Production of Hepatitis B Virus Nucleocapsidlike Core Particles in *Xenopus* Oocytes: Assembly Occurs Mainly in the Cytoplasm and Does Not Require the Nucleus

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The location of hepatitis B virus (HBV) nucleocapsid (core particle) assembly in infected cells remains controversial. Some lines of evidence implicate the nucleus; others favor the cytoplasm. Via injection of a synthetic mRNA encoding the HBV nucleocapsid protein (p21.5), we have expressed both unassembled p21.5 and nucleocapsidlike core particles in *Xenopus* oocytes. Subcellular fractionation reveals that ~91% of the unassembled p21.5 and 95% of the core particles are cytoplasmic, with only 9 and 5%, respectively, in the nucleus. We present evidence showing that unassembled p21.5 equilibrates between nucleus and cytoplasm by passive diffusion and that intact core particles do not enter the nucleus. To examine the role of the nucleus in core particle formation, we expressed p21.5 in surgically anucleate oocytes. We show that anucleate oocytes support efficient core particle formation, indicating that (i) the nucleus is not essential for assembly and (ii) the cytoplasm can assemble most core particles found in oocytes. On the basis of our data, we propose that in oocytes, most core particle assembly (up to 95%) occurs in the cytoplasm, but that at least ~5% of the cellular core particles are assembled in the nucleus and remain there. We discuss the implications of these findings for the formation of replication-competent core particles in infected cells.

The spherical nucleocapsids, or core particles, of human hepatitis B virus (HBV) and related hepadnaviruses provide the environment in which the viral RNA pregenomes are converted by reverse transcription to partially double-stranded DNA molecules (30). The major HBV nucleocapsid structural protein, the 21.5-kDa core protein (p21.5), contains two nuclear localization signals (NLSs) (10, 36) and is often localized to the nuclei of infected cells in immunohistochemical and ultrastructural studies (8, 15, 31, 32). Immunoelectron micrographs reportedly show that the nucleus is the site of core particle assembly (19, 35). However, in persistently infected cells, immature nucleocapsids, which are replication competent but lack viral envelope coats, are found in the cytoplasm (11, 30), as are the minus-strand HBV DNA replication intermediates generated inside such nucleocapsids (4, 5, 6). These observations raise the possibility that specific events in core particle assembly might occur on different sides of the nuclear envelope (15), but this idea has not been directly tested.

In this study, we analyzed the role of the nucleus in core particle assembly by using *Xenopus* oocytes, which produce significant amounts of both nucleocapsidlike core particles and unassembled p21.5 under the direction of a synthetic p21.5 mRNA, and which permit precise nuclear/cytoplasmic fractionation. We show that most p21.5 is cytoplasmic in oocytes, although the nucleus contains small amounts of p21.5 species which apparently enter by diffusion as unassembled p21.5. Further, the nucleus can be completely removed without abrogating core particle assembly. Our data suggest that core particle assembly is mostly cytoplasmic, with a minor fraction occurring in the nucleus.

MATERIALS AND METHODS

Plasmids and RNAs. The HBV DNA used in this study is of subtype *adw* (33). Plasmid pSP64T-C generates a synthetic mRNA (T-C mRNA) which directs high-level translation of p21.5 in frog oocytes. The parent vector pSP64T (20) contains the *Xenopus* β -globin mRNA 5' and 3' untranslated regions, separated by a *Bgl*II cloning site and located downstream from the SP6 polymerase promoter. Briefly, pSP64T-C contains a minimal core gene *StyI* fragment of HBV DNA (nucleotides 1885 to 2466) cloned into the *Bgl*II site of pSP64T. Δ -157 was constructed by using a synthetic oligonucleotide adaptor to replace HBV nucleotides 2369 to 2466 in pSP64T-C. The encoded protein (p18) is contiguous with the first 157 amino acids of p21.5 and contains, in addition, the last two p21.5 residues (Gln-Cys). Δ -149 contains a previously described mutation (28) in which two consecutive termination codons follow Val-149. A *Bgl*II fragment (nucleotides 1987 to 2408) containing this mutation was introduced as a replacement into pSP64T-C. Further details of these constructs and their expression properties will be presented elsewhere (37). Plasmid DNAs were purified by banding twice on cesium chloride gradients. Capped synthetic transcripts were prepared from *SalI*-linearized DNAs, using SP6 polymerase as described previously (29). Transcription reaction mixtures were stored at -70°C until required and then microinjected without further purification.

