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Regulation of Secretion of the Hepatitis B Virus Major Surface Antigen by the PreS-1 Protein

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P24, P30, and P39, the three major surface antigens of the envelope of hepatitis B virus, are co-carboxy-terminal proteins with different amino-terminal extensions. We prompted expression of these proteins in Chinese hamster ovary (CHO) cells by placing the appropriate coding sequence(s) under the control of the simian virus 40 early promoter. P24 and P30 formed 22-nm particles which were efficiently secreted. In contrast, P39 accumulated in a perinuclear structure, presumably the Golgi complex, and was not secreted. Coexpressing P39 and P24 resulted in the localization of both in the perinuclear region and restricted the secretion of P24. We found that P39 must be expressed at a relatively low level to allow efficient secretion of P24 in typical spherical particles. We hypothesize that P39, by inhibiting the formation of spherical particles, helps to induce formation of filamentous particles and mature Hepatitis B virus.

Hepatitis B virus (HBV) causes acute and chronic hepatitis in humans and is causally linked to the most prevalent cancer in the world, hepatocellular carcinoma (3). The 42-nm HBV particle is an enveloped virus (22); this envelope constitutes two other HBV-related spherical and filamentous 22-nm particles. All HBV envelope structures contain three major integral membrane proteins; these proteins, termed surface antigens (HBsAg), are designated P24 (S protein), P30 (preS-2 protein), and P39 (preS-1 protein), reflecting their molecular weights (8, 22). P24 is the major envelope HBsAg. P30 comprises the entire sequence of P24 with an additional 55 amino acids at the amino terminus (pre-S2 region). P24 and P30 have been successfully expressed in heterologous systems for the production of vaccine (12, 13, 16, 19, 24). P39 contains the entire sequence of P30 and an additional 117 amino acids at the amino terminus (the pre-1S region) (25). Thus, these three different HBsAgs are co-carboxy-terminal proteins with different amino-terminal extensions. The function of P39 is unknown. Heermann et al. (8) have shown that P39 represents 10 to 20% of the HBsAg proteins on the 22-nm filamentous particles, but only 1 to 2% of the HBsAg in the spherical particles. It seemed conceivable that P39 plays a role in the morphogenesis of HBV particles. For this paper we studied the functions of P39 through the selective expression of the various HBsAg proteins in Chinese hamster ovary (CHO) cells. Our results indicate that the preS-1 region of P39 facilitates the compartmentalization of HBsAgs within the perinuclear structure, presumably the Golgi complex, and inhibits the secretion of HBsAgs.

MATERIALS AND METHODS

Plasmid constructions and the establishment of transfected cell lines. HBV DNA fragments containing P39 (1.7-kilobase *Bst*E2-*Bam*HI fragment), P30 (1.9-kilobase *Mst*II-*Bgl*II fragment), or P24 (1.3-kilobase *Bam*HI fragment) coding sequences (25) were independently inserted into the *Stu*I/*Bcl*I site of the simian virus 40 (SV40)-derived vector pLSV (10) (P39 DNA) or the *Bgl*II site of pECE1 (7) (P30 and P24 DNA) under the transcriptional control of the SV40 early promoter. The resulting recombinant DNAs were cotransfected with p007LTR (a generous gift of Edison Liu) into Chinese hamster ovary (CHO) cells. p007LTR contains both neomycin and dihydrofolate reductase genes. Transfection of DNA into cells and the selection of cell clones resistant to the neomycin analog, G418, were performed as described before (14). A cell line expressing P39 obtained by G418 selection was treated with increasing concentrations of methotrexate up to 2 μM by a previously described method (2). This treatment increases the expression level of P39 fivefold. CHO-8 and CHO-0.6 cell clones were established by cotransfecting cells with both P24 and P39 sequences at a molecular ratio of 5 to 1.

