Expression of the P-protein of the human hepatitis B virus in a vaccinia virus system and detection of the nucleocapsid-associated P-gene product by radiolabelling at newly introduced phosphorylation sites*

Ralf Bartenschlager[§], Christa Kuhn and Heinz Schaller⁺

Zentrum für Molekulare Biologie Heidelberg, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, FRG

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ABSTRACT

Hepatitis B virus (HBV) contains a particle-associated DNA polymerase/reverse transcriptase activity encoded by the P (*pol*) open reading frame. Due to its low abundance, the corresponding protein has so far escaped direct detection and structural analysis. As a first step to overcome these difficulties, a series of recombinant vaccinia viruses was constructed and used for the synthesis in human hepatoma cells of both the authentic full length protein and of its functional domains. Pulse chase experiments demonstrated that the P-proteins had very short half lives in striking contrast to the viral core protein expressed in parallel with the same system. No evidence was obtained for a specific proteolytic processing of the P-protein as occurring with retroviral *pol* gene products. Overexpression of P-protein by recombinant vaccinia viruses was then employed to develop a highly sensitive detection method based on the *in vitro* phosphorylation of newly introduced target sites for protein kinase A. The usefulness of this method was demonstrated in the analysis of encapsidated P-gene products that were transiently expressed from an appropriately modified HBV genome. The results obtained indicate that the P-protein acts unprocessed, at least during the initial steps of nucleocapsid assembly and reverse transcription, and that a fraction of the P-protein molecules is linked as such to the viral DNA. Direct detection of the hepadnaviral P-protein by *in vitro* phosphorylation should greatly facilitate future analyses on P-protein structure and function.

INTRODUCTION

The hepatitis B viruses (hepadnaviruses), with the human hepatitis B virus (HBV) as their prototype member, are a group of small enveloped DNA viruses that are characterized by a distinct liver

tropism and a narrow host range. Despite containing an encapsidated DNA genome they are classified as viral retroelements since the central step in their replication cycle is the reverse transcription of an RNA pregenome (1). This reaction takes place within the nucleocapsid and is presumably primed by the terminal protein which is covalently attached to the 5' end of the viral minus strand DNA. The enzymatic activities required for initiation of reverse transcription, synthesis of DNA minus and plus strand and removal of the RNA template are encoded in the viral P (*pol*) gene (for a recent review see ref. 2). Genetic analysis has demonstrated that the P-protein, which has a calculated molecular weight of 93.2 kilodalton (kDa), consists of several functional domains, arranged in the order (from the amino- to the carboxyterminus): terminal protein (TP, the putative primer of minus strand DNA synthesis), a non-essential spacer region which can be deleted without affecting enzymatic activities (3, 4), reverse transcriptase/DNA polymerase, and RNase H (4, 5; Fig. 1). In addition, it was recently shown that the P-gene product is essential as a structural component for the efficient packaging of the RNA pregenome into nucleocapsids (6, 7). Use of standard methods that directly detect P-protein(s) have been hindered by its apparently low abundance in infected cells and within virus particles. Thus, detailed genetic analyses of P-protein structure and function so far relied exclusively on indirect methods: the RNA packaging function (6, 7), its coprecipitation with the DNA minus strand (3, 8), and reverse transcriptase/DNA polymerase and RNase H activities (4, 5, 9, 10). Consequently, its biochemical and structural properties remain largely unknown.

As a first step towards the direct characterization of the hepadnaviral P-protein, we used recombinant vaccinia viruses to synthesize the full length protein as well as P-protein fragments that correspond to the different functional domains and to determine their half lives and subcellular localization. Furthermore, by using *in vitro* phosphorylation of slightly modified P-proteins with protein kinase A (PKA), we were able to develop a new detection method sensitive enough to directly demonstrate the nucleocapsid-associated HBV P-gene product.

