Chimeras of Duck and Heron Hepatitis B Viruses Provide Evidence for Functional Interactions between Viral Components of Pregenomic RNA Encapsidation

Kristin M. Ostrow and Daniel D. Loeb*

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received 20 February 2004/Accepted 12 April 2004

Hepadnaviruses, also known as hepatitis B viruses, are DNA viruses that replicate via reverse transcription of an RNA pregenome (pgRNA) (9). The pgRNA is transcribed from the hepadnaviral plasmid present in the nucleus, called covalently closed circular DNA (5, 28). The pgRNA can be translated to produce the viral polymerase, P, and the capsid subunit, C (8, 26). Within the cytoplasm, the pgRNA and P are packaged into capsids in a process known as RNA encapsidation (1, 11). Capsids containing pgRNA and P are competent for reverse transcription of the pgRNA (27). Once DNA synthesis is complete, capsids can be secreted from the cell to produce virions.

There are several viral components important for pgRNA encapsidation (Fig. 1). The C protein is the subunit of the capsid that contains pgRNA. C protein also might contribute to pgRNA encapsidation directly (21). The P protein is required for pgRNA encapsidation, but the reverse transcriptase or DNA-priming activity of P is not required (1, 11, 29, 30). Also, the pgRNA contains cis-acting sequences that contribute to its encapsidation (6, 12, 16). One sequence, called ε, is located near the 5′ end of the pgRNA. ε forms a phylogenetically conserved secondary structure that is important for its function (16, 23, 24). For the human hepatitis B virus (HBV), ε is the only identified sequence on the pgRNA required for encapsidation (16).

For the avian hepadnaviruses, a second sequence located about 900 nucleotides (nt) downstream of ε, called region II, is required for encapsidation (6, 12). Analysis of duck hepatitis B virus (DHBV) region II showed that sequence from nt 420 to 720 contributes to encapsidation, but deletions within a critical sequence of region II from nt 539 to 610 cause a defect in encapsidation that is similar in magnitude to deletion of ε (22). DHBV shares 79% nucleotide identity with heron hepatitis B virus (HHBV), and these viruses have similar encapsidation requirements. The HHBV region II sequence has been roughly mapped to be within nt 398 to 831 (22). Our previous studies have indicated that the intervening sequence between ε and region II could also play a role in pgRNA encapsidation (22). In that work, substitutions or large deletions of the intervening sequence between ε and region II impaired encapsidation. However, smaller deletions within the intervening sequences did not have an effect on encapsidation. There may be functionally redundant sequences within the intervening sequence, or the intervening sequence may be required as a spacer between ε and region II for encapsidation.

Previous work with DHBV has demonstrated that P interacts with ε to facilitate encapsidation of P and the pgRNA (24). It is not known how region II or the intervening sequence between ε and region II contributes to encapsidation; however, it is likely that other interactions are important for encapsidation. One approach to identifying functional interactions is through studies of chimeric viruses. Several groups have used chimeric retroviruses to study the requirements for retroviral encapsidation (3, 4, 7, 17, 19, 25). HHBV/DHBV chimeras have been used successfully to elucidate the interactions between cis-acting sequences that are important for the synthesis of plus-strand DNA (20). Furthermore, our previous work demonstrated that replacing DHBV region II with HHBV region II impaired encapsidation, indicating that HHBV region II is incompatible with one or more DHBV encapsidation components (22). In the present study, we used several chimeras of DHBV and HHBV to identify components that need to
FIG. 1. DHBV encapsidation requires the viral P and C proteins and several cis-acting sequences on the pgRNA: ε, region II, and possibly the intervening sequence between ε and region II. P is represented as an oval, C is represented as a pentagon, and cis-acting sequences are indicated. The terminal redundancy, R, is labeled on pgRNA. For encapsidation, P binds to ε for the packaging of P and pgRNA, but little is known about other required interactions. The binding of P to ε may initiate the cooperative assembly of C subunits around pgRNA. Alternatively, capsid assembly may occur concurrently or before the P-ε interaction. pgRNA encapsidation is a prerequisite for reverse transcription and virion production.

