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Pregenomic RNA Encapsidation Analysis of Eleven Missense and Nonsense Polymerase Mutants of Human Hepatitis B Virus

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We characterized 11 DNA polymerase mutants of human hepatitis B virus (HBV) which contain single missense or nonsense mutations in the various domains within this gene. Except for mutant 738, a tight association between DNA replication and RNA packaging of these missense *pol* mutants was observed. Further analysis of HBV core particle-associated RNA indicated that only the 3.5-kb core-specific RNA, but not the precore-specific RNA, is selectively packaged in this tissue culture system. Previously, we have demonstrated that only the 3.5-kb core-specific RNA can serve as an efficient template for *pol* translation. Taken together, our results suggest that selectivity of HBV RNA packaging occurs as a result of selective translation of *pol*-containing mRNAs. Furthermore, our data suggest that the RNA encapsidation domain of *pol* overlaps with all of the domains of *pol* involved in the synthesis of terminal protein, as well as DNA replication. Finally, on the basis of gradient centrifugation analysis, a *pol* defect appeared to have no negative effect on the assembly or stability of core particles. A new method to assay RNA encapsidation, as well as potential RNase H activity, is reported.

Similar to retroviruses, hepadnaviruses, including human hepatitis B virus (HBV), replicate through an RNA intermediate (9, 21, 28). RNA-dependent DNA synthesis is mediated by a virus-encoded gene product (designated Pol) (Fig. 1). However, unlike retroviruses, the translational strategy of *pol* in HBV (12, 23) and duck hepatitis B virus (4, 24) does not appear to be dependent on ribosomal frameshifting (6). With *cis* rescue (23) and *trans* rescue (4, 24) genetic strategies, it has been demonstrated that translations of *pol* and that of its upstream, overlapping nucleocapsid genes (Fig. 1) appear to be independent from each other.

There are two major HBV-encoded 3.5-kb transcripts that can potentially serve as mRNA templates for Pol synthesis. These two different species of 3.5-kb RNA are referred to as precore- and core-specific mRNAs in this report. The former initiates approximately 25 to 30 nucleotides 5′ upstream from the latter and is therefore able to synthesize the precore protein (31, 32). The latter initiates 3′ downstream from the ATG codon of the precore gene and is responsible mainly for synthesis of the core protein (18, 22). To understand the translational control of *pol* better, it is necessary to determine which 3.5-kb mRNA species is the translational template of *pol*. Using *Xenopus* oocyte microinjection, as well as the rabbit reticulocyte lysate in vitro translation system, we have previously demonstrated that it is the core-specific mRNA which is a more efficient template for Pol protein synthesis (17).

Formation of the HBV nucleocapsid involves the complex association of at least three different components, including pregenomic RNA, core protein, and DNA polymerase. The exact sequence of this complicated recognition process remains unclear. While some reports have proposed that the *pol* gene product is required for RNA encapsidation (1, 11), others have shown that preferential packaging of core-containing RNA by HBV-encoded core protein can occur in an overexpressing *Escherichia coli* system without the presence of polymerase (2). Whether RNA encapsidation has to be mediated via Pol and/or other HBV-specific gene products remains to be clarified.

Using a liver sample from a ground squirrel infected with ground squirrel hepatitis virus, Enders et al. (7) reported preferential packaging of core-specific RNA but not precore-specific RNA in the ground squirrel hepatitis virus animal model. Since hepadnaviruses are similar to each other in many respects, it is expected that in the HBV system in culture, core-specific RNA will probably be encapsidated selectively. Such selectivity clearly depends on highly specific protein-RNA interactions. To explore the molecular basis of the selective encapsidation of core-specific RNA, it will be necessary to define a domain from the *trans*-acting factor which is responsible for recognition of the identified *cis* element for RNA packaging (13).

Frameshift and amber linker insertion mutations located at the various domains of duck hepatitis B virus polymerase appear to have lost the capability of RNA packaging and DNA replication (11). Since these mutations are likely to involve more drastic structural alteration of polymerase, it is quite possible that the protein-folding pathway or stability has been severely affected. Therefore, it is difficult to conclude which specific domain(s) of Pol is required for this selective RNA encapsidation. To map the domain of *pol* responsible for selective RNA encapsidation, it appears to be more ideal to examine the capability of RNA encapsidation in missense *pol* mutants with only minimal structural alteration.

However, one disadvantage of the missense mutation approach is that missense mutation targets only one amino acid at a time; unless it happens to hit a critical amino acid in an effective manner, silent mutation is likely to be the outcome. For example, Bartenschlager et al. (1) have examined the RNA-packaging efficiencies of one reverse transcriptase (RT) and four RNase H missense mutants of HBV. No apparent difference from the wild type was observed. Similarly, Chang et al. (3) have examined the RNA-packaging efficiencies of one RT and five RNase H missense mutants of duck hepatitis B virus. No apparent impairment of RNA encapsidation was found either. The assignment of

