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Multiple Topogenic Sequences Determine the Transmembrane Orientation of Hepatitis B Surface Antigen

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To investigate the mechanisms by which complex membrane proteins achieve their correct transmembrane orientation, we examined in detail the hepatitis B surface antigen for sequences which determine its membrane topology. The results demonstrated the presence of at least two kinds of topogenic elements: an N-terminal uncleaved signal sequence and an internal element containing both signal and stop-transfer functions. Fusion of reporter groups to either end of the protein suggested that both termini are translocated across the membrane bilayer. We propose that this topology is generated by the conjoint action of both elements and involves a specifically oriented membrane insertion event mediated by the internal sequence. The functional properties of each element can be instructively compared with those of simpler membrane proteins and may provide insight into the generation of other complex protein topologies.

Both eucaryotic cells and their subcellular organelles are surrounded by membranes whose lipid bilayers are traversed by a variety of membrane proteins. The proper functioning of the cell requires that each such protein assumes its correct transmembrane orientation. For membrane proteins derived from the rough endoplasmic reticulum (ER), this process begins during translation by interaction of specific protein signal sequences with a signal recognition particle (SRP). The interaction of this complex with the SRP receptor on the ER membrane is followed by the translocation of protein domains across the bilayer (19, 37–39). Events following this step, however, are still poorly understood. Membrane proteins are known to contain hydrophobic "stop-transfer" sequences which terminate the translocation process and may then span the lipid bilayer; further polypeptide synthesis would then occur in the cytoplasmic compartment (7, 26, 40). For polytopic membrane proteins—those which span the bilayer multiple times—it has been proposed that combinations of multiple signal and stop-transfer elements are responsible for the generation of the final topology of the chain (2, 6, 18).

As a model system for the study of membrane protein biogenesis we have used the hepatitis B surface antigen (HBsAg), the principal coat protein of hepatitis B virus (24, 32, 33). The 24-kilodalton (kDa) major surface antigen protein, the product of the viral *S* gene, is of particular interest for several reasons. In addition to being a structural component of the virion, HBsAg (or $p24^s$) is also independently secreted by infected cells as discrete 20-nm subviral particles. These particles are composed primarily of $p24^s$, its glycosylated derivative ($gp27^s$), and host-derived lipid, which appears not to be retained in a morphologically recognizable unit membrane structure. Although HBsAg is ultimately secreted from the cell, we have recently shown that the initial product of synthesis is a transmembrane polypeptide (5; K. Simon, V. Lingappa, and D. Ganem, manuscript in preparation). This form is the earliest detectable intermediate in the pathway of particle assembly, which is envisioned to occur via aggregation and delivery of transmembrane $p24^s$ into the ER lumen (5, 20). The membrane translocation process does not involve cleavage of N-terminal amino acids, and both translocation and export of the protein can proceed in the absence of any other viral gene products (13, 16, 23). Little is known about the nature and location of the sequences which direct the critical initial step of $p24^s$ insertion into the ER membrane. However, our earlier studies of globin-HBsAg fusion proteins constructed in vitro indicated that the molecule contains at least one signal sequence which can function when situated internally in the protein: fusions in which $p24^s$ sequences are either preceded or followed by 100 amino acids of alpha-globin assume a transmembrane configuration (5).

In this study we used a combined genetic and biochemical approach to map and characterize the topogenic signals of HBsAg. Our results revealed the presence of least two distinct kinds of topogenic elements and suggested a model for the orientation of the transmembrane protein resulting from their action.

MATERIALS AND METHODS

Construction of recombinant plasmids containing fused alpha-globin and HBsAg sequences. Procedures used to construct the fusion proteins shown in Fig. 1 have been previously described in detail (5). Cleavage of parent plasmid pSPglo24 (5) with restriction endonuclease *Bal* 1 (D series) or *Xba*I (X series) within the N-terminal region of $p24^s$, followed by treatment with *Bal* 31 exonuclease, cleavage with *Bst*EII within globin to remove intergenic sequences, and religation with T4 ligase was used to construct fusions with fused 5′ globin sequences. Glycoglobin fusion B8* was constructed from B8 by exchanging a homologous 0.33-kilobase *Hin*dIII fragment derived from pSPSG3 (21), containing a 9-amino acid synthetic N-linked glycosylation site inserted at the *Bss*HII site of (144-residue) chimpanzee alpha-globin.

Type K fusions, containing fused 3′ globin sequences, were constructed by cleavage of pSP24glo (5) with *Nco*I at the 5′ end of the globin gene, brief *Bal* 31 exonuclease treatment, and deletion of the intergenic region by cleavage with *Acc*I at the C-terminal end of $p24^s$. A derivative of pSP24glo was used as the parent plasmid to construct C1* and E9* fusions containing C-terminal glycoglobin se-

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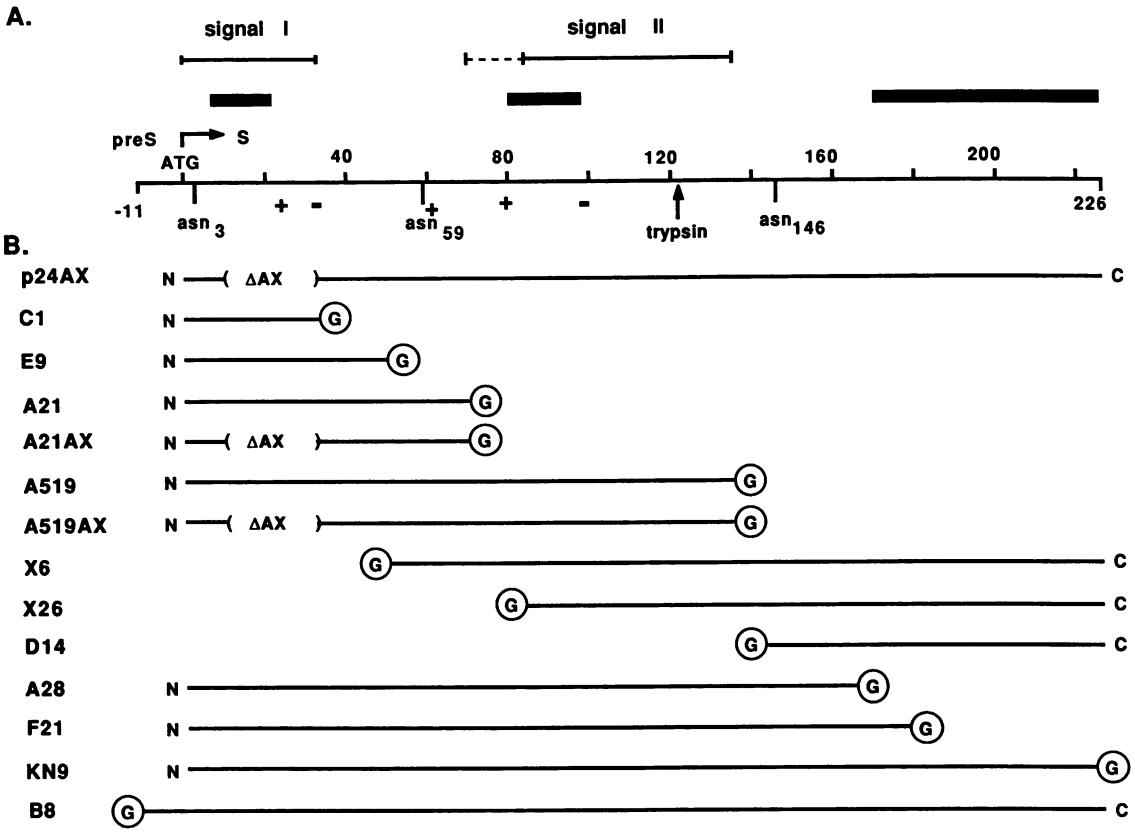