Primer extension experiments. Primer extension experiments were carried out with a synthetic 39-mer comprising nucleotides 123 to 161 of the antisense strand of the HBV genome (25). The details of the experiments are as follows. A 10-μg amount of total cellular RNA (8 μg of CHO-P39 RNA) was incubated with 2×10^6 cpm end-labeled primers in 6 μl of H_2O at 65°C for 5 min. Thereafter, 2 μl of 0.25 M Tris hydrochloride (pH 8.2) containing 0.25 M KCl, 2 mM dithiothreitol, and 40 mM $MgCl_2$ was added, and the mixture (8 μl) was annealed at 42°C for 40 min. The primer extension reaction was then started by adding 2 μl of a mixture containing 5 mM deoxyribonucleotides, 50 mM dithiothreitol, 2 U of RNasin (Promega Biotech) per μl, and 3 U of reverse transcriptase (Life Sciences, Inc.) per μl. After incubation for 1 h at 42°C, the reaction was stopped by adding 10 μl of Maxam-Gilbert dye mix (11). After being heated at 90°C for 2 min, samples were electrophoresed on a 5% sequencing gel.

Preparation of ^{35}S-labeled HBsAgs expressed in CHO cells. CHO cells grown to subconfluency on a 10-cm petri dish were starved for methionine with methionine-free medium for 16 h. Subsequently, 2 ml of methionine-free medium containing 100 μCi of [^{35}S]methionine (Amersham Corp.) per ml were added and the cells were incubated for 6 h. The cells were then washed twice with phosphate-buffered saline and lysed with 200 μl of phosphate-buffered saline containing

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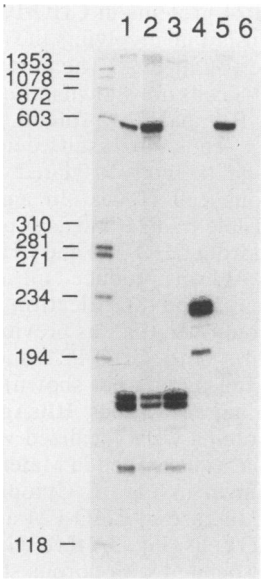

FIG. 1. Primer extension analysis of HBsAg mRNAs expressed in CHO cells. Lanes: 1, CHO-8 (P24/P39 RNA ratio = 8/1); 2, CHO-0.6 (P24/P39 RNA ratio = 0.6/1); 3, CHO-P24 (S protein); 4, CHO-P30 (preS-2); 5, CHO-P39 (preS-1); 6, control CHO cells. The molecular weight marker indicated in base numbers at the left is the *Hae*III-digested φX174 replicative form DNA. Details of the experiments are described in Materials and Methods.

0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. The nuclei were sedimented in a microfuge, and sodium dodecyl sulfate was added (1% final concentration) to the supernatant (about 1 ml). The surface antigens were precipitated from a 200-μl aliquot with a monoclonal antibody against HBsAg (a generous gift of George Kuo, Chiron Corporation).

Immunochemistry. The procedures for cytoplasmic staining have been described previously (14). For cell surface staining, the cells on cover slips were chilled on ice and washed twice with 4°C Ham F-12 medium containing 10% fetal bovine serum and 0.05% NaN_3. The primary antibody (guinea pig anti-HBsAg, 1:100 dilution) was incubated with the cells on the cover slip for 45 min at 4°C. After being washed twice, the cells were treated with the secondary antibody (fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin G [Boehringer-Mannheim Biochemicals, 1:20 dilution]) as in the first incubation. F12 medium was used for washing and antibody dilution.

FACS analysis. In preparation for fluorescence-activated cell-sorting (FACS) analysis, cells (1×10^6 to 2×10^6) were trypsinized with STV (saline containing 0.05% trypsin and 0.02% EDTA) briefly (less than 1 min). The suspended cells were washed twice with Earle balanced salt solution containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.3), 0.1% NaN_3, and 2% heat-inactivated fetal bovine serum. Pilot experiments indicated that treatment of isolated 22-nm HBsAg particles with STV for up to 20 min did not result in a significant loss of antigenicity as measured by an Abbott HBsAg (AUSRIA) diagnostic kit. Incubations with primary and secondary antibody were carried out as described above except that Earle solution was used for washing and antibody dilution. The cells were finally suspended at a concentration of 10^6 cells per ml in Earle solution containing 50 mM HEPES (pH 7.3) and 0.1% NaN_3 for FACS analysis.

Preparation of samples for radioimmunoassays. Fresh medium (10 ml) was overlaid on a confluent layer of cells in a 10-cm petri dish 1 day before the assay. A sample of the medium was assayed by the Abbott AUSRIA diagnostic kit. The cytoplasmic lysate was prepared as described above for the preparation of ^{35}S-labeled HBsAg.

