JOURNAL OF VIROLOGY, Apr. 1996, p. 2277–2285 Vol. 70, No. 4
0022-538X/96/$04.00+0

Protease-Induced Infectivity of Hepatitis B Virus for a Human Hepatoblastoma Cell Line

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Received 21 September 1995/Accepted 21 December 1995

The human hepatoblastoma cell line HepG2 produces and secretes hepatitis B virus (HBV) after transfection of cloned HBV DNA. Intact virions do not infect these cells, although they attach to the surface of the HepG2 cell through binding sites in the pre-S1 domain. Entry of enveloped virions into the cell often requires proteolytic cleavage of a viral surface protein that is involved in fusion between the cell membrane and the viral envelope. Recently, we observed pre-S-independent, nonspecific binding between hepatitis B surface (HBs) particles and HepG2 cells after treatment of HBs antigen particles with V8 protease, which cleaves next to a putative fusion sequence. Chymotrypsin removed this fusion sequence and did not induce binding. In this study, we postulate that lack of a suitable fusion-activating protease was the reason why the HepG2 cells were not susceptible to HBV. To test this hypothesis, virions were partially purified from the plasma of HBV carriers and treated with either staphylococcal V8 or porcine chymotrypsin protease. Protease-digested virus lost reactivity with pre-S2-specific antibody but remained morphologically intact as determined by electron microscopy. After separation from the proteases, virions were incubated with HepG2 cells at pH 5.5. Cultures inoculated with either intact or chymotrypsin-digested virus did not contain detectable levels of intracellular HBV DNA at any time following infection. However, in cultures inoculated with V8-digested virions, HBV-specific products, including covalently closed circular DNA, viral RNA, and viral pre-S2 antigen, could be detected in a time-dependent manner following infection. Immunofluorescence analysis revealed that 10 to 30% of the infected HepG2 cells produced HBV antigen. Persistent secretion of virus by the infected HepG2 cells lasted at least 14 days and was maintained during several reseeding steps. The results show that V8-digested HBV can productively infect tissue cultures of HepG2 cells. It is suggested that proteolysis-dependent exposure of a fusion domain within the envelope protein of HBV is necessary during natural infection.

Considerable progress has been made in the immune prophylaxis of hepatitis B virus (HBV) infection (7) and in the understanding of its replication and pathogenicity at the molecular level (2, 27, 28, 46, 48, 51). However, a convenient in vitro assay for HBV infectivity has been lacking, and the early steps of the viral life cycle are not yet well understood. The human hepatoblastoma cell line HepG2 produces infectious HBV after transient or stable transfection by HBV DNA (1, 40, 42, 43, 45). This cell line is also able to bind HBV via the viral pre-S1 domain (32, 37), but in the experience of most researchers, complete virions are not infectious for HepG2 cells, with one notable exception (3). Primary human hepatocytes are reported to be susceptible to HBV (8, 12, 13, 33, 38), but these systems are not practical because they are short-lived and require primary explanted human liver. Furthermore, reproducibility of infection requires treatment of the cells with substances such as dimethyl sulfoxide (8, 12) or polyethylene glycol (13), which may induce unnatural mechanisms of viral entry.

The nonsusceptibility of HepG2 cells for HBV, despite viral attachment and virus production after transfection, implies that a step between attachment and viral pregenome transcription is blocked. These steps include viral entry, genome release, transport to the nucleus, and genome repair. For many viruses, entry of the genome into a suitable compartment of the target cell is a limiting factor for viability. Entry of enveloped viruses very often requires proteolysis of an envelope protein close to a fusion domain which, in its cleaved form, allows fusion between the viral envelope and either the cellular membrane or, after endocytosis, the endosomal membrane (23, 26, 30, 31, 41, 49). In some systems, such as those involving influenza viruses (41) or paramyxoviruses (30, 31), exogenously added protease may generate infectivity of an otherwise nonviable virus.

As shown in Fig. 1, HBV envelope proteins contain a consensus fusion sequence of four large and two small hydrophobic amino acids between residues 182 and 192 (genotype A). Peptide fragments of this region have been shown to induce fusion of biomembranes in vitro (39). In the present study, we tested the hypothesis that the nonsusceptibility of HepG2 cells for HBV may be due to the lack of exposure of this fusion domain for presentation to target cells. Natural exposure of this region might result from proteolytic cleavage upstream, as is the case with the enveloped viruses listed in Fig. 1C. Experimental exposure of this fusion domain in HBV was achieved by proteolytic digestion with staphylococcal V8 protease, which cleaves at amino acid residue 176 (Fig. 1). V8 digestion of intact virions results in cleavage of large and middle-sized hepatitis B surface (HBs) protein (L and M protein) but spares S except for the extreme amino end (14, 44). Thus, cleavage should result in the removal of preS1 and preS2 domains from particles, with the generation of an exposed potentially fusogenic amino terminus (14, 44). Chymotrypsin also cleaves M and L protein but within the putative fusion sequence (Fig. 1). Proteolysis-induced exposure of the fusogenic region seems to

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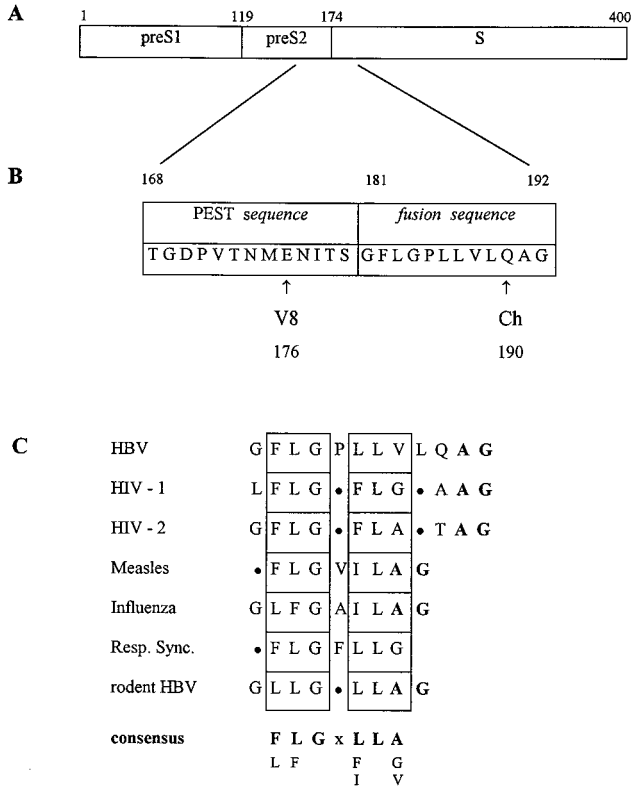

FIG. 1. Map of the HBV envelope protein gene and its putative fusion domain. (A) Linear map of the HBV envelope protein(s). Numbers indicate amino acids along the L protein (derived from genotype A), with individual domains marked. (B) The region specifying amino acids 168 to 192, which spans the pre-S2 and S domains. Amino acids residues are indicated in the box by standard abbreviations. V8 and chymotrypsin cleavage sites are shown. Putative PEST and fusion domains, as proposed by Lu and Gerlich (24), are also shown. (C) Putative HBV fusion domain compared with fusion domains known from other viruses. Common elements are two triads with two large and one small hydrophobic amino acids and an AG motif shown in boldface type. Symbol: ●, gap in the sequence generated by alignment.

mediate binding of HBs particles to cell surfaces independent of specific receptors. Since neither natural HBs antigen (HB sAg) particles nor chymotrypsin-digested particles efficiently bind to the surface of irrelevant cells such as HeLa cells or mouse fibroblast lines but HBsAg particles treated with V8 protease bind to such cells as well as to HepG2 cells (11, 24), the relative infectivity of virions digested with these proteases was tested. This report describes a system for testing the infectivity of HBV in culture, as well as a means of distinguishing input from progeny virus.

