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The P Gene Product of Hepatitis B Virus Is Required as a Structural Component for Genomic RNA Encapsidation

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Encapsidation of the pregenomic RNA into nucleocapsids is a selective process which depends on specific RNA-protein interactions. The signal involved in the packaging of the hepatitis B virus (HBV) RNA pregenome was recently defined as a short sequence located near the 5′ end of that molecule (Junker-Niepmann et al., EMBO J., in press), but it remained an open question which viral proteins are required. Using a genetic approach, we analyzed whether proteins derived from the HBV P gene play an important role in pregenome encapsidation. The results obtained with point mutations, deletions, and insertions scattered throughout the P gene clearly demonstrate that (i) a P gene product containing all functional domains is required both for the encapsidation of HBV pregenomic RNA and for packaging of nonviral RNAs fused to the HBV encapsidation signal, (ii) known enzymatic activities are not involved in the packaging reaction, suggesting that P protein is required as a structural component, and (iii) P protein acts primarily in *cis*, i.e., pregenomic RNAs from which P protein is synthesized are preferentially encapsidated.

Hepatitis B viruses are a group of small enveloped DNA viruses that have the human hepatitis B virus (HBV) as their prototype and are characterized by a narrow host range and a distinct liver tropism (for a review, see reference 6). Despite containing a DNA genome, hepadnaviruses resemble retroviruses in that they replicate via reverse transcription of an RNA pregenome (22). This reaction takes place in a particle which is composed of core proteins, P protein(s), and the RNA pregenome. Thus, packaging of this RNA is an essential step leading to the formation of replication-competent nucleocapsids.

As a first step towards understanding the molecular mechanism of this assembly process, we have recently identified the encapsidation signal on the RNA pregenome as a short sequence located near the 5′ end of this molecule (M. Junker-Niepmann, R. Bartenschlager, and H. Schaller, EMBO J., in press). However, it remained an open question which viral proteins besides the core protein are required. Since the surface proteins are not present in nucleocapsids and since mutations in the X gene have no effect on the formation of replication-competent core particles in transient expression studies (26), we focused our attention on the P gene. Using a system that allows the transient expression of mutated duck hepatitis B virus (DHBV) DNA (1, 16–18) and HBV DNA (9, 14; Junker-Niepmann et al., in press) in HepG2 cells, we analyzed different stop and frameshift mutations in the P gene for their effects on genomic RNA encapsidation. The results clearly demonstrate that a P gene product is required both for packaging of pregenomic RNA and for encapsidation of nonviral RNAs carrying the HBV packaging signal. Furthermore, we could show that packaging function requires all P protein domains but none of the corresponding enzymatic activities. Finally, we found that transcomplementation efficiencies of P gene mutants varied drastically depending on which construct was used as a donor for P protein and that P protein acts primarily in *cis*. These results suggest that P protein is the limiting factor in the packaging reaction.

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MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Boehringer, Mannheim, Federal Republic of Germany (FRG), and New England BioLabs, Beverly, Mass. Radionuclides were purchased from Amersham Buchler, Braunschweig, FRG. Rabbit anti-HBV core antiserum was raised against native or denatured recombinant HBV core antigen (25).

Plasmids. All nucleotide positions given refer to the nomenclature of Pasek et al. (13), beginning with the A residue of the C gene initiation codon.

(i) Basic constructs. The basic constructs used in this analysis, plasmids pMH-34/2922 and pMH3/3143, are shown in Fig. 1 (for a detailed description, see reference 14 and Junker-Niepmann et al., in press). All mutations except δ1 and δ2 were introduced into the wild-type construct pMH-34/2922. Mutations δ1 and δ2 were introduced into a construct in which the RNA pregenome is synthesized under the control of the human metallothionein II_A (MT) promoter (pMH-9/3091 [9; Junker-Niepmann et al., in press]). A summary of the different mutations, including the amino acid changes and the positions in the P protein, is given in Table 1.

(ii) Stop and frameshift P gene mutants. Mutant P11 was constructed by site-directed mutagenesis by a standard method (27), and the nucleotide exchange was confirmed by sequence analysis. Mutant P13 was created by exchanging a 624-bp HBV fragment from pMH-34/2922 from *Nco*I (position 2654) to *Sst*I (in the polylinker, 3′ of the HBV insert ending at position 88) with a 680-bp fragment of DHBV 3 sequence (21) from *Nco*I (position 2354) to *Sst*I (in the polylinker, 3′ of the insert) from a construct containing a full-length DHBV 3 genome cloned via *Eco*RI into a pUC-based vector (G. Radziwill, Diploma thesis, University of Heidelberg, Heidelberg, FRG, 1985). Mutant P14 was created by restriction digestion of plasmid pMH-34/2922 with

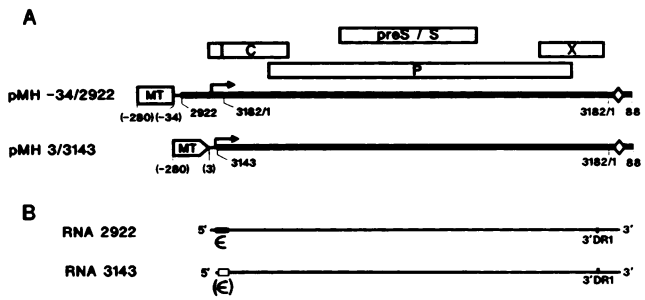

FIG. 1. (A) Schematic presentation of the basic constructs. HBV sequences are shown as a thick line, linker sequences as thin lines (not to scale), and the polyadenylation site as an open diamond. The nonfunctional MT promoter sequence is drawn as an open box in wild-type construct pMH-34/2922 and as an open arrow when functional, as in helper construct pMH3/3143 (Junker-Niepmann et al., in press). Start sites for transcription of overlength RNAs are indicated by arrows. (B) Schematic drawing of the overlength RNAs produced from the constructs shown in panel A. The intact encapsidation signal (ε) is shown as a solid box for the wild-type construct; its truncation in the helper plasmid is indicated by the open box. 3' DR1, 3' copy of direct repeat 1, the signal for initiation of reverse transcription (19).

