

Targeting of the Hepatitis B Virus Precore Protein to the Endoplasmic Reticulum Membrane: After Signal Peptide Cleavage Translocation Can Be Aborted and the Product Released into the Cytoplasm

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Abstract. The major hepatitis B virus (HBV)¹ core protein is a viral structural protein involved in nucleic acid binding. Its coding sequence contains an extension of 29 codons (the "precore" region) at the amino terminus of the protein which is present in a fraction of the viral transcripts. This region is evolutionarily conserved among mammalian and avian HBVs, suggesting it has functional importance, although at least for duck HBV it has been shown to be nonessential for replication of infectious virions.

Using *in vitro* assays for protein translocation across the endoplasmic reticulum membrane, we found that the precore region of the HBV genome encodes a signal sequence. This signal sequence was recognized by signal recognition particle, which targeted the nascent precore protein to the endoplasmic reticulum membrane with efficiencies comparable to those of other mammalian secretory proteins. A 19-amino acid signal peptide was removed by signal peptidase on the luminal side of the microsomal membrane, generating a protein similar to the HBV major core protein, but

containing 10 additional amino acids from the precore region at its amino terminus. Surprisingly, we found that 70–80% of this signal peptidase-cleaved product was localized on the cytoplasmic side of the microsomal vesicles and was not associated with the membranes. We conclude that translocation was aborted by an unknown mechanism, then the protein disengaged from the translocation machinery and was released back into the cytoplasm. Thus, a cytoplasmically disposed protein was created whose amino terminus resulted from signal peptidase cleavage. The remaining 20–30% appeared to be completely translocated into the lumen of the microsomes. A deletion mutant lacking the carboxy-terminal nucleic acid binding domain of the precore protein was similarly partitioned between the lumen of the microsomes and the cytoplasmic compartment, indicating that this highly charged domain is not responsible for the aborted translocation. We discuss the implications of our findings for the protein translocation process and suggest a possible role in the virus life cycle.

HUMAN hepatitis B virus (HBV)¹ is a member of a group of enveloped DNA viruses (hepadna viruses) that use reverse transcription as part of their life cycle (for review see Tiollais et al., 1985; Standring and Rutter, 1986). The HBV virion genome is a circular partially double-stranded DNA of 3.2 kb. Due to its small size, the coding information in the DNA is densely packed into four partially overlapping reading frames. The products of two of these open reading frames are found as the major structural components in the viral particles, the core protein and the

surface protein. The core protein assembles with pre-genomic viral RNA into a core particle, where reverse transcription takes place in the cytoplasm of the infected cells. Core particles are then thought to interact with the cytoplasmic domains of the surface protein, a membrane protein integrated into the endoplasmic reticulum (ER) membrane. Presumably, the surface protein forms patches that bud into the ER lumen enveloping the core particles in the process (Eble et al., 1986). The virion then leaves the cell by passing through its normal secretory pathway. Studies on the biogenesis of HBV have been limited due to the lack, until very recently, of a tissue culture system in which the virus can be replicated. Thus, there is no detailed biochemical knowledge of the steps involved in the HBV assembly process.

The studies described in this paper concern some functional properties of the viral core protein and their potential significance in the viral life cycle. The major protein found

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1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; HBV, hepatitis B virus; K-RM, salt-extracted rough microsomal membranes; RM, microsomal membranes; SRP, signal recognition particle.

in the HBV core is 21 kD in size and contains at its carboxy terminus a stretch of 36 amino acids that are predominantly arginines (47%) thought to be involved in nucleic acid binding (Valenzuela et al., 1980). The open reading frame encoding the core protein contains an amino terminal extension (the "precore" region) of 29 amino acids starting with an AUG codon that precedes the initiating methionine used for the translation of the major core protein (p21; Valenzuela et al., 1980). Mapping of the pregenomic RNA transcripts of the ground squirrel hepatitis virus (closely related to the human HBV), revealed that transcription is initiated at three sites around the first AUG codon of the core open reading frame (Enders et al., 1985). Thus, some of the transcripts initiate between the two AUG codons and, therefore, give rise to a translation product initiating at the second AUG corresponding to the major core protein (p21). Some transcripts initiate upstream of the first AUG codon of the core open reading frame and, therefore, are translated to yield a protein of ~25 kD (p25). Both transcripts are recruited into polyosomes in infected cells (Enders et al., 1987); however p25 protein has not yet been detected *in vivo*. Since the precore region is conserved in the genome of hepadna viruses, it is likely to be of importance, yet it is not essential for viral replication. This was shown by Chang et al. (1987), who demonstrated that the introduction of a frameshift mutation into the precore region did not adversely affect infectivity of a related duck HBV.

The sequence of part of the precore region (Valenzuela et al., 1980; Ou et al., 1986) resembles that of signal sequences that direct proteins across the membrane of the ER (Watson, 1984). In particular, it contains a positively charged amino acid at its amino terminus, followed by a cluster of ~10 hydrophobic residues. Furthermore, the expression of p25 (but not p21) in mammalian cells (Ou et al., 1986; Roossinck et al., 1986; McLachlan et al., 1987) or *Xenopus* oocytes (Standring et al., 1988) results in secretion of core related peptides. While our studies confirm that the precore region functions as an efficient signal peptide in targeting nascent precore protein to the ER membrane and engaging it with the translocation machinery, we observed the unprecedented property that a large proportion of the protein did not complete the translocation process, but was released back into the cytoplasmic compartment.

Materials and Methods

Plasmid Construction

To facilitate the insertion of the HBV core coding sequences into plasmids containing the promoter for the bacteriophage SP6 RNA polymerase, convenient restriction sites were introduced into the HBV genome by site-directed mutagenesis using the double primer method (Zoller and Smith, 1984). The viral sequences TAGGTT and AACTTT (at positions 1757 and 1821 from the unique Eco RI site; Valenzuela et al., 1980) were mutated to the Hind III recognition sequence (AAGCTT) for the construction of pHBVc/p25 and pHBVc/p21, respectively. Double-strand DNA from M13 phage clones containing these mutant HBV genomes were digested with Hind III and Hinc II, and the DNA fragments containing the HBV core sequences were cloned into the Hind III-Hinc II sites in the pSP64 vector polylinker (Krieg and Melton, 1987). For constructing plasmid pHBVc/p25 δ , the arginine codons 179 and 180 (the first positively charged amino acids of the carboxy-terminal nucleic acid binding domain) were changed to the termination codon UGA by site directed mutagenesis (Zoller and Smith, 1984). A Dra I (position 2185)-Eco RI (position 1) viral DNA fragment containing the mutated HBV core coding sequences was transferred

to pHBVc/p25 digested with Eco RI and partially digested with Dra I. The recombinant pHBVc/p25 δ plasmid was selected according to both its restriction map and its capacity to express the mutated protein p25 δ . The introduction of these changes was confirmed by sequencing of the mutated DNA.

Transcription with SP6 Phage Polymerase

The plasmid DNAs were linearized with Eco RI and transcribed in 20- μ l reactions containing 40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 0.5 mM each ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM (G(5')ppp(5')G, 10 mM dithiothreitol, 1,000 U/ml of human placental RNase inhibitor, 0.1 mg/ml of linearized plasmid, and 500 U/ml of SP6 RNA polymerase. The reactions were incubated at 40°C for 60 min, and were terminated by phenol-chloroform extraction. Nucleic acids were ethanol precipitated and the resulting pellet was dissolved in 40 μ l of water.

Translation and Translocation Assays

Wheat germ translation extracts were prepared as previously described (Erickson and Blobel, 1983). Microsomal vesicles (RM) were prepared from canine pancreas as described (Walter and Blobel, 1983a). Ribosomes and signal recognition particle (SRP) were removed from the salt-extracted microsomal membranes (K-RM) by EDTA and high salt treatment (Walter and Blobel, 1983a, b). SRP was purified from RM as previously described (Walter and Blobel, 1983b). Translations were performed at 26°C for 1 h (unless indicated otherwise) as reported (Erickson and Blobel, 1983), except that the magnesium concentration was found optimal at 3.5 mM and that 0.002% Nikkol detergent (octa-ethyleneglycol-n-dodecylether) was included to stabilize SRP activity (Walter and Blobel, 1980). RNA transcripts from 50 ng of plasmid (contained in 1 μ l) were translated in 10- μ l reactions containing 25 μ Ci of [³⁵S]methionine. Translation products were visualized after overnight exposure to X-Omat AR Kodak film of 10-15% gradient SDS-PAGE.