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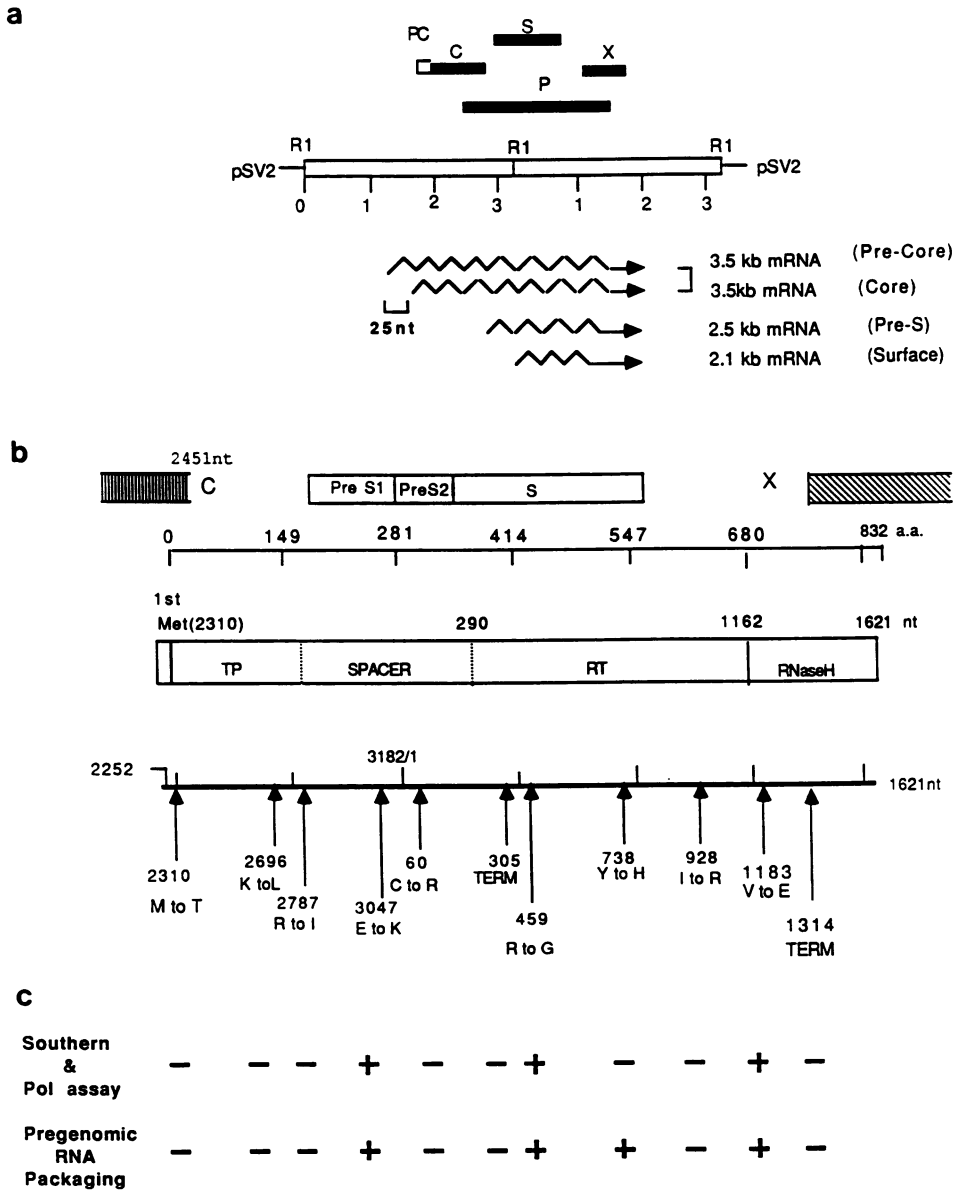

FIG. 1. (a) Transcription map of HBV drawn with a DNA template in a head-to-tail tandem dimer configuration, which represents a circular permutation of a covalently closed circular form. The four different open reading frames are represented by solid bars. The 3.5-kb mRNA encodes the precore (PC), core (C), and polymerase (P) proteins of the virus and also serves as a pregenomic RNA template for RT (see text for a discussion). The 2.5-kb mRNA encodes the presurface antigen (pre-S1), and the 2.1-kb mRNA encodes another presurface antigen (pre-S2) and the surface (S) antigen. The polymerase gene (*pol*) is in the +1 frame with respect to the overlapping nucleocapsid gene (C), and the *X* open reading frame encodes a transactivator (27, 30). nt, nucleotides. (b) The diagram represents an amplified version of the polymerase open reading frame. aa, amino acid; TP, terminal protein. The boundaries of the different domains are tentative. The arrows indicate the positions of different mutations made in the *pol* open reading frame. The one-letter amino acid code has been used to show the structural alteration of missense mutations. TERM indicates introduction of a translational termination codon. (c) Summary of the results of Southern analysis, polymerase assay (8), and pregenomic RNA encapsidation analysis of HBV *pol* mutants.

an RNA encapsidation domain to a specific region of *pol* can be greatly facilitated if subtle missense mutation of *pol* can lead to a profound encapsidation defectiveness phenotype.

To elucidate the complex association of pregenomic RNA, core protein, and DNA polymerase better, we analyzed core particle-associated RNAs from a series of missense point mutations in various domains of the HBV *pol* gene (Fig. 1). Missense mutations in the terminal protein, spacer, and RT domains of HBV polymerase were found to be deficient in RNA encapsidation. The relationship among DNA replication, RNA encapsidation, and *pol* translation in the life cycle of HBV is discussed.

