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Transport of Hepatitis B Virus Precore Protein into the Nucleus after Cleavage of Its Signal Peptide

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The precore and core proteins of hepatitis B virus have identical deduced amino acid sequences other than a 29-residue amino-terminal extension (precore region) on the precore protein. The first 19 of these residues serve as a signal sequence to direct the precore protein to the endoplasmic reticulum, where they are cleaved off with formation of precore protein derivative P22 for secretion. In this report, we show that P22 can alternatively be transported into the nucleus following signal peptide cleavage. Experiments with deletion mutants indicated that this nuclear transport proceeds via the cytosol and is dependent on the amino-terminal portion of P22. Thus, the hepatitis B virus precore protein is a secreted, cytosolic, and nuclear protein.

Hepatitis B virus (HBV) is a small, enveloped DNA virus. The nucleocapsid of the virus comprises the DNA genome, reverse transcriptase activity, and the core protein (P21c). In the HBV genome, the core protein-coding sequence is preceded by an in-phase open reading frame termed the precore region. Translation initiating from the initiation codon of the precore region produces a protein (precore protein) that has the entire core protein sequence plus a 29-residue amino-terminal extension (for a review, see reference 7).

It has been demonstrated that the first 19 residues of the precore region form a signal sequence to direct the precore protein to the endoplasmic reticulum (ER), where they are cleaved off with formation of precore protein derivative P22 (2, 8, 12, 15, 17, 19, 25). P22 is then secreted through the ER and Golgi apparatus (15), with further proteolysis at the arginine-rich carboxy terminus (17). This results in secretion of a heterogeneous population of P22 derivatives serologically defined as e antigen.

As with most secreted proteins, translocation of P22 across the ER membrane is mediated by signal recognition particles (8). However, our previous in vitro studies showed that translocation of P22 is inefficient. After cleavage of the signal peptide, only 30% of P22 was completely translocated into microsomal vesicles and the rest of it was released back into the extravesicular aqueous phase (8).

To confirm this unusual behavior of P22 in intact cells and to understand further its mechanism, we studied the expression of P22 and the core protein in cultured cells. The results presented in this report show that P22, after removal of its signal sequence, can be released from the ER into the cytosol and translocated into the nucleus. Studies with deletion mutants of precore protein showed that this nuclear translocation is dependent on a portion of the precore region, which therefore may function as a nuclear localization signal.

MATERIALS AND METHODS

Construction of DNA plasmids. For construction of pECE-PC, site-directed mutagenesis (17) was used to generate a *Hin*dIII site at nucleotide 1757 (sequence TAGGTT to AAGCTT) of the HBV genome (24). The *Hin*dIII-*Bgl*II HBV DNA fragment containing the entire genomic information was then inserted into the *Hin*dIII-*Bam*HI polylinker site of pSP64 (Promega Biotec) to generate pSP64-PC. The *Hin*dIII-*Sst*I fragment containing the entire HBV sequence was then reisolated from pSP64-PC and cloned into the *Hin*dIII-*Sst*I site of simian virus 40 (SV40)-derived vector pECE (6). The construction of pECE-C was essentially identical to that of pECE-PC, except that the *Hin*dIII site was generated at nucleotide 1821 (sequence AACTTT to AAGCTT), one base pair downstream from the precore ATG.

For construction of pECE-OU3, site-directed mutagenesis was used to delete the sequence 5′ TGCCTTGGGTGG CTTTGGGGC3′. This sequence comprises codons 23 to 29 of the precore region. For construction of pECE-OU4, a silent mutation was created at nucleotide 1889 to generate an *Ava*I site (sequence CTTGGG to CTCGGG). Subsequently, the sequence between the *Hin*dIII and *Ava*I sites was deleted and replaced with the following sequence:

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5′AGCTTCGCACCAGCACCATGTCCAAGCTGTGCC3′
    3′AGCGTGGTCGTGGTACAGGTTCGACACGGAGCC5′
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Thus, in pECE-OU4, the entire signal sequence, except the initiation codon of the precore protein, was deleted.

