JOURNAL OF VIROLOGY, Aug. 1984, p. 346–353
0022-538X/84/080346-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 51, No. 2

Intracellular Transport and Secretion of Hepatitis B Surface Antigen in Mammalian Cells

ERIC J. PATZER,* GERALD R. NAKAMURA, AND AVIMA YAFFE
Genentech, Inc., South San Francisco, California 94080

Received 12 January 1984/Accepted 30 April 1984

The oligosaccharide processing and secretion of hepatitis B surface antigen (HBsAg) was studied in Chinese hamster ovary cells stably transfected with the gene coding HBsAg. HBsAg was secreted from cells with a relatively long half time (ca. 5 h). This appeared to be a characteristic of HBsAg itself, since HBsAg-producing cells infected with vesicular stomatitis virus transported the viral envelope glycoprotein to the cell surface with normal kinetics (half time of ca. 30 min). The secreted HBsAg was comprised of both the unglycosylated (P20) and the glycosylated (G25) polypeptides, characteristic of HBsAg isolated from human serum or secreted from other cell lines (C. W. Crowley, C.-C. Liu, and A. D. Levinson, Mol. Cell. Biol. 3:44–55, 1983; M. F. Dubois, C. Pourcel, S. Rousset, C. Chang, and P. Tiollais, Proc. Natl. Acad. Sci. U.S.A. 77:4549–4553, 1980; C.-C. Liu, D. Yansura, and A. D. Levinson, DNA, 1:213–221, 1982; G. M. Macnab, J. J. Alexander, G. Lecatsas, E. M. Bey, and J. M. Urbanocvicz, Br. J. Cancer, 24:509–515, 1976; A. M. Moriarity, B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer, Proc. Natl. Acad. Sci. U.S.A. 78:2606–2610, 1981; D. L. Peterson, J. Biol. Chem., 256:6975–6983, 1981). The glycosylated polypeptide (GP25) contained complex oligosaccharide chains. Cell-associated HBsAg also was comprised of both an unglycosylated and a glycosylated polypeptide; however, the glycosylated form (GP23) contained only high-mannose oligosaccharide chains. No oligosaccharide processing of the high-mannose chains could be detected within the cells. Thus, most of the time before secretion of HBsAg from cells must have been spent in a pre-Golgi or early Golgi compartment. Glycosylation was inhibited completely by tunicamycin, although unglycosylated particles were still secreted from cells and were antigentic. The secretion and oligosaccharide processing of HBsAg were inhibited with high concentrations of monensin, but at lower concentrations of monensin HBsAg was still secreted, although only half of the oligosaccharide chains were processed to the complex form.

During the acute phase of a hepatitis B virus (HBV) infection and during the chronic carrier state, large quantities of hepatitis B surface antigen (HBsAg) are secreted into the bloodstream. This HBsAg is associated with three types of particles: 42-nm spherical Dane particles, which make up only 10% of the total particles, and either spherical or filamentous particles of ca. 22 nm in diameter (2). The infectious Dane particles consist of a nucleocapsid surrounded by an HBsAg-containing lipoprotein envelope, whereas the two noninfectious 22-nm particles are made up of only the HBsAg-containing lipoprotein envelope. Since the 22-nm particles are made in such abundance, it appears that HBsAg itself can efficiently assemble into a lipoprotein envelope which is secreted from cells without any other HBV proteins. The assembly and secretion also have been shown to occur in tissue culture systems expressing only the HBsAg protein (4, 6, 19, 21, 23).

HBsAg particles are made up of two identical polypeptides (24, 25), except that one is glycosylated and one is unglycosylated. The polypeptides appear to be integrally associated with the particle membrane, as evidenced by their resistance to externally added protease as well as by their hydrophobic character (11, 25). HBsAg particles can be solubilized with detergent in the absence of reductant to form a dimer consisting of one glycosylated and one unglycosylated polypeptide. When 2-mercaptoethanol is added, the dimer can be further dissociated into the two monomeric polypeptides (22).

The ability of HBsAg polypeptides to self-associate and mobilize cellular lipids into lipoprotein particles is a characteristic not shared by many other integral membrane proteins. From electron microscopic studies of HBV-infected liver cells, the HBsAg-containing lipoprotein envelope appears to form in the cytoplasm of cells and to be derived from the endoplasmic reticulum (ER) (7, 17, 32). These studies have identified membranous particles that resemble both Dane particles and 22-nm spherical and filamentous particles within the lumen of the ER (7, 17, 32). In addition, the HBsAg particles appeared to be formed by invagination of the ER membrane, either in the presence of core particles to form mature Dane particles or spontaneously in the absence of core particles to produce 22-nm particles (17). Once inside the ER, the HBsAg particles could be transported through the cell, processed by the Golgi, and secreted in a manner similar to monomeric secretory proteins. One of the major drawbacks of these electron microscopic studies, however, is the lack of quantitation. The fraction of total HBsAg participating in each of these observed events is unknown.

The pathway for intracellular processing and transport to the cell surface has been studied extensively for various secretory proteins as well as integral membrane proteins, such as viral envelope or plasma membrane proteins (16). The transit time for many of these proteins from their site of synthesis to the cell surface has been estimated to occur with a half time of less than 90 min (3, 16, 26, 27, 29). Recently, an alternate pathway for the secretion of adrenocorticotropic hormone was described which had a half time for secretion of 3 to 4 h (14). During their transit to the cell surface many of these glycoproteins undergo a series of oligosaccharide processing steps, which can involve the addition of O-linked oligosaccharide chains as well as the modification of existing asparagine-linked oligosaccharide chains from a high-mannose to a complex form (16). Since the cellular location of

* Corresponding author.

many of these processing enzymes is known, the time of acquisition of these modifications can be used as indirect evidence of the cellular location of the glycoprotein. We have studied the oligosaccharide processing of HBsAg from Chinese hamster ovary (CHO) cells that were stably transfected with the gene encoding HBsAg to obtain information about its biosynthesis and secretion.