⁺ To whom correspondence should be addressed

[§] Present address: F. Hoffmann-La Roche Ltd., Pharmaceutical Research-New Technologies, 4002 Basel, Switzerland

* This is to highlight *in vitro* phosphorylation with protein kinase A: a method to directly detect proteins of low abundance

MATERIALS AND METHODS

Plasmid constructions and production of recombinant vaccinia viruses

For construction of plasmid p11pol (Fig. 1) a KpnI/SacI fragment, which contained the whole P-gene from HBV-2, subtype ayw, (position 407–2902), was excised from plasmid pMTpol (4) and inserted into pATA-18 (11, 12) via KpnI/BamHI (blunt ended). Plasmid p11H1 was derived from plasmid pMTpol by introducing a frameshift into the P-gene open reading frame at position 1280 which led to a stop after 14 further codons in the –1 open reading frame. Finally, a DNA fragment corresponding to HBV position 407–1409 (KpnI/XhoI [blunt ended]) was introduced into pATA-18 using the KpnI/BamHI (blunt ended) restriction sites in the polylinker. To obtain plasmid p11H2 a construct derived from plasmid p11pol was made in which the ATG initiation codon was fused in frame to the P-gene at HBV position 1283. For construction of plasmid p11HBc a HindIII (blunt ended)/BamHI fragment containing HBV sequences from position 3097–1004 was excised from plasmid pMH3/3097 (13) and introduced into the EcoRI (blunt ended)/BamHI cut vector pATA-18. P-gene mutants containing the PKA target sequence (RRXSX; 14) were constructed by site directed mutagenesis (15) using an HBV full length genome cloned into the M13 vector mp18 (4).

The oligonucleotides used were as follows (mismatches to the wildtype sequence are underlined): CCGACCACGCGGCACC for mutant P12, CAAGGTATGTTCCGCGTTTGTC for mutant P17 and CGGAGACTATCGTTGTTAGAC for mutant P18. After confirming the nucleotide exchanges by sequence analysis these were transferred into plasmid p11pol using the following DNA fragments: MroI/XhoI (NT 429–1409) in case of mutant P18, XhoI/SpeI (NT 1409–1961) in case of mutant P17, and NsiI/SacI (NT 2346 up to the polylinker, downstream of NT 88) in case of mutant P12.

Plasmid p11Δ1 was obtained by exchanging the MroI/RsrII (NT 429–2850) fragment from construct p11 pol by the analogous fragment derived from plasmid HBVΔ1 (4). Production and selection of recombinant vaccinia viruses were done as described previously (11, 17, 18) using the wild type vaccinia virus strain WR. Recombinants were plaque purified and amplified by infection of Hela cells. Titers were determined by infection of HU TK⁻ 143 cells (19).

Plasmid pCH3/3097 was derived from plasmid pMH3/3097 (13) by exchanging the human metallothioneine promoter against the CMV IE promoter contained in plasmid pCH3/3142 (16) using the PstI and HindIII restriction sites flanking the promoter sequences. For construction of mutant P12, an EcoRI/RsrII fragment (NT 1280–2850) was transferred from the corresponding M13 clone (see above) to plasmid pCH3/3097 (cf. Fig. 6A). Mutant P12-P2 was obtained by transferring a DNA fragment (EcoRI/RsrII, NT 1280–2850) from missense mutant P2 (4) into the basic construct P12.

Generation of P-protein specific anti-peptide antisera

Chemically synthesized peptides corresponding to the P-protein amino acid sequence 34–51 (peptide P6) or 813–832 (peptide P8) were coupled to keyhole limpet hemocyanine with glutaraldehyde (22). Production of antisera was done by four injections using 200–300 µg of coupled peptide per injection. The antibody titres against the peptides, measured by enzyme-linked immunosorbent assay as described elsewhere (23), were > 1: 64000 for anti-P6 and > 1: 256000 for anti-P8.