For construction of pECE-HGH, the *Bam*HI-*Eco*RI fragment containing the human growth hormone-coding sequence was isolated from p0GH (Nichols) and cloned into the *Bgl*II-*Eco*RI site of pECE.

Cell lines and DNA transfections. COS-7 and CV-1 cell lines were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The standard $CaPO_4$ precipitation method (10) was used to transfect cells. Cells were glycerol shocked for 2 min at 4 to 6 h after transfection. For the establishment of the PC/C10 cell line, subconfluent CV-1 cells were cotransfected with pECE-PC, pECE-C, and p007LTR (16); p007LTR expresses the neomycin resistance gene. More than 10 stable transfectants resistant to the neomycin analog G418 were selected and expanded by using our previously described procedures (16). One of the clones, named PC/C10, was found to express similar levels of P22 and P21c and, thus, was used for further studies.

Subcellular fractionation and radioimmunoprecipitation. Cells infected by recombinant viruses were analyzed 60 to 72 h postinfection, and cells transfected by DNA were analyzed at 48 h posttransfection. Before subcellular fractionation,

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cells were starved for methionine for 2 hours and labeled with [^{35}S]methionine for 1 h. The medium was mixed with 2 volumes of RIPA buffer (10 mM Tris [pH 7.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). The cells were lysed with 10 mM Tris hydrochloride (pH 7.0–150 mM NaCl, 0.5% Nonidet P-40 (lysis buffer) and centrifuged at 3,000 × *g* for 1 min. The supernatant (cytoplasmic fraction), after centrifugation at 15,000 × *g* for 10 min, was mixed with an equal volume of RIPA buffer and used for immunoprecipitation. The nuclear pellet was washed once in lysis buffer and then resuspended in RIPA buffer for immunoprecipitation. Anticore protein serum was raised in rabbits against both native and SDS–2-mercaptoethanol-denatured core protein expressed in *Escherichia coli* (gift of D. Chien, Chiron Corp.). This antiserum has similar affinities for both precore and core proteins, as demonstrated by immunoprecipitation experiments using in vitro-synthesized, [^{35}S]methionine-labeled precore and core proteins (data not shown). The anti-growth hormone serum was a gift of K. R. Sharma (University of California, San Francisco).

Radiosequencing of nuclear P22. Six 60-mm-diameter plates of COS cells infected with SV-PC (15) were labeled with either 1 mCi of [^{35}S]methionine or 2 mCi of [^{3}H]leucine for 1 h. After subcellular fractionation and immunoprecipitation, P22 was purified on a 12.5% polyacrylamide gel. The protein was eluted in 10 mM NH_4HCO_3–0.02% SDS at room temperature overnight and radiosequenced at the Biomolecular Resource Center at the University of California, San Francisco, by standard techniques.

Immunofluorescence. Cells grown on cover slips were transfected by DNA plasmids. At 48 h posttransfection, the cells were fixed in acetone at −20°C for 2 min. Rabbit anti-core protein (1:100 dilution) and rhodamine-conjugated goat anti-rabbit (1:20 dilution; Boehringer Mannheim) sera were used as the primary and secondary antibodies, respectively.

RESULTS

Expression of core and precore proteins in COS cells. We have previously produced two recombinant SV40 viruses, SV-C and SV-PC (15), in whose genomes the coding sequences of the SV40 early genes were replaced with those of the HBV core and precore genes, respectively. This places the expression of the precore and the core proteins under control of the SV40 early promoter. Since these recombinant viruses contain the late region of SV40, they can be propagated in COS-7 cells, a transformed monkey kidney cell line expressing the early genes of SV40 (9). We therefore used these two viral vectors to study expression of the core and precore proteins. After infection with SV-C, core protein (P21c) was detected after radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis (Fig. 1). Subcellular fractionation revealed that P21c was localized in the cytoplasm but not in the nucleus (Fig. 1, lanes 3, 6, and 9).

