reverse transcriptase, known as the P protein.  
Hepadnaviral reverse transcription occurs in cytoplasmic core particles. Core particle formation begins with binding of P to the \( e \) stem loop at the 5' end of the viral pgRNA (5–7). This complex is then encapsidated through polymerization of 120 dimers of C around it (8, 9). Encapsulation appears to be rapid because no intermediates between dimers of C and complete particles have been found in cells (10). Expression of the three viral products needed for encapsidation (pgRNA, C, and P) is closely linked because the pgRNA is also a bicistronic mRNA that encodes C and P (11–14). C and P are both translated by \textit{de novo} initiation from their ORFs on the bicistronic pgRNA (15, 16).

\( \text{C} \) accumulates in cells to easily detectable levels that rise steadily over the first few days of expression in liver or in cultured cells. The accumulation pattern of P has never been directly measured because of the lack of appropriate antibodies. Expression of P is believed to be very low for three reasons. First, P is translated from the downstream ORF of the bicistronic pgRNA. Such ORFs are usually very poorly translated, especially when the context of the initiation codon (17) is suboptimal and there are multiple upstream AUGs, as is the case for P. Second, the best estimate indicates that only one or two \( P \) molecules are within each core particle (18), and thus little P would be needed relative to C for assembly of core particles. Third, using kinase epitope-tagged P, it has been proposed that P is a translational repressor (18). These observations led to the idea that slow translation of P is the rate-limiting step in encapsidation (18).

However, using specific antibodies for P, we found that DHBV P accumulates in the cytoplasm to detectable levels and that the majority of P in cells is not encapsidated (19). Therefore, much more P accumulates in cells than was anticipated. The mechanism of the accumulation of this excess P and its biological function are unknown. P could be translated slowly and have a very long half-life. Alternatively, P could be translated rapidly relative to the demands of P for encapsidation. In any case, the kinetics of P biosynthesis and turnover could play an important role in viral assembly and thus on viral replication, pathology, or antigen presentation by infected cells.

To understand the kinetic mechanism for the expression of excess DHBV P in cells, we performed a quantitative analysis of P and C expression and degradation. These experiments are the first such experiments to directly analyze P using specific antibodies rather than employing surrogate reporter genes, and thus this is the first analysis of the synthesis and degradation of P in the context of ongoing reverse transcription.

**EXPERIMENTAL PROCEDURES**

\textit{Virus and DNA Constructs—}DHBV Type 3 was employed (20). D1.5G is an overlength DHBV3 expression construct containing a 5' duplication of nucleotides 1658–3021 in pbBlueScript(--) (Stratagene). Mutations introduced into D1.5G were dCore (deletion of nucleotides...
2846–2849 that truncates C and blocks encapsidation); P-OF (deletion of nucleotide 424 that truncates P after amino acid 84 (15)); and dIBulge (deletion of nucleotides 2571–2576 that removes the bulge of e and blocks binding of P and encapsidation (21)).

Cell Culture, Transfection, and Infection—LMH cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum. Cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) as recommended by the manufacturer.

Lyse Preparation, Western Blotting, and Immunoprecipitation—LMH cells were lysed in 0.75% or 1% radioimmune precipitation assay buffer (1 x 20 mM Tris, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl) containing 1% phenylmethylsulfonyl fluoride and 3 μg/ml leupeptin on ice for 10 min, and the lysates were clarified at 14,000 × g for 10 min at 4 °C. For immunoprecipitation, antibody was bound to protein A/agarose (Oncogene Research Products) and incubated with lysates at 4 °C overnight. Immunocomplexes were washed three times with 1 ml of 0.75% or 1% radioimmune precipitation assay buffer, and proteins were released with Laemml buffer. Samples were resolved by SDS-PAGE and detected by PhosphorImager analysis or Western blotting.

For Western blotting, proteins were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore) membranes. P was detected with anti-DTP3′-His monoclonal antibody mAb9. Following incubation with the appropriate IgG-alkaline phosphatase conjugate (Promega), P was visualized by incubation with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega) according to the manufacturer’s instructions.