Infection of HepG2 cells and isolation of HBV-specific proteins

HepG2 cells (20, grown in Dulbecco modified minimal essential medium containing 10% fetal calf serum (FCS) at 5% CO₂) were incubated with recombinant or wild type vaccinia viruses at a multiplicity of infection of 10 in FCS free medium for one hour at 37°C. After removal of the inoculum, fresh medium containing FCS was added and cells were incubated for 16 hours. Isolation of proteins from infected cells by immunoprecipitation is described in ref. 18. Briefly, cells were lysed and, after centrifugation of the lysate, proteins contained in the pellet (the insoluble fraction) were dissolved by sonication in the presence of protein sample buffer (200 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2% SDS, 1% 2-Mercaptoethanol (2-ME), 10% sucrose and 0.1% Bromophenol blue). Proteins contained in the supernatant of the cell lysate (the soluble fraction) were T-CA-precipitated as described elsewhere (21) and resolved in protein sample buffer. After boiling, samples were diluted to RIPA buffer (phosphate buffered saline (PBS), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) by adding 20 volumes of RIPA buffer without SDS containing 2 mM Phenylmethylsulfonyl-fluoride, 2 kallikrein inhibitor units Trasylol (Bayer Leverkusen, FRG). Finally, 15 µl of packed protein A sepharose, containing preadsorbed immunoglobulin (from 5–10 µl antiserum), were added and the samples were incubated overnight at 4°C. After several washings with RIPA buffer, samples were either directly loaded onto a protein gel or used for ³²P-labelling with PKA (see below). For competition experiments (e.g. Fig. 3), 10 mg of the corresponding peptide were added to the preadsorbed immunoglobulin 2 hours before the addition of the protein samples. For sedimentation of HBcAg, the supernatant of the cell lysate was loaded on a 200 µl 20% sucrose cushion in a Beckman TLA 100.2 rotor and centrifuged for 60 minutes at 280000g in a TL 100 ultracentrifuge. Finally, the pellet was boiled in protein sample buffer and analyzed by SDS-PAGE. ³⁵S methionine labelling and pulse chase experiments were done as described in ref. 18.

Figure 1. Schematic representation of the basic recombinant vaccinia viruses used for the synthesis of the HBV P (pol) and C (core) proteins. Open reading frames of HBV are shown as stippled bars, HBV specific sequences as solid lines, the 11K promoter as heavy arrow and flanking sequences of the thymidine kinase gene (tk) as dotted lines. HBV nucleotide numbers are given according to the nomenclature of Pasek et al. (32), beginning with the A residue of the C gene initiation codon. TP, terminal protein; sp, spacer region; RT, reverse transcriptase.

Radioactive labelling of P-proteins with PKA and determination of the specific activities

For *in vitro* phosphorylation of P-proteins, immunoprecipitates (see above) were washed three times with RIPA buffer and two times with PKA-buffer (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM MgCl₂). After adding 30 μ l of PKA-buffer, supplemented with 1 mM Dithiothreitol, 20 μ Ci [γ -³²P]ATP and 4 units PKA (catalytic subunit, from bovine heart; Sigma Chemie, München, FRG), samples were incubated for 15 minutes at 30°C, followed by the addition of 4 units of the enzyme and further incubation for 15 minutes. After washing the immunocomplex four times with PBS, 40 ml of protein sample buffer was added. Samples were boiled for 5 minutes, diluted to RIPA buffer, and subjected to immunoprecipitations with different antisera as described above. To determine the specific activities of the variant P-proteins, radioactivities contained in the 90 kDa proteins shown in Fig. 5 were measured by liquid scintillation counting followed by normalization for the input protein as estimated by Western blot analysis and comparison with a dilution series of fusion protein FP492 (Fig. 2A).

Transfection of Huh7 cells and detection of the nucleocapsid-associated P-protein

Transfection of Huh7 cells (25), a human hepatoma cell line which at the time of this analysis gave higher signals in transient

expression than HepG2 cells, was done as described previously for HepG2 cells (4). To detect the nucleocapsid-associated P-protein, cells were lysed 4 days post transfection and core particles were isolated by immunoprecipitation (1. IP) using an antiserum directed against a recombinant HBV core protein (24). Following denaturation of the washed immunocomplex by boiling in TNE containing 2% SDS and 1% 2-ME, P-proteins were isolated, after dilution to RIPA buffer, by immunoprecipitation using a mixture of anti-P6 and anti-P8 antisera (2. IP). Finally, immunoprecipitates were subjected to *in vitro* phosphorylation as described followed by reprecipitation with the anti-peptide antisera. For treatment with micrococcal nuclease (Boehringer Mannheim, Mannheim, FRG) immunocomplexes were washed three times with RIPA buffer and two times with incubation buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM CaCl₂). After the addition of each 5 μ g tRNA and calf-thymus DNA, samples were incubated with 15 U of the enzyme for 20 minutes at 37°C. After quantitative removal of the liquid phase, immunocomplexes were boiled in protein sample buffer and directly analyzed by SDS-PAGE. To determine the transfection efficiency, proteins contained in the supernatant of the 2. IP were concentrated by TCA precipitation and quantitated by Western blot analysis using ¹²⁵I-labelled protein A.