Similarly, SV-PC-infected cells synthesized precore protein in the form of P22 (Fig. 1, lane 5), confirming previous reports that its signal peptide is cleaved off cotranslationally (see also below). Furthermore, as expected, P22 and its proteolytic products were also secreted into the medium (Fig. 1, lane 8). However, fractionation studies revealed that approximately 15% of cell-associated P22 was present in the nuclear fraction rather than the cytoplasmic fraction (Fig. 1, lanes 2 and 5). This is unlikely to be caused by aggregation of P22 into insoluble forms which subsequently copelleted with the nuclei, since core protein and mutant OU3 (see below), which are similar in sequence to P22, were not detected in the nuclear fraction. Furthermore, parallel studies revealed that another secretory protein, human growth hormone, was solely cytoplasmic (Fig. 1, lanes 12 and 14).

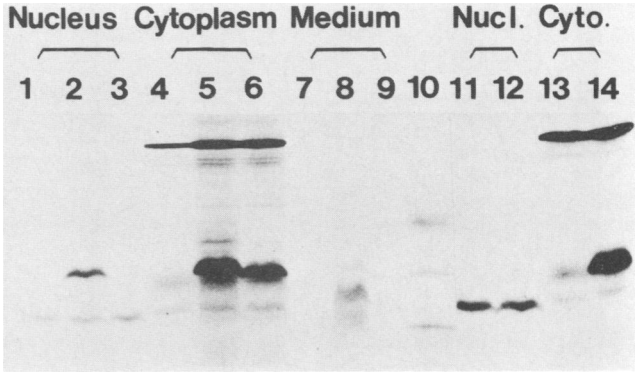

FIG. 1. Subcellular fractionation of precore and core proteins expressed in COS-7 cells. COS-7 cells were mock infected (lanes 1, 4, and 7), infected with SV-PC (lanes 2, 5, and 8) or SV-C (lanes 3, 6, and 9), and divided into nuclear and cytoplasmic fractions. These fractions and the culture medium were subjected to radioimmunoprecipitation with anti-core protein serum and electrophoresed on a 12.5% polyacrylamide gel. Lane 2 shows the P22 band in the nuclear fraction of SV-PC-infected cells. As a control, COS-7 cells were transfected with pECE-HGH, a plasmid expressing human growth hormone (lanes 12 and 14), or mock transfected (lanes 11 and 13), fractionated, and analyzed by radioimmunoprecipitation with anti-growth hormone serum. Lane 12 shows that there was no detectable cross-contaminating growth hormone in the nuclear fraction. (Mature growth hormone has a molecular mass of ~21 kilodaltons [5]). Lane 10 shows molecular mass standards (top to bottom: full-length precore protein, 25 kilodaltons; core protein, 21 kilodaltons; truncated precore protein, 16 kilodaltons). These standards were synthesized in vitro by using reticulocyte lysates and RNA transcripts produced with SP6 RNA polymerase (17).

To confirm the results presented in Fig. 1, we performed immunofluorescence studies. Two DNA plasmids, pECE-PC and pECE-C (20), were constructed by inserting an HBV DNA fragment, starting 60 base pairs upstream (PC construct) or 1 base pair downstream (C construct) from the precore ATG and ending at the *Bgl*II site immediately after the HBV polyadenylation site, into SV40-derived vector pECE (6). In these DNA constructs, expression of the HBV sequence is regulated by the SV40 early promoter. COS cells transfected by pECE-C displayed only cytoplasmic fluorescence (Fig. 2A and B); for pECE-PC-transfected cells, nuclear and cytoplasmic fluorescence was observed in most cells, although cytoplasmic staining was predominant in most of those cells (Fig. 2C). In a few cases, however, nuclear staining was similar in intensity to cytoplasmic staining in pECE-PC-transfected cells (Fig. 2D). Immunofluorescence studies with SV-C- and SV-PC-infected cells yielded similar results (data not shown).

