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A Micromolar Pool of Antigenically Distinct Precursors Is Required To Initiate Cooperative Assembly of Hepatitis B Virus Capsids in *Xenopus* Oocytes

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Assembly of hepatitis B virus capsid-like (core) particles occurs efficiently in a variety of heterologous systems via aggregation of ~180 molecules of a single 21.5-kDa core protein (p21.5), resulting in an icosahedral capsid structure with T=3 symmetry. Recent studies on the assembly of hepatitis B virus core particles in *Xenopus* oocytes suggested that dimers of p21.5 represent the major building block from which capsids are generated. Here we determined the concentration dependence of this assembly process. By injecting serially diluted synthetic p21.5 mRNA into *Xenopus* oocytes, we expressed different levels of intracellular p21.5 and monitored the production of p21.5 dimers and capsids by radiolabeling and immunoprecipitation, by radioimmunoassay, or by quantitative enzyme-linked immunosorbent assay analysis. The data revealed that (i) p21.5 dimers and capsids are antigenically distinct, (ii) capsid assembly is a highly cooperative and concentration-dependent process, and (iii) p21.5 must accumulate to a signature concentration of ~0.7 to 0.8 μM before capsid assembly initiates. This assembly process is strikingly similar to the assembly of RNA bacteriophage R17 as defined by in vitro studies.

Human hepatitis B virus (HBV) and related animal hepadnaviruses are small enveloped, hepatotropic DNA viruses. The 28-nm-diameter spherical T=3 HBV nucleocapsid (core particle) provides the structural environment of the viral replication machinery (for recent reviews, see references 8 and 25). The icosahedral capsid shell contains ~180 subunits of a single viral capsid or core protein of ~21.5 kDa (p21.5). In several heterologous systems, recombinant p21.5 spontaneously assembles into simple capsids containing core protein and RNA (5–7, 14, 15, 23, 32), a property that facilitates the study of HBV capsid assembly.

The 183- or 185-residue (depending on the HBV subtype) p21.5 polypeptide bears at least two major classes of epitopes, the core (HBc) (2) and e (HBe) (19) epitopes. Intact core particles express only HBc epitopes, which appear to be highly sensitive to denaturation and/or limited proteolysis. Dissociation of capsids results in loss of HBc and concomitant appearance of HBe epitopes (18, 21, 30), which are normally latent in capsids.

Although the molecular biology of p21.5 has been well studied (for a recent review, see reference 27), little is known about the assembly of HBV capsids. We recently reconstituted this assembly process in *Xenopus* oocytes programmed with synthetic p21.5 mRNA and showed that oocytes produce two core protein species, nonparticulate (free) p21.5 and assembled capsids, with distinctive chemical and physical properties (36, 38). Nonparticulate p21.5 was found to comprise mainly p21.5 dimers (37), which were demonstrated by pulse-chase experiments to be the precursors for capsid formation (38). In this report, we present an analysis of the capsid assembly process, including a characterization of the antigenic identity of p21.5 dimers versus capsids and determination of the p21.5 concentration requirements for HBV capsid assembly in oocytes.

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MATERIALS AND METHODS

Expression of nonparticulate and particulate p21.5 in oocytes. The synthetic p21.5 mRNA used in this work, microinjection and cultivation of *Xenopus* oocytes, and fractionation of unassembled p21.5 and core particles have all been described earlier (35–38). Briefly, capped p21.5 mRNA was transcribed in vitro from plasmid pSP64T-C (36). This RNA contains the minimal sequence information for p21.5, flanked by noncoding sequences of the *Xenopus* β-globin gene to improve translation efficiency. Concentrated p21.5 RNA was prepared by treating transcription reactions (29) with DNase I (RNase free; Boehringer Mannheim), followed by extraction with phenol-chloroform and precipitation. The purified RNA was dissolved at the desired concentration (typically, 5× to 6×) in nuclease-free H_2O (Promega) containing 5 mM dithiothreitol and RNasin at 400 U/ml (Boehringer Mannheim) and then stored at −70°C.

For RNA dilution experiments, two-step serial dilutions of the stock RNA were prepared directly prior to injection. Batches of 10 to 20 oocytes were cultured 1 to 3 days postinjection at 17°C in modified Barth's solution supplemented with antibiotics as described earlier (28, 29, 36, 38). For synthesis of radiolabeled p21.5 proteins, oocytes were incubated for ~44 h in 0.5 to 2 mCi of [^{35}S]methionine and [^{35}S]cysteine (Expresslabel; NEN) per ml of modified Barth's solution and lysed in ~20 μl of homogenization buffer (50 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 10 mM EDTA) per oocyte. For determination of p21.5 by enzyme-linked immunosorbent assay (ELISA) (see below), oocytes were lysed in PBS (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl). The lysates were clarified (microcentrifuge; 2 × 5 min, 15,000 × *g*, 4°C) prior to further analysis.

Separation of nonparticulate p21.5 and capsids was achieved by subjecting the clarified lysate (2.5 to 12 oocytes) to sucrose gradient centrifugation as described by Zhou and Standring (36). The 14 gradient fractions (~100 μl each) were

analyzed by radioimmunoprecipitation as described previously (28, 38), by radioimmunoassay (RIA), or by ELISA.