Synchronized Translation Assays

Translation reactions of the desired volume were prepared as described above. The reaction was prewarmed at 26°C for 2 min before the addition of the mRNA at time 0 min. To synchronize translation, the initiation inhibitors 7-methylguanosine-5'-monophosphate (at 4 mM final) and edeine (at 5 μ M final) were added at time 2 min. A 10- μ l sample of the reactions was taken at the times indicated in the figures, and the proteins were resolved by SDS-PAGE. The gels were fluorographed with 2,5-diphenyloxazole and exposed to X-Omat AR Kodak film.

Protease Protection Assay

After translation, 10- μ l reactions were transferred to an ice water bath. 1 μ l of 10 mg/ml trypsin or protease K was added and incubated at 0°C for 30 min. 10 U of aprotinin or 1 μ l of 100 mM phenylmethylsulphonyl fluoride (in ethanol), was added to inhibit trypsin or protease K, respectively. The products were precipitated by addition of 1 vol of 20% TCA and resuspended in sample buffer for SDS-PAGE. To the reactions indicated, 1 μ l of 4% Triton X-100 was added before the addition of the proteases.

Microsome Sedimentation Assay

After translation, the reactions were transferred to an ice water bath and potassium acetate was added to a concentration of 500 mM. 10 equivalents of K-RM were added as carrier membranes. The reactions were layered on a 100 μ l cushion containing 500 mM sucrose, 500 mM potassium acetate, and 2 mM magnesium acetate. After centrifugation at 30 psi in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) for 5 min, the proteins from the supernatant and the pellet were TCA precipitated and subjected to SDS-PAGE and autoradiography.

Results

Precore Region of the HBV Core Protein Encodes a Signal Peptide

To address questions concerning the function of the precore region of the HBV core protein we decided to synthesize

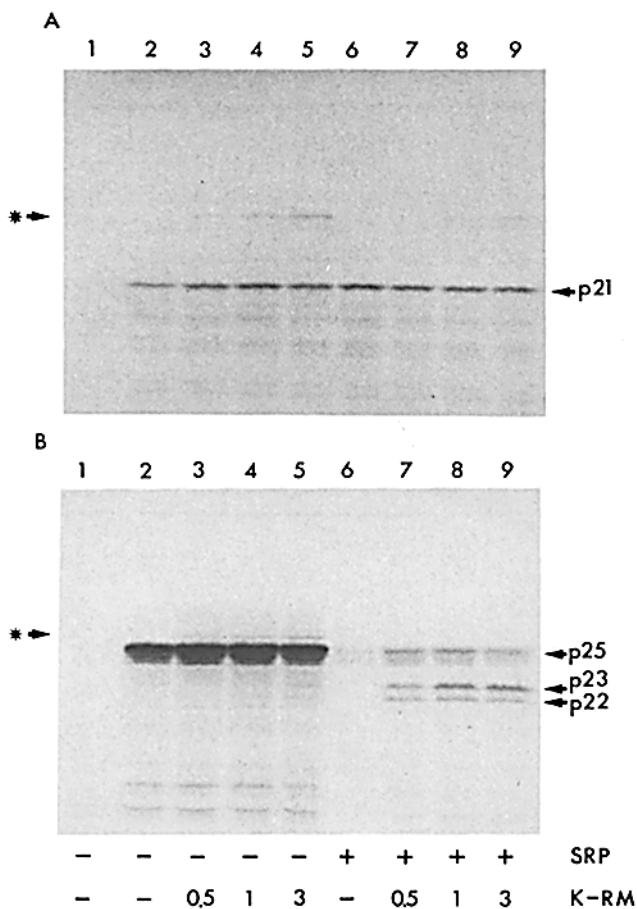


Figure 1. Effect of SRP and K-RM on the translation of p21 and p25. In vitro transcripts from plasmids pHBVc/p21 (A) and pHBVc/p25 (B) were translated by wheat germ extracts in 10- μ l reactions for 1 h at 26°C. The translations were performed with increasing concentrations (0.5–3.0 equivalents per reaction) of salt-extracted microsomal membranes (K-RM, see Materials and Methods), both in the absence (lanes 3–5) or presence (lanes 7–9) of 50 nM SRP. Reactions without RNA (lanes 1) and with mRNA alone (lanes 2) were included as controls. In one reaction only SRP (50 nM) was added (lane 6). The asterisk indicates the product of translation of a minor mRNA contaminant of our K-RM preparation. This protein is not immunoprecipitated with anti-HBV core antibodies (data not shown).

mRNAs that either include the full core protein open reading frame (p25 mRNA) or the open reading frame starting with the second AUG (p21 mRNA). For this purpose, plasmids (pHBVc/p21 and pHBVc/p25, see Materials and Methods for details) were constructed which contain the coding sequences downstream of the bacteriophage SP6 RNA polymerase promoter (Krieg and Melton, 1987). These plasmids were transcribed in vitro as indicated in Materials and Methods, and the resulting transcripts were translated in a cell-free wheat germ extract. Fig. 1 shows the translation products of both the p21 mRNA (A, lane 2) and p25 mRNA (B, lane 2) transcripts. The respective molecular masses of both translation products are in agreement with those predicted from the coding sequences, although, as it will be shown below, the products obtained after the 1-h incubation in the translation extract carried a covalent modification

which increased their respective apparent molecular masses slightly (\sim 1 kD). Both p21 and p25 were immunoprecipitated by anti-HBV core protein antibodies (data not shown), confirming that the products were indeed derived from HBV core protein coding sequences. When the p25 mRNA was translated (Fig. 1 B) hardly any translation products comigrating with p21 were obtained due to the lack of internal initiation at the second AUG codon, i.e., translation is efficiently initiated at the first AUG codon.

To test whether the precore region will function as a signal peptide as suggested (see Introduction), we supplemented the translation reactions with K-RM (Fig. 1, A and B, lanes 3–5). These membranes have been depleted of SRP by the salt treatment (Walter and Blobel, 1980) and thus, as expected, had no effect on the translation of either p21 or p25. However, when the translation reactions were supplemented with purified SRP in addition to K-RM, processing of p25 was observed (Fig. 1 B, lanes 7–9), whereas p21 was not affected (Fig. 1 A, lanes 7–9). Unexpectedly, we found that the SRP-dependent processing of p25 by K-RM yielded a heterogeneous product; two distinct bands with molecular masses of \sim 22 and 23 kD (p22 and p23) were consistently obtained (Fig. 1 B, lanes 7–9). As it is shown below, p23 is derived from p22 by a secondary modification.

Further confirmation that the precore region contains a bona fide signal peptide and is directly recognized by SRP, is given by the result that SRP in the absence of K-RM causes inhibition of p25 synthesis. This effect is due to the ability of SRP to cause an arrest or pausing in the elongation of proteins after a signal sequence has been exposed outside the ribosome (Walter and Blobel, 1981). This elongation arrest is released once the SRP/ribosome/nascent chain complex interacts with the SRP receptor on the surface of the microsomal vesicles (Walter and Blobel, 1981). Although the in vivo significance of the elongation arrest reaction still remains to be determined, it has proven to be a valuable measure of the relative affinity (and thus efficiency) with which a signal sequence is recognized by SRP. Fig. 1 (A and B, lanes 6) shows the qualitative effect of SRP addition on the translation of p21 and p25, respectively. Note, that the synthesis of p25 is severely inhibited, while that of p21 is unaffected. A quantitative comparison (Fig. 2) confirms these results over a range of SRP concentrations. Here, we included globin mRNA as an internal control of a cytoplasmic protein that, lacking a signal peptide, is not affected in its synthesis by SRP (Walter et al., 1981). Fig. 2 B shows a quantitative comparison of the inhibition of p25 with that of two authentic mammalian secretory proteins, preprolactin (pPL) and pregrowth hormone (pGH). While preprolactin synthesis was inhibited by 80% at 10 nM SRP, the synthesis of pregrowth hormone and p25 requires \sim 50 nM SRP to be inhibited to the same level. This indicates that the affinity with which the signal peptide of p25 is recognized by SRP falls into the same range as those observed for signal peptides of at least one mammalian secretory protein.

