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Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome

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Hepadnaviruses, as well as other pararetroviruses, express their *pol* (P) gene product unfused to the preceding core gene implying that these retroelements have developed a mechanism for initiating assembly and replication that is principally different from the one used by retroviruses and retrotransposons. We have analysed this mechanism for the human hepatitis B virus by using a newly developed, highly sensitive detection method based upon radiolabelling of the P protein at newly introduced target sites for protein kinase A. The results obtained demonstrate that polymerase encapsidation depends on the concommittant encapsidation of the HBV RNA pregenome and that packaging of the viral RNA, in turn, depends on the presence of P protein. Loss of P protein encapsidation by mutations inactivating the HBV RNA encapsidation signal ϵ could be compensated by trans-complementation with recombinant RNA molecules carrying the ϵ sequence. Thus, in contrast to retroviral replication, the interaction of the hepadnaviral P protein and the RNA genome at its packaging signal appears to be crucial for initiating the formation of replication-competent nucleocapsids. Furthermore, RNA control of P protein packaging stringently limits the number of polymerase molecules that can be encapsidated.

Key words: human hepatitis B virus/nucleocapsid assembly/P protein packaging/reverse transcription

Introduction

The hepadnaviruses with the human hepatitis B virus (HBV) as their prototype member, comprise a group of small, enveloped DNA viruses whose mode of replication involves reverse transcription of an RNA pregenome (Summers and Mason, 1982). This life cycle places these animal viruses, along with other 'pararetroviruses' such as the plant cauliflower mosaic virus (Hohn and Fütterer, 1991), into the growing family of retroelements that now range from retroviruses and the multitude of retrotransposons in higher and lower eukaryotes to mitochondrial plasmids and bacterial ms DNA (Boeke and Corces 1989; Mason and Seeger, 1991). In all eukaryotic retroelements studied so far, reverse transcription does not occur freely in solution, but requires the prior assembly of the RNA genome with its *gag* and *pol* gene products to form cytoplasmic 'virus-like' particles as an essential step. This assembly step, in particular the coordinate packaging of polymerase and RNA, is therefore crucial for retroreplication to occur. For retrotransposons and retroviruses, this process is governed by the sequence-specific interaction of the capsid *(gag)* protein with the RNA genome, and by the synthesis and passive cointegration and proteolytic maturation of a fused *gag–pol* polyprotein produced by ribosomal frame shifting. Hepatitis B viruses, as well as other pararetroviruses, express their *pol* (P) gene product unfused to the preceding nucleocapsid (core) gene (Chang *et al.*, 1989; Schlicht *et al.*, 1989). This raises the question of how a freely diffusible hepadnaviral reverse transcriptase/DNA polymerase is directed specifically onto the viral RNA template and how promiscuous reverse transcription of cellular RNAs is prevented.

Substantial progress towards answering these questions has been made by mutation and complementation analysis of P gene functions, which revealed that the hepadnaviral P gene product is a multidomain protein that differs from the retroviral *pol* gene product by lacking the protease and integrase domains, and instead containing amino-terminally the DNA terminal protein (TP) involved in the HBV-specific initiation of reverse transcription (Gerlich and Robinson, 1980; Bartenschlager and Schaller, 1988; Radziwill *et al.*, 1990). Moreover, and again in contrast to the retroviral polymerase, the hepadnaviral P protein was found to participate as an essential structural component in the packaging of the RNA pregenome (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990). This latter result implied a specific, direct or indirect interaction of the P protein with the RNA genome, but did not give any clues whether packaging of the P protein itself occurred by the same mechanism. Alternatively, one could envisage an RNA-independent mechanism with the P protein binding to core protein subunits prior to or during nucleocapsid formation.

Differentiation between these two alternatives requires the direct detection of the P protein, which, due to its low abundance, has so far remained elusive. To overcome this problem we have developed a highly sensitive detection method using *in vitro* phosphorylation by protein kinase A (Bartenschlager *et al.*, 1992). Employing this method in combination with transient expression of HBV genomes for the analysis of the nucleocapsid-associated P protein, we have now been able to demonstrate that coencapsidation of a packaging-competent RNA molecule is essential for incorporation of the hepadnaviral P protein into core particles. Thus, hepadnaviruses have developed a strategy for ensuring the initial steps of replication that is fundamentally different from the mechanisms operating in the retroviral life cycle.

Results

Experimental design

So far, direct detection of the hepadnaviral P protein has not been possible due to its low abundance in infected (or