MATERIALS AND METHODS

Isolation of virus and treatment with proteases. Serum (17 ml) containing 2×10^9 HBV DNA molecules per ml from an HBsAg- and HBeAg-positive virus carrier was centrifuged into a 15 to 65% (wt/wt) sucrose gradient at 25,000 rpm and 10°C for 16 h in an SW 28 rotor (Beckman). The HBV DNA-containing fractions between 40 and 47% (wt/wt) sucrose were pooled, dialyzed overnight, and digested either with 1.2 mg of V8 protease per ml in 0.05 M potassium phosphate buffer (pH 7.4) at 37°C overnight or with 88 μg of chymotrypsin per ml in 0.01 M Tris-HCl (pH 7.4)–0.05 M $CaCl_2$ at 37°C for 2 h. The completeness of digestion was determined by enzyme-linked immunosorbent assay (ELISA) with anti-pre-S1 and anti-pre-S2 monoclonal antibodies (MAbs) MA18/7 and Q19/10 as described elsewhere (5, 15). The proteases were removed by a second ultracentrifugation through a 20% sucrose cushion at 36,000 rpm and 10°C for 8 h in an SW41 rotor (Beckman). Virions were resuspended in 150 μl of phosphate-buffered saline (PBS) for infection experiments. The number of virions per milliliter of preparation was determined by quantitative PCR with a European reference plasma for HBV DNA as the standard (10).

Cells and infection. HepG2.2.15 cells were kindly provided by Howard Thomas, St. Mary's Medical College, London, United Kingdom, with the permission of George Acs and were maintained as described in reference 42. HepG2 cells, obtained from the European Collection of Animal Cell Cultures, Porton Down, United Kingdom, were cultured with mixed medium containing 80% RPMI 1640 and 20% Earle's medium 199 supplemented with 10% fetal calf serum. At 5 days after being seeded, when the cells had become semiconfluent, they were briefly washed with mixed medium whose pH had been adjusted to 5.5 by addition of 2-*N*-morpholinoethanesulfonic acid (MES). Thereafter, approximately 10^7 virions per ml in pH 5.5 medium were added and incubated with the cells at 37°C for 12 h. To remove the unabsorbed viruses, the cells were washed with pH 5.5 medium twice, with PBS three times, and with normal medium once and then cultured in mixed medium. The start of this incubation was taken as time zero.

Detection of intracellular HBV DNA. Aliquots of the infected cells were harvested by trypsin treatment each day after infection and were stored at −180°C. Between 2,000 and 4,000 cells per sample were treated with 1 ml of 0.15 M sodium acetate buffer (pH 2.4) at room temperature for 10 min. After two washes with PBS, the cells were suspended in 15 μl of distilled water and 10 μl was analyzed by PCR with primers 176 (GCAGGGGTCCTAGGAATCCTG ATG) and 178 (GGTCACCATATTCTTGGGAACAA), representing nucleotides 191 to 161 and 2815 to 1837, respectively, where base 1 is the deoxycytidine in the *Eco*RI site. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The detection limit was at 10^3 genome copies per assay. For semiquantification, a twofold dilution series of a Eurohep reference sample (10) beginning with 2×10^5 genome copies was run in parallel (see, e.g., Fig. 6A). The addition of 2,000 to 4,000 cells did not disturb the PCR efficiency.

Southern blot analysis. Approximately 10^7 cells were harvested by trypsin digestion after 6 days of infection and were washed twice with PBS. Cells were lysed with 0.05 M Tris-HCl (pH 7.4)–1% sodium dodecyl sulfate (SDS)–0.02 M NaCl–0.02 M EDTA and incubated with 0.5 mg of proteinase K per ml at 37°C overnight. Total DNA was extracted with phenol-chloroform and precipitated with ethanol. In some experiments, samples were enriched for the low-molecular-weight double-stranded DNA species by dissolution in appropriate buffers and passage through the Wizard miniprep DNA purification system, designed for purification of plasmids from the bacterial chromosome (Promega, Madison, Wis.). Buffers and materials used were as described by the manufacturer. DNA eluted from the column was precipitated with ethanol. DNA from the column or from total cellular extraction was resuspended in water, digested with restriction enzymes or left undigested, separated by electrophoresis through either 0.8 or 1.5% agarose gels, and transferred to a cellulose nitrate (E) membrane (Schleicher & Schuell). p2XHBV, an *Escherichia coli* recombinant plasmid containing the entire HBV genome (4), was labelled with ^{32}P by random priming and used as a probe. The specific radioactivity of the probe was 1×10^9 to 2×10^9 cpm/μg. Hybridization was performed in 50% formamide at 42°C overnight, and the test was completed by standard procedures.

Detection of HBV cccDNA by nested PCR. To permit detection of covalently closed circular DNA (cccDNA) in small-scale cell culture, a nested PCR procedure was developed. DNA from approximately 4,000 cells was extracted and precipitated as above and dissolved in 10 μl of water. To degrade the single-stranded region of the HBV DNA and to cleave the genomic DNA at the triple-stranded discontinuity near bases 1923 to 1934, the sample was digested with 20 U of mung bean nuclease in 20 μl of 0.05 M sodium acetate (pH 5.0)–0.03 M NaCl–1 mM $ZnCl_2$ buffer at 37°C for 30 min to remove single-stranded DNA. Primers 147 (nucleotides 1441 to 1460, CTGAATCCCGCG GACGACCC), 153 (nucleotides 1889 to 1867, ACCCAAGGCACAGCTTGG AGG), 148 (nucleotides 1550 to 1571, GTCTGTGCCTTCTCATCTGCC); and 149 (nucleotides 1844 to 1827, AGATGATTAGGCAGAGGTGAAAAA) were used to amplify cccDNA of HBV by nested PCR. Parts of the single-stranded region and the discontinuity were located between the sense and antisense primers. Thus, PCR would amplify only cccDNA which is not sensitive to mung bean nuclease.

Immunofluorescent staining of cultured HepG2 cells. The cells released by trypsin 6 days postinfection (p.i.) were cultured on glass slides. After 1 day, cells were briefly washed with PBS twice and then fixed with 25% paraformaldehyde plus 0.2% Triton X-100 at 37°C for 10 min. The fixed cells were washed with cold PBS for 30 min and incubated with anti-core/e MAb 42B/12 (21) or with anti-pre-S2 MAb P (29) at 37°C for 1 h. After five washes with 5% goat serum plus 2% fish gelatin in PBS, cells were incubated at 37°C for 1 h with rabbit anti-mouse antibody labelled with fluorescein. After five further washes with the same buffer, the cells were covered with 1 M Mowiol 4-88 (Hoechst, Frankfurt/Main, Germany) plus 10% 1,4-diazobicyclo-[2,2,2]-octane and viewed under a microscope with epi-illumination.