Thus, the results presented in Fig. 1 and 2 suggest that after signal sequence cleavage in the ER lumen and chain termination, P22 can be transported into the nucleus, presumably via the cytosol.

Lacks of the signal sequence in nuclear P22. Although nuclear P22 comigrated with cytoplasmic P22 on SDS-polyacrylamide gel, the possibility that nuclear P22 is a different proteolytic cleavage product of the precore protein cannot be ruled out. Therefore, we radiolabeled SV-PC-infected cells with either [^{35}S]methionine or [^{3}H]leucine, gel

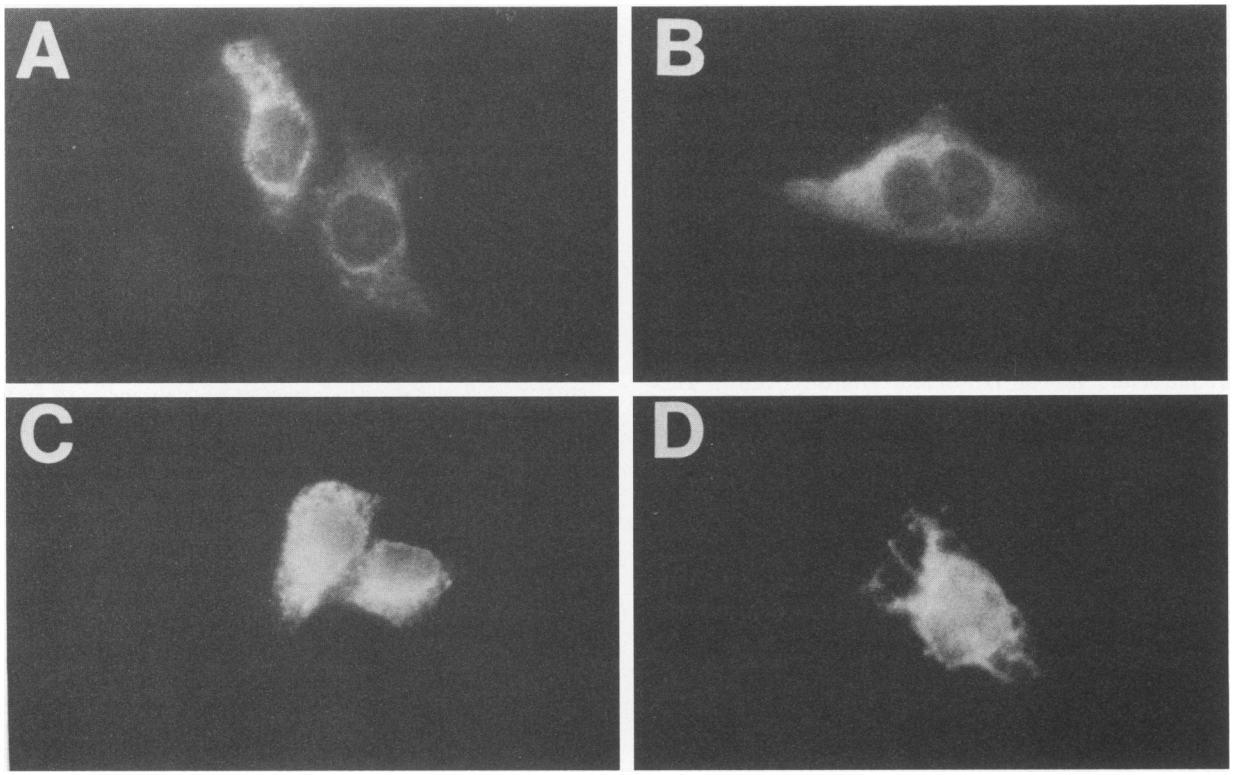

FIG. 2. Immunofluorescence studies of COS cells transfected by pECE-C and pECE-PC. Panels A and B show cells transfected by pECE-C. Panel B shows the staining of a binucleate cell; panels C and D show cells transfected by pECE-PC. Exposure times during photography and printing were identical to eliminate photographic artifacts.

purified the labeled P22 from the nuclear fraction, and performed radiosequencing. With either label, the amino terminus of nuclear P22 was unambiguously localized at Ser-20, i.e., the same as for secreted P22 (Fig. 3) (8, 17). This demonstrates that the signal sequence of nuclear P22 has been cleaved off.