*Eco*RI at position 1280, filling in of the overhangs with Klenow enzyme, and blunt-end ligation, creating a unique *Ase*I site. This led to a frameshift in the C gene open reading frame (C-ORF), which after 14 further codons runs on to a stop. Mutant P15, in which a stop codon was created at position 1967, was constructed in the same way by using the unique *Spe*I restriction site at position 1961. Mutant P16 was isolated from a reaction in which plasmid pMH-34/2922 was cut at the unique *Nco*I site at position 2654, incubated with a high concentration of DNA polymerase I in the presence of deoxynucleoside triphosphates, and blunt-end ligated. This led to a deletion of 23 nucleotides from positions 2639 to 2663, linking the stop codon in the S gene ORF (S-ORF) at position 2664 to the P gene ORF (P-ORF) at position 2638.

(iii) Missense and linker insertion P gene mutants. Construction of mutants P2 to P7 and P9 and P10 has been described (14). Mutant P12 was constructed by site-directed mutagenesis (27).

(iv) Deletion P gene mutants. Construction of deletion mutants HBV δ1 and δ2 is described in reference 14. Mutant δ3 was constructed by J. Salfeld by cutting HBV DNA contained in plasmid pSH2.1 (2) with *Bgl*II at positions 523 and 937, filling in the overhangs with Klenow enzyme, inserting an 8-bp linker with the sequence 5'-GAAGCTTC-3', and blunt-end ligation. This led to a deletion of 410 nucleotides (nt) from HBV and insertion of 8 nt of linker sequence. Finally, an *Mro*I-*Eco*RI fragment (positions 429 to 1280) was transferred into plasmid pMH-34/2922. Mutant δ4 was obtained by cutting pMH-34/2922 with *Xba*I at position 1529 and *Spe*I at position 1961 and religation under sticky-end conditions. This led to an in-phase deletion of 432 nt.

(v) Other constructs. Construction of plasmids pCG-0 and pCHG3134/88 is described in Junker-Niepmann et al. (in press). In brief, plasmid pCG-0 contains the following elements in the 5' to 3' direction: an immediate-early (IE) promoter of human cytomegalovirus (CMV-IE), a polylinker, a 2.8-kb fragment of the *Escherichia coli lacZ* gene, and a 240-bp simian virus 40 (SV40) fragment with the polyadenylation site. Plasmids pCHG3097/88 and pCHG 3134/88 were obtained by insertion of *Hin*dIII-*Bgl*II fragments of HBV sequence into the polylinker of pCG-0. pCHG3097/88 contains HBV sequences from positions 3097 to 88, derived from plasmid pMH3/3097 (Junker-Niepmann et al., in press); pCHG3134/88 contains HBV sequences from positions 3134 to 88, derived from pMH3/3134 (Junker-Niepmann et al., in press). Plasmid pCHG3134/36 was constructed by insertion of a DNA fragment containing the CMV-IE promoter and an HBV segment from positions 3134 to 36 via *Pst*I (in the polylinker, immediately upstream of the CMV-IE promoter) and *Bgl*II into pCG-0. This fragment was obtained by polymerase chain reaction with pCHG3134/88 as the template and a downstream oligonucleotide of the sequence 5'-GTTCGAAGATCTAGAAGCTCCAAATTC-3',

TABLE 1. P gene mutations and their effects on endogenous polymerase activity and genomic RNA encapsidation

Mutation	Mutant	Change[a]	Position(s)[b]	Polymerase activity[c] (% of wild type)	Packaging[d]
Stop/frameshift	P11	L→stop	14	<0.5	−
	P13	HBV/DHBV	750–832/727–786	<0.5	−
	P14	FS	292	<0.5	−
	P15	FS	520	<0.5	−
	P16	FS	744	<0.5	−
Missense	P2	D→H	540	<0.5	+
	P3	E→H	718	10	+
	P4	A→D	725	<0.5	+
	P5	D→V	737	20	+
	P12	G→R	800	20	+
Insertion	P6	G<KLLG>N	44< >45	<0.5	+
	P7	D<RSFD>L	178< >179	<0.5	+
	P9	W<KLPW>L	751< >752	<0.5	+
	P10	R<EASR>G	781< >782	<0.5	+
Deletion	δ1	S< >E	201< >292	40	+
	δ2	F<TSA>L	293< >335	<0.5	−
	δ3	D<RSFD>L	40< >179	<0.5	−
	δ4	S< >S	375< >520	<0.5	−

[a] Amino acid changes (→), cassette exchange (HBV/DHBV), frameshift mutations (FS), insertions and deletions (< >) in the P protein.
[b] Positions in the P protein.
[c] Determination of the enzymatic activity in the endogenous polymerase reaction relative to that obtained with the wild type. Part of these results are taken from reference 14.
[d] Determination of the amounts of encapsidated pregenomic RNA. Symbols: +, no detectable difference from the wild type; −, no detectable signal. Detection limit was 3 to 5% (see Materials and Methods).

which introduces the *BglII* site (underlined) and a nucleotide exchange at position 34 (boldface letter) into the HBV sequence.

Plasmid pMS3C1, a pUC-based vector which was used as a donor of core protein, was constructed by insertion of a synthetic HBV C gene (12) between the MT promoter and the SV40 polyadenylation site (M. Nassal, unpublished). Plasmid pMTpol (14), which was used as a donor of HBV P proteins, is similar to helper construct pMH3/3143 (Fig. 1), but here the MT promoter is fused at position +3 by a synthetic DNA sequence of 20 nt to HBV position 407, the first nucleotide of the P gene.

