Hepatitis B Virus p25 Precore Protein Accumulates in *Xenopus* Oocytes as an Untranslocated Phosphoprotein with an Uncleaved Signal Peptide

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We have analyzed the translocation of hepatitis B virus (HBV) precore (PC) proteins by using *Xenopus* oocytes injected with a synthetic PC mRNA. The PC region is a 29-amino-acid sequence that precedes the 21.5-kDa HBV precore protein (p21.5) and directs the secretion of core-related proteins. The first 19 PC amino acids provide a signal peptide that is cleaved with the resultant translocation of a 22.5-kDa species (p22.5), in which the last 10 PC residues precede the complete p21.5 C polypeptide. Most p22.5 is matured to 16-20 kDa species by carboxyl-terminal proteolytic cleavage prior to secretion. Here we show that some four unexpected PC proteins of 24 to 25 kDa are produced in addition to the secretion products described above. Protease protection and membrane cosedimentation experiments reveal that all PC proteins behave as expected for proteins that are translocated into the lumen of the endoplasmic reticulum except for the single largest PC protein (p25), which is not translocated. Like p21.5, p25 is a phosphoprotein that localizes to the oocyte cytosol and nucleus, and protease digestion studies suggest that the two molecules have similar two-domain structures. Radiosequencing of immobilized p25 demonstrates that it contains the intact PC signal peptide and represents the unprocessed translation product of the entire PC/C locus. Thus, while many HBV PC protein molecules are correctly targeted to intracellular membranes and translocated, a significant fraction of these molecules can evade translocation and processing.

The core (C) gene of hepatitis B virus (HBV) encodes two closely related proteins by virtue of two in-frame ATGs located at its 5' end. Multiple viral transcripts (2, 8) allow the use of both ATGs, which lie 29 amino acids apart, separated by a region known as precore (PC). The first ATG specifies the 21.5-kDa HBV capsid or core (C) protein (p21.5), the sole structural protein of a viral nucleocapsid. The second ATG has the capacity to encode a 25-kDa protein (p25) containing the 29-amino-acid PC sequence fused to the amino terminus of p21.5. However, studies of *Xenopus* oocytes (34) and other systems (9, 19, 23, 24, 26, 38) have established that the primary product of expression of this locus is a 22.5-kDa protein (p22.5), comprising p21.5 preceded by the last 10 PC residues, which is translocated into the lumen of the endoplasmic reticulum (ER). The first 19 PC amino acids provide a cleavable signal peptide (9, 34) which directs this process. Subsequent proteolytic cleavages (34, 36) within the arginine-rich carboxyl-terminal 36 amino acids of p22.5 generate either a 16-kDa species or multiple proteins of 16 to 20 kDa, depending on HBV subtype (28), that are secreted as e antigen (HBeAg), a classic serum marker of HBV infection (17). Secretion of PC proteins via PC sequences is conserved even in the distantly related duck hepatitis B virus (30).

This simple HBeAg secretion model does not explain the fact that uncharacterized PC species of ~25 kDa have been detected sporadically in oocytes (34) and in mammalian cells (12, 13, 32, 33), nor does it explain why PC proteins have been detected in such uncharacterized locations as the cytosol (9), the nucleus (24), stuck in ER membranes (1), or in the plasma membrane (4, 14, 31). Such mislocation of PC proteins poses intriguing mechanistic questions and may have important biologic consequences. Although the PC region is not essential for the viral life cycle (3, 30), there is evidence that plasma membrane (22, 25, 31) or secreted (20) forms of PC proteins may influence the host immune response to HBV infection.

In this study, we have reexamined the expression of PC proteins in *Xenopus* oocytes programmed with a synthetic PC mRNA that directs the efficient translation of some 15 PC proteins, including a population of 24- to 25-kDa PC species. We have conducted a detailed investigation of the translocation, subcellular localization, and phosphorylation of these PC species. We show that all of the 15 PC species behave as translocated proteins except for a single 25-kDa protein that accumulates in oocytes as an untranslocated phosphoprotein. Protein sequencing reveals that p25 contains an uncleaved signal peptide and represents the unprocessed translation product of the PC/C open reading frame (ORF). A mutant PC protein that lacks the arginine-rich carboxyl-terminal protamine domain of p25 also accumulates as a 19-kDa unprocessed precursor, demonstrating that the protamine domain is not responsible for the partial failure to translocate. Our data emphasize the unusual secretory properties of HBV PC proteins.