To characterize further the processing of p25 by microsomal membranes, we determined the exact cleavage site by sequencing the amino terminus of both processed products, p22 and p23. Translation of p25 mRNA in the presence of microsomal membranes (RM; not depleted of SRP, see Materials and Methods) was performed in the presence of [3 H]leucine. p22 and p23 were resolved by preparative

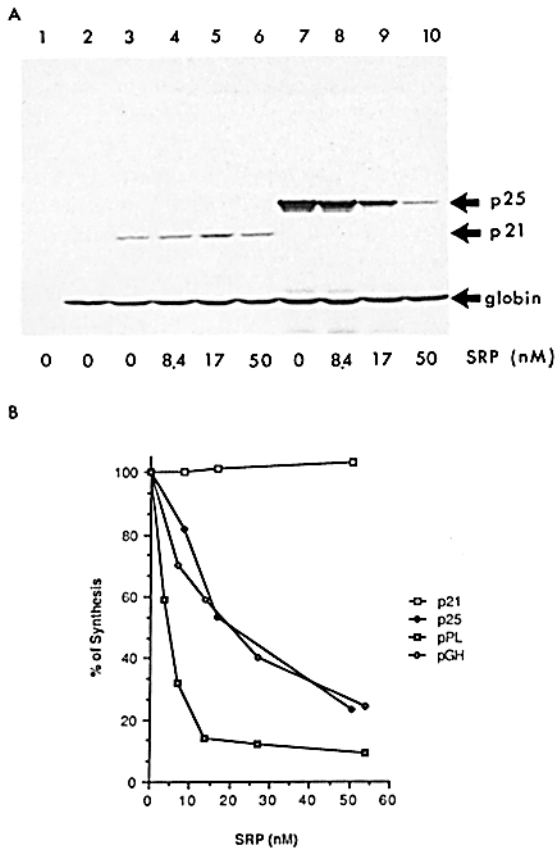


Figure 2. Quantification of SRP-dependent elongation arrest on the translation of p21 and p25. (A) Translations of pHbVc/p21 (lanes 3–6) and pHbVc/p25 (lanes 7–10) mRNAs were performed for 1 h at 26°C, in the presence of increasing concentrations of SRP (as indicated) in 10- μ l reactions. Total rabbit reticulocyte RNA (0.2 OD₂₆₀ per reaction, primarily encoding the cytoplasmic protein globin) was included (lanes 2–10) as an internal control of a protein whose translation is not affected by SRP (Walter et al., 1981). A reaction with no RNA added is shown in lane 1. (B) The relative radioactivity in the bands in A were determined by densitometric scanning of the autoradiograms using scanner (model Ultrosan XL; LKB instruments, Inc., Gaithersburg, MO), under conditions in which the intensities measured were linear with respect to the radioactivity incorporated. The percentage of synthesis indicated was determined as described elsewhere (Garcia et al., 1987). The results of experiments for preprolactin (pPL) and pregrowth hormone (pGH), two secretory proteins, are included for comparison.

SDS-PAGE and electroeluted. After the proteins were tested for their purity in an analytical SDS-PAGE (data not shown), the samples were subjected to sequential Edman degradation. The products released in each degradation cycle were analyzed for their content of radioactivity (Fig. 3). Both processed bands gave an identical sequence pattern with peaks of radioactivity in cycles 3, 5, and 8. This distribution pattern of leucine residues is found only once in the p25 coding sequence with leucines in positions 22, 24, and 27 after the initiating methionine. These results show that both p22 and p23 have the same amino terminus and localize the cleavage site between amino acids 19 and 20 of p25. This processing site is consistent with the empirical consensus rules for signal peptidase cleavage (vonHeijne, 1984), with respect

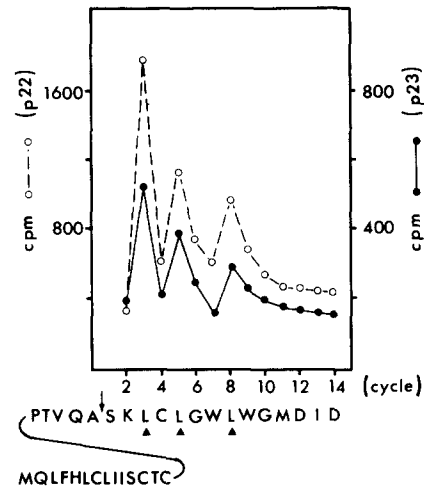


Figure 3. Amino-terminal sequence analysis of p22 and p23. Translation products after 1 h of labeling with [³H]leucine in the presence of microsomal membranes (RM; containing SRP, see Materials and Methods) were resolved by preparative SDS-PAGE. The p22 and p23 protein bands were electroeluted as described (Hunkapiller et al., 1981) and their purity was determined by analytical SDS-PAGE (data not shown). Each labeled protein (~70,000 cpm) was subjected to sequential Edman degradation in a gas phase sequenator. The radioactive content of the products of each cycle was determined. Each point in the figure corresponds to the total radioactivity released per cycle. In the third cycle, 69 and 21% of the radioactivity incorporated per leucine residue was recovered for p22 and p23, respectively. The sequence of the first 33 amino acids of p25 is indicated in the single letter code at the bottom of the figure. The triangles and the arrow indicate the leucine residues (aligned with the radioactive peaks) and the signal peptidase cleavage site, respectively.

to both the distance from the hydrophobic core of the signal peptide and the amino acids found at positions -1 and -3 of the cleavage site. Thus, we conclude that the observed processing of p25 protein is performed by signal peptidase and that at least the amino-terminal portion of the protein has reached the interior of the microsomal vesicles.

p22 Is Posttranslationally Modified to p23

Since both p22 and p23 are products of signal peptidase cleavage (i.e., contain the same amino terminus; see Fig. 3), and are derived from a unique precursor (p25), we reasoned that one could be the product of a secondary modification of the other. To establish a precursor/product relationship between these two proteins, we analyzed the appearance of p22 and p23 at different time points during translation, instead of after 60 min as in the previous experiments. For this purpose, translation reactions were synchronized by the addition of initiation inhibitors after 2 min (see Materials and Methods). Samples were taken at different times and the products were resolved by SDS-PAGE. The results of experiments for both p21 and p25 mRNAs are shown in Fig. 4. For p25, the experiment was carried out both in the absence (Fig. 4 B) or presence (Fig. 4 C) of microsomal vesicles. In the absence of RMs (Fig. 4, A and B), the appearance of the primary translation products for both p21 and p25 were first observed after 8 min of incubation. We observed that both the p21 and p25 translation products at the 8-min time point