RESULTS

Establishment of cell lines that express HBsAgs. HBV DNA fragments containing P24, P30, or P39 coding sequences were independently cloned into an SV40-derived vector (pECE or pLSV) under the transcriptional control of the SV40 early promoter. The resulting SV40-HBV recombinant DNAs were cotransfected with p007LTR, a plasmid containing the neomycin and the dihydrofolate reductase genes, into CHO cells. The procedures for gene transfection into CHO cells and the isolation of cell clones are described in Materials and Methods. The cell clones isolated were screened for the expression of HBV sequences by RNA dot hybridization. The primer extension analysis of HBV RNA expressed in the selected cell clones showed that all the HBV RNA transcripts display the expected transcription initiation sites (Fig. 1). Data from two different cell clones cotransfected with both P24 and P39 sequences are displayed in Fig. 1, lanes 1 and 2. Densitometer scanning indicated that the P24/P39 RNA ratios in these two cell clones are 8 (CHO-8, lane 1) and 0.6 (CHO-0.6, lane 2), respectively. The cell clones transfected with P24 (CHO-P24), P30 (CHO-P30), and P39 (CHO-P39) sequences are also shown (lanes 3, 4, and 5, respectively). With the exception of CHO-P39 cells, all the cell clones expressed similar amounts of HBV RNA sequences inside the cells (less than 10% variation) as quantitated by RNA dot hybridization (Fig. 1; other data not shown). CHO-P39 cells expressed about 15 to 25% less HBV RNA than that of other cell clones.

Expression of HBsAgs in stably transformed CHO cells. The results of HBsAg expression as measured by radioimmunoassays are shown in Table 1. All the transfected cell clones expressed similar levels of intracellular HBsAg activity, but the amount of secreted HBsAg varied widely. Consistent with previous reports, CHO-P24 and CHO-P30 secreted large amounts of HBsAg into the medium (10, 12, 16, 17, 19, 24). In contrast, CHO-P39 secreted no detectable HBsAg into the medium. There was an inverse relationship between the amount of HBsAg secreted and the amount of P39 RNA in CHO-P24, CHO-8, CHO-0.6, and CHO-P39 cells: increasing the relative amount of P39 RNA inside the cells reduced the amount of HBsAg secreted (Table 1). Thus P39 inhibits the secretion of P24. This result was quantitatively confirmed by our recent studies with P39 in *Xenopus* oocytes, which showed that P39 and to a lesser extent P30 inhibit the secretion of P24 in that system (D. N. Standring,

TABLE 1. Expression of HBsAg in CHO cells

CHO clone	Positive/negative ratio[a]	
	Cytoplasm	Medium
P24	14.1	42.2
P30	11.5	41.1
P39	13.4	1.0
8	11.2	32.8
0.6	12.4	8.6
Control	1.3	1.0

[a] Ratio of sample counts per minute to negative-control counts per minute. The results were measured by the Abbott AUSRIA radioimmunoassay kit.

