method (14), and cells were analyzed on day 3 after transfection.

RNA and protein analyses. Total RNA was extracted from transfected cells with RNazolB (Biotecx Corp.), and primer extension with ^{32}P-labeled oligonucleotides was performed as previously described (17, 40). Radioimmunoassay for HBV surface proteins was performed with a commercial kit (Abbott Corp.). Immunofluorescence for surface protein was performed on methanol-fixed cells (15) with a primary rabbit antibody (Accurate Chemical) and a Cy3-labeled sheep anti-rabbit secondary antibody (Sigma Chemical), both at a 1:50 dilution. Immunofluorescence for intermediate filaments was performed with a 1:1 mixture of prediluted monoclonal antibodies against cytokeratin and vimentin (Biogenex lu5 and V9, respectively) and a fluorescein-labeled sheep anti-mouse antibody (Sigma Chemical) at a 1:50 dilution. For Western blotting (immunoblotting) of total cell extracts or secreted particles (15), the primary antibody was a monoclonal anti-preS1 antibody that recognizes amino acid residues 21 to 47 (F35-25 [AMAC]) or a monoclonal anti-S antibody (H166 [Abbott]; courtesy of R. Decker), each used at 1 μg/ml, while the secondary antibody was an alkaline phosphatase-conjugated rabbit anti-mouse antibody (Amersham) used at a 1:5,000 dilution. Detection was done with the ECL chemiluminescence kit from Amersham. Colorimetric assay for β-galactosidase activity was performed by the method of Miller (25).

Characterization of secreted particles. Particles in the medium of transfected cells were precipitated with polyethylene glycol by the protocol of Lenhoff and Summers (20). After resuspension in Dulbecco's phosphate-buffered saline, the concentrated particles were electrophoresed on a 1% nondenaturing agarose gel in Tris-acetate-EDTA buffer and transferred by capillary action to nylon membranes (Schleicher & Schuell) for Southern blotting (20). The probe was the entire HBV DNA genome excised from pHBV1.2 by digestion with restriction endonuclease *Fsp*I and labeled with [α-^{32}P]dCTP by nick translation (34). Alternatively, the secreted particles were reduced and denatured in β-mercaptoethanol–sodium dodecyl sulfate (SDS) sample buffer before electrophoresis on a 10 to 20% polyacrylamide gradient SDS-Tricine gel (Novex) and subsequent Western blotting as described above. For immunoprecipitation of virion particles from spent media, the monoclonal anti-preS1 antibody was used in the protocol of Bruss and Ganem (2). DNA was released from the precipitated material by SDS-proteinase K treatment, electrophoresed on an agarose gel, and processed for Southern blotting as described above.

Electron microscopy. Transfected cells were harvested by trypsinization, fixed in 2.5% glutaraldehyde in phosphate-buffered saline, postfixed with osmium tetroxide, and embedded in Epon. Thin sections were cut at a thickness of approximately 80 nm, stained with lead citrate and uranyl acetate, and examined under a Zeiss 10C transmission electron microscope.

RESULTS

Dysregulated surface gene transcription from a mutant HBV genome. To determine the effect of a deletion in the S promoter region on HBV surface gene transcription, we utilized a plasmid that contains wild-type HBV strain adw2 genomic DNA (courtesy of T. J. Liang). This plasmid, pHBV1.2, gives rise to virion particles upon transfection into well-differentiated hepatoma cells grown in culture (1). A 129-bp deletion in the S promoter was introduced into pHBV1.2 (Fig. 1) to generate mutant 1. This deletion has been found in two patients, one from Japan (38) and one from Italy (35), and removes amino acid residues 56 to 98 from the large surface protein and is accompanied by substitution of a lysine residue for arginine residue 99. The wild-type and mutant plasmids were transfected into HuH-7 hepatoma cells, and the amount of preS1 and S transcripts was quantitated by primer extension. As seen in Fig. 2B, mutant 1 gave rise to low levels of S transcripts (lane 2), while large amounts of these transcripts were synthesized from the wild-type plasmid (lane 1). On the other hand, mutant 1 gave rise to more than three times as much of the preS1 transcripts as did the wild-type plasmid (Fig. 2A). These are the expected results, as the deletion in mutant 1 spans the CCAAT element of the S promoter (Fig. 1). We have previously shown that this element not only is the major positive-acting *cis* element of the S promoter (41) but is also necessary for posttranscriptional down-regulation of