Metabolic Labeling and Pulse-chase Determination of Half-life of P and C—Transfected LMH cells were washed twice with DMEM lacking methionine and cysteine (labeling medium) and pulsed with labeling medium supplemented with 120 μCi/ml EasyTag Express (Promega) and 1% fetal bovine serum. For pulse-chase experiments, cells were labeled for 15 min (for P) or 3 h (for C), rapidly washed twice with DMEM/F12, and then fed with DMEM/F12 containing 10% fetal bovine serum. Great effort was taken to perform these steps as rapidly as possible, and all media were equilibrated to 37 °C prior to use. Cells were incubated at 37 °C overnight. Cells were incubated at 37 °C overnight. Immunoprecipitation, antibody was bound to protein A/G-agarose (Oncogene Research Products) and incubated with lysates at 4 °C overnight. Immunocomplexes were washed three times with 1 ml of 0.75% or 1% radioimmune precipitation assay buffer, and proteins were released with Laemml buffer. Samples were resolved by SDS-PAGE and detected by PhosphorImager analysis or Western blotting.

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The solution to this equation is $y(t) = y_0 e^{-\lambda t}$, where $y_0$ is the instantaneous synthesis rate, $\lambda$ is the decay constant, and $t$ is time. The radioactivity detected by immunoprecipitation (A) is the area under the decay curve for the labeling period (T), as shown in Equation 1.

$$A = \int_0^T y_0 e^{-\lambda t} dt$$

The solution to this equation is $y(t) = A/[1/(1/\lambda) - (1/\lambda)e^{-\lambda T}]$. Solving for $\lambda$ in terms of half-life ($T_{1/2}$) yields $\lambda = -\ln(0.5)/T_{1/2}$. The instantaneous translation rate, $y_0$, was calculated from these equations. We then assumed that the translation rate remained constant during the labeling period and multiplied $y_0$ by $T$ to yield the total amount of P synthesized.

RESULTS

The Half-lives of P and C Are Vastly Different—The half-life of P was determined by transfecting LMH cells with DHBV expression constructs and performing pulse-chase experiments at day 1 or 3 after transfection. There are no cell lines infectable by DHBV, but transfection of LMH chicken hepatoma cells with expression constructs for DHBV (e.g. D1.5G) produces viral core particles competent for reverse transcription and results in release of infectious virions from the cells (22). Transfected LMH cells were metabolically labeled with [35S]methionine/cysteine for 15 min, washed twice with non-radioactive medium, supplied with non-radioactive medium containing 10% fetal bovine serum, and incubated for various times. At each time point, cells were lysed, P was immunoprecipitated, and radioactivity in P was determined by phosphorimage analysis. This assay measures the half-life of nonencapsidated P because encapsidated P cannot be precipitated with anti-P antibodies (19). A representative experiment for wild-type D1.5G-transfected cells at day 3 after transfection is shown in Fig. 1. Table I shows that nonencapsidated P had a very short half-life of 2.3 h at day 1 and 15 min at day 3.

We were unable to measure the half-life of C because it was exceptionally stable. A representative experiment with D1.5G-transfected cells at 3 days after transfection is in Fig. 1. Chase periods as long as 29 h revealed no reproducible diminution of the radioactivity immunoprecipitated. The amount of radioactive C immunoprecipitated in pulse-chase experiments could be reduced by secretion of virus particles or by degradation of C. The persistence of radioactive C indicates that C is stable in this system and that its degradation and secretion rates can be ignored in kinetic analyses. The negligible secretion rate is consistent with the low rate of production of mature viruses from LMH cells at early times after transfection (data not shown).

P Is Translated at 10% the Rate of C—P and C are translated from the bicistronic pgRNA (11–14). The downstream position of the P ORF on the pgRNA, the suboptimal Kozak context (17) of the DHBV P AUG (UAUAUGG), and the presence of 15 AUG codons between the mRNA cap and the P AUG (four of which are in identical or equivalent Kozak contexts to the P AUG) lead to the prediction that P would be translated a great deal less frequently than C. To test this prediction, LMH cells were transfected with a wild-type DHBV expression vector (D1.5G) and labeled with [35S]methionine/cysteine for 1.5 or 4 h prior to lysis at 1 or 3 days after transfection. P and C were immunoprecipitated from equal fractions of the lysates, and radioactivity incorporated into the proteins was measured by PhosphorImager analysis of the gels exposed simultaneously to the same plate. A representative experiment is shown in Fig. 2.