MATERIALS AND METHODS

DNA transfections. Cells expressing HBsAg were obtained by transfecting CHO DHFR$^-$ cells (31) with a derivative of plasmid p342E (4), into which was inserted a 660-base-pair restriction fragment of a DHFR cDNA (from *Fnu*4HI to *Bgl*II; 28). Cells with a DHFR$^+$ phenotype were selected as described previously (28). Individual DHFR$^+$ clones were isolated, most of which expressed HBsAg as determined by quantitative radioimmunoassay (4). Clone 21.8 was selected for further analysis of the expression and secretion of HBsAg.

Cells and radiolabeling. Cells were grown as monolayer cultures in 10-cm plastic dishes (Corning Glass Works) in Ham F-12 medium supplemented with 10% fetal bovine serum and 50 μg of gentamycin per ml. For carbohydrate labeling, 1 mCi of [^{3}H]glucosamine (Amersham Corp.) was added to 10 ml of glucose-free RPMI medium in the absence of serum, and monolayers of cells were allowed to incorporate label overnight at 37°C. Overnight protein labeling was accomplished in monolayer cultures at 37°C with 2 mCi of [^{35}S]methionine ([^{35}S]met; Amersham) in 10 ml of methionine-free RPMI medium in the absence of serum. Pulse-labeling was performed with 2 ml of methionine-free RPMI medium per 10-cm dish containing 0.5 mCi of [^{35}S]met. The cells were subsequently chased in 3 ml of Ham F-12 medium supplemented with 2 mM methionine, 10% fetal bovine serum, and 50 μg of gentamycin per ml.

Stock solutions of tunicamycin were prepared in phosphate-buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.016 M phosphate [pH 9]) at 1 mg/ml. Cells were pretreated with tunicamycin for 4 h at 37°C before adding the radiolabel. Monensin was prepared in ethanol at a concentration of 2 mM and was added at the same time as the radiolabel.

Virus infection. 21.8 cells in monolayer cultures in 10-cm plastic dishes were infected with vesicular stomatitis virus (VSV, Indiana serotype) at 37°C in 2 ml of medium at a multiplicity of 10 PFU per cell in the presence of 5 μg of actinomycin D per ml. At 5 h postinfection the cells were washed three times with PBS (pH 7.2), scraped from monolayers, and resuspended in 0.5 ml of methionine-free RPMI medium. The cells were pulse-labeled for 5 min with [^{35}S]met, washed once with PBS, and resuspended in 3 ml of Ham F-12 medium supplemented with 10% fetal bovine serum and 2 mM methionine.

Protease treatment of cells. After the chase period, VSV-infected 21.8 cells were resuspended in PBS and divided into two 0.5-ml portions. One portion was treated with 1 mg of chymotrypsin (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK] treated; Sigma Chemical Co.), and both were incubated at 37°C for 10 min. Proteolysis was inhibited by the addition of 2 mM phenylmethylsulfonyl fluoride in ethanol (final ethanol concentration, 1%). Cells were lysed by resuspending them in PBS containing 0.1 mg of bovine serum albumin (BSA) per ml and 1% Nonidet P-40. Radiolabeled proteins were precipitated with 10% trichloroacetic acid, and the precipitates were solubilized in sample buffer containing 1 M Tris base to neutralize the trichloroacetic acid.

Immunoprecipitation. Cells were prepared for immunoprecipitation by the following procedure. They were washed three times in PBS, scraped from the monolayer, and sedimented at 600 × *g* for 5 min. The pellet was resuspended in 10 ml of swelling buffer (15 mM KCl, 15 mM magnesium acetate, 1 mM dithiothreitol, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.5]) and incubated for 5 min at 4°C. The swollen cells were sedimented at 600 × *g* for 5 min and resuspended in 0.9 ml of swelling buffer. One microliter of 0.2 M phenylmethylsulfonyl fluoride was added, and the cells were homogenized with 20 strokes of a Dounce homogenizer. Ninety microliters of 10× extract buffer (450 mM HEPES-KOH [pH 7.5], 750 mM KCl, 50 mM magnesium acetate, 10 mM dithiothreitol) was added, and the suspension was sedimented at 600 × *g* for 5 min to produce the postnuclear supernatant (PNS).

Radiolabeled HBsAg was immunoprecipitated from the PNS or the cell culture medium by initially adding 1/10 volume of 2% Tween–10 mg of BSA per ml–2.5 M NaCl in PBS and incubating for 15 min at room temperature. After the subsequent addition of guinea pig antibody to HBsAg diluted in PBS, the samples were incubated for 2 h at room temperature. Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals) were preadsorbed with a PNS extract from the DHFR$^-$ parental cells before resuspension in PBS containing 1 mg of BSA per ml. The adsorbed beads were used to immunoprecipitate antigen bound to antibody during a 1-h incubation at 37°C. The resulting pellet was washed three times with PBS containing 0.2% Tween–0.2% deoxycholate. Bound antigen was released by boiling for 3 min in sample buffer (0.0625 M Tris [pH 6.8], 2% SDS, 2.5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue). The released antigen was electrophoresed on 10% polyacrylamide gels and subjected to autoradiography. Molecular weight standards were purchased from Amersham as a ^{14}C-methylated protein mixture containing polypeptides from 14,300 to 200,000.

Glycosidase treatments. HBsAg was treated with endo-β-*N*-acetylglucosaminidase H (endo H) and endo-β-*N*-acetylglucosaminidase F (endo F) after immunoprecipitation with protein A-Sepharose beads. The bound antigen was washed an additional time with 0.001 M Tris-hydrochloride (pH 8)–0.001 M phenylmethylsulfonyl fluoride, resuspended in 10 μl of 0.1 M Tris (pH 6.8)–1% sodium dodecyl sulfate (SDS), and boiled for 2 min. For endo H treatment, 40 μl of 0.1 M sodium acetate (pH 5.5) and 3 μl of endo H (1 U/ml) were added, and the samples were incubated for 16 to 18 h at 37°C. For endo F treatment, 40 μl of 0.1 M sodium phosphate (pH 6.1), 1% Nonidet P-40, and 5 μl of endo F (provided by J. Elder) were added, and the samples were incubated for 16 to 18 h at 37°C (8). The endo F-treated samples subsequently received 10 μl of 20% SDS, and both endo H- and endo F-treated samples received an additional 20 μl of 4× sample buffer before boiling and electrophoresis on 10% polyacrylamide gels.