FIG. 1. Structural features of p24^s and maps of fusion proteins and AX deletion mutants. (A) A diagram of the HBsAg amino acid sequence from residues −11 (in the pre-*S* region), through the start of the p24^s sequence (ATG) at Met-1, to the end of the surface antigen (*S*) gene of human hepatitis B virus (32), Ile-226. Also indicated are potential N-linked glycosylation sites at the indicated asparagine residues and the characterized trypsin cleavage site at residue 122. The locations of charged residues 24, 33, and 73 and also of His-60, which are conserved throughout the hepadnavirus family (28, 30, 31), are shown (+ or −) under the diagram. The approximate locations of conserved hydrophobic regions (solid bars) and HBsAg topogenic domains, signal I or signal II (see text), are indicated above the diagram. (B) Regions of HBsAg sequences within the indicated fusion proteins containing either N- or C-terminally fused alpha-globin domains (indicated by G) are shown relative to the diagram above, as is the AX deletion mutant of p24^s (top). AX deletion mutants lacked the indicated region, residues 10 to 32, enclosed in parenthesis.

quences. This parent plasmid contains a 0.1-kilobase (*Ava*I to *Bst*XI) segment containing the Asn-Gly-Ser glycosylation site of rat lactalbumin (41 residues) inserted in the *Bst*EII site of globin. The parent plasmid was digested with *Nco*I, followed by brief *Bal*31 treatment and cleavage by *Xba*I (C series) or *Bal*1 (E series) within p24^s to remove intergenic sequences before religation. The glycoglobin glycosylation site of E9* was removed by cleavage at nearby flanking *Bst*EII sites to form E9. The p24-glycoglobin parent plasmid was digested with *Acc*I before *Bal* 31 treatment and joined to the *Bss*HII site of globin in a similar manner to construct F-series fusions.

Plasmid constructions for the expression of p24^s and its AX derivative employed plasmid pSP24H (5). Deletion mutants designated AX were constructed by digestion with *Avr*II and *Xba*I and religation. The fusion junctions of all constructs shown in Fig. 1 were defined by DNA sequence analysis by the dideoxy method of Sanger et al. (27).

Membrane translocation assays. Transcription of recombinant SP6 plasmids, programming of wheat germ cell extracts with in vitro transcripts in the presence of canine pancreatic membranes, and analysis of [^{35}S]methionine-labeled translation products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography were performed as previously described (5). Posttranslational enzymatic treatments and radioimmunoprecipitation were also conducted as before (5), except endoglycosidase H (endo H) treatment after proteolytic digestion (0.1 to 0.6 mg of proteinase K per ml at 25°C for 1 h) was performed by dilution of 8 μl of SDS-treated proteolytic digest into 200 μl of 0.1 M sodium citrate (pH 5.5)–0.1% SDS, followed by incubation with 2 μl of enzyme (1 U/μl; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 37°C overnight. The mixture was then precipitated by the addition of 50 μl of 99% trichloroacetic acid–20 mM KCl for 20 min at 0°C, followed by 5 min of centrifugation (10,000 × *g*), washing, and resuspension in SDS loading buffer as described before (5). Tests for membrane cosedimentation were performed by the procedure of Perara and Lingappa (22). When glycosylation was not observed (i.e., fusion D14), membrane sedimentation behavior was judged as compared with a control experiment in which membranes were added posttranslationally.

Tests for posttranslational membrane translocation were performed either by late membrane addition as previously described (5) or (fusions A519AX, E9, B8*, and D14) by the use of chemical inhibitors of translation initiation and elongation. After 75 min of incubation at 25°C, reaction mixtures were brought to 10^{-4} M aurintricarboxylic acid, incubated

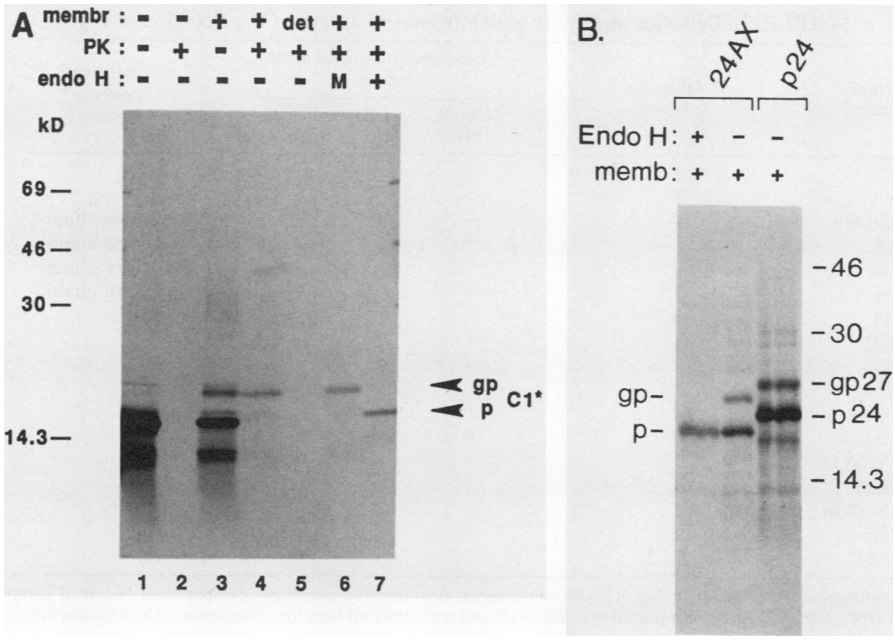

FIG. 2. HBsAg contains at least two signal sequences. (A) The N-terminal region contains a signal sequence. In vitro transcripts of fusion plasmid C1* were used to program a cell-free translation system. Translations were performed either in the absence (lanes 1 and 2) or in the presence (lanes 3 to 7) of microsomal membrane vesicles (membr). After translation, radiolabeled products were either exposed (lanes 2 and 4 to 7) or not exposed (lanes 1 and 3) to proteinase K (PK) digestion before electrophoresis through SDS-polyacrylamide gels. Vesicles in lanes 5 were disrupted by a nonionic detergent (1% Nikkol) before proteolysis (det). Protease-resistant products were either treated (lane 7) or mock incubated (lane 6) with endo H before electrophoresis and autoradiography. The positions of the full-size glycosylated (gp) and unglycosylated (p) translation products are indicated. (B) Deletion of signal I reveals additional signals. In vitro transcripts from p24 (right lane) and p24AX (left and center lanes) were used to program wheat germ translation extracts supplemented with microsomal membranes, and the products were displayed by SDS-polyacrylamide gel electrophoresis before (center and right lanes) or after (left lane) endo H digestion. p and gp denote positions of the unglycosylated and glycosylated chains, respectively, of the 24AX protein. Numbers on right are in kilodaltons.

for an additional 15 min, and then brought to 10^{-4} M in emetine, at which time membranes were added and incubation continued for a further 90 min before analysis.

RESULTS

Experimental strategy. The amino acid sequence of $p24^s$ reveals several relevant features of the molecule which are schematically depicted in Fig. 1A. The molecule contains three major hydrophobic domains, indicated by the black bars above the diagram: one N terminal (residues 7 to 22), one central (residues 80 to 98), and one C terminal (residues 170 to 226). Three potential sites for N-linked glycosylation exist (asparagines at positions 3, 59, and 146), but only one of these (Asn-146) is glycosylated in vivo (25). Similarly, although the molecule contains numerous potential tryptic cleavage sites, only one (Lys-122) is recognized by the enzyme (24). These features provide useful landmarks with which to recognize their respective regions of the protein.