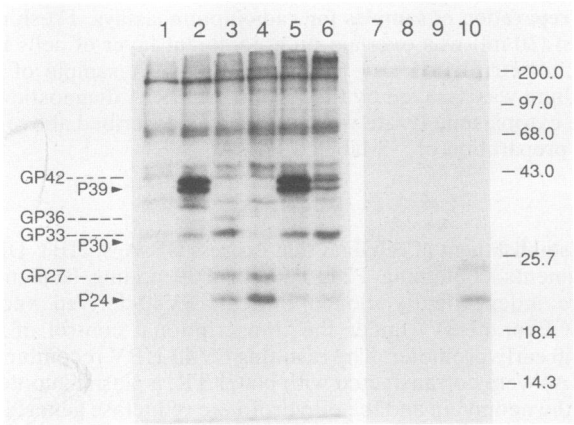

FIG. 2. Regulated expression of HBsAgs (P24, P30, and P39) in CHO cells. Lanes 1 to 6 are cell lysates, and 7 to 10 are incubation medium. Lanes: 1 and 7, control CHO cells; 2 and 8, CHO-P39; 3 and 9, CHO-P30; 4 and 10, CHO-P24; 5, CHO-0.6; 6, CHO-8. The positions on the gel of P39, P30, and P24 were verified by running in parallel samples of P39, P30, and P24 translated in vitro (data not shown). CHO-8 cells expressed P24 and P39 proteins at a ratio of 1:2.6 (P24/P39 RNA ratio = 8:1), and CHO-0.6 expressed these two proteins at a ratio of 1:8.1 (P24/P39 RNA ratio = 0.6:1). In these two cell clones, therefore, P39 proteins were overrepresented and P24 proteins were underrepresented with respect to mRNA content. In another experiment in which cells were starved for methionine for a shorter period of time (4 versus 16 h) before labeling, the protein and RNA ratios were similar (data not shown). The discrepancy between protein and RNA ratios could result from different stabilities of the P39 and P24 mRNAs during the starvation period. GP, Glycoprotein. Molecular weights are indicated in thousands on the right.

J.-H. Ou, and W. J. Rutter, Proc. Natl. Acad. Sci. USA, in press). In addition, F. V. Chisari and colleagues (personal communication) have observed that in transgenic mice harboring the entire preS-S coding region, activation of preS transcription via a linked metallothionein promoter dramatically reduces the level of HBsAg in the serum.

The results of immunoprecipitation of the HBsAg proteins expressed in CHO cells are shown in Fig. 2. Lanes 1 to 6 display the intracellular HBsAg proteins; lanes 7 to 10 show the proteins precipitated from the medium. P39, P30, and P24 protein bands were identified by running in parallel a sample containing in-vitro-translated P39, P30, and P24 (data not shown). Two major HBsAg proteins were precipitated from the cellular extract of CHO-P39 cells (lane 2). The lower-molecular-weight species (arrowhead) comigrated with in-vitro-translated P39 and thus probably represent P39. The higher-molecular-weight species is presumably the glycosylated form of P39 (GP42) reported by Heermann et al. (8). CHO-P24 (lane 4) produced two major HBsAg proteins. The lower-molecular-weight protein comigrated with P24 translated in vitro and therefore presumably represents P24 produced in vivo. The higher-molecular-weight protein has the characteristics of glycosylated P24 (gp27) reported by other groups (8, 17). CHO-P30 produced four major species of HBsAg (lane 3). The two higher-molecular-weight proteins migrated slightly more slowly than did P30 and therefore presumably represent the two different glycosylated forms of P30, viz., gp33 and gp36 (8). The lower-molecular-weight proteins comigrated with P24 and GP27. Since the expression of the P30 sequence in CHO-P30 cells was controlled by the SV40 early promoter, which directs only the transcription of P30 mRNA (Fig. 1, lane 4), the expression of P24 proteins in CHO-P30 cells thus must be a result of translation involving the internal initiation codon of P24 in the P30 mRNA. The immunoprecipitates of CHO-0.6 and CHO-8 cells are shown in Fig. 2, lanes 5 and 6, respectively. Both P39 and P24 proteins were produced in these two cell lines. Thus our results demonstrate that the HBsAg proteins and their glycosylated forms (8, 17) are expressed in various cell clones. In agreement with the results shown in Table 1, P24 and P30 proteins were produced and secreted from CHO-P24 and CHO-P30 cells (lanes 9 and 10), whereas P39 was produced intracellularly but was not secreted (lanes 2 and 8). Electron microscopy with negative staining confirmed that, as previously reported (10, 12, 16, 17, 19, 24), both CHO-P24 and CHO-P30 cells produce 22-nm particles (data not shown).

Cellular localization of various HBsAgs expressed. The various HBsAg proteins were localized within the cells by immunofluorescence as described in Materials and Methods. The results are shown in Fig. 3. Cytoplasmic staining of HBsAgs was more intense in CHO-P24 and CHO-P30 cells than in control CHO cells (Fig. 3A through C). Furthermore, the staining was associated with fibrous structures, presumably the endoplasmic reticulum (ER). This observation is consistent with the results of studies suggesting that HBsAgs are membrane proteins which form 22-nm particles by a budding process occurring in the ER (6, 9, 26). In contrast, in CHO-P39 cells, most of the HBsAg staining was associated with the perinuclear region in Golgi-like structures rather than in the ER (Fig. 3D). CHO-8 and CHO-0.6 cells (Fig. 3E and F, respectively) displayed HBsAg staining which was somewhat different from that of CHO-P24, CHO-P30, or CHO-P39 cells. In both CHO-8.0 and CHO-0.6 cells, intense labeling was seen in the perinuclear region, and less was seen in fibrous structures. Furthermore, the insoluble globular structures (Fig. 3F) related to the staining pattern were evident by phase-contrast microscopy (data not shown). P39 thus apparently restricted the localization of HBsAgs to these cellular structures; this may explain the inhibition by P39 of the secretion of HBsAg particles.