HBsAg was sedimented from the tissue culture medium at 49,000 rpm for 2 h in an SW50.1 rotor before treatment with neuraminidase. The pellet was resuspended in 20 μl of 1× extract buffer, diluted in 50 μl of 0.1 M sodium phosphate (pH 5.3), and treated with 20 μl of neuraminidase (1 U/ml; *Arthrobacter ureafaciens*; Calbiochem-Behring) for 16 to 18 h at 37°C. The treated sample was immunoprecipitated and electrophoresed in polyacrylamide gels as described above.

Velocity sedimentation of HBsAg. HBsAg-containing medium was harvested from 21.8 cells maintained overnight at 37°C in the presence of 2 μg of tunicamycin per ml. Medium (400 ml) was centrifuged at 600 × *g* for 5 min and then

concentrated 200-fold with an Amicon YM30 membrane. A 0.1-ml portion of the concentrated medium was layered on top of a 9-ml 5 to 20% sucrose gradient and centrifuged in a SW41 rotor at 39,000 rpm for 3 h. The gradient fractions were collected with a Buchler auto densi-flow IIc.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was a solid-phase assay used to determine HBsAg concentrations. Microtiter wells (96 well; Dynatech Laboratories, Inc.) were coated overnight at 4°C with 2.5 μg of monoclonal antibody to HBsAg (subtype ad; Hybritech) per ml. BSA (10 mg/ml) was added for 1 h at room temperature to inhibit any nonspecific sticking. Samples containing HBsAg were diluted in PBS containing 10 mg of BSA per ml and 0.5% Tween 20 and incubated for 2 h at room temperature. Peroxidase-labeled monoclonal antibody to HBsAg (subtype ad; Hybritech) was incubated for 1 h at room temperature. The substrate, *o*-phenylenediamine (Sigma Chemical Co.), was incubated for 10 min at room temperature and stopped with H_2SO_4. The optical density was measured at 490 nm.

RESULTS

Expression and secretion of HBsAg in CHO 21.8 cells. HBsAg has been expressed in a variety of cell types (4, 6, 19, 21, 23), and in each case the HBsAg was secreted into the cell culture medium. The particle size and density, when examined, were similar to the values reported for authentic 22-nm particles isolated from the serum of patients infected with HBV (5, 12). We have been studying the expression and secretion of HBsAg in CHO cells transfected with the gene encoding HBsAg. The expression from clone 21.8 initially was detected by cytoplasmic immunofluorescence and quantified by radioimmunoassay (Ausria II-125; Abbott Laboratories) and ELISA. About 200 ng of HBsAg per ml accumulated in the cell culture medium during an overnight incubation at 37°C. Secreted HBsAg had a density (1.2 g/cm^3) and sedimentation rate (see unglycoslyated HBsAg in Fig. 3) that were similar to those of HBsAg from other cell culture systems and serum-derived HBsAg particles (5, 12, 19, 23).

The rate of secretion of HBsAg from cells was determined by pulse-chase experiments. Cells were pulse-labeled with [^{35}S]met for 20 min and then chased with unlabeled medium under conditions in which no further incorporation of radiolabel occurred. After the initial 20-min pulse, all of the immunoprecipitated HBsAg polypeptides were cell associated in the PNS fraction (Fig. 1A, lanes 3 and 4). There were two specific HBsAg polypeptides in the PNS fraction designated P20 and GP23 (Fig. 1A, lanes 3 and 5). Other higher-molecular-weight polypeptide bands were nonspecific contaminants, illustrated by using preimmune serum with HBsAg-producing 21.8 cells (Fig. 1A, lane 2) or immune serum with nonproducing parental CHO DHFR$^-$ cells (Figure 1A, lane 1). As chase times increased, more of the HBsAg polypeptides were secreted into the medium, as depicted in gel lanes 3 through 8 (Fig. 1A). A comparison of the HBsAg polypeptides in the PNS and medium fractions (Fig. 1) revealed that P20 was detected in cells and in the medium, whereas GP23 was detected only in cells and GP25 was detected only in the medium. This exclusive localization for GP23 and GP25 was true at all times during the chase period and suggested a product-precursor relationship between these two polypeptides.

The amount of radiolabeled HBsAg in each polypeptide band was determined by scanning the autoradiograph with a densitometer. The areas for each polypeptide band were used to calculate the amount of HBsAg in the PNS (GP23 and P20) and medium (GP25 and P20) fractions as a percentage of the total HBsAg in both fractions. Total radiolabeled HBsAg did not increase during the chase period and had a