Oocyte handling and microinjection. Oocytes were collected and cultured in modified Barth's solution containing antibiotics (MBSH) by standard procedures (9). Oocytes were injected as described previously (28, 29) except that a programmable microinjection device (Sutter Instruments, San Rafael, Calif.) allowed very reproducible delivery of 30 nl of RNA into each oocyte. Unlabeled oocytes were cultured in MBSH for 1 to 3 days postinjection. Metabolic labeling was initiated within 3 to 5 h of injection by placing the oocytes in MBSH containing 0.5 to 2 mCi of [³⁵S]-me-

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thionine and -cysteine (Expresslabel; ICN) per ml and was continued for 24 to 48 h. Groups of 10 to 30 oocytes were homogenized in homogenization buffer (50 mM Tris hydrochloride [pH 7.5], 1% Nonidet P-40, 10 mM EDTA).

Oocyte enucleation. Enucleation was performed under MBSH at $\times 15$ magnification. A superficial slit was carefully introduced in the center of the oocyte animal pole with a 23- or 27-gauge needle. The nucleus was extruded through the slit by squeezing the oocyte gently around the middle with forceps. Anucleate oocytes were allowed to recover under fresh MBSH with antibiotics for 1 to 3 h prior to RNA microinjection.

Preparation of nuclear and cytoplasmic extracts. Oocytes were precooled by incubation on ice for 10 min prior to enucleation (see above). The isolated nuclei were rapidly stripped of traces of adhering yellow residual cytoplasm by repeated pipetting through a standard 200- μ l Eppendorf tip and then immediately transferred to ice-cold homogenization buffer. The cytoplasmic compartments were collected separately on ice. All damaged nuclei were discarded along with their corresponding cytoplasm. From 10 to 25 of each compartment were collected prior to homogenization. The subcellular extracts were stored at -70°C until analyzed as described below.

Fractionation and analysis of core proteins. The particulate and nonparticulate forms of p21.5 present in either 1 to 2 oocytes (for intact oocytes, anucleate oocytes, and cytoplasmic compartments) or 11 to 24 nuclei were resolved on sucrose gradients. Portions (200 μ l) of clarified (15,000 $\times$ g, 5 min) extract containing the appropriate amount of oocyte proteins were layered onto 1.4-ml step gradients of sucrose (10 to 60%) in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–1 mM EDTA and centrifuged for 30 min at 4°C and 55,000 rpm (201,000 $\times$ g) in a TLS-55 swinging-bucket rotor on a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, Calif.). Fractions (~ 100 μ l) were collected from the top of the gradient and analyzed by immunoprecipitation with polyclonal anticore antiserum (Dako Corp.) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (28) or by radioimmunoassay (RIA) using an HBe diagnostic kit as described by the manufacturer (Abbott Laboratories, North Chicago, Ill.). The RIA data are presented as counts per minute and are corrected for background unless otherwise stated.

Preparation of core particles for microinjection. Core particles from about 200 ^{35}S -labeled, T-C mRNA-injected oocytes were purified by a single round of sucrose gradient centrifugation essentially as described above. Peak particle fractions were pelleted by centrifugation at 100,000 rpm (412,000 $\times$ g) for 1 h at 4°C in a Beckman TLA-100.3 rotor. The pellets were carefully resuspended in 100 μ l of Tris-EDTA TE buffer, and ~ 30 nl of this preparation was injected into each recipient oocyte. After incubation for 24 h, oocytes were enucleated and p21.5 was immunoprecipitated from subcellular extracts corresponding to 2 cytoplasmic or 24 nuclei without fractionation through sucrose. An electrophoretically homogeneous preparation of recombinant core particles (0.5 mg/ml; subtype *adw*) from *Escherichia coli* was a gift from Chiron Corp. (Emeryville, Calif.).