Determination of HBc antigen and HBe antigen activities by RIA. Aliquots (10 μl) of the gradient fractions were diluted to 200 μl with PBS and assayed with a commercial HBe/anti-HBe diagnostic kit (HBe [rDNA]; Abbott Laboratories) as recommended by the manufacturer. The RIA data are given as 10^3 counts per minute corrected for the background.

p21.5 ELISAs. For HBe- and HBc-specific ELISAs, immunoplates (Nunc) were coated overnight at 4°C with either mouse monoclonal anti-e (MAEI; 200 ng per well) or anti-c (18C $E_{11}B_{12}$; 500 ng per well) immunoglobulin G in 50 mM sodium carbonate buffer (pH 9.6). Both antibodies were a gift of E. Korec (Institute of Molecular Genetics, Prague, Czechoslovakia). For simultaneous measurement of HBe and HBc (HBe/c ELISA), plates were coated with 1 μg of polyclonal sheep anti-HBe/c immunoglobulin G (generated by R. Thomssen and W. H. Gerlich, University of Göttingen, Göttingen, Germany) per well. After three washes with PBS–0.1% (vol/vol) Tween 20, plates were blocked with 10% (vol/vol) newborn calf serum (GIBCO) in TNE (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA) for 1 h at room temperature, washed as described above, and then incubated for 2 h at 37°C with one of the following samples: (i) 20-μl portions of gradient fractions, (ii) two-step serial dilutions of pooled intermediate or capsid fractions, or (iii) two-step serial dilutions of clarified total-oocyte lysates. All sample volumes were adjusted to 100 μl with 1% (wt/vol) bovine serum albumin in PBS. After six washes with PBS–0.1% (vol/vol) Tween 20 and addition of 100 μl of diluted rabbit anti-c/e serum (1/2,000 in 10% newborn calf serum–TNE; Dako Corp.), the plates were incubated for a further 1 h at 37°C. After being washed, the plates were incubated for 1 h at 37°C with a 1/5,000 dilution of horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Cappel) in 10% newborn calf serum–TNE and washed six times with PBS–0.1% (vol/vol) Tween 20. Color development was initiated by adding 1 mg of *o*-phenylenediamine (Zymed) per ml and 0.0125% H_2O_2 in 22 mM citric acid–50 mM sodium phosphate (pH 5.1). A_{490} was measured with a microplate reader (Thermomax; Molecular Devices Corporation).

Preparation of p21.5 concentration standards and quantitation of p21.5 species. Pure recombinant HBc-bearing capsids from *Escherichia coli* containing 0.5 mg of core proteins per ml (a gift from M.-A. Selby and P. Valenzuela of Chiron Corporation, Emeryville, Calif.) provided a concentration standard for the quantitation of native p21.5 capsids (native standard). Two-step serial dilutions of this core particle stock covering a concentration range of 50 to 0.39 ng/ml were prepared in sample diluent (1% bovine serum albumin–PBS) and run in the HBc- and HBe/c-specific ELISAs. The lower limit of detection with these ELISAs was ~50 pg of capsids.

An HBe-positive p21.5 concentration standard (denatured standard) was generated by chemically disrupting capsids (17). Portions of the *E. coli* capsid stock (diluted to 50 μg/ml in PBS) were boiled for 10 min with 6% (vol/vol) 2-mercaptoethanol and 2% (wt/vol) sodium dodecyl sulfate (SDS). The standard was stored at −20°C in small aliquots. Two-step serial dilutions ranging from 25 to 0.2 ng/ml were empirically found to give the best linear absorbance values over 3 to 5 concentration steps in both the HBe/c- and HBe-specific ELISAs.

For quantitative estimation of the p21.5 species synthesized in oocytes, serially diluted samples of total oocyte extracts or of individual or pooled gradient fractions were assayed by ELISA as described above, along with dilution series of the native and denatured standards. Typically, 3 to 5 sample dilutions gave absorbance readings in the range appropriate for reliable quantitation, and the final concentration estimates represent means derived from these values. Analysis of standard curves and test samples, background correction (given by the mean values of about eight negative controls), conversion of absorbances into actual protein concentrations (in nanograms per oocyte), and calculation of the mean concentration values were performed with Softmax ELISA software (Molecular Devices Corp.).

The reproducibility of these ELISAs was checked by assaying samples in duplicate; the p21.5 concentration estimates from duplicates routinely agreed to within 3 to 6%. Fractionated gradients were often frozen after the initial ELISA analysis and later reassayed; moreover, the p21.5 content of important samples was rechecked in some cases by repeating the entire fractionation and ELISA procedure with a frozen sample of the original oocyte lysate. These reanalyses yielded p21.5 concentration estimates that were within 3 to 10% of the original estimates for capsids and within 20% for unassembled p21.5 species.

RESULTS

Strategy for analyzing the capsid assembly process. Expression of p21.5 in *Xenopus* oocytes generates both nonparticulate (free) and particulate p21.5 species (36, 38) that can be resolved on 10 to 60% sucrose gradients (see below). The free p21.5 was found to comprise mainly (or even exclusively) p21.5 dimers (37). Pulse-chase analysis (38) established that essentially the entire dimer population can be chased into capsids. Thus, p21.5 dimers are true precursors or building blocks for capsid assembly (rather than an assembly byproduct or a product of misfolding or disassembly).

To determine the dependence of HBV capsid assembly on the concentration of precursors, we elected to vary the intracellular concentration of p21.5 and monitor the resultant production of free p21.5 and capsids. This was accomplished by RNA dilution series experiments; oocytes were injected with serially diluted preparations of p21.5 RNA, resulting in commensurate reductions in p21.5 synthesis (see below). Lysates from p21.5-expressing oocytes were subsequently clarified and fractionated through minigradients of 10 to 60% (wt/vol) sucrose to yield characteristic p21.5 profiles, in which free p21.5 dimers in the first 4 (of 14) gradient fractions were separated from a peak of capsids in fractions 7 through 11. Control experiments (data not shown) established that clarification of the oocyte lysates did not result in significant losses of p21.5, and data given later in this report established that loss of either p21.5 species was minimal during gradient fractionation. Depending on the experiment, p21.5 was detected by radiolabeling and immunoprecipitation or by RIA or quantitated by ELISA.