RESULTS

Construction of recombinant vaccinia viruses

The basic constructs used in this study all contain the strong 11K vaccinia virus (vv) late promoter (11, Fig. 1). Recombinant v11pol contains the whole P-gene (codon 1–832) starting with the first ATG codon at position 407. Recombinant v11H1 allows

Figure 2. Detection of HBV specific P-proteins in HepG2 cells, infected with P-gene recombinant vv. (A) Schematic representation of the HBV P-gene showing the position of the deletion present in recombinant v11Δ1 as well as the positions of peptides P6 and P8, and of fusionprotein FP492 used to generate P-protein specific antisera. (B) Detection of ³⁵S-methionine labelled HBV P-proteins from HepG2 cells infected with wild type vaccinia virus or with the recombinants v11pol (lane 2) or v11Δ1 (lane 3). Proteins were immunoprecipitated using anti-FP492 antiserum. (C) Characterization of the P-proteins using anti-peptide antisera anti-P6 or anti-P8. Proteins initially isolated as described in B (lanes 1 and 5) were reprecipitated using the antisera indicated (lanes 2 to 4, 6 to 8). Numbers between (B) and (C) refer to the size of marker proteins in kilodaltons.

Figure 3. Detection of HBV P and C gene products after metabolic labelling of HepG2 cells infected with the various recombinants indicated or wild type (wt) vaccinia virus. Cells were labelled for one hour at 16 hours post infection with ³⁵S-methionine. Proteins isolated as described in Materials and Methods were subjected to immunoprecipitations using either anti-P6 or anti-P8 antisera in the presence (+) or absence (-) of the competing homologous peptide. In the case of the core gene-recombinant, only one half of the lysate was used for the immunoprecipitation (IP, lane 9) while the other half was subjected to an ultracentrifugation and the dissolved pellet was analyzed in parallel (UC, lane 10). HBV specific bands are marked with an arrow. Numbers refer to the size of marker proteins in kilodaltons.

the synthesis of the terminal protein (TP) including the spacer region (amino acid 1–295 plus 14 aminoacids in the –1 frame, see Materials and Methods), while recombinant v11H2 directs the expression of the reverse transcriptase/RNase H domain(s) (aminoacid 295–832). Synthesis of the HBV core protein was obtained with recombinant v11HBc containing the whole C gene (codon 1–183). All HBV sequences were introduced into a vv-wild type using homologous recombination, mediated by the flanking sequences of the thymidine kinase gene (26). Infections were carried out with HepG2 cells, a well differentiated human hepatoma cell line (20) which allows the synthesis of infectious HBV virions upon transfection with cloned viral DNA (27).

Synthesis of HBV P-gene product in HepG2 cells

For the synthesis of the full length P-protein, recombinant v11pol was used to infect HepG2 cells as described previously (18, 28). 16 hours after infection, cells were labelled with ^{35}S methionine for 60 minutes, lysed and insoluble material and nuclei were removed by centrifugation. To reduce the background of labelled vv proteins adsorbing to protein A, proteins were denatured as described in Materials and Methods and subjected to immunoprecipitation using an antiserum directed against a fusionprotein containing aminoacid 30–201 of the HBV P-protein (anti-FP492, Fig. 2A; ref. 24). Finally, proteins were separated by electrophoresis through a 10% polyacrylamide gel and detected

by autoradiography. As shown in Fig. 2B lane 2, a protein could be detected with an apparent molecular weight of about 90 kDa which was not present in lysates of cells infected with a vv-wild type (lane 1). [Proteins present in lane 1 and all other lanes were vv-encoded late polypeptides present as contaminants in the immunoprecipitates and are difficult to remove by washing, despite denaturation, due to their high affinity for protein A (12)]. To ascertain whether the 90 kDa protein was the HBV P-protein, HepG2 cells were also infected with a recombinant bearing a deletion of 270 nucleotides in the non-essential spacer region (recombinant v11 Δ 1, Fig. 2A, and ref. 4), and analyzed as described above. As expected, an immunoreactive protein could be precipitated which displayed the reduced molecular weight of about 80 kDa (Fig 2B, lane 3).