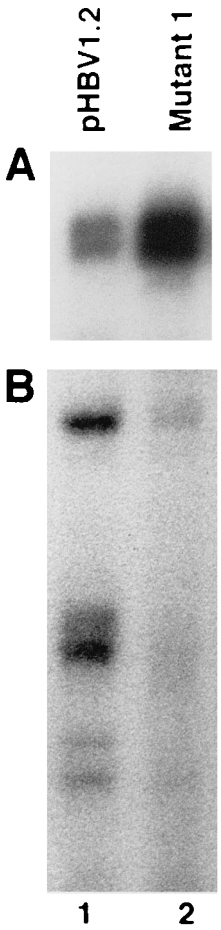

FIG. 2. Primer extension analysis of preS1 (A) and S (B) RNA levels in cells transfected with either wild-type pHBV1.2 or mutant 1. Note that five major start sites are specified by the S promoter (41).

preS1 transcripts (22, 23). Thus, its absence in mutant 1 would simultaneously lead to decreased S transcript levels and increased preS1 transcript levels. In addition, the changes in transcript levels shown in Fig. 2 were not due to the presence of other, unexpected mutations introduced during the mutagenesis process, since a separately generated clone of mutant 1 gave rise to the identical amount of S and preS1 transcripts (data not shown).

Lack of surface protein secretion by mutant 1. The amount of S transcripts present in mutant 1-transfected cells was approximately 10% of that in cells transfected with pHBV1.2, as estimated by digital video imaging. Therefore, in the absence of other complications, one would expect mutant 1-transfected cells to secrete a commensurate amount of surface proteins into the medium. However, a radioimmunoassay revealed that they actually secreted essentially no surface proteins (<1% of the amount of surface protein secreted by cells transfected with pHBV1.2, which is not statistically significantly above the background) (Fig. 3). This result cannot be attributed to the inability of the Abbott radioimmunoassay to detect the particles secreted by mutant 1, since Western blotting also detected no surface proteins in the medium of cells transfected with mutant 1, with either a monoclonal antibody against an S epitope (Fig. 4, lane 2) or a monoclonal antibody against a preS1 epitope (Fig. 4, lane 4). This discrepancy between S transcript levels and surface protein secretion suggests that the small amount of

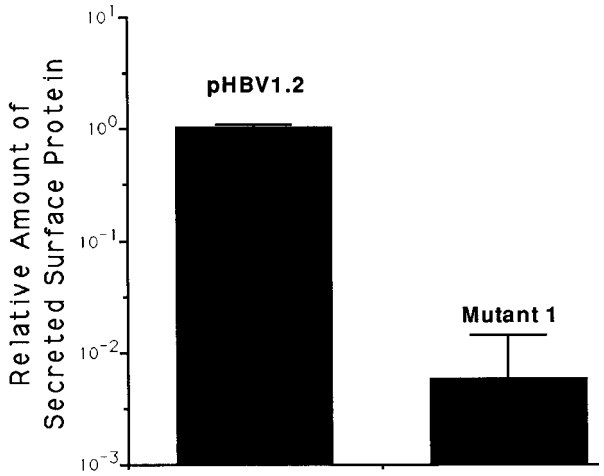

FIG. 3. Quantitation by radioimmunoassay of surface protein secreted from cells transfected with pHBV1.2 or mutant 1. Note the logarithmic scale used to accommodate the large difference between the two values. The results shown are the mean plus the standard deviation of three independent transfections, normalized to the value for pHBV1.2-transfected cells.

middle and small surface proteins synthesized in these cells was not secreted into the medium, presumably because of a secretory block mediated by the large surface protein.

Nevertheless, the lack of detectable surface protein in the medium raised the alternative possibility of a defect in the synthesis of surface proteins in mutant 1-transfected cells, possibly because of other mutations unintentionally introduced during the in vitro mutagenesis process. However, we were able to rule out this possibility, since Western blotting of cel-