MATERIALS AND METHODS

Site-directed mutagenesis and dimerization of HBV DNA. The procedure for site-directed mutagenesis was adapted from that of Kunkel (14) and described in detail elsewhere

TABLE 1. Oligonucleotides used to construct HBV mutants

Nucleotide no. of mutation position (HBV *ayw*)	Oligonucleotide sequence 5′ to 3′[a]
2310	GAC CAC CAA ACG CCC CTATC
2696	AAG GGT ATT CTA CCT TATTA
2787	TTA TAT AAG ATA GAA ACAAC
3047	TTT TGG GGT GAA GCC CTCAG
60	GTA TTT CCC CGC TGG TGGCT
305	CGT GTG TCT TGA CCA AAATT
459	GGA CTA TCA GGG TAT GTTGC
738	GCT TTC AGT CAT ATG GATGA
928	GAA CAC ATC AGA CAA AAAAT
1183	CTG TGC CAA GAG TTT GCTGA
1314	AGC AGG TCT TGA GCA AACAT

[a] The asterisks indicate positions where base substitutions were introduced into wild-type HBV.

(23). The oligonucleotides used to construct HBV mutants are described in Table 1.

To dimerize the HBV genome efficiently, either downstream or upstream *Eco*RI sites of pSV2ANeo-HBV (25) were eliminated, resulting in a pSV2ANeo-HBV monomer with either a single upstream *Eco*RI site (MC-3) or a single downstream *Eco*RI site (MC-1). Construction of these plasmids and the method of dimerization of mutant HBV DNAs have been described previously (23). Mutants 2310, 2696, 2787, and 3047 have been cloned into MC-3, while mutants 60, 305, 459, 738, 928, 1183, and 1314 were cloned into MC-1.

Cell culture and transfection. The human hepatoma cell line Huh7 was maintained in 10% fetal bovine serum in Dulbecco modified medium at 37°C in the presence of 5.5% CO_2. The calcium phosphate transfection procedure used has been detailed elsewhere (25). Briefly, 10^6 cells per 10-cm-diameter dish were transfected with 10 μg of either wild-type or mutant dimer DNA plus 30 μg of human genomic DNA as the carrier. Donor DNA was removed at approximately 6 h posttransfection, and cells were fed with fresh Dulbecco modified medium containing 10% fetal bovine serum.

Isolation of core particles. The procedure used to prepare intracellular core particles was adapted from Pugh et al. (19) and Lavine et al. (15). At 5 days posttransfection, cells from one 10-cm-diameter dish (6×10^6 cells) were lysed at 37°C for 10 min in 1 ml of buffer containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 50 mM NaCl, 0.25% Nonidet P-40, and 8% sucrose. The lysate was then spun in a microcentrifuge for 2 min, and the supernatant was collected in another tube. The supernatant was made to 8 mM $CaCl_2$ and 6 mM $MgCl_2$, followed by digestion with 30 U of micrococcal nuclease and 1 U of DNase 1 for 15 min at 37°C. The crude core particles were precipitated by adding 330 μl of 26% polyethylene glycol (molecular weight, 8,000) in 1.5 M NaCl and 60 mM EDTA. After incubation for 1 h at 4°C, the crude core particles were pelleted by being spun in a microcentrifuge for 4 min.

Preparation of RNA. The RNA isolation method used was that of Chomczynski and Sacchi (5). Briefly, the core pellet was first dissolved in 0.1 ml of denaturation solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Following dissolution, 0.01 ml of 2 M sodium acetate (pH 4.0), and 0.1 ml of water-saturated phenol were added, each time with mixing of the solution by inversion. Finally, 0.02 ml of a chloroform-isoamyl alcohol mixture (49:1) was added and vortexed for 10 s. The whole mixture was put on ice for 15 min and then centrifuged for 15 min at 4°C. The resulting aqueous phase was collected in another tube, and RNA was precipitated by adding an equal volume of isopropanol. For isolation of total RNA, cells from one 10-cm-diameter dish were lysed in 2 ml of denaturation solution and the volumes of the rest of the solutions were adjusted accordingly.

DNase and RNase digestion of core-associated RNA. Core-associated RNA isolated from one 10-cm-diameter dish was digested with either 15 U of RNase-free DNase (Boehringer Mannheim Co.) for 15 min at 37°C or a mixture of 40 μg of RNase A per ml and 20 μg of RNase T1 per ml for 30 min at 37°C. After digestion, the DNase-digested sample was phenol extracted and ethanol precipitated. The RNase-digested sample was made 0.5% with sodium dodecyl sulfate and then digested with proteinase K (0.125 mg/ml) for 15 min at 37°C. The RNA was then phenol extracted and ethanol precipitated.