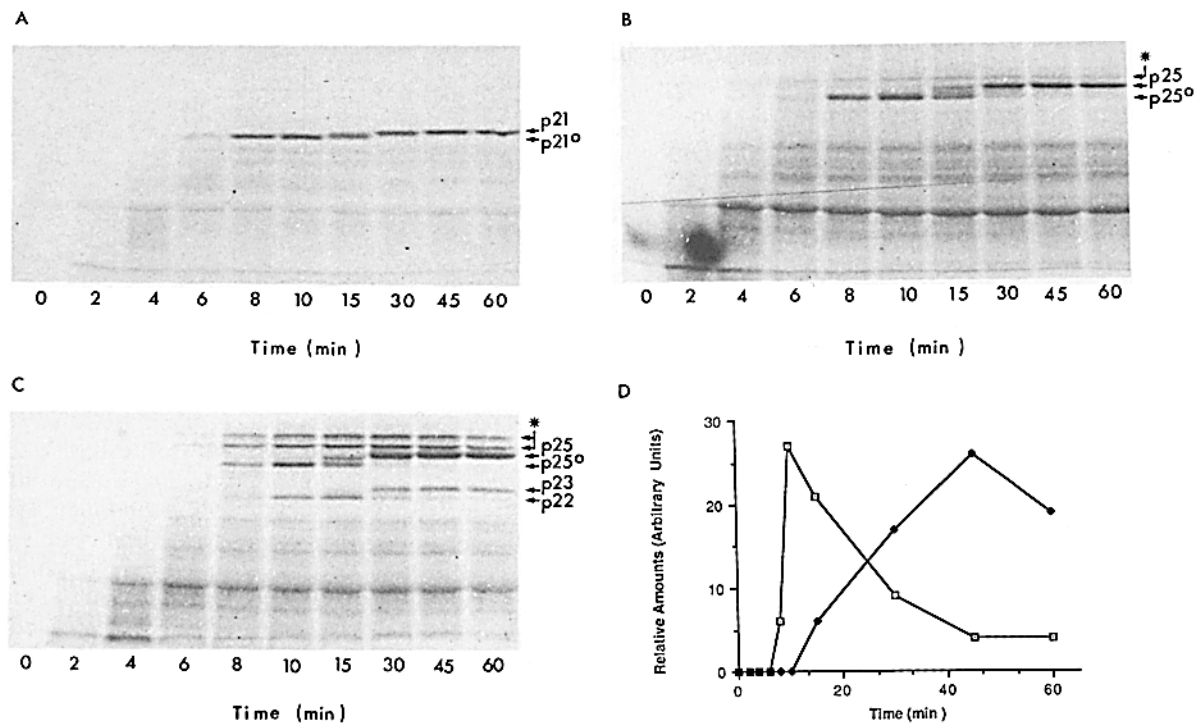


Figure 4. Time course in synchronized translations of p21 and p25. Samples of synchronized translations (see Materials and Methods) were taken at 0, 2, 4, 6, 8, 10, 15, 30, 45, and 60 min of incubation and the products were analyzed by SDS-PAGE. (A) The results obtained for the pHBVc/p21 mRNA. p21^o indicates the unmodified p21 protein. Translation of the pHBVc/p25 mRNA was performed in the absence (B) or the presence (C) of microsomal membranes (RM; containing SRP, see Materials and Methods). As for p21, p25^o indicates the unmodified p25 protein. The bands observed above p25 (B and C, asterisk), correspond to the products of minor mRNA contaminants in our extracts. They are not immunoprecipitated with anti-HBV core antibodies (as in Fig. 1). D shows a quantitative analysis of the results obtained for p22 (□) and p23 (◆). To obtain this data, a longer exposure than the one shown in C was scanned, as for Fig. 2.

migrated slightly faster on the gel than the products obtained after a 60-min incubation. A shift to the slower migrating species was observed ~15 min of incubation. This shift was not due to a discrete pause in elongation. It occurred independently of ongoing protein synthesis since in control experiments where 100 μ M cycloheximide was added after 8 min of incubation, identical results were obtained (data not shown). Thus we conclude that the shift in electrophoretic mobility was produced by a posttranslational modification of both p21 and p25. This modification was independent of the presence of microsomal membranes in the reactions (Fig. 4, A and B) and therefore must have been catalyzed by a cytoplasmic enzyme(s).

Presently we do not know the chemical nature of this post-translational modification. However, since it is carried out by a cytoplasmic enzyme(s) it provided us with a convenient marker for the topographical disposition of the products of signal peptidase cleavage with respect to the microsomal membrane (see also below). Fig. 4 C shows that p22 was formed first as the primary cleavage product. The bulk of p22 was subsequently modified and converted to p23. Again, the conversion was insensitive to the addition of cycloheximide at 8 min of incubation (data not shown) and, therefore, corresponds to a posttranslational modification of the signal peptidase cleaved form of p25. However, in marked contrast with what we observed for p21 and p25, not all p22 is converted to p23 during the time extent of our analysis. A quantitative

analysis of the time course of appearance of these two proteins is shown in Fig. 4 D. Note that after 45 min no more of the remaining p22 (~20%) was converted to p23. These results suggest that initially two distinct populations of p22 were formed: ~80% of p22 was not completely translocated across the membrane and, therefore, was accessible to the cytoplasmic enzyme(s) that converted it to p23, whereas ~20% was completely translocated and therefore not modified.

Topography of p22 and p23

To establish directly the topography of p22 and p23 with respect to the microsomal membrane, we used two additional experimental approaches: protease protection by the lipid bilayer (Fig. 5) and cosedimentation with the microsomal vesicles (Fig. 6). Proteins that are not translocated or, as is the case for some integral membrane proteins, are only partially translocated across the lipid bilayer will remain sensitive to externally added proteases while those that are completely in the lumen of the microsomal vesicles will be protected from proteolytic digestion. Fig. 5 shows the results for translation products obtained after 60-min incubations with two different proteases, trypsin (A) and protease K (B). Although p25 is completely digested in both cases, the resulting pattern of bands is complex because both proteases leave discrete fragments that are resistant to further digestion