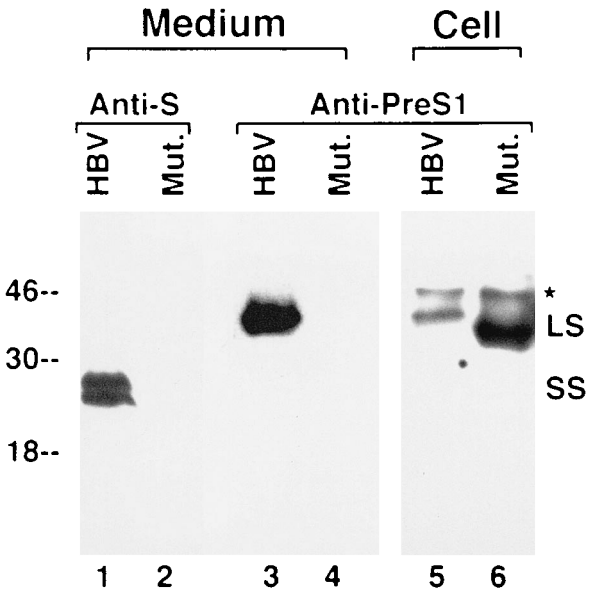

FIG. 4. Western blotting of surface protein secreted into the medium of transfected cells (lanes 1 to 4) or present within transfected cells (lanes 5 and 6). The primary antibodies used were a monoclonal anti-S antibody (lanes 1 and 2) and a monoclonal anti-preS1 antibody (lanes 3 to 6). LS and SS indicate the migration positions of the large and small surface proteins, respectively. Note that mutant 1 (Mut.) gave rise to a slightly smaller large surface protein than the wild-type virus (HBV), as expected. *, a cellular protein that cross-reacts with the anti-preS1 antibody. The numbers on the left are the approximate molecular masses, in kilodaltons, of prestained marker proteins (Gibco-BRL).

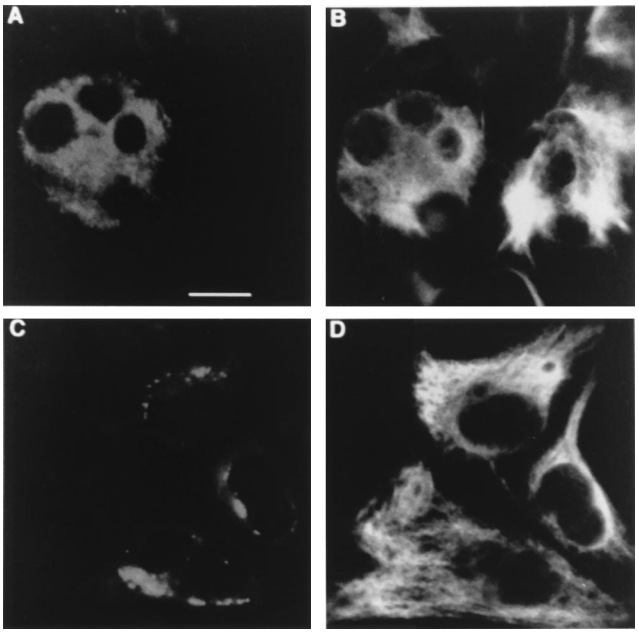

FIG. 5. Immunofluorescence staining of surface proteins in cells transfected with pHBV1.2 (A) or mutant 1 (C). Staining of intermediate filaments in the same set of cells is shown in panels B and D, respectively, to allow visualization of the entire cytoplasm. Note that not all of the cells in panels A and B were transfected. Bar, 20 μm.

lular extracts revealed that mutant-transfected cells contained a significant amount of a large surface protein (Fig. 4, lane 6) slightly smaller than the wild-type large surface protein present within and secreted from wild-type-transfected cells (Fig. 4, lanes 5 and 3, respectively). To localize this intracellular surface protein, we performed immunofluorescence analysis of cells transfected with either wild-type or mutant 1 HBV DNA. In the cells transfected with wild-type DNA, there was a diffuse granular-reticular staining of the cytoplasm for surface proteins, consistent with their presence throughout the entire secretory pathway (compare Fig. 5A and B, which shows the entire cytoplasm of the same cells stained by antibodies against intermediate filaments). In contrast, the cells transfected with mutant 1 DNA showed blobs of intense perinuclear staining without staining of the periphery of the cytoplasm (compare Fig. 5C and D, which shows the entire cytoplasm of the same cells stained by antibodies against intermediate filaments). We have shown that this is the staining pattern expected for surface proteins accumulated within the endoplasmic reticulum-Golgi intermediate compartment (17, 37). Therefore, it appears that in these cells the small amount of residual surface proteins synthesized was stuck in the intermediate compartment rather than secreted.