**MATERIALS AND METHODS**

SP6 plasmids and in vitro transcription. The PC/C ORF occupies nucleotides (nt) 1817 to 2459 relative to the unique viral EcoRI site in the HBV genome (subtype adw) used in this work (37). To construct plasmid pSP64T-PC, a 787-bp FspI-HincII HBV fragment (nt 1806 to 2592) was ligated into BglII-linearized, filled-in plasmid pSP64T (15). Upon in vitro transcription (see below), pSP64T-PC gives a synthetic

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mRNA of ~1,060 nt in which the PC gene is embedded between the 5'– and 3'–flanking regions of the Xenopus β-globin gene which have been shown to enhance translation. Plasmid pSP64T-PC5 contains a previously described mutation (9) which introduces a stop codon after Val-178. This mutation was subcloned into plasmid pSP64T-PC so as to replace the parental sequence and create pSP64T-PC8. Plasmid pSP64T-C contains a minimal C gene Styl-I-Styl fragment (nt 1885 to 2466) cloned into pSP64T. Plasmid pSP64T-Tag (constructed by S. L. Zhou) contains a 2.7-kb BamHI-BglII fragment of simian virus 40 (SV40) DNA (including the SV40 T-antigen coding sequence) cloned into the BglII site of pSP64T. The plasmids containing the three different HBV surface antigen coding sequences will be described elsewhere. An SP6 plasmid containing the bovine preprolactin coding sequence was provided by P. Walter’s laboratory (University of California at San Francisco). Plasmids pSP64T-PC5 were purified by standard procedures followed by two rounds of cesium chloride banding. Capped synthetic transcripts were prepared from Sall-linearized DNAs, using SP6 polymerase (Boehringer Mannheim) and an in vitro transcription kit (Promega) as described previously (35). Transcription reaction mixtures were stored at -70°C until required and then thawed and injected into oocytes without further purification.

*Xenopus oocyte methodology*. Oocyte handling, microinjection, and labeling were performed by standard procedures (5, 6) as described previously (34, 35). A programmable microinjection system (Sutter Instruments, Novato, Calif.) was used in some experiments, allowing delivery of a very reproducible volume (~30 nl) into each oocyte cytoplasm. For metabolic labeling, batches of 10 to 50 oocytes were cultured for 12 to 36 h in 300- to 500-μl aliquots of modified Barth’s solution plus antibiotics (MBSH) supplemented with [35S]methionine and -cysteine (2 to 2 mCi/ml; [35S]-Translabel [ICN] or Expresslabel [NEN]) or carrier-free [32P]P (2 to 3 mCi/ml; NEN). Oocytes were homogenized in buffer (HB) containing 50 mM Tris hydrochloride (pH 7.5), 1% Nonidet P-40, and 10 mM EDTA. PC proteins were immunoprecipitated as described previously (34), using a commercial rabbit antiserum against antiserum (Dako), and resolved on 15% polyacrylamide–sodium dodecyl sulfate (SDS) gels unless otherwise stated. The gels were enhanced with 1 M sodium salicylate, dried, and exposed to X-ray film (Kodak) as described for individual experiments.

**Protease protection and membrane fractionation studies.** For protease protection studies, batches of ~30 oocytes (injected with either C or PC mRNA) were labeled for ~24 h in ~500 μl of MBSH containing either 0.5 mCi of [35S]-Translabel or 0.7 mCi of [32P], and then homogenized on ice in 800 μl of protease protection buffer (PPB), containing 10% sucrose, 150 mM NaCl, 2 mM KCl, 10 mM magnesium acetate, 200 mM Tris chloride (pH 7.5), and 1 mM dithiothreitol. The lysates were centrifuged for 1 min (Eppendorf microfuge; 15,000 x g) to remove yolk proteins, and then aliquots (100 μl) were incubated on ice for 3 h in a final volume of 170 μl containing 1X PPB plus 10 mM CaCl₂ and the appropriate additions of protease K and/or Triton X-100. Reactions were terminated by rapid addition of phenylmethylsulfonyl fluoride-SDS-Tris hydrochloride (pH 9.5) to final concentrations of 10 mM, 1%, and 100 mM, respectively, followed by immediate boiling for 5 min. Proteins were recovered by immunoprecipitation.