Enhancement of nuclear transport of P22 by deletion of the precore signal sequence. To understand the mechanism that regulates nuclear localization of P22, we created a deletion mutant plasmid, pECE-OU4, from plasmid pECE-PC. In pECE-OU4, the entire signal sequence, except Met-1 of the precore protein (Gln-2 to Ala-19), was deleted (Fig. 3C). This DNA construct was transiently transfected into COS-7 cells for expression studies, and the expressed product was analyzed by radioimmunoprecipitation.

Control transfections with plasmids expressing either precore (pECE-PC) or core (pECE-C) protein confirmed that P22 but not P21c can be translocated into the nucleus (Fig. 4, lanes 2, 3, 7, and 8). Deletion of the precore signal sequence in pECE-OU4 enhanced the amount of P22 in the nuclear fraction (lanes 5 and 9) up to about 30% of the total P22 expressed. Presumably, the increased nuclear localization resulted from lack of loss of mutated P22 through translocation into the ER lumen.

Transport of P22 into the nuclei of pECE-OU4-transfected cells was further confirmed by immunofluorescence studies. Nuclear staining was detected in most of the transfected cells (Fig. 5A and B). This indicates that P22 can be actively transported into the nucleus. Interestingly, some of the cells transfected by pECE-OU4 showed both cytoplasmic and nuclear staining (Fig. 5C) or even predominant cytoplasmic fluorescence (Fig. 5D). This result is reminiscent of clinical

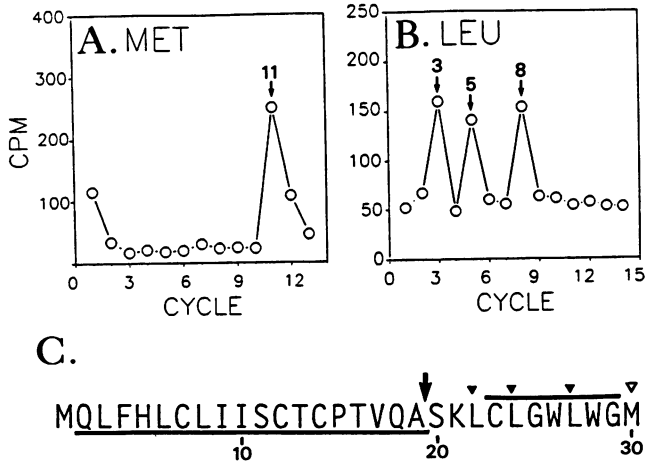

FIG. 3. Amino-terminal sequencing of nuclear P22. (A) Nuclear P22 was labeled with [^{35}S]methionine and purified as described in Materials and Methods. The protein was subjected to Edman degradation, and the amount of radioactivity released from 13 cycles is shown. The arrow highlights the only methionine residue detected (cycle 11). (B) Nuclear P22 labeled with [^{3}H]leucine was similarly sequenced, and the data from 14 cycles of Edman degradation are shown. The arrows point to the three leucine residues detected (cycles 3, 5, and 8). (C) The sequence of the amino-terminal portion of the precore protein is given in the single-letter code. The arrow points to the peptide bond cleaved by the signal peptidase to give rise to P22. The open arrowhead indicates the methionine residue detected in part A; the solid arrowheads indicate the leucine residues detected in part B. The overline and underline indicate the deleted residues in pECE-OU3 and pECE-OU4, respectively.