![Fig. 1. Representative pulse-chase experiments to determine $T_{1/2}$ for P and C. LMH cells were transfected with D1.5G. At day 3 after transfection, the cells were labeled for 15 min (for P) or 3 h (for C) with [35S]methionine/cysteine and incubated in non-radioactive medium for the indicated times, and then the cells were lysed, P or C was immunoprecipitated.](http://www.jbc.org/Downloaded from)
The total amounts of P and C synthesized during the labeling period were calculated from the radioactivity immunoprecipitated, the labeling time, the half-life of P (25.3 min for day 1 or 15.2 min for day 3; Table I), and the numbers of methionine and cysteine residues in P and C. Dividing the total amount of P by the total amount of C yielded a molar P/C translation ratio of 0.10 ± 0.020 at day 1 and 0.11 ± 0.001 at day 3 (Table II). A measure of the validity of this approach is that it should be insensitive to the length of the labeling period, and indeed, the values for the day 1 P/C ratio in Table II reveal little difference when the labeling time was 1.5 or 4 h. Therefore, P is translated 10-fold less rapidly than C, despite the downstream location of the P ORF on the bicistronic mRNA, the suboptimal Kozak context of the P AUG, and the presence of 15 other AUGs upstream of the P AUG.

P Is Rarely Encapsidated—The level of nonencapsidated P in cells exceeds the amount of encapsidated P by 3-fold at day 3 after transfection (19). Because encapsidation greatly stabilizes P, this implies that very few P molecules are encapsidated. We tested this hypothesis by examining the decay kinetics of P using mutations to DHBV that blocked encapsidation.

The disappearance of P in pulse-chase experiments measured by immunoprecipitation could occur by either encapsidation or degradation. If the rates of encapsidation and degradation are appreciably different, and if both processes affect significant fractions of P, then the decay curve of nonencapsidated P would be biphasic. To determine whether disappearance of nonencapsidated P is monophasic or biphasic, LMH cells were transfected with DHBV or core-deleted DHBV (DHBV(dlCore)) expression constructs, the cultures were metabolized of P, this implies that very few P molecules are encapsidated. We tested this hypothesis by examining the decay kinetics of P using mutations to DHBV that blocked encapsidation.

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The decay profile for nonencapsidated P was monophasic when P was expressed from the wild-type genome on day 1 after transfection, shortly after encapsidation begins (Fig. 3A; linear correlation coefficient 0.978). The decay profile for nonencapsidated P from the wild-type genome was also linear at day 3 after transfection, when the encapsidation reaction is well established (Fig. 3B; linear correlation coefficient 0.979). Next, we measured the decay profile of nonencapsidated P expressed from DHBV(dlCore), where encapsidation is blocked because C is not produced. The decay profile at day 3 after transfection for nonencapsidated P expressed from DHBV(dlCore) was also linear (Fig. 3C; linear correlation coefficient 0.980). These data indicate that the decay of nonencapsidated P is monophasic and that the turnover rates of P are the same from wild-type and encapsidation-defective DHBV. Therefore, the fraction of P molecules that becomes encapsidated is too small to affect the decay kinetics of P.

DISCUSSION

These data reveal that the kinetic mechanism for the accumulation of excess P is rapid translation coupled with inefficient encapsidation and that the steady-state level of P in the cells is limited by the short half-life of P. These experiments are the first to analyze P in the context of active reverse transcription because previous quantitative analyses of the synthesis or degradation of P relied on in vitro systems or employed replication-deficient constructs in which P was replaced by a
encapsidation-deficient.

ysis. P was quantitated by PhosphorImager analysis. DHBV(dlCore) is stability of the nonencapsidated P was determined by pulse-chase anal-

transfected with DHBV expression constructs, and 1 or 3 days later, the 
maker gene (11, 14, 23-28).

DHBV P was translated from the pgRNA at 10% the rate of 
C (i.e. P/C = 0.1). This relatively high rate is similar to the P/C rate of 0.25–0.33 that was determined using a P-LacZ fusion gene initiating at the P AUG (23). The P/C translation rate for HBV has been measured at 0.1 by employing a reporter gene for HBV P (24), indicating that rapid synthesis of P is probably conserved between the avian and mammalian hepadnaviruses. The unusually high translation rate of P and the overlap between the P and C ORFs raises the possibility that translation of the P and C ORFs could interfere with each other. Given that the P AUG is probably located by the ribosomes through an unusual mechanism (14, 26), we suspect the pgRNA may be segregated into two translational pools. One of these pools would produce C, and the other would produce P. Because encapsidation appears to occur in cis (i.e. P binds to the same mRNA molecule from which it was translated (6)), this implies that those pgRNA molecules that produce C would not be encapsidated.