To separate the cytosolic and translocated forms of PC proteins by pelleting the membranes, 15 injected oocytes were [35S] labeled for 15 h and then homogenized in 600 μl of PPB. The lysate was centrifuged for 1 min (Eppendorf microfuge, 15,000 x g) to remove yolk, and a sample (50 μl) was retained as the unfraccionated (total) lysate. A further 50 μl of total lysate (~1.25 oocytes) was spun in a Beckman airfuge at 4°C for 20 min, using a 30° A-100 rotor (166,800 x g). The supernatant (cytosolic fraction) was carefully removed from the pellet (membrane fraction). The membrane pellet was resuspended in 200 μl of HB, and the total and cytosolic fractions were adjusted to a final volume of 200 μl with HB prior to recovery of PC proteins by immunoprecipitation and analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

For step gradient fractionation of cytosolic and membrane proteins as described by Colman (6), batches of 46 to 52 oocytes were injected with either C or PC mRNA, labeled for 12 h, and then homogenized in 600 μl of PPB. One third of this lysate was layered onto a gradient consisting of 1-ml steps of 20 and 50% (wt/vol) sucrose in PPB and centrifuged at 20,000 rpm (16,500 x g) for 30 min at 20°C in a TLA 100.3 rotor and TL100 centrifuge (Beckman). Fractions (~200 μl) were taken from the top of the gradient, and half of each fraction was analyzed by immunoprecipitation and SDS-PAGE. The cytosolic protein fractions (fractions 1 and 2, ~10% sucrose) from an identical gradient were pooled, layered onto a 2-ml linear sucrose gradient (15 to 60%), and centrifuged at 70,000 rpm (265,000 x g) for 30 min at 20°C in the TLA 100.3 rotor. Fractions (24) were collected from the top of the gradient, and every second fraction was analyzed by immunoprecipitation and SDS-PAGE.

**Nuclear and cytoplasmic fractionation.** Oocytes were injected with the appropriate mRNA, labeled for 15 h, and then manually enucleated as described previously (40). The isolated nuclei were rapidly freed from residual cytoplasm by repeated pipeting. Nuclear and cytoplasmic compartments from 11 to 12 oocytes were collected separately on ice in 400 μl of HB. Damaged nuclei were discarded along with their corresponding cytoplasm. Aliquots of lysate representing equal volumes of each compartment (either 5.5 nuclei or 0.5 cytoplasm) were subjected to immunoprecipitation to recover the proteins, which were analyzed by SDS-PAGE and autoradiography.

**Radiosequencing of PC proteins.** For the radiosequencing of p25 (p19 was sequenced by a very similar protocol), 30 injected oocytes were labeled for 30 h in 1.6 ml of MBSH containing 3 mCi of [35S]lysine (Amersham), homogenized in 1 ml of buffer containing 1% Triton X-100, 0.3% SDS, and 20 mM Tris chloride (pH 7.5), and immunoprecipitated by addition of 1 μl of rabbit anticore antisera. The PC protein–antibody complex was collected on Pansorbin (Calbiochem) and resolved by electrophoresis on a 12.5% SDS-gel. Electrophoretic transfer onto an Immobilon-P polyvinylidene difluoride membrane (Millipore) was carried out at 0.25 A (50 V) for 2 h in transfer buffer (25 mM Tris base, 192 mM glycine, 15% methanol). The membrane was briefly rinsed with water, air dried, and tape to a paper sheet. The paper was taped to X-ray film, keyed to the film with a needle, and exposed for 2 h at room temperature. The portion of membrane containing the highest-molecular-mass p25 PC species was precisely located from the autoradiogram, excised, and subjected to multiple cycles of Edman degradation reactions (with the membrane providing the solid support) to determine the amino-terminal sequence of the immobilized p25 protein. The eluate from each cycle was collected and counted in a liquid scintillation counter after addition of scintillation cocktail.
The lysates (lanes 1 to 3) or media (lanes 4 to 6). Oocytes were injected with undiluted (lanes 1 and 4) or eightfold-diluted PC mRNA (lanes 2 and 5) or with no RNA (lanes 3 and 6). The 3S-PC proteins recovered from approximately three oocytes were loaded onto each lane. The autoradiogram was exposed for 12 h in the presence of an intensifying screen at -70°C. Positions of prestained molecular mass markers (in kilodaltons) are indicated at the right, and positions of the p24-25, p22-23, and p16-20 size classes of PC proteins are shown at the left.