To localize the regions of the molecule containing topogenic information, we constructed the fusion proteins shown in Fig. 1B. In this approach, regions of the $p24^s$ molecule to be tested were fused to a reporter group, the nonsecreted protein alpha-globin (denoted by G in Fig. 1); our previous studies have established that globin chains are not translocated across membranes unless linked to topogenically active (signal) sequences (3, 14). Recombinant SP6 plasmids containing these fusions were transcribed in vitro, and the resulting mRNAs were used to program an in vitro translation system containing translocation-competent microsomal membrane vesicles. Membrane translocation of the radiolabeled products was assessed (i) by glycosylation of the product, as judged by gel electrophoresis and endo H digestion; since enzymes for N-linked carbohydrate addition are only found in ER vesicles, only translocated protein domains can be glycosylated; (ii) by cosedimentation of the product with the membrane vesicle fraction; or (iii) by protection of translocated domains from attack by exogenous proteases. In selected cases (designated by asterisks in Table 1) a sequence containing a glycosylation signal was introduced into the globin-coding region to facilitate the detection of translocation of this reporter group. (This modified reporter group is referred to as glycoglobin.)

We note that in contrast to the in vivo situation, in which less than 50% of translocated $p24^s$ chains are glycosylated, our previous results (5) indicate that translocated chains are efficiently glycosylated in vitro (although the overall efficiency of chain translocation may vary). Thus, unglycosylated $p24^s$ is not protected from trypsin treatment, while $gp27^s$ is fully protected from cleavage with this enzyme. Therefore, unglycosylated chains provide an internal control for the proteolysis and sedimentation assays just described.

Identification and mapping of an N-terminal signal sequence in HBsAg. Since many secretory and membrane proteins have N-terminal signal sequences (37–39), we first examined the translocation competence of fusion proteins containing various lengths of N-terminal $p24^s$ sequences fused to downstream globin domains (mutants C1, E9, and A21 in Fig. 1). The results of the analysis of mutant C1* are representative of this class of fusions and are shown in Fig. 2A; the data on the other fusions are summarized in Table 1. Fusion C1* contains the first 32 amino acids of $p24^s$ linked to gly-

TABLE 1. Behavior of HBsAg-globin fusions in assays of translocation in vitro

Mutant[a]	Fusion junction[b]	Glycosylation[c]	Cosedimentation with membranes		Proteinase K-protected species[d]	Phenotype[e]
			Neutral pH	Alkaline pH		
$p24^s$	None	1	+	+	R_0	Transmembrane
$p24^s$AX	None	1			R_0	Transmembrane
C1*	S32/Glu/G9	1	+	−	Entire chain	Secretory
E9*	S50/G6	1	+	−	Entire chain	Secretory
E9	S50/G6	0			Entire chain	Secretory
A21	S70/G4	1	+	−	Entire chain	Secretory
A21AX	S70/G4	0			None	Nontranslocated
A519	S135/G1	1			R_2	Transmembrane
A519AX	S135/G1	1	+	+	R_2	Transmembrane
X6	G109/S51	1			R_0	Transmembrane
X26	G109/S84	1			R_0	Transmembrane
D14	G108/S143	0	−	−	None	Nontranslocated
A28	S165/Val/G2	1	+	+	R_2	Transmembrane
F21*	S178/G20	1, 2			R_2	Transmembrane
KN9	S224/Ser/G6	1			R_0, R_2	Transmembrane
B8	S224/G6	1			R_0	Transmembrane
B8*	G97/S-11	1, 2			R_0	Transmembrane

[a] Mutants listed are diagrammed schematically in Fig. 1, with the asterisk denoting the presence of a potential N-linked glycosylation site within globin sequences. Some results for $p24^s$, A28, and B8 were reported previously (5) and are tabulated here for comparison. AX indicates deletion of the *Avr*II-*Xba*I region of $p24^s$ spanning codons 10 to 32.

[b] Fusion junctions are indicated as follows. S denotes $p24^s$, G denotes globin, numbers denote amino acid residue of each parental species present at fusion junction (denoted by slash). Thus, S32/G9 indicates that residue 32 of $p24^s$ is fused to codon 9 of globin. Residues listed between slashes correspond to codons created during recombination.

[c] 1, Singly, and 2, doubly glycosylated chains; 0, no glycosylation, as judged by the ca. 3 kDa per glycosylation molecular weight shift after endo H digestion.

[d] The anti-HBsAg immunoreactive R_0 fragments comigrate with the 18-kDa product of proteinase K digestion of translocated wild-type $p24^s$. The designation R_2 denotes a class of protected fragments of various sizes containing both $p24^s$- and globin-immunoreactive determinants in constructs bearing globin fused to C-terminal regions of $p24^s$ (see text). Glycosylation of R_0 and R_2 fragments was verified by treatment with endo H (except A519 and A519AX, in which Asn-146 is absent).

[e] A summary assessment of the phenotype of each mutant is indicated. Secretory denotes full protease protection after proteolysis, with translocated chains within vesicles at neutral pH but released from vesicles by alkali. Transmembrane denotes the presence of only partial protease resistance after translocation, with the translocated product remaining associated with the membrane even at alkaline pH.

coglobin. Translation of C1* RNA in vitro in the absence of membranes resulted in a ca. 16-kDa product (designated p in Fig. 2A, lane 1); a smaller product of ca. 12 kDa possibly resulting from internal translational initiation on this message was also produced. In the absence of microsomes, the C1* protein was fully sensitive to exogenous proteinase K (Fig. 2A, lane 2). When membranes were present during the translation, however, an additional species (designated gp in Fig. 2A) was seen (lane 3). This species was preferentially protected from proteinase K digestion (lane 4), unless the vesicle membranes were first disrupted with nonionic detergent (lane 5), suggesting that the entire polypeptide was translocated into the vesicle lumen. The mobility shift of this species is due to its glycosylation by lumenal enzymes. When the protected species (gp) was treated with endo H, it was quantitatively converted back to the unglycosylated form (lane 7); both the glycosylated and unglycosylated species exhibited the expected antiglobin immunoreactivity (data not shown). (Fusion proteins containing only N-terminal $p24^s$ sequences were not immunoreactive with antisera to native HBsAg particles.)

Identical behavior was observed for the larger fusion proteins E9* and A21 (Table 1). In the fusion A21, which contains one potential N-linked glycosylation site at Asn-59 within $p24^s$ and none within globin, we again observed glycosylation (Table 1), confirming directly the translocation of N-terminal $p24^s$ domains (Asn-59 is not normally glycosylated in wild-type $p24^s$; this finding will be considered further in the Discussion). Since the endpoint of the N-terminal $p24^s$ sequences of fusion C1* is at residue 32, we conclude from the above data that an N-terminal uncleaved signal (hereafter referred to as signal I) directs the translocation of N-terminal regions of $p24^s$ as well as of fused C-terminal globin domains. When residues 10 through 32 were removed from fusion A21 (which extends to residue 70) by deleting sequences between the *Avr*II and *Xba*I sites of the *S* gene, this in-phase AX deletion resulted in a loss of signal function, as no glycosylation or protease protection of fusion A21AX was observed (Table 1). This result confirms the location of a signal sequence in the first 32 amino acid residues of $p24^s$, the first 9 of which are not sufficient to maintain signal function.

