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The N-Terminal (pre-S2) Domain of a Hepatitis B Virus Surface Glycoprotein Is Translocated across Membranes by Downstream Signal Sequences

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The coding region for the hepatitis B virus surface antigens contains three in-phase ATG codons which direct the synthesis of three related polypeptides. The 24-kilodalton major surface (or S) glycoprotein is initiated at the most distal ATG and is a transmembrane protein whose translocation across the bilayer is mediated by at least two uncleaved signal sequences. The product of the next upstream ATG is the 31-kilodalton pre-S2 protein, which contains 55 additional amino acids attached to the N terminus of the S protein. This pre-S2-specific domain is translocated into the endoplasmic reticulum. Using a coupled in vitro translation-translocation system, we showed that (i) the pre-S2 domain itself lacks functional signal sequence activity, (ii) its translocation across the endoplasmic reticulum membrane is mediated by downstream signals within the S domain, and (iii) the N-terminal signal sequence of the S protein can translocate upstream protein domains in the absence of other signals. The hepatitis B virus pre-S2 protein is an example of a natural protein which displays upstream domain translocation, a phenomenon whose existence was originally inferred from the behavior of synthetic fusion proteins in vitro.

The correct functioning of cells and organelles requires that the proteins that traverse cellular membranes assume the correct transmembrane orientation. For most such proteins this process begins with the cotranslational recognition of specific "signal" sequences on the nascent polypeptide by the a signal recognition particle. The interaction of the resulting complex with the signal recognition particle receptor on the membrane of the endoplasmic reticulum (ER) is followed by the translocation of the ensuing protein domains across the lipid bilayer (30). Signal sequences may be located at the N terminus of a polypeptide or internally in the chain; typically, they function to translocate the downstream domains of the protein.

Recently, using a genetically engineered fusion protein in an in vitro translocation assay, Perara and Lingappa (19) demonstrated that signal sequences that usually translocate downstream domains could in certain contexts translocate domains upstream of the signal. Specifically, sequences from chimpanzee alpha-globin were fused to the N terminus of the secretory protein prolactin; this placed the normally N-terminal signal sequence of prolactin in an internal position. In this location, the prolactin signal proved to be able to translocate both globin and prolactin domains into the ER lumen. Because the function of topogenic elements could be altered by their relocation to different contexts in such artificial chimeric proteins (12, 13, 17), we have asked whether there are any naturally occurring proteins which display upstream domain transport by internal signals. We report here that the N-terminal pre-S2 domain of one of the surface glycoproteins of hepatitis B virus (HBV) is transported across the ER membrane in such a manner.

The surface glycoproteins of HBV are encoded by a large open reading frame that contains three in-phase AUG codons (8). Translation from each start codon results in the synthesis of three related polypeptides. The smallest of these, the 24-kilodalton (kDa) S protein, is synthesized from the innermost AUG of the frame and is the most abundant of the viral coat proteins (11). In addition to its role as a component of the viral coat, this protein is also independently secreted from cells as a subviral lipoprotein particle (9, 22, 23). We have recently shown that this secretory process is initiated by the action of two distinct signal sequences, one (termed signal I) at the N terminus and the other (signal II) located internally. The initial product of synthesis is a transmembrane protein which spans the bilayer at least twice; this transmembrane form is then extruded from the ER membrane into the lumen to give rise to the secreted form (6, 7, 26).

Translation initiation in the so-called pre-S coding region upstream of the S gene (11, 14) results in the production of two additional proteins. The smaller of these, the pre-S2 protein, is a 31-kDa polypeptide (p31) composed of the S domain plus an added 55-amino-acid pre-S2 domain at its N terminus (Fig. 1). The pre-S2 protein is found on both viral and subviral particles in infected serum (14, 28). Inspection of the predicted amino acid sequence of the pre-S2 domain reveals a relatively hydrophilic domain with few if any of the features traditionally ascribed to signal sequences. However, this domain must be translocated into the ER lumen in vivo because it contains an N-linked high-mannose carbohydrate linked to an asparagine residue at position 4 of the chain (11, 28). Accordingly, we wondered whether its topogenesis might be effected by one or more of the signals we had previously defined in the downstream S domain.