Transmission electron microscopy of mutant 1-transfected cells revealed dilated perinuclear vesicles within the cytoplasm of many of the cells (7 of the 50 counted) (Fig. 6A, arrowheads). These smooth vesicles contained closely packed arrays of particles slightly greater than 20 nm in diameter, whose morphology is consistent with that of subviral (capsid-deficient) particles composed of surface proteins. Some of these particles had a spherical outline, while a few appeared as filaments (Fig. 6A, inset). Such vesicles are similar to those found within ground-glass cells in the livers of infected humans (Fig. 6B) and transgenic mice (5), although the particles in those cells are usually more loosely packed and the filaments appear to be longer (5, 11). No such particle-laden vesicles were seen in 50 untransfected cells and 50 cells transfected with wild-type HBV DNA examined at the same time.

Again, the amount of secreted surface proteins, the pattern of immunofluorescent staining, and the ultrastructural appearance of transfected cells were identical when a different clone of mutant 1 was used to transfect HuH-7 cells (data not shown). Therefore, all of these findings are specific to the S promoter deletion and cannot be ascribed to other mutations within the HBV genome unintentionally generated during mutagenesis.

Virion secretion by mutant 1-transfected cells. Since mutant 1-transfected cells did not secrete detectable amounts of subviral surface protein particles, we anticipated that no virion particles would be secreted either. Surprisingly, however, Southern blotting of concentrated media electrophoresed on native agarose gels consistently detected HBV DNA migrating at the same position as apparent virion particles secreted from cells transfected with wild-type HBV DNA (Fig. 7, lanes 2 and 3, respectively). The electrophoretic mobility of these particles was also similar to that of authentic HBV virions in serum of an infected person (courtesy of T. Wright, San Francisco Veterans Affairs Medical Center) (Fig. 7, lane 1), although there was a slight difference in mobility that may be attributable to differences in lipid composition and/or adsorbed serum proteins.

These mutant particles appeared to have an intact lipid coat by two criteria. First, treatment with Nonidet P-40 (NP-40) caused a large increase in their electrophoretic mobility, similar to particles secreted from wild-type-transfected cells, as well as serum particles (Fig. 7, lanes 4 to 6); this shift corresponds to the release of naked core particles from detergent-induced disruption of the viral envelope, as has been shown by Lenhoff and Summers (20). Second, the DNA contained in these particles was resistant to DNase I digestion, except after pretreatment with both proteinase K and NP-40 (Fig. 8). Therefore, they cannot be naked core particles, which are sensitive to proteinase K digestion in the absence of NP-40 and which can sometimes be found in the medium of transfected cells (20). In addition, we showed that these particles were coated with the large surface protein, since they were immunoprecipitated by a monoclonal antibody against a preS1 epitope (Fig. 9, lane 1) but not by an irrelevant monoclonal antibody (Fig. 9, lane 3). Therefore, these particles have all of the hallmarks of bona fide virion particles.

Since ground-glass cells are fragile and die easily (6, 13), it was possible that the virion particles released by mutant 1-transfected cells resulted from cell lysis rather than true secretion. To examine this possibility, we quantitated the amount of β-galactosidase released from cells cotransfected with a plasmid that expresses this nonsecretable enzyme. Very little β-galactosidase, which is localized in the cytosol, was released into the medium of these cells (0.05% of the amount within the cells), and it was identical to the amount found in the medium of cells transfected with wild-type HBV DNA. Therefore, we can rule out nonspecific release of intracellular virion particles because of cellular damage.

The DNA contained within NP-40-treated particles released from mutant 1-transfected cells could be labeled with the endogenous polymerase reaction (data not shown), which utilizes the HBV polymerase present within the particles to extend the partially replicated DNA present in virion particles (2). Therefore, the polymerase synthesized by mutant 1 was functionally active, despite the internal deletion engendered by the 129-bp deletion (the entire surface ORF is embedded within the polymerase ORF). This result was not unexpected, since this de-