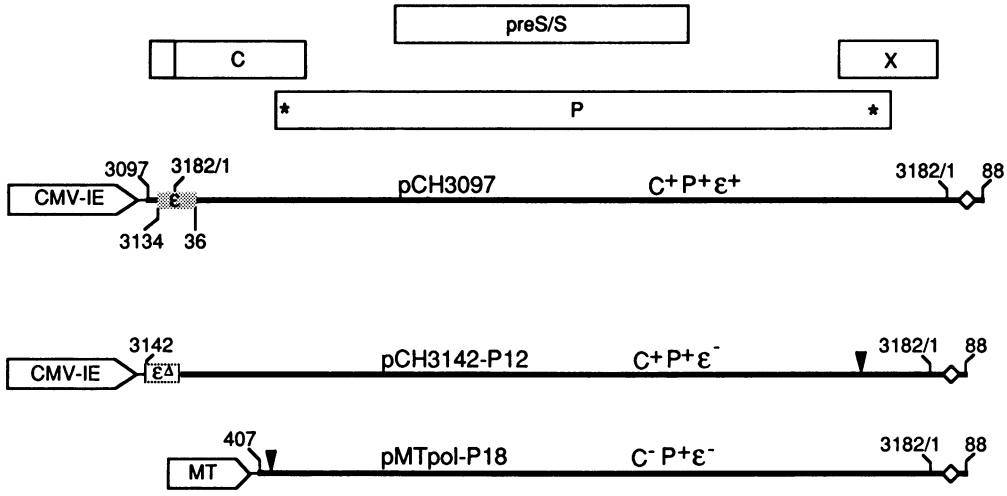

Fig. 1. Schematic presentation of the basic constructs used for transient expression of HBV genomes. HBV sequences are drawn as heavy lines; the HBV polyadenylation signal as open diamond; the human cytomegalovirus immediate early promoter (CMV IE) and the human metallothioneine promoter (MT) are drawn as open arrows; the positions of the PKA target sequences (RRXSX; Edelman *et al.*, 1987), introduced by site directed mutagenesis, are indicated by stars or arrowheads; the 85 nucleotide long RNA encapsidation signal (ε), contained in the wild type construct pCH3097, is drawn as a stippled bar; the partially deleted, non-functional encapsidation signal (ε^{Δ}, lacking the HBV sequence 5′ of position 3142) contained in construct pCH3142-P12, is drawn as an open bar. Numbers are given according to the nomenclature of Pasek *et al.* (1979), starting with the A residue of the core gene start codon.

transfected) cells or within the viral particle. To overcome this problem, we have recently developed a sensitive detection method based on the *in vitro* phosphorylation by protein kinase A (PKA), which allows ^{32}P labelling to high specific activities, thereby increasing the sensitivity of P protein detection by two orders of magnitude (Bartenschlager *et al.*, 1992). These target sites were introduced into the terminal regions of the P gene contained in an HBV genome, which is transcribed under the control of the strong human cytomegalovirus immediate early promoter (plasmid pCH3097; Figure 1). After transfection into a human hepatoma cell line, this construct directs the synthesis of replication competent virus-like particles and therefore it is referred to as the wild type. For P gene trans-complementation, two suitably modified helper plasmids, pCH3142 and pMT pol (Figure 1), were used to direct expression of phosphorylatable P proteins from HBV RNAs lacking a functional encapsidation signal and therefore not competing for the viral proteins involved in capsid formation (Junker-Niepmann *et al.*, 1990).

Identification and characterization of the nucleocapsid-associated HBV P protein

An outline of the procedure used to detect the nucleocapsid-associated P protein is given in Figure 2. P proteins from purified core particles were immunoprecipitated using a mixture of anti-peptide antisera directed against amino-terminal (anti-P6) or carboxy-terminal (anti-P8) sequences of the P protein (Figure 3A). While the supernatant of this immunoprecipitation was used for quantification of core protein by Western blot analysis, precipitated P proteins were radiolabelled with PKA and [γ-^{32}P]ATP. Finally, P proteins were reprecipitated with the same anti-P antisera and analysed by SDS−PAGE.

The result of such an experiment is presented in Figure 3B. Analysis of nucleocapsids produced from the carboxy-terminal variant P12 revealed that the major P protein detected with this method was a 90 kDa full length product. The identity of this protein was demonstrated (i)

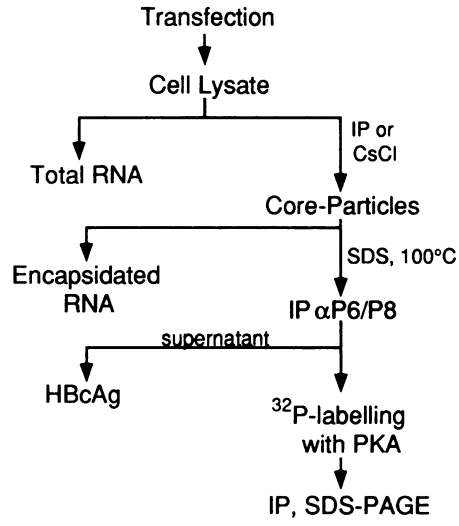

Fig. 2. Flow chart presentation of the method used to detect and to quantify the HBV P protein as well as encapsidated RNA and core protein (HBcAg). IP, immunoprecipitation; CsCl, Caesium chloride density gradient centrifugation; PKA, protein kinase A. RNAs present either in the total cell lysate or within the nucleocapsid were quantified using RNase protection analyses as described previously (Bartenschlager *et al.*, 1990). For further details see text.

by its absence in mutant P11, which carried a stop codon at the very beginning of the P gene (lanes 1 and 2) and (ii) by a strong reduction of its immunoprecipitation by competition with the homologous peptides P6 and P8 (lane 4). To prove further that the ^{32}P-labelled 90 kDa protein detected was a P gene product, mutant PΔ1-P12, which was derived from construct P12 by introducing a 90 amino acid deletion into the spacer region (Radziwill *et al.*, 1990; Table I) was analysed in parallel. As shown in lane 5, a protein with an expected size of ~80 kDa was detected and again immunoprecipitation could be competed with the homologous peptides (lane 6).