Primer extension analysis. A 5′-end-labeled 22-nucleotide synthetic oligonucleotide (nucleotides 1980 to 2001) was used as a primer. Approximately 10^5 cpm (0.1 pmol) was lyophilized with core-associated RNA isolated from one 10-cm-diameter dish. The dried pellet was dissolved in 30 μl of hybridization buffer containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% formamide and allowed to anneal at 30°C overnight. After annealing, 170 μl of 0.3 M sodium acetate and 500 μl of ethanol were added for precipitation. The washed and dried pellet was then dissolved in 24 μl of reverse transcription buffer (50 mM Tris hydrochloride [pH 8.3], 6 mM $MgCl_2$, 100 mM NaCl, 10 mM dithiothreitol, a 0.4 mM four-deoxynucleoside triphosphate mixture, 50 μg of dactinomycin per ml, 10 U of human placental RNase inhibitor [BRL]) and incubated with 12 U of avian myeloblastosis virus RT (Molecular Genetic Resources Inc.) for 90 min at 42°C. The reaction was terminated by addition of 1 μl of 0.5 M EDTA, and the RNA was digested with 1 μg of pancreatic RNase A for 30 min at 37°C. A 100-μl volume of 2.5 M ammonium acetate was then added, followed by phenol extraction and ethanol precipitation. The washed and dried pellet was dissolved in 3 μl of TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), and 4 μl of loading buffer (80% formamide, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added. A 3-μl portion of each sample was analyzed on a 6% polyacrylamide sequencing gel.

Sedimentation and density gradient analysis of intracellular core particles. Cells transfected with either wild-type or mutated HBV dimer DNA were lysed in 0.5 ml of chilled extraction buffer (20 mM Tris hydrochloride [pH 7.4], 7 mM MgSO4, 50 mM NaCl, 0.1% 2-mercaptoethanol, 100 μg of bovine serum albumin per ml, 0.25 M sucrose) with a Dounce homogenizer. The homogenate was cleared by spinning in a microcentrifuge for 15 min at 4°C. The cleared homogenate was then loaded either on a sucrose gradient (5

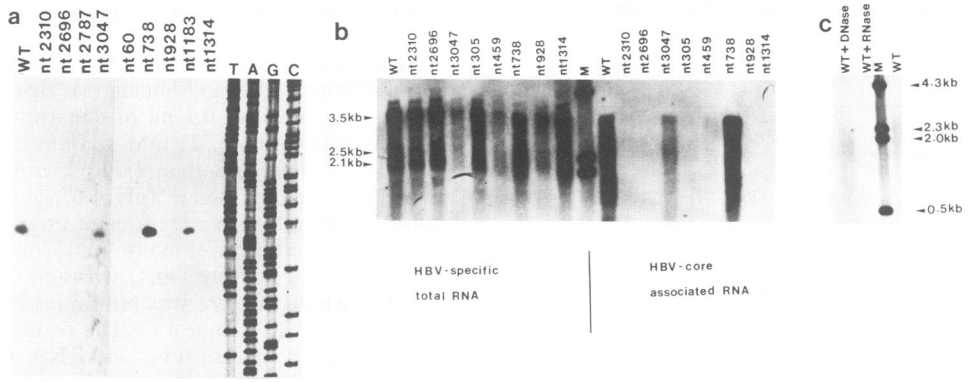

FIG. 2. Primer extension analysis of core-associated RNA shows strong association between HBV *pol* defects and RNA packaging. Core-associated RNA from one 10-cm-diameter dish was used for each sample. Size markers were prepared by using the same primer in a sequencing reaction with an HBV wild-type (WT) DNA template. The arrowhead indicates the calculated position of the extended product of the core-specific transcript. The nucleotide number on the top of each lane indicates the position of the base changed (Fig. 1b). (b) Packaging-defective HBV *pol* mutants express all three major transcripts of HBV. At 5 days posttransfection, cells from one-half of a 10-cm-diameter dish were harvested and total cellular RNA was prepared while core-associated RNA was isolated from the other half as described in Materials and Methods. Twenty micrograms of total RNA, as well as core-associated RNAs isolated from one-half of a 10-cm-diameter dish, was used for Northern blot analysis. The three major HBV-specific transcripts are indicated on the left. Mutants 2787, 60, and 1183 were assayed by primer extension only, while mutants 305 and 459 were assayed by Northern blotting only. (c) DNase and RNase sensitivity of core-associated RNA. Core-associated RNA isolated from one 10-cm-diameter dish transfected with wild-type HBV DNA was subjected to either RNase-free DNase I (Boehringer Mannheim Co.) or RNase digestion as described in Materials and Methods and analyzed by Northern blot analysis.

ml of 15 to 30% [wt/vol] sucrose in extraction buffer) or a cesium chloride gradient (5 ml of 20 to 50% cesium chloride in 10 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA). The sucrose gradients were run at 32,000 rpm for 6 h at 4°C in a Beckman SW50.1 rotor. The cesium chloride gradients were run at 35,000 rpm for 16 h at 4°C in a Beckman SW50.1 rotor. Fractions (200 μl) were collected from the bottom of the gradient. Alternate fractions were used for core-e antigen analysis with a kit from Abbott Laboratories. Sucrose fractions were used for assay directly, while cesium chloride fractions were desalted by dialysis through an Amicon filter (type 10) with four changes of buffer.

Northern (RNA) blot analysis. RNA samples were denatured with glyoxal and then run on a 1% agarose gel (16). RNAs were then transferred to a nylon membrane filter and probed with a vector-free ^{32}P-labeled full-length HBV DNA fragment.