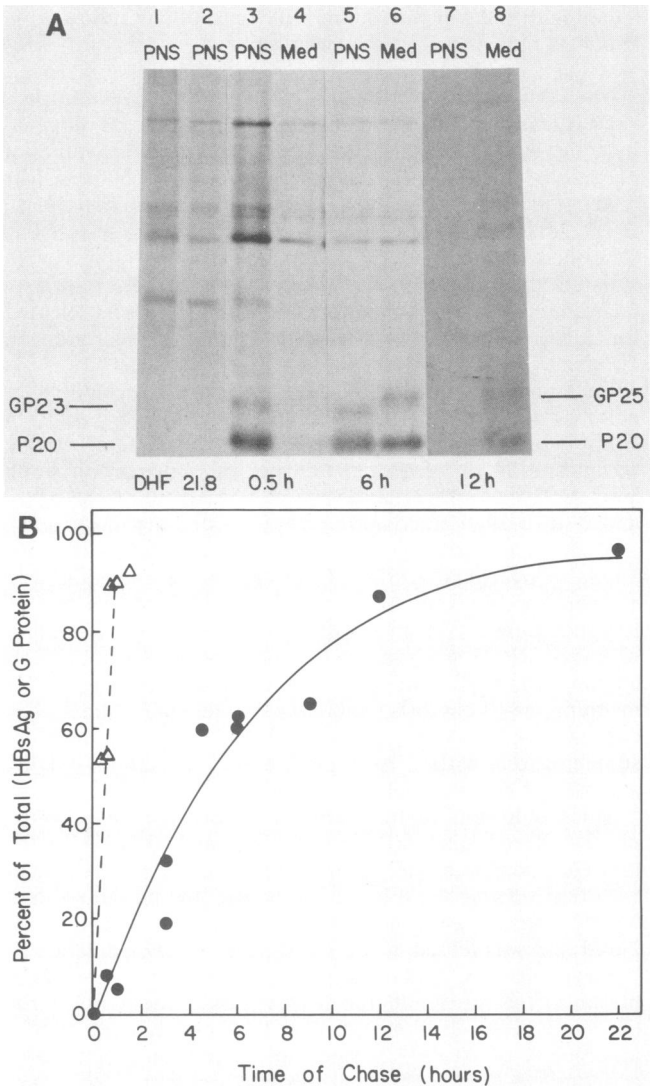

FIG. 1. Kinetics of secretion of HBsAg polypeptides (●) and G protein of VSV (△). To radiolabel HBsAg for autoradiography, 21.8 cells were pulse-labeled for 20 min with [^{35}S]met and chased in unlabeled medium (Med) containing 2 mM methionine for up to 22 h. At each time point the PNS or medium fractions were immunoprecipitated with anti-HBsAg antisera and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (A). The HBsAg polypeptides were quantified by scanning densitometry, and the amount of HBsAg (both glycosylated and unglycosylated) in the medium (●) was calculated as a percentage of total HBsAg in both fractions (B). Total HBsAg was constant throughout the entire chase period. To label G protein, infected 21.8 cells were pulse-labeled with [^{35}S]met for 5 min and chased for up to 90 min. At each time point half of the cells were treated with chymotrypsin to proteolytically degrade any G protein on the cell surface and half of the cells were untreated. The cells were lysed with 1% Nonidet P-40–0.1 mg of BSA per ml, and radiolabeled proteins were precipitated with 10% trichloroacetic acid. The precipitates were resolubilized in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The percentage of G protein on the cell surface (△) was determined as 100 − [(G protein resistant to proteolysis/total G protein) × 100] (B). DHF, CHO DHFR$^-$ cells.

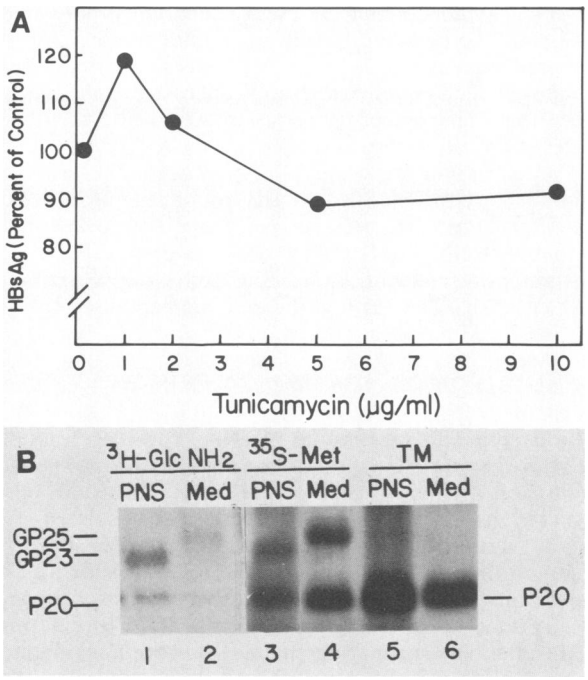

FIG. 2. Effect of tunicamycin (TM) treatment on HBsAg expression and glycosylation in 21.8 cells. The amount of HBsAg that accumulated in the growth media of 21.8 cells during an overnight incubation at 37°C was quantified by ELISA, normalized to the amount of HBsAg secreted in the absence of tunicamycin, and plotted as a function of increasing concentrations of tunicamycin. HBsAg polypeptides radiolabeled with [^{3}H]glucosamine (lanes 1 and 2) or [^{35}S]met (lanes 3 through 6) in the absence (lanes 1 through 4) or presence (lanes 5 and 6) of 2 μg of tunicamycin per ml were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

coefficient of variation of 30 to 50%. In addition, both the glycosylated and unglycosylated polypeptides were secreted at approximately the same rate. The solid line in Fig. 1B represents the percentage of HBsAg that was secreted into the medium. This curve reveals that newly synthesized HBsAg takes at least 22 h to be completely cleared from the cell. The secretion of HBsAg exhibited first-order kinetics and, using a semilogarithmic plot, could be represented by a straight line with a correlation coefficient of 0.988 (graph not shown). The half time for secretion determined from this plot was about 5 h.

The slow rate of secretion of HBsAg could be due to a unique characteristic of the 21.8 cells or a characteristic of HBsAg itself. To distinguish between these two possibilities, we infected 21.8 cells with VSV and studied the rate of appearance of the envelope glycoprotein (G protein) on the cell surface. This is known to occur ca. 10 times faster than the rate of secretion we measured for HBsAg in 21.8 cells (28). 21.8 cells infected with VSV were pulse-labeled with [^{35}S]met, and the amount of time required for G protein to reach the cell surface was determined by its sensitivity to hydrolysis by externally added protease (18). The dotted line in Fig. 1B reveals that G protein was transported to the cell surface significantly faster than the secretion of HBsAg. The slow rate of secretion of HBsAg, therefore, appears to be a unique characteristic of HBsAg.

The P20 and GP25 polypeptides secreted into the cell culture media comigrated on SDS-polyacrylamide gels with the unglycosylated and glycosylated polypeptides of authentic serum-derived HBsAg, respectively (data not shown). The unglycosylated and glycosylated polypeptides of serum-derived HBsAg have been shown to be identical, except that the slower-migrating form contains carbohydrate (24). Cells producing HBsAg were biosynthetically labeled with [^{3}H]glucosamine to determine whether the two slower-migrating polypeptides in the PNS and medium also contained carbohydrate. The incorporation of [^{3}H]glucosamine into GP23 and GP25 is illustrated in lanes 1 and 2 of Fig. 2. A second polypeptide in the PNS that was biosynthetically labeled with [^{3}H]glucosamine migrated at about the same rate as P20 (Fig. 2). It is unlikely that P20 is glycosylated since previous studies with serum-derived HBsAg have not revealed any carbohydrate in this polypeptide (24), nor has treatment of recombinant HBsAg with tunicamycin or glycosidases (see below) produced any change in the migration rate of P20. In addition, only GP23 was labeled after radiolabeling with [^{3}H]mannose (data not shown). This labeled polypeptide that migrates at the P20 position is due either to nonspecific trapping of a non-HBsAg glycoprotein in the immunoprecipitate or to metabolic breakdown of [^{3}H]glucosamine into radiolabeled amino acids during the overnight incubation. If the metabolic breakdown were slow, this would explain why no radiolabeled P20 had reached the medium.