RESULTS

Expression and analysis of p21.5 in *Xenopus* oocytes. The expression of p21.5 in the absence of other viral proteins drives the production of 28-nm nucleocapsidlike core particles in several heterologous systems (3, 13, 18, 21). Such

FIG. 1. Production of ^{35}S -labeled p21.5 species in frog oocytes. Proteins from about two oocytes were fractionated on a 10 to 60% sucrose gradient, and 14 fractions were collected as described in Materials and Methods. After immunoprecipitation from the fractions, ^{35}S -labeled p21.5 was resolved by SDS-PAGE on a 12.5% gel. The dried gel was exposed to film for 15 h at -70°C . The lanes represent individual gradient fractions from the top of the gradient (lane 1) to the bottom (lane 14). Positions of molecular mass markers (in kilodaltons) are indicated at the right, and the position of p21.5 is shown at the left.

particles, which contain ~ 180 molecules of p21.5 but lack the HBV pregenome mRNA and reverse transcriptase, provide a simple system for examining core particle assembly, and we set out to generate them in oocytes by using a synthetic mRNA (T-C mRNA) that encodes only p21.5.

In practice, T-C mRNA has the valuable property of yielding significant quantities of both nonparticulate (free) p21.5 and core particles which can be resolved on sucrose gradients. In a typical experiment (Fig. 1), injected oocytes were metabolically labeled with [^{35}S]methionine and -cysteine, and two oocytes' worth of extract was fractionated on a 10 to 60% sucrose gradient. After immunoprecipitation from the fractions, p21.5 was resolved by SDS-PAGE. The autoradiogram provides a direct representation of the gradient profile. A peak of free p21.5 is found mainly in the first four fractions at the top of the gradient (lanes 1 to 4), with a second peak, representing core particles, in fractions 7 to 11 (the ~ 35 -kDa species in these lanes is a p21.5 dimer); control oocyte gradients show no p21.5 or p35 bands (data not shown). The ratio of free to particulate p21.5 varies somewhat among experiments, and pulse-chase labeling demonstrates that these species exhibit a precursor-product relationship (38). A detailed characterization of oocyte core particles and the assembly process will be presented elsewhere (37).

We also used RIA directed against HBV core antigen (HBcAg) and e antigen, antigenic determinants carried by p21.5, to detect unassembled and particulate core proteins in the gradient fractions. HBcAg gradient profiles are qualitatively similar to ^{35}S -p21.5 profiles (see subsequent figures) and provide a convenient way to estimate both species quantitatively.

Characterization of nuclear and cytoplasmic core proteins. With care, the large nucleus (also known as the germinal vesicle) can be removed intact from the oocyte by manual enucleation and stripped of residual traces of adhering cytoplasm by repeated pipetting. The nuclear and cytoplasmic compartments can be separated with almost no cross-contamination and only minimal loss of cytoplasm. We used this approach to determine the relative concentration of nonparticulate and particulate p21.5 in each subcellular compartment.

Initial single-oocyte fractionation experiments showed

FIG. 2. Comparison of cytoplasmic and nuclear oocyte core proteins. Subcellular extracts were prepared from metabolically labeled oocytes as described in Materials and Methods. The ^{35}S -p21.5 proteins from 1 cytoplasm (A) or 11 nuclei (B) were resolved on sucrose gradients, immunoprecipitated from the gradient fractions, resolved by SDS-PAGE, and exposed to X-ray film at -70°C for 3 days, using an intensifying screen. Lanes 1 to 14 represent gradient fractions 1 (top) to 14 (bottom), respectively. Positions of molecular mass markers (in kilodaltons) are indicated at the right, and the position of p21.5 is shown at the left. A subcellular fractionation experiment comparable to that described above was analyzed by RIA (C) to reveal the HBcAg and e antigen activity present in the gradient fractions obtained when 1 cytoplasm (open squares) or 11 nuclei (closed diamonds) were fractionated through sucrose. Approximately 40% of each gradient fraction was used for the RIA.

that most p21.5 is cytoplasmic under conditions in which simian virus 40 T antigen or the human immunodeficiency virus Tat protein accumulates in the nucleus (data not shown). Since the effective volume (16) of the oocyte cytoplasm (~ 500 nl) is ~ 11 times that of the nucleus (~ 45 nl), we decided to analyze equal volumes of nucleoplasm and cytoplasm (i.e., ~ 11 nuclei versus 1 cytoplasm). The data that follow show the relative concentrations of the p21.5 species in each compartment.

