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Hepatitis B Surface Antigen: an Unusual Secreted Protein Initially Synthesized as a Transmembrane Polypeptide

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Hepatitis B surface antigen (HBsAg), the major coat protein of hepatitis B virus, is also secreted from cells as a subviral particle, without concomitant cleavage of N-terminal amino acid sequences. We examined this unusual export process in a cell-free system and showed that the initial product of HBsAg biosynthesis is an integral transmembrane protein, with most or all of its C-terminal half on the lumenal side of the endoplasmic reticulum membrane. To study the nature of its topogenic signals, we synthesized fusion proteins between HBsAg and the nonsecreted protein alpha-globin. Fusion proteins in which approximately 100 amino acids of globin preceded all HBsAg sequences were successfully translocated in vitro; the same domain as in the wild-type HBsAg was transported into the vesicle lumen. Fusions in which the entire globin domain was C terminal were able to translocate both the C-terminal region of HBsAg and its attached globin domain. Thus, uncleaved signal sequences in $p24^s$ function to direct portions of the molecule across the membrane and are able to perform this function even when positioned in an internal protein domain.

One of the unique features of hepatitis B virus (HBV) infection is that, in addition to the elaboration of HBV virions, infected cells also synthesize and secrete massive quantities of small subviral particles (35). These particles lack nucleic acid and are composed principally of the major viral coat protein, known as hepatitis B surface antigen (HBsAg), in close association with host-derived lipids (12, 34). Although a great deal is known about the structure of HBsAg, relatively little attention has been paid to the mechanisms by which this protein is exported from cells. However, what information is available suggests that this process has features which may be of considerable interest.

HBsAg is a hydrophobic protein of 24 kilodaltons (kDa) which exists in serum in both an unglycosylated form (termed $p24^s$) and its glycosylated derivative ($gp27^s$) (33). Sequence analysis of cloned HBV DNA (11, 30, 42) has revealed that the coding region for $p24^s$ is preceded by an in-phase, contiguous open reading frame, termed presurface (or pre-S), which is capable of encoding at least two larger proteins containing $p24^s$ sequences at their C termini (Fig. 1A). These proteins, $p31^{pre\text{-}S}$ and $p39^{pre\text{-}S}$, are synthesized in small quantities in vivo and, like $p24^s$, are also glycosylated, assembled into particles, and secreted (14, 23, 40). No precursor-product relationship exists between these larger proteins and $p24^s$. Each is synthesized by an independent translation initiation at its corresponding AUG codon (32). In addition, when $p24^s$ coding sequences alone are cloned into simian virus 40-based expression vectors, they can direct the normal synthesis and export of 22-nm HBsAg particles (18, 22). Thus, pre-S sequences are not required for the synthesis of $p24^s$ and cannot serve as signal sequences to direct its secretion; such signals must reside within the $p24^s$ coding region itself. Interestingly, comparison of the N-terminal amino acid sequence of secreted $p24^s$ (34) with the HBV DNA sequence reveals that, unlike the export of most proteins (2), no amino acids are cleaved from the polypeptide during export.

These unusual features led us to examine the export of the surface-pre-S protein family in more detail, since this process raises interesting questions concerning the position dependence of topogenic signal sequences (1). Although the location of export signals within the $p24^s$ molecule is not yet known (given the absence of signal cleavage), such elements must be situated internally within the larger pre-S proteins. Can export information within the $p24^s$ sequence function when so positioned, and if so, for which protein domains can it mediate transmembrane translocation?

As a first step in addressing these issues, we have studied the transmembrane transport of $p24^s$ in a cell-free translation-coupled translocation system (43). Using this system we demonstrated that this exported protein is not, as might have been predicted, directly secreted as such into the endoplasmic reticulum (ER) lumen. Rather, it is initially synthesized as an integral transmembrane protein in a process which results in the translocation of most or all of the C-terminal half of the molecule into the ER lumen. Using genetically engineered fusions between HBsAg and the cytoplasmic protein alpha-globin, we showed that HBsAg sequences can mediate the translocation of C-terminal domains of the hybrid polypeptide when positioned at either its N or C terminus. Thus, in HBsAg, uncleaved signal sequences function to direct domains in the C-terminal half of the molecule across the membrane and can perform this function even when positioned in an internal protein domain.

MATERIALS AND METHODS

Plasmid constructions. Plasmids pSP65 and pSP64, containing the bacteriophage SP6 promoter, were obtained from Promega Biotech. All restriction enzymes were purchased from New England BioLabs, Inc. and used according to the directions of the manufacturer. Plasmid pSP24H was derived by *Bam*HI and *Eco*RI cleavage of pSP65, followed by ligation of the cleavage products to the 2.0-kilobase fragment of HBV DNA generated by cleavage of pEC63 (42) with *Eco*RI and *Bgl*II. Plasmid p125E is a derivative of pSP64 which carries downstream of its SP6 promoter a 600-base-pair chimpanzee alpha-globin cDNA fused to the signal sequence of *E. coli* β-lactamase (20). Into the unique *Bgl*II site upstream of the

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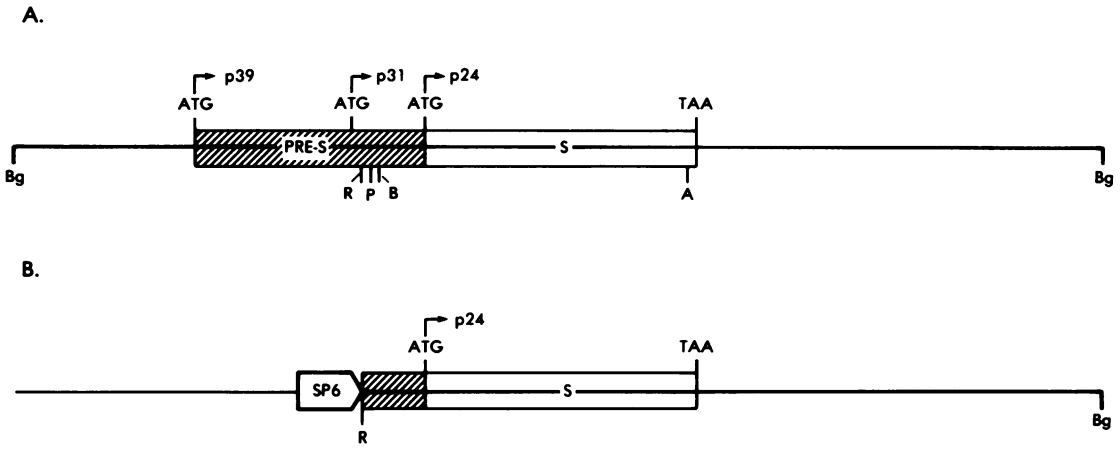

FIG. 1. Pre-S and surface antigen coding region of HBV. (A) The 2.8-kilobase *Bgl*II subgenomic fragment of HBV with contiguous open reading frame containing both pre-S (▨) and S antigen (▭, S) regions. The initiation codons for the three overlapping HBsAg-derived polypeptides $p39^{pre\text{-}S}$, $p31^{pre\text{-}S}$, and $p24^{s}$, at respective nucleotide positions 2858, 3214, or 158 of the HBV genome are shown, as is the termination codon at position 836 (genomic coordinates are relative to the unique *Eco*RI site at position 1). Restriction endonuclease sites are indicated as R (*Eco*RI), P (*Pst*I), B (*Bam*HI), Bg (*Bgl*II), and A (*Acc*I). (B) Recombinant plasmid pSP24H, containing a functional bacteriophage SP6 promoter (SP6, with arrow indicating transcriptional orientation), 5′ to the 2.0-kilobase *Eco*RI to *Bgl*II HBV subgenomic fragment containing the entire $p24^{s}$ gene.