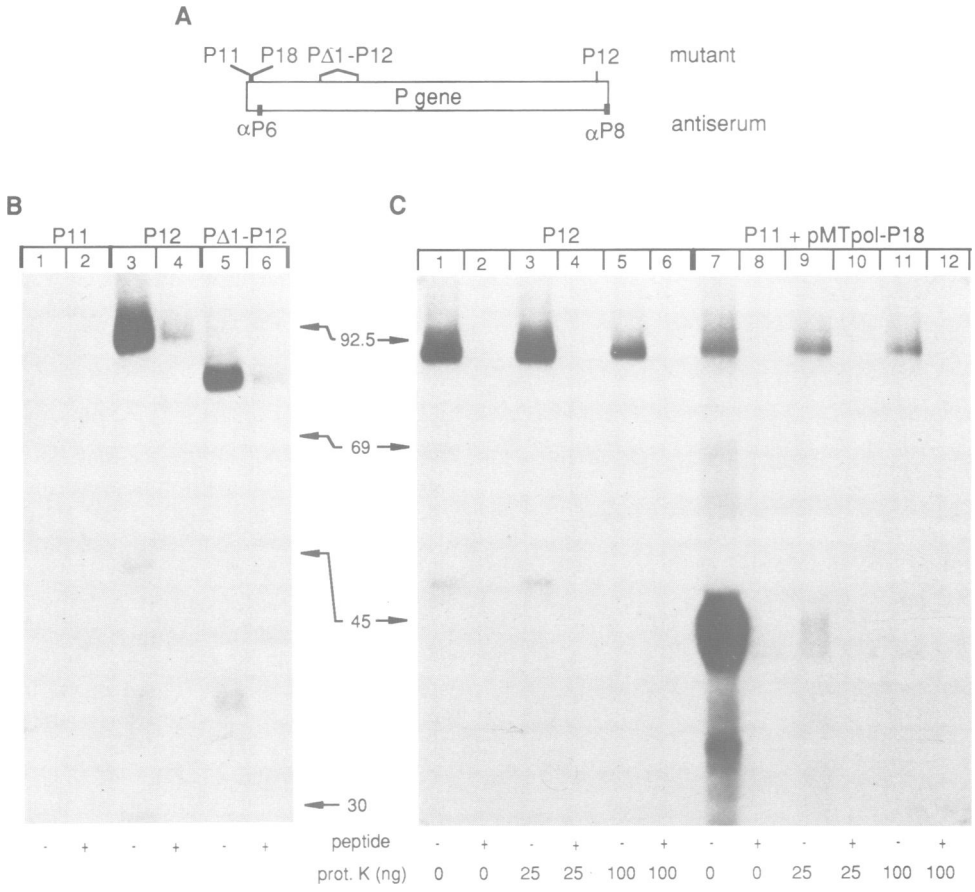

Fig. 3. Identification and characterization of the HBV P gene product. **(A)** Schematic presentation of the P gene indicating position and type of mutations and the positions of the peptides used to elicit P-specific antisera. **(B)** Detection of the HBV P protein after *in vitro* phosphorylation. Constructs shown in panel A were transfected into Huh7 cells and P proteins were isolated from the cell lysate as described in Materials and methods. Following the phosphorylation step, radiolabelled P proteins were immunoprecipitated in the presence (+) or absence (−) of the homologous antigenic peptides. **(C)** Protease protection assay with core particles isolated from Huh7 cells transfected with construct P12 (lanes 1−6) or with a mixture of stop mutant P11 and the P gene construct pMTpol-P18 (lanes 7−12; compare with Figure 1). Nucleocapsids were incubated with or without proteinase K (prot. K) as indicated prior to denaturation and immunoprecipitation of the P protein. All other steps were as in B. Numbers between B and C refer to the size of marker proteins in kDa.

To show that the detected P proteins were truly encapsidated rather than only tightly adsorbed to the outside of the nucleocapsid, a protease protection experiment was performed. When core particles produced from pCH3097-P12 were isolated by immunoprecipitation, treated with proteinase K prior to denaturation and analysed as described before, a significant loss of the 90 kDa P protein was only observed at high protease concentrations (Figure 3C, lanes 1−6) indicating that the protein was located within the relatively protease-resistant nucleocapsid. In these experiments, a convenient internal control for the protease sensitivity of proteins bound to the outside of the nucleocapsid was provided by a phosphorylatable protein of ~43 kDa (Figure 3C, lane 7), which was produced, in addition to the full size 90 kDa protein, as a byproduct from the P-transcomplementing construct pMT pol-P18 (Figure 1). This protein, most likely a P−preS−S fusion produced from a spliced RNA and specifically binding to core particles (R.Bartenschlager, unpublished observations), was found to be rapidly degraded even with low amounts of proteinase K, whereas no significant loss of the 90 kDa P protein was detected (Figure 3C, lanes 7−12). Taken together, these results clearly demonstrate that the full length P protein detected was contained in the nucleocapsid.

Table I. P gene mutations and their effects on genomic RNA encapsidation, endogenous polymerase activity and P protein packaging

Mutant	Amino acid changes[a]	Position[b]	Polymerase activity (%)[c]	RNA packaging[d]	P protein packaging[e]
P18	L→S (T→I)	12 (147)	10	+	+
P12	G→R	800	25	+	+
P1	YP→SA	133/134	1	−	−
P2	D→H	540	0	+	+
P19	Y→F	633	1	+	+
P11	L→Stop	14	0	−	−
PΔ1	S<>E	201<>292	40	+	+

[a]Changes in the P protein and, in case of mutant P18, also in the core protein (T→I).
[b]Positions in the P protein and, in case of mutant P18, also in the core protein.
[c]Determination of the enzymatic activities relative to those of the wild type using the endogenous polymerase reaction. The data for P1, P2 and PΔ1 are taken from Radziwill *et al.* (1990), for P12 and P18 from Bartenschlager *et al.* (1992).
[d]Encapsidated RNA pregenome detected by an RNase protection assay. The data for P2, P11 and PΔ1 are taken from Bartenschlager *et al.* (1990).
[e]Encapsidated P protein measured by *in vitro* phosphorylation.