Detection of polyadenylated HBV RNA. About 4,000 cells were lysed with 4 M guanidinium thiocyanate–7% β-mercaptoethanol, and mRNA was extracted with phenol-chloroform (1:1), precipitated with 75% cold ethanol, washed with 75% cold ethanol, and dissolved in 20 μl of water. The samples were digested with 40 U of RNase-free DNase in the presence of 20 U of RNasin in 40 μl of 0.1 M sodium acetate (pH 7.5)–0.06 M NaCl–5 mM $MgCl_2$ buffer at 37°C for 1 h. DNase was removed by phenol-chloroform extraction and ethanol precipitation.

TABLE 1. Removal of pre-S domains from virions following digestion with protease

Protease treatment[a]	Binding of following MAb[b]:		
	MA 18/7 (anti-pre-S1)	Q19/10 (anti-pre-S2)	C20/02 (anti-S)
None	1.92 ± 0.00	1.90 ± 0.04	1.86 ± 0.05
V8 protease	0.16 ± 0.01	0.06 ± 0.01	1.94 ± 0.06
Chymotrypsin	0.02 ± 0.01	0.01 ± 0.00	1.96 ± 0.04

[a] Virions were prepared and digested with proteases as described in Materials and Methods.

[b] After microtiter plates were coated with the indicated MAb, the same amounts of virions (purified from proteases) were incubated and binding was detected by using a polyvalent peroxidase-conjugated anti-S antibody (5). Values are the optical density of the ELISA product at 492 nm ± standard deviation. Binding of MAbs was less than 0.01 in the absence of antigen.

For reverse transcription, the mRNA pellet was dissolved in 12 μl of water, 1 μl of oligo(dT)$_{25}$ (7.5 μM) was added, and the mixture was heated to 70°C for 10 min. Then, 1 μl of 10 mM deoxynucleoside triphosphate, 2 μl of 0.1 M dithiothreitol, and 4 μl of 5× reverse transcriptase buffer (0.25 M Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.375 mM KCl) were added. After 2 min at 37°C, 1 μl (200 U) of reverse transcriptase and 1 μl of RNasin (20 U) were added. The total reaction volume of 21 μl was incubated at 37°C for 1 h. The reverse transcriptase activity was destroyed by heating to 95°C for 10 min, and the primer pair 148 and 149 was used to amplify the cDNA.

Detection of secreted HBV. Culture medium of infected cells was changed daily, and 1.0 ml was centrifuged at low speed to remove insoluble materials. Anti-pre-S2 MAb Q19/10 (1 μg) was added to the supernatant, and the mixture was incubated at 37°C for 2 h. Then, 30 μl of protein G agarose beads preabsorbed with 2% bovine serum albumin in PBS was added, and the mixture was incubated at 4°C for 1 h and sedimented. After two washings with 0.5% Tween 20–PBS, two with 0.1% Tween 20–PBS, and one with PBS, 15 μl of 1% Nonidet P-40–1% β-mercaptoethanol was added and the mixture was heated to 95°C for 5 min to release HBV DNA. PCR with primer sets 94 plus 97 (1305 to 1285, TGCGAGCAAAACAAGCGGCAT, and 2718 to 1738, AGTTAATCATTACT TCCAAAC) and 176 plus 178 was used to detect DNA from immunoprecipitated virions.

RESULTS

Effect of V8 and chymotrypsin proteases on virion structure. As shown in Fig. 1, a stretch of 11 amino acids between position 182 and 192 close to the transition from pre-S2 to S contains three motifs which are also present in the known fusion domains of human immunodeficiency virus types 1 and 2, paramyxoviruses, and myxoviruses. These are two triads consisting of two large and one small hydrophobic amino acid and an AG motif close to or overlapping with the second triad. This putative fusion sequence is also present in hepadnaviruses from rodents. Upstream of this fusion domain is a stretch of 13 amino acids rich in P, E or D, S, and T. This PEST sequence is characteristic of many proteins that are rapidly degraded within the cell (24). The fusion-inducing viruses in Fig. 1C also contain upstream of their fusion domain a protease cleavage site, although of a different specificity. The putative fusion sequence of HBV is bracketed by chymotrypsin and V8 protease cleavage sites. Previous studies have shown that L and M proteins present in virions and subviral particles are cleaved by V8 protease at glutamic acid 2 next to the amino end of the S domains (Fig. 1B) (14, 44). Chymotrypsin cleaves the HBs proteins present in intact virions 14 amino acids downstream of the V8 site (Fig. 1) (24). Thus, V8 and chymotrypsin digestion of virions should remove only the pre-S domains and a small part of the S domain and leave the major part of the S domain intact. Since it is believed that the S domain is sufficient to maintain the morphology of both HBsAg and the virion, it was reasoned that V8 and chymotrypsin cleavage would leave the particle structure essentially unaltered (24). To test this hypothesis, virions were prepared and digested with either protease V8 or chymotrypsin. The integrity of the pre-S1, pre-S2, and S antigenic domains following digestion was evaluated by an ELISA with pre-S1-, pre-S2-, and S-specific antibodies. The results of the ELISA are summarized in Table 1 and show that both V8 protease and chymotrypsin removed the pre-S domains from the virion surface while the S epitopes remained (Table 1). The morphological properties of digested virions were determined by electron microscopy of particles resolved through CsCl gradients. As shown in Fig. 2, virions digested with either V8 or chymotrypsin (Fig. 2B and C) retained the spherical appearance and a diameter (40 to 45 nm) characteristic of undigested control particles (Fig. 2A). The three preparations showed similar amounts of virus-like particles at a concentration of approximately 10^7 virions per ml. Although this concentration resulted in fields of only one or two virions per photograph, hundreds of virions were examined. These data thus show that although V8 and chymotrypsin digestion of virions removes the pre-S epitopes, the morphological integrity of the particles is maintained.