RESULTS

As shown in Fig. 2, upon transfection of the tandem dimer of the HBV mutant genomes into Huh7 hepatoma cells, we analyzed the total RNA and the core particle-associated RNA prepared from the transfected culture by primer extension and Northern blot analysis.

Analysis of encapsidated RNA by primer extension. Mutants 2310, 2696, 2787, 60, 928, and 1314 exhibited no detectable signals of encapsidated RNA by primer extension analysis (Fig. 1 and 2a). In contrast, the wild type, as well as mutants 3047, 738, and 1183, whose mutations are in the spacer, RT, and RNase H domains, respectively, exhibited detectable levels of encapsidated RNA (Fig. 1 and 2a). The signal was RNA template specific, since the primer extension reactions were performed in the presence of dactinomycin (50 μg/ml). Furthermore, the primer-extended product resulted in a band at a position corresponding to the known 5′ initiation sites of the 3.5-kb core-specific mRNA (31, 32), rather than to the 5′ end of the short (+) strand of HBV DNA.

Analysis of encapsidated RNA by Northern blot. Another way to assay core particle-associated RNA is by Northern blot analysis. As shown in Fig. 2b, all of the mutants exhibited significant levels of HBV-specific total RNA (8). However, only mutants 3047, 459, and 738 exhibited detectable levels of encapsidated 3.5-kb RNA. Mutants 2310, 2696, 305, 928, and 1314 exhibited no detectable signal of encapsidated RNA despite having a total HBV-specific RNA level that appeared to be similar to that of the wild type. In a control experiment, when an exogenous RNA marker was mixed, before RNA extraction, with the core particles isolated from *pol* mutants, discrete RNA marker signals were observed on a Northern blot (26). Therefore, the smearing signals of the encapsidated HBV RNA in the wild type and mutants 3047, 459, and 738 (Fig. 2b) are more likely due to the degradation activity of Pol-associated RNase H during the process of DNA replication (for details, see the Discussion) rather than contamination of other species of HBV-specific RNAs and artifactual degradation during experimental manipulations.

These encapsidated RNA signals as detected by Northern analysis are resistant to RNase-free DNase (Boehringer Mannheim Co.) digestion and sensitive to DNase-free RNase digestion (Fig. 2c), suggesting that they are indeed of RNA origin. The results from Northern blot analysis (Fig. 2b) appear to be consistent with those from primer extension (Fig. 2a). Taken together, they show that mutants 3047, 459, 738, and 1183 are competent to encapsidate the pregenomic RNA, indicating that structural alterations in these positions are tolerable. In contrast, subtle missense mutants 2310, 2696, 2787, 60, and 928 and nonsense mutants 305 and 1314 apparently have lost the ability for pregenomic RNA packaging.

Selective packaging of core-specific RNA. Our primer extension results (Fig. 2a) indicate that only the core-specific mRNA, not the precore-specific mRNA, is packaged. How-