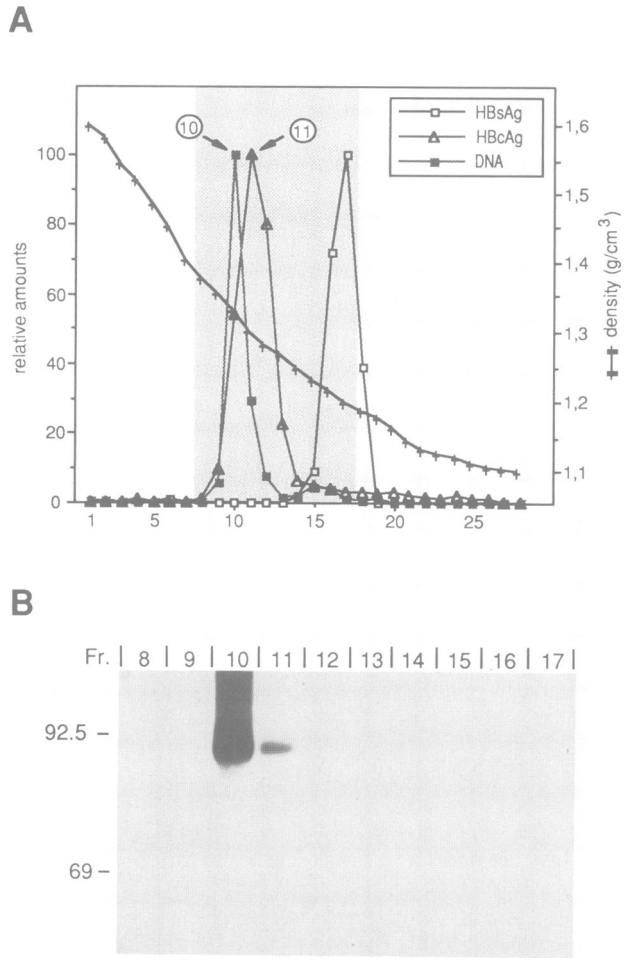

Fig. 4. Distribution of the HBV P protein in nucleocapsids purified by CsCl density gradient centrifugation. **(A)** Particles released into the medium from transfected Huh7 cells were concentrated by pelleting, redissolved and fractionated by CsCl density gradient centrifugation. Fractions were analysed for the presence of HBV-specific DNA, HBcAg, HBsAg and density profile (see Materials and methods). The shaded area indicates the fractions analysed for the presence of the P protein. **(B)** Detection of the P protein in fractions 8–17. P protein was immunoprecipitated and radiolabelled according to the protocol described in Figure 2. Numbers beside the gel refer to the size of marker proteins in kDa.

Pregenomic RNA participates in P protein encapsidation

As outlined in the Introduction, packaging of the hepadnaviral P protein can be accomplished principally either via binding to the RNA pregenome or via binding to one or several core protein molecules, i.e. independent from the pregenome. To differentiate between these two possible mechanisms, core particles released from Huh7 cells transfected with pCH3097-P12 were banded in a CsCl density gradient and analysed for the presence of phosphorylatable P protein, the core protein (HBcAg), the surface protein (HBsAg) and HBV-specific DNA (see Materials and methods). The majority of core particles banded at a density of 1.30 g/cm^3 (Figure 4A, fraction 11). Analysis of the fractions by DNA dot blot revealed that only a minority of these nucleocapsids, banding at a density of 1.33 g/cm^3 (fraction 10), contained high amounts of HBV-specific DNA. To analyse which subpopulation of core particles contained the P protein, *in vitro* phosphorylations were performed as described above and the proteins were analysed by SDS–PAGE and autoradiography. As shown in Figure 4B, the peak of the P protein was found to coincide with the peak of viral DNA (fraction 10), i.e. outside the core protein peak in fractions 11 and 12. Thus, we conclude that the RNA pregenome is an essential prerequisite for P protein encapsidation.

In addition, the above analysis also allowed us to make an approximate estimation of the molar ratio of P protein and viral DNA in a fraction enriched for DNA-containing particles. By using the specific activity of vaccinia virus expressed HBV-P12 P protein ($\sim 6\times10^5$ c.p.m. ^{32}P/pmol protein; Bartenschlager *et al.*, 1992) and an HBV DNA standard for quantification (see Materials and Methods), we calculate that fraction 10 contained ~7 fmol of P protein and 10 fmol of (full length) HBV DNA. This calculation does not take into account that some core particles contained incomplete DNA genomes or RNA pregenomes (also banding at the same position in the gradient, unpublished observations), which both would lead to an underestimation of the average molar DNA content. On the other hand, P protein quantification is based on the specific activity of template-free, vaccinia virus expressed P12 P protein, which might be a considerably better substrate for PKA phosphorylation and therefore led to an underestimation of the amounts of core-associated P protein. Although the relative influence of these counteracting factors on our calculation is not known, the molar ratio of 0.7 polymerase per HBV DNA molecule observed, is consistent with the assumed low copy number of the hepadnaviral P protein and suggests that there is probably only one or two polymerase molecule(s) per nucleic acid containing core particle.

A missense mutation in the P gene affecting the RNA packaging function impairs P protein encapsidation

If P protein encapsidation was indeed mediated by binding to the RNA, P gene mutants defective for the RNA packaging function would be predicted not to direct P protein encapsidation. To test this hypothesis, a series of missense mutations located in different P gene domains and affecting different P protein functions were transferred into pCH3097-P12 and analysed for their effects on genomic RNA encapsidation and P protein packaging (Figure 5A; Table I). As shown in Figure 5B by the relative amounts of pregenomic RNA detectable by RNase protection analysis, TP mutant P1 was defective for RNA packaging (lane 7), while no impairment of RNA encapsidation was detected in case of the other mutations located in the TP domain (P19, lane 6) or in the RT domain (P2, lane 8). Analysis of the amounts of encapsidated P protein revealed no significant difference between the RNA packaging competent mutants P19 and P2 and the parental construct P12 (Figure 5C, lanes 2, 3 and 5). However, core particles produced from mutant P1 lacked detectable amounts of P protein although core protein synthesis remained essentially unchanged (Figures 5C and D, lane 4). Thus, the results from this genetic analysis confirm the results of the physical analysis described above and suggest that packaging of pregenomic RNA and P protein are closely coupled and probably mutually dependent.