Analysis of HBsAgs present on the cell surface of CHO cell transformants. Immunofluorescence studies showed that there are very few HBsAg molecules on the cell surface of any of the CHO cells expressing HBsAg. The lack of detectable cell surface immunofluorescence for CHO-P39 cells is illustrated in Fig. 3G and H; similar results were found with CHO-P24 and CHO-P30 cells (data not shown). FACS experiments were also carried out, as illustrated in Fig. 4 for P39. Indirect immunolabeling and cell sorting generated nearly congruent profiles for CHO-P39 and control cells. Similar data were obtained in experiments with CHO-P24 and CHO-P30 cells (data not shown). The low concentration of HBsAg on the cell surface may have resulted from the inefficient transport of HBsAg to the cell surface or a short lifetime there. There is considerable circumstantial evidence suggesting that HBV may not be intrinsically cytolytic and that the HBV-infected liver cells are lysed by a cellular immune response (5). Since we detected few molecules on the cell membrane, HBsAg may play a lesser role than the core antigen (HBcAg) in generating the cellular immune response. (HBcAg is found abundantly on the cell membranes of HBV-infected hepatocytes [23].) This hypothesis is supported by the observation that during acute infections with HBV, liver necrosis is coincident with the appearance of immunoglobulin M anti-HBcAg in the serum and precedes the appearance of anti-HBsAg (21).