Qualitative analysis of the capsid assembly process. In initial experiments (Fig. 1), oocytes were injected with dilutions of either a 1× standard (panels A to C) or a 10×-concentrated (panels D to F) p21.5 mRNA preparation and labeled metabolically with [^{35}S]Met and [^{35}S]Cys. The ^{35}S-labeled p21.5 species were resolved on gradients (see above), immunoprecipitated from the fractions with rabbit anti-core antibody, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

The autoradiograms revealed that increasing amounts of input RNA elicited strikingly different accumulation re-

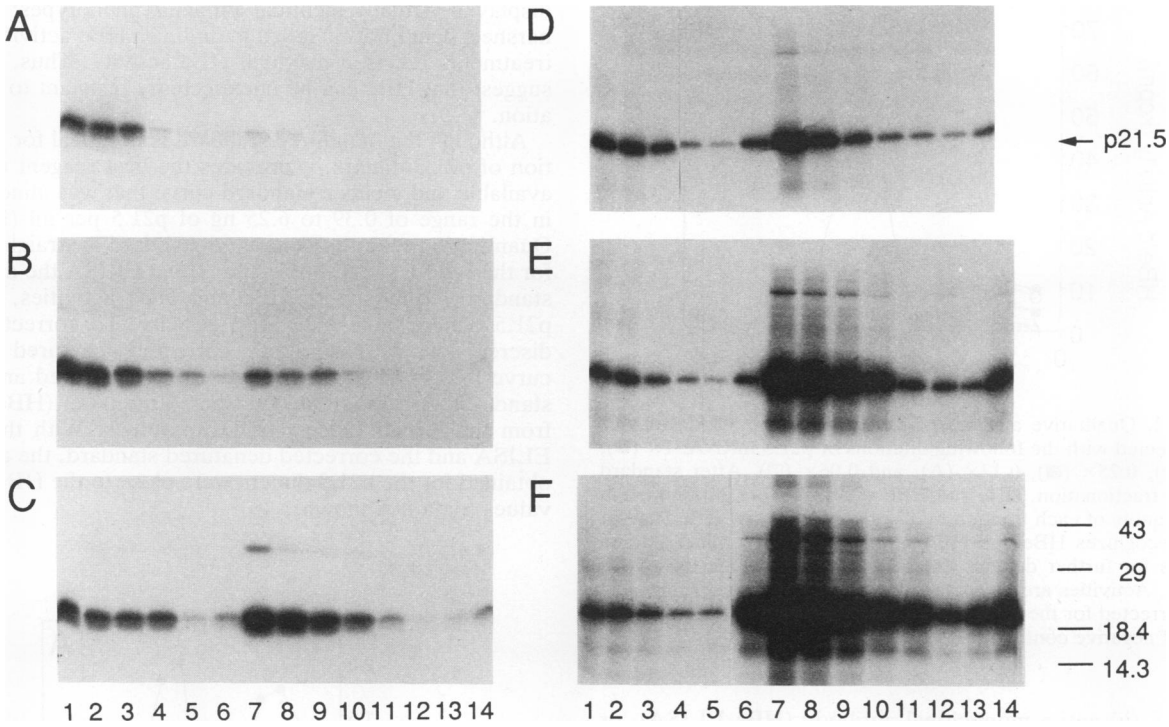

FIG. 1. Analysis of nonparticulate ^{35}S-labeled p21.5 and capsids on sucrose gradients. Lysates were prepared from ^{35}S-labeled oocytes injected with the following concentrations of p21.5 mRNAs: 0.25× (A), 0.5× (B), 1× (C), 1× (D), 5× (E), and 10× (F). These mRNAs were prepared by serial dilution of 1× (A to C) and 10× (D to F) transcription mixtures, respectively. Lysates were fractionated on gradients of 10 to 60% sucrose (for further details, see Materials and Methods). Nonparticulate and capsid forms of p21.5 were immunoprecipitated from the fractions with rabbit anti-p21.5 serum and analyzed by SDS-PAGE. The autoradiograms show gradient fractions arranged from the top (lane 1) to the bottom (lane 14); only 13 fractions were collected in panel D. For clarity, the positions of prestained molecular mass markers (sizes are in kilodaltons) are indicated only at the right of panel F and the position of p21.5 is indicated at the right of panel D.

sponses for free p21.5 dimers (lanes 1 to 4) versus capsids (lanes 7 to 12). The 0.25× (A), 0.5× (B), and 1× (C) RNAs and the 1× (D), 5× (E), and 10× (F) RNAs yielded apparently similar amounts of free ^{35}S-labeled p21.5 dimers (as judged from the signal intensities). In contrast, capsid production was minimal for the 0.25× RNA (A) and rose dramatically with each increase in input RNA.

A third dilution experiment (Fig. 2)—in which oocytes were injected with 1× (●), 0.5× (○), 0.25× (■), 0.12× (△), or 0.06× (□) p21.5 mRNA transcription reaction mixtures—was analyzed by an RIA that detects both the HBe and HBc epitopes carried by the p21.5 polypeptide (see below). Only the 1× and 0.5× mRNAs generated detectable levels of capsids (fractions 7 to 9), and the capsid signal was strikingly lower for the 0.5× RNA. These two RNAs yielded similar amounts of p21.5 dimer activity (fractions 1 to 3), but this diminished rapidly for the subsequent dilutions.

The above-described experiments indicate that capsid assembly proceeds in two distinct phases. The initial phase is accumulation of free p21.5, presumably in the form of p21.5 dimers. In the second phase, the free p21.5 levels begin to saturate and capsid formation initiates, with most further free p21.5 synthesis being channeled into capsid production. The experimental results in Fig. 2 depict both phases of the assembly process, whereas the data in Fig. 1 focus mainly on the second phase.

Antigenic identity of p21.5 species and evaluation of standards for their quantitation. The above-described approaches are not well suited to determining the concentration of p21.5 dimers required to establish capsid assembly. Detection of dimers was not very reproducible by RIA (data not shown). Quantitation by metabolic labeling is problematic, since we do not know (i) the relative efficiencies with which dimers and capsids can be immunoprecipitated, (ii) the specific activity of the label in these species (oocytes have large endogenous pools of amino acids), and (iii) the relative kinetics of uptake of labeled amino acids versus dimer synthesis and capsid assembly. (During the 48-h labeling period a substantial amount of unlabeled dimer synthesis occurs before the ^{35}S label has had time to equilibrate throughout the oocyte, and the rate of label uptake must decrease before the end of the experiment; thus, for kinetic reasons, the specific activities of the dimer precursors and capsid products are likely to differ.)