The protease protection experiments of Fig. 2A suggested that fusions containing signal I have a secretory phenotype; i.e., they are translocated entirely into the vesicle lumen. To confirm this, we examined the translocated glycoproteins for cosedimentation with membrane vesicles. Figure 3A shows representative data for the signal I-containing fusion E9*. Under neutral pH conditions, the translocated product cosedimented with the membranes (lane 2). When the vesicles were opened by alkaline carbonate treatment, the translocated species were released into the supernatant (lanes 3 and 4); only a small amount remained with the membrane pellet, presumably owing to nonspecific sticking of the hydrophobic N terminus to the membranes. Thus, the E9* fusion protein appears to be fully translocated into the vesicle lumen. As summarized in Table 1, secretory behavior was also observed for fusions C1* and A21, which leads us to conclude that no stop-transfer function exists in the entire N-terminal region of the molecule (i.e., to the A21 endpoint at residue 70).

HBsAg contains a second, internal signal. To determine

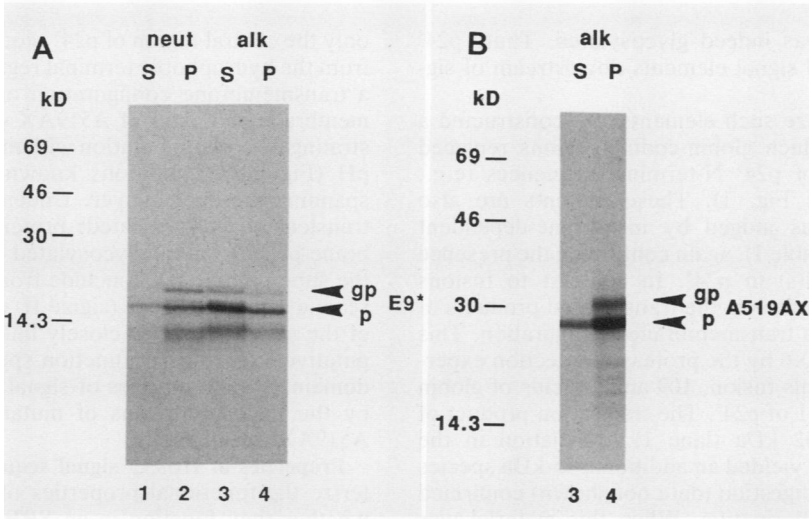

FIG. 3. Membrane sedimentation behavior of HBsAG-globin fusion proteins exhibiting secretory or transmembrane characteristics. In vitro transcripts of fusion plasmids E9* (A) or A519AX (B) were translated in the presence of microsomal membranes. Translation products were diluted 250-fold into either isotonic sucrose buffer (neut; lanes 1 and 2) or 0.1 M sodium carbonate, pH 11.5 (alk; lanes 3 and 4). After sedimentation at 250,000 × g, radiolabeled products derived from either the neutralized supernatant solution (S; lanes 1 and 3) or the neutralized, resuspended membrane pellets (P; lanes 2 and 4) were immunoprecipitated with antiglobin antiserum before electrophoresis. The positions of the glycosylated (gp) and unglycosylated (p) full-length fusion proteins are indicated. The behavior of secretory fusion E9* resembled that of bovine prolactin, a control secretory protein (not shown). kD, kilodaltons.

whether p24^s contains additional topogenic information outside of signal I, we deleted this sequence from the wild-type molecule (mutant p24AX) and examined the ability of the resulting species to be translocated in vitro. As expected, translation of p24AX in the absence of membranes generated a species ca. 2 kDa smaller than p24^s (data not shown), consistent with the size of the deletion. When membranes were present (Fig. 2B), an additional band with the mobility predicted for the glycosylated form of the molecule was present; treatment of this preparation with endo H confirmed

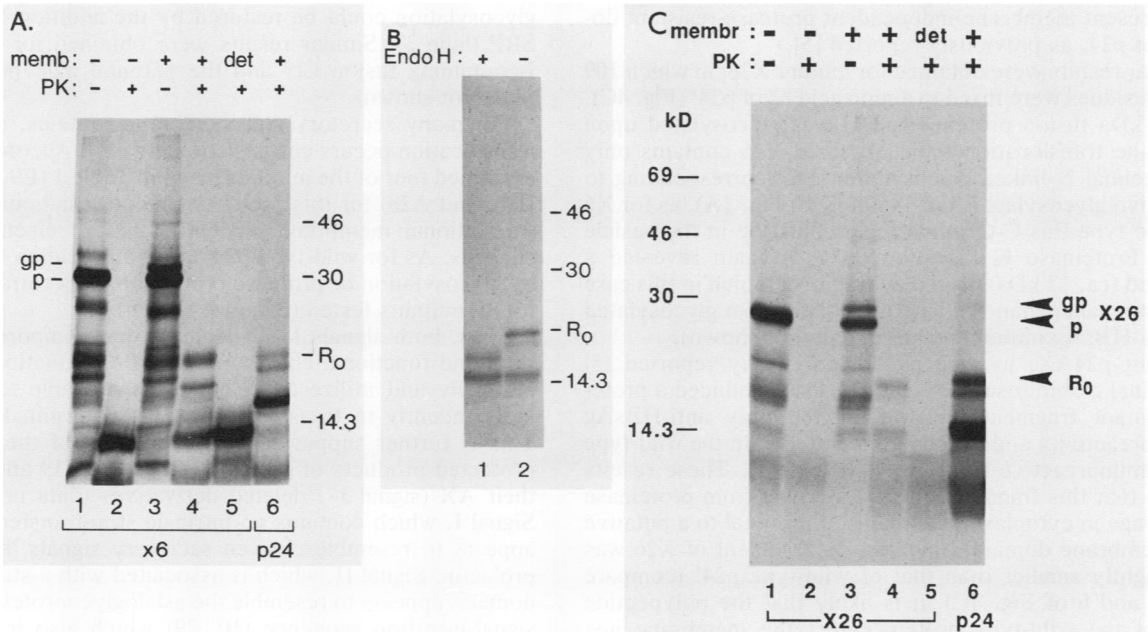

FIG. 4. Globin-p24^s fusions lacking signal I are transmembrane proteins. (A and C) In vitro transcripts of fusion X6 (A) or X26 (C) were used to program cell-free translation systems. Translations were performed in either the absence (lanes 1 and 2) or presence (lanes 3 to 5) of microsomal membrane vesicles (membr). After translation, the products were displayed by SDS-polyacrylamide gel electrophoresis either before (lanes 1 and 3) or after (lanes 2, 4, and 5) proteinase K (PK) digestion. In lanes 6, the pattern of proteinase K-resistant products of digestion of translocated p24^s (wild type) is displayed for comparison. R_0 denotes the position of the protease-protected (translocated) domain of gp27^s. kD, kilodaltons. (B) The protease-protected R_0 fragment is glycosylated. p24^s transcripts were translated in the presence of membranes; the translocated products were treated with proteinase K and then analyzed by SDS-polyacrylamide gel electrophoresis before (lane 2) or after (lane 1) endo H digestion. Identical results were obtained for the R_0 fragment derived from fusion X6 (not shown). Numbers on right side of panels A and B are in kilodaltons.

that the upper band was indeed glycosylated. Thus, $p24^s$ must contain additional signal elements downstream of signal I.