globin coding region was inserted the 1.4-kilobase *Bam* fragment of HBV DNA carrying the $p24^{s}$ coding region. The resulting plasmid, pSP24*glo*, contains three *Acc*I sites, two at the 3′ end of $p24^{s}$ and one in the polylinker distal to the globin coding region. The latter site was inactivated by filling in the adjacent unique *Sal*I site, resulting in a plasmid which may be cleaved at the 3′ end of $p24^{s}$ by *Acc*I. For fusion A28 construction, 0.7 μg of plasmid DNA was cleaved with this enzyme and treated with exonuclease *Bal* 31 (1 U for 60 s at 22°C), and the nuclease was inactivated by phenol extraction. Product DNA was recleaved with *Nco*I, ends were repaired by incubation in 10 mM Tris–10 mM $MgCl_2$–1 mM dithiothreitol with 100 μM of deoxynucleoside triphosphates and the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim Biochemicals). This resulted in deletion of the intergenic region between p24 and globin, including β-lactamase signal sequences. Ends were religated with T4 DNA ligase for 12 h at 22°C, and the fusion plasmids were recloned and screened as described in the text. For construction of globin-surface fusions (e.g., B8), pSP*glo*24 was constructed as follows: (i) the β-lactamase signal sequence of pSP125E was deleted by cleavage with *Bgl*II and *Nco*I, by end repair with the Klenow enzyme, and by recloning; (ii) into the *Pst*I and *Sma*I sites of the polylinker 3′ to the globin sequences was cloned a 950-base-pair HBV *Pst*I-*Stu*I fragment bearing the p24 coding region. Deletion of the β-lactamase signal sequence was verified by restriction mapping and by functional tests in the coupled translation-translocation assay: pSP*glo*24 SP6 transcripts synthesized wild-type globin chains (lacking the N-terminal bacterial signal) and were not translocated on addition of membrane vesicles (unpublished data).

Transcription-linked translation. Plasmids containing the SP6 bacteriophage promoter were transcribed in vitro in a 50-μl reaction mixture containing 0.1 mg of plasmid DNA per ml; 40 mM Tris hydrochloride (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM dithiothreitol; 0.5 mM each ATP, CTP, GTP, and UTP; 1.2 U of RNase inhibitor (Promega) per ml; 250 μg of calf liver tRNA (Boehringer) per ml; and 50 to 60 U of SP6 polymerase (Promega). Reactions were incubated for 1.5 h at 40°C and then chilled at 0°C or stored at −70°C before use.

Aliquots of transcription reactions were diluted into 4 volumes of translation reaction premix at 0°C for final concentrations of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5), 140 mM potassium acetate, 3 mM dithiothreitol, 2.2 mM magnesium acetate plus $MgCl_2$, 10 mM Tris (pH 7.0 to 7.5), 0.4 mM spermidine, 1 mM ATP and GTP, 10 mM creatine phosphate, 40 mM each of 19 L-amino acids minus methionine, 1 mCi of [^{35}S]methionine per ml (1,200 Ci/mmol), 0.1 mg of calf liver tRNA per ml, 20 μg of creatine kinase (Boehringer) per ml, 1 U of RNase inhibitor per ml, and 20% (vol/vol) wheat germ extract prepared according to the method of Erickson and Blobel (9). In addition, some reactions contained canine pancreatic membranes prepared by the method of Walter and Blobel (43) at a concentration of 2.5 A_{280} U/ml. Translations were incubated for 1.5 to 2.5 h at 24°C. Samples (2 μl) to be analyzed directly for total products were precipitated at 0°C with 20% trichloroacetic acid, washed with ethanol-ether (1:1), suspended in 10 to 15 μl of sodium dodecyl sulfate (SDS) loading buffer (4% SDS, 0.1 M Tris [pH 8.9], 2 mM EDTA, 15% sucrose, 0.1% bromophenol blue, 0.5 M dithiothreitol), and boiled for 2 min before SDS-polyacrylamide gel electrophoresis and fluorography by a modification of the method of Bonner and Laskey (4).

Posttranslational enzymatic treatments. Aliquots (2 μl) of translation reactions to be examined for glycosylation were diluted into 50 μl of 0.1 M sodium citrate (pH 5.5)–0.1% SDS and heated at 100°C for 2 min (36). After return to ambient temperature, 2 μl of endoglycosidase H (New England Nuclear Corp.; 45 μg/ml) were added, and the mixture was incubated at 37°C overnight, followed by trichloroacetic acid precipitation, as described above.

Translation reactions to be digested with protease were brought to 10 mM $CaCl_2$, and microsomal membranes were added posttranslationally. Protease was predigested at 10 times the final concentration for 30 min at 37°C in 10 mM Tris (pH 8)–10 mM $CaCl_2$ and then added to reaction mixtures (at the final temperature) for a final concentration of 0.1 mg of

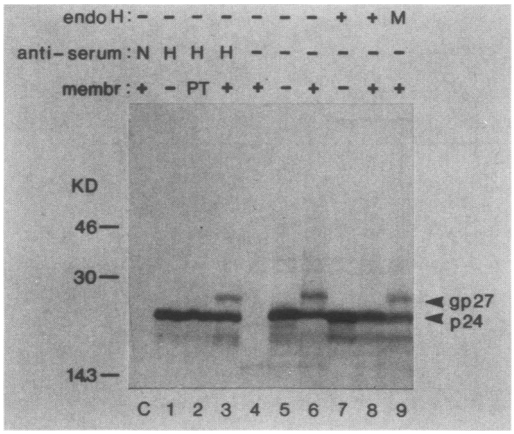

FIG. 2. Co-translational membrane translocation of p24^s. In vitro transcripts of plasmid pSP24H were used to program an in vitro translation system as outlined in the text; radiolabeled translation products were electrophoresed through polyacrylamide-SDS gels either before (lanes 4 to 9) or after (lanes C and 1 to 3) immunoprecipitation with the indicated antisera (N, nonimmune; H, anti-HBsAg). Translations were carried out in the absence (lanes 1, 5, and 7) or presence (lanes C, 3, 4, 6, 8, and 9) of microsomal vesicles (membr); vesicles in lane 2 were added posttranslationally (PT). The products of synthesis in the absence (lane 7) and presence (lane 8) of membranes were digested with endoglycosidase H before electrophoresis; M designates mock addition of endoglycosidase H (lane 9). In lane 4, no mRNA was added to the translation reaction.

enzyme per ml. Digestions were performed for 1 h at 24°C or at 0°C. Trypsin proteolysis was terminated by addition of aprotinin (Boehringer) to 1 mg/ml and subsequent trichloroacetic acid precipitation. Proteinase K digests were terminated by the addition of phenylmethylsulfonyl fluoride to 2 mM and by rapid dilution into 3 volumes of boiling 1% SDS–0.1 M Tris (pH 8.9) for 5 min. Aliquots to be examined for total products were subsequently diluted into 3 volumes of SDS loading buffer and reheated as above, whereas aliquots to be immunoprecipitated were diluted into 20 volumes of 1% Triton X-100–0.1 M Tris (pH 8)–0.1 M NaCl–10 mM EDTA–1 mM phenylmethylsulfonyl fluoride before addition of antisera.