The sucrose gradient profiles of the ^{35}S -p21.5 present in equal volumes of cytoplasm (Fig. 2A) and nucleoplasm (Fig. 2B) are very comparable. Very similar levels of free p21.5 (lanes 1 to 3) are seen in both compartments, and the levels of particulate p21.5 (lanes 7 to 11) are similar, although scrutiny of the band intensities suggests that the nucleus contains a slightly lower concentration of core particles.

Sucrose gradient profiles (Fig. 2C) of the HBcAg activity present in 1 oocyte cytoplasm or 11 nuclei confirm the results presented above. The concentration of free p21.5 (fractions 1 to 4) is almost identical in both compartments, but the level of core particle HBcAg (fractions 7 to 12) is clearly lower in the nucleus. Quantitatively, core particles comprise $\sim 67\%$ of the total cytoplasmic HBcAg but only 49% of the nuclear HBcAg, and the concentration of core particles is $\sim 50\%$ lower in the nucleus than in the cytoplasm.

From the data for HBcAg, we can estimate the p21.5 content of an individual oocyte (i.e., one cytoplasm and one nucleus): 91% of the free p21.5 and $\sim 95\%$ of the core particles are in the cytoplasm; only 9 and 5%, respectively, are in the nucleus.

Subcellular localization of mutant core proteins. The foregoing data show no indication that p21.5 accumulates in the oocyte nucleus; indeed, the cytoplasm contains modestly ($\sim 25\%$) higher p21.5 as a result of the reduced nuclear

particle level (Fig. 2C). However, the arginine-rich carboxyl-terminal tail of p21.5 (Fig. 3A) reportedly contains duplicate NLSs (10, 36) that can direct p21.5 to the nucleus (10). The boundaries of these NLSs have been partially mapped (10), and the relevant sequences are underlined in Fig. 3A. To determine whether these NLSs influence p21.5 localization in oocytes, we examined the subcellular distribution of two p21.5 mutants. Mutant Δ -157 encodes an ~ 18 -kDa protein (p18) comprising the first 157 and last 2 residues of p21.5 (i.e., amino acids 158 to 183 are deleted), while Δ -149 encodes a 17-kDa core protein (p17) that ends at Val-149 and lacks the Arg tail region (Fig. 3A). Thus, p18 contains one NLS and p17 contains none. Both p18 and p17 should be able to form core particles (3, 13).

The subcellular distribution profile of Δ -157 shows that 1 cytoplasm (Fig. 3B) and 11 nuclei (Fig. 3C) contain very similar levels of both free (lanes 1 to 3) and particulate (lanes 6 to 9) forms of ^{35}S -p18 (an ~ 32 -kDa dimer is seen in some lanes). For Δ -149, the profiles again reveal very similar amounts of immunoprecipitable ^{35}S -p17 in the cytoplasmic (Fig. 3D) and nuclear (Fig. 3E) compartments, but p17 is found mainly in the first five or six gradient fractions (lanes 1 to 6) and there is no discrete peak of particles in this experiment.

These data generally confirm the results for p21.5 in Fig. 2. For p18 in particular, the species produced and their subcellular distribution pattern are essentially identical to those seen for p21.5. Particle formation is apparently relatively inefficient for p17, but the same uniform subcellular distribution pattern is again seen for the p17 species observed. We conclude that the putative NLS motifs in p21.5 do not influence its subcellular distribution.

Transport properties of core particles. Nuclear core particles could be assembled in the nucleus from intranuclear

FIG. 3. Subcellular localization of mutant core proteins p18 and p17. (A) Structure of the wild-type and mutant core proteins. The top line shows the carboxyl terminus of p21.5 (amino acids 146 to 185), with the two NLSs underlined. Subsequent lines show the termini of mutants Δ -157 (p18) and Δ -149 (p17). (B to D) Sucrose gradient profiles of cytoplasmic (B and D) and nuclear (C and E) proteins from Δ -157 (B and C) or Δ -149 (D and E). Fractionation was done as described in Materials and Methods and the legend to Fig. 1. Autoradiography was for 12 h at 22°C (Δ -157) or for 2 days at -70°C with an intensifying screen (Δ -149). The positions of p17 and p18 are indicated at the left, and those of molecular mass markers (in kilodaltons) are indicated at the right.