Three observations indicate that the proportion of P molecules that become encapsidated is very small. First, blocking encapsidation did not affect the decay profile of P or the intra-
cellular accumulation of P (Fig. 3), and thus the proportion of P that was encapsidated must be much smaller than the proportion that was not encapsidated. Although we cannot directly determine the level of encapsidation that would be required to leave a mark on the decay pattern of nonencapsidated P, we estimate that the sensitivity limit of these assays would be about 10–20% of the total P synthesized. Second, we previously demonstrated that the amount of nonencapsidated P exceeds the amount of encapsidated P in cells by 3-fold at day 3 after transfection (19). Here, we found that nonencapsidated P has a half-life of only 15 min, whereas encapsidated P must be as stable as the core particle, which has a half-life of many hours to days. This indicates that the fraction of P molecules that are encapsidated must be considerably below the 10–20% estimated detection limit of our kinetic assays. Third, much more P was made than the minimum needed to assemble core particles. At day 3 after transfection in LMH cells (when encapsi-
dation is well established), P is synthesized 10% as rapidly as C. However, core particles contain 240 C molecules (8, 9) and 1–2 P molecules (18), so the P translation rate was 12–24-fold higher than the minimum required to supply P for core particles.

The kinetic mechanism for the metabolism of P is in Fig. 4. In this mechanism, newly synthesized P (P_new) is made from amino acids (AA) to produce P_new. P_new can then either interact with cellular components to become nonencapsidated P (P_ne) or be encapsidated (P_encap). Secretion of capsids containing P results in virion-associated P (P_virus). The flow of P through this mechanism is controlled by the translation rate (K_Synth), the rate of association of P_new with cellular components (K_Encap), the rate of degradation of P_ne (K_Deg), the rate of disintegration of capsids (K_Disint), and the secretion rate of core particles as virions (K_Sec).

The half-life of P drops from 25.3 ± 4.0 min at day 1 after transfection to 15.2 ± 1.5 min on day 3 after transfection (Table I). Because the half-life of a protein is inversely proportional to its degradation rate, this 40% reduction in the stability of P implies that degradation of P is regulated. The reduction in stability of P is independent of the encapsidation reaction (Table I), indicating that the destabilization of P is not controlled by the level of encapsidation. The mechanism for the turnover of P is unknown, but it may involve the ubiquitin/proteasome pathway because addition of the proteasome inhibitor MG-132 increases the steady-state level of P in cells 2–3-fold (data not shown).

These data disprove the accepted model that slow translation of P is limiting for encapsidation (18). Because encapsidation

FIG. 3. P decays with monophasic kinetics. LMH cells were transfected with DHBV expression constructs, and 1 or 3 days later, the stability of the nonencapsidated P was determined by pulse-chase analy-

FIG. 4. Kinetic mechanism for metabolism of P. P is translated from amino acids (AA) to produce P_new. P_new can then either interact with cellular components to become nonencapsidated P (P_ne) or be encapsidated (P_encap). Secretion of capsids containing P results in virion-associated P (P_virus). The flow of P through this mechanism is controlled by the translation rate (K_Synth), the rate of association of P_new with cellular components (K_Encap), the rate of degradation of P_ne (K_Deg), the rate of disintegration of capsids (K_Disint), and the secretion rate of core particles as virions (K_Sec).
requires binding of P to e on the pgRNA (5–7), our data imply either that the intracellular concentration of P and the pgRNA combined with the P:e binding constant do not favor P:e binding or that only a small amount of P ever binds to the cellular chaperones that are needed for P to bind to e (29–31).

The large amount of cytoplasmic P must be the source of antigen resulting in the strong and early immune response to P, which can be as rapid and strong as the response to C, a major viral structural protein (32–35). Production of this excess of P is a liability to the virus through induction of additional immune pressure. The hepadnaviral replication strategy is to establish a persistent infection lasting decades, and the presence of an avoidable liability such as production of a large excess of P implies that the nonencapsidated P may be important for the virus. We have speculated that P may play a regulatory role on cellular or viral functions in addition to synthesizing the viral genome (19). These results support this hypothesis by demonstrating that P is primarily a cytoplasmic protein that is only occasionally encapsidated.

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