RESULTS

Expression of PC proteins in frog oocytes. Synthetic PC mRNA directs the production of a complex and variable pattern of both intracellular and secreted 3S-PC proteins in metabolically labeled oocytes (Fig. 1). Up to 15 intracellular 3S-PC proteins, falling into three main size classes, are immunoprecipitated from the lysates of oocytes injected with undiluted (lane 1) or eightfold-diluted (lane 2) PC mRNA but not from control lysate (lane 3). The corresponding immunoprecipitable PC proteins secreted into the medium surrounding the oocytes are shown in lanes 4 to 6.

For the intracellular PC proteins, the largest size class (p24-25) contains two discrete species of ~24 and 25 kDa in this experiment (lane 2), but other experiments reported here suggest that there are two additional ~24-kDa proteins in this size class. The middle size class (p22-23) contains two main species of ~22 and 23 kDa (lane 2), but two minor, slightly smaller species of around 21.5 kDa are also seen (one of which may be the p21.5 C protein; see below). The 22- to 23-kDa species were previously collectively characterized as translocated proteins that lack the 19-amino-acid PC signal peptide (34). The exact relationship between p22 and p23 in oocytes is unknown, but one or other of these species certainly represents the initial PC translocation product (predicted molecular mass of 22.5 kDa). The smallest size class (p16-20) contains at least seven distinct translocated proteins of 16 to 20 kDa that are believed to be derived from p22-23 by cleavages within the carboxyl-terminal premate domain (34).

The approximately eight secreted HBeAg proteins immunoprecipitated from the medium surrounding PC RNA-injected (Fig. 1, lanes 4 and 5) but not control (lane 6) oocytes contain members of the p22-23 and p16-20 PC size classes. Even after 2 days, only low levels of HBeAg proteins are seen in the oocyte medium, and the secretion of PC proteins is very inefficient compared with the secretion of prolactin (data not shown). Despite the abundance of intracellular p24-25 species in many experiments, we never observe these proteins secreted into the medium. One curious feature of HBeAg secretion in oocytes is that there is no obvious linkage between the level of intracellular and secreted PC proteins. Thus, the substantial increase in the level of intracellular PC proteins seen in lane 1 versus lane 2 is not accompanied by any obvious increase in HBeAg secretion (cf. lanes 4 and 5), although there is a shift in the pattern of HBeAg proteins.

Comparing these data with our previous work, we note that the profiles of PC proteins observed appear similar except that the p24-25 species were previously seen infrequently (e.g., see Fig. 1 in reference 34) and thus escaped characterization but are observed consistently in the present experiments. This difference is probably related to the greater than 20-fold increase in PC protein expression observed with the improved Sp6 construct used in this work (data not shown). Comparison of the PC proteins generated by higher (Fig. 1, lane 1) or lower (lane 2) amounts of injected PC RNA provides support for this notion; elevated PC mRNA levels cause an increase in both the p24-25 and p22-23 PC species, but the major increase occurs in the p24-25 size class. The present Sp6 PC construct is based on the vector pSP64T (15) and generates an RNA of ~1,000 nt containing a minimal PC coding sequence embedded between the -70-nt 5'-flanking and the -200-nt 3'-flanking sequence of β-globin, which serve to augment translation (15). The earlier construct generated a much longer transcript (~3 kb) which lacks the β-globin flanks.

Phosphorylation of C and PC proteins. An initial goal of this work was to determine whether we could detect the cytoplasmic forms of p22-23 seen in vitro, where as much as of 70 to 80% of the p22 is initially translocated across the ER membrane but is subsequently released back into the cytosol, despite removal of the signal peptide (9). In oocytes, our protease protection experiments (34) had suggested that most p22-23 is translocated, but such experiments are hard to quantitate and some cytosolic p22-23 (perhaps up to 30% of the total) could have been missed in this analysis.