Finally, the HBV P-proteins isolated by the first immunoprecipitation with anti-FP492 were subjected to a reprecipitation with anti-peptide antisera specific for the amino- or carboxyterminal region of the P-protein. As shown in Fig. 2C, both the full length protein and the shortened protein from recombinant v11 Δ 1 were recognized by both antisera with high efficiency (compare lanes 1 and 5 with lanes 2–4 and 6–8). These results clearly demonstrate that the major P-gene products detected in the recombinant vv-infected HepG2 cells were full length proteins whose sizes were in good agreement with the predicted molecular weights. We noted that the pattern

Figure 4. Determination of the half lives of the P and C gene products. 16 hours post infection cells were labelled for 20 minutes with ^{35}S methionine and harvested at different time points after replacement with non-radioactive medium (chase). Finally, proteins contained in the soluble and the insoluble fractions (see Materials and Methods) were immunoprecipitated with the anti-FP492 antiserum (cf. Fig. 2) or the anti-HBc antiserum and analyzed by gel electrophoresis and autoradiography. The results obtained with the P-gene recombinant vv and the core gene recombinant vv are shown in (A) and (C), respectively. A quantitative evaluation of the half lives of the P and core protein is shown in (B) and (D), respectively. The radioactivities in the HBV-specific proteins were quantitated, normalized relative to the values obtained at time zero, and plotted semi-logarithmically against the chase time. Note the different time scales in A and B compared to C and D (minutes versus hours). A summary of the half lives for all C and P-gene products is given in Table 1.

of immunoprecipitated proteins differed, depending on which antiserum was used, with respect to the presence of minor protein species. In particular, immunoprecipitations with the antiserum directed against carboxyterminal sequences of the P-protein, detected only full length products (Fig. 2C, lanes 4 and 8) while a series of shortened proteins was detected in immunoprecipitations with antisera recognizing aminoterminal sequences of the P-protein (lanes 1–3, 5–7). Therefore, these shorter, minor proteins represent carboxyterminally truncated P-proteins.

To correlate the structural and biochemical properties of the full length P-protein with those of its various functional domains and the core protein, recombinants containing the appropriate HBV sequences under the control of the vv 11K promoter (Fig. 1) were analyzed as described above. As shown in Fig. 3, the expected HBV proteins could be detected as demonstrated by: (i) the appropriate molecular weights (lanes 1, 4, 6, and 9); (ii) their absence in vv wild type infected cells (lanes 3 and 8); (iii) their absence in the immunoprecipitates containing the homologous competing peptides (lanes 2, 5, and 7); and (iiii) the particulate structure of the core protein as determined by sedimentation through a sucrose cushion (lane 10) and by determination of its buoyant density of about 1.32 g/cm³ in CsCl density gradient (not shown).

Stabilities of the HBV P- and C-gene products

To assess the stabilities of the HBV-specific proteins, pulse chase experiments were performed. HepG2 cells were infected with the C-gene and P-gene recombinants and were labelled 16 hours later with ³⁵S methionine for 20 minutes. After replacement by medium containing non-radioactive methionine, cells were harvested at different time points by lysis in NP40 containing buffer. Proteins contained in the insoluble fraction were dissolved as described in Materials and Methods and subjected to immunoprecipitations in parallel to proteins from the soluble fractions. These were finally analyzed by SDS-PAGE and autoradiography. The results shown in Fig. 4A and B demonstrate that the full length P-protein contained in the soluble fraction decayed rapidly with an apparent half life of about 40 minutes. In contrast, a minor fraction present in the insoluble material (10–20% of the total P-protein) displayed a somewhat increased stability with an apparent half life of about 70 minutes (cf. Table 1). Similar half lives were obtained for the amino- and carboxyterminal P-protein segments in the soluble fractions. However, the amounts of P-protein present in the insoluble material were either much higher as in the case of the aminoterminal domain (up to 50% of the total P-protein) or below the detection limit as with the carboxyterminal domain (cf. Table 1).

Table 1. Determination of the half lives of the P- and C-gene products expressed from the recombinant vaccinia viruses.

recombinant ^a	$t_{1/2}$ (min.) ^b	
	soluble	insoluble
v11pol	40 ± 5	70 ± 5
v11H1	45 ± 5	170 ± 10
v11H2	35 ± 5	n.d.
v11HBc	> 1320	–

^a see Figure 1.

^b mean values from 2 different experiments analyzed as described in Fig. 4. Symbols: n.d., not detectable; –, not applicable, since amounts are increasing with time.