FIG. 2. Experimental strategy for analyzing encapsidation of hepadnavirus chimeras. A test plasmid and an internal standard plasmid were cotransfected into LMH cells. This figure represents the pgRNA of DHBV and the DHBV internal standard. Nucleotide coordinates of the 5′ end, region II, and the position of the P32P mutation are indicated. The test plasmid is the wild-type reference or the chimeric variant whose encapsidation was tested. All test plasmids are null for the synthesis of P and C proteins. The internal standard provided P32P and C in trans. After three days, A and C fractions of RNA were isolated from the cells. RPA was performed to detect the test RNA and internal standard RNA in the A and C fractions. To detect the chimeric RNAs, two probes, one with DHBV sequence and one with HHBV sequence, were used simultaneously. Also, the P32P substitution in the DHBV and HHBV internal standards allowed the internal standard RNA to be distinguished from the test RNA in the RPA. Encapsidation was measured by comparing the level of test RNA found in the C fraction, normalized to the internal standard in the C fraction, to the level of test RNA found in the A fraction, normalized to the internal standard in the A fraction.

MATERIALS AND METHODS

Plasmid constructs. The designations of all test constructs, including wild-type references or chimeras, are boldfaced. Chimeras were given designations corresponding to the sequence that was substituted. All chimeric plasmids were generated from two parental plasmids, DHBV P-ε-C and HHBV P-ε-C, referred to below as DHBV and HHBV, respectively (22). DHBV contains 1.5 tandem copies of the DHBV3 genome. DHBV is null for the synthesis of P because of a 1-nt deletion at nt 424 which produces a unique PstI restriction site at nt 420 and is null for the synthesis of C because of a 4-nt deletion from nt 2646 to 2849. HHBV contains 1.4 tandem copies of the HHBV4 genome. HHBV is null for the synthesis of P because of a 1-nt substitution at nt 182, and it is null for the synthesis of C because of a 1-nt deletion at nt 2690. The HHBV/DHBV chimeras (see Fig. 4A, 5A, and 6A) were made by substituting the analogous HHBV sequence in DHBV. HHBV RII (see Fig. 6A) has HHBV nt 426 to 717 replacing the DHBV sequence by using the PstI site at nt 420 and the EcoRV site at nt 718. HHBV ε (see Fig. 4A) has HHBV sequence from nt 1787 to 2652 replacing the DHBV sequence from nt 1660 to 2646 in the 5′ copy of the redundancy in DHBV. See Fig. 5A for diagrams of the following chimeras: HHBV ε/RII, HHBV 5′ third, HHBV ε 5′ to 327/RII, HHBV ε/RII and HHBV ε/RII and HHBV. HHBV 5′ third contains HHBV sequence from nt 1787 to 3027, HHBV sequence from nt 1 to 717 replacing DHBV nt 1660 to 3021, and DHBV nt 1 to 717 in DHBV, but with the P-null and C-null mutations of HHBV. HHBV 5′ to 327/RII has HHBV nt 2653 to 3027 replacing DHBV nt 2647 to 3021 in HHBV ε/RII. HHBV ε/RII and HHBV has HHBV nt 1 to 425 replacing DHBV nt 1 to 425 in HHBV ε/RII. HHBV ε/RII and HHBV has the single substitution of HHBV nt 1 to 717 for DHBV nt 1 to 717.

The DHBV/HHBV chimeras (see Fig. 7A) were made by substituting the analogous DHBV sequence in HHBV. See Fig. 7A for diagrams of DHBV RII, DHBV ε, DHBV ε/RII, and DHBV 5′ third. DHBV RII contains DHBV sequence from nt 425 to 722 replacing HHBV sequence at the same positions. DHBV ε has DHBV sequence from nt 1660 to 2669 replacing HHBV sequence from nt 1787 to 2675 and contains the HHBV P- and C-null mutations. DHBV ε/RII has the DHBV substitutions of both DHBV RII and DHBV ε in HHBV. HHBV 5′ third has HHBV sequence from nt 1660 to 3021, DHBV nt 1 to 722 replacing HHBV nt 1787 to 3027, and HHBV nt 1 to 722 in HHBV. The details for the molecular cloning of any plasmid construct will be provided upon request.