For more reliable quantitation, we turned to ELISAs employing antibodies directed against two classes of epitopes (core and e) carried by the p21.5 polypeptide. Capsids characteristically express core epitopes (HBc), while e epitopes (HBe) are latent in capsids but are released upon their disruption. However, the antigenic character of the unassembled p21.5 dimers is not known and this complicates the ELISA analysis. We therefore used ELISAs monospecific for HBe and HBc to compare the antigenicity of p21.5 dimers and capsids (Fig. 3).

p21.5-expressing oocytes were fractionated on a standard sucrose gradient. Aliquots of the fractions were diluted and assayed in parallel by using the following antibodies to capture p21.5: (i) anti-e/c polyclonal antibody (HBe/c

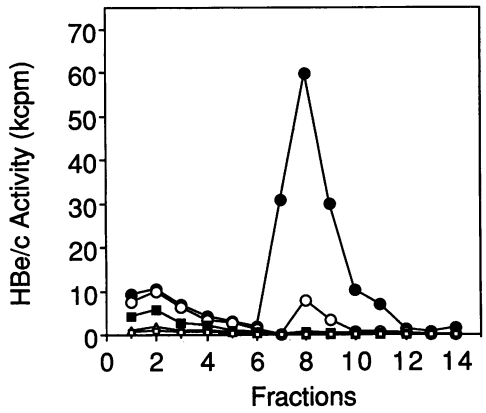

FIG. 2. Qualitative analysis of p21.5 species by RIA. Oocytes were injected with the following dilutions of p21.5 mRNA: 1× (●), 0.5× (○), 0.25× (■), 0.12× (△), and 0.06× (□). After standard gradient fractionation, HBe and HBc activities were determined in 10-μl aliquots of each fraction by using a commercial HBe RIA kit which recognizes HBe and HBc determinants. See Materials and Methods for further details. Fraction 1 represents the top of the gradient. Activities are given as 10^3 counts per minute (kcpm) and were corrected for the background by subtraction of the mean of a series of negative controls.

ELISA), (ii) anti-e monoclonal antibody (HBe ELISA), or (iii) anti-c monoclonal antibody (HBc ELISA). A polyclonal rabbit anti-p21.5 serum that recognizes multiple epitopes on p21.5 (data not shown) was then used to detect the captured antigens. The HBe/c assay (Fig. 3A) yielded the typical profile with both p21.5 dimers and capsids. The HBe ELISA (Fig. 3B) detected p21.5 dimers (fractions 1 to 6) but failed to recognize capsids. Conversely, the HBc ELISA (Fig. 3C) detected capsids (fractions 8 to 10) but not dimers. Thus, the two p21.5 species are antigenically distinct; unassembled dimers express HBe but not HBc, while capsids display HBc but not HBe.

In light of these results, quantitation of p21.5 dimers and capsids demands different types of p21.5 concentration standards. For quantitation of capsids, a very pure preparation of recombinant capsids (of known concentration) from *E. coli* provided a suitable standard (the native standard), which yielded an almost linear standard curve for 0.78 to 12.5 ng of p21.5 per ml in the HBc ELISA (Fig. 4A) or the HBe/c ELISA (data not shown). Core particles from *Saccharomyces cerevisiae* (14) gave an essentially identical curve (data not shown) and provided independent verification of the concentration of the *E. coli* capsids.

As expected, the native standard displayed very little HBe activity (Fig. 4B). An HBe-positive standard (the denatured standard) was generated by boiling the *E. coli* capsids with 6% 2-mercaptoethanol and 2% SDS, a procedure reported to release HBe immunoreactivity with concomitant loss of HBc (17, 20). Successful disruption of capsids and release of HBe activity was confirmed by the HBe ELISA (Fig. 4B) and by nonreducing SDS-PAGE (data not shown), in which the denatured standard ran mainly as dissociated p21.5 monomers. However, surprisingly, the HBc ELISA indicated that this denaturation did not destroy or even significantly diminish HBc immunoreactivity (Fig. 4A). In further experiments (data not shown), dilute solutions of *E. coli* capsids (50 μg/ml) were denatured with a wide range of concentrations of 2-mercaptoethanol (0.3 to 6% [vol/vol]) and SDS (0.1 to 5% [wt/vol]) and assayed by ELISA. All of the preparations displayed virtually identical antigenic phenotypes; even the harshest denaturation failed to diminish HBc activity, but all treatments released maximal HBe activity. Thus, our data suggest that HBc can be unexpectedly resistant to denaturation.

Although the denatured standard is not ideal for quantitation of p21.5 dimers, it provides the best reagent currently available and yields a standard curve that was almost linear in the range of 0.39 to 6.25 ng of p21.5 per ml (Fig. 4B). Quantitation with the denatured standard is straightforward for the HBe ELISA, but in the HBe/c ELISA the denatured standard exhibits both HBe and HBc activities, whereas p21.5 dimers have only HBe activity. To correct for this discrepancy, we generated a corrected denatured standard curve by assaying serial dilutions of denatured and native standards in parallel and subtracted the latter (HBc) values from the former (HBe plus HBc) values. With the HBe/c ELISA and the corrected denatured standard, the estimates obtained for the p21.5 dimers were close to the HBe ELISA values (data not shown).

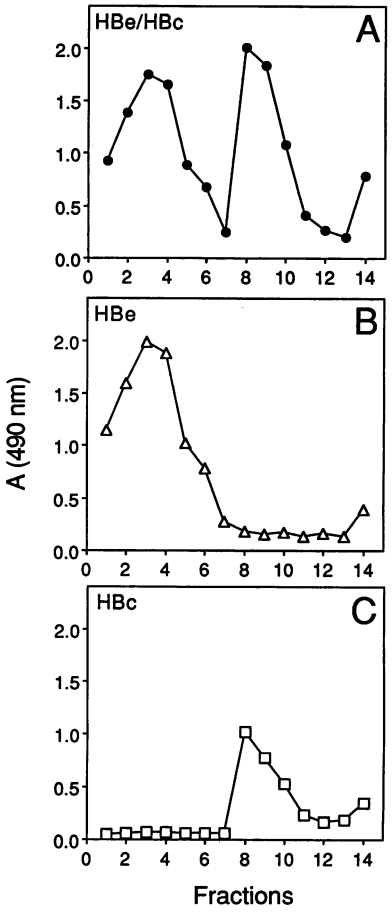

FIG. 3. Antigenic characterization of p21.5 assembly intermediates and capsids. Oocytes were injected with 6× p21.5 mRNA and fractionated into free p21.5 and capsids on a standard sucrose gradient. The 14 fractions (fraction 1 is the top gradient fraction) were analyzed in parallel by using ELISAs against the determinants HBe and HBc (A), HBe (B), and HBc (C). The data are presented as A_{490} values. Further experimental details are provided in Materials and Methods.

