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Research Article

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Targeted Transfection and Expression of Hepatitis B Viral DNA in Human Hepatoma Cells

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Abstract

A soluble DNA carrier system consisting of an asialoglycoprotein covalently linked to poly-L-lysine was used to bind DNA and deliver hepatitis B virus (HBV) DNA constructs to asialoglycoprotein receptor-positive human hepatoma cells. 4 d after transfection with surface or core gene expression constructs, HBsAg and HBeAg in the media were measured to be 16 ng/ml and 32 U/ml per 10^7 cells, respectively. Antigen production was completely inhibited by the addition of an excess of asialoorosomucoid. On the other hand, asialoglycoprotein receptor-negative human hepatoma cells, SK-Hep1, did not produce any viral antigens under identical conditions after incubation with HBV DNA complexed to a conjugate composed of asialoorosomucoid and poly-L-lysine. Using a complete HBV genome construct, HBsAg and HBeAg levels reached 16 ng/ml and 16 U/ml per 10^7 cells, respectively. Northern blots revealed characteristic HBV RNA transcripts including 3.5-, 2.4-, and 2.1-kb fragments. Intracellular and extracellular HBV DNA sequences including relaxed circular, linear and single stranded forms were detected by Southern blot hybridization. Finally, 42-nm Dane particles purified from the spent culture medium were visualized by electron microscopy. This study demonstrates that a targetable DNA carrier system can transfect HBV DNA in vitro resulting in the production of complete HBV virions. (*J. Clin. Invest.* 1993. 91:1241–1246.)

Key words: hepatitis B virus • replication • targeted expression • asialoglycoprotein

Introduction

Studies on the pathogenesis of hepatitis B virus (HBV)[1] infection have been hampered by the narrow host range of the virus which limits in vivo models to higher primates. The chimpanzee and gibbons are the only animals in which HBV experimental infection has been successfully established. Because of its scarcity and expense in handling, the usefulness of this model is relatively limited. The demonstration of HBV replication in several hepatoma cell lines after transfection of HBV DNA has been instrumental in elucidating the replication pathway of HBV (1, 2). However, in vivo behavior may not be inferred reliably from these in vitro observations. The discovery and characterization of HBV-like viruses in other animals has permitted the establishment of new models for the study of the biology of hepadnaviruses, and indeed, these studies have provided much needed insights into the pathogenesis of HBV infection (3, 4). However, the basic physiology and immunology of hepatitis in these animals remain largely unknown, and many of the molecular and immunological tools to study these hepadnaviruses are yet to be developed. In addition, information obtained in these animal models may not actually reflect the pathogenesis of HBV infection in humans.

In this study, we have taken advantage of a soluble DNA carrier system which has been used to target a foreign gene specifically to liver in vitro and vivo via asialoglycoprotein receptors of hepatocytes (5, 6). This DNA carrier system consists of a galactose-terminal (asialo-) glycoprotein covalently linked to poly-L-lysine which in turn binds to DNA in a strong noncovalent interaction. Previous studies have shown that foreign genes can be successfully targeted to, and expressed in the liver using this delivery system (6, 7). In addition, persistent expression of a foreign gene can be achieved by partial hepatectomy (7). The objective of the experiments described below were to determine whether this system can be used to target HBV DNA via asialoglycoprotein receptors and obtain viral gene expression and replication in human hepatocellular carcinoma cell lines.

Methods

Cells and cell culture. Human hepatoma cell lines HuH-7 (8) and SK-Hep1 (9) were maintained in plastic dishes containing modified Eagle's medium (MEM) (Gibco Laboratories, Grand Island, NY) plus 10% fetal bovine serum at 37°C and 5% CO_2. As positive controls, HuH-7 and SK-Hep1 cells were grown to 50% confluence and transfected with DNA using a modified $CaPO_4$ transfection method (10).

HBV plasmids. A replication-competent construct of HBV genome, adw R9 (1.2 × length of HBV genome) (11) was cloned into pGEM7Zf(+) (Promega Biotec, Madison, WI). To express the HBV surface gene, a 1,982-bp fragment of HBV genome from EcoRI (nt 0) to BglII (nt 1982) containing the major surface antigen open reading

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1. *Abbreviations used in this paper:* AsOR, asialoorosomucoid; HBV, hepatitis B virus; HuH, human hepatoma cells.