To examine the translocation of the pre-S2 protein, we used an in vitro assay of membrane transport. The coding region for the protein was cloned downstream of an SP6 promoter and transcribed in vitro with SP6 polymerase (15). The resulting synthetic mRNA was used to program an in vitro translation system supplemented with microsomal

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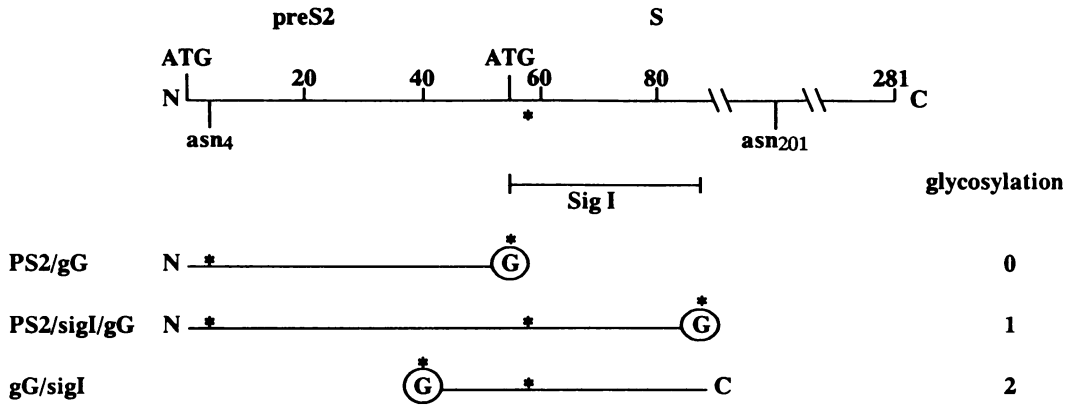

FIG. 1. Schematic diagram of the pre-S2 S region and structures of the gene fusions used in this study. The organization of the pre-S2/S region is outlined schematically at the top; numbers refer to amino acid residues in p31. Below are depicted the structures of HBV-globin gene fusions referred to in the text. Solid lines indicate HBV sequences; an encircled G denotes a globin-coding region. Sig I indicates the location of the first signal sequence in the S region. Potential glycosylation sites are indicated by *, and the number of experimentally observed glycosylations is indicated at the right. asn denotes an in vivo glycosylation site.

membrane vesicles; the latter contain all components required for the correct translocation of membrane or secretory proteins across the bilayer (29). Translocation was assayed by glycosylation of the radiolabeled product, as judged by gel electrophoresis before and after endoglycosidase H (endo H) digestion; since enzymes for N-linked carbohydrate addition are localized to the vesicle lumen, only translocated protein domains can be glycosylated. Also, translocated domains can be identified by their protection from the action of exogenous proteases.

The pre-S2 domain is translocated in vitro. The pre-S2 proteins found in the sera of HBV-infected individuals bear endo H-sensitive carbohydrates linked to the Asn residue at position 4 of the pre-S2 domain, in addition to the glycosylation within the S domain common to all HBV surface proteins (11, 22, 23, 28) (Fig. 1). We first set out to show that the in vitro system described above faithfully mirrors the in vivo translocation of this protein. Plasmid pSP31 bearing the pre-S2/S region downstream from the SP6 promoter was transcribed and translated in vitro in the presence of [^{35}S]methionine, and the radiolabeled translation products were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A).

When the translation was carried out in the absence of microsomal membranes, the major product was the 31-kDa pre-S2 protein (not shown, but cf. Fig. 2A, lane 1). A minor species of 24 kDa was also produced, corresponding to the S protein derived from internal translational initiation; additional, more minor species resulted from translation starts at more internal AUG codons within S. (The identity of these species has been confirmed by immunoprecipitation with anti-hepatitis B surface antisera [data not shown].) Both p31 and p24 were fully sensitive to exogenous proteinase K digestion (lane 2), yielding only small fragments that represent the limit digest products of the protein (6). When translation was conducted in the presence of membrane vesicles, however, several new bands arose (lane 3). The two uppermost species corresponded to the mono- and diglycosylated derivatives of p31, known as gp33 and gp36, respectively (11, 21); this was confirmed by their sensitivity to endo H digestion, which resulted in their conversion to p31 (lane 1). The pattern of glycosylated forms closely paralleled the pre-S2 forms described previously in authentic HBV infection in vivo (11). Glycosylation of the internally initiated S protein was also observed under these conditions (lane 3), as expected.