To further characterize such elements, we constructed a family of fusions in which globin-coding regions replaced progressive deletions of $p24^s$ N-terminal sequences (e.g., mutants X6 and X26; Fig. 1). These mutants are also translocated in vitro as judged by membrane-dependent glycosylation (Fig. 4; Table 1), again confirming the presence of an additional signal(s) in $p24^s$. In contrast to fusions bearing only signal I, however, the translocated products of these mutants were in a transmembrane configuration. This is indicated for mutant X6 by the protease protection experiments of Fig. 4A. In this fusion, 109 amino acids of globin were fused to residue 51 of $p24^s$. The translation product of this mutant was ca. 32 kDa (lane 1); translation in the presence of membranes yielded an additional 35-kDa species (lane 3) which endo H digestion (data not shown) confirmed to be its glycosylated derivative. When this material was exposed to proteinase K (lane 4), no full-length species were protected. Rather, the major protected fragment was ca. 18 kDa and exactly comigrated with the fragment (R_0) generated by similar proteolysis of translocated wild-type $p24^s$ (Fig. 4A, lane 6) (5). As expected, this fragment was reactive with anti-$p24^s$ antibody, but not with antiglobin (Fig. 4B; data not shown), indicating that it derived from the C-terminal portion of the molecule. Thus, X6 is a transmembrane glycoprotein; the identical portion of the $p24^s$ sequence was protected in both X6 and the wild type. To confirm that this protected species is indeed translocated, we showed the R_0 fragment to be fully sensitive to endo H treatment (Fig. 4B). (The lower-molecular-weight resistant species in proteinase K digests were poorly characterized but represent membrane-independent protease-resistant domains of p24, as previously reported [5].)

Similar results were obtained for mutant X26, in which 109 globin residues were fused to amino acid 84 of $p24^s$ (Fig. 4C). The 29-kDa fusion protein (lane 1) was glycosylated upon membrane translocation (lane 3); since X26 contains only one potential N-linked glycosylation site (corresponding to the in vivo glycosylated Asn-146 of $p24^s$; Fig. 1A), as for X6 and wild type this C-terminal region must be in the vesicle lumen. Proteinase K digestion (lane 4) again revealed a truncated (ca. 17 kDa) protected fragment which in this case was just smaller than R_0 (lane 6) and was also glycosylated and anti-HBsAg immunoreactive (data not shown).

Mutant p24AX, as well as the previously reported (5) N-terminal globin fusion B8 (Fig. 1), also produced a protease-resistant fragment which exhibited only anti-HBsAg immunoreactivity and exactly comigrated with the wild-type $p24^s$-immunoreactive fragment R_0 (Table 1). These results confirm that this fragment must be derived from proteinase K cleavage in cytoplasmic domains N terminal to a putative transmembrane domain. Since the R_0 fragment of X26 was only slightly smaller than that of wild-type $p24^s$ (compare lanes 4 and 6 of Fig. 4C), it is likely that the polypeptide chain of the wild-type protein enters the membrane just upstream of the X26 endpoint, which maps at residue 84, near the beginning of the internal hydrophobic domain (residues 80 to 98) of $p24^s$.

To further localize the second $p24^s$ signal, we constructed a fusion protein containing deletions of both N- and C-terminal hydrophobic regions, but retaining the central hydrophobic domain (mutant A519AX; Fig. 1). As summarized in Table 1, A519AX was found to be glycosylated (see also Fig. 5B) and partially protease protected, indicating that only the central region of $p24^s$ sequences, without assistance from the hydrophobic terminal regions, is sufficient to confer a transmembrane configuration on the protein. The transmembrane character of A519AX was confirmed by demonstrating its cosedimentation with membranes even at alkaline pH (Fig. 3B), conditions known to remove proteins not spanning the lipid bilayer. Under these conditions, all the translocated (glycosylated) protein remained in the membrane pellet; only unglycosylated chains were found free in the supernatant. We conclude from the above results that a second signal sequence (signal II) exists in the central region of the molecule and is closely linked or coextensive with a putative stop-transfer function specifying a transmembrane domain. The boundaries of signal II are defined genetically by the fusion junctions of mutants X26 (residue 84) and A519AX (residue 136).

Properties of HBsAg signal sequences. To further characterize the functional properties of these signals, we asked whether they function in an SRP-mediated fashion, analogous to previously characterized eucaryotic signal sequences (1, 37, 38). As an initial test of this, we asked whether the translocation of HBsAg could be arrested by SRP in the absence of microsomal membranes. Such translational arrest of $p24^s$, relative to a control (globin) present in the same reaction, was indeed observed in the absence of membranes (Fig. 5A, lane 2); no arrest was observed in the presence of intact membranes containing SRP receptor (lane 3). Next, both fusion Cl* and A519AX, containing signal I or II, respectively, and wild-type $p24^s$ were tested for the stimulation of translocation by SRP. Representative data for A519AX are shown in Fig. 5B. When translation was done in the presence of salt-washed membranes, which contain no SRP, no glycosylation was observed (lane 2); however, glycosylation could be restored by the addition of purified SRP (lane 3). Similar results were obtained for the signal I-containing fusion Cl* and the parental $p24^s$ polypeptide (data not shown).

For many secretory and membrane proteins, membrane translocation occurs cotranslationally (21). Accordingly, we examined four of the mutants listed in Table 1 (E9, A519AX, B8*, and A28) for this property by cotranslational or posttranslational membrane incubation before electrophoretic analysis. As for wild-type $p24^s$ (5), translocation (as judged by glycosylation or protease protection) was cotranslational for all mutants tested (data not shown).

Thus, both signals I and II share many important structural and functional characteristics. Both function cotranslationally and utilize SRP, and both are able to function independently to translocate flanking C-terminal domains. This is further supported by the identity of the protease-protected products of parental $p24^s$ and A519 and those of their AX (signal I^-) deleted derivatives (data not shown). Signal I, which contains no intrinsic stop-transfer function, appears to resemble cleaved secretory signals like that of prolactin. Signal II, which is associated with a stop-transfer domain, appears to resemble the asialoglycoprotein receptor signal-insertion sequence (10, 29) which also translocates C-terminal-flanking domains.

Translocation of both N- and C-terminal domains of $p24^s$. The presence of an N-terminal secretory signal led us to ask whether the extreme N-terminus of $p24^s$ was itself translocated. To do this, we attached a reporter group to this region of $p24^s$ by constructing fusion B8* (Fig. 1), which contains glycoglobin fused just upstream of the N terminus of $p24^s$ (in the pre-S region). This molecule therefore contained reporter glycosylation sites in both its N-terminal (globin) and