In contrast to the short half lives of the P-gene products, we could not detect a significant decay of the core protein over a chase of up to 22 hours (Fig. 4C and D). A slow decrease in the amount of core protein in the soluble fraction was paralleled by its increase in the insoluble fraction. Indirect immunofluorescence studies (not shown) strongly suggest that this is due to transport of the core protein to the nucleus, which is the major component of the insoluble fraction. Predominantly cytoplasmic staining was detectable in cells infected with the full length P-gene recombinant and only late in infection does the P-protein appear to accumulate at the nuclear periphery (not shown).

A new method for the sensitive detection of the HBV P-gene product: construction of phosphorylatable P-proteins

As mentioned in the introduction, studies about the hepadnaviral P-protein(s) so far have been hampered by the lack of a sensitive direct detection system. To overcome this problem, we used the recombinant vv-expressed P-protein as a model to develop a new system that allows radiolabelling of the P-protein to a high specific activity. This method, originally introduced for the direct detection of interferon proteins (29), uses the phosphorylation of proteins with PKA and [γ -³²P]ATP.

Figure 5. Detection of vv-expressed HBV P-protein after *in vitro* phosphorylation by PKA. (A) Schematic presentation of the P-gene showing the positions of the PKA target sequences introduced into the various P-gene recombinants as well as the target sequences for the anti-peptide antisera P6 and P8. (B) Detection of the radiolabelled P-proteins after gel electrophoresis and autoradiography. Proteins immunoprecipitated with the anti-FP492 antiserum (Fig. 2) were ³²P-labelled with PKA, reprecipitated with anti-peptide antisera, and separated by SDS-PAGE. Numbers besides the gel refer to the size of marker proteins in kilodaltons.

Since many proteins contain target sites for this enzyme which are not used efficiently, we introduced the consensus target sequence for the enzyme (RRXSX; 14) to achieve a high specific activity labelling of the P-protein. In addition, as efficient labelling of a protein depends on the accessibility of the target sequence for the enzyme, we introduced the sequence at various positions of the P-gene (Fig. 5A, Materials and Methods). The corresponding recombinants were then used for infection of HepG2 cells in parallel with the recombinant expressing the unaltered P-protein and proteins were isolated by immunoprecipitation with the anti-FP492 antiserum (cf. Fig. 2A) as described above. For a quantitative evaluation of the specific activities of the different P-proteins, immunoprecipitates were divided into two portions: One portion was used to determine by Western blot analysis the amounts of P-proteins precipitated relative to a standard dilution series of the FP492-fusion protein (not shown). The other portion (while still bound to protein A sepharose) was used for the *in vitro* phosphorylation of the P-proteins with PKA (see Materials and Methods). Finally the radiolabelled proteins were reprecipitated using two different anti-peptide antisera (Fig. 5A). As shown in Fig. 5B, all P-proteins tested were radioactively labelled by this procedure. However, the strength of the signal obtained differed drastically depending on both the presence of the consensus target sequence itself and the position of this sequence within the P-protein. Calculations of the specific activities show that the wild type P-protein was only poorly labelled (ca. 6×10^3 cpm per pmol protein). This value was increased by a factor of 50 when the PKA consensus target sequence was introduced into the aminoterminal of the P-protein (ca. 3×10^5 cpm per pmol P-protein from recombinant v11P18) or by a factor of 100 in case of the P-protein containing the PKA target sequence at the carboxyterminus (ca. 6×10^5 cpm per pmol P-protein from recombinant v11P12). However, the same target sequence was only poorly phosphorylated when introduced into the central part of the P-protein (ca. 4×10^4 cpm per pmol P-protein from recombinant v11P17) indicating that the sequence was not well accessible for the protein kinase at this position.

By combining the position-specific radiolabelling of the P-protein with immunoprecipitations by antisera recognizing terminal sequences of the P-protein, we observed different protein patterns. As exemplified for the labelled P-protein expressed from recombinant v11P18, a series of bands was observed when the antiserum directed against the aminoterminal P-protein sequence was used (Fig. 5B, lane 1). In contrast, when the antiserum raised against the last 18 amino acids of the P-protein was used, only the full length product was detectable (lane 2) showing that the fragments observed by immunoprecipitation with the anti-aminoterminal antiserum (lane 1) represent carboxyterminally truncated P-proteins. In addition, almost exclusively full length P-proteins were detected when the anti-carboxyterminus antiserum was used for the isolation of the P-protein from cells infected with recombinant v11P12 as well as for the reprecipitation after *in vitro* phosphorylation (not shown). Thus, the major P-protein detected in the recombinant vv-infected cells was the full length P-gene product and this protein was degraded preferentially from the carboxyterminus.