Additional evidence of glycosylation was provided by the drug tunicamycin, which is known to inhibit the formation of asparagine-linked oligosaccharides by blocking the addition of *N*-acetylglucosamine onto dolichyl-phosphate (15). Up to 10 μg of tunicamycin per ml added to the cell culture medium during an overnight incubation resulted in little or no loss of secreted HBsAg detected by ELISA (Fig. 2). When cells were radiolabeled with [^{35}S]met in the presence of 2 μg of tunicamycin per ml, however, only P20 was detected in either the PNS or medium fractions (Fig. 2, cf. lanes 3 and 4 with 5 and 6). Treatment with tunicamycin resulted in an increase in the amount of radiolabeled P20 equal to the amount of radiolabel in the missing GP23 and GP25 polypeptide bands (determined by scanning densitometry of Fig. 2). Hence, HBsAg was still synthesized and secreted at normal levels in the presence of tunicamycin, but all of it was unglycosylated. This inhibition of glycosylation by tunicamycin means that the oligosaccharide chain(s) on HBsAg must be linked via asparagine residues and is solely responsible for the differences in apparent molecular weights between the glycosylated GP25 and GP23, and the unglycosylated P20.

The HBsAg that was secreted in the presence of 2 μg of tunicamycin per ml was analyzed by velocity sedimentation in a 5 to 20% sucrose gradient to determine whether it was still in particle form. The sedimentation rate of this unglycosylated HBsAg was compared to serum-derived HBsAg particles that were radiolabeled with Na^{125}I and Iodo-gen (Pierce Chemical Co.) (Fig. 3). The peak fractions of both the serum-derived HBsAg and unglycosylated HBsAg (assayed by ELISA) were nearly coincident, indicating a similar particle size for both forms. The oligosaccharide chain(s) does not appear to be necessary for the formation and secretion of authentic HBsAg particles or for antigenic reactivity in the ELISA.

Oligosaccharide chains on HBsAg. HBsAg was treated with several glycosidases to gain some structural information about the oligosaccharide chain(s). Asparagine-linked oligosaccharides exist in one of two basic forms: high-mannose chains, which are hydrolyzed by both endo H and endo F, or complex chains, which are hydrolyzed only by endo F (8).

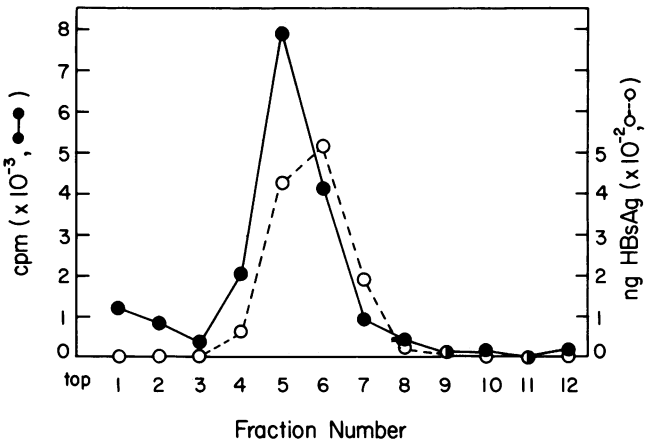

FIG. 3. Velocity sedimentation of unglycosylated HBsAg particles. HBsAg particles secreted from 21.8 cells in the presence of 2 μg of tunicamycin per ml (○) were concentrated with an Amicon YM30 membrane and layered onto a 5 to 20% sucrose gradient. Authentic HBsAg particles purified from human serum (kindly provided by D. Peterson, Medical College of Virginia) were radiolabeled with $Na^{125}I$ and Iodo-gen (Pierce) and layered onto the same gradient (●). After centrifugation for 2 h at 39,000 rpm in a SW41 rotor, gradient fractions were collected and assayed for HBsAg activity by ELISA and with a gamma counter. The amount of ^{125}I-HBsAg tracer added to the gradients was not measurable by ELISA. Recovery from the gradients was 63% of the added HBsAg by ELISA.

Treatment of the PNS and medium from cells labeled with [^{35}S]met with both of these endoglycosidases resulted in the gel pattern illustrated in lanes 1 through 4 of Fig. 4. GP23 was not detected after treatment with either endo H or endo F. There was a concomitant increase in the amount of radiolabeled polypeptide in the unglycosylated P20 band, indicating that the oligosaccharide chains on GP23 were hydrolyzed by both enzymes and therefore are of the high-mannose form. In contrast, GP25 changed in migration rate only after treatment with endo F, indicating that its oligosaccharide chain(s) is of the complex form.

[^{35}S]met-labeled HBsAg in the medium also was treated with neuraminidase to determine whether the complex oligosaccharide chain(s) on GP25 contained terminal sialic acid residues (Fig. 4, lane 6). There was a slight increase in the migration rate of GP25 after treatment, although it did not migrate as fast as GP23 (cf. untreated PNS and medium in lanes 5 and 7, respectively, with lane 6; Fig. 4). Some of the difference in apparent molecular weight between GP25 and GP23 is due to terminal sialic acid residues. Whether the remaining difference is due to sialic acid residues resistant to hydrolysis by neuraminidase or to other carbohydrate differences between these two forms has not been established.

During pulse-chase experiments, GP23 was observed only in the cell-associated fraction and GP25 was observed in the medium. Since some of the difference in apparent molecular weight between these two polypeptides is due to the addition of sialic acid, which is the terminal step in oligosaccharide processing, we wanted to know whether any earlier Golgi-associated processing steps had occurred which may not affect the migration rate of GP23 on polyacrylamide gels. Hydrolysis by endo H was used as the assay of high-mannose oligosaccharide chains on GP23 during a pulse-chase experiment. Figure 5 reveals that GP23 remained endo H sensitive throughout the entire chase period, providing additional evidence that no oligosaccharide processing of GP23 could be detected before secretion from the cell.