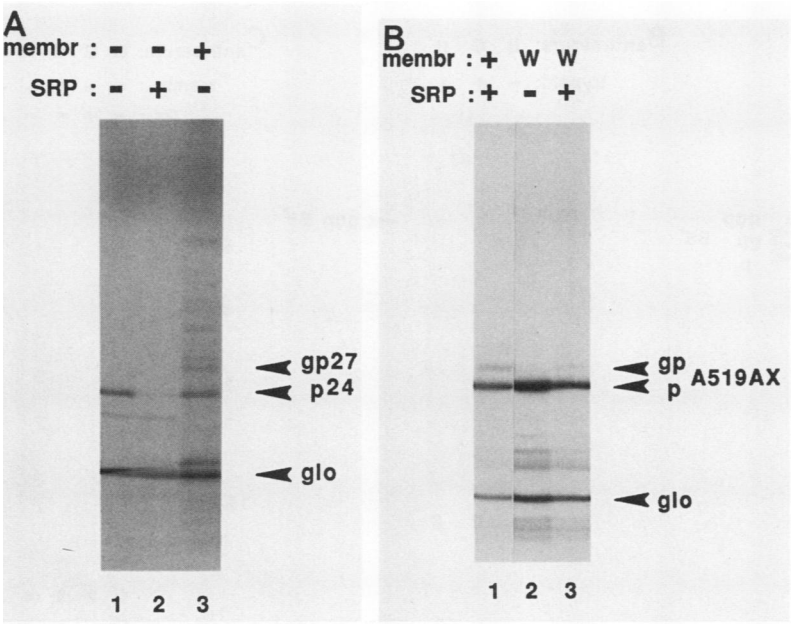

FIG. 5. Interaction of HBsAg signal sequences with SRP. Plasmids containing the wild-type p24^s gene (A) or fusion protein A519AX (B) were transcribed and translated in vitro. In addition, wild-type alpha-globin transcripts were present in these translation reactions, and the position of the alpha-globin translation product is indicated (glo). (A) Elongation arrest of p24^s translation by exogenous addition of SRP. Translations were done in the absence (lanes 1 and 2) or presence (lane 3) of microsomal membranes (membr). The reaction mixture in lane 2 contained, in addition, 10 nM SRP. A similar reaction containing both salt-washed membranes (36) and SRP resembled lane 3 (data not shown). (B) Dependence of fusion A519AX glycosylation on SRP. Translations were done in the presence of whole microsomal membranes (membr; lane 1) or an equivalent amount of salt-washed membranes (W), with (lane 3) or without (lane 2) added 10 nM SRP. The residual amount of glycosylation exhibited by salt-washed membranes in the absence of SRP is comparable to background levels observed for the processing of bovine preprolactin (not shown).

C-terminal (p24^s) domains. This mutant was translocated in vitro, and the products were examined before or after trypsin exposure. This enzyme, unlike proteinase K, was expected to cleave only within globin sequences, since the one p24^s cleavage site (Lys-122) should be protected after translocation into membrane vesicles, as previously shown for wild-type p24^s (5). Translocation of fusion B8* (Fig. 6A), resulted in the appearance of a doubly glycosylated species (ggp) (lane 3) not observed for B8 (5), suggesting that glycosylation of the site within globin had occurred. In addition, when translocated B8* was digested with trypsin (Fig. 6B), the doubly glycosylated species, exhibiting both antiglobin and anti-HBsAg immunoreactivity (lanes 2 and 3), was preferentially protected from proteolytic cleavage, confirming that the glycosylated N-terminal globin domains, as well as C-terminal HBsAg sequences near the trypsin site at Lys-122, were indeed translocated into membrane vesicles. As expected, singly or unglycosylated forms, which had not undergone simultaneous translocation of both regions, were not protected from trypsinolysis. Translocation of the N-terminal globin domain is relatively inefficient, which may account for our earlier inability to detect it in mutant B8 using less sensitive assays based on proteinase K protection (5); however, trypsin digestion experiments with this mutant (data not shown) produced results analogous to those depicted for B8*.

To determine the disposition of the C terminus of p24^s, we examined the translocation of a series of p24^s-globin fusions with globin (or glycoglobin) domains at the C terminus of the fusion (mutants A28, F21*, and KN9 in Fig. 1). All these mutants were glycosylated in the presence of membranes (at a p24 glycosylation site) and exhibited after proteinase K proteolysis a class of membrane-protected C-terminal fragments, collectively referred to as R_2 in Table 1, which were glycosylated and were both anti-HBsAG and antiglobin immunoreactive (Table 1) (5). These fragments were analogous to the R_0 fragment of wild-type p24^s, being derived by proteolytic cleavage in cytoplasmic HBsAg domains N terminal to signal II. The results for fusion KN9, which contained all but the two most C-terminal residues of p24^s, are shown in Fig. 6C. The presence of dually immunoreactive fragment R_2 (lanes 5 and 6) indicated that the extreme C terminus of p24^s (with its attached globin domain) was translocated. The cotranslocation of the globin domains in such constructs was further affirmed by glycoglobin fusion F21* (Fig. 1; Table 1), which was found to be doubly glycosylated.

DISCUSSION

Existence of multiple topogenic signals within p24^s. These studies indicated that multiple topogenic elements exist within the p24^s molecule and that these signals differ in their functions and are experimentally separable. Signal I resides in a hydrophobic domain within the first 30 amino acids, contains no intrinsic stop-transfer activity, and is able to confer a secretory phenotype upon downstream domains. In these respects it resembles conventional signal sequences except for its inability to be cleaved by signal peptidase. Signal II, by contrast, resides internally in the sequence: by genetic analysis it must reside between the endpoints of mutants X26 (residue 84) and A519AX (residue 136). This region includes a highly conserved and extremely hydrophobic domain (residues 80 to 98; Fig. 1), is able to translocate downstream sequences, and confers a transmembrane orientation on the resulting product. Thus far we have been