We then asked whether, like the S protein, the pre-S2 protein also exists in a transmembrane form and, if so, whether the topology of the homologous C-terminal (S) domains was similar in the two proteins. This was done by exposing the translocated products to digestion with exogenous proteinase K, which will attack regions of the protein exposed on the cytoplasmic surface of the membrane vesicles. Lane S shows the protected fragments derived from exposure of translocated p24 (S protein) to such proteolysis (6). As we have described previously (6, 7), several protected bands were observed; the fragment termed R_0 is the signature fragment of the transmembrane form of p24 and is a C-terminal species derived from cleavage at cytoplasmically disposed sites within the first 80 residues of the S domain. The pattern of protected species for the proteolyzed pre-S2 translocation products (Fig. 2A, lane 4) was similar to that of the S protein, including the generation of fragment R_0. We conclude that, as expected, p31 spans the bilayer and the addition of the pre-S2 domain had little impact on the topology of the S domain of the molecule.

The presence of the doubly glycosylated form of the pre-S2 protein strongly suggested that the pre-S2 domain had also been translocated into the vesicle lumen. To confirm this, we examined the susceptibility of the translocated protein to proteolysis by exogenous trypsin. Trypsin is known to cleave the S protein only once, at Lys-122 (22); in the transmembrane form of the protein, this site (located in the R_0 fragment mentioned above) is translocated into the ER lumen, rendering the entire protein trypsin resistant (6). Since the pre-S2 domain is known to harbor trypsin sites (28), exposure of the translocated version of p31 to this enzyme provided a simple and powerful test of whether this domain had been translocated. If the pre-S2 domain is within the vesicle, then the entire p31 protein should be resistant to trypsin. Figure 2B shows the results of such an experiment. Lane 2 displays the products of translation in the presence of membranes, again showing the presence of gp33 and gp36. As can be seen in lane 3, after proteolysis under conditions in which all of the unglycosylated (untranslocated) p31 was digested, a detectable proportion of both gp33 and gp36 was fully protected from tryptic attack. Although the efficiency of recovery of protected fragments in this experiment was