Radioimmunoprecipitations. Reaction of radiolabeled antigens with 4 μl of anti-HBsAg (Calbiochem-Behring) or antiglobin (Cooper Biomedical, Inc.) was performed in RIPA buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM Tris [pH 8], 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) at 4°C overnight. After incubation with protein A-Sepharose (Pharmacia) for 1 h, reaction mixtures were centrifuged at 10,000 × *g* for 5 min through a cushion of 35% (wt/vol) sucrose in RIPA buffer. Pellets were washed three times with RIPA buffer and two times with 1 mM Tris (pH 8)–0.1 mM EDTA–1 mM phenylmethylsulfonyl fluoride). The last wash was transferred to a fresh tube to avoid pelleted aggregates, and the final pellet was dried in vacuo before suspension in SDS loading buffer and electrophoresis, as described above.

RESULTS

In vitro translation of p24^s. To study p24^s translocation we employed an in vitro coupled transcription-translation system analogous to those previously described (20, 43) for the analysis of secretory and membrane proteins. In this system, synthetic p24^s mRNA is generated by in vitro transcription with bacteriophage SP6 polymerase (27) of a recombinant plasmid in which p24^s coding sequences are cloned downstream of a bacteriophage SP6 promoter (Fig. 1B); the resulting RNA is then used to program an in vitro translation extract (derived from wheat germ embryos) with or without added canine pancreatic microsomal vesicles. Together, these components contain all of the enzymatic machinery required for the transmembrane translocation of proteins. Translation products are radiolabeled with [^{35}S]methionine and identified by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Translocation of the protein into the lumen of the vesicle can be assayed in two ways: (i) since the enzymatic apparatus for glycosylation is localized to the interior of the vesicle, glycosylation of p24^s to gp27^s (detected by the corresponding electrophoretic mobility shift) indicates translocation into the lumen; and (ii) protein domains transported into the lumen of the intact vesicle become resistant to attack by exogenously added proteases under conditions where untranslocated domains are digested.

In the absence of added membranes (Fig. 2, lane 5), the major radiolabeled product of translation of synthetic p24^s mRNA was a protein of 24 kDa, which immunoprecipitation with anti-HBsAg antibodies confirmed to be p24^s (lane 1). When an identical translation was carried out in the presence of microsomes (lane 6), in addition to the 24-kDa species, a new species of 27 kDa was detected; this latter species, which typically accounts for 15 to 25% of the products, was also immunoprecipitable with anti-HBsAg (lane 3). To confirm that this new species was indeed gp27^s, the N-linked glycosylation product of p24^s, we treated the translation products made in the presence of microsomes with endoglycosidase H, which cleaves asparagine-linked high-mannose carbohydrate chains (41). This treatment resulted in the disappearance of the 27-kDa species with an increase in the intensity of the p24^s band (Fig. 4, lanes 8 and 9). We next examined whether glycosylation of p24^s could proceed posttranslationally. In this experiment, translation was performed without membranes for 2.5 h; at this point, when no further incorporation of [^{35}S]methionine label was detectable (data not shown), microsomal vesicles were added, and incubation continued for an additional 1 h. Electrophoretic analysis of the products (lane 2, and Fig. 3, lane 3) revealed no gp27^s under these conditions, indicating that (i) no leakage of glycosylation enzymes outside of the vesicles occurs during incubation and that (ii) translocation-glycosylation of p24^s is cotranslational. Preliminary experiments indicate that this cotranslational process involves interaction with signal recognition particle; addition of signal recognition particle in vitro translation extracts programmed with p24^s mRNA results in translation arrest (44), which is relieved by the addition of microsomal membranes (unpublished data).

To confirm that gp27^s was indeed translocated into or across the membrane of the vesicle, we next examined the translocated products for resistance to exogenous trypsin (Fig. 3). Under standard conditions trypsin is known to cleave p24^s only once, after lysine 122, despite the large number of other *arg* and *lys* residues in the protein (33). When p24^s made in the absence of membranes (lane 2) was exposed to trypsin, p24^s was completely cleaved to its expected limit products (lane 1). When membranes were present during translation, gp27^s was seen as expected (lane 4). When the labeled translocated products were exposed to trypsin (lane 5), gp27^s remained intact under conditions in which p24^s was virtually completely cleaved. If, however,

after translation in the presence of membranes, the vesicles were first disrupted with nonionic detergent (Nikkol) before trypsin exposure, both gp27^s and p24^s were cleaved (lane 6). Taken together, the data shown in Fig. 2 and 3 demonstrate that the cotranslational glycosylation of p24^s is accompanied by translocation of at least the protein domain surrounding *lys*122 (the trypsin site) and *asn*146 (the glycosylation site) into the lumen of the vesicle.

Translocated p24^s is an integral transmembrane protein. As noted above, since trypsin cleaves HBsAg at only one site, use of trypsin resistance as a criterion of membrane transport only probes the translocation of a limited region of the molecule. To determine if the entire molecule is translocated into the lumen (as for other secretory proteins), we next assayed the translocated products for resistance to proteinase K, a protease of lesser sequence specificity which was expected to attack the molecule at a different array of sites. This was indeed so (Fig. 4); labeled p24^s synthesized in the absence of membranes (lane 1) was cleaved to different (and somewhat smaller) products after the addition of exogenous proteinase K (lanes 2 and 3). Interestingly, even this potent protease did not cleave HBsAg to oligopeptide products under these conditions, but rather generated a series of discrete limit digest products—another reflection of the known protease resistance of this polypeptide (33). Although these fragments were not efficiently immunoprecipitated by polyclonal antisera to native HBsAg particles (Fig. 6, lanes 7 and 9), they could be detected in longer exposures of such gels (data not shown). Translation of HBsAg mRNA in the presence of microsomal vesicles (lane 4) again generated the expected products, p24^s and gp27^s; however, when these products were exposed to exogenous proteinase K, neither product was fully protected. Rather, in addition to limit products of p24^s digestion, a protected band (R_0), ca. 7 kDa smaller than gp27^s, was observed (lane 5) which was immunoreactive with anti-HBsAg antibodies (lane 8). (The quantitative distribution of the limit digest products is reproducibly altered by the presence of membranes, even when added posttranslation, as in lane 3; this effect, presumed to result from altered cleavage efficiency at certain sites, has previously been observed with other translocated proteins in vitro [21] and does not alter the central conclusions of these experiments.) To ensure that this finding did not result from limited permeability of our vesicle preparations to proteinase K (or failure to completely inactivate the enzyme before opening the vesicles), we carried out control experiments in parallel with prolactin, a secretory protein known to be translocated entirely into the vesicle lumen. Addition of proteinase K to this preparation, under conditions identical to those used for p24^s and gp27^s, completely degraded preprolactin, but did not cleave the mature (translocated) prolactin (data not shown). For both p24^s (lane 6) and prolactin (not shown), the protected fragment was attacked only when proteinase K was added after disruption of the vesicle preparation with Nikkol.

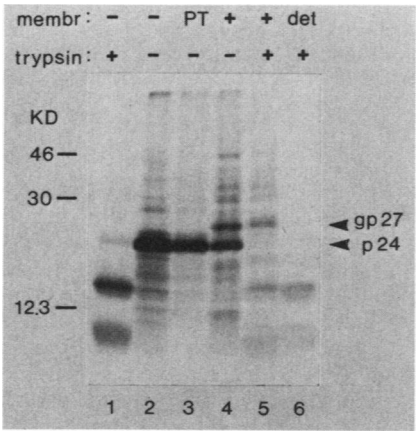

FIG. 3. Resistance of translocated p24^s to trypsin cleavage. Transcripts of pSP24H were translated in vitro as described in the legend to Fig. 2, and radiolabeled products that were made in the absence (lanes 1 and 2) or presence (lanes 4 to 6) of microsomal vesicles were either exposed (lanes 1, 5, and 6) or not exposed (lanes 2 to 4) to trypsin before electrophoresis and autoradiography; vesicles in lane 3 were added posttranslationally (PT). In lane 6, after translation in the presence of membranes (membr), the vesicles were disrupted by detergent (det) before exposure to trypsin.