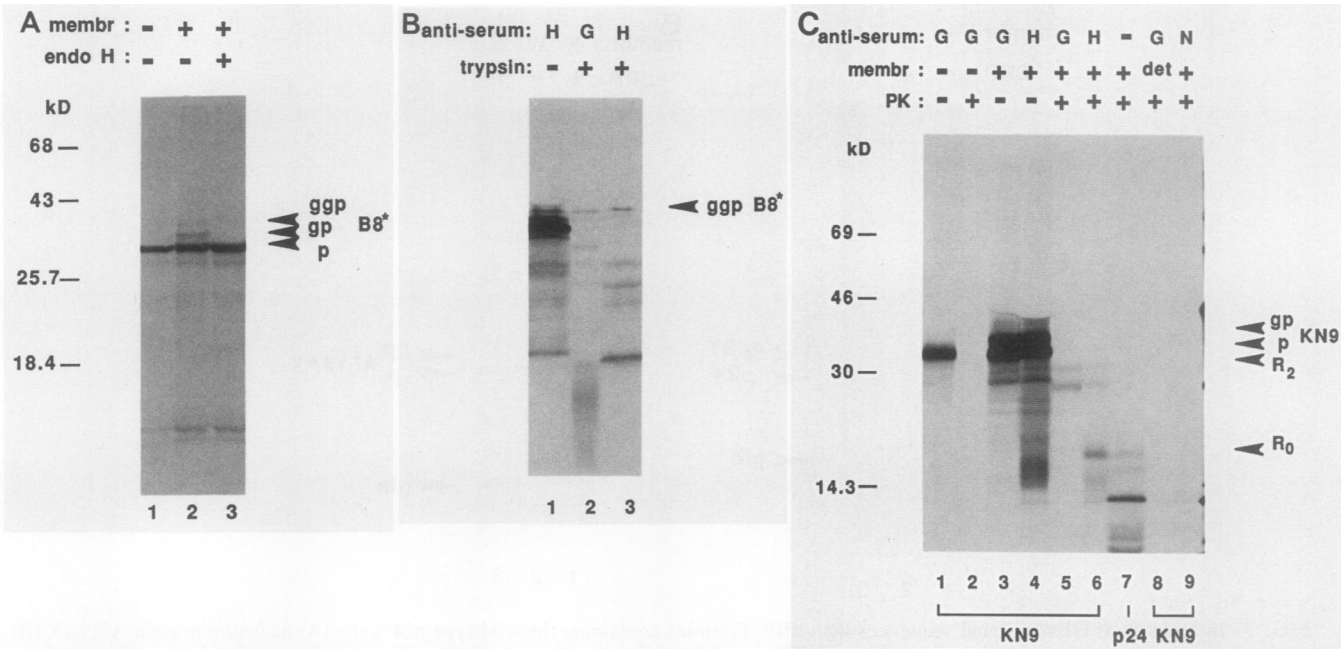

FIG. 6. Translocation of the terminal domains of HBsAg-globin fusions containing the complete p24^s gene. (A) Membrane translocation of fusion B8*. Translations of fusion B8* mRNA were done in the absence (lane 1) or presence (lanes 2 and 3) of microsomal membranes (membr), followed by treatment (lane 3) or mock incubation (lane 2) with endo H. The positions of the singly (gp) and doubly (ggp) glycosylated derivatives of the primary translation product (p) are indicated. The band marked ggp was not present in a control translation reaction containing membranes but not exogenously added mRNA (not shown). kD, kilodaltons. (B) Protease protection of translocated B8* proteins. Membrane-translocated translation products of fusion B8* were digested (lanes 2 and 3) or mock digested (lane 1) with 0.6 mg of trypsin (Boehringer) per ml for 1 h at 25°C in manner similar to that described in Table 1 for proteinase K. As for proteinase K digests (5), trypsinolysis reactions were terminated by transferring to boiling SDS and immunoprecipitated at 4°C with either antiglobin (G) serum (lane 2) or anti-HBsAg (H) serum (lanes 1 and 3) before electrophoresis. The band marked ggp was not present after proteolysis, if translations were done in the absence of membranes, or if membranes were added posttranslationally, or if nonionic detergent was used to disrupt membranes before digestion (not shown). (C) Translocation of C-terminal domains. Translation products of fusion KN9 made in the presence or absence of membranes (membr), as indicated above each lane, were either digested (lanes 2 and 5 to 9) or mock digested (lanes 1, 3, and 4) with proteinase K (PK) before immunoprecipitation with antiglobin (G) serum (lanes 1, 2, 3, 5, and 8), anti-HBsAG (H) serum (lanes 4 and 6), or nonimmune (N) serum (lane 9) before electrophoresis. For comparison, a similar digest (without immunoprecipitation) for wild-type p24^s is shown in lane 7. The positions of the primary translation product of the fusion protein (p), its glycosylated derivative (gp), and the membrane-protected fragment (R_2) are indicated, as well as the immunoreactive membrane-protected fragment (R_0) of wild-type p24^s. An additional membrane-protected band of slightly greater mobility than R_2, referred to as R_1 in a previous report (5), is not observed in all mutants of this series.

unable to separate the translocation signal from that required for membrane insertion; additional fusions with endpoints within this region will be required to determine whether these functions can be physically uncoupled. In most respects, signal II resembles the internal elements of so-called type II membrane proteins, such as the transferrin and asialoglycoprotein receptors (15, 29, 41), which similarly translocate C-terminal-flanking domains while conferring on the protein a transmembrane orientation (N-terminus cytoplasmic, C-terminus lumenal). That this is also the orientation conferred by signal II is indicated by the phenotype of numerous mutants. Transmembrane mutants with N-terminal deletions (p24AX) or substitutions (X6) still reveal the same proteinase K-protected fragment (R_0) as wild type (Table 1; Fig. 4), indicating that C-terminal domains are translocated; when regions immediately C terminal to signal II are marked with globin reporter groups (e.g., mutants A28, F21*) the reporter groups are also rendered protease resistant (5). Therefore, the (cytoplasmic) proteinase K cleavage site(s) must be N terminal to signal II, though its precise location has not been directly determined.

Both signal I and II contain several conserved structural features (Fig. 1), including a hydrophobic domain followed by specific charged residues, which are also found in the corresponding sequences of all the members of the hepadnavirus family (28, 30, 31). In fact, the regions spanning both signals overlap regions of generally high sequence homology with even the most divergent hepadnavirus, duck hepatitis B virus (30).

Thus, at least two of the three known hydrophobic domains of p24^s can be shown to contain topogenic information. Efforts to locate such information within the third (C-terminal) hydrophobic region have been less extensive. When this region was isolated downstream of globin sequences (mutant D14; Fig. 1), no association with membranes could be observed by cosedimentation, no protection of globin sequences occurred, and the normal p24 glycosylation site at Asn-146 was not glycosylated (Table 1). However, in full-length fusion KN9, translocation of the fused C-terminal globin domain was somewhat inefficient. This was evidenced by the presence of fragment R_0 after proteolysis (compare lanes 6 and 7 of Fig. 6C), which suggests that some chain translocations stopped in the C-terminal hydrophobic region of p24^s. It is possible that this C-terminal region has some degree of nonspecific stop-transfer activity in vitro owing to its very hydrophobic character (4). Alter-

natively, the existence in this region of an additional, inefficient stop-transfer–signal element which can only translocate downstream reporter groups could also explain these results; further studies of this region are required (see below).

Transmembrane topology of p24^s. As noted previously, transmembrane p24^s is a recently recognized intermediate in the pathway of surface antigen particle formation (5). As such, little direct information is available concerning its transmembrane orientation. However, our data, together with in vivo information about the structure of HBsAg particles, can be used to formulate a preliminary model for the disposition of p24^s chains in the membrane.

(i) In vivo data on p24^s topology. Although multiple potential glycosylation sites are present in p24^s (Fig. 1), only one (Asn-146) is used in vivo (25). This indicates that in transmembrane p24^s this site must be lumenal. Very few direct in vivo data on the disposition of the N-terminal region of p24^s exist. The potential glycosylation sites at Asn-3 and Asn-59 are not used, but it is unknown whether this reflects their failure to enter the lumen or their assumption of a conformation unfavorable for glycosylation. The latter would not be surprising, since conformational effects in HBsAg have long been known. For example, although the molecule contains numerous potential trypsin cleavage sites, only one of these (Lys-122) is susceptible to tryptic attack, even under dissociating conditions (24); similarly, antibodies raised to native HBsAg particles recognize denatured p24^s polypeptides poorly or not at all (35).

Although no other in vivo information about the N terminus of p24^s exists, virally infected cells produce an additional class of independently expressed, related polypeptides, known as pre-S proteins, which provide additional topological landmarks. Fifty-five codons upstream of the p24^s ATG is an additional initiation codon and coding region (termed pre-S2) which is in phase with the p24^s open reading frame. Translation from this upstream ATG results in the synthesis of a 31-kDa polypeptide (p31$^{pre\text{-}S}$) which adds an additional domain of 55 largely hydrophilic amino acids to the N terminus of p24^s (33). This protein is made in vivo and, like p24^s, is secreted from cells (without cleavage) in particulate form (9, 23). Importantly, the Asn residue at position 4 of this chain is efficiently glycosylated in vivo (33). Thus, the pre-S domain must also be disposed in the ER lumen. The pre-S2 domain of p31 is also efficiently translocated in vitro, but examination of pre-S–globin fusions bearing only this region have revealed no signal sequence activity (B. Eble, V. Lingappa, and D. Ganem, manuscript in preparation), implying that its disposition is mediated by signals within p24^s.

(ii) In vitro correlates and model building. When a reporter group (glycoglobin) is appended to the N terminus of p24^s, the reporter group is translocated (Fig. 6). Since the reporter group itself lacks signal activity, this suggests that the N terminus of p24 is itself lumenal. The identical behavior of the analogous protein p31$^{pre\text{-}S}$ in vivo strongly supports this conclusion. Similarly, when globin reporters are located C terminally (mutants A28, F21, KN9; Fig. 1), they too are translocated. To place both termini in the lumen, the molecule must span the bilayer at least twice.