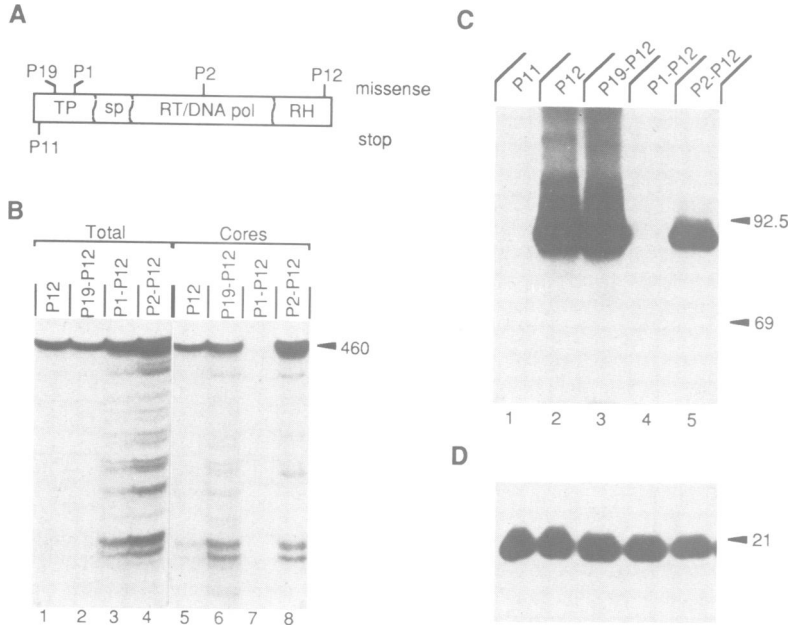

Fig. 5. Analysis of missense mutations in the P gene for their effects on P protein and RNA encapsidation. **(A)** Localization of the mutations with respect to the various functional domains of the P protein. Symbols: TP, terminal protein; sp, non-essential spacer region; RT−DNA pol, reverse transcriptase/DNA polymerase; RH, RNase H. **(B)** RNase protection assay with total cytoplasmic RNA (Total), or encapsidated RNA (Cores) obtained after transient expression in Huh7 cells of the various mutants or their basic construct P12. The arrowhead marks the 460 nucleotide fragment protected by the HBV RNA. **(C)** Detection of the encapsidated P protein by SDS−PAGE after PKA phosphorylation. **(D)** Control of the transfection efficiency by Western blot analysis of the core protein (HBcAg; compare with Figure 2). Numbers beside the gels in C and D refer to the size of marker proteins in kDa.

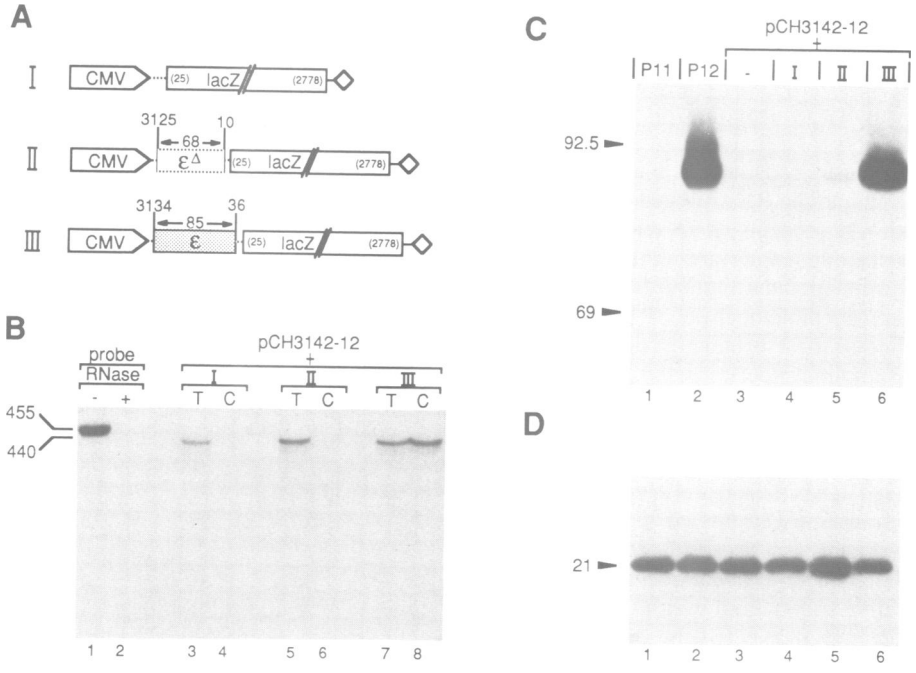

Fig. 6. Dependence on a functional encapsidation signal of P protein packaging. **(A)** Schematic presentation of the basic constructs used for the synthesis of *lacZ* or HBV−*lacZ* chimeric RNAs. HBV sequences are shown as stippled bars, non-viral sequences as open bars and the SV40 polyadenylation signal as an open diamond. Numbers refer to the map positions in the HBV genome or the *lacZ* gene. The size of the HBV fragments inserted between the CMV promoter and the *lacZ* sequence is given in base pairs. **(B)** Detection of *lacZ*-specific RNAs in total cytoplasmic extracts (T) or in nucleocapsids (C) by RNase protection after cotransfection of plasmid pCH3142-P12 (compare with Figure 1) and the constructs shown in panel (A) (for details see Materials and methods). 1/100 of the input RNA probe is shown in lane 1, after treatment with RNase in lane 2. Numbers beside the gel refer to the lengths of the RNA fragments in nucleotides. **(C)** Detection of the encapsidated P protein after transient expression of phosphorylation variant P12 (lane 2), or the encapsidation deficient construct pCH3142-P12 (compare with Figure 1) either alone (lane 3) or together with the constructs shown in panel A (lanes 4−6). The result obtained with stop mutant P11 is included as a negative control (lane 1). **(D)** Comparison of core protein (HBcAg) produced by Western blot analysis (compare with Figure 2).