Cell cultures and transfection. The chicken hepatoma cell line LMH was cultured as described previously (10). DNA transfections were performed by the calcium phosphate precipitation method. For each transfection, 7 μg of test plasmid and 3 μg of internal standard plasmid were transfected.

Isolation of viral RNA. Three days posttransfection, LMH cells were washed with 2 mM HEPES (pH 7.45), 150 mM NaCl, and 0.5 mM EGTA. Cytoplasmic lysates were prepared by treatment with Nonidet P-40 lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 0.2% Nonidet P-40), and nuclei were pelleted. Half of the lysate was used for the isolation of cytoplasmic poly(A) RNA (A RNA), and the other half was used to isolate capsid RNA. The cytoplasmic poly(A) RNA was isolated by using oligo(dT) cellulose (New England Biolabs) as previously described (22). Capsid RNA was isolated by treating the lysate with micrococcal nuclease ( Worthington) as previously described (22).

RPA. Riboprobes were transcribed in vitro from linearized DNA templates by using T7 RNA polymerase and were labeled with [α-32P]UTP. Riboprobes were gel purified, and RNase protection analysis (RPA) was carried out as described previously (22). RNA from 1/10 of a transfected plate was used in each analysis. The probes in the RPA were designed to detect only pgRNA and not subgenomic RNA. However, the 5′ half of the pgRNA, which is unique to the pgRNA, was the region replaced with DHBV or HHBV, and none of the sequence within this region was common to all the variants. Consequently, we used two riboprobes in every RPA: one that was derived from DHBV (nt 420 to 722) and one that was derived from HHBV (nt 426 to 727). The test RNA from the HHBV probes detected only HHBV sequence and.

From: "Avian Hepadnavirus RNA Encapsulation" Published in VOL. 78, 2004 AVIAN HEPADNAVIRUS RNA ENCAPSIDATION 8781

Downloaded from http://jvi.asm.org/ on October 23, 2017 by guest
not HHBV sequence and that the HHBV probe detected only HHBV sequence and not DHBV sequence. The DNA template for the HHBV probe contains a PstI restriction site engineered 5′ of nt 426 and an EcoRV site engineered 3′ of nt 718. Some HHBV/DHBV chimeras contain HHBV substitutions with these engineered restriction sites, and consequently, the chimeric RNA will protect more of the probe than wild-type HHBV sequence.

The probes annealed over a region in the RNA that was different in the internal standard RNA versus the test RNA (as previously described [22]). The DHBV internal standard contained a substitution of 5 out of 7 nt from nt 451 to 457, and the HHBV internal standard contained a substitution of 4 out of 6 nt from nt 452 to 457. These substitutions changed amino acid 96 in the P protein from tyrosine to phenylalanine, and the remaining nucleotide changes were silent in the P gene. The substitutions in the DHBV and HHBV internal standards allowed the internal standard RNA to be distinguished from the test RNA in the RPA.

![Diagram](http://jvi.asm.org/)

**FIG. 3.** DHBV P and C support efficient encapsidation of HHBV pgRNA, but HHBV P and C do not support efficient encapsidation of DHBV pgRNA. (A) RPA of DHBV and HHBV cotransfected with the DHBV internal standard. The positions of test RNA, internal standard RNA, and the probe are indicated. Lane 1, probes undigested; lane 2, probes digested; lanes 3 and 4, test RNA alone or internal standard RNA alone; lanes 5 and 7, cytoplasmic poly(A) fraction of RNA (A RNA); lanes 6 and 8, capsid fraction of RNA (C RNA). The source of the replication proteins is adjacent to ovals (P protein) and pentagons (C protein). The histogram compares the encapsidation efficiency of HHBV with that of DHBV using DHBV P and C proteins. Results are averages and standard deviations from six independent analyses. (B) RPA of HHBV and DHBV cotransfected with the HHBV internal standard. Lanes are as explained in the legend to panel A and as indicated. The histogram compares the encapsidation efficiency of DHBV with that of HHBV using HHBV P and C proteins. Results are averages and standard deviations from four independent analyses.