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frame was inserted into the multiple cloning site of pJ3Ω expression vector (12) which uses the SV40 enhancer/promoter to express the inserted gene, as the pSVHBVSurf (Fig. 1). Similarly, the HBV core region from HincII (nt 1684) to HaeIII (nt 2602) was inserted to express the c/e antigen. To construct this plasmid, the complete HBV genome was initially cleaved with HaeIII enzyme, and then digested with HincII and BclI following the addition of a BclI linker. A 918-bp fragment containing two heterologous ends (a blunt end from HaeII restriction and a 3′ overhang end with BclI/BglII compatible sequence) was isolated and inserted between the SmaI and BglII sites of the pJ3Ω vector, as the pSVHBVCore (Fig. 1). The nucleotide numbering was based on EcoRI site as nucleotide position zero in the published HBV sequences (Genbank). DH5α cells (Bethesda Research Laboratories, Bethesda, MD) carrying these plasmid were grown and lysed as per standard protocols. All plasmids were purified through cesium chloride/ethidium bromide equilibrium gradient centrifugation.

Assays for asialoglycoprotein receptor activity. Asialoglycoprotein receptor activity of HuH-7 and SK-Hep1 cell lines was determined by uptake studies of ^{125}I-asialoorosomucoid (13). In brief, 35-mm tissue culture dishes (Falcon Plastics Co., Oxnard, CA) were seeded generally 6 d before assay ($0.3–0.5 \times 10^6$ cells/dish). Confluent cells were incubated in serum-free MEM at 37°C for 12 h to eliminate residual asialoglycoproteins that might have been present in the serum-containing medium. Then, 3 μg/ml of ^{125}I-AsOR was added and the dishes were immediately placed at 37°C. At regular time intervals, dishes were placed at 4°C, then rinsed with PBS. Surface-bound asialoorosomucoid was removed by PBS, pH 5.0, 1 mM EDTA, followed by harvesting of cells with 0.1 N NaOH. The bound and internalized radioactivity was determined using a gamma counter. Nonspecific uptake was determined in the presence of a 100-fold excess of unlabeled asialoorosomucoid (AsOR), and specific uptake was determined as the difference between total and nonspecific accumulation of radioactivity in the cell lysates.

Targeted transfection. A targetable DNA carrier system was prepared by chemically coupling asialoorosomucoid (AsOR) to poly-L-lysine (M_r = 59,000) as described previously (5, 6). Transfection of cells with HBV DNA was accomplished as described previously (5–7). HuH-7 and SK-Hep1 cells were grown separately in 60-mm plastic dishes to half-confluence. Growth medium was replaced with MEM containing 3 mM Ca^{2+}, 0.1 μg/ml biotin, and cells incubated for 8 h at 37°C to eliminate residual asialoglycoprotein that may have been present in the medium. Cells were then changed to medium plus AsOR-poly-L-lysine-HBV DNA complex (5 μg/ml, based on DNA), complex plus 100-fold molar excess of AsOR, or uncomplexed HBV DNA alone (5 μg/ml). After 24 h, cells were changed to medium plus 10% fetal bovine serum.

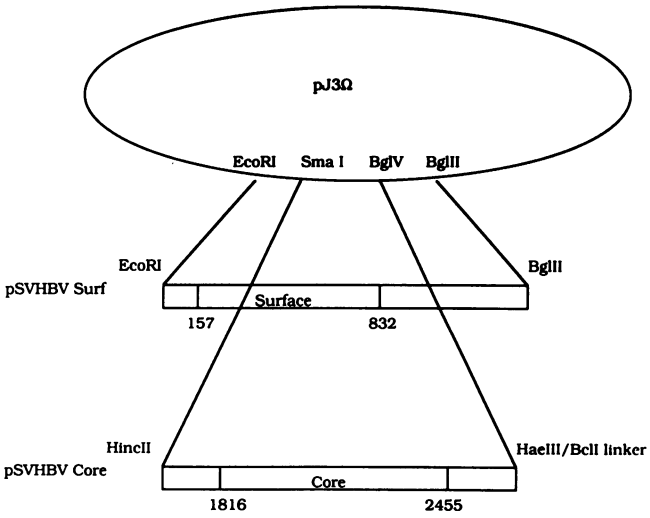

Figure 1. HBV surface antigen and core antigen constructs. A 1,982-bp fragment of HBV genome from EcoRI to BglII sites containing the major surface open reading frame was inserted into the multiple cloning site of pJ3Ω expression vector. Similarly, the HBV core region from HincII to HaeIII was inserted to express the c/e antigen as described in Methods.