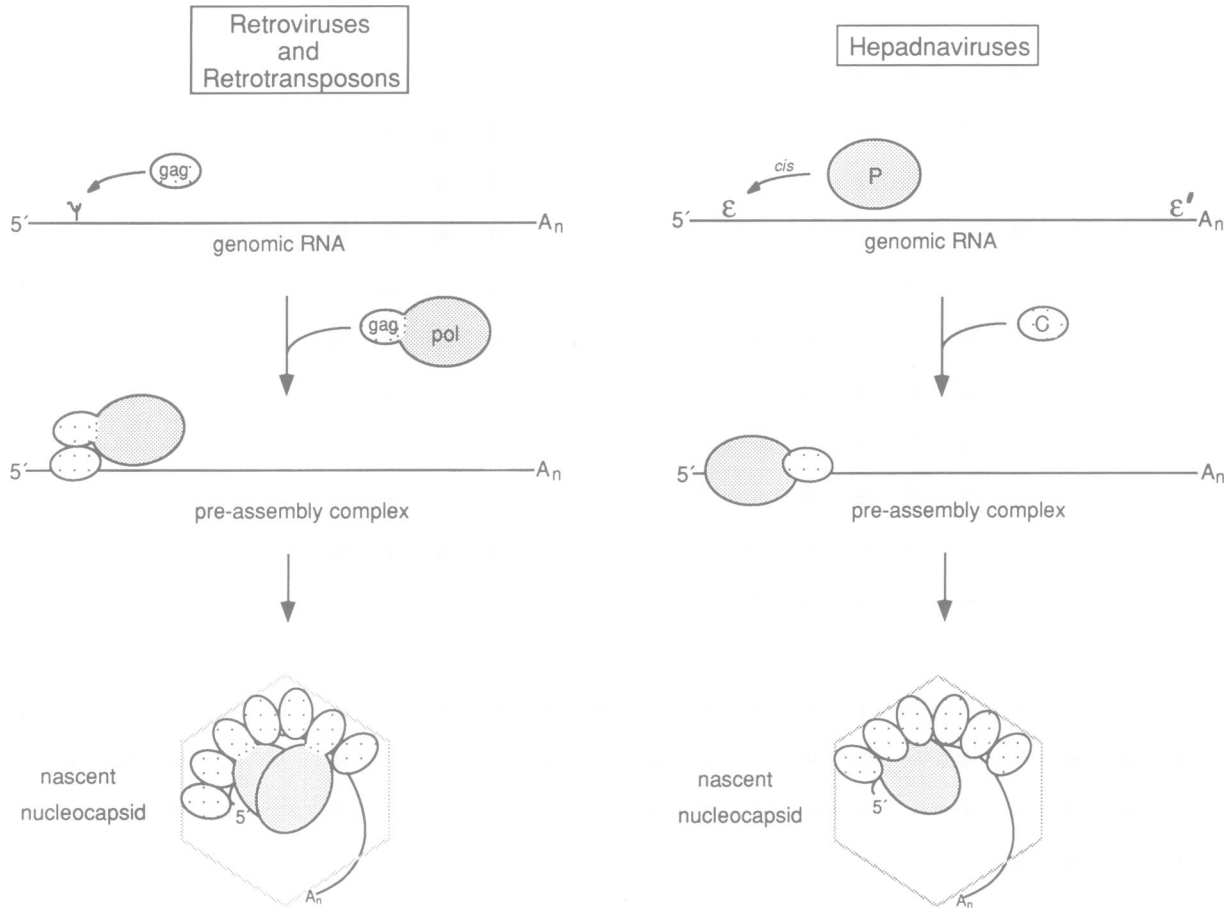

Fig. 7. Schematic presentation of a model for HBV polymerase encapsidation. For details see text.

A functional ϵ sequence is essential for efficient P protein packaging

All data described so far are consistent with a model in which binding of the P protein to the RNA pregenome ensures the packaging of both components into nucleocapsids. In addition, since packaging of a non-viral RNA can be mediated *in cis* by a functional ϵ sequence (Bartenschlager *et al.*, 1990; Junker-Niepmann *et al.*, 1990), it seemed plausible that binding of the P protein occurred at this site. To evaluate this assumption experimentally, a construct was made (pCH3142-P12; Figure 1) that allows the expression of phosphorylatable P protein from an RNA pregenome which, due to a deletion of some 40 nucleotides at its 5′-end, is packaging deficient (Junker-Niepmann *et al.*, 1990). When this construct was transiently expressed in Huh7 cells and analysed as described above, no encapsidated P protein could be detected in the core fraction, in contrast to core particles isolated from cells transfected with pCH3097-P12 (Figure 6C, lanes 3 and 2, respectively) and expressed in comparable amounts (Figure 6D). To prove that the phenotype observed was due to the absence of a functional encapsidation signal, a trans-complementation experiment was performed by cotransfecting pCH3142-P12 with a construct that directs the transcription of a functional ϵ sequence fused to the 5′ end of a 3 kb *lacZ*−RNA (Figure 6A, construct III). As negative controls, two additional trans-complementations were carried out using constructs either lacking the ϵ sequence (construct I) or carrying a truncated, non-functional variant (construct II). Core particles were isolated by immunoprecipitation and the immunocomplexes were divided in two portions. One portion was used to quantify the amount of encapsidated *lacZ*−RNA relative to the amount of *lacZ*−RNA in total cytoplasmic extracts (Figure 6B) and the other portion was used to determine the amount of encapsidated P protein. *lacZ*-specific RNAs were present in the cytoplasm in comparable amounts in all cases (Figure 6B, lanes 3, 5 and 7). However, examination of the corresponding core fractions revealed that packaging of these RNAs depended strictly on the presence of a functional encapsidation signal (construct III, lane 8) consistent with our previous data (Bartenschlager *et al.*, 1990; Junker-Niepmann *et al.*, 1990). Analogous results were obtained when the same core fractions were also assayed for the presence of encapsidated P protein. Trans-complementation of plasmid pCH3142-P12 with the *lacZ* constructs I or II, which lack a functional ϵ sequence, did not restore P protein packaging (Figure 6C, lanes 4 and 5). In contrast, packaging of the P protein occurred with the ϵ−*lacZ* construct III (lane 6), demonstrating that a functional RNA encapsidation signal was essential for packaging of the hepadnaviral P protein into nucleocapsids.