All newly created sequences of the constructs described above were confirmed by sequence analysis.

Cell culture, transfections, and preparation of RNAs. Cultivation of HepG2 cells and DNA transfections were performed as described earlier (14).

Preparation of RNAs from transfected HepG2 cells was essentially done as described elsewhere (Junker-Niepmann et al., in press). In brief, cells from one 10-cm dish were lysed in 1 ml of HNEN (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.6], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 U of RNasin [Promega Biotec, Madison, Wis.] per ml), and total cytoplasmic RNA was prepared from 10% of the crude lysate by sodium dodecyl sulfate (SDS)-proteinase K treatment (1% SDS and 500 μg of enzyme per ml for 60 min at 37°C), followed by phenol and phenol-chloroform extractions. After isopropanol precipitation, pellets were dissolved in DNase digestion buffer (10 mM HEPES [pH 7.6], 10 mM $MgCl_2$, 40 U of RNasin per ml) and incubated with RNase-free DNase for 20 min at 37°C, followed by one phenol-chloroform extraction and isopropanol precipitation. From the remaining 90% of the lysate, core particles were isolated by immunoprecipitation and, after incubation of the immunocomplexes with micrococcal nuclease (Boehringer, Mannheim, FRG; 1 U per reaction mix for 20 min in the presence of 10 μg of carrier tRNA in a total volume of 200 μl), digested with proteinase K in the presence of 1% SDS–12.5 mM EDTA for 60 min at 37°C, followed by phenol and phenol-chloroform extractions and isopropanol precipitation.

RNase protection assays. Analysis of RNA was done exactly as described earlier (18) except that RNA probes were in most cases cut out of a preparative gel.

Synthesis of high-specific activity RNA probes was done as described elsewhere (11). For synthesis of probe A, a T7 promoter cassette of synthetic DNA was inserted into the *AvaI* site at HBV position 533, resulting in construct pTH540a (Junker-Niepmann et al., in press). This plasmid was cut with *RsaI* at position 79 to allow transcription of a 460-nt-long HBV antisense RNA containing an 8-nt long nonmatching linker sequence at its 5' end. For generation of probe B, shown in Fig. 7, a 949-bp-long *AvaI* (blunt end)-*SstII* (sticky end) fragment (from position 537 in the HBV sequence to position −152 in the MT element; see Fig. 1) from mutant P11 was inserted into an analogously treated Bluescript vector (Stratagene, Heidelberg, FRG). The resulting plasmid was digested with *StyI* at HBV position 3164, and antisense RNAs of 586 nt in length (556-nt HBV sequence plus 30-nt nonmatching linker sequence) were generated from the T7 promoter. Synthesis of the *lacZ* antisense RNA (see Fig. 6) was directed from an SP6 promoter located next to the *SstI* site at position 1948 to the *ClaI* site at position 837 (Junker-Niepmann et al., in press).

Determination of the detection limit of some RNase protection assays was done by serial dilution of a portion of probe RNA protected by encapsidated wild-type RNA and analysis of the different dilutions in parallel on the sequencing gel. The highest dilution which gave a detectable signal was taken as the detection limit.

To assay for proper reaction conditions and to obtain size markers of the original sequence, RNase protection assays were done in parallel with HBV RNAs generated in vitro. Plus-strand RNAs of wild-type sequence were obtained from an HBV insert (positions 3182 to 1280) cloned in the sense orientation behind an SP6 promoter (pHTW3182 [23]) after cleavage with *SspI* at HBV position 768 (e.g., Fig. 2, lane 22). RNAs of mutant P11 sequence were synthesized in vitro from an *XhoI*-cut Bluescript vector containing an HBV insert of P11 origin cloned via *SstII* (position −152 in the MT element) and *XhoI* (position 1409 in the HBV sequence) sites in the sense orientation behind the T3 promoter (e.g., Fig. 2, lane 21).

Endogenous polymerase assay. Core particles were prepared as described above, and after removal of micrococcal nuclease by several washings with HNEN, endogenous polymerase assays were performed as recently described (Junker-Niepmann et al., in press), including a treatment with avian myeloblastosis virus reverse transcriptase to complete gap filling. Finally, labeled DNA products were analyzed by electrophoresis in a 1.2% agarose gel containing 0.1% SDS (3).

RESULTS

To analyze whether P protein is required for genomic RNA encapsidation, a series of stop and frameshift mutations scattered throughout the P-ORF were constructed (Fig. 2A and Table 1). These mutations were introduced into a plasmid containing a 10% overlength HBV-2 genome of proven infectivity (5, 24), starting at position 2922 and ending at position 88, which is about 70 nt behind the HBV polyadenylation signal. Due to a 3'-terminal truncation to position −34, removing the TATA box, the 270-bp fragment of the MT promoter upstream of the HBV sequence is not functional. Since this construct therefore directs transcription of the RNA pregenome from the authentic core gene promoter, it is referred to as the wild type.

To ensure that the defects observed were caused by the mutation in the viral protein and not by defects in the mutated RNA pregenome, transcomplementations with wild-type P protein were included, with pMH3/3143 as a helper construct (Fig. 1). In this plasmid, a functional MT promoter is fused in position +3 to HBV position 3143 via a short linker sequence of 12 nt. Thus, HBV RNA transcribed from this construct lacks part of the encapsidation signal, located between positions 3134 and 36 (ε, Fig. 1B; see below), and is preceded by 15 nt of nonviral sequence. Although defective itself for RNA pregenome encapsidation, this construct efficiently provides all viral proteins in *trans*.