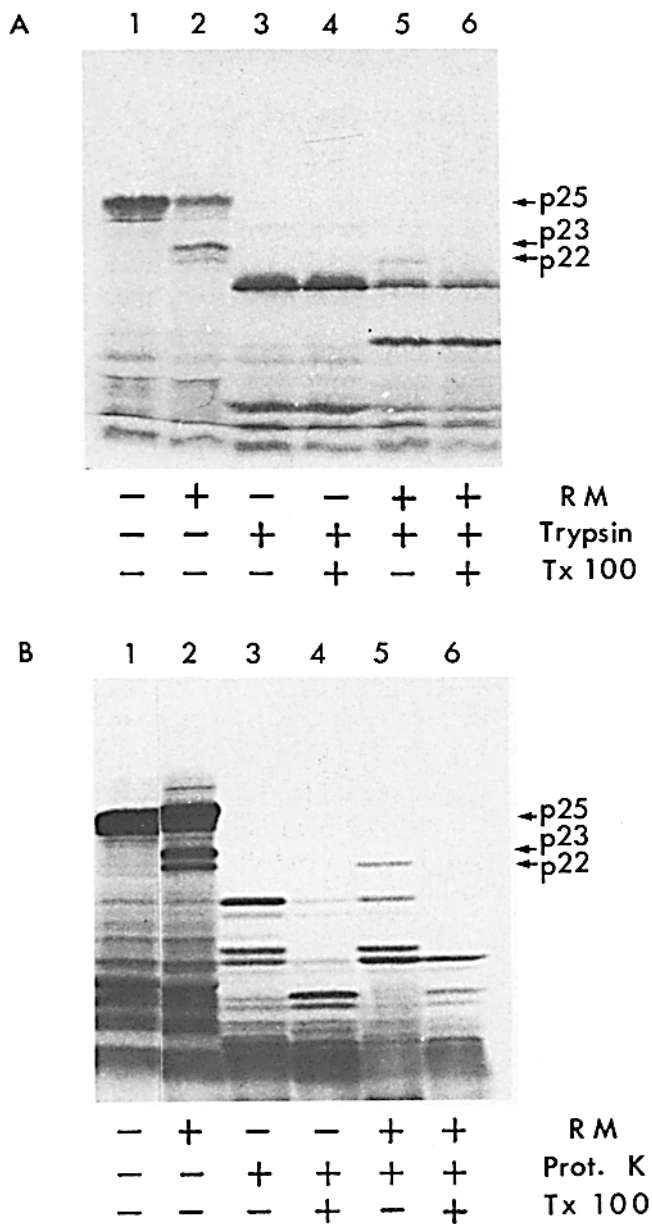


Figure 5. Protease protection analysis. The pHBVc/p25 mRNA was translated for 1 h at 26°C, in the absence (lanes 1, 3, and 4) or presence (lanes 2, 5, and 6) of microsomal membranes (RM; containing SRP, see Materials and Methods). The products of a 10- μ l translation were treated (lanes 3–6) with trypsin (A) or protease K (B) at 0°C for 30 min. Triton X-100 (0.4% final) was added to the reactions in lanes 4 and 6, before the protease addition. After the proteases were inhibited (see Materials and Methods), the products were resolved by SDS-PAGE. The positions at which p22, p23, and p25 migrate are indicated. These results are representative of five experiments carried out independently. Note that the fraction of p22 that is resistant to proteases depends on the degree to which cytoplasmically disposed p22 had been modified to p23 (see Figs. 4 and 7). In the unsynchronized translations shown here, the modification has not reached completion and its extent is different for the experiments of A and B; thus p22 is not equally protected. These results also allowed us to map the protease sensitive site(s). Digestion of p25 with trypsin and protease K yields a 20- and 18-kD fragment, respectively, which are very resistant to further digestion. Trypsin digestion of a mix of p25, p22, and p23 (A, lanes 5 and 6) results in an additional fragment of \sim 17–18 kD. Since p25 and p23 differ on their amino terminus due to the removal of the

even in the absence of microsomal membranes (Fig. 5, A and B, lanes 3; see also Takahashi et al., 1983). Furthermore, the addition of the nonionic detergent Triton X-100 allows further digestion of these fragments by protease K (Fig. 5 B, lane 4). When translation of p25 was carried out in the presence of microsomal membranes (Fig. 5, A and B, lanes 2, 5, and 6), we found that p22 was protected from proteases (Fig. 5, A and B, lanes 5) unless the membranes were disrupted with detergent (Fig. 5, A and B, lanes 6). These results indicate that p22 after 1 h of incubation was completely translocated. In contrast p23 was entirely sensitive to both proteases, indicating that it was exposed on the cytoplasmic side of the microsomal membranes. Thus, these results are consistent with the observed modification pattern of the signal peptidase cleaved products of p25 (see above).

The cosedimentation assays shown in Fig. 6 further confirm this conclusion. Translation products obtained after 1 h of incubation were layered on top of a sucrose cushion (see Materials and Methods). Membrane (pellet) and cytoplasmic (supernatant) fractions were separated by centrifugation (see Materials and Methods). In this assay, p21 always remained in the supernatant fraction (Fig. 6 A, compare lanes 4 and 5). This result is expected since p21 does not contain a signal peptide and therefore will not be targeted to the microsomal membrane during translation. The same result was obtained for the bulk of residual p25 that has not been targeted and cleaved by signal peptidase (Fig. 6 A, compare lanes 8 and 9). In contrast p22 behaved as expected for a protein that is translocated into the microsomal vesicles; the bulk of p22 cosedimented with the membrane vesicles and was recovered in the pellet fraction (Fig. 6 A, compare lanes 8 and 9; also see Fig. 6 B for quantification). Under identical conditions, *in vitro* synthesized and processed preprolactin is quantitatively recovered in the pellet fraction (data not shown). p23, on the other hand, was quantitatively recovered in the supernatant fraction (Fig. 6 A, lanes 8 and 9 and B). Thus, in spite of the fact that both p22 and p23 are the result of proteolytic processing of p25 by signal peptidase and, therefore, must have been, at least partially, translocated across the membrane to contact the active site of the enzyme, only p22 is found associated with the vesicles. Surprisingly, p23 appears to be localized free in the cytoplasm as indicated by its sedimentation behavior, its modification by cytoplasmic enzyme(s), and its susceptibility to proteases.

In summary, we can conclude that only a fraction of p22 (\sim 20–30%) was completely translocated across the microsomal membranes and located in the lumen of the vesicles, whereas translocation of the bulk of p22 (70–80%) appears to be aborted after being processed by signal peptidase and the protein is released into the cytoplasm. The fraction of p22 that was sequestered inside of the microsomal vesicles corresponds to the fraction of p22 in Fig. 4, C and D that was not modified to p23 even upon prolonged incubations. Con-

signal peptide, it follows that the protease-sensitive site(s) lies \sim 5–6 kD from the carboxy terminus of both proteins. We have tested other proteases (elastase, protease V8, thermolysin, and subtilysin; each at 1 mg/ml) and found that they also cleaved the protein in approximately the same location. This indicates that the core protein has at its amino terminus a domain that is extremely resistant to proteases (see also Takahashi et al., 1983), but that the carboxy-terminal portion can easily be digested.

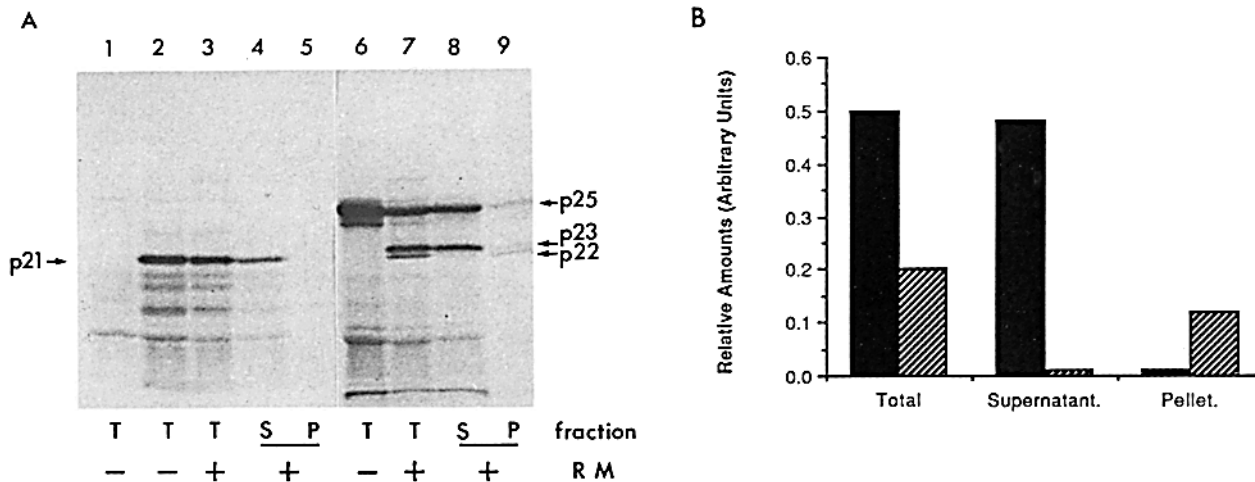


Figure 6. Sedimentation analysis. (A) Translations of pHbVc/p21 (lanes 2–5) or pHbVc/p25 (lanes 6–9) mRNAs were carried out for 1 h at 26°C, in the absence (lanes 2 and 6) or presence (lanes 2–5 and 7–9) of microsomal membranes (RM; containing SRP). Translation products made in the presence of RM were fractionated into a supernatant (lanes 4 and 8, corresponding to the cytoplasmic fraction) and a pellet (lanes 5 and 9, corresponding to the microsomal membrane fraction) fraction by ultracentrifugation in an airfuge (see Materials and Methods). (B) Quantitative representation of the distribution of p22 (▨) and p23 (■) in the supernatant and pellet fractions (lanes 7–9 from A). The total amounts of p22 and p23 indicated, correspond to the products obtained without fractionation. To obtain these data, a longer exposure than the one shown in A was scanned as indicated in Fig. 2. These results are representative of five independent experiments. Some loss of p22 (~10–20%) was observed and is likely due to incomplete recovery of the pellet fraction. Qualitatively very similar results were obtained when the ionic strength was reduced by a 1:10 dilution with distilled water, or increased (by addition of potassium acetate to 1 M), or when the translation products were treated with 2 M urea before the fractionation procedure.

versely, the fraction released into the cytoplasmic compartment became accessible to the same cytoplasmic enzymes that modify p21 and p25 in the absence of microsomal membranes (see Fig. 4, A and B) and, therefore, was converted to p23 (see model in Fig. 9).