Assays for HBV antigens. Aliquots were removed after 4 d and assayed for hepatitis B surface antigen using a monoclonal immunoradiometric assay (Centocor, Malvern, PA). The HBsAg was quantitated by serial dilution of the sample by factors of two and multiplying the dilution factor required to bring the sample to below the cut-off value of the assay by 50 pg/ml, which is the detection limit of this assay (14). The culture medium sample was also assayed for HBeAg using HBe (rDNA) kit (Abbott Laboratories, Chicago, IL). HBeAg was quantitated by serial dilution of the sample by factors of two, and the dilution factor required to bring the sample below the cut-off value of the assay was arbitrarily designated as the number of units of HBeAg per ml. For a study on the time course of surface antigen appearance, 200-μl aliquots of medium were removed daily and assayed as described above.

Detection of viral replication. 4 d after transfection with either $CaPO_4$ precipitation or targeted delivery via asialoglycoprotein receptors, HuH-7 cells were harvested for viral DNA and RNA analysis. The secreted HBV viral particles were purified from the conditioned culture medium and analyzed as described previously (11, 15). Viral replicative intermediates associated with intracellular core particles were isolated and analyzed as described previously (11, 15). RNA was prepared by a guanidine-isothiocyanate-acid-phenol method (16) analyzed by formaldehyde agarose gel, and hybridized with HBV specific probes.

Purification and concentration of HBV particles from cell culture medium. Conditioned cell culture medium from HuH-7 cells transfected by targeted delivery was centrifuged at 5,000 rpm for 1 h at 4°C as described previously (17). The clarified medium was then layered on a 5-ml 25% sucrose cushion in TEN buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4) and centrifuged at 25,000 rpm for 16 h at 4°C in an SW28 rotor. The pellet was suspended in TEN buffer. Isopycnic centrifugation of the sedimented particles was performed in a 20–50% continuous cesium chloride gradient at 35,000 rpm for 16 h at 4°C in an SW41 rotor (Beckman Instruments, Palo Alto, CA). Fractions were collected from the bottom of the polyallomer tubes and the density was determined with a refractometer.

Electron microscopy. Fractions from a cesium chloride gradient were dialyzed against TEN buffer. Samples with buoyant densities between 1.24 g/ml and 1.28 g/ml were applied to carbon-coated copper grids. 1% Uranyl acetate was added for negative staining (17). The preparations were observed with a Zeiss EM 10 electron microscope.

Results

Asialoglycoprotein receptor activity of HuH-7 and SK-Hep1 human hepatoma cells. Incubation of SK-Hep1 cells with a labeled asialoglycoprotein ligand failed to result in significant uptake at 37°C even after 2 h. In contrast, HuH-7 cells took up the ligand at a rate of 20 ng/10^6 cells in the first hour and this rate remained linear for at least 2 h at 37°C (Fig. 2). Based on these data, HuH-7 cells were designated asialoglycoprotein receptor-positive while the SK-Hep1 cell line were labeled receptor-negative.

Targeted expression of HBV antigens using surface and core gene constructs. The two plasmids which contain either the surface or the core gene constructs under the control of a heterologous promoter (SV40) were incubated with HuH-7 and SK-Hep1 cells using either the AsOR-DNA complex or the $CaPO_4$ precipitation method. The inclusion of the precore sequence with the core gene construct allowed the detection of gene expression by measuring e antigen in the culture media.