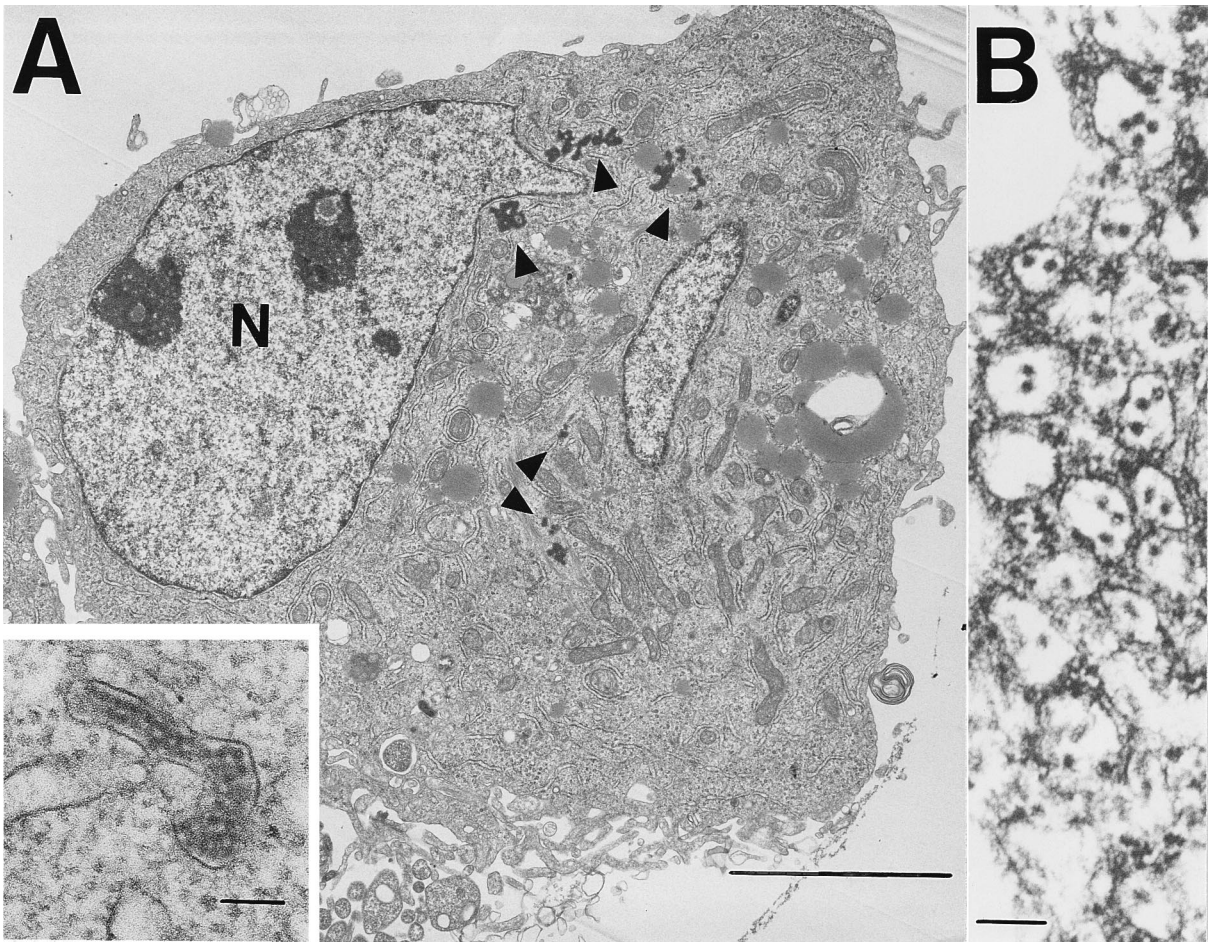

FIG. 6. (A) Transmission electron micrograph of dilated perinuclear smooth vesicles (arrowheads) in cells transfected with mutant 1. N, nucleus. Bar, 5 μm. The inset is a higher-power view that reveals that these vesicles contain spherical and occasional filamentous particles approximately 20 nm in diameter. Bar, 100 nm. (B) Transmission electron micrograph of a ground-glass cell from the liver biopsy specimen of a chronic HBV carrier, showing similar intravesicular particles. Bar, 100 nm.

letion lies within the so-called spacer region of the polymerase protein that appears to be dispensable for its activity (32).

Finally, it should also be noted that both of the independently generated clones of mutant 1 gave rise to replication-competent particles, as determined by Southern blotting and the endogenous polymerase reaction (data not shown).

How is it that mutant 1 gives rise to virions but not subviral particles? One possibility is that the mutant large surface protein has specifically lost the ability to block virion secretion. Another possibility is that the levels of large surface protein expression in the transfected cells are not high enough to block virion secretion. To sort out these possibilities, we cotransfected mutant 1 into HuH-7 cells with one of two simian virus 40 early promoter-driven plasmids: pSVLM-S-, which expresses high levels of the wild-type large surface protein, or pSVE5M-S-, which expresses the same internally deleted large surface protein as mutant 1. It should be noted that neither plasmid expresses a middle or small surface protein (3), which might have confounded the analysis. As a control, pUC19 was cotransfected. Particles from the media of the cotransfected cells were then electrophoresed on a native gel and characterized by Southern blotting for HBV DNA. As shown in Fig. 10, lanes 2 and 3, cotransfection of either plasmid eliminated virion particle secretion by mutant 1. Therefore, it appears that the second explanation is correct; i.e., large surface protein expression by mutant 1 is sufficient to block subviral particle secretion but not to block virion secretion. However, this result does not entirely rule out the possibility that the mutant large surface protein is also somewhat less efficient at blocking virion secretion than is the wild-type protein.