Discussion

In the present study we have investigated the nature and hierarchy of the interactions that control and ascertain the coordinate uptake of the RNA pregenome and the DNA polymerase−reverse transcriptase of the pararetrovirus HBV

into intracellular, replication-competent core particles. By using a novel technique allowing direct detection of the polymerase protein, we demonstrate that (i) encapsidation of polymerase and genomic RNA are tightly coupled, (ii) polymerase encapsidation requires in the RNA the presence of the ε sequence that we previously defined as being essential for RNA packaging and (iii) only very few (possibly only one) P protein molecules are encapsidated along with the RNA genome. These observations suggest a model for the hepadnaviral assembly process that rationalizes our present findings and that also incorporates recent observations demonstrating that the P gene product is essential for the encapsidation of RNA molecules carrying a functional ε sequence near their 5′ end (Bartenschlager *et al.*, 1990, Hirsch *et al.*, 1990). According to this model (Figure 7), HBV assembly is initiated by the direct binding of the P protein to the ε sequence in the RNA genome. The resulting RNA−P protein complex is then further stabilized by one or several core protein molecules and finally the assembly complex is converted by the essentially sequence-independent addition of further core protein subunits into the mature core particle capable of initiating reverse transcription. Previous complementation experiments revealed that the P protein acts preferentially *in cis* (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990) and therefore the initial P protein−ε RNA interaction appears to be essentially irreversible indicating very tight binding and/or rapid addition of core protein subunits.

Such a mechanism of nucleocapsid assembly differs strikingly from the analogous events in retroviral reverse transcription where packaging of genomic RNA is mediated by proteins encoded in the *gag* gene only (Gorelick *et al.*, 1988; Oertle and Spahr, 1990; Luban and Goff, 1991). In contrast to hepadnaviral assembly, encapsidation of the retroviral *pol* protein is accomplished independently from genomic RNA by synthesis of a *gag−pol* polyprotein precursor followed by coassembly into the nucleocapsid via the *gag* moiety (Dickson *et al.*, 1982). By this mechanism, packaging of the retroviral *pol* protein can occur in the absence of genomic RNA (Levin *et al.*, 1974) and lead to 20−70 *pol* protein molecules per particle (Panet *et al.*, 1975; Krakower *et al.*, 1977).

A much lower copy number of the hepadnaviral P protein has always been assumed to be present in the viral or subviral core particle due to the difficulties to detect it with conventional methods. Considering only the minority of cores containing P protein and viral DNA, our data suggest that there are very few, possibly only one, P protein molecules per core particle. This is again consistent with an assembly model in which stoichiometric binding of the P protein, possibly in the form of a dimer as in retroviruses, to the ε sequence determines the number of polymerase molecules encapsidated. [It should be noted in this context that there is also a second copy of the ε sequence in the 3′-terminal redundancy of the HBV RNA genome (ε′; Figure 7) that was, however, not functional in RNA encapsidation upon transient HBV genome expression (Junker-Niepmann *et al.*, 1990). On the other hand, Koechel *et al.* (1991) recently reported the binding of RNA molecules containing a 3′-proximal ε sequence to a β-galactosidase−P-fusion protein in a cell free system.]

By being able to detect directly the polymerase protein as encapsidated in the core particle, we can also readdress the longstanding issue whether the hepadnaviral P protein requires proteolytic activation to exert its several functions during HBV genome replication. Together with initial results from an earlier report (Bartenschlager *et al.*, 1992), the data presented here provide direct experimental evidence that the HBV P gene product is contained in the nucleocapsid as an unprocessed 90 kDa protein and suggest that initiation of reverse transcription and covalent linkage of the polymerase to its DNA product do not significantly alter its primary structure. These observations are consistent with a genetic analysis demonstrating that the intact HBV P gene product is required as an essential structural component for genomic RNA encapsidation (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990). They also support the notion that the hepadnaviral P protein, in contrast to the retroviral polymerase precursor, can perform its functions as a multidomain polypeptide without requiring proteolytic processing (Bartenschlager and Schaller, 1988).

Finally, the proposed assembly scheme also offers an attractive mechanism for coordinating translation and packaging of the HBV RNA genome. Early in replication, this molecule serves primarily as mRNA for translation of the core gene, while P gene translation seems to be a rare event (Jean-Jean *et al.*, 1989). Late in replication, however, high affinity binding of P protein to the ε sequence (containing the core gene AUG codon) is predicted to downregulate core gene translation (Nassal *et al.*, 1990). Preliminary data from cotransfection experiments with increasing amounts of a P protein producing plasmid indicate that this is indeed the case (R.Bartenschlager, unpublished results). Thus, in initiating formation of a pre-assembly complex, the P protein may also function as a translational repressor facilitating the assembly process by withdrawing the pregenome from the pool of translatable mRNAs.