Since the cytosolic capsid protein p21.5 is a phosphoprotein (27), we decided to test whether phosphorylation might provide a specific marker for cytosolic PC proteins. Oocytes were injected with C or PC mRNA and labeled with either 32P or [35S]methionine and [32S]cysteine, and the autoradiographic profile of labeled proteins was compared following immunoprecipitation and SDS-PAGE (Fig. 2). The p21.5 capsid protein is labeled by both 35S (lane 1) or 32P (lane 4), as expected. The profile of 35S-PC proteins (lane 2) displays strong bands of p24-25 and p22-23, with a small amount of p16-20. To our surprise, the only 32P-labeled PC protein comigrates with p25 (lane 3). Much longer exposures (data not shown) reveal traces of 32P species that comigrate with p23 and p21.5, but p24 and p22 are not labeled.

Assuming that phosphorylation occurs mainly in the cytosol (or nucleus), these data imply that most p22-23 proteins are translocated but that a significant fraction of p25 appears to be cytosolic. These conclusions are validated by more direct methods (see below).

Protease protection analysis of the topography of C and PC proteins. To clarify the topography of PC proteins with respect to the ER membrane, we prepared oocyte sucrose lysates (containing a mixture of intact membrane vesicles plus cytosolic and nuclear components) and then added protease K, allowing us to distinguish PC species that are sequestered within ER membranes (translocated), and thus protected from protease, from cytosolic PC species that are accessible to protease (Fig. 3).

Since core-related proteins are intrinsically resistant to proteolysis (9, 34), we first performed control protease
digestion experiments on sucrose lysates containing the 35S-labeled p21.5 capsid protein (Fig. 3A) which is found in the oocyte cytosol and nucleus (40). The 21.5-kDa C protein (lane 1) is cleaved by proteinase K (lanes 2 and 4) into about five protease-resistant species with an average molecular mass of 14 kDa that are not fully degraded by 730 µg of protease per ml (lane 4) or when the nonionic detergent Triton X-100 is present during proteolysis (lane 3). In contrast, 32P-labeled p21.5 (lane 5) is completely protease sensitive in the absence (lane 6) or presence (lane 7) of detergent. Since phosphorylation of p21.5 in oocytes is restricted to the 36-amino-acid proteaminelike region found at the p21.5 carboxyl terminus (33a), the differential protease sensitivity of the 32P- and 35S-labeled molecules can readily be explained in terms of a two-domain capsid protein structure. The Arg-rich proamine region is readily digested by proteases (eliminating the 32P label), while the remainder of the molecule (120 to 149 residues) forms a tightly folded, protease-resistant structure which carries [35S]Met and [35S]Cys residues.

We next investigated the effect of proteinase K on sucrose lysates prepared from PC oocytes (Fig. 3B). Prominent 32P bands corresponding to the p24-25 and p22-23 size classes are immunoprecipitated from untreated PC lysate (lane 1) but not from control lysate (lane 4). These PC species differ sharply in their response to protease (lane 2), which leaves p22-23 untouched but selectively eliminates most of the upper (p25) portion of the p24-25 band, disclosing an underlying minor population of protease-resistant PC species of ~24 kDa. Cleavage of 35S-p25 yields ~14-kDa protease-resistant fragments that comigrate with the equivalent p21.5 fragments (Fig. 3A, lanes 2 and 4) and a small amount of a ~19-kDa fragment. Both p22-23 and p24 are rendered protease sensitive upon detergent disruption of membranes (lane 3), suggesting that they are indeed translocated. The only significantly 32P-labeled PC species (lane 5) clearly aligns with p25 (the prominent p22-23 species seen in lane 1 are not phosphorylated), and 32P-p25 is degraded by protease in the absence (lane 6) or presence (lane 7) of detergent, suggesting that this species is not translocated.

Thus, these data confirm the phosphorylation data reported above and provide direct evidence that most or all of the 32P species is untranslocated, in contrast to p22-23 and p24, which are clearly translocated and thus inaccessible to proteases. The results further suggest that p21.5 and p25 possess the same basic two-domain protein structure in which the 36-amino-acid proamine region protrudes from a tightly folded, protease-resistant domain comprising most of the first 149 amino acids of the C-protein sequence.