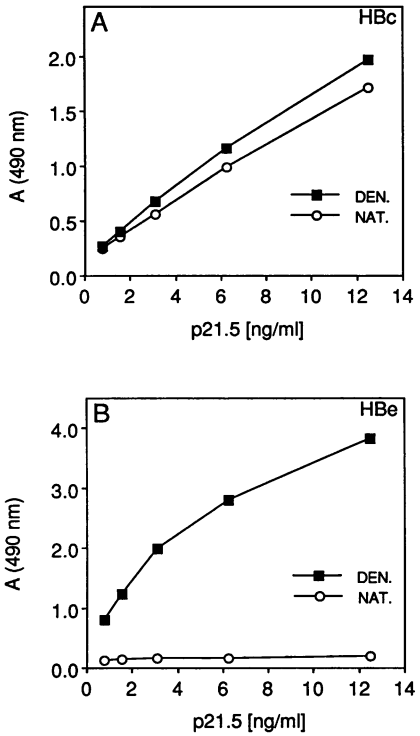

FIG. 4. HBe and HBc immunoreactivities of native and denatured p21.5 standards. HBc (A)- and HBe (B)-specific ELISAs were used to compare native (NAT.) and SDS–2-mercaptoethanol-denatured (DEN.) standards derived from the same preparation of *E. coli* capsids (see Materials and Methods). Serial dilutions of both standards (0.78 to 12.5 ng of p21.5 per ml) were assayed in parallel. The data are raw A_{490} values.

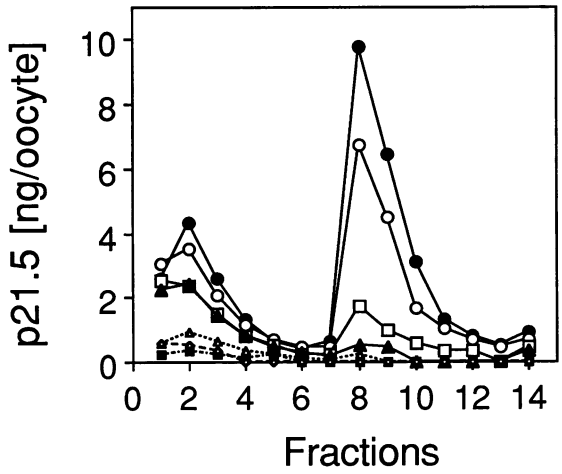

FIG. 5. Analysis of p21.5 assembly intermediates and capsids by ELISA. Oocytes were injected with a dilution series of 6× (●), 3× (○), 1.5× (□), 0.75× (▲), 0.38× (△), 0.19× (◇), and 0.09× (■) p21.5 mRNA. Lysates were fractionated on standard sucrose gradients. The p21.5 present in each fraction was estimated with the HBe/c ELISA by using the native standard for the capsid fractions and the corrected denatured standard for the nonparticulate p21.5 fractions. The data are given as nanograms per oocyte. Further details are provided in Materials and Methods.

Capsid formation initiates as the free p21.5 approaches micromolar concentrations. We next conducted a detailed quantitative analysis of the production of p21.5 dimers and capsids in a single RNA dilution series experiment (no. 289). Oocytes were microinjected with seven RNA dilutions covering a 64-fold concentration range (6× to 0.09×) and homogenized in PBS, and the p21.5 species were fractionated on sucrose gradients. The gradient profiles determined by HBe/c ELISA (Fig. 5) are similar to the earlier RIA profiles (Fig. 2) and further confirm that capsid production (fractions 7 to 12) requires prior accumulation of p21.5 dimer intermediates (fractions 1 to 5).

To quantitate the dimers and capsids more accurately, we pooled the respective gradient fractions and estimated their p21.5 contents (in nanograms per oocyte) with the HBe/c (Fig. 6A), HBe (Fig. 6B), and HBc (Fig. 6C) ELISAs in conjunction with the appropriate standards (see above and the legend to Fig. 6). The data reveal that the overall shape of the accumulation profile for dimers appears to be sigmoidal; the dimer levels (hatched) increased rapidly for the four most dilute RNAs and then more slowly for the remaining RNAs, and they finally reached a plateau at around 9 (HBe/c ELISA) or 16 (HBe ELISA) ng of p21.5 per oocyte. The reason for the variance between these estimates is unclear; the HBe and HBe/c ELISAs usually yielded much closer estimates of the p21.5 dimer concentrations (see below and Table 1).

The accumulation profile of capsids was strikingly different. Capsids were not detected for the three lowest RNA concentrations and first appeared (Fig. 6A and C) at 0.75× RNA. Thereafter, capsid production rose rapidly with increasing RNA concentrations with no sign of saturation. For capsids, the HBe/c (Fig. 6A) and HBc (Fig. 6C) ELISAs gave comparable concentration estimates.

We were concerned that significant losses of either p21.5 species might have occurred during gradient fractionation. To address this issue, and to test whether the p21.5 species could be measured directly in crude lysates, we analyzed unfractionated total oocyte lysates with the dimer-specific HBe (Fig. 6D) and the capsid-specific HBc (Fig. 6E) ELISAs. The resulting data are essentially identical to the data from the pooled gradient fraction (Fig. 6B and C), suggesting that (i) p21.5 dimers and capsids can be estimated directly in crude oocyte lysates and (ii) fractionation of oocyte extracts results in remarkably low losses of either species.