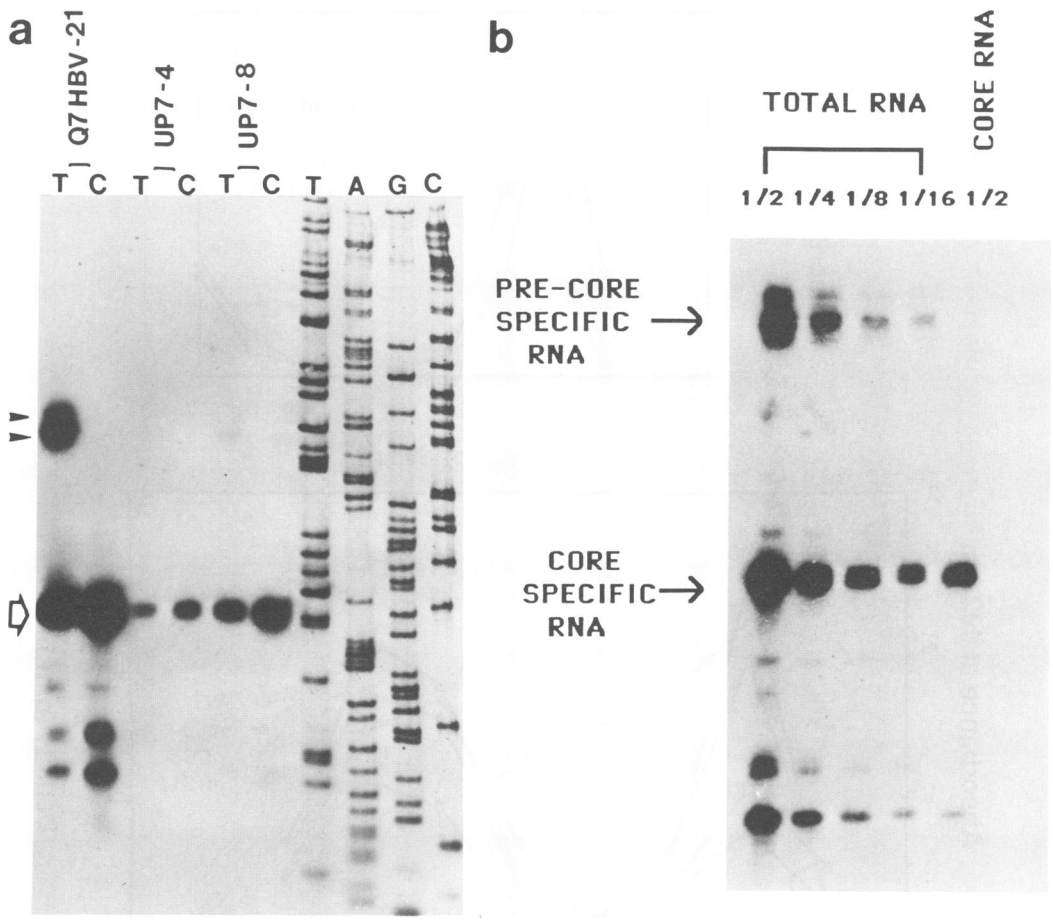

FIG. 3. Selective packaging of core-specific 3.5-kb RNA demonstrated by primer extension analysis using three different HBV-producing cell lines. Q7HBV-21 is a high-titer HBV-producing hepatoma cell line (25). UP7-4 and UP7-8 are two independent clones obtained by stable transfection of the HepG2 human hepatoma cell line (26). Approximately 50 μg of total RNA (one-fifth of a 10-cm-diameter dish) and one-half of the core-associated RNA from a 10-cm-diameter dish, approximately 1 day postconfluence for Q7HBV-21 and 10 days postconfluence for UP7-4 and UP7-8, were used for primer extension analysis. T represents total RNA, and C represents core-associated RNA. The open arrow indicates the position of the extended product from the core-specific transcript, and the solid arrowhead indicates that of the precore-specific transcript. (b) Approximately 10 to 20% of HBV core-specific RNA is selectively packaged. Total and core-associated RNAs were each isolated from one 10-cm-diameter dish of Q7HBV-21 cells (25). Aliquots of 1/2, 1/4, 1/8, and 1/16 of the total RNA and 1/2 of the core-associated RNA were quantitatively compared by primer extension analysis.

ever, it is possible that the level of the precore-specific RNA is too low to be detectable in transient transfection assays.

We therefore analyzed the total intracellular, as well as particle-associated, HBV-specific RNAs prepared from several stable HBV-producing cell lines of rat Morris hepatoma and human HepG2 hepatoblastoma origins (25). As shown in Fig. 3, although the precore-specific mRNA was rather abundant in the total intracellular RNA fraction of cell line Q7HBV-21, weakly detectable in UP7-8, and undetectable in UP7-4, we were unable to detect any encapsidated precore-specific RNA, even in the Q7HBV-21 cell line.

Quantitation of the packaging efficiency of core-specific RNA. The selective packaging (Fig. 3a) and selective translation of *pol* (17) from the same core-specific mRNA species suggest potential competition for the core-specific mRNA between translation and packaging. Quantitation of the percentage of encapsidated core-specific RNA would help to address this issue.

In HBV-producing HepG2 cell lines (eg., UP7-4 and UP7-8 in Fig. 3a), HBV replication appears to increase with time after the cell density has reached confluence (26, 29). To avoid confusion from this phenomenon of aging-dependent HBV replication in HBV-producing HepG2 cell lines, we used instead a system of an HBV-producing Morris hepatoma cell line (25) for quantitation of encapsidated pregenomic RNA. Significant levels of HBV transcription and replication can be easily detected in this system, even before the cell density has reached confluence. Since the experiment shown in Fig. 3a was not performed in a quantitative manner, as shown in Fig. 3b, a more quantitative comparison of core-specific mRNA between the particle-associated fraction and the total intracellular pool in Q7HBV-21 (25) demonstrated that approximately 10 to 20% of the core-specific mRNA is encapsidated (Fig. 3b).

Sedimentation and buoyant density gradient analysis of core particles. Although the results shown in Fig. 2 are consistent with involvement of the Pol protein in encapsidation (11), an alternative explanation suggests an overall decrease in the yield of subviral core particles produced by these *pol* mutants without changing the specific packaging efficiency per viral particle. To differentiate between these two possibilities, we compared the core-e antigens in the medium be-