Infection of HepG2 cells and detection of secreted progeny. The ability of protease-digested virions to enter and replicate within tissue culture cells was determined by incubation of HepG2 cells with digested virions. Briefly, HepG2 cells were incubated overnight at pH 5.5 with approximately 100 undigested or V8- or chymotrypsin-digested virions per cell (as in Materials and Methods). Low-pH medium was replaced with standard growth medium, and cultures were maintained, as usual, until needed. Initial evaluation of the infection system was performed by PCR, which is highly sensitive and permits the use of small amounts of material. The PCR used here could detect as few as 10^3 HBV genomes per assay. For initial analysis of intracellular HBV DNA, 10^3 cells were harvested 6 days after infection, potentially adhering HBV was removed by washing at pH 2.4, and HBV sequences were amplified with virus-specific primers and resolved by agarose gel electrophoresis (see Materials and Methods). These results are shown in Fig. 3A. The 592-nucleotide fragment is the expected amplification product and is easily detected in DNA derived from HepG2.2.15 cells but not uninfected HepG2 cells. Since HepG2.2.15 cells are stably transfected HepG2 cells that persistently produce HBV progeny (42) and uninfected HepG2 cells do not contain HBV sequences, these results demonstrate the specificity of this PCR. HepG2 cells incubated with HBV either left intact (∅ in Fig. 3) or digested with chymotrypsin (Ch) contained no detectable viral DNA. Since this PCR detects 10^3 viral genomes and analysis was performed on 10^3 cells, these results suggest that less than one HBV genome per

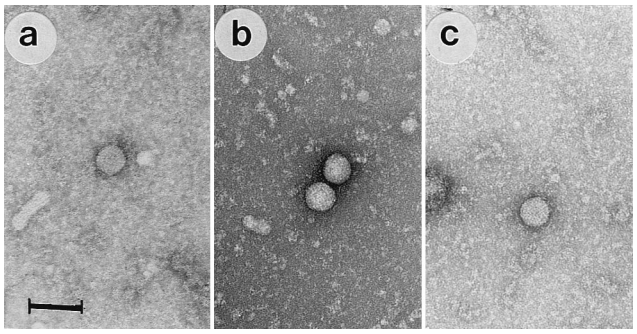

FIG. 2. Morphology of protease-treated virions. Virions were purified and treated with proteases as described in Materials and Methods, stained with uranyl acetate, and examined by electron microscopy. (a) Untreated virus; (b) virus treated with V8 protease; (c) virus treated with chymotrypsin. Bar, 100 nm.

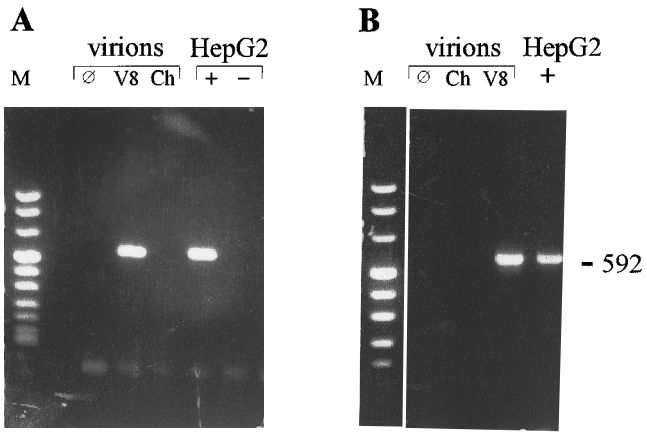

FIG. 3. Detection of HBV DNA by PCR in HepG2 cells infected with V8 protease-treated virions. (A) HepG2 cells were infected with untreated (∅), V8 protease-treated (V8), or chymotrypsin-treated (Ch) virions and were harvested by trypsinization on day 6 p.i. Cells were washed with pH 2.4 buffer (which removes most unabsorbed HBV [data not shown]) and analyzed by standard PCR, which generated the expected 592-bp DNA. The culture medium from HepG2 cells and HepG2.215 cells (which produce HBV) served as negative (−) and positive (+) controls, respectively. M, molecular weight markers. (B) A 1-ml volume of medium collected on day 6 p.i. was immunoprecipitated with anti-pre-S2 MAb Q19/10, and the precipitate was analyzed by nested PCR.

cell was present and that viral entry (or persistence) had not efficiently occurred. On the other hand, an HBV-specific amplification product, comigrating with the HepG2.2.15 cell-derived band, is clearly detectable in HepG2 cells which had been inoculated with V8 protease-digested virus. This suggested that V8-digested virus, unlike the chymotrypsin-digested particles, had gained entry and either replicated or been maintained within the target cells.

The possibility that the infected cells containing intracellular HBV secreted viral DNA was initially tested by immunoprecipitation of particles from 1.0 ml of culture medium (from approximately 10^6 cells) collected 6 days after inoculation. Immunoprecipitation was performed with MAb specific for pre-S2. Since the pre-S2 domain was removed from the inoculating virus by digestion with either V8 or chymotrypsin (Table 1), this method was specific for progeny virus, which would have acquired intact pre-S2 domains during de novo synthesis. The HBV-specific DNA present in the immunoprecipitates was detected by PCR and is shown in Fig. 3B. Primers and methods were as in Fig. 3A. No detectable viral DNA was immunoprecipitated from medium collected from HepG2 cells left uninfected or inoculated with chymotrypsin-digested virus. On the other hand, a clear PCR product, at the expected size of 592 bp, comigrating with material from the HepG2.2.15 cell control was generated from immunoprecipitates of medium from HepG2 cells inoculated with V8 protease-digested virus. These results suggest that HepG2 cells inoculated with virus digested with V8 but not with chymotrypsin took up HBV (Fig. 3A) and secreted progeny HBV (Fig. 3B) which had gained pre-S2 domains.

HBV DNA in HepG2 cells maintained in the presence of a secretion inhibitor. *N*-Butyldeoxynorjirimycin (NBDNJ) inhibits the endoplasmic glycan-processing enzyme, glucosidase I (50). Glucosidase I processes nascent glycoproteins and is necessary for HepG2.2.15 cells to secrete enveloped HBV virions (25). If the appearance of HBV DNA in the medium of infected HepG2 cells (Fig. 3C) was the result of de novo viral gene product synthesis, secretion should be prevented by NBDNJ. Therefore, the ability of HBV to be secreted from infected cells maintained in NBDNJ following infection was determined by Southern blot analysis of viral DNA in the