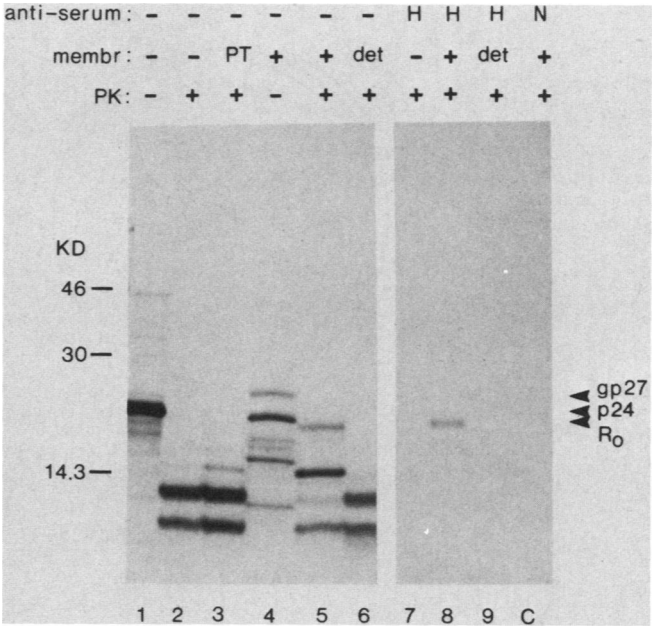

FIG. 4. Proteolysis of translocated p24^s by proteinase K. In vitro transcripts of pSP24H were translated in vitro in the presence (lanes 4 to 6 and 8, 9, and C) or absence (lanes 1, 2, and 7) of microsomal membranes (membr); in lane 3, membranes were added posttranslationally (PT). After translation the products were either exposed (lanes 2, 3, 5 to 9, and C) or not exposed (lanes 1 and 4) to proteinase K (PK), and the resulting products were electrophoresed directly (left panel) or after immunoprecipitation with either anti-HBsAg (H) or nonimmune (N) serum (right panel). In lanes 6 and 9, products translated in the presence of microsomes were treated with detergent (det) to disrupt the vesicles before proteolysis. R_0 denotes the protease-resistant fragment of gp27^s resulting from membrane translocation.

The demonstration that gp27^s is only partially protected from proteinase K (Fig. 4) indicates that only a portion of the molecule is translocated into the vesicle lumen; that is, that transported HBsAg is a transmembrane protein which spans the membrane at least once. Together with the results of Fig. 2 and 3, the data indicate that the translocated portion of the polypeptide must include the trypsin and glycosylation sites in the C-terminal half of the molecule. Further experiments to define the topology of transmembrane HBsAg will be presented below.

p24^s transport signals can function in an internal protein domain. As noted above, p24^s is one member of a nested set of proteins which includes p31$^{pre\text{-}S}$ and p39$^{pre\text{-}S}$, all of which

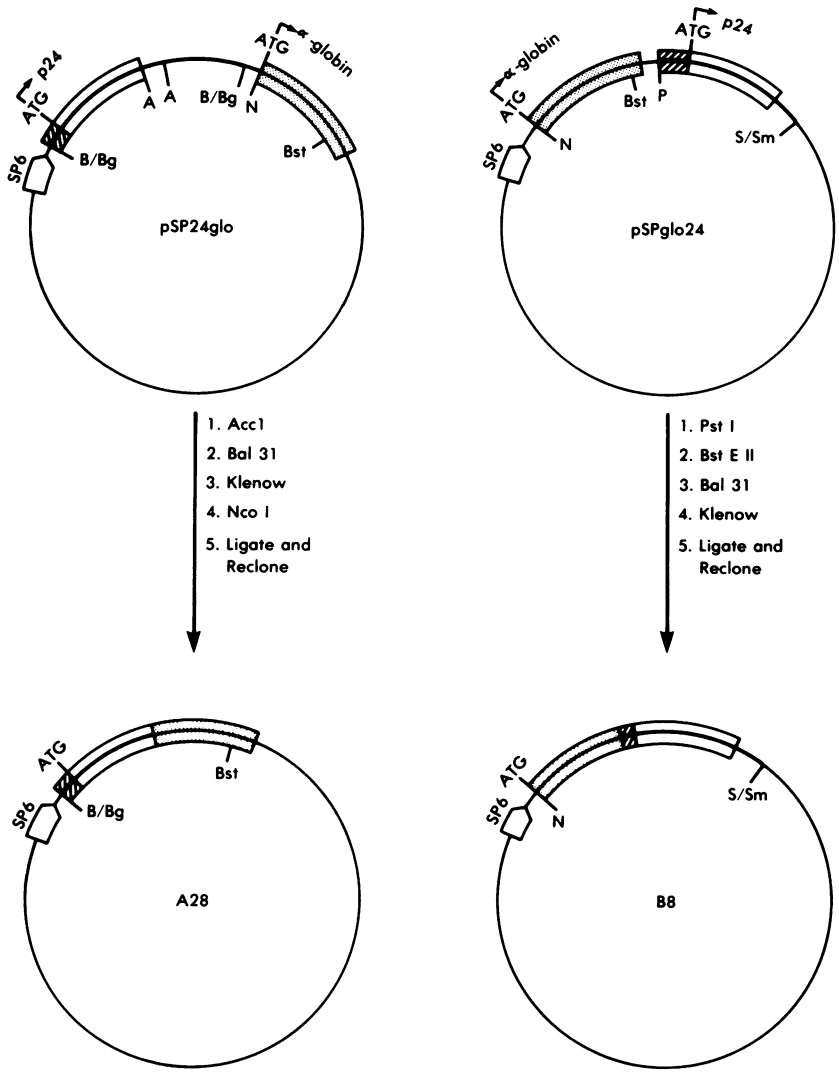

FIG. 5. Construction of recombinant plasmids containing fused globin and HBsAg sequences. Parent plasmids (upper panels), constructed as described in Materials and Methods, contained the complete $p24^s$ (▭) and alpha-globin (▨) genes and were treated as described in the text to generate recombinants in which globin sequences were fused to either the N-terminal (B8, lower right panel) or the C-terminal (A28, lower left panel) ends of HBsAg. Enzymatic steps as described in Materials and Methods are listed for each construction. Restriction endonuclease sites are indicated as in Fig. 1, with additional enzymes *Nco*I (N), *Bst*EII (Bst), *Stu*I (S), and *Sma*I (Sm) indicated. pre-S sequences (▭) are noted.

contain $p24^s$ sequences at their C termini. Since $p24^s$ contains sequences which mediate transmembrane insertion, we sought to explore whether such topogenic sequences could retain their function when presented in an internal protein domain, analogous to their position in the pre-S proteins. Accordingly, we decided to test fusion proteins constructed by joining HBsAg amino acid sequences to those of the nonsecreted cytoplasmic protein alpha-globin. This polypeptide has no topogenic sequences of its own, but previous studies (20) have shown that the entire globin polypeptide can be transported across the vesicle membrane if fused downstream of (heterologous) N-terminal signal sequences; this indicates that no sequences inhibitory to translocation exist within the molecule, making it an ideal fusion partner for these experiments. In the first set of experiments, fusion proteins were synthesized which contained alpha-globin sequences at their N termini and HBsAg domains at their C termini. These proteins were generated by first preparing in vitro a recombinant plasmid (pSP*glo*24) in which surface antigen coding sequences were cloned 3′ to a chimpanzee alpha-globin cDNA clone, which in turn was positioned immediately downstream of the phage SP6 promoter (Fig. 5). To fuse the coding regions, we cleaved the recombinant with *Bst*EII, which cuts once within the 3′ end of the globin gene, and with *Pst*I, which cleaves in the distal pre-S region; the cleaved plasmid was further resected with Bal 31 nuclease, and the resulting shortened products were recloned in *E. coli*. Candidate fusion proteins were identified by transcribing individual recombinant plasmids in vitro with SP6 polymerase, followed by in vitro translation of the resulting hybrid mRNAs. Radiolabeled translation products of the appropriate size were screened for in-phase fusions by immunoprecipitation with antiglobin and anti-HbsAg antisera; this allowed the rapid screening of many recombinants without the need for DNA sequencing. The fusion junctions of in-phase chimeras were determined by restriction map-