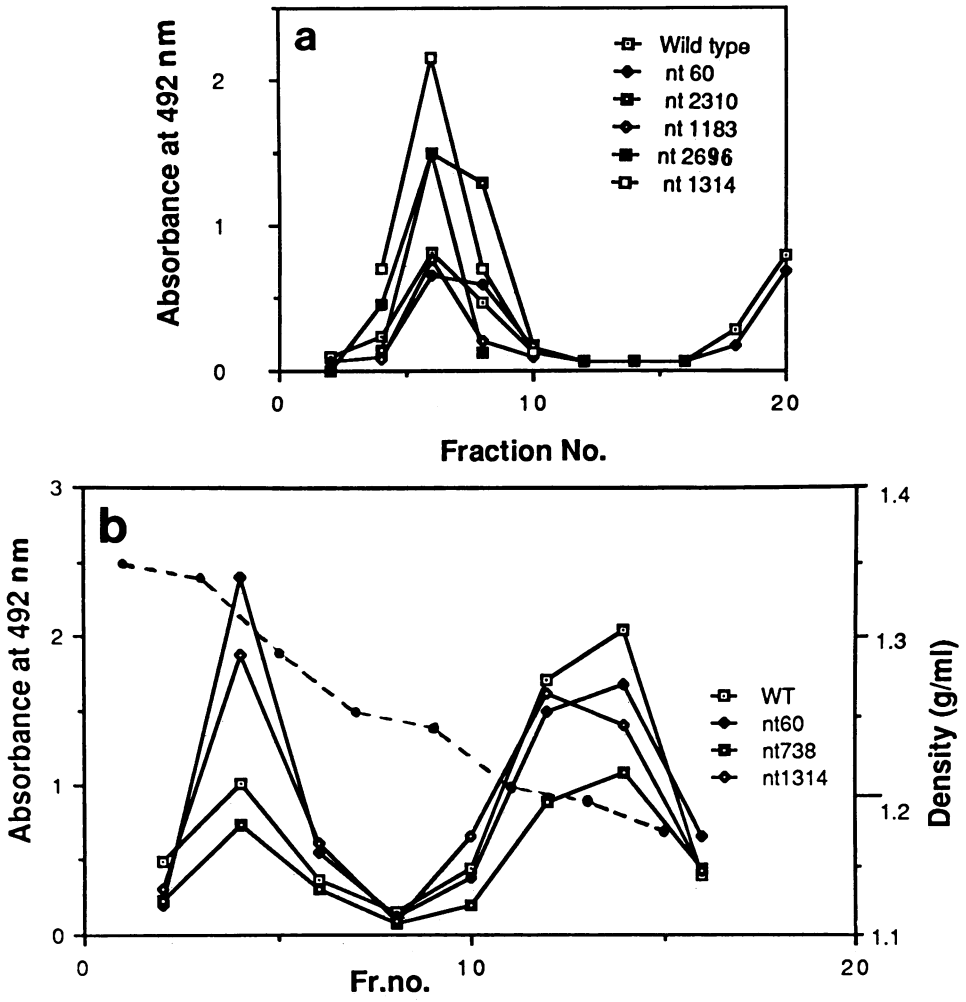

FIG. 4. Sedimentation and density gradient analysis indicate no apparent effect of *pol* mutation on core particle assembly. Intracellular core particles from one 10-cm-diameter dish transfected with 5 μg of respective mutant or wild-type DNA were subjected to either sucrose density gradient (a) or cesium chloride gradient (b) centrifugation. Alternative fractions were used for core-e antigen analysis as recommended by the manufacturer.

tween the mutants and the wild type (26). In addition, when the intracellular incompletely matured subviral particles from both wild-type and mutant transfected cultures were analyzed by sucrose and cesium chloride gradient centrifugation, equivalent levels of core particles, as analyzed by a semiquantitative enzyme immunoassay of core-e antigen, were observed to peak at the same position (ca. 1.34 g/ml) (Fig. 4). The same level of intracellular core protein between the wild type and mutants has also been observed when immunoprecipitation with rabbit anti-core polyclonal antibody was performed (26). The material banding at 1.2 g/ml is most likely intracellular nascent Dane particles on their way out to be secreted into the medium.

DISCUSSION

General correlation between DNA replication and RNA encapsidation. When we compared these RNA encapsidation results (Fig. 2) with the results of DNA replication and polymerase activity associated with these point mutants of *pol* (8), a good but not perfect correlation was evident (Fig. 1). Although the signals of encapsidated RNA in mutants 459 (Fig. 2b) and 3047 (Fig. 2a and b) are relatively weaker than that of the wild type, the mutants are clearly positive in RNA encapsidation. All three mutants (3047, 459, and 1183) competent in RNA encapsidation also are capable of DNA replication (8; Fig. 1). Similarly, all seven mutants defective in RNA encapsidation also fail to perform DNA replication and exhibit no polymerase activity (8; Fig. 1).

Requirement of polymerase in RNA packaging. The correlation between DNA replication and RNA encapsidation could be due to a number of reasons. It may be that (i) the encapsidation signal is ablated by the mutations, (ii) the stability of the pregenomic RNA is altered by the mutations; (iii) the synthesis, stability, or assembly of the nucleocapsid is impaired by the mutations; (iv) replication is a prerequisite of encapsidation; or (v) a functional Pol protein is required for both replication and encapsidation.

The *cis* element on the pregenomic RNA responsible for packaging has been mapped to a narrow region near its 5′ end (13). A short segment of 85 nucleotides from the *cis* element is sufficient for encapsidation of heterologous nonviral RNA (13). As shown in Fig. 1, all of the point mutations introduced into the *pol* gene of HBV are located outside the

encapsidation signal. Therefore, the failure of those *pol*-defective mutants to perform RNA packaging (Fig. 2) is not due to fortuitous mutational perturbation of the *cis* element of encapsidation. Additionally, the steady-state levels of HBV-specific RNA transcribed by these mutants (except mutant 3047) appear to be similar to the wild-type level (Fig. 2b). Furthermore, there is no apparent effect of these *pol* mutations on the synthesis or stability of core protein or particles (Fig. 4; see also discussions below). Finally, we consider it highly unlikely that efficient DNA replication, if it is a prerequisite to RNA encapsidation, can take place before complete formation of the nucleocapsid. We prefer the interpretation which suggests that the RNA encapsidation defectiveness phenotype is due to a defect of a *trans*-acting *pol* gene product required for DNA replication, as well as RNA encapsidation.