DISCUSSION

We have presented data showing that a deletion in the S promoter region of HBV DNA leads to decreased S transcript levels and increased preS1 transcript levels. Consequently, there is overexpression of the large surface protein in transfected cells, such that the secretion of subviral surface protein particles becomes blocked. The surface proteins then accumulate in the form of 20-nm particles in smooth cytoplasmic vesicles. These features are similar, but not identical, to the ultrastructural features of ground-glass cells found in chronic hepatitis B. Since the identical mutation has been found in the circulating virion particles of two chronically infected people on two different continents (35, 38), and since similar deletions in the S promoter have been found in other people in different parts of the world (8, 12, 26), it seems possible that such mutants constitute one contributing cause of ground-glass hepatocytes during chronic hepatitis B. The fact that ground-glass cells usually are found late in infection in a random

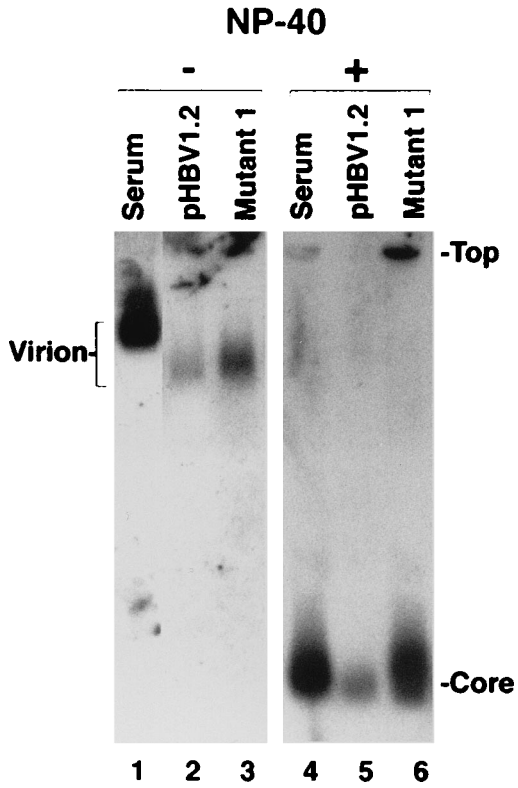

FIG. 7. Southern blotting of virion particles before and after NP-40 treatment. Serum from a high-titer HBV patient (courtesy of T. Wright) and concentrated particles from cells transfected with pHBV1.2 or mutant 1 were electrophoresed on a native 1% agarose gel (20), transferred to a nylon membrane, and probed with HBV DNA. The material in lanes 4 to 6 was treated with 1% NP-40 for 1 h before electrophoresis, which removed any lipid-containing envelope and liberated core (nucleocapsid) particles from intact HBV virions (20).