Comparison of capsid assembly in different experiments. In the above-described experiment (no. 289), detectable production of capsids was first seen in response to 0.75× RNA when unassembled p21.5 had accumulated to between 6 and 12 ng per oocyte (depending on the exact quantitation procedure used). We next asked whether the concentration dependence of capsid assembly varies from experiment to experiment or whether a specific concentration of unassembled p21.5 dimers is a characteristic of capsid assembly. Both the HBe/c and HBe ELISAs were used to quantitate the unassembled p21.5 dimers present in total lysates originating from experiment 289 (see above) and three other independent RNA dilution series experiments which were conducted over a 1-year period and involved different batches of oocytes and synthetic p21.5 RNA (Table 1). Capsid production was analyzed by subjecting either gradient-fractionated particle pools or crude oocyte lysates to RIA or ELISA (Table 1).

It is apparent from the data that different experiments exhibited significant variations in p21.5 expression and cap-

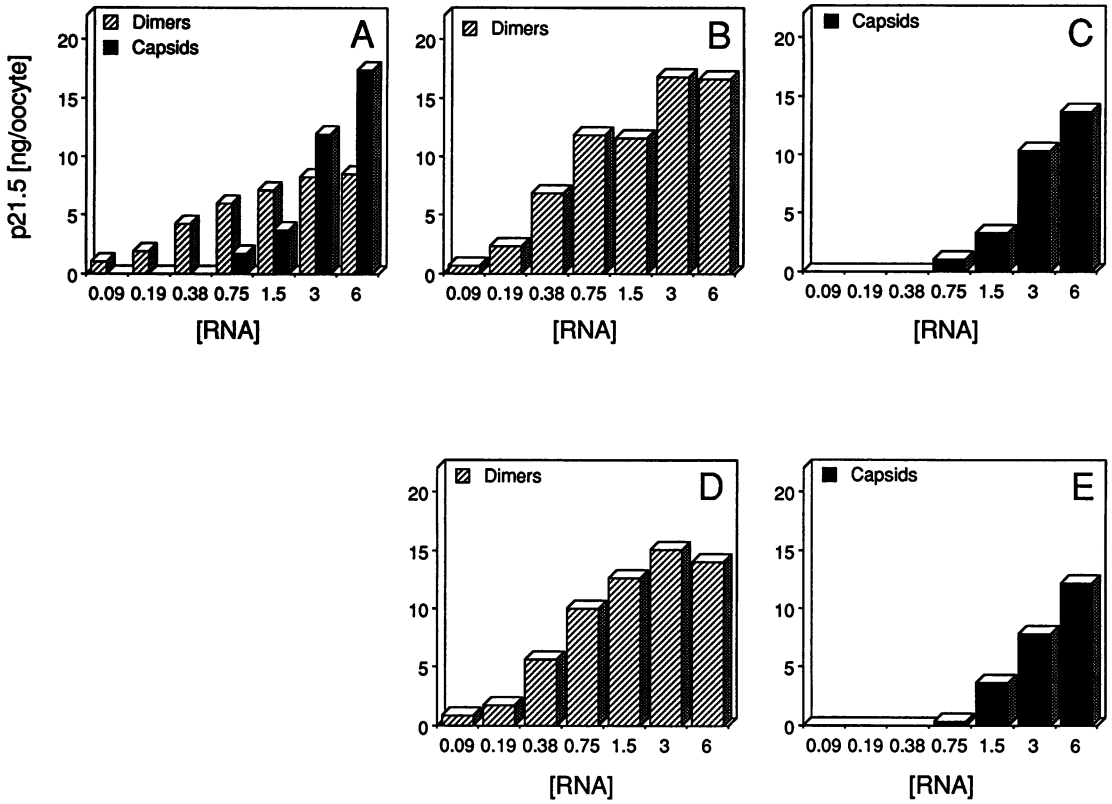

FIG. 6. Quantitation of p21.5 assembly intermediates and capsids. The RNA dilution series experiment (6× to 0.09× RNA) shown in Fig. 5 provided the material for this ELISA analysis. The p21.5 (in nanograms per oocyte) present in the form of dimers (hatched) or capsids (filled) was quantitated in either pooled gradient fractions (A to C) or unfractionated oocyte lysates (D and E). Serially diluted samples were analyzed in parallel with HBe/c (A), HBe (B and D), and HBc (C and E) ELISAs (see text). The following standards were used for calibration: denatured (B and D), native (C, E, and capsid pools in A) and corrected denatured (dimer pools in A). Further details can be found in Materials and Methods and elsewhere in the text. The gradient fraction pools were prepared by combining fractions 1 to 5 for p21.5 dimers and 7 to 12 for capsids.

sid assembly. A plateau of dimer production was reached in response to the more concentrated RNAs in experiments 177 and 289, but not in the remaining experiments. In one experiment (no. 284), even 5× concentrated mRNA failed to solicit capsid production. This experiment, however, was atypical; in our experience, standard (1×-concentrated) p21.5 mRNA generally drove significant capsid production (data not shown).

Despite the variations in p21.5 expression levels, the data from all four experiments are consistent with the picture of capsid assembly derived in the preceding sections and support the idea that a signature threshold concentration of p21.5 dimers is required to initiate capsid assembly. The estimates of p21.5 dimer concentration obtained from the HBe and HBe/c ELISAs were generally in good agreement throughout the range of p21.5 concentrations (e.g., see experiment 177); only in experiment 289 did the two assays differ significantly. The average of the HBe and HBe/c ELISA estimates for the concentration of p21.5 intermediates at which capsid production was first detected in each experiment were ~8.1 ± 0.1 (no. 177), 7.1 ± 0.9 (no. 193), and 7.6 ± 2.4 (no. 289) nanograms per oocyte. Thus, capsid assembly appears to initiate once an oocyte has accumulated a pool of ~7 to 8 ng of p21.5.

Several precautions were taken to reduce errors in our ELISA concentration measurements. We analyzed independent experiments with both intermediate and capsid-specific ELISAs, checked p21.5 concentrations with both bacterium- and yeast-derived capsid standards, performed quantitation assays in duplicate, reassayed critical samples, and quantitated p21.5 species in total extracts, as well as in gradient fractions (gradient fractionation resulted in negligible loss of either p21.5 species). All of these approaches gave results which were consistent and generally agreed remarkably well, thus increasing our confidence in the estimates.