Assay for RNA encapsidation and potential RNase H activity. As shown in the Northern blot of Fig. 2b and c, HBV-specific, RNase-sensitive signals can be detected from core particle-associated RNA. The smearing pattern of encapsidated HBV-specific RNAs of mutants 3047, 459, and 738 and the wild type suggests that it is likely due to processive degradation of the RNA moiety from 3′ to 5′ by polymerase-associated RNase H activity. A control experiment done by adding an exogenous RNA reference marker to the polyethylene glycol-isolated core particles before RNA extraction (see Materials and Methods) indicates that the exogenous RNA marker remains intact throughout the procedures of RNA preparation (26). We cannot rule out the possibility that core-associated pregenomic RNA was degraded by cellular RNase before addition of RNA extraction buffer. It would be very helpful if we could examine the encapsidated RNA from an RNase H mutant which is positive in RNA packaging and defective in RNase H activity, as reported by Radziwill et al. (20).

Mutant 738: uncoupling between replication and encapsidation. RT domain mutant 738 contains a missense substitution which changes the most-conserved YMDD motif into HMDD (Fig. 1). This mutant does not appear to exhibit any normal DNA replicative intermediates on Southern blot analysis, nor does it contain any detectable polymerase activity (8). However, its RNA encapsidation activity appears to be similar to or higher than that of the wild type.

One RNase H missense mutant (designated P4) and one RT missense mutant (designated P2) reported by Bartenschlager et al. (1) were found to be polymerase activity defective yet packaging positive. Similarly, in the duck hepatitis B virus system, Chang et al. (3, 11) have reported RT and RNase H mutants that are defective at DNA synthesis but encapsidation competent. Our results of mutant 738 appear to substantiate these reports (1, 3, 11) that DNA replication and RNA encapsidation are uncouplable events.

Coupling between RNA encapsidation and Pol translation. Previously, we have demonstrated that the core-specific mRNA, but not the precore-specific mRNA, can serve as a good template for Pol translation (17). Selective packaging (Fig. 3a) and selective translation of Pol suggest that translation and packaging of Pol are two intimately coupled events. The essence of this hypothesis is that translation and packaging are cooperative rather than competing events. On the basis of asymmetric complementation results, *cis* preference of pregenomic RNA packaging has been proposed (11). This phenomenon is consistent with the hypothesis that translation and packaging of Pol are tightly coupled.

The targeting strategy of most retroviral polymerase is to form a Gag-Pol fusion protein through ribosomal frameshifting (6, 10). In hepadnaviruses, translation of Pol appears to be independent of core protein synthesis (4, 12, 23, 24). This hypothesis that translation and packaging of polymerase are coupled events in hepadnaviruses is particularly appealing, since it offers an explanation of how trace amounts of Pol protein and pregenomic RNA can be brought into proximity for productive RNA-protein interaction and packaging.

Effect of *pol* mutation on production and assembly of core particles. Although the results reported here, as well as those from others (1, 3, 11), indicate that the *pol* gene product is required for RNA packaging, a recent report (2) indicates that HBV-specific core protein expressed in *E. coli* at a nonphysiologically high concentration can preferentially package core protein RNA in the absence of any HBV-specific Pol protein. The apparent discrepancy concerning whether polymerase is required for HBV pregenomic RNA encapsidation can be explained if the failure of Pol encapsidation is due to a negative effect of *pol* mutation on the synthesis or stability of core protein or on the assembly or stability of core particles.

This interpretation was tested as shown in Fig. 4. Neither the number nor the sedimentation value and density of core particles appeared to alter significantly in our Pol-defective mutants. Our gradient analysis results (Fig. 4) suggest that the morphogenesis of both core particles and Dane particles can take place even in the absence of encapsidated RNA, a functional *pol* gene product, or a binary complex between RNA and polymerase. In addition, the defect of *pol* has no apparent direct or indirect effect on core protein expression as analyzed by immunoprecipitation (26).

Encapsidation and replication domains of Pol overlap extensively. As shown in Fig. 1 and 2, four different missense mutations within the *pol* gene, with a single amino acid substitution, resulted in failure of DNA replication, as well as RNA encapsidation. These four mutations include presumably subtle structural alterations in the terminal protein domain (mutants 2696 and 2787), the spacer (mutant 60), and the RT domain (mutant 928). In addition, mut ion 1314 within the RNase H domain causes failure to p kage the pregenomic RNA. Therefore, it is possible tha all four domains of Pol are required for packaging. Considering protein with a three-dimensional structure, it is perhaps not surprising that the RNA encapsidation and DNA replication domains of Pol are highly overlapped and were therefore difficult to dissociate (see the data summarized in Fig. 1). Alternatively, the Pol protein may be exceedingly sensitive to any subtle conformational change which may lead to improper protein folding and loss of replication, as well as packaging activity. Although this conclusion appears to be consistent with that of Bartenschlager et al. (1), it remains to be shown whether it is also true of other hepadnaviruses.

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