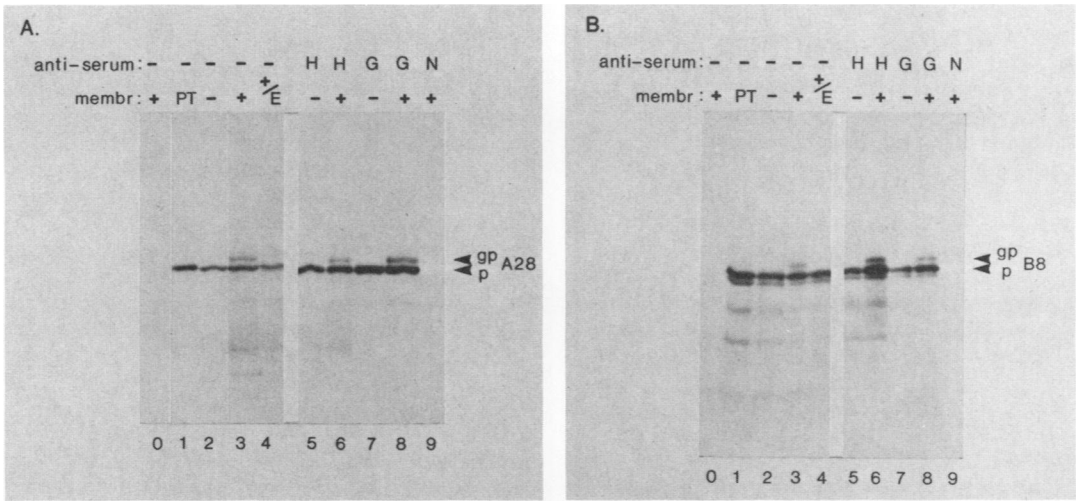

FIG. 6. Cotranslational membrane translocation of HBsAg-globin fusion proteins. Fusion plasmids A28 and B8 (Fig. 2) were transcribed and translated in vitro in the presence or absence of microsomal membranes (membr) and assayed for translocation-dependent glycosylation as in Fig. 4. Translation products of fusion A28 (A) or B8 (B) made in the absence (lanes 2, 5, and 7) or presence (lanes 3, 4, 6, 8, and 9) of vesicles were electrophoresed directly (lanes 1 to 4) or after immunoprecipitation (lanes 5 to 9) with antisera to HBsAg (H) or to alpha-globin (G) or with nonimmune (N) serum. In lane 0, no mRNA was added to the translation reaction; in lane 1, membranes were added posttranslationally (PT). In lane 4, total products synthesized in the presence of membranes were treated with endoglycosidase H (E) before electrophoresis. The positions of the unglycosylated (p) and glycosylated (gp) polypeptides are indicated (arrows).

ping and, where indicated, by DNA sequencing, to precisely locate the globin-HBsAg boundary.

Three in-phase fusions with fusion junctions in the distal pre-S region were recovered. All three were then examined in the in vitro translocation assay previously described, and representative data for fusion B8 are shown in Fig. 6B. Translation of B8 mRNA in the absence of membranes gave rise to a radiolabeled product of ca. 34 kDa (lane 2) which was precipitable with both antiglobin (lane 7) and anti-HBsAg (lane 5) antisera. When translation was performed in the presence of microsomes, in addition to the 34-kDa band another, more slowly migrating band of ca. 37 kDa was seen (lane 3), which was also reactive with both antisera (lanes 8 and 6), as would be expected for the glycosylated (translocated) derivative of the fusion protein. To verify this, we treated the translocated products with endoglycosidase H (lane 4); the putative glycosylated product was indeed sensitive to endoglycosidase H digestion. As for wild-type $p24^s$, translocation was cotranslational: addition of membranes posttranslation did not result in the appearance of glycosylated products (lane 1).

All three globin-HBV fusions behaved similarly in the translocation assay (data not shown). Mutant B8, which contained the most extensive deletion of HBV DNA, was sequenced by the method of Maxam and Gilbert (25), which revealed the fusion to join codon 97 of globin to a point 11 codons upstream of the p24 initiator methionine. More recently we have isolated and characterized fusions extending into $p24^s$ coding sequences, thus eliminating all pre-S residues. Similar analysis reveals that even a fusion joining 109 residues of globin to amino acid 51 of $p24^s$ is translocated in vitro (unpublished data). Since the alpha-globin sequence lacks potential sites for N-linked glycosylation, the observed glycosylation of the fusion proteins must reflect carbohydrate addition to the $p24^s$ domain, presumably at *asn*146. Thus, these studies indicate that translocation of the C-terminal region of the fusion protein can be effected by topogenic signals within $p24^s$, even when such signals are positioned in an internal protein domain. Further studies on the transmembrane orientation of these fusions are presented below.

Since the preceding studies indicate that substantial alterations at the N terminus of $p24^s$ do not abolish signal function, it was of interest to determine if additions of protein to the C terminus would likewise be tolerated. Accordingly, we generated gene fusions between $p24^s$ and globin in a recombinant SP6 plasmid vector (pSP24*glo*) in which the gene order is 5′–SP6 promoter–$p24^s$–globin–3′. Figure 5 outlines the strategy used for fusing globin sequences to the distal portion of the $p24^s$ coding region in this vector. As before, candidate fusion plasmids were screened by coupled in vitro transcription-translation for protein products of the expected size that were immunoreactive with both globin and HBsAg antisera. Of four in-phase fusions initially recovered, two (mutants A23 and A28) were shown by restriction mapping (A23) or DNA sequencing (A28) to have preserved the p24 glycosylation site required for the glycosylation assay.

These recombinants produced fusion proteins of 36 kDa (A23) and 32 kDa (A28). Both underwent cotranslational glycosylation upon translation in the presence of microsomes (see Fig. 6A for data on A28). DNA sequence analysis of mutant A28 indicated that, in the protein encoded by this mutant, residue 165 of $p24^s$ is fused to the second amino acid of alpha-globin.