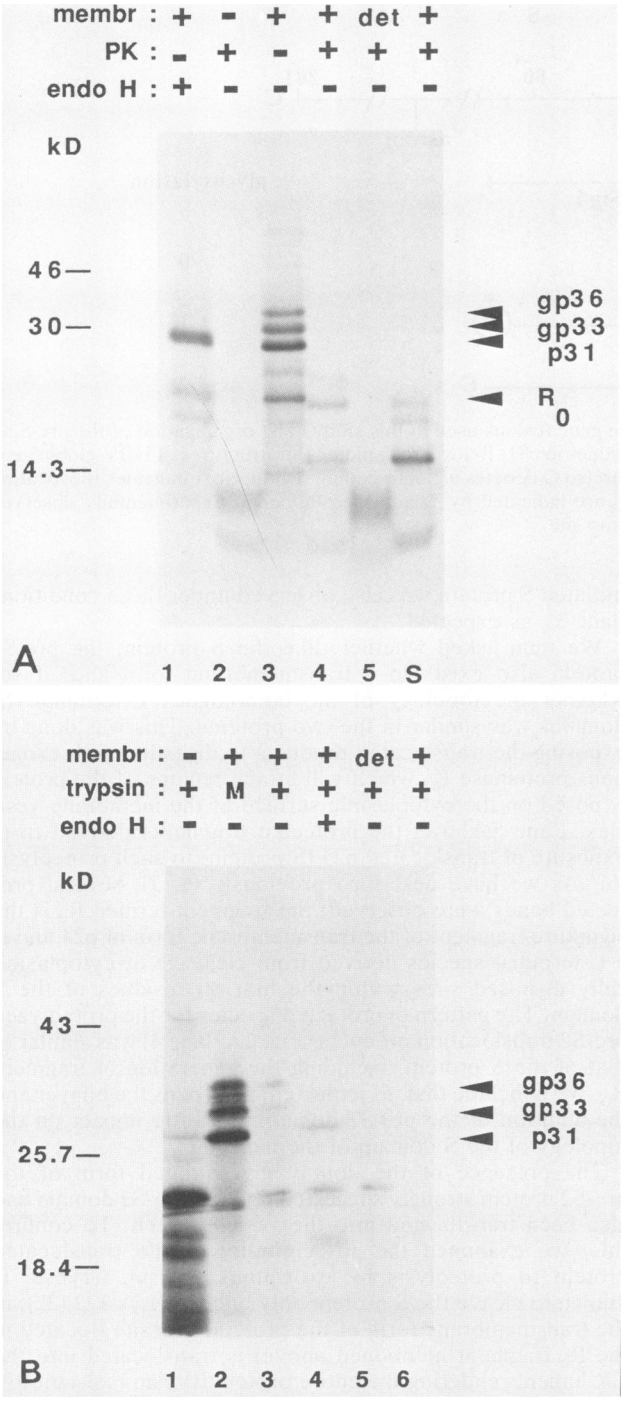

FIG. 2. Membrane translocation of the pre-S2 protein in vitro. (A) Glycosylation and proteinase K protection assays. SP6 pre-S2 (lanes 1 to 5) or SP6-S (lane S) recombinants were translated in vitro in the presence (+) or absence (−) of microsomal membranes (membr), as indicated at the top, and analyzed by SDS-PAGE. Following translocation, products in lanes 2 and 5 were exposed to proteinase K (PK) as indicated by (+). (Detergent disruption of the vesicle prior to proteolysis is indicated by det.) After extraction from the membranes, products in lane 1 were exposed to endo H. p31, gp33, and gp36 refer to pre-S2 chains containing no, one, or two high-mannose carbohydrate chains, respectively; fragment R_0 is discussed in the text. In vitro transcription-translocation, proteinase K digestion, and SDS-PAGE were carried out as described in detail previously (6, 7). (B) Trypsin protection assay. pre-S2 products translated in vitro in the presence or absence of membranes as indicated in the key were either digested (+) or mock-digested (M) with trypsin as described previously (6) and then immunoprecipitated with either anti-hepatitis B surface antigen (lanes 1 to 5) or nonimmune (lane 6) antisera prior to SDS-PAGE. In lane 4, the protected products were digested with endo H prior to electrophoresis. Conditions for endo H digestion were as outlined in reference 26.

low (ca. 20%), direct proof that these bands represent genuinely translocated species is shown in lane 5: disruption of the vesicle bilayer with detergent prior to proteolysis resulted in complete degradation of all three species. When these protected species were treated with endo H (lane 4), they were quantitatively converted to p31, confirming that they represent the glycosylated forms of the pre-S2 protein. (In repeated experiments, we observed variable protection of the monoglycosylated species gp33, but protection of the doubly glycosylated gp36 was always observed.) These studies document that the pre-S2 domain is indeed translocated into the ER lumen.

The pre-S2 region does not contain a signal sequence. We next asked whether the pre-S2 domain possesses a signal sequence which could mediate its membrane translocation. To do this, it was necessary to isolate the pre-S2 domain away from its linked S sequences, since the latter are known to harbor two efficient and complex topogenic signals (7). This was accomplished by constructing chimeric genes in which the pre-S2 region was fused to coding sequences for alpha-globin so as to generate in-frame fusion proteins (cf. Fig. 1). The alpha-globin gene was chosen because its product is a well-characterized soluble protein which is not usually transported across membranes but which can be translocated if linked to signal sequences from heterologous proteins. It thus can serve as a reporter group for topogenic signals. We have used this approach successfully in the past to identify such signals in a variety of proteins, including the S protein of HBV (6, 7).