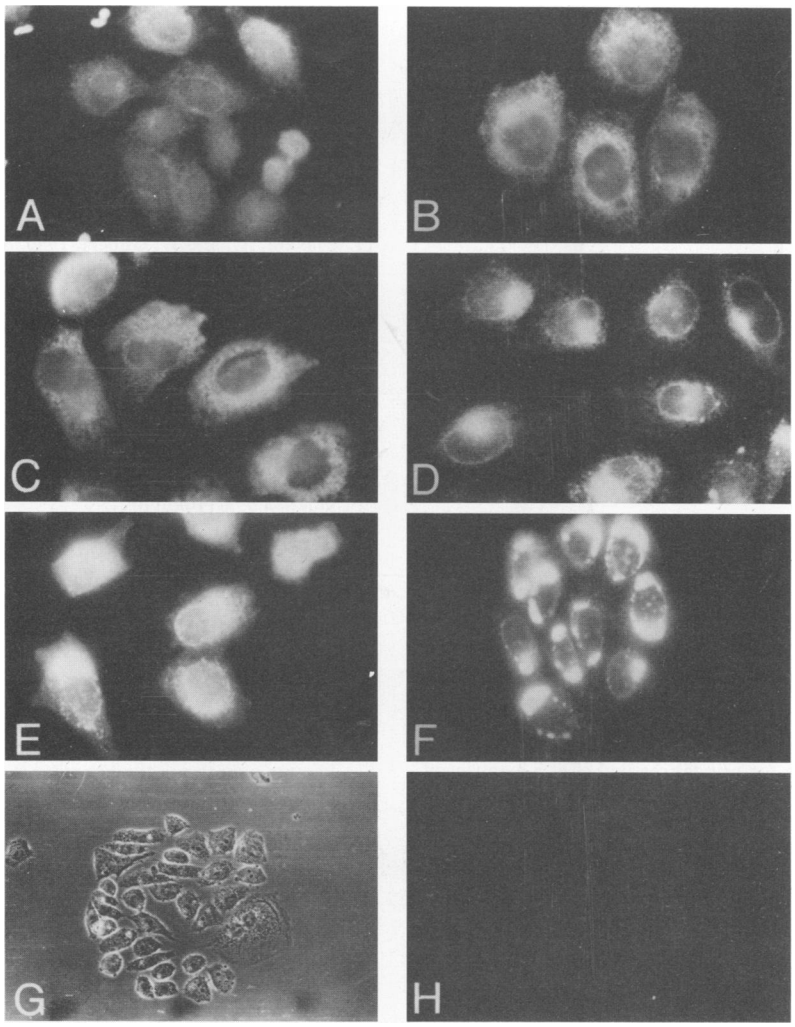

FIG. 3. Effects of P39 on the cellular compartmentalization of HBsAg. Shown are cytoplasmic (A to F) and cell surface (G and H) staining of HBsAg. (A) CHO-control cells; (B) CHO-P24; (C) CHO-P30; (D) CHO-P39; (E) CHO-8; (F) CHO-0.6; (G) CHO-P39 (a phase-contrast photomicrograph of panel H); (H) CHO-P39, absence of cell surface staining of HBsAg. The procedures for immunofluorescence staining are described in Materials and Methods. P24 and P30 appear associated with fibrous structures in the cytoplasm. P39 is restricted to the perinuclear region. Coexpression of P39 and P24 changed the pattern of HBsAg staining, which became localized within perinuclear structures visible by phase-contrast microscopy.

DISCUSSION

We have shown in this paper that in contrast to P24 and P30, which form 22-nm particles and are efficiently secreted, P39 accumulates at the Golgi complex and is not secreted. P39 also causes P24 to accumulate in the Golgi complex and inhibits secretion of HBsAg particles. Our finding that P24 and P30 are associated with ER is consistent with the previous report that transport of HBsAg through the ER may be rate limiting in the secretion process (17). This hypothesis was based on the observation that the glycosylated form of intracellular P24 contains high mannose oligosaccharide chains and that of the secreted P24 contains complex oligosaccharide chains (17). The P39-imposed restriction on the transport of HBsAg through the ER may be due to the association of these molecules with P39 via intermolecular (S-S?) linkages in the Golgi complex. This in turn could restrict transport and secretion of HBsAg 22-nm spherical particles and may thus favor the formation of the more complicated structures, i.e., the 22-nm filamentous forms and the virus itself. This is consistent with the preferential localization of P39 in filamentous particles and mature HBV (8). In the context of its putative role, it is significant that the expression of P39 is controlled by a separate promoter from that of P24 and P30 (4, 15, 18, 20); thus its expression can be independently regulated. This could result in phasing the formation of the different viral structures during the virus life cycle. We have, however, been unable to find filamentous particles in the medium of either CHO-8 or CHO-0.6 cells by electron microscopy. This contrasts with the Alexander hepatoma cell line (PLC/PRF/5) (1), in which filamentous particles can be observed (data not shown), even though P39 mRNA represents only 2% of the HBsAg mRNA (15). Thus P39 and P24 are not the only factors required for the formation of filamentous particles: P30 may also be required or, alternatively, filament formation may somehow be associated with the peculiarities of the secretion mechanism in liver cells. Further studies of the expression of selected viral

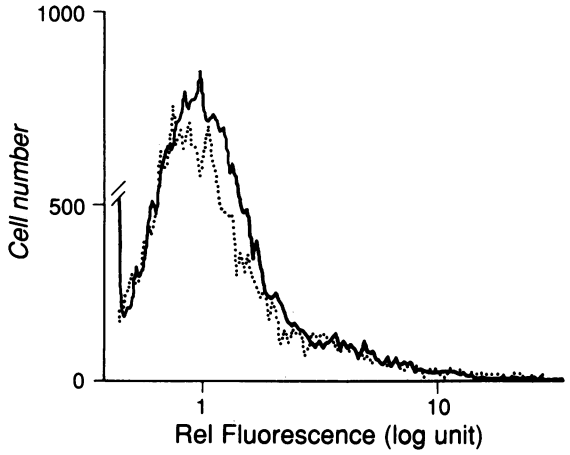

FIG. 4. HBsAgs on the cell surface of CHO cells as detected by FACS. . . . . ., Control cells; ——, CHO-P39 cells. Similar studies were carried out with CHO-P24, CHO-P30, CHO-8, and CHO-0.6 cells (see Results). Rel, Relative.

mRNAs may help to resolve this issue and illuminate other complexities of the formation of viral structures and the pathology of HBV, the smallest known human DNA virus.

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