Monensin treatment of cells. Monensin is a carboxylic ionophore which equilibrates cations across membranes and blocks the transport of secretory proteins and membrane glycoproteins as they travel through the Golgi (30). It has been reported that the site of inhibition occurs in the middle Golgi stacks (13). We treated HBsAg-producing 21.8 cells with increasing concentrations of monensin to determine whether secretion of HBsAg also was inhibited (Fig. 6). As the monensin concentration increased, the secretion of [^{35}S]met-labeled HBsAg into the medium decreased (Fig. 6). At 1 and 5 μM monensin there appeared to be a build-up of cell-associated HBsAg as the amount secreted diminished. This inhibition of secretion affected both the unglycosylated and glycosylated HBsAg equally. At 10 μM monensin there was a decrease in the amount of HBsAg in both fractions. The HBsAg secreted in the presence of intermediate concentrations of monensin (0.5 and 1 μM) consisted of an unglycosylated polypeptide (P20) and a glycosylated polypeptide with the same migration rate as GP23 that was sensitive to hydrolysis with endo F (data not shown). This was the only time that GP23 was observed extracellularly. This secreted GP23 was treated with endo H to determine whether the oligosaccharide chain(s) was the high-mannose or complex form (Fig. 6, lanes 11 and 12). Quantitation of the treated and untreated HBsAg by scanning densitometry indicated that 50% of GP23 was sensitive to endo H and 50% was resistant (data not shown). Apparently, in the presence of intermediate concentrations of monensin, half of the glycosylated HBsAg that was secreted was processed by

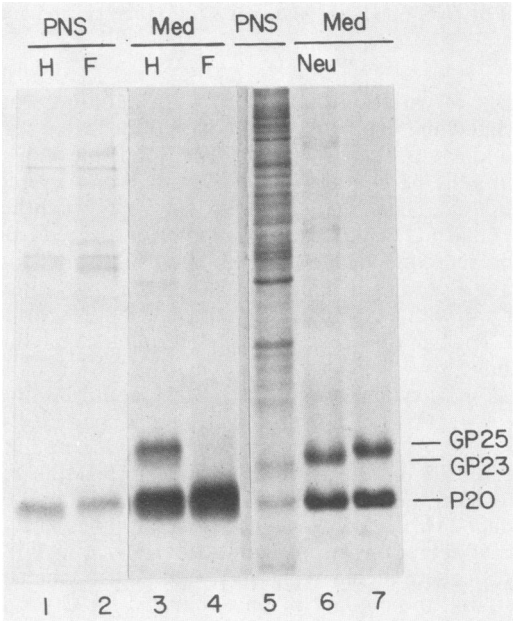

FIG. 4. SDS-polyacrylamide gel electrophoresis of [^{35}S]met-labeled HBsAg treated with endo H, endo F, or neuraminidase. 21.8 cells were radiolabeled overnight (16 to 18 h) at 37°C with [^{35}S]met, and the medium (Med) fraction was treated with neuraminidase (Neu, lane 6) before immunoprecipitation, or with endo H (H, lanes 1 and 3) or endo F (F, lanes 2 and 4) after immunoprecipitation. Lanes 5 and 7 are untreated PNS and medium, respectively. The samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiographed.

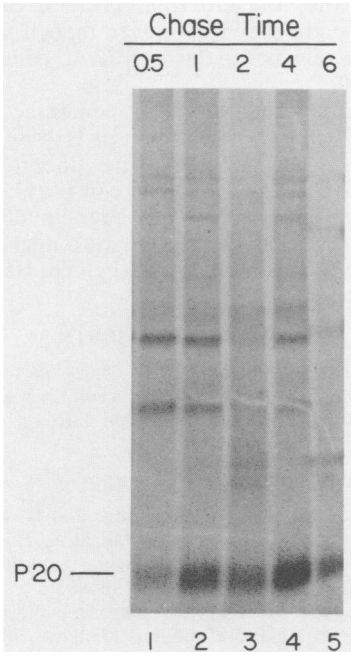

FIG. 5. SDS-polyacrylamide gel electrophoresis of pulse-labeled HBsAg treated with endo H after increasing chase times. 21.8 cells were pulse-labeled with [^{35}S]met for 20 min and subsequently incubated at 37°C in medium containing 2 mM unlabeled methionine for the times indicated. The PNS fraction was immunoprecipitated with anti-HBsAg antiserum and treated with endo H before SDS-polyacrylamide gel electrophoresis and autoradiography.

Golgi-associated enzymes to a form no longer sensitive to endo H hydrolysis (20), whereas the remainder either was not processed by Golgi enzymes or was only processed by early Golgi enzymes (20) and remained endo H sensitive. The cell-associated HBsAg was still completely sensitive to endo H hydrolysis (data not shown).

DISCUSSION

Transfection of CHO DHFR$^-$ cells with the gene encoding HBsAg resulted in the secretion of 22-nm particles containing both the unglycosylated (P20) and glycosylated (GP25) forms of HBsAg. Other cell lines have been used for the expression of HBsAg with similar results (4, 6, 19, 23). In each case 22-nm particles of similar size and density to those of human serum-derived particles were secreted from cells. HBsAg apparently has the ability to mobilize membrane lipids in cells into spherical or filamentous particles of 22 nm in diameter. This is not an artifact of cell culture systems containing recombinant HBsAg genes or of cells expressing only HBsAg in the absence of the other HBV proteins. Electron microscopic studies have reported 22-nm spheres and filaments in liver cells obtained from biopsies of HBV-infected individuals (7, 17, 32). In addition, 22-nm particles are the major HBV-related particles in the sera of patients infected with HBV (2).