DISCUSSION

Recent studies with *Xenopus* oocytes have offered the first view of how simple HBV capsids are assembled. This process appears to proceed via aggregation of p21.5 dimers without extensive accumulation of detectable intermediates (36–38). This report describes a more detailed analysis of this assembly process and contains three new elements: (i) the demonstration that dimers and capsids are antigenically distinct, (ii) qualitative studies indicating that core particle assembly is a highly concentration-dependent and cooperative process, and (iii) an estimate of the concentration of p21.5 required to initiate capsid formation. These data strengthen the analogy between the assembly pathways seen for HBV capsids in oocytes and for simple RNA viruses,

TABLE 1. Quantitative comparison of p21.5 dimer assembly intermediate and capsid concentrations among individual experiments

Expt no.	[RNA] (×)	Concn (ng/oocyte) of:		
		Intermediates		Capsids (anti-e/c or anti-c)
		Anti-e/c	Anti-e	
177	1.00	9.99	9.00	++[a]
	0.50	8.02	8.13	+
	0.25	3.56	3.87	ND[b]
	0.12	1.57	1.59	ND
	0.06	1.13	1.01	ND
193	1.00	7.95	6.38	1.76[c]
	0.50	3.07	2.64	ND
	0.25	2.75	2.20	ND
	0.12	1.17	0.58	ND
	0.06	0.56	0.10	ND
284	5.00	4.46	6.44	ND[c]
	2.50	1.44	2.04	ND
	1.25	0.31	0.31	ND
	1.00	0.56	0.57	ND
289	6.00	11.02	13.98	12.41[d]
	3.00	8.25	15.04	7.80
	1.50	6.20	12.62	3.64
	0.75	5.20	9.98	0.34
	0.38	3.23	5.67	ND
	0.19	1.45	1.78	ND
	0.09	0.55	0.86	ND

[a] Assessed from gradient-fractionated p21.5 by RIA and expressed in arbitrary units (+, low; ++, medium).
[b] ND, not detected.
[c] Assessed from gradient-fractionated p21.5 by HBe/c-ELISA.
[d] Assessed from crude lysates by HBc-ELISA.

such as *E. coli* bacteriophage R17, which have been determined from in vitro reconstitution studies.

Antigenic characterization of capsid assembly and disassembly. A byproduct of our ELISA studies on the concentration dependence of core particle assembly was the demonstration that p21.5 dimers express HBe but not HBc epitopes, while capsids have the reciprocal antigenicity. It was not previously realized that dimers and capsids are antigenically distinct and that capsid assembly is accompanied by masking of HBe and elaboration of HBc. This finding was confirmed by using several different HBe- and HBc-specific antibodies (data not shown). Mechanistically, loss of HBe activity may be a simple consequence of HBe epitopes becoming buried as dimers aggregate into capsids. We envision the HBc epitope as discontinuous, probably involving residues contributed by adjacent p21.5 subunits which are brought together only as dimers aggregate. An alternative, although perhaps less persuasive, explanation for these epitope interconversions is that they are caused by conformational changes in the p21.5 polypeptide that accompany capsid assembly.

Although we failed to detect p21.5 multimers intermediate in size between dimers and capsids in a recent biochemical characterization of the precursors for capsid assembly (37), it could be argued that the low-resolution methods used in this study permitted a significant fraction of higher p21.5 multimers to escape detection. The HBc-specific ELISA offers a potentially powerful method for detecting such species, since aggregated p21.5 dimers, in particular, might be expected to carry HBc epitopes (see above). Our failure to detect HBc activity in the population of unassembled p21.5 species is therefore a further indication that there are few, if any, higher multimers of p21.5 hidden within the free p21.5 dimer pool.

To obtain quantitation standards suitable for estimation of HBe-expressing dimers, we dissociated HBc-positive capsids. It is generally thought that chemically induced disassembly of capsids is accompanied by destruction of HBc epitopes and the emergence of cryptic HBe epitopes (18, 21, 26, 30)—essentially the converse of capsid assembly. Thus, we were surprised to find that preparations of *E. coli*-derived capsids that had been disrupted with 2-mercaptoethanol and SDS retained almost full HBc activity despite being highly HBe immunoreactive. Even the severest denaturation conditions tested failed either to release additional HBe activity from our preparations or to strip them of HBc epitopes. Sporadic claims from other groups support the idea that disrupted capsids can retain HBc activity (5, 17, 31). How the resistance of the HBc epitope to denaturation is accomplished in molecular terms is an intriguing issue that warrants further investigation.

Qualitative analysis of the process of HBV capsid assembly. Earlier work (38) established the utility of oocytes for studying the concentration dependence of capsid assembly by showing (i) that both p21.5 capsids and dimers can be simultaneously observed and (ii) that capsid assembly can be prevented by lowering the intracellular concentration of p21.5 (by diluting the injected RNA). Here we created a wide range of p21.5 concentrations by injecting oocytes with stepwise serial dilutions of p21.5 mRNA. The effectiveness of this strategy for manipulating the capsid assembly process was readily apparent throughout this work (e.g., Fig. 1 and Table 1). In practice, we observed capsid assembly in most of the experiments involving concentrated or 1× preparations of p21.5 mRNA, whereas diluted RNA preparations yielded mostly unassembled p21.5 (Table 1 and data not shown). Thus, the oocyte system appears to have the valuable, if fortuitous, property of expressing p21.5 at concentrations particularly suited for studying HBV capsid assembly.

All of the RNA dilution series experiments gave the same qualitative picture of capsid assembly, regardless of the method of analysis. HBV capsid assembly is a biphasic process. The initial phase involves accumulation of stable p21.5 dimers (37) with little or no detectable production of capsids. Dimer accumulation continues until the pool of dimers begins to saturate. Shortly before the plateau of dimer production is reached, there is a sharp transition to the second phase, in which capsid assembly initiates. Beyond this transition point, there is little further increase in the size of the dimer pool and most additional p21.5 synthesis becomes channeled into capsid production.