RESULTS

**Rationale and experimental design.** DHBV encapsidation requires several viral components: the P and C proteins and the cis-acting sequences in pgRNA, including ε, region II, and possibly portions of the intervening sequence between ε and region II (Fig. 1). Our aim was to learn how these viral components contribute in concert to the packaging of pgRNA into capsids. We made chimeras of DHBV and HHBV to identify which encapsidation components were required to be derived from the same species for encapsidation. If a chimeric substitution disrupts encapsidation, it suggests that there is an incompatibility between the substitution and the other encapsidation components. By understanding what components need to be derived from the same virus, we can gain insight into how the viral components interact and function in encapsidation.

We used a quantitative assay that has been previously described to measure the level of RNA encapsidation of the chimeras (Fig. 2) [22]. LMH cells were cotransfected with a test plasmid and an internal standard plasmid. The test plasmid expressed the pgRNA of the wild-type reference or the chimeric variant. All test plasmids were null for P and C protein expression (P<sub>C</sub>/H11002) to avoid the production of chimeric viral proteins. Therefore, we do not underestimate pgRNA encapsidation because of a loss of RNA due to DNA synthesis. The internal standard plasmid also served as a control for possible variations during the isolation and measurement of RNA. Three days posttransfection, cytoplasmic poly(A) RNA (A fraction) and capsid RNA (C fraction) were isolated from cells, and the test and internal standard RNAs were examined by RPA (as described in Materials and Methods). Encapsulation efficiency was calculated as the level of test RNA, normalized to internal standard RNA, detected in the C fraction divided by the level of test RNA, normalized to internal standard RNA, detected in the A fraction.
DHBV P and C support encapsidation of the HHBV pgRNA, but HHBV P and C do not encapsidate the DHBV pgRNA at high levels. An interspecies complementation was performed in order to understand the ability of DHBV and HHBV pgRNAs and proteins to support encapsidation of each other. We asked how well the DHBV P and C proteins encapsidate the HHBV pgRNA compared to the DHBV pgRNA, and how well HHBV P and C encapsidate the DHBV pgRNA compared to the HHBV pgRNA. We learned that HHBV was encapsidated with DHBV P and C at the same level as DHBV (99% ± 19%) (Fig. 3A; see also Fig. 4A). This result indicated that DHBV P and C were functionally compatible with the HHBV pgRNA for encapsidation. On the other hand, DHBV was not encapsidated with HHBV P and C at the same level as HHBV (19% ± 4%) (Fig. 3B and 4A). One possible reason why the DHBV pgRNA is not encapsidated at high levels by HHBV P and C may be that the process of HHBV encapsidation is more efficient than DHBV. However, analysis of the level of C RNA compared to A RNA of the HHBV internal standard and the DHBV internal standard demonstrated that these two viruses package their RNA into capsids at similar levels (compare C to A RNA levels for HHBV and DHBV internal standards [Fig. 3 and 4; see also Fig. 5 to 7; data not shown]). Therefore, the DHBV pgRNA is encapsidated at low levels with HHBV P and C because of incompatibilities between the DHBV pgRNA and one or both of the HHBV proteins.

An ε chimera demonstrates incompatibilities of HHBV P and C proteins with a DHBV sequence downstream of ε. The reduced ability of the HHBV proteins to encapsidate the DHBV pgRNA was potentially the result of a disrupted RNA-protein interaction. It is known that P interacts with ε for encapsidation. Possibly, the DHBV ε could not interact efficiently with the HHBV P protein. Thus, we replaced the DHBV ε with HHBV ε in DHBV (Fig. 4A, HHBV ε) and assayed encapsidation using DHBV and HHBV proteins. Interestingly, HHBV ε was also deficient in encapsidation when HHBV P and C were used, relative to encapsidation of HHBV (22% ± 3% [Fig. 4A and B]). On the other hand, HHBV ε was efficiently encapsidated by using DHBV P and C (102% ± 3%...
indicating that HHBV ε is compatible with the DHBV sequence downstream of ε and the DHBV proteins. Together, the results in Fig. 4B and C indicate an incompatibility between HHBV P and/or C and the DHBV sequence downstream of ε. Possibly, a viral protein(s) interacts with the sequence downstream of ε, which contains the intervening sequence and region II, for the encapsidation of pgRNA. P and/or C is required to be compatible with HHBV and a large sequence containing region II.