Transmembrane orientation of $p24^s$-globin fusion proteins. We next sought to define the transmembrane orientation of the translocated fusion proteins to determine which protein domains were translocated under the influence of $p24^s$ signals. Our analysis exploited the availability of active fusions with globin-immunoreactive domains at either the N or C terminus; since the two halves of each fusion protein are differentially reactive with anti-HBsAg and antiglobin, these reagents can be used as probes for determining which portions of the translocated fusions are protected from protease attack. Accordingly, mutants A28 (N-HBsAg-globin-C) and B8 (N-globin-HBsAg-C) were selected for analysis; each mutant was transcribed with SP6 polymerase

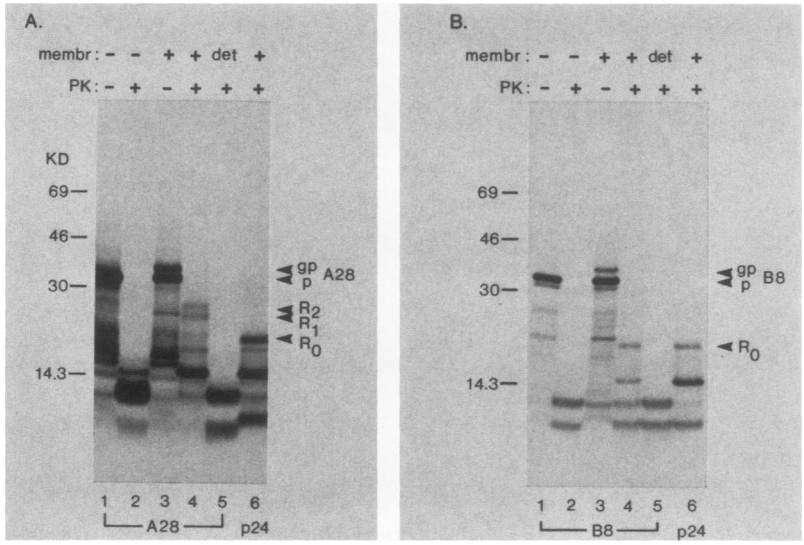

FIG. 7. Proteinase K treatment of translocated HBsAg-globin fusion proteins. Translation products of fusions A28 (A) and B8 (B) made in the presence or absence of membranes (membr) as indicated above each figure were either exposed (lanes 2, 4, and 5) or not exposed (lanes 1 and 3) to proteinase K (PK) before electrophoresis; in both panels, products were analyzed without immunoprecipitation. Protease-protected fragments from fusion A28 (R_1 and R_2) and B8 (R_0) are indicated, as are the positions of the full-size-glycosylated (gp) and unglycosylated (p) proteins. In lane 6 of each panel, the pattern of proteinase K digestion products of wild-type translocated $p24^s$ is displayed for comparison. Lanes are marked (det) for detergent treatment.

in vitro, and the product RNA was translated in vitro with or without added microsomes. Products were then treated with proteinase K, and the remaining protected species were identified by SDS-polyacrylamide gel electrophoresis before (Fig. 7) or after (Fig. 8) immunoprecipitation with anti-HBsAg or antiglobin. Figure 7A shows the results of such an experiment for mutant A28. In this case, protease treatment of the translocated fusion protein (lane 4) resulted, as expected, in the production of truncated species (labeled R_1 and R_2), indicating that the fusion was not entirely sequestered within the vesicle. Immunoprecipitation of the protected fragments with antiglobin serum (Fig. 8A) or anti-HBsAg (not shown) revealed that the protected species harbor determinants of both proteins. As for the R_0 fragment of p24, the R_1 and R_2 fragments were also sensitive to endoglycosidase H (not shown). These data indicate that, as

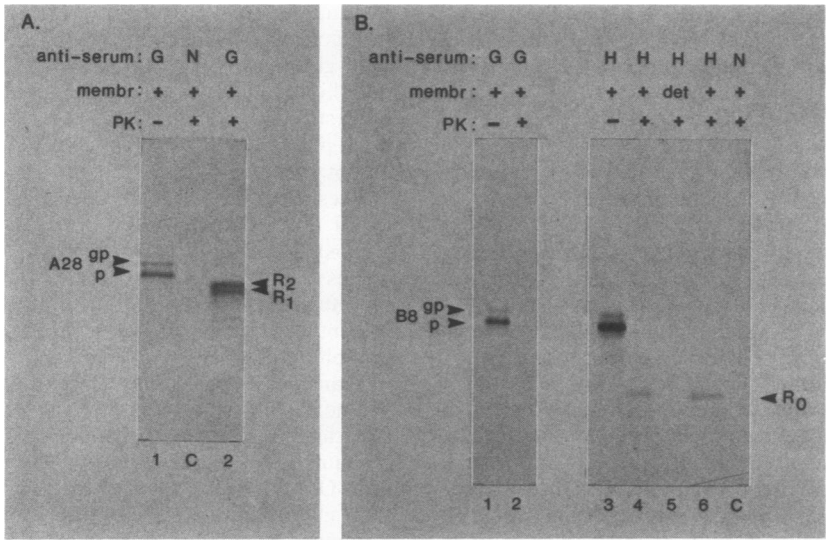

FIG. 8. Immunoreactivity of the proteinase K-resistant fragments of HBsAg-globin fusion polypeptides. (A) Translation products of fusion gene A28 made in the presence of microsomal vesicles were either exposed (lanes C and 2) or not exposed (lane 1) to proteinase K (PK), then immunoprecipitated with nonimmune (N) or antiglobin (G) antisera before electrophoresis in polyacrylamide-SDS gels. (B) Translation products of gene fusion B8 were prepared in the presence of membranes and analyzed either before (lanes 1 and 3) or after (lanes 2, 4, 5, and C) proteinase K digestion. After proteolysis, samples were immunoprecipitated with antiglobin (G) sera (lanes 1 and 2), anti-HBsAg (H) (lanes 3 to 5), or nonimmune (N) serum (lane C). In lane 6, the proteinase K-resistant fragment (R_0) of translocated $p24^s$ was precipitated with anti-HBsAg and electrophoresed in parallel for comparison.

for $p24^s$ itself, the HBsAg signal mediates translocation of at least the C-terminal portion of the molecule and confirms that there is no block to the transport of globin sequences in this system. Comparison of the molecular weights of the A28 polypeptides before and after proteinase K treatment indicates that, as in wild-type $p24^s$, approximately 7 kDa of protein was removed by protease attack.

When the globin domain was at the N terminus of the hybrid polypeptide (mutant B), protease treatment of the translocated species also produced a truncated protected fragment (R_0) (Fig. 7B, lane 4). Two points, however, are noteworthy: (i) the size of this fragment was identical to that resulting from the protease treatment of translocated $p24^s$ itself (lane 6), and (ii) the fragment was reactive only with anti-HBsAg and not with antiglobin (Fig. 8B). Thus, in this case, at least some and perhaps most of the fused globin domain was removed from mutant B8 by cleavage within N-terminal regions accessible to protease attack; hence these regions were not sequestered within the vesicle lumen or membrane. As was true for translocated wild-type HBsAg, the protease-protected fragment of fusion protein B8 was sensitive to endoglycosidase H digestion (data not shown). The implications of these findings for HBsAg organization and biosynthesis are considered below.

DISCUSSION

These results demonstrate (i) that $p24^s$, a protein destined for export from cells, is initially synthesized as a transmembrane polypeptide; (ii) that the newly synthesized product in the microsomal membrane is oriented with C-terminal regions in the vesicle lumen; and (iii) that topogenic signals within $p24^s$ sequences can still mediate the translocation of appropriate domains despite substantial changes in their relative position in the primary sequence: most notably, these signals can function even when located in an internal protein domain. These findings have several implications for HBV morphogenesis and may be relevant to a number of more general aspects of protein translocation across membranes.

Transmembrane HBsAg polypeptides: an intermediate in HBsAg export? The data reported here provide the first experimental evidence that the initial product of HBsAg biosynthesis is a transmembrane polypeptide. Although these experiments have been conducted in a cell-free system, preliminary in vivo experiments in amphibian oocytes programmed with synthetic mRNA for p24-globin fusion proteins have yielded similar results (K. Simon, unpublished data), suggesting that, as in other published cases (17, 38), this in vitro system faithfully reproduces the events involved in membrane translocation in vivo. If this is so, then transmembrane HBsAg is likely to be an intermediate in the biogenesis of the exported 22-nm particle.