Detection of the nucleocapsid-associated HBV P-protein

To test whether the *in vitro* phosphorylation of altered P-proteins also allowed the detection of the much less abundant P-gene product present within the nucleocapsid, the carboxyterminally

located PKA consensus target sequence, P12, which allowed labelling of the P-protein to the highest specific activity, was transferred into an HBV genome which was transcribed under the control of the strong human cytomegalovirus immediate early promoter (Fig. 6A). This construct was transfected into Huh7 cells, a well differentiated human hepatoma cell line (25), and first analyzed for potential effects on the various P-protein functions caused by the introduction of the PKA target sequence. Using RNase protection assays and endogenous polymerase reactions as described (6) we found that mutant P12 was unaffected with respect to genomic RNA encapsidation, while the endogenous polymerase reaction was reduced about a factor of four.

As the introduced amino acid exchange did not severely affect the relevant P-protein functions, mutant P12 was used next to detect the particle-associated P-protein: nucleocapsids were isolated from transfected cells by immunoprecipitation, denatured by boiling in SDS containing buffer and P-proteins were isolated

Figure 6. Detection of the nucleocapsid-associated P-protein in transient expression. (A) Schematic representation of the basic construct used for transfection of Huh7 cells. A terminally redundant HBV genome is preceded by the CMV IE promoter (open arrow) and contains a polyadenylation signal (open diamond) near its 3'-end. (B) Detection of the encapsidated P-protein after *in vitro* phosphorylation with PKA. Phosphorylated P-proteins were reprecipitated and analyzed with (+) or without (-) digestion with micrococcal nuclease. Proteins were analyzed by electrophoresis through a 7% polyacrylamide gel and autoradiography. As size marker of identical sequence, an aliquot of the phosphorylated P-protein obtained from v11P12-infected cells was loaded in parallel (lane 7). Numbers beside the gel refer to the size of marker proteins in kDa. (C) Transfection efficiency was quantitated by Western blot analysis of core-protein as described in Materials and Methods.

by immunoprecipitation using a mixture of the anti-peptide antisera (cf. Fig. 5). Following *in vitro* phosphorylation with PKA and [γ - 32 P] ATP, P-proteins were reprecipitated with the same antiserum mixture and analyzed by SDS-PAGE. The result of this experiment is shown in Fig. 6B. Analysis of core particles obtained from wild type DNA transfected cells did not give any specific P-protein signal (lanes 1 and 2) in spite of high synthesis of genomic RNA (core gene/P-gene mRNA) as evidenced by the high amounts of core protein detected in Western blots (Fig 6C, lane 1). In contrast, when variant P12 DNA was transfected, we could clearly detect a 90 kDa protein (lane 3) which comigrated with the phosphorylated P-protein produced by recombinant v11P12 (lane 7) and which could be competed in an immunoprecipitation containing the homologous peptides (not shown). In addition to this major 90 kDa P-protein, we consistently observed several P-specific proteins of slower electrophoretic mobilities. These proteins most likely represent P-gene products covalently bound to the viral nucleic acid since they could be shifted to the position of the 90 kDa protein after treatment with micrococcal nuclease (lane 4). The same result was obtained after treatment with DNaseI whereas treatment with RNaseA did not change the pattern (not shown) strongly suggesting that the slowly migrating P-protein species represent P-protein molecules that are covalently linked to newly synthesized viral DNA. To further substantiate this assumption, a previously described P-gene mutant (P2; ref. 4), in which the exchange of a highly conserved aminoacid in the reverse transcriptase domain eliminates the enzymatic activity of the protein, was combined with phosphorylation variant P12 (mutant P12-P2, see Materials and Methods), and this construct was analyzed in parallel. As shown in lanes 5 and 6, no additional signal besides the major band at about 90 kDa was detected suggesting that the DNA-linked P-proteins observed with construct P12 correlated with DNA replication. Therefore, we conclude that at least during the initial steps of replication the hepadnaviral P-gene product acts as an unprocessed molecule. In addition, these observations confirm the notion that the P-protein is covalently linked (presumably after serving as a primer for reverse transcription) as a full length protein to the growing DNA minus strand (3, 8). On the other hand, a large fraction of the P-protein detected was found to be not covalently linked to DNA. This raises the question whether these molecules come from core particles not yet engaged in replication, or whether more than one molecule is present in the replication competent particle.