Stop and frameshift mutations in the HBV P gene drastically reduce genomic RNA encapsidation. To assay for the effects of nonsense mutations in the P gene on packaging of the RNA pregenome, mutated HBV genomes were transiently expressed in HepG2 cells, and RNAs transcribed from these constructs were then analyzed for the fraction packaged into cores in nuclease protection experiments as recently described (Junker-Niepmann et al., in press). Briefly, in all experiments described below, cells were lysed 4 days post-transfection and 10% of the crude lysate was used to prepare total cytoplasmic RNA, whereas core particles were isolated

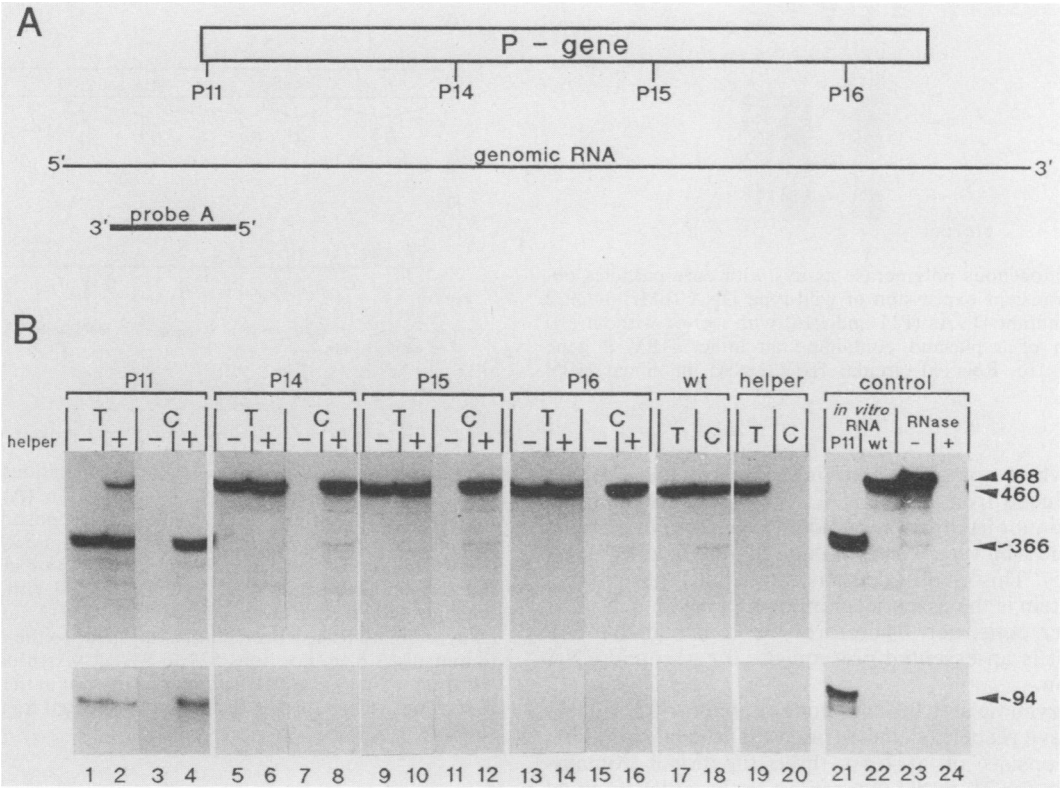

FIG. 2. Analysis of P gene stop and frameshift mutations for their effects on genomic RNA encapsidation. (A) Localization of the different mutations (see Table 1) with respect to the P gene, the genomic RNA, and probe A. (B) RNase protection assays with total cytoplasmic RNA (T) and RNA in core particles (C) after transient expression of the mutant DNAs with (+) or without (−) cotransfection of helper plasmid pMH3/3143. The fragments shown in lanes 21 and 22 were obtained in parallel by hybridization of the probe to in vitro-transcribed RNAs of mutant P11 (lane 21) or wild type (wt, lane 22) origin. 1/50 of the input probe is shown in lane 23, the result of an RNase treatment of the unprotected probe in lane 24. Numbers refer to the lengths of the RNA fragments in nucleotides.

from the remaining 90% by immunoprecipitation with an antiserum specific for the core protein. HBV-specific genomic RNAs present in both preparations were then quantitated by nuclease protection analysis with a ^{32}P-labeled riboprobe covering 460 nt near the 5′ end of the pregenome (Fig. 2A).

The result of such an experiment is presented in Fig. 2B. Analysis of total cytoplasmic RNA revealed that all mutants produced RNA pregenomes in amounts comparable to our wild type (compare lanes 1, 5, 9, and 13 with lane 17). However, in the corresponding core fractions, these signals from the P gene mutants were not detected (lanes 3, 7, 11, and 15), indicating a drastic reduction in packaging efficiency. Since we estimate the detection limit of the assay to be about 3 to 5%, this means that packaging of RNA genomes in these mutants is reduced by at least a factor of 20.

The defect in RNA encapsidation observed could be compensated for by cotransfection with the helper plasmid pMH3/3143 (lanes 4, 8, 12, and 16), which provides all viral proteins in *trans* but which is itself not encapsidated when transfected either alone (lane 20) or in a mixture with a P^- mutant. This was exemplified by mutant P11 (lane 1), whose RNA contained a nucleotide exchange in the region protected by the probe (Fig. 2A). The probe was therefore cleaved at the resulting mismatch into two fragments of about 366 and 94 nt, and the signals obtained could therefore be distinguished from the signals produced by the helper RNA (upper band in lane 2). Analysis of the corresponding core fraction (lane 4) revealed that RNA of exclusively mutant origin was encapsidated, whereas signals corresponding to RNAs of the helper were missing. Essentially the same results were obtained with probes that allowed the specific detection of RNAs produced from the three other P gene mutants (not shown). Thus, the signals obtained in the transcomplementations with the other mutants (lanes 8, 12, and 16) should also be of P^- origin.