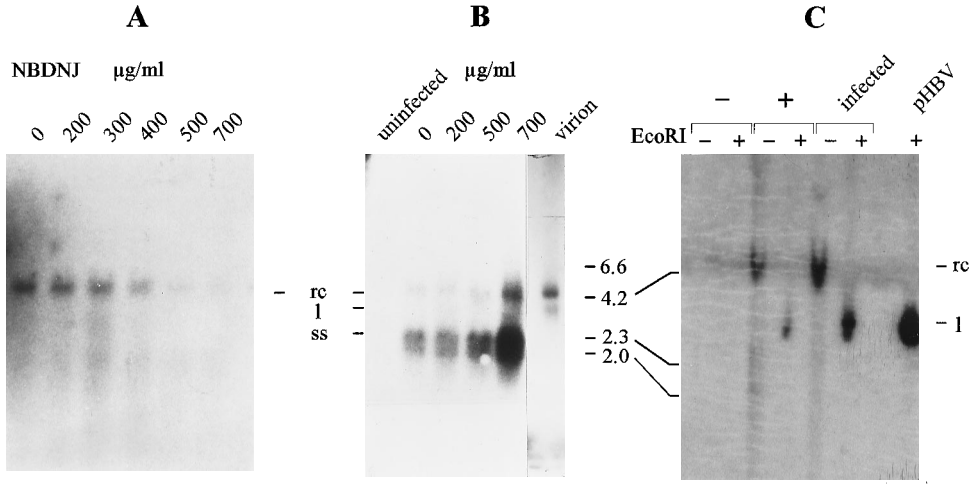

FIG. 4. Southern blot analysis of HBV DNA produced by HepG2 cells after infection with V8 protease-treated virions on day 5 p.i. (A) HBV in medium from 10^6 cells maintained in the absence or presence of 200 to 700 µg of NBDNJ per ml was sedimented by ultracentrifugation and analyzed by Southern blotting as in reference 4. (B) About 10^7 infected cells were harvested by trypsinization, lysed with SDS buffer, and digested with proteinase K. Extracted DNA was digested with *Hin*dIII (which does not cut HBV DNA), resolved by electrophoresis, transferred to Hybond membrane, and probed with a ^{32}P-labeled plasmid containing HBV DNA sequences (4). Parallel cultures were incubated in the absence or presence of 200, 500, or 700 µg of NBDNJ per ml. The right-hand lane shows the DNA extracted from ca. 10^8 plasma-derived virions. (C) DNA extracted from 10^7 HepG2 cells 5 days after infection with V8 protease-digested HBV, as in panels A and B, was passed over Wizard columns to enrich for low-molecular-weight double-stranded DNA. Eluted DNA was left undigested or digested with *Eco*RI (to linearize HBV genomes) and resolved by electrophoresis and Southern blotting followed by hybridization to radioactive HBV probes, as above. Uninfected HepG2 cells (−) and HepG2.2.15 cells (+) were used as controls. In panels A and B, 0.8% agarose gels were used; in panel C, a 1.5% gel was used. Molecular weight markers (*Hin*dIII-digested lambda DNA) are shown, as are the apparent mobilities of the rc, linear (l), and single-stranded (ss) HBV DNA.

culture medium (Fig. 4A). DNA isolated from the medium of HepG2 cells inoculated with V8-digested virus (lanes 0 through 700 μg of NBDNJ per ml) was similar in mobility to the relaxed circular (rc) species derived from the inoculum (Fig. 4B, lane virion). Note that the amount of HBV DNA recovered from the medium of infected cells decreased as a function of the concentration of NBDNJ. Little detectable virus is recovered from cells incubated with the highest NBDNJ concentration (compare lanes 0 and 700). NBDNJ was not toxic at any concentration used, as measured by the dimethylthiazol diphenyltetrazolium bromide (MTT [16]) assay and the ability of cells to secrete labelled albumin (data not shown). These results suggest that (i) a significant number of intact viral DNA genomes are present in the medium of HepG2 cells 6 days after inoculation and (ii) the appearance of these genomes depends on de novo glycosylation processing, implying that the detected genomes are derived from virus which has gained a new envelope from the infected cells.

During the synthesis of progeny viral genomes, cells infected with HBV accumulate genomic structures that are distinct from those present in the virion and are representative of different replication steps (20, 43, 47). For example, cccDNA is derived from the input viral genome, which is a relaxed circle. Linear genomes are also a characteristic form and migrate as 3.2-kb bands. Single-stranded HBV DNA is generated in large amounts during genome replication and migrates close to or together with cccDNA. To determine if the various genomic structures of HBV DNA accumulated in cells inoculated with V8-digested HBV, total DNA was prepared from 10^7 HepG2 cells 6 days after infection, digested with restriction endonuclease *Hin*dIII, resolved in 1.5% agarose gels, and Southern blotted. Blots were hybridized with radioactively labelled plasmid containing HBV sequences, and the results are shown in Fig. 4B. *Hin*dIII does not cleave within the HBV genome and was used to digest host chromosomal DNA to facilitate loading large amounts of cellular DNA onto the gel. Figure 4B shows that cells inoculated with V8-digested HBV accumulate various genomic forms of viral DNA. The abundance of these forms increases as a function of NBDNJ concentration, presumably because secretion of intact virus has been prevented (4). On the basis of the position of the largest band in comparison with virion-derived DNA, the most slowly migrating species is assumed to be a relaxed circle. The signal strength of that relaxed-circular (rc) band was lower than the signal generated by 10^8 virion-derived genomes. If secretion of HBV was inhibited by 700 μg of NBDNJ per ml (Fig. 4B), the amount of rc HBV DNA within cells exceeded the amount in the reference sample, suggesting that in this case more than 10 copies of rc DNA per infected cell were present. The faster-migrating forms of HBV DNA are probably linear and single-stranded DNA and possibly cccDNA. The single-stranded nature of the DNA migrating near the 2.0-kb marker was confirmed by its cytoplasmic location and its resistance to digestion with *Eco*RI (data not shown).

To test the circularity of the largest HBV DNA from these cells, cellular DNA extracts were enriched for low-molecular-weight double-stranded DNA by being passed through Wizard columns (see Materials and Methods). This low-molecular-weight DNA was resolved in a 0.8% agarose gel, Southern blotted, and probed as in Fig. 4A and B. Figure 4C shows that the presumed rc form of the HBV DNA was, as expected, linearized to a 3.2-kb species by the single-cutting enzyme *Eco*RI. A weak signal of cccDNA was detectable in the original autoradiogram at an apparent DNA size of 2 kb; this signal disappeared after *Eco*RI digestion. Since the cccDNA was difficult to detect by Southern blotting, it is further considered

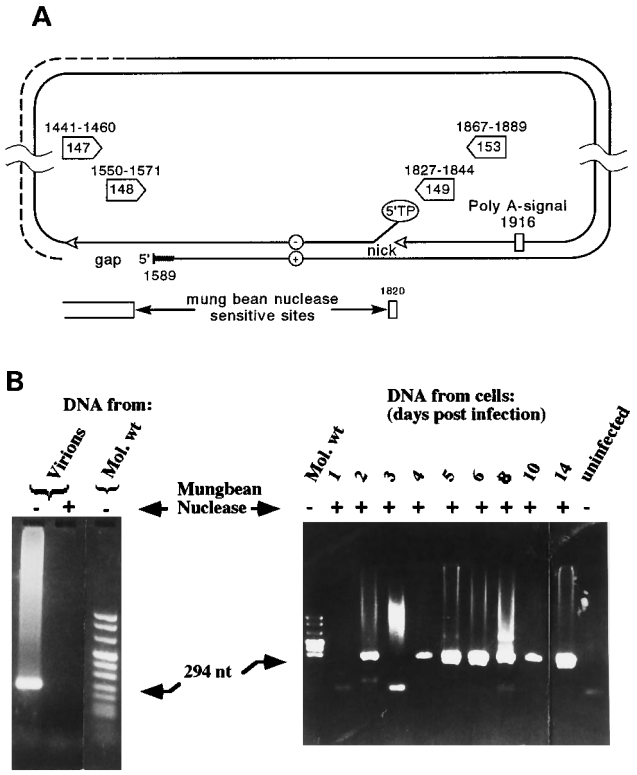

FIG. 5. Detection and kinetics of cccDNA in HepG2 cells infected with V8 protease-treated virions. (A) Map of mung bean nuclease digestion sites and positions of primers for nested PCR. Mung bean nuclease cleaves the single-stranded region of the HBV genome and at the gap in the minus strand. Primer pair 147 and 153 amplifies the DNA from nucleotides 1441 to 1889; primer pair 148 and 149 amplifies the DNA from nucleotides 1550 to 1844, which includes the triple-stranded and a part of the single-stranded region of HBV DNA. The figure is not drawn to scale. 5′TP, terminal protein. (B) Nested PCR generated the expected 294-bp products in extracts from infected HepG2 cells. Bands obtained on day 3 p.i. are nonspecific. DNA from input virions served as a control for effectiveness of the mung bean nuclease digestion (lanes marked "Virions"). Mol. wt, molecular weight markers.

in the next section. Taken together, these data show that HepG2 cells infected with V8-digested HBV accumulate intracellular viral DNA, which is different in structure from that present in the inoculum and resembles the multiple replicative forms found in HepG2.2.15 cells. Moreover, these forms increase in abundance in the presence of NBDNJ, a compound which prevents secretion of virus. The amount of rc HBV DNA in the infected cell culture was similar to that in HepG2.2.15 cells.