Available electron microscopic evidence, while limited, suggests that 22-nm particles form within ER cisternae in infected cells (13, 16, 29). Biochemical studies showing that high-mannose N-linked carbohydrates on HBsAg are processed to an endoglycosidase H-resistant form indicate that antigens destined for export likely traverse the Golgi apparatus (31). Interestingly, the export of HBsAg proceeds much more slowly than does the processing of conventional secretory and membrane proteins, and analysis of the glycosylation pattern of accumulated intracellular $p24^s$ suggests a substantial delay in a pre- or early-Golgi processing step (31). If transmembrane $p24^s$ on rough ER membranes is a precursor to particle formation, then major structural reorganization must occur to allow particle assembly: aggregation of $p24^s$ (and pre-S protein) monomers, exclusion of other host proteins, and (presumably) substantial reorganization of associated lipids are likely to be required. One model for how particle formation might occur is shown in Fig. 9B. These complex morphogenetic events could account at least in part for the observed delay in particle export.

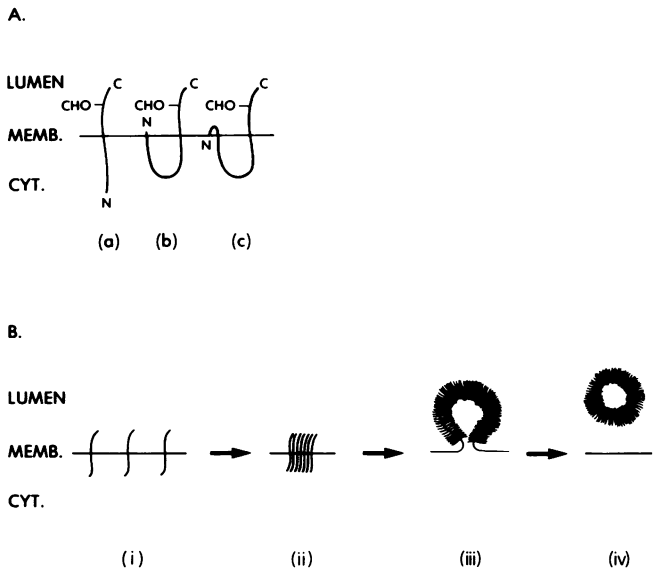

FIG. 9. (A) Possible fine structures of transmembrane $p24^s$. Three models for the fine structure of transmembrane $p24^s$ are shown. In all cases, the C-terminal end of the molecule (C), including the site of the N-linked oligosaccharide addition (CHO), protrudes into the vesicle (ER) lumen. The N terminus of the molecule may be free in the cytoplasm (CYT.) (model a) or loop back across the vesicle membrane (memb.) (models b and c). (B) One model for HBsAg particle formation. In this model, particle formation is envisioned to begin with the transmembrane insertion of HBsAg polypeptides (i), depicted here (for simplicity only) as in orientation a of Fig. 9A. Subsequently, monomers undergo aggregation in the plane of the bilayer (ii), concomitant with or followed by exclusion of host membrane proteins and reorganization of host lipid components. Budding (iii) and detachment (iv) from the membrane result in the delivery of the newly formed particle into the ER lumen.

We know of no other eucaryotic export process described to date which exactly resembles that of HBsAg, though transmembrane intermediates in the export of coat proteins of filamentous bacteriophages have been previously observed (37, 45). Many analogies are apparent with envelope glycoproteins of budding animal RNA viruses (e.g., insertion into membranes and exclusion of host proteins), but in these cases, the resulting exported virus, unlike HBsAg particles, contains matrix or nucleocapsid proteins which are responsible for the recognition of the envelope proteins, a step which is required for budding to occur. In addition, unlike HBsAg, the budded product retains a morphologically recognizable unit membrane structure derived from the host. In some respects, HBsAg particles are formally analogous to certain serum lipoprotein complexes normally involved in lipid transport and metabolism (e.g., very-low-density lipoprotein). Like HBsAg, such very-low-density lipoprotein particles are composed of apoproteins complexed with lipids and are known to be assembled within liver cells, from which they are exported into serum (10, 39); however, it is not known whether these apparent similarities reflect a common

mode of biogenesis. Among the nonparticulate exported proteins, at least two have been identified in which the initial product of synthesis is thought to be a transmembrane polypeptide: the secretory component of immunoglobulin A (28) and the transforming growth factor alpha (19). However, in both these cases the secreted form of the protein is liberated from its membrane-bound precursor by proteolytic cleavage.

Transmembrane orientation of translocated HBsAg. Our studies indicate that at least the portion of $p24^s$ which includes *lys*122 (the trypsin site) and *asn*146 (the glycosylation site) is sequestered within the vesicle lumen. The protection of the globin domain from protease in fusion A28 indicates that the translocated segment of HBsAg must extend at least to amino acid 165 (the fusion junction); detailed studies are underway to determine if the extreme C terminus of $p24^s$ is also translocated. The experiments indicate that at least one transmembrane domain must be present upstream of *lys*122. Inspection of the predicted amino acid sequence of $p24^s$ reveals an extremely hydrophobic segment (from residues 80 to 100) which is likely to mediate this function.

The fact that the protease-protected domains of fusion B8 do not include identifiable globin sequences demonstrates that at least some domains N terminal to the transmembrane region are exposed on the cytoplasmic face of the membrane. However, the precise disposition of this N-terminal region with respect to the membrane remains to be clarified. Models in which single (Fig. 9A, model a) or multiple (Fig. 9A, models b and c) transmembrane segments exist are equally compatible with the data presented here. Although the failure of N-terminal sequences in fusion B8 to be protected from protease might argue against model b (Fig. 9A), we consider it possible that such upstream globin domains might be translocated inefficiently relative to the wild-type N terminus and hence might be accessible to digestion. Further experiments to determine the fine structue of transmembrane HBsAg are in progress; it will be of considerable interest to compare its transmembrane topology with those of other proteins known to harbor uncleaved or internal signals (3, 5–8, 15, 24, 26).

Topogenic signals in $p24^s$ in variety of contexts. Because $p24^s$ sequences are positioned internally in two distinct pre-S proteins (14) during natural HBV infection, we asked whether topogenic signals in $p24^s$ could function when positioned in an internal domain. Since the role of pre-S sequences themselves in translocation is uncertain, we replaced them with coding sequences (from alpha-globin) known to be devoid of signal function but nonetheless capable of being transported across the lipid bilayer when coupled to an active signal (20). Our findings (Fig. 6 and 7) that hybrid globin-$p24^s$ proteins were translocated demonstrate that $p24^S$ sequences can function to translocate downstream protein domains even when positioned behind almost 100 amino acids of globin. The ability of HBsAg topogenic signals to function from within an internal protein domain may reflect an important property of signal sequences which direct the topology of other integral transmembrane proteins, especially those whose internal regions span the lipid bilayer many times; the existence of internal signal sequences has been proposed (1) to be responsible for the internal looping across the membrane of such "polytopic" membrane proteins.

As noted earlier, pre-S sequences are not required for $p24^s$ translocation. However, this does not exclude a role for pre-S sequences in the topogenesis of the larger pre-S proteins ($p31^{pre\text{-}S}$ and $p39^{pre\text{-}S}$). Our data with globin-$p24^s$ fusions (e.g., B8) make it likely that, in the natural pre-S proteins, at least the C-terminal regions of the p24 domain will be translocated into the ER lumen. However, the orientation of the pre-S domains themselves may depend upon the presence or absence of additional topogenic signals within these sequences.