Although sedimentation analysis of cell lysates showed that all P gene mutants produced normal amounts of particulate core protein (not shown), we could not completely exclude the possibility that the helper effect obtained in the cotransfection experiments with the C^+ P^+ construct was caused by core protein provided in *trans*. This was ruled out by the *trans* complementation experiment shown in Fig. 3, in which a construct containing solely the HBV P gene downstream of the MT promoter (pMTpol, see Materials and Methods) was used as a donor of P protein. Upon cotransfection of mutant P11 or P16 with this construct, core particles were isolated as described above and subjected to the endogenous polymerase reaction. This assay can be used as an indirect measurement of pregenome encapsidation, as it determines the incorporation of radioactively labeled deoxyribonucleotides into incomplete viral genomes contained in nucleocapsids.

As expected, no signals were obtained when mutated DNAs were transfected alone (Fig. 3, lanes 1 and 3).

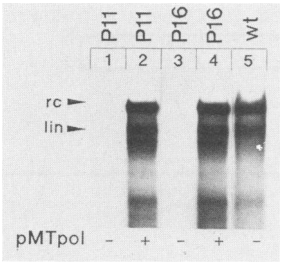

FIG. 3. Endogenous polymerase assays with core particles obtained after transient expression of wild-type DNA (pMH-34/2922 [wt]) and of mutant DNAs (P11 and P16) with (+) or without (−) cotransfection of a plasmid containing an intact HBV P gene (pMTpol [14]). rc, Relaxed circular HBV DNA; lin, linear HBV DNA.

However, when complemented in *trans* by the HBV P protein produced from an mRNA, which because of the 5′ truncation cannot itself be encapsidated, signals comparable to those in the wild type were obtained (compare lanes 2 and 4 with lane 5). This clearly demonstrates that P protein and not core protein is the essential component provided in *trans* by the helper construct. Therefore, we conclude that a P gene product is an essential prerequisite for genomic RNA encapsidation.

Known enzymatic activities are not essential for RNA packaging. We have recently demonstrated that the hepadnaviral P protein consists of at least three functional domains mapping from the N- to the C-terminus in the order terminal protein, DNA polymerase-reverse transcriptase, and RNase H, the first two domains being separated by a spacer element which can be largely deleted without causing loss of enzymatic activity (Fig. 4) (1, 14). Using a genetic approach, we demonstrated that mutations in any of these domains led to a drastic reduction of enzymatic activity as measured by the endogenous polymerase reaction (Table 1). Furthermore, the importance of certain amino acid sequence motifs for

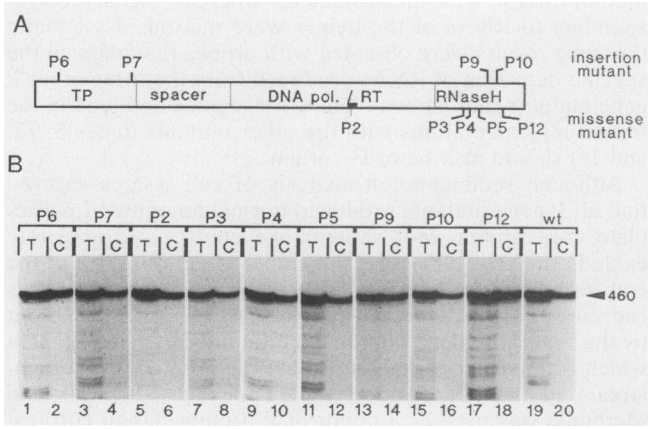

FIG. 4. Analysis of missense mutations in the HBV P gene for ability to produce RNA-containing nucleocapsids. (A) Schematic presentation of the P gene indicating the type and position of the different mutations with respect to the predicted functional domains. TP, Terminal protein; DNA pol./RT, DNA polymerase/reverse transcriptase. The black box at the position of mutant P2 indicates the YMDD sequence motif. (B) RNase protection assay with total cytoplasmic (T) and encapsidated (C) RNA obtained after transient expression of mutant and wild-type (wt) DNA in HepG2 cells. The size of the protected fragment is given in nucleotides.

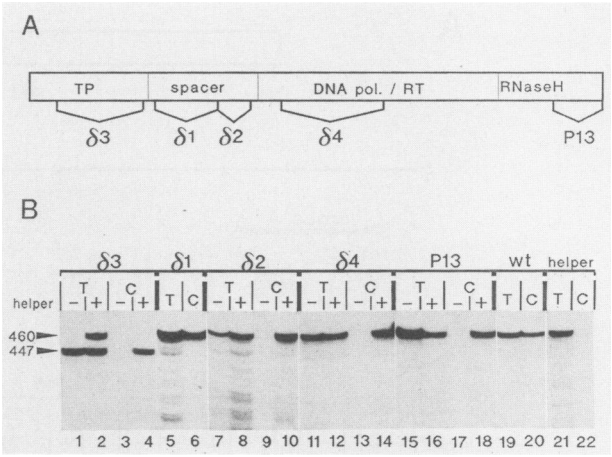

FIG. 5. Analysis of in-phase deletion mutations in the HBV P gene for effects on genomic RNA encapsidation. (A) Localization of the different mutations with respect to the postulated functional domains (see Fig. 4). (B) Detection of pregenomic RNA of wild-type (wt) or mutant origin detectable in cores (C) or in the total RNA fraction (T). Mutant DNAs were transfected either alone (−) or together with helper plasmid pMH3/3143 (+), and RNAs were analyzed in an RNase protection assay. Due to the internal deletion in mutant δ3 from positions 528 to 937, RNAs produced from this construct lack 13 nt of HBV sequence present at the 5′ end of probe A starting at position 540. Therefore, protected fragments are 13 nt shorter.

reverse transcriptase and RNase H functions was demonstrated (14); first, a highly conserved YMDD motif (changed in mutant P2) was found to be absolutely essential for DNA polymerase activity, and second, amino acid changes affecting a sequence important for RNase H function (mutants P3 and P5) eliminated DNA plus-strand synthesis and reduced overall DNA repair synthesis by a factor of 5 to 10 (Table 1).

