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Hepadnavirus P Protein Utilizes a Tyrosine Residue in the TP Domain To Prime Reverse Transcription

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Hepadnavirus DNA minus strands are covalently linked at their 5′ terminus to the viral P gene product, which has been taken to indicate that the hepadnaviral polymerase polypeptide itself also functions as a protein primer for initiating reverse transcription of the RNA pregenome. The present study confirms this indication by identifying the nucleotide-linked amino acid in the P protein sequence of the duck hepatitis B virus (DHBV). In a first set of experiments, mutational analysis of three phylogenetically conserved tyrosine residues in the DNA terminal (TP) domain indicated that of these, only tyrosine 96 was essential for both viral DNA synthesis in transfected cells and priming of DNA synthesis in a cell-free system. This assignment was confirmed by direct biochemical analysis: tryptic peptides from the DHBV P protein, ^{32}P labelled at the priming amino acid by the initiating dGTP and additionally labelled internally by [^{35}S]methionine, were isolated and analyzed in parallel to reference peptides synthesized chemically and ^{33}P labelled by a tyrosine kinase. Mobility in high-performance liquid chromatography, as well as the release in stepwise amino acid sequencing of phospholabel and of [^{35}S]methionine, identified the priming amino acid unequivocally as the tyrosine in the sequence $_{91}$KLSGLYQMK$_{99}$, which is located in the center of the TP domain. Conserved sequence motifs surrounding Tyr-96 allow the prediction of the priming tyrosine in other hepadnaviruses. Weak sequence similarity to picornavirus genome-linked polypeptides (VPgs) and similar gene organization suggest a common origin for the mechanisms that use protein priming to initiate synthesis of viral DNA genomes or RNA genomes from an RNA template.

Hepadnaviruses, or hepatitis B viruses, form a family of small, enveloped animal viruses that are characterized by a distinct liver tropism, by a narrow host range, and by containing a small, partially double-stranded DNA genome which replicates via reverse transcription of an RNA pregenome (6, 20). Hepadnaviruses are thus similar to the RNA-containing retroviruses and to retrotransposons in basic life cycle and genome organization. Their replication does, however, not require integration into the host genome, nor does its polymerase, the hepadnaviral P gene product, need proteolytic cleavage for maturation. Consequently, the P protein lacks integrase and protease domains; instead it carries, amino terminally and separated from the catalytic domains by a spacer region, an additional domain that is unique to hepadnaviruses (15, 16) (Fig. 1). Using a panel of domain-specific anti-P antisera, we have previously provided evidence for the duck hepatitis B virus (DHBV) that this P protein domain is covalently linked to the 5′ end of the viral DNA minus strand. This finding identified a long-known genome-linked protein, of unknown origin and of suspected primer function (7, 14), as virus encoded and led to the proposal that the P protein carried in itself with this DNA terminal protein domain (designated TP, in analogy to the DNA terminal proteins of adenoviruses and bacteriophage ϕ29; for a recent review, see reference 17) the primer for hepadnavirus reverse transcription (4). Further experimental evidence supporting this proposal was recently presented by Wang and Seeger, who demonstrated that P protein-primed reverse transcription could be initiated with DHBV P protein produced by cell-free translation (21).

The nature of the hepadnavirus TP-DNA linkage had already been characterized preliminarily on the basis of its alkali resistance as probably involving a phosphodiester bond to a tyrosine and as being located in a short tryptic peptide (4, 7, 14). We have now used these characteristics to define potentially priming tyrosine residues as targets for a genetic mapping of the primer site within the DHBV P protein. As functional assays, both the DNA polymerase activity associated with core particles produced in transfected cultured cells and the in vitro priming of DNA synthesis were employed. Additionally, in a direct biochemical approach, the priming amino acid was identified by amino acid sequencing of tryptic peptides produced from P protein radiolabelled by the priming nucleotide.

MATERIALS AND METHODS

Plasmids. For transient expression experiments, plasmid pOL16 (18) was used as the basic construct. Plasmids pOL16-P15, pOL16-P16, pOL16-P17, and pOL16-P18 were created by oligonucleotide-directed site-specific mutagenesis (25). Plasmids pOL16-P15, pOL16-P16, and pOL16-P17 bear A-to-T mutations at nucleotide positions 456, 678, and 711, respectively, leading to Y-to-F exchanges in the P open reading frame at amino acid positions 96, 170, and 181, respectively. Plasmid pOL16-P18 bears a G-to-T exchange at nucleotide position 1706, leading to a D-to-H exchange at amino acid position 513 (2). For in vitro experiments, plasmid pT7/AMV-pol16 was constructed from plasmid pMT-HBVpol (16) by removing the hepatitis B virus polymerase gene and inserting chemically synthesized cassettes specifying a T7 promoter element (19) and an untranslated alfalfa mosaic virus leader sequence (10) in front of a full-length DHBV type 16 (DHBV16) polymerase