To confirm this notion, we analyzed the protease sensitivity of the products obtained at different time points during translation. Reactions were programmed with p25 mRNA and incubated in the presence of microsomal membranes. Samples of the reaction were removed at 10, 15, 30, and 60 min and transferred to an ice water bath. Half of each sample was analyzed directly by SDS-PAGE and the other half was treated with protease K before electrophoresis (Fig. 7 A). Both unmodified (p25^o) and modified p25 were completely sensitive to protease K. Only p22 was resistant to the protease at all time points examined (Fig. 7 A, lanes 5–8), whereas p23 was completely degraded. A quantitative analysis of these results demonstrates (Fig. 7 B), however, that only a fraction of p22 (~30%) at the early time points was protected by the lipid bilayer. In similar experiments using preprolactin as control, we found that processed preprolactin was quantitatively protected at all the time points examined (data not shown). Thus, the lack of protection of the bulk of p22 at early time point was not due to an intrinsic leakiness of the microsomal vesicles but rather was specific for p22.

During the course of our experiments, we observed that the modification reaction of the HBV core proteins was ATP dependent. Thus, the modification reaction could be blocked by depletion of ATP at an early time point after the appearance of p22 and p25. Depletion of ATP was performed by the addition of an excess of glycerol kinase and glycerol at 10 min after translation (see legend to Fig. 7). The results of such an experiment are shown in Fig. 7 C. In this case,

as in Fig. 7 A, only p22 was protected from protease at all the time points examined, whereas, the unmodified form of p25 was protease sensitive. However, a quantitative analysis (Fig. 7 D) shows that only ~30% of the p22 protein was protected from protease, i.e., was translocated into the microsomal vesicles. This result demonstrates that the modification reaction is a secondary process that has no effect on the localization of the processed forms of p25.

A Processed Form of p25 Lacking the Nucleic Acid Binding Domain Is also Partitioned between the Lumen of the Microsomal Vesicles and the Cytoplasm

Because of the very unusual behavior of p22, we were intrigued by the possibility that the extremely charged carboxy-terminal domain could adversely affect the translocation process. Thus, this domain could be the responsible for the aborted translocation. To investigate this possibility, a deletion mutant was constructed (pHBVc/p25 δ ; see Materials and Methods for details) containing a termination codon precisely at the amino-terminal boundary of the charged cluster. When the p25 δ mRNA was translated in the absence of microsomal membranes, a major translation product of ~17 kD (p25 δ) was observed (Fig. 8 A, lane 1). If microsomal membranes were present during translation a corresponding processed form (p22 δ) of ~15 kD was also observed (Fig. 8 A, lane 2). Only the cosedimentation assay could be used to determine the topography of p22 δ because p22 δ and p25 δ are resistant to both trypsin and protease K (the cleavage sites for both proteases map near the position where the termination codon was introduced; see legend to Fig. 5). The cosedimentation assay was performed as in Fig. 6. As expected, residual p25 δ remains in the supernatant (Fig. 8 A,

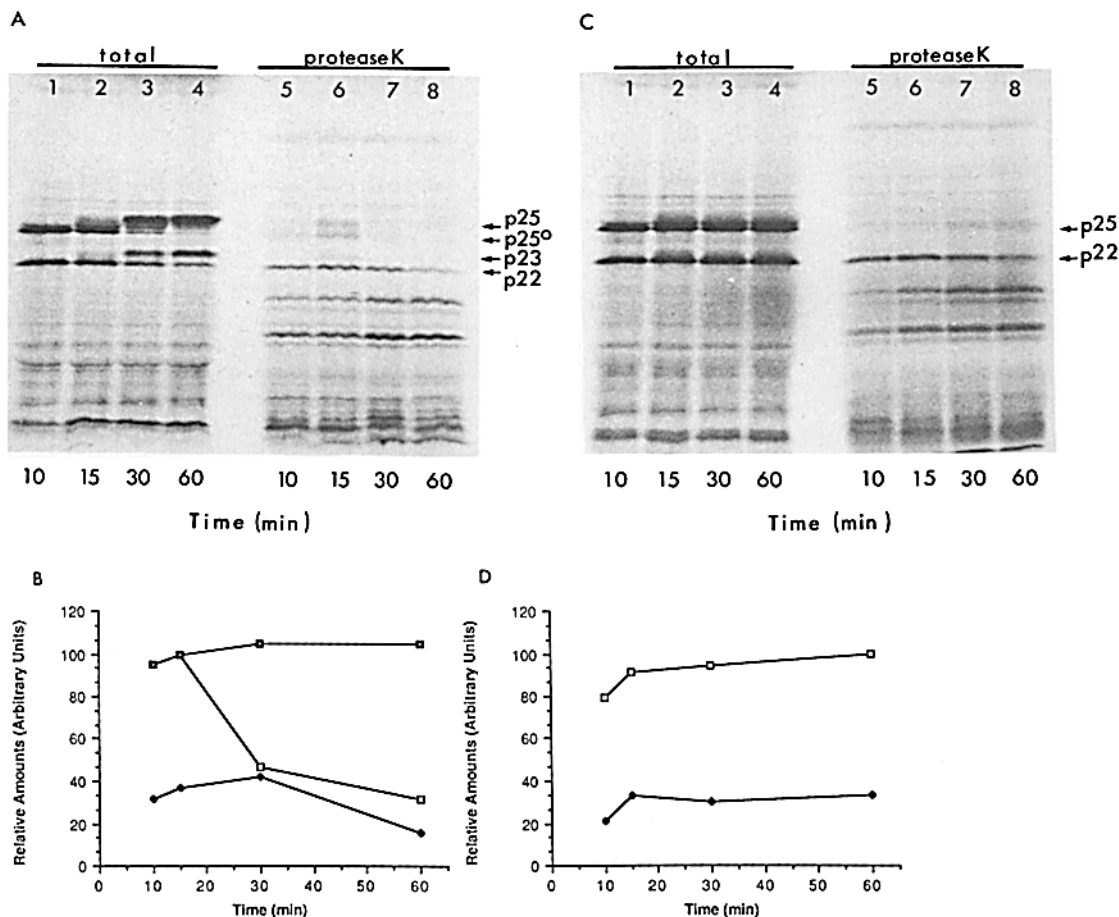


Figure 7. Protease protection analysis during time course of translations. Samples (20 μ l) of synchronized translations were taken at 10, 15, 30, and 60 min of incubation. Each sample was divided in two: one-half was directly subjected to SDS-PAGE (lanes 1–4), and the other half was treated with protease K before electrophoresis (lanes 5–8). In the experiment shown in C, *Escherichia coli* glycerol kinase was added to a final concentration of 250 μ g/ml (from a stock of 10 mg/ml in 50% glycerol) at 10 min of incubation. B and D show the quantification of the experiments in A and C, respectively. The relative amounts of p22 (□), p22 plus p23 (□), and protected p22 (◆) were determined as for Fig. 2.

compare lanes 3 and 4). Interestingly, the bulk of p22 δ also remained in the supernatant (Fig. 8 A, compare lanes 3 and 4), indicating that, as was for p22, a large fraction of the protein was not translocated. Fig. 8 B shows a quantitative analysis which indicates that ~20–30% of p22 δ is found in the pellet fraction. Thus, we can conclude that removal of the nucleic acid binding domain does not affect the partitioning of the signal peptidase processed form between the lumen of the microsomal vesicles and the cytoplasmic compartment.

Interestingly, in this experiment no heterogeneity in the electrophoretic mobility of the p25 δ and p22 δ protein bands was observed. In a time course of translation neither protein showed any shift in the electrophoretic mobility during incubations similar to those of Fig. 4 (data not shown). Since both proteins are found in the cytoplasmic fraction (where the modifying enzyme[s] resides), this result indicates that, in contrast to the other HBV core proteins, p22 δ and p25 δ are not substrates for the modifying enzyme(s) described above. The result suggests that the site of modification reside in the nucleic acid binding domain of the HBV core proteins. This result also confirms that the abortion of translocation and the release of the protein into the cytoplasm occurs independent of its modification.