Detection of cccDNA. Although HepG2 cells infected with V8 protease-digested virus contained multiple forms of viral DNA which were indicative of genomic replication, the sensitivity of the Southern blot studies (Fig. 4) did not permit unambiguous detection of cccDNA. The virion-associated form of HBV DNA contains a single-stranded gap and a short triple-stranded region at the 5′ end of the minus DNA strand (Fig. 5A). Both these regions are sensitive to single-strand-specific nucleases such as mung bean nuclease (22), whereas cccDNA is resistant. We tested both virions and infected cultures for the presence of cccDNA by PCR amplification of DNA extracts following mung bean nuclease digestion. The primers for this assay were selected such that they were separated by the triple-stranded region and the gap (Fig. 5A). Since the copy number of cccDNA was too low to be detected reli-

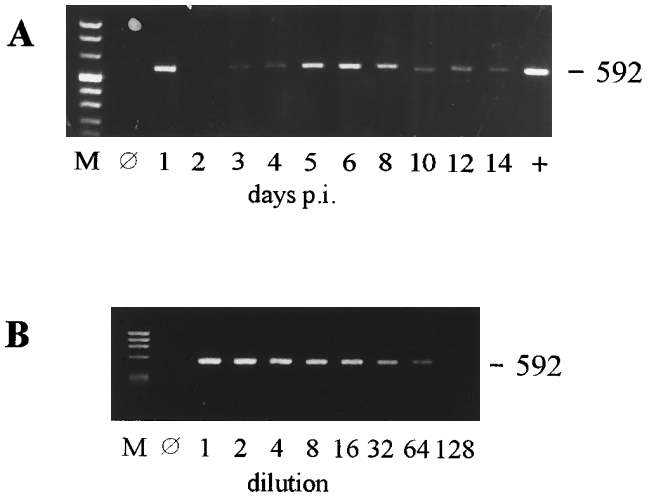

FIG. 6. Kinetics and amount of HBV DNA appearing within HepG2 cells infected with V8-treated virions. (A) Parallel cultures were harvested by trypsinization on the indicated days p.i. Intracellular HBV DNA from 2,000 to 4,000 cells was analyzed by standard PCR as described in Materials and Methods. Extracts from uninfected HepG2 cells (∅) or HepG2.2.15 cells (+) served as controls. (B) PCR of a twofold dilution series of the Eurohep reference sample for HBV particles. The reference plasma was prediluted to 2×10^5 genomes per assay corresponding to the start of the dilution series. At longer exposure, the 1:128 dilution was also positive.

ably by standard PCR, nested PCR was performed. As a control for the sensitivity of virion-derived rc DNA to mung bean nuclease, DNA was isolated from virions and mixed with whole-cell DNA isolated from 10^4 uninfected HepG2 cells (to simulate infected cell conditions) and either mock digested or digested with mung bean endonuclease prior to PCR amplification. As shown in Fig. 5B (left panel), virion-derived DNA cannot be amplified if first digested with mung bean nuclease. This is consistent with the rc or linear nature of virion-derived DNA. The failure to amplify virion DNA following mung bean nuclease digestion was not due to a lack of sensitivity in the system, since DNA not digested with mung bean nuclease could readily be detected (Fig. 5B, left panel). HBV DNA isolated from infected cells was tested for the presence of mung bean nuclease-resistant HBV-specific DNA as a function of time following inoculation (Fig. 5B). Mung bean nuclease-resistant HBV DNA was not detected until 2 days p.i. and was then detected consistently between days 4 and 14 p.i. Anomalous bands at 3 and 8 days are presumably due to occasional false priming in the reaction and have not been reproduced. Overall, the appearance of mung bean nuclease-resistant HBV DNA, in a time-dependent fashion, in HepG2 cells infected with V8-digested virus suggests that cccDNA has been synthesized.

Kinetics and amount of HBV DNA synthesis. The time-dependent appearance of cccDNA raised the question about the time following infection at which de novo-synthesized intracellular and secreted HBV DNA first appears and then declines. The appearance of HBV DNA within the infected HepG2 cells is shown in Fig. 6 as a function of time p.i. HBV DNA was amplified from whole-cell DNA isolated from infected cells (Fig. 6A). On day 1, the inoculated cultures obviously contained input virions despite extensive washings. On day 2, the standard PCR assay became negative or, in some experiments, borderline positive. This suggests that on the average, less than one HBV DNA molecule per cell was present, since analysis was performed on 10^3 cells and this PCR could detect 10^3 HBV genomes. However, on day 3, HBV DNA became detectable, and on days 5 and 6, it reached levels comparable to 10^5 genome copies from the Eurohep reference plasma, as shown in Fig. 6B. Thus, at least 100 genome copies were replicated from one cccDNA molecule. As shown in Fig. 4B, the major part of this DNA may be present as single-stranded replicative intermediate. After days 5 and 6, the amount of intracellular HBV DNA decreased again but remained positive until day 14 at approximately 10 copies per cell. HBV DNA was not detected at any time in cell cultures which were inoculated with untreated or chymotrypsin-treated cells (data not shown).

Kinetics of HBV secretion. The kinetics of HBV DNA secretion is presented in Fig. 7A. Secreted virions were immunoprecipitated with a pre-S2-specific antibody, and HBV DNA was detected in the immunoprecipitates by nested PCR. On days 1 and 2, HBV DNA was not detectable by this assay, as expected since any remaining input virus would be devoid of pre-S2 epitopes as a result of V8 protease digestion. Signals for secreted HBV DNA were found from days 3 through 14 with a peak at days 5 and 6. This parallels the appearance of intracellular HBV DNA (Fig. 7A) and lags behind the appearance of intracellular cccDNA (Fig. 5B).

Antigenic structure of secreted HBV. The virions harvested on day 6 were also precipitated by MAb MA18/7 against pre-S1 and by the conformation-dependent antibody C20/02 against the S domain. A core antigen-specific antibody, 42B12, did not precipitate the secreted virions unless they were treated with detergent (Fig. 7B). The PCR signals of the immune precipitates from culture medium with envelope antibodies were comparable to that of 10^5 virions in the reference sample lane (Fig. 7B, lane 1) or to the supernatant of HepG2.2.15. The weaker signal for the immunoprecipitate of core particles may be due to an incomplete removal of the HBs envelope from the virions.