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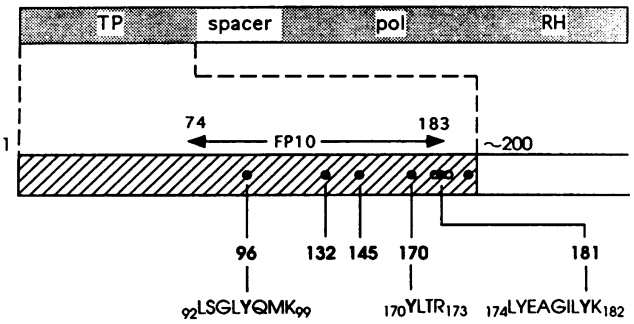

FIG. 1. Domain structure of the DHBV P protein and tryptic peptides containing candidate tyrosines for nucleotide linkage. The polymerase open reading frame encodes a protein of 786 amino acids starting with the terminal protein (TP) followed by a nonessential spacer, the DNA polymerase (pol), and the RNase H (RH) domain. FP10 delimits the amino acid sequence that specifies an antiserum recognizing DNA-linked proteolytic TP fragments (4). Tyrosine residues located in the TP region are shown by open and closed circles, the latter indicating residues conserved between hepadnaviruses. The amino acid sequences of tryptic peptides containing tyrosine residue 96, 170, or 181 are shown below.

gene. The deoxyoligonucleotides used for construction of the T7 promoter element were AGCTCTAATACGACTCACTA TAGGGAA and AGCTTTCCCTATAGTGAGTCGTAT TAG, and those used for construction of the alfalfa mosaic virus leader were AGCTTGTTTTTATTTTTAATTTTCTT TCAAATACTTCCAC and ATGGTGGAAAGTATTTGAA AGAAAATTAAAAATAAAAACA. The above-mentioned DHBV P gene mutations were transferred as restriction fragments into the expression plasmid.

The encapsidation-deficient helper construct pCD4 contains, under control of the cytomegalovirus immediate-early promoter, an overlength DHBV16 genome spanning positions 2579 to 3021 (2). All newly created sequences of the constructs described above were confirmed by sequence analysis.

Functional assays. RNase protection assays were essentially done as described elsewhere (11, 13). The antisense RNA probe used had a length of 419 nucleotides, consisting of a 400-nucleotide DHBV sequence and a 19-nucleotide nonhomologous linker sequence; it specifically hybridizes to the 5′ region of genomic DHBV RNA (Fig. 2A). Endogenous polymerase assays were performed as previously described (11). Quantitation of the labelled DNA product is based on the material excluded upon gel filtration on G100 (16). Transfection efficiency was determined by Western blot (immunoblot) analysis of DHBe antigen secreted into the medium.

Labelling and purification of reference peptides. Two synthetic peptides, LSGLYQMK and LYEAGILYK, representing products of total tryptic digestion of DHBV polymerase (Fig. 1), were used as references. Tyrosine residues within these peptides were labelled with recombinant *fyn* kinase (a kind gift from Geraldine Twonley and Sarah Courtneige, European Molecular Biology Laboratory, Heidelberg) by adding 1 μl of the enzyme preparation (10 mg/ml) to a solution of 25 μg of the peptide in 9 μl of labelling buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4), 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.3 mM ATP, and 10 μCi of [γ-^{33}P]ATP. After 5 min of incubation at 30°C, the reaction was stopped by adding 1 μl of 0.5M EDTA solution (pH 7.4).

The labelled reference peptides were purified by a unidirectional run on CEL-300 thin-layer chromatography plates (Macherey & Nagel) with a mixture of 785 ml of *n*-butanol, 607 ml of pyridine, 122 ml of glacial acetic acid, and 486 ml of H_2O as running buffer (5). Following separation, the radioactive peptide spots were scraped from the plate and eluted with running buffer. The organic solvents were removed by repeated drying of the samples under vacuum and dissolving the soluble material in water.