Accordingly, we constructed the fusion PS2/gG, in which the first 52 residues of the pre-S2 domain are fused to residue 20 of globin (Fig. 1). To facilitate the detection of low levels of translocation, we engineered into the globin region of this construct a glycosylation site (from rat lactalbumin) which allowed the monitoring of translocation by the addition of N-linked carbohydrate. (We have shown previously [19] that this site is efficiently glycosylated in its new context when translocated into the vesicle lumen; hereafter, we refer to the resulting globin chain as "glycoglobin.") SP6 transcripts of this construct were translated in the presence or absence of membrane vesicles, and the products were examined by SDS-PAGE (Fig. 3). In the absence of microsomes, the fusion protein migrated as a ca. 17-kDa species (lane 1) which was fully sensitive to exogenous proteinase K (lane 2). (Again, minor products migrating more rapidly than the 14-kDa marker arose from aberrant internal translation initiation.) When membranes were present during the translation (lane 3), the same 17-kDa species was seen, with no evidence of additional glycosylated species. Exposure of this product to proteinase K again resulted in complete degradation, confirming that the chain had not been translocated (lane 4). In addition, we examined the ability of the fusion protein made in the presence of microsomes to cosediment with the membranes following translation; consistent with the proteolysis findings, no association of the products with the membranes was found (data not shown).

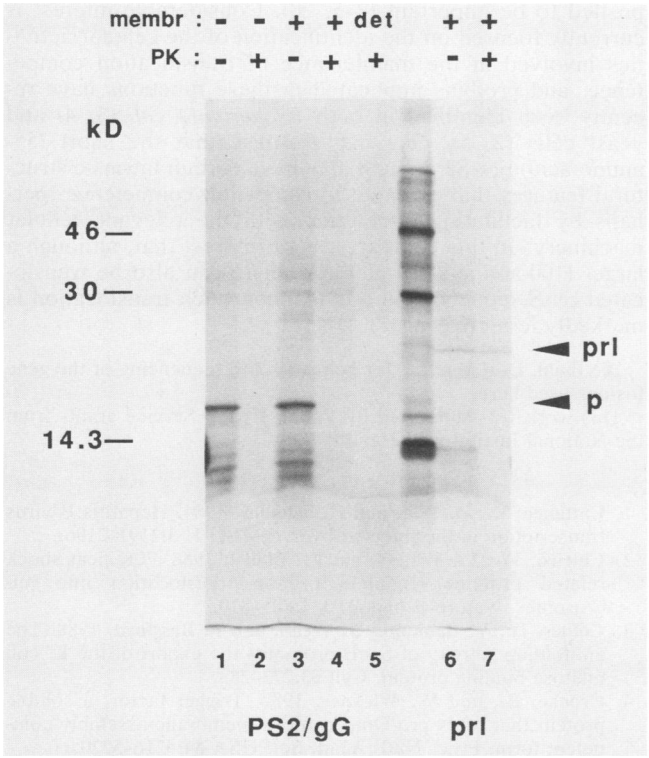

FIG. 3. Evidence that the pre-S2 region lacks signal sequence activity: autoradiogram of SDS-polyacrylamide gels of in vitro translocation assays. (Lanes 1 to 5) Translocation of fusion PS2/gG. (Lanes 6 and 7) Translocation of bovine preprolactin. Key at the top indicates the presence (+) or absence (−) of membranes (membr) during translation and whether (+) or not (−) the products were exposed to proteinase K (PK) following translocation. p, Position of the PS2/gG fusion protein; prl, position of prolactin.

In parallel experiments, we monitored the translocation activity of the microsomes to assure that this result was not due to inactive or disrupted membrane vesicles. In vitro transcripts for bovine preprolactin were translated in the presence of membranes (lane 6); this showed the presence of processed prolactin (i.e., the chain whose signal sequence had been removed), indicating translocation into the vesicle. This was confirmed by showing that the processed prolactin chains were resistant to exogenous proteinase K attack (lane 7).