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LITERATURE CITED

1. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77**:1496–1500.
2. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. J. Cell Biol. **67**:852–862.
3. **Blok, J., G. M. Air, W. Laver, C. Ward, G. Lilley, E. F. Woods, C. Roxburgh, and A. Inglis.** 1982. Studies on the size, chemical composition, and partial sequence of the neuraminidase from type A influenza viruses show that the N-terminal region of the NA is not processed and serves to anchor the NA in the viral membrane. Virology **119**:109–121.
4. **Bonner, W., and R. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
5. **Bos, T., A. Davis, and D. Nayak.** 1984. NH_2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. Proc. Natl. Acad. Sci. USA **81**: 2327–2331.
6. **Brunner, J., H. Hauser, H. Braun, K. Wilson, H. Wacker, B. O'Neill, and G. Semenza.** 1979. The mode of association of the enzyme complex sucrase-isolmaltase with the intestinal brush border membrane. J. Biol. Chem. **254**:1821–1828.
7. **Claesson, L., D. Larhammar, L. Rask, and P. Peterson.** 1983. cDNA clone for the human invariant gamma-chain of class II histocompatibility antigens and its implications for the protein structure. Proc. Natl. Acad. Sci. USA **80**:7395–7399.
8. **Drickamer, K.** 1981. Complete amino acid sequence of a membrane receptor for glycoproteins. J. Biol. Chem. **256**:5827–5839.
9. **Erickson, A., and G. Blobel.** 1983. Cell-free translation of messenger RNA in a wheat germ system. Methods Enzymol. **96**:38–49.
10. **Forte, T.** 1984. Primary hepatocytes in monolayer culture: a model for studies on lipoprotein metabolism. Annu. Rev. Physiol. **46**:403–415.
11. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charney.** 1979. Nucleotide sequence of hepatitis B virus genome (subtype ayw) cloned in *E. coli*. Nature (London) **281**:646–650.
12. **Gavilanes, F., J. Gonzalez-Ros, and D. 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.
13. **Gerber, M., S. Hadziyannis, C. Vissoulis, F. Schaffner, F. Paronetto, and H. Popper.** 1974. Electron microscopy and immunoelectronmicroscopy of cytoplasmic hepatitis B antigen in hepatocytes. Am. J. Pathol. **75**:489–502.
14. **Heermann, K. H., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-*s* sequence. J. Virol. **52**:396–402.
15. **Holland, E., J. Leung, and K. Drickamer.** 1984. Rat liver asialoglycoprotein receptor lacks a cleavable NH_2-terminal signal sequence. Proc. Natl. Acad. Sci. USA **81**:7338–7342.
16. **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 HBcAg in serum. Hepatology 1:392–397.
17. **Katz, F., and H. Lodish.** 1979. Transmembrane biogenesis of the vesicular stomatitis virus glycoprotein. J. Cell Biol. **80**:416–426.
18. **Laub, O., L. B. Rall, M. Truett, Y. Shaul, D. N. Standring, P. Valenzuela, and W. J. Rutter.** 1983. Synthesis of hepatitis B surface antigen in mammalian cells: expression of the entire gene and the coding region. J. Virol. **48**:271–280.
19. **Lee, D., T. Rose, N. Webb, and G. Todaro.** 1985. Cloning and sequence analysis of a cDNA for rat transforming growth factor-alpha. Nature (London) **313**:489–491.
20. **Lingappa, V., J. Chaidez, C. S. Yost, and J. Hedgepeth.** 1984. Determinants for protein localization: beta-lactamase signal sequence directs globin across microsomal membranes. Proc. Natl. Acad. Sci. USA **81**:456–460.
21. **Lingappa, V., D. Shields, S. Woo, and G. Blobel.** 1978. Nascent chicken ovalbumin contains the functional equivalent of a signal sequence. J. Cell Biol. **79**:567–572.
22. **Liu, C. C., D. Yansura, and A. Levinson.** 1982. Direct expression of hepatitis B surface antigen in monkey cells from an SV40 vector. DNA **1**:213–221.
23. **Machida, A., S. Kishimoto, H. Ohumura, H. Miyamoto, K. Baba, K. Oda, T. Nakamura, and Y. Miyakawa.** 1983. A hepatitis B surface antigen polypeptide (p31) with the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology **85**:268–274.
24. **Maroux, S., and D. Louvard.** 1976. On the hydrophobic part of aminopeptidases and maltases which bind the enzyme to the intestinal brush border membrane. Biochim. Biophys. Acta **419**:189–195.
25. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
26. **McClelland, A., L. Kuhn, and F. Ruddle.** 1984. The human transferrin receptor gene: genomic organization and the complete primary structure of the receptor deduced from a cDNA sequence. Cell **39**:267–274.
27. **Melton, D., P. Krieg, M. Rebogliati, T. Maniatis, K. Zinn, and M. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
28. **Mostov, K., and G. Blobel.** 1982. A transmembrane precursor of secretory component, the receptor for transcellular transport of polymeric immunoglobulins. J. Biol. Chem. **257**:11816–11821.
29. **Paronetto, F., and H. Popper.** 1974. Electron microscopy and immunoelectronmicroscopy of cytoplasmic hepatitis B antigen in hepatocytes. Am. J. Pathol. **75**:489–502.
30. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. Mackay, G. Leadbetter, and K. Murray.** 1978. Hepatitis B virus genes and their expression in *E. coli*. Nature (London) **282**:575–579.
31. **Patzer, E. J., G. R. Nakamura, and A. Yaffe.** 1984. Intracellular transport and secretion of hepatitis B surface antigen in mammalian cells. J. Virol. **51**:346–353.
32. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1985. A frameshift mutation in their pre-S region of the human hepatitis B virus genome allows production of surface antigen particles but eliminates binding to polymerized albumin. Proc. Natl. Acad. Sci. USA **82**:3440–3444.
33. **Peterson, D.** 1981. Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J. Biol. Chem. **256**:6975–6983.
34. **Peterson, D., I. Roberts, and G. Vyas.** 1977. Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **74**:1530–1534.
35. **Robinson, W. S., and L. I. Lutwick.** 1976. The virus of hepatitis type B. N. Engl. J. Med. **295**:1168–1175, 1232–1236.
36. **Rothman, J., F. Katz, and H. Lodish.** 1978. Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. Cell **15**:1447–1454.
37. **Russel, M., and P. Model.** 1982. Filamentous phage pre-coat is an integral membrane protein: analysis by a new method of membrane preparation. Cell **28**:177–184.
38. **Sabatini, D., G. Kribech, T. Morimoto, and M. Adesnick.** 1982. Mechanisms for the incorporation of proteins into membranes and organelles. J. Cell Biol. **92**:1–22.
39. **Schaefer, E., and R. Levy.** 1985. Pathogenesis and management of lipoprotein disorders. N. Engl. J. Med. **312**:1300–1310.
40. **Stibbe, W., and W. H. Gerlich.** 1983. Structural relationships between minor and major proteins of hepatitis B surface antigen. J. Virol. **46**:626–628.
41. **Tarentino, A., and F. Maley.** 1974. Purification and properties of an endobeta-*N*-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–817.
42. **Valenzuela, P., M. Quiroga, J. Zaldivar, P. Gray, and W. J. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. ICN-UCLA Symp. Mol. Cell. Biol. **18**:57–70.
43. **Walter, P., and G. Blobel.** 1983. Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol. **96**:84–93.
44. **Walter, P., R. Gilmore, and G. Blobel.** 1984. Protein translocation across the endoplasmic reticulum. Cell **38**:5–8.
45. **Webster, R., and J. Cashman.** 1978. Morphogenesis of the filamentous single-stranded DNA phages. *In* D. Denhardt, D. Dressler, and D. Ray (ed.), The single-stranded DNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.