The highly cooperative and concentration-dependent nature of the HBV capsid assembly process has mechanistic implications. For instance, our data seem incompatible with any model that invokes highly localized (and therefore concentration-independent) interactions between p21.5 dimers and the mRNA which directed their synthesis. Nor do they appear to favor models requiring formation of a discrete series of progressively more complex subcapsid intermediates. On the other hand, they do seem consistent with a model in which a distinct initiation event directs a freely diffusible population of p21.5 dimers to aggregate once a specific concentration is reached (cf. R17 phage assembly [see below]).

Quantitative aspects of p21.5 synthesis and assembly. Quantitation of p21.5 by ELISA was undertaken for three reasons. First, we simply wanted to know how much p21.5 is synthesized in oocytes. In experiment 289, for example, the extent of p21.5 production varied from ~0.55 to ~23.43 ng per oocyte, depending on the input RNA concentration. In this case, a 64-fold range of input RNA elicited a 43-fold range of p21.5 expression, revealing an almost linear relationship between p21.5 synthesis and the input RNA concentration (whether the product is exclusively dimers or a mixture of dimers and capsids) and confirming the effectiveness of the RNA dilution series strategy. The quantitation data also reveal that the extents of p21.5 synthesis and capsid assembly varied between experiments (Table 1), presumably because of quality fluctuations among batches of RNA and also oocytes.

Our second goal was to estimate the size of the pool of p21.5 dimers required for initiation of capsid assembly. Estimates from several different experiments suggest that this value corresponds to ~7 to 8 ng of p21.5 per oocyte. Given that most (>90%) of the p21.5 dimers and the bulk (~95%) of capsid assembly localize to the oocyte cytosol (36) with an effective volume of ~500 nl (9), our data suggest that the signature concentration of p21.5 required to initiate capsid assembly is ~14 to 16 μg/ml (0.7 to 0.8 μM).

The accuracy of this estimate is compromised by the lack of chemically pure dimers with which to calibrate the ELISA. The substitute calibration standard (denatured capsids) may have a different HBe activity per p21.5 polypeptide chain compared with p21.5 dimers. Thus, it is formally possible that dimer estimates differ from the real values by an unknown, but presumably constant, factor. Unfortunately, estimation of dimers by the metabolic labeling or RIA approach is no more reliable (see Results) and estimates from immunoblotting (data not shown) are compromised by the large amount of endogenous proteins and the small amount of p21.5 dimers in the oocyte (although this method has verified the expected levels of p21.5 expression for oocytes engaged in efficient capsid production). However, none of these alternative approaches contradict the ELISA data. Indeed, our analyses of a given experiment by the qualitative metabolic labeling or quantitative ELISA approach always suggested comparable extents of capsid assembly and comparable dimer/capsid ratios (data not shown).

Thus, we feel that the error introduced into the pool size estimates by use of denatured capsid standards is unlikely to be major. Other possible sources of inaccuracy, such as slight fluctuations in the local cytoplasmic p21.5 concentration due to uneven diffusion (either of p21.5 or of the injected mRNA) and the fact that capsid assembly takes place at 17°C in oocytes versus 37°C in cultured animal cells, are probably relatively minor. While the pool size of p21.5 required for capsid assembly may be off by, say, two- to perhaps fourfold from our 0.7 to 0.8 μM estimate, we are confident that the dimer pool size is close to 1 μM, rather than 1 nM or 1 mM. A more precise quantitation of the concentration dependence of HBV capsid assembly will likely have to await the availability of pure p21.5 dimers and an in vitro reconstitution study.

The final reason for our interest in quantitation is that it paves the way for dissection of the contribution of specific interactions to the assembly process. The 0.7 to 0.8 μM value reported in this study reflects the pool of p21.5 required to assemble simple HBV capsids containing not only p21.5 dimers but also RNA, as judged by their density on cesium chloride gradients (data not shown). Capsids containing little or no RNA are assembled from p21.5 molecules from which the protamine-like nucleic acid-binding region has been deleted (5, 12). The relative size of the dimer pool required to assemble such RNA-deficient capsids may give clues as to whether RNA plays a direct role in capsid assembly. Similarly, it will be interesting to determine whether coexpression of the viral polymerase and pregenome RNA causes an alteration in dimer pool size; such an effect may shed light on the way these components become drawn into the assembly process. The oocyte system appears ideal for addressing these issues. Moreover, relative rather than absolute estimates of dimer pool size will be sufficient.

Comparison with the assembly of bacteriophage R17. High-resolution electron micrographs (22, 34) suggest that the HBV capsid is a structure with T=3 symmetry that is constructed from 180 p21.5 capsid protein subunits. Many small positive-strand RNA plant and insect viruses, as well as bacteriophages and animal picornaviruses, share this simple capsid architecture (1, 10, 11, 13, 16, 24). The nucleocapsids of vertebrate picornaviruses, such as poliovirus, mengovirus, and foot-and-mouth disease virus, are assembled from protomers containing one subunit of each of distinct VP0, VP1, and VP3 capsid proteins. Five of these protomers subsequently aggregate into stable pentameric intermediates from which the capsid is ultimately assembled. Thus, picornavirus assembly proceeds via an ordered pathway involving a discrete series of intermediates (24).

On the other hand, T=3 plant viruses, such as tomato bushy stunt virus (10), or RNA coliphages, such as R17 (3, 4), are assembled from 90 chemically identical dimers. In vitro reconstitution studies have recently defined the R17 bacteriophage assembly process in detail (3, 4). R17 capsid formation appears to be a highly concentration-dependent and cooperative coassembly process involving dimers of the ~25-kDa coat protein and RNA (33). Phage assembly first occurs as dimer concentrations reach 1 μM and proceeds by cooperative polymerization of 90 dimers around the ~3.6-kb R17 RNA genome with no obvious accumulation of intermediates. Interactions between the dimers and RNA appear to initiate R17 assembly.

De novo assembly of simple HBV capsids, as defined in our oocyte studies, appears to be quite different from picornavirus assembly but is strikingly similar to phage R17 assembly in regard to (i) the use of dimer building blocks, (ii) the concentration requirements for assembly, (iii) cooperativity, and (iv) lack of accumulation of readily detectable intermediates (37). This analogy between the R17 and HBV assembly processes may furnish valuable clues to HBV capsid assembly.

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