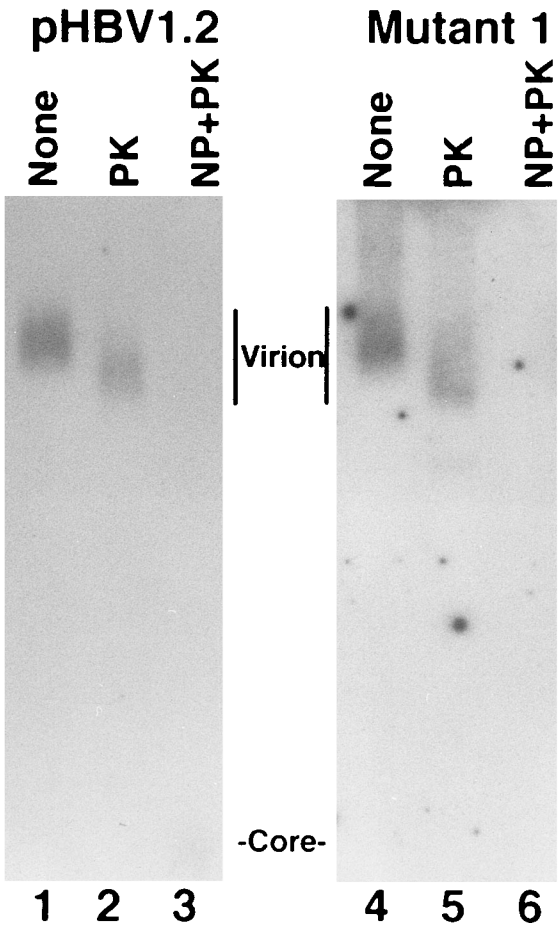

FIG. 8. DNase I resistance of virion particles from the medium of cells transfected with either pHBV1.2 or mutant 1. Concentrated particles were digested with DNase I, either with no pretreatment (lanes 1 and 4), pretreated with proteinase K (PK) alone (lanes 2 and 5), or pretreated with NP-40 (NP) and proteinase K (lanes 3 and 6). Digested particles were electrophoresed on a native 1% agarose gel, transferred to a nylon membrane, and probed with HBV DNA (20). Protease digestion of duck HBV virions is known to cause an increase in electrophoretic mobility (20), and, as shown here, the same phenomenon appears to hold true for HBV, presumably because of digestion of adsorbed serum proteins and/or the exposed portion of the surface proteins. The expected migration position of core particles is indicated.

pattern in the liver is consistent with this hypothesis, as the mutants presumably arise by a stochastic process during chronic infection. Naturally, these results do not preclude the possibility that there are other causes of ground-glass cells. Indeed, we have previously demonstrated that integrated HBV genomes may be another such cause (17).

The ultrastructural features of the impacted subviral particles within the transfected cells are similar, but not identical, to those of the particles in ground-glass cells of human patients (compare Fig. 6A and B). Specifically, both show particles slightly greater than 20 nm in diameter within small, smooth vesicles and the particles are either spherical or filamentous in profile. However, the particles in transfected cells tend to be closely packed and the filaments tend to be scattered and short. On the other hand, in patients the particles tend to be packed more loosely and filaments can be quite long and numerous. The reason for these differences is not clear. We have found that cells transfected with plasmids that overexpress the wild-type large surface protein also contain particles similar to those seen in mutant 1-transfected cells (37). Therefore, the internal deletion present in the large surface protein of mutant 1 cannot account for the difference. Perhaps the morphology of the particles in patients has been altered by being present over long periods of time (months to years versus 1 to 2 days for the transfected-cell particles).

A surprising finding from our studies is the secretion of apparent virion particles from mutant-transfected cells at a rate slightly higher than that from wild-type-transfected cells. These particles are indistinguishable from particles secreted by wild-type-transfected cells, as judged by electrophoretic mobility on native gels, sensitivity to nonionic detergents, and the presence of large surface protein on the surface. Furthermore, they contain viral DNA and express endogenous polymerase activity (data not shown). Therefore, they cannot be aberrant virus-like particles that do not have a virus-encoded envelope. This was unexpected, since it has been reported that overexpression of wild-type large surface protein blocks the secretion of virion particles (2). The explanation seems to be that higher levels of large surface protein are needed to block virion secretion, compared with subviral particle secretion, and the amount of large surface protein expressed by mutant 1 is not large enough to block virion secretion. Therefore, it appears that active DNA replication and virion secretion can occur in cells incapable of secreting subviral surface protein particles.

Finally, the slight advantage of the mutant in virion release, together with possible immune system-mediated host selection pressures against the wild-type virus, can explain the fact that

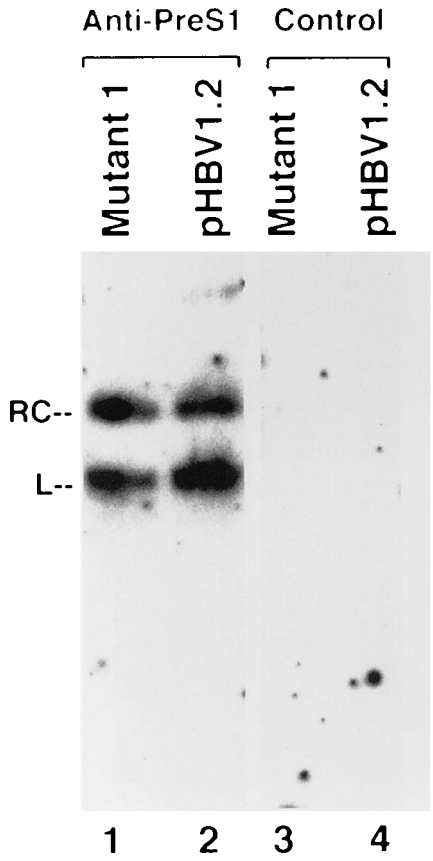

FIG. 9. Immunoprecipitation of virions produced from cells transfected with mutant 1 or pHBV1.2, followed by Southern blot detection. Spent media were precipitated with a monoclonal antibody against preS1 residues 21 to 47, which is an epitope exposed on the surface of virion particles (2, 9). RC and L indicate the migration positions of relaxed circular and linear HBV DNAs, respectively. As a negative control, an identical volume of medium was precipitated with an irrelevant monoclonal antibody produced by a mineral oil-induced plasmacytoma (MOPC141; Sigma Chemical).