Discussion

Implications for the Viral Life Cycle

Using in vitro protein translation and translocation assays we have determined that the precore region of the HBV genome encodes a functional signal sequence that directs nascent p25 to the membrane of the endoplasmic reticulum. This targeting event is indistinguishable in its molecular requirements (i. e., its SRP and SRP receptor dependence) from that observed for other secretory and membrane proteins. Once targeted to the ER membrane, p25 is engaged in the translocation machinery and in the process becomes a substrate for signal peptidase after the cleavage site has been exposed on the luminal side of the membrane. Removal of the signal peptide creates p22. This protein differs from the “major” core protein (p21) initiated at the second AUG codon of the open reading frame since it contains 10 additional amino acids at its amino terminus.

This finding is consistent with the observation that forms of core protein can indeed be secreted in vivo. Proteolytic fragments of the core protein (of ~15 kD) can be found in the serum of infected patients. These fragments have been

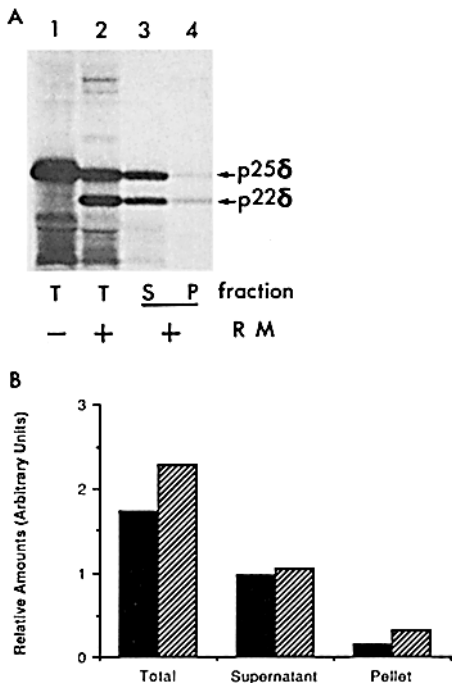


Figure 8. Sedimentation analysis of p22 δ and p25 δ . (A) Translations of pHBVc/p25 δ mRNA were performed in the absence (lane 1) or presence (lanes 2–4) of microsomal membranes (RM; containing SRP, see Materials and Methods). Translation reactions done in the presence of RM were fractionated into a supernatant (lane 3) and a pellet fraction (lane 4) as indicated in Materials and Methods. (B) The quantification of the amounts of p22 δ (▨) and p25 δ (■) found in an unfractionated reaction, in the supernatant, and in the pellet fractions. The intensity of each protein band was determined as in Fig. 2. However, to compare both proteins in this case the intensity of p22 δ was multiplied by 1.5 to account for the loss of one of the three methionines due to removal of the signal peptide.

immunologically defined as HBV “e” antigen which is distinguishable from core antigenicity. More direct evidence of secretion of core related products (similar to those found in the serum of infected patients) comes from expression of its complete coding sequence in mammalian cells (Ou et al., 1986; Roossinck et al., 1986; McLachlan et al., 1987) and *Xenopus* oocytes (Standring et al., 1988). Furthermore, these secreted fragments contain the same amino terminus as the signal peptidase cleaved p22 (Standring et al., 1988). It follows that the second proteolytic processing event (to generate 15-kD products) occurs at the carboxy terminus of the protein. Since we observe intact translocated p22 in the lumen of the microsomal vesicles, this secondary cleavage event is likely to occur in a later compartment of the secretory pathway. It is possible that this processing is performed by the same enzyme that processes pro-proteins in the Golgi apparatus, or secretory vesicles (that cleaves at Lys-Arg or Arg-Arg residues), since the fragment removed is very rich in arginines. Taken together, these studies demonstrate that the signal peptide in the precore region is responsible for the targeting of p25 to the ER and, thus, the secretion of the HBV “e” antigen.

Much to our surprise, we found that a large fraction of p22 was released into the cytoplasm (where it became modified to p23) after being targeted to the ER and processed by signal peptidase. We have shown that this property is specific for p22, since in similar experiments preprolactin (a typical secretory protein) remains stable inside of the microsomal vesicles (data not shown). We speculate that our results may reflect a novel cellular mechanism used by HBV to obtain different forms of a protein with respect to its structure and/or intracellular localization. At present we do not know that aborted translocation of p25 does indeed occur in vivo. There is, however, indirect evidence, since forms of core protein with molecular masses \sim 23 kD can be observed in intact virions isolated from the serum of infected patients

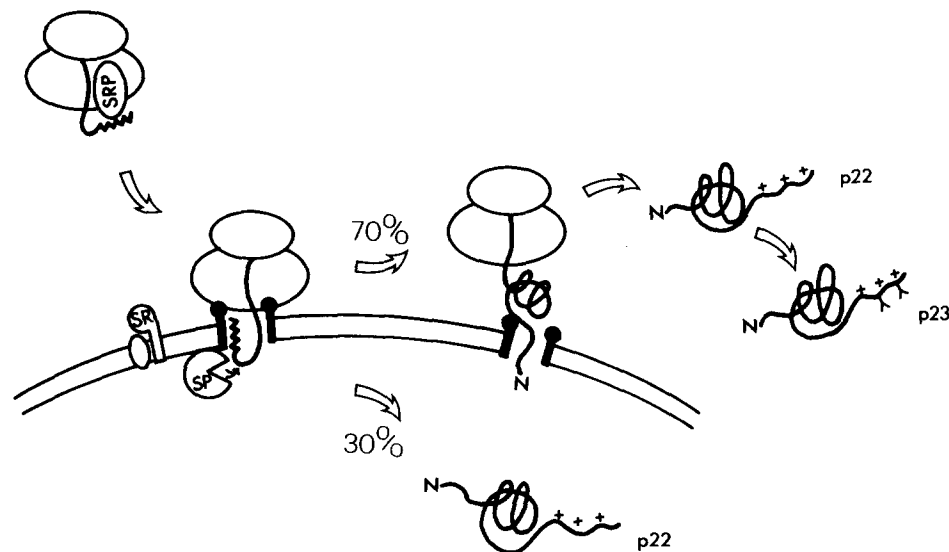


Figure 9. Model for the targeting of p25 to the ER membrane. SRP recognizes the signal peptide of p25 as the protein is being synthesized by free ribosomes. The binding of SRP results in the arrest of the synthesis of p25. This inhibition of translation is released when the arrested complex interacts (through SRP) with the SRP receptor (SR) in the membrane of the ER. A translocation competent junction of the ribosome to the membrane is established. During translocation of p25, the cleavage site becomes accessible to signal peptidase (SP). However, translocation of 70% of the protein that has been cleaved by SP is aborted and the protein is released into the cytoplasm. Only 30% of the processed p25 is correctly translocated and is found in the lumen of the microsomal vesicles.

(Gerlich, W., personal communication) and upon expression of p25 mRNA in *Xenopus* oocytes (Strandring, D., personal communication), respectively. Unfortunately, amino-terminal sequence information of these proteins is not yet available and will be required to confirm their correspondence to p23. Most interestingly, the 23-kD form found in intact virions is not present in core particles isolated from infected cells (Gerlich, W., personal communication) hinting that this protein may be acquired by the virus during the assembly/budding process (see below).

What purpose could the virus accomplish by producing a signal peptidase processed form and localizing part of the protein in the cytoplasm? While we do not know the answer to this question, we can discuss a few possibilities. For example, the partial translocation of a portion of the core protein across the ER membrane may put this protein into proper configuration to interact with other viral components on the cytoplasmic side of the membrane and thereby affect its assembly. p22 thus localized in the vicinity of the ER could interact with membrane integrated surface protein and subsequently become "copolymerized" into already assembled or partially assembled core particles. Thus a minor amount of p22 could provide a physical link between the core particles and the viral envelope. Such an interaction may facilitate assembly and give the virus a growth advantage, but as discussed above this is unlikely to be essential for the assembly process. Furthermore, but not necessarily exclusive, considering that p22 has 10 more amino acids at its amino terminus than p21, p22 may have novel properties that could allow the virus to use the protein for a function(s) in the cytoplasm, secretory pathway, and/or blood stream that still remains to be discovered.