nonparticulate p21.5, but they could alternatively be transported into the nucleus after cytoplasmic assembly. Such transport would presumably be NLS mediated since 28-nm particles exceed the size limit for diffusion (27). To assess the feasibility of transport, we injected recombinant core particles into the oocyte vegetal hemisphere, well away from the nucleus, and monitored their ability to enter the nucleus. Both radiolabeled p21.5 particles from oocytes and unlabeled core particles derived from *E. coli* were tested in this manner. In the oocyte particles p21.5 is phosphorylated within the Arg tail region, while *E. coli* particles appear to be unmodified (16a). Phosphorylation could conceivably influence the activity of the NLSs.

Radiolabeled p21.5 core particles from oocytes were purified on a sucrose gradient, concentrated, and injected into oocytes. After 24 h of incubation, subcellular fractions were prepared and p21.5 was immunoprecipitated directly from 24 nuclei or 2 cytoplasms and resolved by SDS-PAGE (Fig. 4A). Almost all of the p21.5 is recovered from the cytoplasm (lane C), with only a trace in the nucleoplasm (lane N).

For the *E. coli* particles, ~ 15 ng of a highly purified preparation was injected into each oocyte cytoplasm. After 24 h of incubation followed by subcellular fractionation, the contents of 1 cytoplasm and 11 nuclei were fractionated on sucrose gradients and assayed for HBcAg (Fig. 4B). A peak of core particles (fractions 8 to 12) is seen in the cytoplasm,

while the nuclear HBcAg activity shows no clear peak and is essentially indistinguishable from the assay background (not shown). No peak of free p21.5 is seen in either compartment, suggesting that core particles remain intact in the cytoplasm of oocytes.

These experiments show that cytoplasmic core particles neither enter the nucleus nor significantly associate with the nuclear envelope. From the data for HBcAg, we estimate that the nuclear core particle concentration is at most 10% of the cytoplasmic concentration and probably much lower. Thus, in a single oocyte, $\leq 1\%$ of introduced core particles becomes associated with the nucleus, an amount that could easily represent contamination. We conclude that there is no significant nuclear influx of core particles from the cytoplasm, so that nuclear core particles are probably assembled in the nucleus.

The nucleus is not required for core particle assembly. It is formally possible that cytoplasmic core particles are assembled in the nucleus and exported to the cytoplasm. We therefore tested whether the nucleus is essential for core particle assembly by surgically removing it to create anucleate oocytes, which can still support the translation of exogenous T-C mRNA.

The ^{35}S -p21.5 species from one intact (Fig. 5A) or one anucleate (Fig. 5B) oocyte were fractionated through sucrose and analyzed by immunoprecipitation and SDS-

FIG. 4. Subcellular distribution of introduced core particles. (A) Autoradiogram showing the nuclear/cytoplasmic partitioning of ^{35}S -labeled core particles 24 h after injection into the cytoplasm of oocytes. ^{35}S -labeled p21.5 was immunoprecipitated from 2 oocytes' worth of cytoplasm (lane C) or 24 oocytes' worth of nuclei (lane N) and resolved by SDS-PAGE. The gel was exposed to film for 1 week at -70°C with an intensifying screen. (B) Analysis of the subcellular distribution of injected *E. coli* core particles. Either 1 oocytes' worth of cytoplasmic proteins (C; squares) or 11 oocytes' worth of nuclear proteins (N; diamonds) was fractionated on sucrose gradients. HbAg was determined by RIA and is presented without subtraction of background ($\sim 1,000$ cpm in this experiment). For details of the core particle preparations, see Materials and Methods.

PAGE. Both free (lanes 1 to 3; by error fraction 1 was not loaded onto the gel shown in Fig. 5B) and particulate (lanes 6 to 11) forms of p21.5 are evident in these profiles. The overall appearance of these species is little changed by enucleation, although anucleate oocytes appear to produce slightly lower amounts of p21.5 proteins.