There is some suggestive evidence from electron microscopic studies that HBsAg particles in the liver form intracellularly (7, 17, 32), possibly by the incorporation of HBsAg into the ER membrane and invagination of the ER membrane producing HBsAg particles in the lumen of the ER (17). These intracellular particles would contain HBsAg integrally

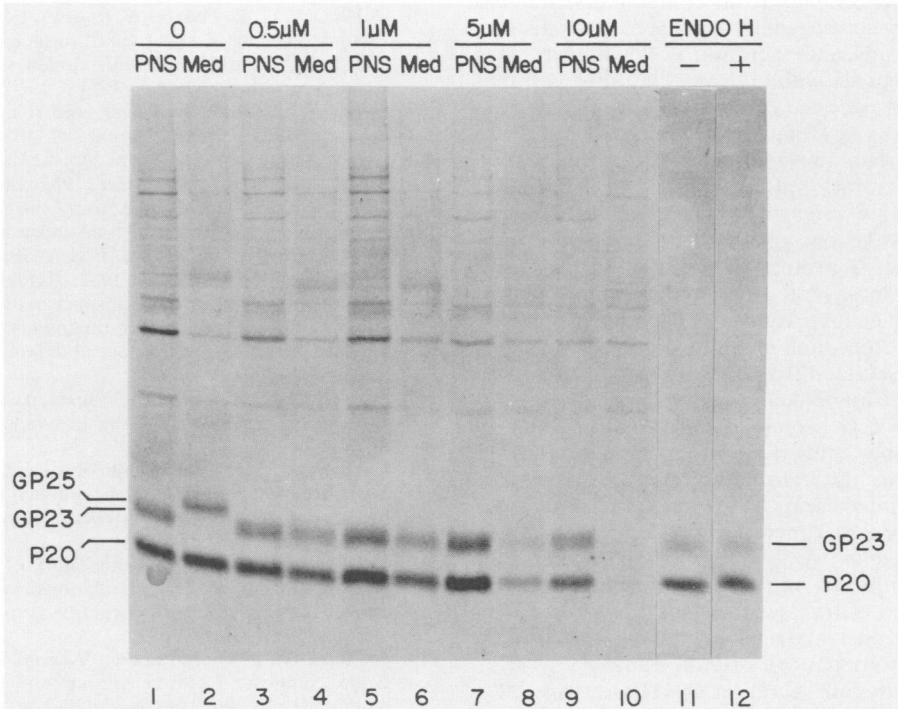

FIG. 6. SDS-polyacrylamide gel electrophoresis of HBsAg from monensin-treated cells. 21.8 cells were radiolabeled overnight (16 to 18 h) at 37°C with [^{35}S]met in the presence of monensin at the concentrations listed for lanes 1 through 10. The PNS and medium fractions were immunoprecipitated before SDS-polyacrylamide gel electrophoresis and autoradiography. Immunoprecipitates of the medium from cells treated with 0.5 μM monensin were electrophoresed on lanes 11 and 12. Lane 12 was treated with endo H to determine the percentage of GP23 that contained high-mannose oligosaccharide chains.

associated with the particle membrane, and the entire particle could be secreted from the cell by a pathway similar to that used by soluble monomeric secretory proteins. In this respect the intracellular transport of an integral membrane protein such as HBsAg would have the characteristics of a secretory protein. In certain disease states, however, HBsAg also has been detected on the cell surface (1, 10, 32).

Preliminary studies have indicated that HBsAg cannot be detected on the surface of 21.8 cells by indirect immunofluorescence (data not shown). In addition, the kinetics of secretion of HBsAg from 21.8 cells and the time of processing of its oligosaccharide chains argue that either HBsAg is not incorporated into the plasma membrane or its residence time in the plasma membrane is very short relative to its total residence time within the cell (half time of ca. 5 h). The secreted HBsAg particles contain a glycosylated polypeptide (GP25) with a complex oligosaccharide chain(s) that was processed by the Golgi. This form (GP25) was only detected after secretion from the cells, and its secretion was blocked by high concentrations of monensin. Cell-associated HBsAg, however, is made up of a glycosylated polypeptide (GP23) with high-mannose oligosaccharide chains, indicating that it resides in a pre-Golgi or early Golgi compartment. Since GP23 is the only glycosylated form detected in 21.8 cells, even during a pulse-chase experiment, it appears that transport through the Golgi and from the Golgi to the cell surface must occur very rapidly and immediately before its appearance in the media.

In the presence of intermediate concentrations (0.5 μM) of monensin, the HBsAg that was secreted contained a glycosylated polypeptide with the migration rate of GP23, but only half of it was hydrolyzed by endo H. The remainder appeared to be an intermediate in oligosaccharide processing that was no longer in the high-mannose form (20) but was not fully processed to the complex form containing sialic acid (GP25). Whether this heterogeneity represents two distinct pathways for processing and secretion is not known. It is clear from the experiments with tunicamycin, however, that carbohydrate was not necessary for particle formation.

The secretion of HBsAg from 21.8 cells occurred at a 5 to 10-fold slower rate than those of many other membrane glycoproteins and secretory proteins studied to date (3, 16, 26, 27, 29). This slow rate of secretion is an inherent characteristic of HBsAg because 21.8 cells infected with VSV transported the G protein to the cell surface with normal kinetics (29). In several other studies, similar differences in rates of intracellular transport have been reported for both secretory proteins and membrane glycoproteins (9, 20, 26, 29). Proteins secreted from rat and human hepatoma cells (20, 29), two murine leukemia virus membrane glycoproteins (9), and VSV G protein with and without its 79 COOH-terminal amino acids (26) in each case showed marked differences in the rate of intracellular transport which could be accounted for by a slow rate of transfer from the ER to the Golgi compartment (9, 20, 26, 29). Once the protein reached the Golgi it was processed and transported to the cell surface within 15 min (9). It was postulated that these differential rates of transfer from the ER to the Golgi indicate that this transport step must be mediated by specific receptors, rather than by passive or bulk transfer (9, 20).

Based on the kinetics of secretion of HBsAg from 21.8 cells and the sensitivity of cell-associated GP23 to endo H hydrolysis, the rate-limiting step in the secretion of HBsAg also seems to be transfer from ER to Golgi or from an early Golgi compartment to a late Golgi compartment. We cannot eliminate the latter possibility by using sensitivity to endo H hydrolysis as the sole criterion, because oligosaccharide chains remain endo H sensitive even though some trimming of the mannose residues in the early Golgi compartments has taken place (20).