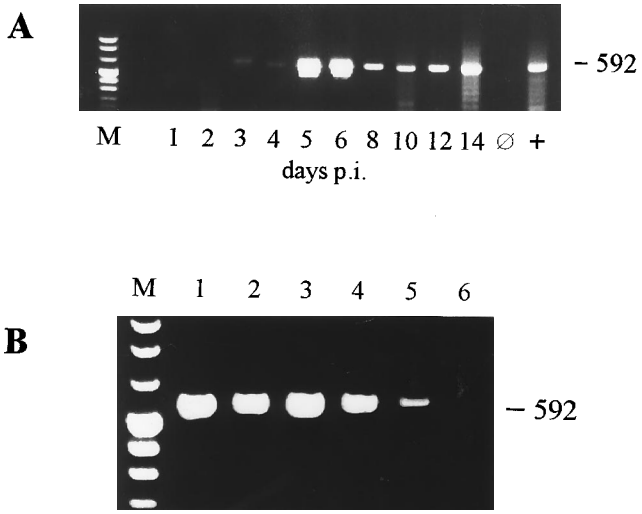

FIG. 7. Kinetics and antigenic structure of virions secreted by infected HepG2 cells. (A) Secreted virion DNA in 1 ml of culture fluid was detected by immunoprecipitation with anti-pre-S2 MAb Q19/10 and nested PCR at the indicated times. The medium of noninfected HepG2 cells (∅) and HepG2.2.15 cells (+) served as negative and positive controls, respectively. (B) PCR of immunoprecipitated HBV from day 6 p.i. with pre-S1 MAb MA18/7 (lane 2) pre-S2 MAb Q19/10 (lane 3), S MAb C20/02 (lane 4), and HBV core MAb 42B12 (lanes 5 and 6). For lane 5, the virions were pretreated with 1% Tween 20. Lane 1 shows the results for 2×10^5 genomes from the Eurohep reference samples, which were immunoprecipitated with MAb C20/02.

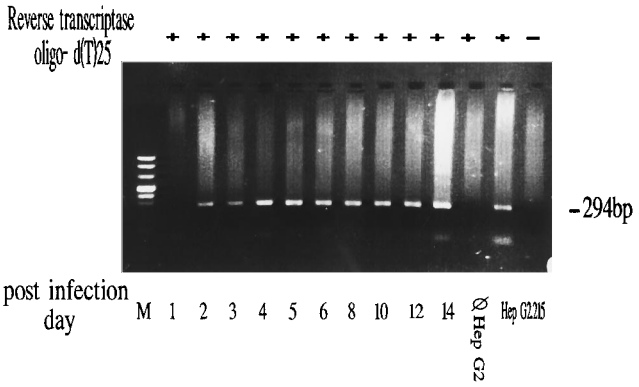

FIG. 8. Kinetics of polyadenylated HBV RNA in infected HepG2 cells. RNA was extracted with guanidinium thiocyanate and phenol-chloroform at the indicated times. HBV DNA was digested with DNase. Polyadenylated RNA was reverse transcribed with oligo(dT) as the primer, and cDNA was amplified by PCR. RNA from HepG2.215 was used as a positive control. An RNA-specific negative control without oligo(dT) and reverse transcriptase with RNA extract from HepG2.2.15 is shown in the last lane. The negative result shows that HBV DNA was completely digested by DNase.

HBV RNA in HepG2 cells infected with V8-digested virus. If the appearance of viral DNA in the medium of infected cells was due to de novo synthesis of progeny, viral RNA should appear in a time-dependent fashion. Therefore, the kinetics of the appearance of polyadenylated viral RNA within the HepG2 cells as a function of time after inoculation was studied by reverse transcriptase and subsequent PCR of the cDNA. As shown in Fig. 8, 1 day after infection, HBV RNA was undetectable by reverse transcription-PCR; however, from 2 to 14 days after infection, HBV RNA was readily detectable. The kinetics of HBV RNA appearance parallel the appearance of cccDNA (Fig. 5C). A control reaction with HepG2.2.15 cells showed that the signal of the reverse transcription-PCR depended on RT and oligo(dT) as primer and was not due to HBV DNA (compare lanes HepG2 2.2.5 + and −).

HBV antigens within HepG2 cells infected with V8-digested virus. To estimate the relative number of infected cells producing viral antigen, following infection with V8 protease-digested virus, cells were examined for the production of HBV antigens by immunofluorescence following inoculation (Fig. 9). The infected cells contained HBV core antigen (Fig. 9A) and pre-S2 antigen (Fig. 9B) as detected by immunofluorescence staining of the permeabilized cell cultures. Cells inoculated with undigested virions did not show a core- or pre-S2-specific immunostaining (Fig. 9C and D). At least 10,000 infected cells were examined, and approximately 10 to 30% of the cells in a culture were positive. The intracellular pattern of staining was similar to that obtained with HepG2.2.15 cells. A larger percentage of HepG2.2.15 cells was positive, but the staining intensity was on the average weaker (data not shown). The detection of pre-S2 antigen was consistent with the hypothesis that not only were the V8-treated virions taken up by the cells but also that de novo viral protein synthesis had occurred, since the input virions no longer contained detectable levels of pre-S2 antigen (Table 1). Parenthetically, there was no evidence of increased cytopathology within infected cultures compared with uninfected controls.

DISCUSSION

The results of this study agree with those of Qiao et al. (37), in that HepG2 cells are not susceptible to natural serum-derived HBV virions at a multiplicity of infection of approximately 100 particles per cell. The infection of HepG2 cells with serum-derived virions reported by Bchini et al. (3) may be due to the much larger inoculum used, the different chemical treatment of the cells, or perhaps to a difference in the subclone of HepG2 cells used. Significantly, however, we did observe infection when the virions were treated with V8 protease but not chymotrypsin and the infection was performed at pH 5.5. We have determined elsewhere that V8 protease cleaves L and M proteins at amino acid 176 and chymotrypsin cleaves 14 amino acids downstream, as shown in Fig. 1 (see also references 14 and 24). It is suggested that exposure of the amino acids between these cleavage sites plays a critical role in mediating the infectivity of the virus in tissue culture. Since this stretch of amino acids contains a consensus fusion sequence (Fig. 1), it is tempting to speculate that in nature, exposure of an envelope protein fusion function plays a role in infectivity. In this regard it is interesting that Rodriguez et al. (39) and we (unpublished data) have recently observed that peptide fragments of the HBV envelope protein containing amino acids 164 to 220 of L protein mediate fusion of biomembranes in vitro.

Optimal HBV infectivity requires low-pH medium during the incubation of virus with cells. The necessity of low pH for infection is also consistent with the hypothesis that fusion between the virus and host membranes is promoted by exposure of fusion domains in the envelope antigen. Several other viral envelope proteins, e.g., from influenza virus, also require low pH for induction of fusion.

It appears unlikely that infectivity in our system involves cell surface receptors specific for the pre-S-mediated HBV attachment observed by others (6, 9, 32, 34, 37), since the surface-exposed pre-S domains are removed by V8 protease digestion. We favor a model whereby V8-digested virions bind via S-region interactions (17, 38) with possible fusion receptors, although more work is needed to verify this speculation.