One possible explanation for these negative results is that the pre-S2 region might contain sequences inhibitory to the translocation of globin domains in an assay of this type or that the fused globin sequences might inhibit putative signals from functioning. To rule out this explanation, we constructed fusion PS2/sigI/gG (Fig. 1), in which the entire pre-S2 region plus the first 29 residues of the S domain (including signal I) are fused to residue 20 of glycoglobin. This construct, which is identical to PS2/gG save for the addition of signal I, was translocated across the vesicle membrane (as judged by glycosylation) with an efficiency comparable to that of many other globin fusions we have studied (Fig. 1) (B. Eble, unpublished data). We conclude that the pre-S2 domain is not capable of initiating domain translocation across membranes because it lacks a functional signal sequence. Its translocation in the wild-type pre-S2 protein must therefore be due to the action of downstream topogenic signals in the S region.

Signal I of the S region can translocate upstream domains. Our previous work (7) has demonstrated that the S region contains at least two topogenically active elements, signals I and II. The former is located within the first 30 amino acids of the protein, serves to initiate the translocation of the chain, and contains no intrinsic stop-transfer activity. Signal II maps to residues 80 to 100, is extremely hydrophobic, and acts to both halt the translocation of the upstream domains initiated by signal I and initiate the translocation of downstream domains; in these regards, it resembles the internal signal/stop-transfer sequences of several other class II transmembrane proteins (7). To define better the nature of the elements responsible for the translocation of the pre-S2 domain, we have tested fusion proteins bearing these elements for their ability to translocate upstream domains.

In a previous study (7), we examined fusion B8*, in which a glycoglobin domain was fused to the N terminus of the S region, creating in effect a pre-S2 analog. This fusion behaved similarly to the pre-S2 protein itself: it was doubly glycosylated (although the efficiency of double glycosylation was much lower than for the pre-S2 protein itself), and the glycosylated globin domain was protected from trypsin. This result confirms that sequences within the S region can translocate upstream domains which themselves lack intrinsic signal sequences.

Next we assessed the ability of signal I alone to mediate the translocation of upstream protein domains. To do this, we generated a deletion mutant derivative of B8* by excising the S region distal to signal I with *Xba*I and *Acc*I. After repair of the termini and religation, the resulting fusion (designated gG/sigI in Fig. 1) contained only the glycoglobin domain followed by signal I and four additional amino acids (Thr-Phe-Lys-Pro) resulting from the frameshift generated at the *Xba*/*Acc*I junction. When SP6 transcripts of this fusion were translated in a reticulocyte extract, they gave rise to a ca. 14-kDa fusion protein (Fig. 4, lane 1) which was reactive with both antiglobin and anti-hepatitis B surface antigen sera (data not shown). Translation in the presence of membranes gave rise to two additional bands (lane 2), which endo H digestion (lane 3) confirmed to be the mono- and diglycosylated forms of the polypeptide.

These data indicate that the pre-S2 protein, like its S counterpart, is synthesized as an integral transmembrane protein and that the transmembrane disposition of its S region is similar to that of the 24-kDa S protein itself. Analysis of glycosylation pattern and protease protection experiments indicated that the pre-S2 domain itself appears to project from the N terminus of S into the lumen of the ER. A model for the transmembrane toplogy of the pre-S2 protein is shown in Fig. 5. The lumenal disposition of the 55-amino-acid pre-S2 domain is that predicted by the model proposed previously (26) for the generation of subviral particles, in which budding of the transmembrane form of the surface glycoproteins generates lumenal particles. In such a scheme, domains which project into the ER lumen wind up on the surface of the particle. That this is indeed the location of the pre-S2 domain on hepatitis B surface antigen particles in vivo is shown by (i) its accessibility to pre-S2-specific antibodies, (ii) its differential susceptibility to exogenous proteases (28), and (iii) its ability to bind large polymers of human serum albumin (14, 21).