Our data strongly suggest that one transmembrane domain must lie within signal II, presumably in the very hydrophobic region of residues 80 to 98, with its C terminus projecting into the ER lumen. To place the N terminus in the lumen, the other domain would therefore have to lie within the first 80 amino acids. Interestingly, the first 70 amino acids of the polypeptide contain no demonstrable stop-transfer activity, as judged by the secretory phenotype of mutants C1, E9, and A21 (Fig. 1), so that a precise definition of the borders of the first membrane-spanning region cannot be derived from this approach. Two possibilities can be envisioned: either a traditional stop-transfer element exists in residues 60 to 80 (disrupted in A21 by the deletion endpoint at residue 70) or sequences lacking intrinsic stop-transfer activity can come to span the bilayer. Further experiments are in progress to examine these possibilities.

On the basis of these in vitro findings and the available in vivo data, we propose two tentative models for the transmembrane disposition of p24^s (Fig. 7). As discussed above, in both models both the extreme N and C termini protrude into the ER lumen, and at least two transmembrane domains (one in signal II, one N terminal to it) are present. The models differ in the C-terminal region regarding the disposition of the third hydrophobic domain and reflect remaining uncertainties about the presence or absence of additional signals in this region of the molecule (see above). If a signal II-like element was present here, an additional (protease-resistant) loop through the membrane could be present (Fig. 7A, dashed line). However, if such an element does not exist, then the configuration shown by the solid line would be suggested. In any case, it seems likely that this very hydrophobic region will be affiliated with the membrane in some fashion.

(iii) Limitations of this approach. In this work we used fusion proteins and deletion mutants to isolate individual functional elements within p24^s and to make inferences about their behavior in the intact protein. Although powerful, this approach has several limitations. Since fusion proteins are experimental constructs there is no assurance that their properties will mimic those of the native molecule. When topogenic elements are taken out of their normal contents, and particularly when these transplantations are radical, they may function differently than when in their proper context (11, 12, 17). To minimize these potential problems we generated a large number of mutants with various degrees of deletion and substitution. In general, as noted above, the behavior of the fusions has been remarkably consistent, with similar fusions behaving similarly both to one another and to the wild-type p24^s parent. Importantly, the picture which emerges from study of the fusions accurately conforms to the known topogenic landmarks of the proteins in vivo.

To extend these in vivo correlations we have recently begun to examine the behavior of several of the mutants reported here in cultured cells transfected with the mutant constructions. Initial experiments indicate that mouse L cells harboring the signal I deletion 24AX are unable to export HBsAg, while control cells bearing the wild-type gene do so efficiently (D. Ganem, unpublished data).

However, some findings remain unexplained. For example, in mutants A519 and A519AX an Asn residue in the p24 N terminus is glycosylated. Although this finding is compatible with the models of Fig. 7A, we do not know why this site is used in the fusion and not in the wild-type molecule; perhaps an altered conformation assumed by the fusion is responsible. In any case, it is the potential for occurrences of this sort that limits the resolution of this approach, which complements but does not substitute for direct structural analysis.

Topogenesis of p24^s: some mechanistic possibilities. The model of Fig. 7A and our observation that multiple kinds of topogenic sequences are present in p24^s suggest several considerations as to how complex topologies of this sort are

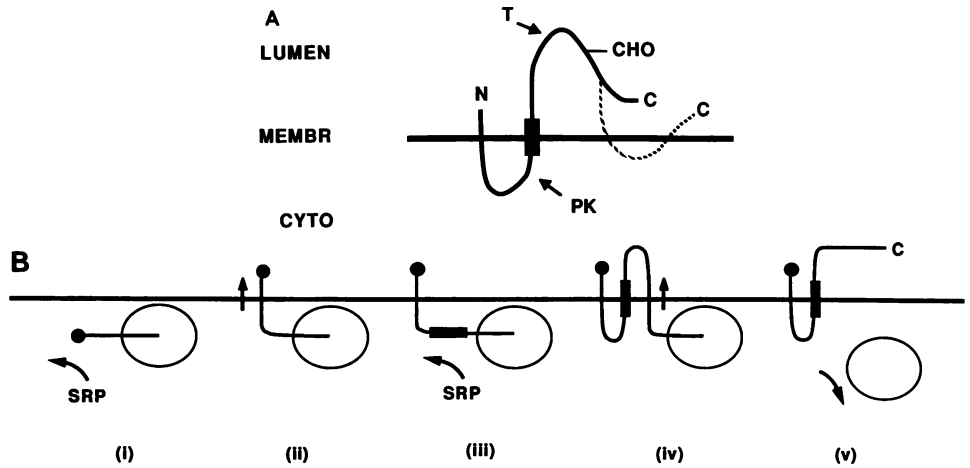

FIG. 7A. Models of the transmembrane topology of HBsAg $p24^s$, as discussed in the text. The amino (N)- and carboxy (C)-terminal ends of the molecule, the site of N-linked oligosaccharide addition (CHO), and a membrane-protected trypsin (T) cleavage site (5) protrude into the vesicle (ER) lumen, whereas the region of proteinase K (PK) cleavage is cytoplasmic (CYTO). The central hydrophobic domain (filled box), mapped in Fig. 1, is depicted as spanning the lipid bilayer (MEMBR), but the precise location of an upstream transmembrane domain is undetermined. An alternative model (dotted line) indicating a C-terminal cytoplasmic domain, as discussed in the text, is also shown. (B) Model for the functioning of multiple different signal sequences. In this schematic model, discussed in the text, an N-terminal signal (filled circle) emerging from the ribosome (large oval) interacts with SRP (i), to initiate translocation of N-terminal domains (ii). An internal signal (filled bar) interacts with SRP (iii) and inserts within the membrane in a C-terminal end-first orientation (iv). Translocation of C-terminal domains by the internal signal (iv) completes the generation of the final tritopic configuration (v). Although for illustrative purposes signal I is depicted as within the ER lumen, it may in fact partially or entirely span the bilayer.

generated. We presume that chain translocation will be initiated by signal I upon its emergence from the ribosome, as schematized in Fig. 7B and as observed for many conventional secretory proteins. It is noteworthy that in this case, as in a previous report (22), the signal sequence need not remain in a transmembrane configuration after translocation. In addition, the properties of signal I indicate that, in this case, hydrophobicity per se is not sufficient to stop the transfer of chains across the ER membrane. The key step in the generation of the complex topology of $p24^s$ is the subsequent emergence of signal II. This element is functionally complex and can mediate both stop-transfer and signal functions (Fig. 3B). In this view, this internal element can stop the progressive translocation of upstream domains (Fig. 7B, panel ii) by inserting into the bilayer in a specific (C-terminal end first) orientation (Fig. 7B, panel iv). Both signal functions may be mediated by SRP, as depicted schematically in Fig. 7B, panels i and iii, although we do not know whether the second signal need employ SRP when functioning from within its normal context following an N-terminal signal. After insertion, the membrane translocation function would commence translocation of C-terminal domains to complete topogenesis. Asymmetric structural features of the internal element (or its interaction with components in the membrane) as opposed to nonspecific hydrophobicity (4) are required to produce the specific (C terminal toward lumen) orientation of the insertion event. More speculatively, the ability of signal II to interact with the membrane in this fashion might also be involved in the positioning of the first transmembrane region. One simple notion might be that whatever region is spanning the membrane when signal II is engaged will be "locked" in a transmembrane configuration. Alternatively, specific regions in the first 80 residues might cause a pause in translocation (rather than an overt stop), allowing signal II to emerge and fix this configuration by its own membrane interaction. Such locking could be mediated by interactions of signal II with specific receptors or other elements of the translocation apparatus (8). If so, then a discrete stop-transfer function might not be required to establish the first transmembrane domain. Experiments to evaluate this possibility are in progress.

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