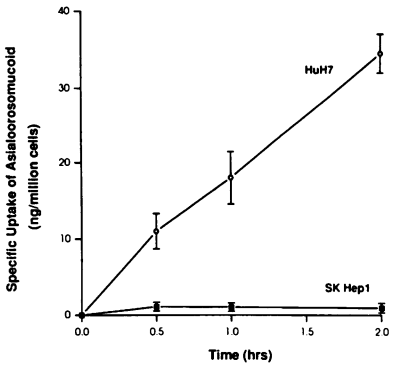

Figure 2. Assay for asialoglycoprotein receptor activity in HuH-7 and SK-Hep1 cells. In brief, confluent cells were incubated in serum-free MEM at 37°C for 12 h to eliminate residual asialoglycoproteins that might have been present in the serum-containing medium. Then, 3 μg/ml of ^{125}I-AsOR was added and the dishes were immediately placed at 37°C. At regular time intervals, dishes were placed at 4°C, then rinsed with PBS. Surface-bound asialoorosomucoid was removed by PBS, pH 5.0, 1 mM EDTA, followed by harvesting of cells with 0.1 N NaOH. The bound and internalized radioactivity was determined using a gamma counter. Nonspecific uptake was determined in the presence of a 100-fold excess of unlabeled asialoorosomucoid, and specific uptake was determined as the difference between total and nonspecific accumulation of radioactivity in the cell lysates. Assays were performed in triplicate and the results expressed as means±SEM in units of nanograms per million cells as a function of time.

As shown in Table I, HuH-7 cells exposed to plasmid DNA alone failed to produce detectable HBsAg or HBeAg in the media. In contrast, HuH-7 cells treated with the complexed surface or core gene constructs resulted in the production of HBsAg and HBeAg at concentrations of 16 ng/ml and 32 U/ml/10^7 cells, respectively. Addition of a 100-fold molar excess of AsOR to the complex in the incubation media led to complete inhibition of HBsAg and HBeAg production. This competitive inhibition of complex uptake by a large molar excess of AsOR indicates that recognition of the complex by HuH-7 cells was directed by the asialoglycoprotein component of the complex. Exposure of SK-Hep1 asialoglycoprotein receptor–negative cells to the complexed constructs failed to produce detectable HBsAg or HBeAg. However, when constructs were added to SK-Hep1 cells in the form of $CaPO_4$ precipitates, significant antigen production was obtained. The concentrations of HBsAg and HBeAg in the media were 32 ng/ml and 32 U/ml per 10^7 cells, respectively. This indicates that SK-Hep1 cells were able to express viral genes and that the absence of antigen production by SK-Hep1 cells treated with the complexed constructs was due to the lack of complex uptake.

Table I. Targeted Expression of HBV Antigens Using Surface and Core Expression Constructs

Plasmid: pSVHBVSurface				
Receptor			HBsAg	HBeAg
Cells	*Status*	*Additions*	*ng/ml*	*U/ml*
HuH-7	(+)	DNA alone	<0.05*	<1
HuH-7	(+)	DNA-AsOR complex	16	<1
HuH-7	(+)	DNA AsOR complex + 100-fold excess AsOR	<0.05	<1
HuH-7	(+)	DNA as $CaPO_4$ precipitate	64	<1
SK-Hep1	(−)	DNA-AsOR complex	<0.05	<1
SK-Hep1	(−)	DNA as $CaPO_4$ precipitate	32	<1
Plasmid: pSVBHBVCore				
HuH-7	(+)	DNA alone	<0.05	<1
HuH-7	(+)	DNA-AsOR complex	<0.05	32
HuH-7	(+)	DNA AsOR complex + 100-fold excess AsOR	<0.05	<1
HuH-7	(+)	DNA as $CaPO_4$ precipitate	<0.05	128
SK-Hep1	(−)	AsOR-PL-DNA complex	<0.05	<1
SK-Hep1	(−)	DNA as $CaPO_4$ precipitate	<0.05	32

* 0.05 ng/ml corresponds to the lowest detectable signal. Complexed HBV DNA was incubated with HuH-7 or SK-Hep1 cells in 60 mm dishes. A total of 10 μg of complex (based on DNA) was applied and 5 μg of DNA used for the $CaPO_4$ transfection method. Aliquots of culture medium were collected and assayed for HBsAg and HBeAg 4 d after transfection. HBsAg and HBeAg were quantitated per 10^7 cells as described in Methods. The results shown are representative of three separate experiments.