**Membrane cosedimentation experiments.** An independent topographical analysis was performed by centrifuging sucrose lysates from 35S-PC oocytes so as to pellet intact membrane vesicles with their complement of membrane-associated PC proteins while leaving the bulk of the cytosolic proteins in the supernatant. The 35S-PC proteins present in the unfractonated lysate, the cytosolic fraction, and the membrane fraction were compared following immunoprecipitation and SDS-PAGE (Fig. 4). The total lysate (lane 1) contains similar amounts of p22-23 and p24-25 species (with a distinct upper band of p25), along with small amounts of p16-20. Comparing the total lysate with the cytoplasmic (lane 2) and membrane (lane 3) fractions, it is apparent that the bulk of the p22-23 and p16-20 species cosediment with the membranes, but the p24-25 species show a divided localization pattern. Some three diffuse bands, representing smaller (~24-kDa) members of the size class, cosediment with the membranes (lane 3), but the sharp p25 band fractions cleanly with the cytosolic proteins (lane 2).

In a second type of cosedimentation experiment (Fig. 5), we centrifuged crude 35S-PC oocyte sucrose lysates through steps of 20 and 50% sucrose under conditions in which cytosolic proteins float in the 10% sucrose at the top of the gradient while membrane vesicles sediment to the interface between sucrose layers (6). The cytosolic PC species were reanalyzed on a 10 to 60% sucrose gradient to determine whether they were present in nonparticulate or particulate form.

As a control, we analyzed the behavior of 35S-p21.5 C...
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Fig. 4. Analysis of the PC proteins present in unfractionated (lane 1) oocyte lysate or in cytosolic (lane 2) or membrane (lane 3) fractions. The procedures used for membrane pelleting and for preparation of the different fractions are described in Materials and Methods. Each lane shows the 35S-PC proteins recovered from the equivalent of one oocyte. The dried gel was exposed to X-ray film for 1 week at room temperature. Positions of prestained protein markers (in kilodaltons) are depicted at the right; positions of the different size classes of PC proteins are indicated by arrows at the left.

For PC proteins (Fig. 5C), most of the p22-23, p24, and p16-20 comigrate with the membranes (lanes 6 and 7) on the sucrose step gradient. Only small amounts of PC proteins are in the cytosolic fractions (lanes 1 and 2). In comparison with the membrane PC proteins, the cytosolic species are heavily depleted in p22-23 and p24 but enriched in p25 (which was relatively weak in this experiment) and in a species of ~21.5 kDa, which is a very minor product of PC expression that may in fact be the capsid protein. On the second gradient (Fig. 5D), these cytosolic PC proteins band mainly in the upper gradient fractions (lanes 1 to 5) with no clear particle peak.

In summary, the two different cosedimentation experiments confirm that p25 is an untranslocated protein. The remaining PC proteins, including approximately three types of p24 proteins, behave as membrane-associated (presumably translocated) species. Both experiments show clearly that only a minor fraction of the total p22-23 (~5%) is found in the cytosolic fraction, and we cannot rule out the possibility that this material arose by leakage from ruptured membrane vesicles. The cytosolic PC species are not incorporated into particles, but since p21.5 particle formation is highly dependent on p21.5 concentration (41), this result may simply reflect insufficient PC protein levels (which are much lower than those observed for p21.5) rather than inability to form particles.

Partitioning of PC proteins between the nucleus and cytoplasm. Two recent reports show that the protamin-like domain at the carboxyl termini of C and PC proteins contains two potential nuclear localization signals, which reportedly direct the nuclear accumulation of PC proteins in cultured cells (7, 39). To determine whether p25 or p22-23 species accumulate in the oocyte nucleus, we manually divided oocytes into nuclear and cytoplasmic fractions (the latter contains both the cytosol and the membrane-enclosed secre-