As the endogenous polymerase assay depends on the formation of replication-competent nucleocapsids, it was not clear from this analysis whether all of these mutations in the different functional P domains affected only enzymatic activities or whether these alterations were at least in part due to a reduction in the efficiency of RNA encapsidation. Therefore, these mutants and additionally constructed missense and linker insertion mutants, listed in Table 1, were transfected into HepG2 cells, and RNA encapsidation was analyzed as described above, again with probe A (Fig. 2A). The results presented in Fig. 4 clearly show that all mutants produced core particles containing pregenomic RNA in amounts similar to that produced by the wild type (lane 20). Interestingly, linker insertions in the N-terminal domain of the P protein, which encodes the DNA terminal protein (mutants P6 and P7, [1]) also had no effect on genomic RNA encapsidation but again did not support HBV DNA synthesis. Thus, the DNA polymerase and RNase H activities as well as the predicted priming function are not required for RNA encapsidation. This demonstrates that the packaging function of the P protein can be genetically separated from its enzymatic activities.

Packaging function requires all P protein domains. Next we investigated whether genomic RNA encapsidation can be ascribed to a specific P protein segment or whether all domains are required. To address this question, a series of in-phase deletions affecting each of the different domains were constructed (Fig. 5A and Table 1; see Materials and

Methods). While in mutants δ1 to δ4 variable portions of the P gene were internally deleted, in mutant P13 the last 83 amino acids of the HBV P protein were exchanged for an equivalent part of the DHBV P protein. Again these DNAs were transfected into HepG2 cells and analyzed for their ability to produce RNA-containing core particles as measured with an RNase protection assay with probe A (Fig. 2A).

As shown in Fig. 5B, all mutants produced pregenomic RNA in amounts comparable to the wild type (compare lanes 1, 5, 7, 11, and 15 with lane 19). However, with the exception of mutant δ1 (lane 6), no signals were detectable in the corresponding core fractions (lanes 3, 9, 13, and 17), demonstrating that these deletions in P protein led to a drastic reduction in packaging efficiency. Again, this defect could be compensated for by *trans* complementation with the helper construct pMH3/3143 (lanes 4, 10, 14, and 18), which itself is not encapsidated (lane 22).

In this analysis, the only mutant which had no effect on RNA packaging was δ1, in which 90 amino acids of the spacer region were deleted. This is in accord with our previous finding that this deletion had no effect on the endogenous polymerase reaction (14). Assuming that all other mutations did not drastically reduce P protein stability, these results indicate that, except for part of the spacer region, all domains of the HBV P protein are required for genomic RNA encapsidation.

Packaging of nonviral RNAs fused to the encapsidation signal of HBV requires P protein. In the following analysis, we investigated whether the target of P protein action is the RNA sequence recently identified as the HBV encapsidation signal (Junker-Niepmann et al., in press) or whether flanking HBV sequences are required. To address this question, a hybrid construct was used in which a 137-nt HBV sequence cassette containing the encapsidation signal was cloned between the CMV-IE promoter and a 2.8-kb fragment of the *E. coli lacZ* gene (pCHG3134/88, Fig. 6A and Materials and Methods). Furthermore, two constructs either containing additional 5′ HBV sequences (pCHG3097/88) or lacking flanking 3′ HBV sequences (pCHG3134/36) were analyzed. The parent construct pCG-0, lacking any HBV sequence, was used as a negative control. These constructs were transfected into HepG2 cells together with a plasmid directing the expression of HBV core protein (pMS3C1, see Materials and Methods), and *lacZ* RNAs present in the total RNA fraction and in core particles were analyzed by a nuclease protection assay with an appropriate RNA (Fig. 6A). In addition, a plasmid directing the expression of the P gene (pMTpol, Fig. 3) was added as indicated in Fig. 6B to assay for the effects of P protein on RNA encapsidation.

Analysis of the total RNA fraction revealed that RNAs produced from the four different constructs were present in similar amounts (lanes 1 to 5, 11, and 12). In the corresponding core fractions, however, signals were only detectable when the P protein-expressing plasmid had been included in the transfections with the constructs containing the HBV encapsidation signal (lanes 8, 10, and 14). These results clearly demonstrate that P gene products are also required for the encapsidation of chimeric RNAs containing only the HBV packaging signal. Although, in this experiment, the amounts of encapsidated RNAs derived from pCHG3134/36 were about twofold lower than the amounts of packaged RNAs produced from the other two hybrid constructs, this result demonstrates in addition that the HBV encapsidation signal can be as short as 85 nt. This is in accord with a recent