By analysing the mechanism of polymerase encapsidation, we thus find that hepadnaviruses follow a strategy for coordinating gene expression and nucleocapsid assembly that is novel in many aspects among retroelements. It will be interesting to see whether this alternative strategy is common to other pararetroviruses.

Materials and methods

Plasmid constructions

The basic constructs, pCH3097, pMTpol and pCH3142, are described in Bartenschlager *et al.* (1992), Radziwill *et al.* (1990) and Junker-Niepmann *et al.* (1990). Construction of the HBV−*lac*Z hybrid plasmids (Figure 6) is described in Bartenschlager *et al.* (1990). P gene mutants, P1, P2, P11 and PΔ1, are described in Radziwill *et al.* (1990) and Bartenschlager *et al.* (1990). Mutant P19 was generated by site-directed mutagenesis (Zoller and Smith, 1984) using the following oligonucleotide (mismatch to the wild type sequence underlined): CTGGGCTTTTTTCTTCTA. The carboxy-terminal PKA target sequence from variant P12 was introduced into the various HBV constructs by substituting the *Eco*RI−*Rsr*II HBV DNA segments (nucleotides 1280−2853) of the mutants by the analogous fragment from pCH3097-P12.

Cell culture and transfections

Cultivation of Huh7 cells and transfections were done as described in Radziwill *et al.* (1990) with slight modifications. In brief, ~3×10^6 cells were seeded in 75 cm^2 dishes 24 h before transfection. To prepare the calcium phosphate precipitate, 20 μg plasmid DNA (15 μg of each plasmid for cotransfection) were ethanol-precipitated and dissolved in 450 μl distilled water plus 50 μl 2.5 M $CaCl_2$. After the addition of 500 μl 2×HBS (280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4, pH 7.1), samples were incubated 20 min at room temperature and added dropwise into the medium. 16 h post-transfection, cells were washed and fresh medium was added. Three days later cells were harvested as described by Bartenschlager *et al.* (1990).

Analysis of the P protein by in vitro phosphorylation and proteinase K treatment of nucleocapsids

Isolation of the P protein by immunoprecipitation and *in vitro* phosphorylation of the P protein was done as outlined in Figure 2 and described in Bartenschlager *et al.* (1992). Briefly, core particles isolated from the cell lysate by immunoprecipitation were denatured by boiling in TNE (10 mM Tris–HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA) containing 2% SDS and 1% 2-mercaptoethanol (2-ME) and, after dilution to RIPA buffer (phosphate buffered saline (PBS), 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS), P proteins were isolated by immunoprecipitation using a mixture of antisera directed against P-specific peptides (Figure 3). After intensive washing of the immunocomplex, P proteins were radiolabelled with PKA and [γ-^{32}P]ATP, washed several times with PBS and, after denaturation, reprecipitated with P-specific antisera. Finally, phosphorylated proteins were analysed by SDS–PAGE and autoradiography. Treatment of the immunoprecipitated nucleocapsids with proteinase K was carried out in TNE containing different amounts of the enzyme. After 20 min at 37°C, 2 mM phenylmethylsulfonylfluoride and 200 U of Trasylol (a kallikrein inhibitor; Bayer) were added followed by the addition of 2% SDS and 1% 2-ME. After boiling for 5 min samples were subjected to immunoprecipitation and analysed as described above.

Purification of core particles by CsCl density gradient centrifugation and ELISA analysis of the fractions

To increase the amount of core particles, transfected cells were maintained in culture for 2 weeks and the medium containing particles (possibly released by lysis) was collected every third day and stored at 4°C. Pooled media were clarified by centrifugation for 30 min at 15 000 r.p.m. in a Sorvall SS34 rotor and the core particles were concentrated from the supernatant by pelleting in a 45TI rotor (Beckman) at 35 000 r.p.m. for 4 h. Pellets were redissolved in 2 ml TNE and loaded onto a CsCl step gradient in an SW40 rotor, as described by Junker *et al.* (1987). After 36 h at 36 000 r.p.m., 28 fractions of 450 μl were collected from the bottom of the tube and 10 μl of each fraction was assayed for HBcAg using an enzyme-linked immunosorbent assay as recently described (Birnbaum and Nassal, 1990). The amount of HBV-specific DNA was determined in 50 μl aliquots by dot blot analysis using a ^{32}P-labelled HBV DNA probe. Radioactivity in each dot was determined by liquid scintillation counting and quantified relative to a dilution series of plasmid pCH3097; from this, the 100% value in Figure 4 (fraction 10) corresponds to 4580 c.p.m. ^{32}P or 10 fmol of HBV DNA. Determination of the density was done by weighing 100 μl of each fraction. To detect the P protein, core particles contained in 400 μl of each fraction were denatured and the P protein was isolated by immunoprecipitation. Following *in vitro* phosphorylation P proteins were reprecipitated and analysed by SDS–PAGE.

To quantify the amount of radiolabelled P protein, bands were cut out of the gel and the radioactivity that was contained herein was measured by liquid scintillation.

RNase protection analysis

RNase protection analyses were done as described recently (Bartenschlager *et al.*, 1990). The RNA probe used to detect HBV-specific RNAs (Figure 5B) was generated from plasmid pTH540a after cleavage with *Rsa*I (Junker-Niepmann *et al.*, 1990); it comprises sequences from nucleotides 79–533. Detection of *lacZ*-specific RNAs (Figure 6B) was done with an RNA probe complementary to sequences from *lacZ* nucleotides 1506–1948 using the *Rsa*I-cut plasmid pMG-1 (Junker-Niepmann *et al.*, 1990).

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