Fig. 5. Sucrose gradient analysis of C (A and B) and PC (C and D) proteins. For experimental details, see Materials and Methods. Sucrose lysates derived from ~16 C (A) or PC (C) oocytes were first fractionated on step gradients to separate the cytosolic proteins (lanes 1 and 2) from the membrane fractions (lanes 6 and 7). The 10 lanes represent the 10 fractions from the top (10% sucrose; lane 1) to the bottom (50% sucrose; lane 10) of the gradient. The pooled cytosolic fractions were analyzed on 15 to 60% sucrose gradients (B and D) to determine whether C (B) and PC (D) proteins were assembled into particles. The autoradiograms show the immunoprecipitated proteins present in every second fraction from the first 20 (out of 24) fractions (top of gradient corresponds to lane 1). Autoradiography was for 2 days at room temperature (A and C) or 3 days at -70°C with a screen (B and D). Positions of p21.5 and the p22-23 and p24-25 species are indicated in the center.
The protein.

We favored the notion that p25 represents a posttranslationally modified version of the cytoplasmic p22-23 species that result, at least in vitro, from aborted translocation (9).

These two possibilities were distinguished by radiosequencing the amino terminus of immobilized [35S]Cys-labeled p25, using an adaptation of the method described by Matsudaira (18). The intact signal peptide should generate a characteristic [35S]Cys signature in cycles 7, 12, and 14, but a species that lacks the 19-amino-acid PC signal peptide should display only one [35S]Cys peak at cycle 4 (Fig. 7A).

Immunoprecipitated PC proteins from [35S]Cys-labeled oocytes were resolved by SDS-PAGE and electrophoblated onto a polyvinylidene difluoride membrane. The p24-25 region, detected by autoradiography, displayed the characteristic pattern of a relatively sharp p25 band above diffuse p24 species. The membrane segment containing p25 was carefully excised and subjected to 21 Edman degradation cycles. The radiosequenese profile (Fig. 7B) displays [35S]Cys peaks in cycles 7, 12, and 14, demonstrating beyond doubt that p25 contains the PC signal peptide.

The 36-amino-acid proteamlike region at the carboxy terminus of p25 contains 17 Arg residues and is an obvious candidate for an element that might interfere with PC translocation. However, a PC8 mutant that precisely lacks the protamine region directs translation (Fig. 7C) of a major ~19-kDa protein (p19, roughly the size expected for an unprocessed PC8 protein) and a ~16-kDa protein (p16, roughly the size expected for a molecule that lacks the signal peptide). Production of p19, which was not seen in our earlier study (34), again appears dependent on the use of a translationally efficient SP6 vector. The radiosequencing strategy described for p25 was therefore used to examine the amino terminus of [35S]Cys-labeled p19 (Fig. 7C). After 24 cycles of Edman degradation, the resulting profile displays Cys residues in cycles 7, 12, 14, and 23, confirming the presence of an intact signal peptide.

Thus, the full-length translation products of both the PC and PC8 mRNAs accumulate in the cell as preproteins with uncleaved signal peptides. Evidently the Arg-rich protamine region of p25 is not the element responsible for keeping p25 untranslocated.

**DISCUSSION**

In this report we have reanalyzed the HBeAg secretion pathway, using *Xenopus* oocytes injected with a synthetic PC mRNA. Most of our data are consistent with the generally accepted model for HBeAg secretion (see introduction) and confirm the existence of translocated p22-23 and p16-20 species that are ultimately secreted (particularly p16-20) into the oocyte medium as HBeAg. However, this simple secretion model does not account for all of the approximately 15 intracellular PC proteins seen in this work and, in particular, provides no explanation for the existence of approximately four PC proteins of 24 to 25 kDa, larger than the largest anticipated PC species (p22-23). These p24-25 species were detected sporadically in our previous studies (34) but are seen consistently here. We believe that accumulation of intracellular p24-25 proteins requires efficient expression of PC proteins (cf. lanes 1 and 2 in Fig. 1), and the synthetic mRNA used in this work, which contains a minimal PC coding embedded within the translationally enhancing β-globin flanking sequences (15), directs more efficient translation than our earlier mRNA.