These results were verified by a comparable enucleation experiment which was assayed for HbAg (Fig. 5C). The HbAg profiles confirm that intact and anucleate oocytes produce both particulate (fractions 7 to 13) and free HbAg (fractions 1 to 5). Quantitatively, the HbAg activity found

in anucleate oocytes is only $\sim 77\%$ of the intact oocyte HbAg, the decrease presumably being the result of the minor surgical injury. Since any reduction in overall p21.5 synthesis is accompanied by a sharp decline in core particle levels, presumably because of the highly cooperative nature of particle formation (37), it is not surprising that the HbAg deficit in anucleate oocytes is concentrated in the core particle population.

We conclude that the nucleus is dispensable for core particle formation. From the data for anucleate oocytes in Fig. 5C, we estimate that the cellular cytoplasm has the

FIG. 5. Production of core proteins by intact versus anucleate oocytes. Sucrose gradient-fractionated ^{35}S -p21.5 from intact (A) or anucleate (B) oocytes was recovered by immunoprecipitation and resolved by SDS-PAGE. The gel was exposed to film for 3 days at -70°C . (C) HbAg analysis of the core proteins produced by intact (squares) or anucleate (diamonds) oocytes after sucrose gradient fractionation.

capacity to assemble at least 62% of the core particles found in intact oocytes. For the reason discussed above, this figure almost certainly underestimates the assembly capacity of the cytoplasm.

DISCUSSION

Under the direction of a synthetic mRNA (T-C mRNA) encoding the HBV p21.5 core protein, *Xenopus* oocytes produce both nucleocapsidlike particles and unassembled (free) p21.5. This system provides a powerful means for studying viral nucleocapsid assembly. In this study, we used the very clean separation of nucleus and cytoplasm attainable in oocytes to analyze the subcellular localization and nuclear transport properties of p21.5 or its associated antigenic determinant. We also investigated the subcellular location of core particle assembly and the role of the nucleus in this process.

Subcellular localization of p21.5 and core particles. Subcellular fractionation (Fig. 2) reveals that the oocyte nucleus and cytoplasm contain similar concentrations of free p21.5 and that the core particle concentration is about twofold higher in the cytoplasm. This means that the nucleus, which represents 8 to 10% of the total oocyte volume, contains only about 9% of the total free p21.5 and about 5% of the core particles. The cytoplasm contains roughly 91 and 95%, respectively, of the two p21.5 species, so that p21.5 proteins are mainly cytoplasmic. Experiments involving subcellular dissection and analysis of individual oocytes confirm that p21.5 is cytoplasmic under all conditions of time and concentration tested, whereas simian virus 40 T antigen or the human immunodeficiency virus Tat protein accumulates up to ~30-fold in the nucleus (38).

Role of the nucleus and location of core particle assembly. Since the distinctly cytoplasmic character of p21.5 seen here contrasts with reports that core particles are assembled in the nucleus (19, 35), we tested whether the nucleus is essential for core particle assembly by using anucleate oocytes, which are pure cytoplasmic compartments created by surgical removal of the nucleus. Our results show that anucleate oocytes produce at least 62% of the core particles found in intact oocytes (Fig. 5). Thus, core particle assembly is not mechanistically dependent on the nucleus. Further, the cytoplasm has sufficient assembly capacity to produce at least 62%, and perhaps all (see above), of the core particles found in intact oocytes.

The fact that the nucleus is not required for core particle assembly does not imply that particle assembly is excluded from the nucleus. Indeed, the inability of core particles to enter the nucleus (Fig. 4) leads us to conclude that the minor core particle population (5%) seen in the nucleus must have been assembled there. There is precedent for the nuclear assembly of nucleocapsids for a number of viruses (12, 34).

Thus, HBV core particles are assembled in both the cytoplasm and nucleus. We propose a simple model for the assembly process which explains the subcellular distribution of core particles seen in this study. Free p21.5 is synthesized in the cytoplasm, equilibrates between the nucleus and cytoplasm by diffusion (see below), and assembles into independent populations of core particles in both compartments. This model predicts that each compartment should contain a quantity of core particles proportional to its size, i.e., roughly 92% in the cytoplasm and 8% in the nucleus, which is close to what is observed except that the level of nuclear core particles (5%) is slightly lower than anticipated. This could be explained by kinetic trapping of free p21.5 into

cytoplasmic core particles, by efflux of core particles from the nucleus, or by preferential losses.