In the process of the membrane translocation studies, we noticed that all cytoplasmic forms of HBV core protein (p21, p25, and p22 after release from the membrane vesicles) were covalently modified in the translation extract. The modification did not appear to be peculiar to the wheat germ extract since similar experiments performed in a rabbit reticulocyte lysate translation system showed similar modification patterns (data not shown). Therefore, we conclude that the HBV core proteins serve as a substrate for a modification enzyme(s) that is(are) present in cytoplasm of cells as divergent as plants and mammals. We suggest that core protein may be similarly modified by a corresponding enzyme(s) in the host cell cytoplasm prior to its assembly into viral particles. We have not determined the chemical nature of the modification, but we know that the reaction requires ATP (Fig. 7). It has been described that the major core protein is phosphorylated *in vivo* (Roossinck and Siddiqui, 1987). We tested if the modification observed in our assays was phosphorylation by treating the translation products with alkaline phosphatase. Although the electrophoretic mobility of modified p25 was slightly increased by this treatment (indicating that it is indeed phosphorylated), it still migrated significantly slower than the primary translation product (p25^o, data not shown). Thus, we conclude that the HBV core proteins can carry at least two different modifications, phosphorylation and the unknown modification described here. This latter modification is likely to occur within the charged carboxy-terminal domain, because p25^o showed no shift in mobility even upon prolonged incubation with the translation extracts. Alterna-

tively, the carboxy-terminal domain could be required for substrate recognition by the modifying enzymes.

Implications for the Protein Translocation Process

Regardless of the implications for the HBV life cycle, the study of p25 as a translocation substrate allowed us to address a variety of questions concerning the mechanism of protein translocation *per se*. First it should be noted that a fraction of p22 is completely translocated across the microsomal membrane *in vitro* (see above), consistent with the observation that HBV core protein related polypeptides are secreted *in vivo*. We are confident that translocation occurred to completion, since the carboxy-terminal tail of p25 (the most protease sensitive part of the protein) was not removed in the protease protection assays and was not a substrate for the cytoplasmic modification enzyme(s). It is unlikely that the observed protease resistance was due to an interaction of the arginine-rich region with negatively charged phospholipid head groups, since no interaction of p25 with membranes was observed when microsomal vesicles were added posttranslationally (data not shown). Detection of completely translocated p22 is of particular interest, since the carboxy terminus of the protein comprises the nucleic acid binding domain and has a very high charge density (36 amino acids, 17 of which are arginines). It has been a long-standing debate whether translocation of a protein across the membrane occurs through a protein pore (i.e., a hydrophilic environment) or whether the chain passes directly through the hydrophobic interior of the lipid bilayer. Our findings clearly favor protein translocation in a nonlipid environment. Even if one assumes a completely stretched out conformation of p25 during translocation (i.e., only ~12 amino acids would be required to span the membrane), still, temporarily, up to eight arginine residues would need to reside simultaneously within the bilayer. Thus if translocation were to proceed directly through a lipid environment, this could only be achieved at a significant energy cost.

The most unusual aspect of the translocation of p25 is our observation that the translocation process can be aborted after the protein has been processed by signal peptidase. Existing evidence in eukaryotes suggests that the catalytic site of signal peptidase is localized on the luminal side of the membrane, i.e., signal peptidase activity is latent unless either the vesicles are dissolved in detergent or the substrate protein is translocated across the bilayer. It follows that p25 must have been at least partially translocated across the membrane to become processed. At some stage between processing and completion of protein synthesis the translocation process is aborted for a large fraction of the nascent polypeptide chains. We must assume that abortion of translocation is caused by some unusual characteristic of p25, since all other substrates tested (with the exception of integral membrane proteins) are completely translocated. It is possible that this unusual behavior is determined by some unknown feature of the signal peptide. However, we consider this possibility unlikely since the signal peptide behaves normally in its interactions with SRP or the translocation machinery before its cleavage. Therefore, if the signal peptide determines this behavior, we must assume that it has a yet undefined function in the translocation process after its cleavage by signal peptidase.

An alternative mechanism to explain the abortion of translocation of p22 would assume, in analogy with the process of the insertion of integral membrane proteins, that translocation is terminated in response to the recognition by the translocation machinery of "stop-transfer" sequences in p25 (Blobel, 1980; Yost et al., 1983). However, p25 does not contain sequence stretches hydrophobic enough to resemble "classical" stop-transfer sequences. Yet it is still possible that a sequence within nascent p25 could interact (albeit with poor affinity, thereby giving rise to only 80% efficiency of stop-transfer) with a site in the translocation apparatus that normally interacts with stop-transfer sequences and in doing so triggers the nascent protein to disengage from the translocation machinery. Whereas a membrane protein at this stage would attain a stable integrated configuration, p25 lacking sufficiently hydrophobic sequences would slip back into the cytoplasm.

A conceptually different alternative to explain the mechanism that causes aborted translocation, is that certain regions of p25 are simply difficult to translocate. We ruled out that such an interference could be caused by the charge clusters in the carboxy-terminal nucleic acid binding domain (see above). Although there are no other unusual features apparent in p25, the possibility remains that portions of the protein may fold before translocation into a very tight domain (such as, for example, the protease resistant portion of core protein) that is then translocation incompetent. In order for this mechanism to be plausible, there would need to be sufficient slack in the nascent polypeptide chain between ribosome and membrane to allow it to assume a folded structure. In other words, at some stage after signal peptidase cleavage elongation must be considerably faster than translocation, and the two respective processes may, in fact, not be as tightly coupled as generally assumed. Presently, we cannot distinguish between these various possibilities, but we hope that through the construction of appropriate fusion proteins we will be able to map the cause for the peculiar behavior of p25 to defined determinants within its primary sequence and thereby learn about the underlying molecular mechanism.

Signal peptidase cleavage in the absence of translocation was previously observed in other experimental situations involving posttranslational assays. Thus it was demonstrated that the signal peptide of maltose binding protein can be removed by purified bacterial signal peptidase that has been reconstituted into liposomes (Ohno-Iwashita et al., 1984). The protein in this case was not translocated into the lumen of the vesicles and the processed protein was not found associated with the lipid bilayer. Similarly, prepromellitin can be processed but not be translocated across mammalian microsomal membranes that have been trypsinized or alkylated (Zimmerman and Mollay, 1986). In both of these cases it is likely that parts of the translocation machinery required for the translocation event beyond signal peptidase cleavage were either absent or rendered nonfunctional. Thus both of these observations differ from the one described here performed with intact and unperturbed microsomal vesicles and that were shown to be co-translational. Yet they demonstrate that at least for some proteins the initial membrane insertion that leads to peptidase cleavage can be uncoupled from the subsequent translocation events.

We wish to thank Ms. Mila Dayanghirang for technical assistance, Dr. A.

Patthey for helping with the protein sequencing, Dr. W. Gerlich for communication of results before publication, D. Ganem, L. Lauffer, V. Siegel, and D. Standing for helpful discussions, and Drs. D. Ganem, N. Green, V. Lingappa, T. Rapoport, V. Siegel, D. Standing, and D. Zimmerman for critical reading of the manuscript.

P. D. Garcia receives a scholarship from the Weingart Program in Developmental Genetics. This work was supported by grants GM-32384 to P. Walter and AI-19744 to W. J. Rutter.

Received for publication 23 September 1987, and in revised form 9 November 1987.

Note Added in Proof: After submission of this manuscript, we learned that Bruss and Gerlich have also observed that the precore protein fails to be translocated (Bruss, V., and W. Gerlich. 1988. *Virology*. In press).

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