DISCUSSION

In this study we demonstrate synthesis of both the authentic HBV P-protein and fragments thereof corresponding to its different functional domains in a human hepatoma cell line. We were able to obtain P-proteins in amounts easily detectable, but the overall expression was low. As estimated from Western blot analysis, only about 10^4 full length protein molecules per cell were present 16 hours post infection, and this number could not be increased significantly by prolonged incubation. In contrast, at the same time about 10^6 core protein molecules per cell were detected in cells infected with recombinant virus expressing the HBV core gene. This difference reflects the unusually short half life of the free P-protein which, in turn, ensures (together with the low frequency of P-gene translation initiation from the

genomic RNA and P-protein packaging in cis) that there is very little free P-protein in the HBV-infected cell. Furthermore, as the protein is degraded preferentially from the carboxyterminus, this will rapidly inactivate its enzymatic activities and thereby prevent the risk of promiscuous reverse transcription of cellular RNAs. Finally, carboxyterminal degradation is expected to rapidly inactivate P-protein cytotoxicity and to produce aminoterminal P-protein fragments shown to interfere with the cellular response to interferons (30).

Although the P-protein yields obtained were relatively low, the amounts available were sufficient to attempt characterization of the DNA polymerase/reverse transcriptase and RNaseH activities residing in the protein. However, despite numerous attempts with the full size protein or with the fragment containing the catalytic domains (produced by recombinant v11H2, see Fig. 1), we were not able to detect any of these activities neither by measurement of incorporation of radioactivity into DNA substrates in solution nor in activity gel analysis as described by others (9, 10; 33).

Using the *in vitro* phosphorylation of accordingly modified P-proteins, we were able to establish a highly sensitive, direct detection method. The advantages of this technique are several: It allows (1) the radiolabelling of P-proteins to high specific activities, (2) labelling at defined positions in the polypeptide chain, and (3) a significant reduction of the background since coprecipitated non-phosphorylatable proteins will not be detected (this explains why the late vv-proteins, always present as contaminants in the immunoprecipitations of 35 S labelled proteins (cf. Fig. 2 and 3), are not detectable as 32 P labelled species in Fig. 5B). Thus, as little as 5 picogramms of P-protein (i.e. ca. 0.5 fmol, corresponding to about 50 cpm in case of the terminally labelled P-proteins) can still be detected with high fidelity; this increases the sensitivity over the currently available standard methods for direct protein detection by at least two orders of magnitude. Potential disadvantages of our method should not be ignored: the introduction of the consensus target sequence for PKA may affect enzymatic functions, and quantitations may be difficult for proteins for which no reference exists, e.g. protein species produced by post-translational modifications affecting, directly or indirectly, the target sequence for phosphorylation.

As shown previously for α -interferon (29) and here for the HBV P-protein, introduction of phosphorylation sites provides a relatively simple and sensitive method for detecting protein molecules of low abundance. Our results demonstrate furthermore that the efficiency of labelling depends on the accessibility of the target sequence for PKA and that the terminal regions of the target protein are labelled preferentially. Considering the formation of replication competent nucleocapsids, the modifications introduced into these regions had only minor effects on the biological activity of the P-protein. Therefore, it appears feasible to further improve the sensitivity of the method by introducing multiple target sites at different positions in the protein sequence and thereby also the probability to detect unexpected cleavage products. Finally, it should be pointed out that the use of our method for analyzing the fate of the HBV P-protein during the viral replication cycle is presently limited to transfection experiments which only allow to study the steps leading from cloned viral DNA genomes to infectious virions (27). As long as there is no tissue culture system that allows propagation of mutated genomes and infection experiments, animal virus models will provide an alternative assay for phosphorylatable P-proteins able to mediate the hepadnaviral

replication cycle. In any case, *in vitro* phosphorylation of the hepadnaviral P-protein should provide a powerful tool to characterize its structure and function.

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