Unlike the other secretory and membrane glycoproteins discussed (9, 20, 26, 29), secretion of HBsAg also requires assembly into 22-nm macromolecular lipoprotein particles. If this assembly occurs within the cell (7, 17, 32), then the rate-determining step for secretion might involve this assembly process rather than interaction with a specific receptor (9, 20). We are currently studying when the HBsAg particles form within cells.

ACKNOWLEDGMENTS

We thank A. D. Levinson and C. C. Simonsen for providing the HBsAg-expressing 21.8 and the DHFR$^-$ cell lines and D. Peterson for providing the purified serum-derived HBsAg. We also thank Jeanne Arch for typing the manuscript.

LITERATURE CITED

1. **Alberti, A., G. Realdi, F. Tremolada, and G. P. Spina.** 1976. Liver cell surface localization of hepatitis B antigen and of immunoglobulins in acute and chronic hepatitis and in liver cirrhosis. Clin. Exp. Immunol. **25**:396–402.
2. **Almeida, J. D.** 1971. Electron microscopic observations and speculations on Australia antigen. Postgrad. Med. J. **47**:484–487.
3. **Braell, W. A., and H. F. Lodish.** 1981. Biosynthesis of the erythrocyte anion transport protein. J. Biol. Chem. **256**:11337–11344.
4. **Crowley, C. W., C.-C. Liu, and A. D. Levinson.** 1983. Plasmid-directed synthesis of hepatitis B surface antigen in monkey cells. Mol. Cell. Biol. **3**:44–55.
5. **Dreesman, G. R., F. B. Hollinger, J. R. Suriano, R. S. Fujioka, J. P. Brunschwig, and J. L. Melnick.** 1972. Biophysical and biochemical heterogeneity of purified hepatitis B antigen. J. Virol. **10**:469–476.
6. **Dubois, M. F., C. Pourcel, S. Rousset, C. Chany, and P. Tiollais.** 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:4549–4553.
7. **Dunn, A. E. G., R. L. Peters, and I. L. Schweitzer.** 1973. An ultrastructural study of liver explants from infants with vertically transmitted hepatitis. Exp. Mol. Pathol. **19**:113–126.
8. **Elder, J. H., and S. Alexander.** 1982. Endo-β-*N*-acetylglucosaminidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **79**:4540–4544.
9. **Fitting, T., and D. Kabat.** 1982. Evidence for a glycoprotein "signal" involved in the transport between subcellular organelles. Two membrane glycoproteins encoded by murine leukemia virus reach the cell surface at different rates. J. Biol. Chem. **257**:14011–14017.
10. **Furuta, S., K. Kiyosawa, A. Nagata, Y. Akahane, and M. Oda.** 1975. HBsAg on cell membrane in symptom-free carrier. Lancet **ii**:227.
11. **Gavilanes, F., J. M. Gonzalez-Ros, and D. L. Peterson.** 1982. Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins. J. Biol. Chem. **257**:7770–7777.
12. **Gerlich, W. H., M. A. Feitelson, P. L. Marion, and W. S. Robinson.** 1980. Structural relationships between the surface antigens of ground squirrel hepatitis virus and human hepatitis B virus. J. Virol. **36**:787–795.
13. **Griffiths, G., P. Quinn, and G. Warren.** 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from *medial* to *trans* Golgi cisternae in baby hamster kidney cells infected with Semliki Forest virus. J. Cell. Biol. **96**:835–850.
14. **Gumbiner, B., and R. B. Kelly.** 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell **28**:51–59.

15. **Heifetz, A., R. W. Keenan, and A. D. Elbein.** 1979. Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase. Biochemistry **18**:2186–2192.
16. **Hubbard, S. C., and R. J. Ivatt.** 1981. Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **50**:555–583.
17. **Kamimura, T., A. Yoshikawa, F. Ichida, and H. Sasaki.** 1981. Electron microscopic studies of Dane particles in hepatocytes with special reference to intracellular development of Dane particles and their relation with HBeAg in serum. Hepatology **1**:392–397.
18. **Knipe, D. M., H. F. Lodish, and D. Baltimore.** 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. J. Virol. **21**:1121–1127.
19. **Liu, C.-C., D. Yansura, and A. D. Levinson.** 1982. Direct expression of hepatitis B surface antigen in monkey cells from an SV40 vector. DNA **1**:213–221.
20. **Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous.** 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. Nature (London) **304**:80–83.
21. **Macnab, G. M., J. J. Alexander, G. Lecatsas, E. M. Bey, and J. M. Urbanocvicz.** 1976. Hepatitis B surface antigen produced by a human hepatoma cell line. Br. J. Cancer **24**:509–515.
22. **Mishiro, S., M. Imai, K. Takahashi, A. Machida, T. Gotanda, Y. Miyakawa, and M. Mayumi.** 1980. A 49,000-dalton glycopeptide bearing all antigenic determinants and full immunogenicity of 22-nm hepatitis B surface antigen particles. J. Immunol. **124**:1589–1593.
23. **Moriarity, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer.** 1981. Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. Proc. Natl. Acad. Sci. U.S.A. **78**:2606–2610.
24. **Peterson, D. L.** 1981. Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J. Biol. Chem. **256**:6975–6983.
25. **Peterson, D. L., N. Nath, and F. Gavilanes.** 1982. Structure of hepatitis B surface antigen. Correlation of subtype with amino acid sequence and location of the carbohydrate moiety. J. Biol. Chem. **257**:10414–10420.
26. **Rose, J. K., and J. E. Bergmann.** 1982. Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. Cell **30**:753–762.
27. **Ruddon, R. W., C. A. Hanson, and N. J. Addison.** 1979. Synthesis and processing of human chorionic gonadotropin subunits in cultured choriocarcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **76**:5143–5147.
28. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. U.S.A. **80**:2495–2499.
29. **Strous, G. J. A. M., and H. F. Lodish.** 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. Cell **22**:709–717.
30. **Tartakoff, A. M.** 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell **32**:1026–1028.
31. **Urlaub, G., and L. A. Chasin.** 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. U.S.A. **77**:4216–4220.
32. **Yamada, G., L. E. Feinberg, and P. K. Nakane.** 1978. Hepatitis B. Cytologic localization of virus antigens and the role of the immune response. Hum. Pathol. **9**:93–109.