Targeted expression of HBV antigens using a complete HBV genome. To determine whether genomic HBV DNA could be targeted to cells via asialoglycoprotein receptors with subsequent viral replication, a complete HBV viral genome construct, adw R9, which is capable of directing HBV replication, was complexed with the AsOR-PL carrier and incubated with HuH-7 and SK-Hep1 cells. Table II shows that HuH-7 cells which were either untreated or exposed to viral DNA alone failed to produce detectable HBsAg and HBeAg. However, HuH-7 cells treated with AsOR-HBV DNA complex resulted in the production of HBsAg at a concentration of 6.4 ng/ml per 10^7 cells. The addition of a 100-fold excess of an asialoglycoprotein to compete with the complex for receptor uptake resulted in inhibition of HBsAg and HBeAg production to below the limits of detectability. Finally, exposure of complexed HBV DNA to SK-Hep1, asialoglycoprotein receptor–negative cells, resulted in no significant production of HBsAg and HBeAg under identical conditions. In contrast to the previous results of SK-Hep1 cells transfected with the SV40 driven constructs, the SK-Hep1 cells, after $CaPO_4$ transfection of

Table II. Targeted Expression of HBV Antigens Using a Complete HBV Genome

Cells	Additions	HBsAg	HBeAg
		ng/ml	*U/ml*
HuH-7	None	<0.05	<1
HuH-7	HBV DNA alone	<0.05	<1
HuH-7	HBV DNA-AsOR complex	6.4	16
HuH-7	HBV DNA-AsOR complex + 100-fold excess AsOR	<0.05	<1
HuH-7	HBV DNA-AsOR + $CaPO_4$ precepitation	25.6	64
SK Hep1	None	<0.05	<1
SK Hep1	HBV DNA alone	<0.05	<1
SK Hep1	HBV DNA-AsOR complex	<0.05	<1

Complexed HBV DNA was incubated with HuH-7 or SK-Hep1 cells in 60-mm dishes as described in the text. A total of 10 μg of complex (based on DNA) was applied. For the $CaPO_4$ transfection method, 5 μg of HBV DNA was used. Aliquots of culture medium were collected and assayed for HBsAg and HBeAg which are expressed as 10^7 cells 4 d after transfection as described in Methods. The results shown are representative of three separate experiments.

HBV DNA, did not produce any significant quantity of HBV antigens (Table II). This could have been due to the absence of liver specific cellular factors necessary for the transcription of HBV genome in SK-Hep1 cells, which is a poorly differentiated fibroblastoid hepatoma cell line (9).

A study on the time course of the appearance of surface antigen in the medium of HuH-7 cells exposed to ASOR-PL-DNA revealed that HBsAg concentration increased nearly linearly through the first 4 d: 0.52±0.10, 1.4±0.20, 2.8±0.40, and 6.9±0.4 ng/ml/10^7 cells on days 1–4, respectively. Thereafter, antigen concentration rose more slowly, reaching 8.4±0.20 ng/ml/10^7 cells on day 7.

Identification of HBV-specific transcripts and replicative forms. After targeted transfection of HBV DNA into the HuH-7 cells, the cell lysates were analyzed for viral RNA by Northern blots (Fig. 3 *A*). No viral RNA was detected in cells exposed to the HBV DNA plasmid (Fig. 3 *A*, lane *1*). In contrast, characteristic viral RNA transcripts including the pregenomic 3.5-, and subgenomic 2.4- and 2.1-kb RNA were detected in AsOR-PL-HBV DNA complex-treated cells (Fig. 3 *A*, lane *2*), and in $CaPO_4$-treated positive controls (lane *3*). Core-associated viral replicative intermediates (panel *B*, lane *2*) and virion-associated complete viral genomes (panel *B*, lane *5*), ranging from single-stranded, double-stranded linear to relaxed circular forms, were detected in the cell lysate and culture medium respectively. Viral RNA and DNA were also analyzed in $CaPO_4$-transfected cells for comparison (panel *A*, lane *3;* panel *B*, lanes *3* and *6*). Parallel to the HBV antigen productions, there were much higher levels of HBV RNA and DNA in $CaPO_4$ transfected cells. In contrast, cell lysates from experiment in which excess ASOR was added with the DNA complex did not show any replicative intermediates (data not shown).