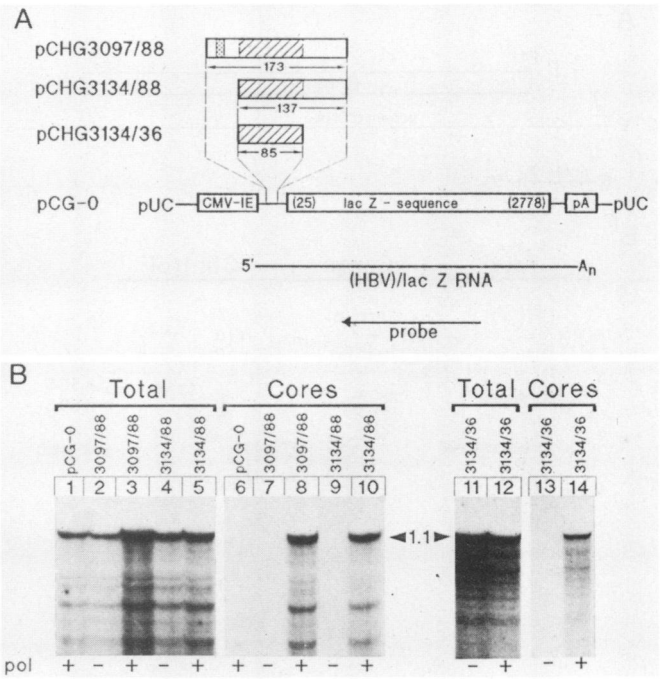

FIG. 6. Analysis of P protein for its effects on *trans* packaging of nonviral RNAs fused to the HBV encapsidation signal. (A) Schematic presentation of the basic construct pCG-0 and of the HBV-*lacZ* hybrid constructs pCHG3097/88, pCHG3134/88, and pCHG3134/36. These constructs were derived from pCG-0 by insertion of HBV sequences of variable lengths (given in base pairs; see Materials and Methods). The encapsidation signal is drawn as a hatched box, and the 5′ copy of direct repeat 1 is shown as a dotted box. The 3-kb transcript starting from the CMV-IE promoter and terminating at the SV40 polyadenylation signal (pA) and the position of the *lacZ*-specific antisense RNA probe are drawn below. (B) Detection of *lacZ*-specific RNAs present in the total cytoplasmic extract or in core particles. DNAs from the four different constructs shown in panel A were transfected into HepG2 cells together with a plasmid directing the expression of the HBV C gene and, as indicated by +, a plasmid containing an intact HBV P gene. The protected fragment of about 1.1 kb is marked. Note that the result shown on the right side of panel B was obtained from a different experiment.

prediction from sequence homologies between mammalian hepadnaviruses (Junker-Niepmann et al., in press).

P protein acts primarily in *cis*. Originally developed during a detailed analysis of the expression strategy of the DHBV P gene (4, 17), *trans* complementations of HBV gene mutants are now widely used to examine different aspects of hepadnaviral replication. During the course of this work we found that the packaging efficiency of *trans*-complemented genomes varied drastically depending on the construct used as a donor of viral P proteins. While *trans* packaging was very effective with the packaging-defective helper pMH3/3143 (Fig. 1), this was not the case with the wild-type construct pMH-34/2922 (Fig. 1), in which a strong preference for encapsidation of wild-type RNAs was consistently observed. A representative result of such an experiment is shown in Fig. 7, in which stop mutant P11 was transfected into HepG2 cells alone or in a mixture with either the helper construct or the wild type.

To be able to detect even a very low fraction of mutant RNA, we used a probe homologous to the P11 RNA (Fig. 7A). This probe contains a mismatch to the RNAs synthe-

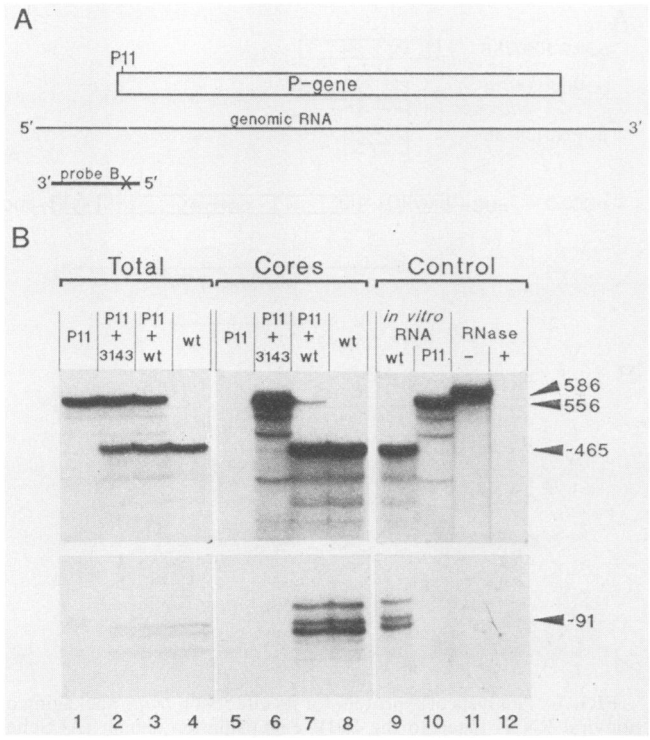

FIG. 7. Analysis of *trans* complementation efficiencies. (A) Schematic presentation of the P gene with stop mutant P11 and the position of probe B relative to the RNA pregenome. (B) Analysis of RNAs detectable in the total RNA fraction and in core particles after transfection of HepG2 cells with mutant P11 alone (lanes 1 and 5) or with a mixture of either helper construct pMH3/3143 plus mutant P11 (lanes 2 and 6) or wild-type construct pMH-34/2922 plus mutant P11 (lanes 3 and 7) or with wild-type DNA alone (lanes 4 and 8). Fragments shown in lane 9 and 10 were obtained in parallel by hybridization of the probe to in vitro-transcribed RNAs of wild-type (wt) or mutant P11 sequence. 1/100 of the input probe is shown in lane 11; the unprotected probe treated with RNases is shown in lane 12. Because of the small amounts of encapsidated P11 RNA present in lane 7, a five-times-longer exposure of the signals obtained with packaged RNA compared with the signals obtained with total RNA is shown. Sizes are shown to the right (in nucleotides).

sized from the helper and the wild-type constructs, and therefore RNAs protected by wild-type genomes are cleaved into two fragments of about 465 and 91 nt.

As shown in the left panel of Fig. 7B, all RNAs were present in about equal amounts in the total RNA fraction; no signal was detectable in the core fraction when mutant P11 was transfected alone (lane 5), and this defect was compensated for by cotransfection with the helper construct, which itself is not encapsidated (lane 6). In contrast, cotransfection of mutant P11 with our wild type revealed that predominantly wild-type RNA was encapsidated (lane 7, lower bands), whereas RNA from the mutant was only poorly detectable (upper band in lane 7), although both RNAs were present in equal amounts in the total RNA fraction (lane 3). Essentially the same result was obtained when a mutant with a frameshift mutation in the middle of the core gene, cloned into our wild-type plasmid pMH-34/2922, was used as a donor of P protein (not shown). This preference for encapsidation of P^+ RNAs was observed consistently, although the results obtained varied between 10% of encapsidated P^- RNA, as in the experiment shown in Fig. 7, up to 25%. These data suggest that P protein acts primarily on the RNA pregenome, which is used as mRNA for its synthesis.