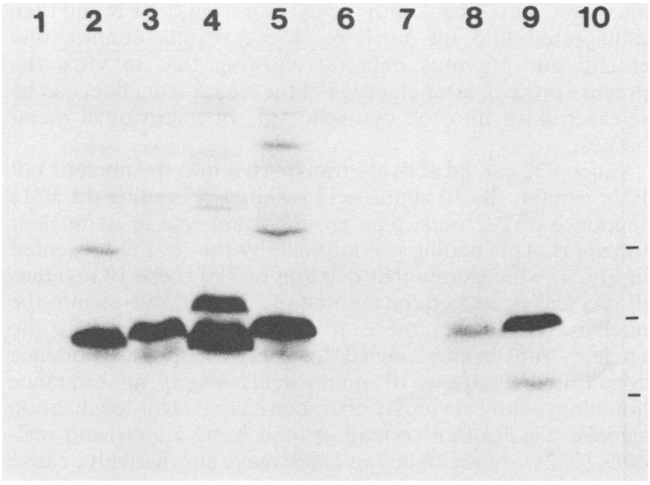

FIG. 4. Deletion analysis of the precore region. COS-7 cells mock transfected (lanes 1 and 6) or transfected with pECE-C (lanes 2 and 7), pECE-PC (lanes 3 and 8), pECE-OU3 (lanes 4 and 10), or pECE-OU4 (lanes 5 and 9) were labeled with [^{35}S]methionine and separated into cytoplasmic (lanes 1 to 5) and nuclear (lanes 6 to 10) fractions. The core and precore proteins were then immunoprecipitated with anti-core protein serum. The ticks on the right indicate the positions of the molecular weight markers as described in the legend to Fig. 1.

findings which revealed staining of precore-core protein in the nuclei, the cytoplasm, or both of HBV-infected liver cells (3, 11, 13, 18). The reason for this differential distribution of P22 in cells is unclear. It may be related to the cell cycle or the expression of other HBV gene products. Experiments are being conducted to investigate this finding.

Putative nuclear localization signal in the precore region. The only sequence difference between P21c and P22 is the presence of 10 extra residues at the amino terminus of P22 (corresponding to residues 20 to 29 of the uncleaved precore protein; Fig. 3C). This region may thus constitute a nuclear localization signal. To test this hypothesis, another deletion mutant plasmid, pECE-OU3, was created. In pECE-OU3, residues Cys-23 to Gly-29, which include most of the putative nuclear localization signal of P22, were deleted (Fig. 3C). This DNA construct was then transfected into COS cells for expression studies. Deletion of these seven amino acid residues reduced the amount of nuclear P22 to an almost undetectable level (Fig. 4, lanes 4 and 10). These results are consistent with the notion that the amino-terminal portion of P22 is important in nuclear localization.

Besides the P22 band, an additional protein band with slower mobility on the gel was also present in the cytoplasmic fraction of cells transfected with pECE-OU3 (Fig. 4, lane 4). The nature of this protein band is unclear, but it may be the uncleaved precursor of mutated P22, since deletion of the residues following the signal sequence may have reduced the efficiency of cleavage by the signal peptidase.