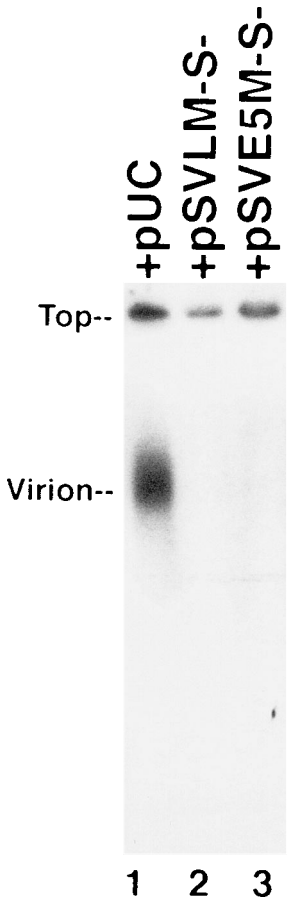

FIG. 10. Blockage of virion secretion by mutant 1-transfected cells with additional expression of large surface protein from a cotransfected plasmid. Cells were transfected with 5 μg of mutant 1 DNA and 5 μg of each of the indicated plasmids, and virions in the spent media were concentrated, electrophoresed on an agarose gel, and detected by Southern blotting for HBV DNA. Plasmid pSVLM-S-produces the full-length large surface protein (3), while pSVE5M-S-produces a large surface protein with an internal deletion, like that produced by mutant 1.

S promoter mutants have been observed as a major form of circulating HBV particles in the sera of chronically infected people (8, 12, 26, 35, 38). Our results also raise the intriguing possibility that such mutants may even be infectious, since the receptor-binding region of the large surface protein has been mapped to residues 21 to 47, upstream of the deleted region (27, 30, 31). If so, it is, at first glance, puzzling that such mutants are not dominant in nature. We speculate that the reason is that these mutants are cytopathic, since they would cause ground-glass cells, which are prone to injury and death (6, 13). Indeed, we have not been able to obtain cells stably transfected with the mutant (as opposed to wild-type pHBV1.2), presumably because the accumulation of intracellular surface protein is injurious to the host cell (37). For pathogens such as HBV that are transmitted predominantly vertically by chronically infected organisms, there is strong evolutionary pressure to select for benevolence (7). Hence, despite the apparent short-term advantage of these mutants in terms of replication, they may actually be at a severe disadvantage during natural transmission. A possibly similar but more extreme situation has been found experimentally to be true for a duck HBV mutant characterized by Lenhoff et al. (19, 21). This mutant, which replicates better than the wild-type virus in single-round infection studies in vitro, has a partially defective large surface protein, such that there is sufficient overaccumulation of episomal viral DNA in host cell nuclei to injure the infected cells (21). Preliminary results indicate that when this mutant is used to infect ducks, there seems to be strong selective pressure against it, such that phenotypically wild-type viruses (presumably revertants) become recoverable from the infected animals within a few weeks (19). We believe that a similar scenario can explain why mutant 1 and similar HBV mutants do not become dominant, although in this case the competition with the wild-type virus may be at the whole-organism rather than at the individual-hepatocyte level. However, definitive answers to this and other questions regarding the natural history of S promoter mutants and the role of these mutants in HBV-related disease pathogenesis await further experiments, possibly with animal models.

ACKNOWLEDGMENTS

We thank J. Summers for many helpful discussions and for permission to cite unpublished data; R. Decker for monoclonal anti-S antibody; J. H. Ou and P. Gripon for critical reading of the manuscript; K. C. Lim for performing radioimmunoassays and preparing plasmids; V. Bruss, J. Chan, and T. J. Liang for plasmids; and T. Wright for the patient serum sample.

Oligonucleotides were synthesized on a shared instrument purchased with UCSF Academic Senate Opportunity Funds. This work was supported by NIH grant R01CA55578 and a VA Merit Review grant.

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