Here we report the characterization of the single largest PC species (p25) seen in oocytes. Much to our surprise, protein sequencing reveals that p25 contains an intact PC...
signal peptide and represents the unprocessed translation product of the entire PC/C ORF. A mutant PC protein (PC8) that precisely lacks the protamine domain also accumulates as a precursor protein (p19) with an uncleaved signal peptide in oocytes. Despite the presence of the uncleaved signal peptide, p25 is not translocated into the lumen of the ER and does not appear to associate with intracellular membranes. Untranslocated p25 has properties that are closer to those of the p21.5 capsid protein than to those of the remaining PC proteins. Thus, p25, like p21.5, is a phosphoprotein that is found in the oocyte cytosol and nucleus. Moreover, protease digestion studies (Fig. 3) reveal that p21.5 and p25 possess very similar two-domain structures, in which the 36-amino-acid protamine region protrudes from a tightly folded protease-resistant domain.

Five lines of evidence confirm the topographical difference between untranslocated p25 and the remaining PC proteins, including three or so species of ~24 kDa, which are translocated. Protease protection analysis (Fig. 3) and two different membrane sedimentation experiments (Fig. 4 and 5) provide direct evidence that only p25 escapes segregation into membrane vesicles. Nuclear/cytoplasmic fractionation studies reveal that p25, like the p21.5 capsid protein (40), behaves as a protein that equilibrates passively between the cytosol and the nucleus. All other PC species, as well as unrelated secreted proteins, segregate with the cytoplasm, as expected for proteins confined within the membrane boundaries of secretory organelles located in the cytoplasmic compartment. Finally, p21.5 and p25 are phosphoproteins, but the translocated PC species are not, suggesting that phosphorylation is largely restricted to the oocyte cytosol (or possibly the nucleus).

Although cleavage of the PC signal peptide is usually efficient in transfected animal cells, there have been several reports of uncharacterized ~25-kDa PC species (11, 12, 13, 32, 33). We speculate that high-level PC protein expression may again be responsible for the phenomenon of p25 accumulation in these cases. In yeast (21) and insect (16) cells, in which PC protein expression levels are extremely high, p25 is the sole PC product, although insect cells, in particular, secrete proteins efficiently under the direction of mammalian signal peptides (16). Frog oocytes appear to give results that are intermediate between these systems.

This work was initiated in part to look for the cytosolic forms of p22-23 that result in vitro from aborted translocation (9), but our cosedimentation and phosphorylation experiments suggest that there is only a minor population of such species in oocytes, making it difficult to further characterize them or to rule out the possibility that they arise by leakage from damaged membrane vesicles. Despite this difference, the oocyte and in vitro systems agree on two important points. First, the translocation of PC proteins seems to pose a difficult problem for the cellular secretion machinery. Second, cytosolic PC proteins, albeit different ones, are found in both systems.

At present we know relatively little either about the biological role of PC proteins or about the expression of p25 and other PC proteins during different types of HBV infection, making it difficult to assess the potential significance of untranslocated p25 with respect to the biology of HBV. However, we can propose two possible implications of our findings. First, recent studies in transfected mouse fibroblasts have shown that p25 can become incorporated into chimeric nucleocapsids and virions (33) which display diminished stability and reduced plus-strand DNA synthesis compared with their wild-type counterparts. We speculate that this incorporation of untranslocated p25 proteins into HBV particles may serve as a mechanism to modulate virion production or infectivity, perhaps during specific stages of HBV infection or in response to factors such as the host...
immune response. Second, PC proteins have been shown to become localized to the outer membrane of cultured animal cells (29, 31). Similar proteins in hepatocytes may serve as the target for the immune response to HBV (22, 25). Perhaps the accumulation of untranslocated p25 represents another manifestation of some unusual feature of the translocation pathway for PC proteins that also serves to divert a fraction of these proteins to the plasma membrane.

The existence of cytosolic unprocessed p25 is highly intriguing from the point of view of translocation mechanism. We note that preprolactin is expressed at higher levels than PC proteins in oocytes, yet signal peptide cleavage, translocation, and mature prolactin secretion are all so highly efficient that no unprocessed preprolactin is detected (e.g., Fig. 6), suggesting that the oocyte has ample excess translocation capacity for typical secreted proteins. The partial translocation observed for PC proteins has little precedent as far as we are aware. At present we do not know whether p25 translocation was initiated and subsequently aborted, or whether the cytosolic p25 has eluded recognized by the cellular translocation machinery. Further studies are in progress to address the mechanistic basis for the accumulation of untranslocated p25 and to determine whether specific p25 sequences play a role in this process.

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