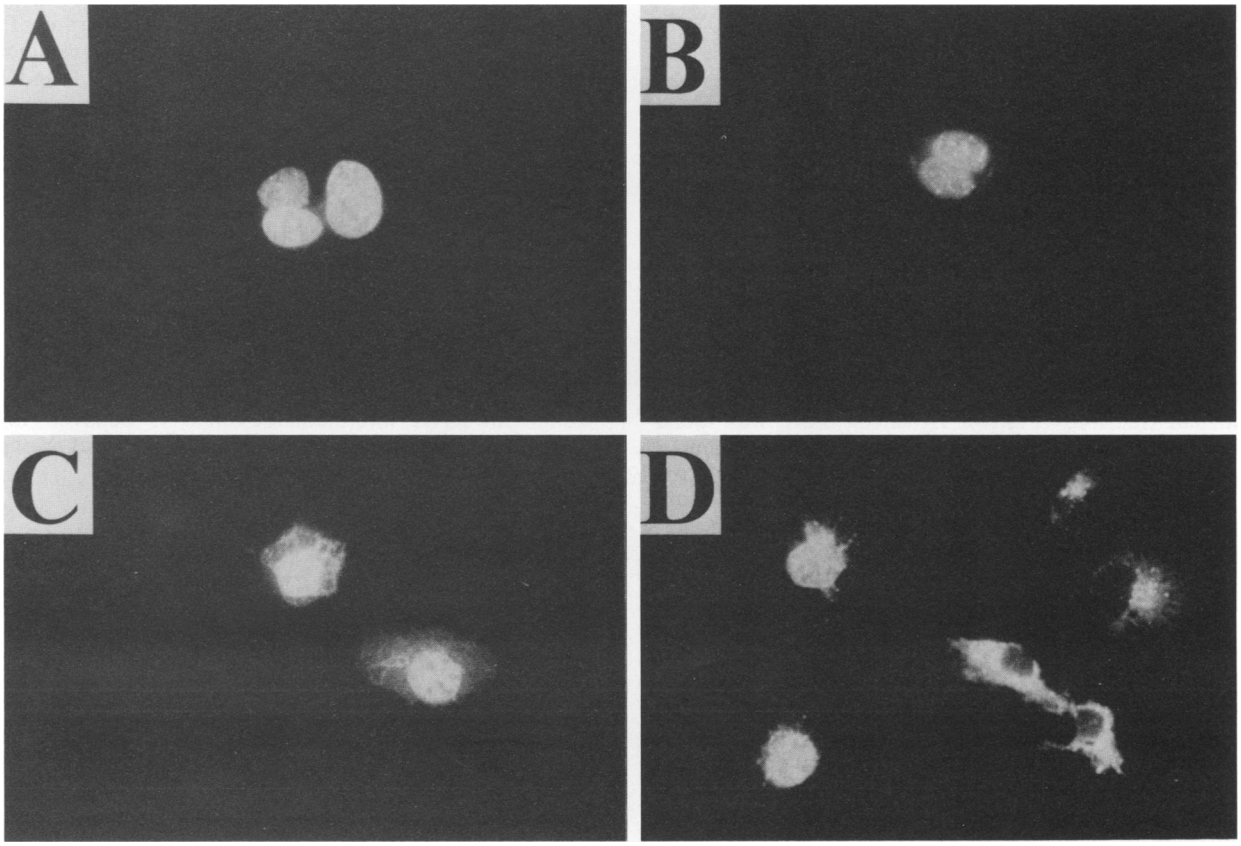

FIG. 5. Immunofluorescence of P22 in COS cells transfected by pECE-OU4. The procedure for immunofluorescence is described in Materials and Methods. In panel B, nuclear staining of a binucleate cell is clearly visible.

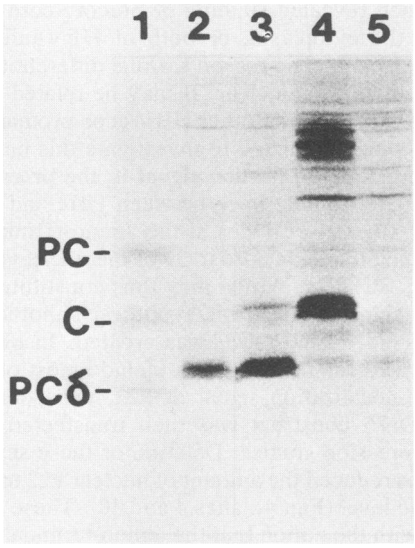

FIG. 6. Subcellular localization of P22 and P21c in PC/C10 cells. Cloning of the PC/C10 cell line, which stably expresses both P22 and P21c proteins, is described in Materials and Methods. The cells were labeled with [^{35}S]methionine, fractionated, immunoprecipitated with anti-core protein serum, and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes: 1, molecular mass standards (PC, 25 kilodaltons; C, 21 kilodaltons; PCδ, 16 kilodaltons); 2, nuclear fraction of parental untransfected CV-1 cells; 3, nuclear fraction of PC/C10 cells; 4, cytoplasmic fraction of PC/C10 cells; 5, cytoplasmic fraction of untransfected CV-1 cells. P22 but not P21c was found in the nuclear fraction.