We tested additional chimeras to locate the sequence downstream of ε that was incompatible with the HHBV proteins (Fig. 5). We found that having ε and region II derived from HHBV was not sufficient to restore encapsidation to the same level as that of HHBV. HHBV ε/RII was encapsidated at low levels by using HHBV P and C compared to encapsidation of HHBV (30% ± 8% [Fig. 5A and B]). Extending the HHBV ε substitution 3′-wards in HHBV 5′-3027/RII did not increase encapsidation using HHBV P and C, either (15% ± 1% [Fig. 5A and C]). However, extending the region II substitution 5′-wards in HHBV ε/1-717 restored encapsidation using HHBV P and C (87% ± 10% [Fig. 5A and C]), demonstrating that HHBV P and C need to be compatible with nt 1 to 717. Having only nt 1 to 717 derived from HHBV in the single chimera HHBV 1-717 was not sufficient to restore encapsidation using HHBV P and C (44% ± 3% [Fig. 5A and C]), indicating that both ε and nt 1 to 717 needed to be derived from HHBV for encapsidation using HHBV P and C. The finding that HHBV 1-717 exhibited impaired encapsidation, but HHBV ε/1-717 restored encapsidation using HHBV P and C, provides evidence that ε needs to be compatible with a viral protein for encapsidation. Also, region II and/or the intervening sequence is required to be compatible with P and C, since adding HHBV sequence from nt 1 to 717 restores encapsida-

FIG. 5. HHBV ε and nt 1 to 717 are required for efficient encapsidation using HHBV P and C. (A) Schematic of pgRNAs synthesized by test plasmids of HHBV or HHBV/DHBV chimeras and their encapsidation efficiency using HHBV P and C and normalized to HHBV. Nucleotide coordinates at substitution boundaries of the chimeras represent the HHBV position. Results are averages and standard deviations from four independent analyses for all variants, except for HHBV 1-717, results for which represent five independent analyses. (B and C) RPAs of HHBV/DHBV chimeras cotransfected with the HHBV internal standard. Lanes are as explained in the legend to Fig. 3A and as indicated.
tion of HHBV ε. These results are consistent with one or more viral proteins interacting directly or indirectly with ε and with a large sequence containing region II and the intervening sequence to facilitate pgRNA encapsidation.

Compatibility of cis-acting sequences within region II and possibly the intervening sequence is required for encapsidation. In contrast to the DHBV pgRNA and HHBV P and C, the HHBV pgRNA was efficiently encapsidated by using DHBV P and C. Thus, DHBV P and C are compatible with HHBV cis-acting sequences for encapsidation. If a chimeric substitution disrupted encapsidation using DHBV P and C, this result would identify cis-acting sequences that potentially interact for encapsidation. Accordingly, we tested encapsidation of the chimeras by using DHBV P and C to identify incompatibilities between cis-acting sequences. Interestingly, HHBV ε/RII, HHBV ε'-3027/RII, and HHBV RII showed impaired encapsidation when DHBV P and C were used (33% ± 4%, 32% ± 5%, and 22% ± 7%, respectively [Fig. 6]). In contrast, HHBV ε'-1-717 was encapsidated at a level similar to that of DHBV by using DHBV P and C (96% ± 4% [Fig. 6A]). Together, these results indicate that DHBV sequence from nt 1 to 425 is not fully functional with one or more HHBV cis-acting sequences. Adding back HHBV sequence from nt 1 to 425 with HHBV ε and region II in the chimera HHBV ε/1-717 restored encapsidation using DHBV P and C (84% ± 12% [Fig. 6A and C]). But having only nt 1 to 425 and region II derived from HHBV in the chimera HHBV 1-717 was sufficient to restore encapsidation using DHBV P and C (88% ± 17% [Fig. 6A and C]), showing that nt 1 to 425 are required to be compatible with nt 426 to 717. The sequence from nt 1 to 717 contains region II and part of the intervening sequence between ε and region II. Possibly, an RNA-RNA interaction occurs within region II and the intervening sequence to contribute to encapsidation.