DNA isolated from transfected cells was still present after digestion with Dpn1 (not shown). This indicates that the DNA extracted from the cell lysates was predominantly mammalian in origin, and not bacterial in origin (18). In addition, the relaxed circular form of complete HBV genome can be converted to linear form after treatment with EcoRI, which has a single recognition site in the HBV genome (not shown).

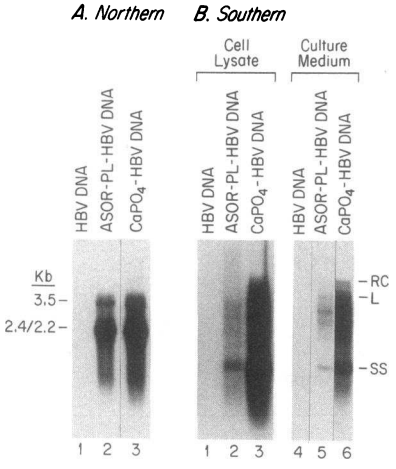

Figure 3. HBV transcription and replication in HuH-7 cells after targeted delivery. HuH-7 cells were plated and transfected with HBV DNA with either AsOR complex or $CaPO_4$ precipitation method. 4 d later, viral RNA and DNA were isolated and analyzed as per Methods. Panels *A* and *B* showed Northern and Southern blot analysis, respectively. In panel *A*, lane 1 showed HBV DNA alone, lane 2 HBV DNA-AsOR complex, and lane 3 HBV DNA/$CaPO_4$ precipitation. In panel B, lanes 1 and 4 were HBV DNA alone, lanes 2 and 5 HBV DNA-AsOR complex, and lanes 3 and 6 HBV DNA/$CaPO_4$ precipitation.

Identification of HBV particles by electron microscopy. Gradient fractions with densities between 1.18 g/ml and 1.22 g/ml which contains the HBsAg particles, were examined by electron microscopy. Several double-shelled particles, 42 nm in size, were observed along with many 22-nm spherical particles, (Fig. 4). These were identical in appearance to particles isolated from media conditioned by cell lines permanently transfected previously with HBV DNA in a nontargeted fashion (19). No particles could be identified in gradient fractions containing media from nontransfected HuH-7 cells.

Discussion

Establishment of an in vivo model for HBV infection has remained a central issue to the study of HBV pathogenesis. Due to the narrow host range of the hepadnaviruses, only their respective natural host or closely related species can be infected (1–3). Many investigators have shown previously that hepadnaviruses are able to replicate efficiently in hepatocytes or hepatoma cell lines from other species after artificial introduction (transfection) of viral genomes into these cells (15, 20). Shih et al. have demonstrated HBV replication in rat hepatoma cells stably transfected with HBV DNA using $CaPO_4$ precipitation (15). Furthermore, direct injection of HBV DNA into the liver of mice resulted in production of HBV viral antigens several months later (21). Another group using the liposome delivery system was able to introduce HBV DNA into the mouse liver and obtain HBsAg expression (22) and hepatitis (23). These data support the likelihood that the species specificity and tissue tropism probably depend on the interaction between the virus and specific cell-surface receptors. To test this hypothesis, HBV DNA in the form of a complex targetable to asialoglycoprotein receptors was used to transfect an AsOR receptor-bearing hepatoma cell line.

The HuH-7 cell line is a well-differentiated human hepatoma cell line, as indicated by cell morphology and ability to synthesize liver-specific enzymes and plasma proteins (8). HuH-7 cells have been shown to be negative for HBV infection and for integrated HBV DNA (24). They have been transiently transfected in a nontargeted fashion, with cloned HBV DNA by $CaPO_4$ precipitation with demonstration of characteristic transcripts, replicative intermediates and mature viral particles (24). In a similar transfection experiment of HuH-7 cells by the transient technique, Chang et al. demonstrated Dane-like particles in the culture media (25). Of interest, these investigators showed very little expression of HBsAg and no detectable HBeAg in SK-Hep1 cells transfected by $CaPO_4$ precipitation. This agrees with our findings and is probably due to the poorly differentiated state of this cell line.