Failure of coexpression of precore and core proteins to lead to translocation of core protein into the nucleus. Previous reports have shown that both P21c and P22 can be assembled into capsidlike particles in COS-7 (15) and other cells (4, 14, 23), raising the possibility that the core protein in the presence of P22 could be translocated into the nucleus by formation of mixed particles with P22. Since COS-7 cells transfected with pECE-PC did not synthesize detectable amounts of P21c, we cotransfected plasmids expressing core and precore proteins (pECE-C and pECE-PC, respectively) into CV-1 cells (parental line of COS-7) and obtained a stably transfected line (PC/C10) that synthesizes comparable amounts of P21c and P22 (Fig. 6). As in transiently transfected COS-7 cells, P22 was found both in the cytoplasmic and nuclear fractions (Fig. 6). However, P21c was found exclusively in the cytoplasmic fraction, indicating that it cannot be passively transported into the nucleus in the presence of P22. Since both P21c and P22 are assembled into capsidlike particles in these cells (Ou et al., manuscript in preparation), P22 must be transported into the nucleus in the form of monomers, or P21c and P22 are segregated into discrete populations of particles.

DISCUSSION

In this report we have shown that precore protein derivative P22 is a nuclear protein as well as a secreted protein. Furthermore, since nuclear P22 lacks the signal sequence (Fig. 3), nuclear transport must occur after partial translocation into the endoplasmic reticulum. However, nuclear transport of P22 apparently does not require the signal peptide, since deleting the signal peptide enhances nuclear transport of P22 (Fig. 4 and 5). Therefore, a portion of P22 must be released back into the cytosol from the ER and then transported into the nucleus. These results confirm and extend our previous data (8) showing that in vitro the precore protein, after cleavage of the signal sequence, can be released back into the cytosolic side of microsomal membranes.

Since P22 can be actively transported into the nucleus but P21c cannot, the 10 amino acid residues preceding the P21c sequence of P22 must play an important role in its nuclear transport. This finding is confirmed by the results presented in Fig. 4, which show that deletion of 7 of these 10 residues dramatically reduced the amount of P22 transported into the nucleus. Therefore, these 10 residues may serve as the nuclear translocation signal for P22. Since the sequence constituted by these 10 amino acids shares no sequence homology with previously characterized nuclear localization signals, which often contain several basic amino acid residues (1, 21), these 10 amino acids may, alternatively, cause a conformational change to expose an internal nuclear localization signal. In this regard, it is interesting that the carboxy terminus of P22 is rich in arginine residues and thus may contain nuclear localization signals. We are currently trying to distinguish between these two possibilities by linking different sequence domains of P22 to β-galactosidase. Our preliminary results indicate that the 10 amino acid residues and a sequence downstream are both important for nuclear localization. Experiments are being conducted to further study the mechanism of this nuclear transport.

A significant number of HBV capsid particles can be detected in the nuclei of HBV-infected liver cells (3, 11, 13, 18). The nature and function of these particles are unclear. Since we have demonstrated here that precore protein derivative P22 can be a nuclear protein and core protein P21c is not, our results suggest that the nuclear core particles in infected hepatocytes are constituted of P22 rather than P21c. We cannot rule out the possibility, however, that during the infection cycle of HBV, some other viral factors can induce nuclear transport of P21c. Nevertheless, the fact that a significant proportion of P22 is retained within the cell nucleus indicates that P22 plays an important role in the HBV life cycle. One possible function of the precore protein may be to transport daughter viral genomes from the cytosol into the nucleus, where they can serve to direct the synthesis of additional viral RNA and eventually increase the number of progeny particles produced by the infected cells. This is necessary, since the HBV genomic DNA cannot be directly replicated into daughter DNA molecules (22) but must go through an RNA intermediate which is believed to be reverse transcribed only in capsid particles. Further experiments are required to verify this hypothesis.

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