DISCUSSION

The aim of this study was to analyze whether proteins derived from the HBV P gene are required for genomic RNA encapsidation. By analyzing a series of different P gene mutants for their ability to produce RNA-containing nucleocapsids, we found that a P gene product containing all of the functional domains is an absolute requirement for packaging of RNAs carrying the HBV encapsidation signal and that the packaging function of the P protein can be separated genetically from its enzymatic activities.

These findings appear to be true for all hepadnaviruses, since similar results with respect to P protein requirement and *cis* action were recently obtained with the most distantly related hepatitis B virus, DHBV (7; R. Bartenschlager, Ph.D. thesis, University of Heidelberg, Heidelberg, FRG, 1990). Despite the overall similarity of these results, there was a significant difference in the efficiencies of genomic RNA encapsidation. While wild-type HBV consistently encapsidated only about 10% of the cytoplasmic HBV RNA pregenome into core particles, wild-type DHBV encapsidated about 50 to 70% of the cytoplasmic DHBV RNA pregenome (7; R. Bartenschlager, Ph.D. thesis). As we used the same experimental conditions in both systems, this difference seems to be significant.

In working with DHBV, we also observed a strong nonspecific binding of RNA to the DHBV core protein (R. Bartenschlager, Ph.D. thesis). This property, which originally masked the requirement of RNA packaging for P gene products in our DHBV system, could only be overcome by extensive treatments of the immunoprecipitated core particles with micrococcal nuclease (three cycles of a 20-min incubation with at least 30 U of enzyme per reaction in each cycle), which was not necessary when we used the HBV system (see Materials and Methods).

Despite these differences, the results obtained with both systems clearly demonstrate that a P gene product is essential for hepadnaviral RNA packaging. This finding invites speculation about the assembly process leading to the formation of replication-competent nucleocapsids.

(i) We (14, 17) and others (4, 8, 15) have recently shown that the hepadnaviral P protein is not synthesized via a core-polymerase fusion protein as in the case of retroviruses, but rather by internal initiation at the P gene AUG. This raises the question of how P protein is packaged into nucleocapsids, since it, unlike the retroviral reverse transcriptase, cannot be incorporated into nascent particles by coassembly of such fusion proteins with core protein molecules. An alternative, which seems to be realized in hepadnaviruses, could be that the P protein binds as an essential component of a packaging complex to an RNA carrying the appropriate encapsidation signal and in this way is copackaged along with the pregenome into nucleocapsids. According to this model, one would expect that the formation of such a packaging complex is a prerequisite not only for the selective incorporation of the RNA pregenome but also for packaging of P protein into core particles, i.e., the presence of the appropriate RNA would be essential for P protein packaging. Such a mechanism of capsid assembly appears to differ from retroviral assembly in two ways. First, polymerase seems to be dispensable for RNA packaging in murine retroviruses (20), since several Pol^- mutants produce viral particles containing normal amounts of 70S RNA. Second,

genomic RNA is apparently not required for polymerase incorporation. Cells treated with dactinomycin produced, from intracellular pools of viral proteins, particles containing normal amounts of enzymatically active reverse transcriptase but lacking genomic RNA (10).

(ii) Another aspect regarding HBV nucleocapsid assembly comes from the observation that P protein acts primarily in *cis* on the RNA genome. Using constructs in which P protein is synthesized from RNAs lacking a functional encapsidation signal (pMTpol or helper pMH3/3143), we obtained high efficiencies of *trans* complementation, indicating that diffusible P proteins are able to mediate RNA encapsidation in *trans*. In contrast, efficiencies were very low when our wild-type construct (pMH-34/2922) was used as a donor of P protein. The fact that essentially the same result was recently described for the DHBV system (7) suggests that asymmetric complementation is a general property of hepadnaviruses. We do not know the reason for this finding, but one plausible explanation is that P protein is normally present only in limiting amounts. This could be due to low expression, a short half-life (with a vaccinia virus expression system, it was found in preliminary experiments that the HBV P protein has a half-life of only about 30 min [R. Bartenschlager, Ph.D. thesis]) and a high affinity to the RNA target sequence. Due to such properties, P protein would preferentially act on the RNA from which it is made, and only when overexpressed, in particular from constructs lacking ε, would the quantities of diffusible P protein be high enough to allow efficient *trans* complementation. This suggests that P protein is the limiting factor of the packaging reaction.

(iii) Although we are still far from a detailed understanding of the mechanism of the packaging reaction on the molecular level, the components required are now defined: core protein, P protein with all of the functional domains, and the encapsidation signal in the RNA pregenome, which we have now identified as an 85-nt sequence located near the pregenome's 5′ end. The fact that this sequence cassette is sufficient to direct encapsidation of nonviral RNAs into HBV nucleocapsids argues against an RNA packaging model in which P protein binding, involving distal HBV sequences, alters RNA secondary structure in such a way that the encapsidation signal becomes exposed. We therefore presently favor a model in which as a first step in core assembly, P protein(s) binds directly to ε, either alone or as a complex with one or several core protein molecules. It is notable that, for initiation of reverse transcription, P protein would have to be directed from the 5′ to the 3′ end of the RNA pregenome, since target sequences for RNA packaging (ε) and initiation of reverse transcription (the 3′ copy of direct repeat 1, see Fig. 1B) (19) reside 3 kb apart. This may require a specific RNA structure present in the nucleocapsid.

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