Using the AsOR-DNA complex, we were able to achieve targeted transfection of genomic or subgenomic HBV DNA expression constructs into human hepatoma cells containing AsOR receptor and obtain high levels of viral gene expression. After targeted delivery, analysis of cellular RNA for viral transcripts revealed three species of RNA, 3.5-, 2.4- and 2.1-kb fragments. These are compatible with the most abundant viral mRNA transcripts detected in HBV-infected liver tissue (1, 2, 26) and in HBV DNA-transfected HuH-7 cells (24). Production of viral replicative intermediates and complete HBV particles (Dane particles) were evident in these cells as well.

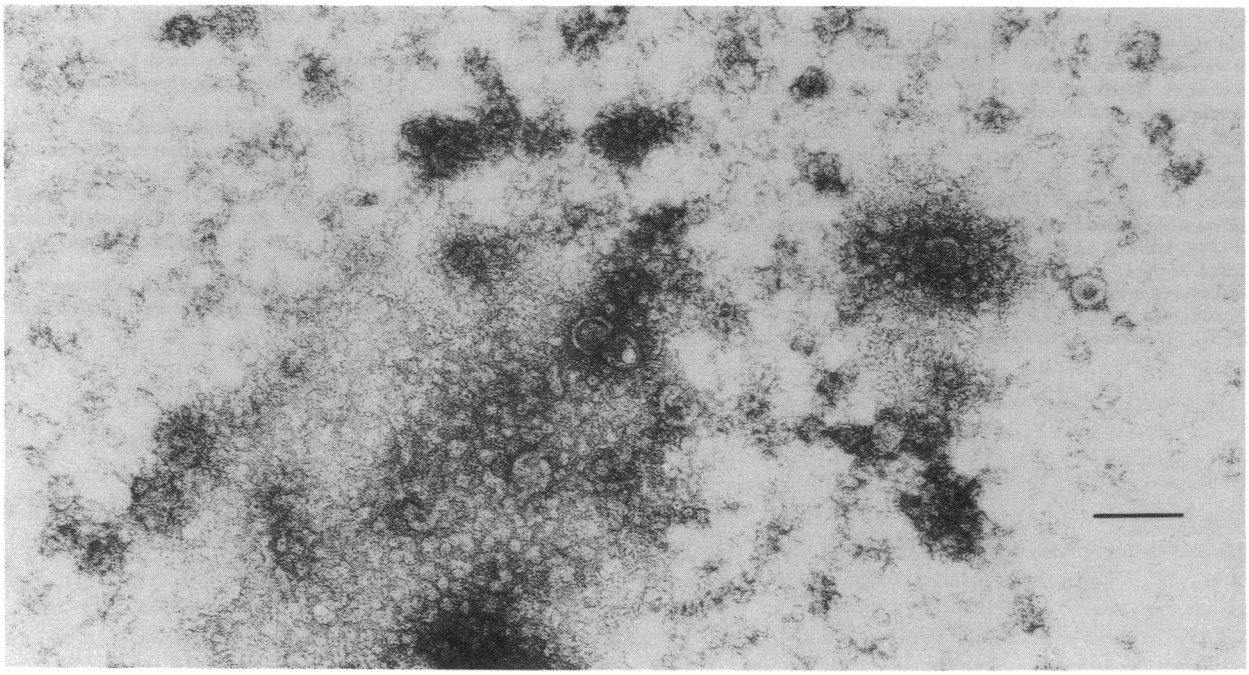

Figure 4. An electron micrograph of HBV particles in the media of HBV-complex treated cells. 4 d after transfection culture medium was collected, HBV particles were concentrated and purified as described in Methods. Samples were stained with 0.1% uranyl acetate and visualized by electron microscopy. (Bar indicates 100 nm).

Our data indicate that HBV DNA in the form of a soluble complex targetable to asialoglycoprotein receptors can result in transfection into hepatoma cell lines containing asialoglycoprotein receptors leading to viral antigen production, transcription, replication, and secretion of complete virions. The results indicate that under appropriate conditions, viral DNA can be internalized by an unnatural receptor system and still express viral-specific antigens and concomitant viral replication. Because of the solubility and targetability of the asialoglycoprotein-based DNA delivery system, it is likely that HBV DNA can be targeted to hepatocytes in vivo. This could provide a means to develop a convenient rodent model of human hepatitis B viral infection for the study of the molecular events that occur during acute and chronic hepatitis B viral infection as well as convenient means for evaluating novel anti-viral agents.

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