Reciprocal chimeras corroborate findings that intervening sequence needs to be compatible with region II for high levels of encapsidation. The analysis for which results are shown in Fig. 6 shows that the sequence from nt 1 to 717 in HHBV must be compatible for high levels of encapsidation. Since region II has not been well defined for HHBV, we do not know if the area that was contributing to function within HHBV nt 1 to 717 consists of region II alone or includes part of the intervening sequence as well as region II. On the other hand, the ε and region II sequences have been better defined for DHBV than for HHBV (22). Thus, we might better understand the contributions of cis-acting sequences within nt 1 to 717 if we studied

FIG. 6. An interaction within region II and/or the intervening sequence may contribute to encapsidation. (A) pgRNAs of DHBV and HHBV/DHBV chimeras and their encapsidation efficiency using DHBV P and C and normalized to DHBV. Nucleotide coordinates at substitution boundaries of the chimeras represent the HHBV position. Results are averages and standard deviations from four independent experiments for all chimeras except HHBV ε/1-717, results for which represent five independent analyses. (B and C) RPAs of HHBV/DHBV chimeras cotransfected with the DHBV internal standard. Lanes are as explained in the legend to Fig. 3A and as indicated.
experiments for all chimeras except

Results are averages and standard deviations from four independent

pared to that of

with the DHBV internal standard, and encapsidation was com-

pared with the HHBV backbone. These chimeras (Fig. 7) were cotransfected

contained DHBV encapsidation sequences substituted in an

HHBV backbone. We tested chimeras that con-

trained DHBV encapsidation sequences substituted in an

HHBV backbone. These chimeras (Fig. 7) were cotransfected with the DHBV internal standard, and encapsidation efficiency was compared to that of DHBV.

Results are averages and standard deviations from four independent experiments for all chimeras except DHBV RII, which was analyzed five times. (B) RPA of reciprocal chimeras cotransfected with the DHBV internal standard. Lanes are as explained in the legend to Fig. 3A and as indicated.

FIG. 7. Reciprocal DHBV/HHBV chimeras show that the inter-

vening sequence needs to be compatible with region II for encapsi-

dation. (A) Schematic of pgRNAs of reciprocal chimeras with DHBV

capsidation sequences substituted into HHBV. Nucleotide coordi-

nates at substitution boundaries of the chimeras represent the DHBV

position. These chimeras were cotransfected with the DHBV internal standard, and encapsidation efficiency was compared to that of DHBV.

They in the context of DHBV. We tested chimeras that con-

tained DHBV encapsidation sequences substituted in an

HHBV backbone. These chimeras (Fig. 7) were cotransfected with the DHBV internal standard, and encapsidation was com-

pared to that of DHBV. A chimera containing DHBV ε and region II, DHBV ε/RII, exhibited impaired encapsidation compared to that of DHBV (42% ± 2% [Fig. 7]). Having the 5’ third derived from DHBV in DHBV 5’ third restored encapsi-
dation to higher levels (85% ± 9% [Fig. 7]). These results show that the HHBV intervening sequence is incompatible with DHBV ε and region II. Having ε and the intervening sequence derived from the same virus in DHBV RII was not

sufficient to restore encapsidation (46% ± 7% [Fig. 7]). How-

ever, having the intervening sequence and region II derived from the same virus, in DHBV ε, increased encapsidation over that of DHBV ε/RII (76% ± 5% [Fig. 7]). These results show that the intervening sequences are required to be compatible with region II to create a functional unit for pgRNA encapsi-
dation. This corroborates the findings depicted in Fig. 6 that suggest that an RNA-RNA interaction between the intervening sequence and region II contributes to encapsidation.

DISCUSSION

Our analysis of hepadnaviral encapsidation using chimeric viruses has provided information about functional interactions required for encapsidation of DHBV and HHBV pgRNA. We learned that the DHBV pgRNA was encapsidated with HHBV P and C at a level significantly lower than that of the HHBV pgRNA. Therefore, one or more incompatibilities exist be-
tween the DHBV pgRNA and the HHBV P and/or C proteins.