Fusion sequences react with still unidentified ubiquitous structures in cell membranes. Thus, V8 protease-treated virions should be able to enter all kinds of cells. We have previously shown that the immortalized fetal mouse hepatocyte line FMH 202 produces HBV after transfection with dimeric HBV DNA (18). Untreated virions are not infectious for this cell line and do not attach to it, but V8-treated virions can enter the cell and replicate in it (24a). From our data, we postulate that HepG2 cells and, by implication, most permanent hepatocyte-derived lines are nonsusceptible to HBV, because they do not provide a sufficiently active protease for cleavage of the M and/or L envelope protein. Whether a protease, acting at a site close to the V8 site, cleaves M and/or L protein during in vivo infection is unknown. However, it should be kept in mind that the liver is a major site of protein uptake and degradation (19).

Several lines of evidence suggest that the appearance of HBV DNA in the medium of HepG2 cells inoculated with V8-digested virus is the result of the de novo synthesis. First, viral DNA in the medium was immunoprecipitated with MAb specific for pre-S2 and was not detectable until 3 days after inoculation. Since pre-S2 domains were removed by V8 protease digestion, virus in the inoculum would not be efficiently precipitated. Second, the lack of virus in the medium on days 1 and 2 suggests that time for new virus synthesis was necessary. Third, the inhibition of virus secretion and the accumulation of intracellular forms of viral DNA following incubation of cultures with NBDNJ also provide evidence that de novo synthesis of virus and active secretion of enveloped virus were occurring. NBDNJ inhibits HBV secretion from HepG2.2.15 cells (4) by inhibiting endoplasmic reticulum glucosidase I (25). Since glucosidase I processes nascent glycoproteins in the en-

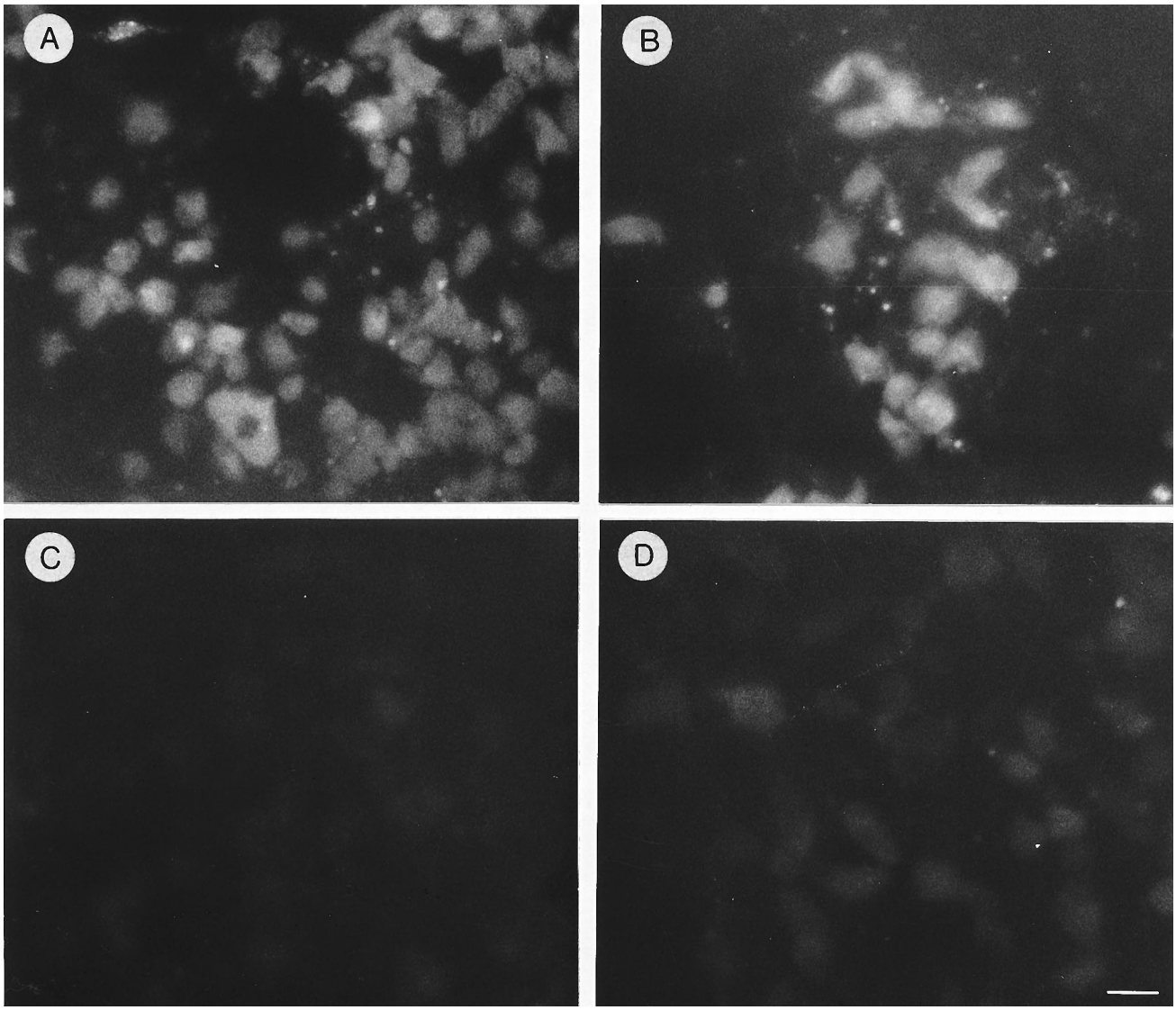

FIG. 9. Detection of HBV core and pre-S2 antigen in HepG2 cells infected with V8-digested HBV. Cells were inoculated with either undigested HBV (C and D) or V8-digested HBV (A and B) and probed with either mouse MAb anti-core (A and C) or anti-pre-S2 (B and D) antibody, followed by incubation with fluorescein labeled anti-mouse antibody. Bar in panel D, 10 μm.

doplasmic reticulum, it would be expected to be necessary for virus secretion only if (i) new protein synthesis was necessary and (ii) enveloped virus was secreted.

Infection as described here may be a more authentic way than transfection to deliver the HBV genome to a target cell. In our system, at least 10% of the cells took up viral DNA and expressed viral gene products. The infected cells are particularly useful for the study of the early steps in viral replication. Furthermore, we have observed that HepG2 cells infected with HBV in this manner, in contrast to HepG2.2.15 cells (35) but in agreement with natural infection (36), do not secrete nonenveloped nucleocapsids. The infected cells secreted virus containing all three envelope domains for up to 40 days after inoculation (data not shown). However, this artificial infection system still does not reach the efficiency of in vivo infection, because only 1 in 10^3 virions or less of the inoculum enters the replication cycle. The ultimate relevance of this mode of infection to the natural infection of the liver by HBV therefore remains to be determined.

ACKNOWLEDGMENTS

We thank B. Boschek for performing electron microscopy and G. Caspari for providing HBV-containing plasma.

This study was supported by Sonderforschungsbereich 249 of Deutsche Forschungsgemeinschaft, NATO, and The Hepatitis B Foundation (of America).

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