In keeping with its overall hydrophilic character, the pre-S2 region appears to lack a functional signal sequence. It is therefore apparent that its translocation into the ER lumen must be mediated by downstream signals. This translocation must occur posttranslationally, since the pre-S2 domain

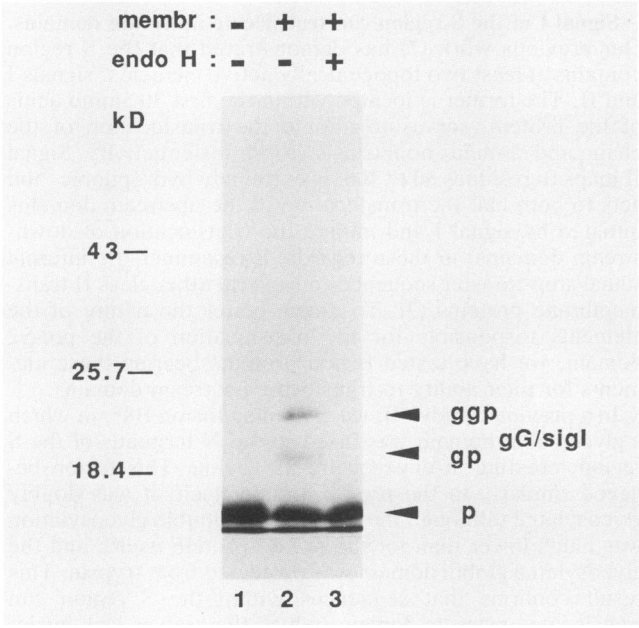

FIG. 4. Evidence that signal I can translocate upstream domains: autoradiograms of in vitro translocation reactions of gG/sigI. Key indicates membrane addition or post-translocation endo H digestion as in Fig. 2 and 3. All products were immunoprecipitated with antiglobin antibodies prior to electrophoresis. p, gp, and ggp denote forms of the corresponding proteins bearing no, one, or two N-linked carbohydrate chains, respectively.

must be synthesized before its responsible signal element. Although most protein transport across membranes in vivo is cotranslational, recent experiments indicate that such a coupling is not obligatory in vitro (10, 18–20, 24). A key variable appears to be the involvement of the ribosome or other cellular components in the maintenance of "translocation competence," a poorly characterized state in which the maintenance of the polypeptide in an unfolded state is

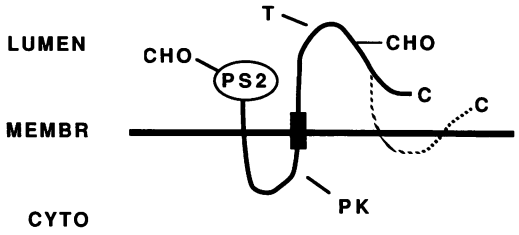

FIG. 5. Model for transmembrane disposition of the pre-S2 polypeptide. The ER membrane is shown as a horizontal line, with the ER lumen depicted above the line and the cell cytoplasm (CYTO) shown below. The S domain of the chain is shown as a heavy line traversing the membrane. As demonstrated previously (7), the S domain must span the membrane at least twice; it remains unclear whether the C-terminal region of the molecule makes additional transmembrane passages (cf. reference 7), so a potential alternate structure of this region is presented as a dashed line. The N-terminal pre-S2 domain (PS2) is shown projecting into the lumen of the ER, where it is glycosylated and protected from exogenous trypsin. T and PK denote the positions of the major cleavage sites in the S domain for trypsin and proteinase K, respectively. Cleavage of either S or pre-S2 proteins with proteinase K yields the identical C-terminal fragment (denoted R_0 in Fig. 2). CHO indicates the position of the N-linked carbohydrate in the S and pre-S2 domains.

posited to be important (2–5, 20). Considerable interest is currently focused on the identification of the cellular activities involved in the maintenance of translocation competence, and proteins implicated in these functions have recently been identified in both *Escherichia coli* (3, 4) and yeast cells (2, 5). One may surmise that the short (55-amino-acid) pre-S2 domain may have certain intrinsic structural features that promote translocation competence, perhaps by facilitating interaction with the relevant cellular machinery. In this context, it is of interest that, although a larger (100-amino-acid) globin domain can also be translocated by S sequences in this manner, such translocation is markedly less efficient (7).

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