We substituted HHBV sequence in the DHBV pgRNA to show that P and/or C needs to be compatible with ε and a large sequence containing region II for encapsidation. We also showed that the HHBV pgRNA was encapsidated at normal levels by using DHBV P and C. We used this relationship to identify cis-acting interactions for encapsidation. These studies demonstrate that sequence from nt 1 to 425 must be compatible with the adjacent sequence containing region II (nt 426 to 717) for efficient encapsidation. Reciprocal chimeras with DHBV substituted in the HHBV pgRNA showed that the intervening sequence needs to be compatible with region II for wild-type levels of encapsidation. This work also corroborates previous findings that suggested that ε and region II are not sufficient for DHBV encapsidation (22), and it further implicates the intervening sequence between ε and region II as playing a role in the encapsidation of avian hepadnaviruses.

It is possible that interactions between region II and the intervening sequence might organize and/or fold the pgRNA into a conformation that allows the pgRNA to fit more readily into the interior of the nucleocapsid. These putative interactions could potentially be augmented through additional inter-
actions with the C and/or P protein. More information is needed about secondary and tertiary conformations of the pgRNA during the encapsidation process.

Furthermore, it is unclear whether the intervening sequence and region II contain multiple discrete elements that function independently or if the intervening sequence and region II are part of a single functional unit. The finding that the intervening sequence and region II need to be derived from the same virus suggests that they are acting in concert.

A model that is consistent with our findings is that a pgRNA conformation formed by the intervening sequence and region II interacts with P for encapsidation. Findings from other studies are consistent with the idea of P interacting with this region of the pgRNA. A previous report suggested that P may bind DHBV sequence within nt 142 to 828 and regulate C transla-
tion in vitro (13). This observation may be related to pgRNA encapsidation in cells. P interacting with nt 142 to 828 could be required for pgRNA encapsidation and downregulation of C translation. Alternatively, downregulation of translation may be important to promote pgRNA encapsidation. It is possible
that several processes, such as translation or RNA degradation, contribute to the efficiency of pgRNA encapsidation. An interaction between P and the intervening sequence and region II might be involved in several molecular events that direct pgRNA down the encapsidation pathway.

It is thought that only one molecule of P is encapsidated into each capsid particle (2). Possibly, a ternary complex between P, ε, and the sequence containing region II facilitates pgRNA encapsidation. This ternary complex could be important for inhibiting the translation of P and C and promoting pgRNA encapsidation. Alternatively, two molecules of P could be encapsidated by one binding to ε and one binding to the intervening sequence and region II. The P-ε interaction is facilitated by host chaperones (14, 15), and other P-pgRNA interactions could have similar requirements. The P-pgRNA complex may be packaged into capsids through interactions between P and C. HBV P and C have been shown to interact in insect cells (18). However, it is not known if DHBV P and C can also interact or if interactions between P and C are important for encapsidation.

Another possibility that can be suggested from our results is that C binds in a specific manner to the intervening sequence and region II. Binding of C to this sequence could initiate assembly of the capsid around the pgRNA and P. There would still be a requirement for recognition by C of the P-ε RNP, since pgRNA encapsidation does not occur efficiently in the absence of P (1, 11). Further studies to identify important RNA structures that might form within the intervening sequence and region II, and to test if P or C binds to this region of the pgRNA, will be very useful.

Given that HBV does not require sequences in addition to ε for pgRNA encapsidation (16), it is likely that HBV uses an alternative mechanism to substitute for the function of the seemingly complex array of cis-acting sequences of avian hepadnaviruses. By first understanding the role of the additional sequences of avian hepadnaviruses, we can figure out how HBV has solved this problem.

ACKNOWLEDGMENTS

We thank members of the Loeb laboratory, Paul Ahlquist, and Mike Janda for helpful discussions. Thanks to Katy Haines for critical review of the manuscript. We thank Megs Maguire for making the molecular clone DHBVε.

This work was supported by NIH grants PO1 CA22443 and P30 CA14520.

REFERENCES