Implications for the nuclear transport of p21.5. Clustered SPRRR motifs, found in the Arg-rich carboxyl-terminal domain of p21.5, resemble known NLSs that direct proteins to the nucleus (27). Two recent reports suggest that this carboxyl-terminal domain contains duplicate NLSs (Fig. 3A) that can direct nuclear transport and accumulation of proteins in cultured cells (10, 36), although in one case p21.5 itself is cytoplasmic and the NLSs act only on heterologous proteins or precore proteins (36). Our data do not support the idea of NLS-facilitated transport of p21.5 in oocytes. The absence of core particle import into the nucleus and the equilibration of free p21.5 between cytoplasm and nucleus are consistent with the idea that passive diffusion governs p21.5 localization. The absence of a functional NLS in p21.5 is supported by the fact that comparable uniform subcellular distribution patterns are observed for mutant core proteins which lack one (p18) or both (p17) NLS motifs (Fig. 3B to E). Since passive diffusion through the nuclear pore is restricted to molecules of 9.0 nm (≤ 60 kDa) or less (27) in diameter, p21.5 presumably enters the nucleus as a species no larger than a trimer.

Despite the cytoplasmic localization of p21.5 seen in oocytes and in animal cells (see introduction), other studies have provided evidence for nuclear localization of core proteins. Nuclear HBcAg, detected by immunostaining in cells or tissue sections, is a classic marker of HBV infection, although it can be argued that the presence of nuclear HBcAg does not necessarily imply p21.5 accumulation in the nucleus, particularly since cytoplasmic HBcAg may be sensitive to fixation (6, 25). More persuasively, biochemical evidence for NLSs directing free p21.5 to the nucleus has been presented in one case (10). These observations suggest that p21.5 may be able to change its localization. Recent evidence shows that nuclear localization can be regulated (27) by masking of NLSs for proteins such as *Drosophila* dorsal (24) and transcription factor NF- κ B (1). Posttranslational modification has been implicated in the relocation of these proteins from the cytoplasm to the nucleus (14, 24, 27), and phosphorylation reportedly modulates the activity of a simian virus 40 T-antigen NLS (22). Since p21.5 is phosphorylated in mammalian cells (23) and in oocytes (27a) within the NLS region (27a), we speculate that the variable localization of p21.5 in different systems, and even reports that p21.5 localization correlates with specific states of hepatitis disease (8, 19), may be attributable to varying extents of phosphorylation. We are currently investigating this possibility.

Implications for the HBV life cycle. Our finding that most core particles assemble in the cytoplasm makes biological sense for the production of replication-competent nucleocapsids in infected cells. To construct such functional nucleocapsids, the core protein shell must incorporate two other viral components, the pregenome RNA and the reverse transcriptase product of the *pol* gene. Both p21.5 and the *pol* protein(s) are translated from the pregenomic RNA (7, 26), and recent genetic evidence indicates that the *pol* gene product(s) mediates pregenome RNA encapsidation (2, 17). An obvious advantage of cytoplasmic assembly is that it allows close spatial and temporal coupling between the translation and packaging of pregenome RNA, as has been envisaged in the *cis*-packaging model (11). The localized high concentrations of these three components at the translation site should drive their interaction, perhaps resulting in a p21.5-*pol* protein-pregenome RNA nucleation complex

which can be completed by addition of p21.5. The resultant cytoplasmic nucleocapsids are ideally positioned to link with the viral surface envelope proteins in the endoplasmic reticulum membrane and form infectious virions.

In oocytes, p21.5 species and core particle assembly remain cytoplasmic under conditions in which there is clearly no coupling of assembly to either pregenome RNA packaging or viral replication. Thus, one intriguing explanation for the accumulation of nuclear core proteins, namely, that they represent excess core proteins "unrelated to the virus replication strategy" (6), is not supported by our study. Although we do see a minor population of nuclear core particles in oocytes, the potential significance of this observation in terms of the virus life cycle in infected cells remains to be determined.

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