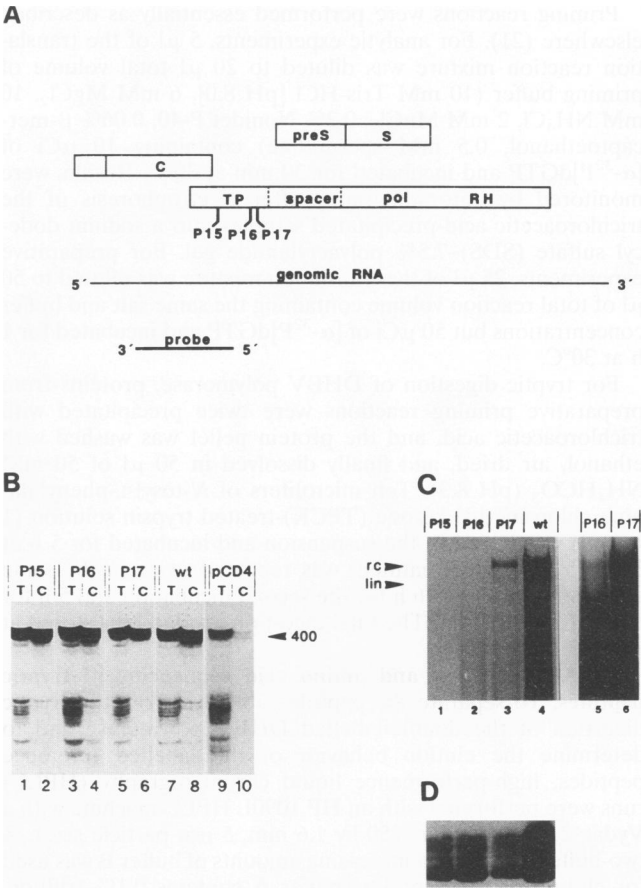

FIG. 2. Effects of targeted single-amino-acid exchanges in DHBV P protein on in vivo DNA synthesis activity and RNA encapsidation function. (A) Genomic RNA and proteins encoded by the overlength DHBV genome contained in the pOL16 construct. Positions of polymerase point mutations and the antisense RNA probe with a homology region to the RNA pregenome of 400 nucleotides are indicated. C, core; other components are labelled as in Fig. 1. (B) RNase protection assay of DHBV P gene mutants. Total (T) and encapsidated (C) RNAs were determined as described, with the encapsidation-deficient helper construct pCD4 as negative control (2). (C) DNA synthesis by DHBV polymerase in the endogenous polymerase assay. Lanes 1 to 3 show tyrosine mutants compared with the wild type (lane 4); lanes 5 and 6 are longer exposures of lanes 2 and 3). The positions reached after agarose gel electrophoresis by relaxed circular (rc) and linear (lin) forms of DHBV DNA are indicated. (D) Western blot analysis of DHBe antigen in the medium used to determine transfection efficiencies and to normalize the values obtained in the experiments shown in panel C.

Labelling and tryptic digestion of in vitro-translated DHBV polymerase. In vitro translation of DHBV polymerase was performed in a nuclease-treated reticulocyte-lysate according to the protocol of the supplier (Promega). RNA encoding DHBV P protein was transcribed in vitro according to standard protocols (19), in the absence or presence of [^{35}S]methionine, after linearization of in vitro expression plasmids with *Sac*I.

Priming reactions were performed essentially as described elsewhere (21). For analytic experiments, 5 μl of the translation reaction mixture was diluted to 20 μl total volume of priming buffer (10 mM Tris-HCl [pH 8.0], 6 mM $MgCl_2$, 10 mM NH_4Cl, 2 mM $MnCl_2$, 0.2% Nonidet P-40, 0.06% β-mercaptoethanol, 0.5 mM spermidine) containing 10 μCi of [α-^{32}P]dGTP and incubated for 30 min at 30°C. Results were monitored by autoradiography after electrophoresis of the trichloroacetic acid-precipitated samples into a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel. For preparative experiments, 25 μl of the translation mixture was diluted to 50 μl of total reaction volume containing the same salt and buffer concentrations but 50 μCi of [α-^{32}P]dGTP and incubated for 1 h at 30°C.

For tryptic digestion of DHBV polymerase, proteins from preparative priming reactions were twice precipitated with trichloroacetic acid, and the protein pellet was washed with ethanol, air dried, and finally dissolved in 50 μl of 50 mM NH_4HCO_3 (pH 8.3). Ten microliters of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin solution (1 mg/ml) was added to the suspension and incubated for 5 h at 37°C (5). Trypsin treatment was repeated twice in the same way with periods of 16 h for the second incubation and 5 h for the third incubation. The final digestion product was stored at −20°C.

HPLC separation and amino acid sequencing of tryptic peptides. To separate the peptides obtained from the tryptic digestion of the double-labelled DHBV polymerase and to determine the elution behavior of the labelled reference peptides, high-performance liquid chromatography (HPLC) runs were performed with an HP 1090L HPLC machine with a Vydac 218TP column (250 by 1.6 mm, 5 μm particle size). A two-buffer system with increasing amounts of buffer B was used for elution of the peptides (buffer A contains 0.1% trifluoracetic acid in H_2O, and buffer B contains 0.07% trifluoracetic acid in acetonitrile). The elution profile was monitored by liquid scintillation counting (in the case of the ^{33}P-labelled reference peptides) or by determination of Cerenkov counts (in the case of the tryptic cleavage products of DHBV P protein). The positions of the labelled amino acids within a tryptic peptide was determined by a method for automated peptide sequencers described in detail by Aebersold et al. (1). Released radioactivity was monitored by liquid scintillation counting; different energy channels were used to differentiate between ^{35}S and ^{32}P radioactivity.

RESULTS

Tyrosine 96 is essential for DNA synthesis. In previous work (4), we had observed that DNA-linked DHBV P protein fragments were preferentially immunoprecipitated by an antiserum raised against the TP amino acid sequence between positions 74 and 183 (anti-FP10; see Fig. 1). In addition, we had characterized and preliminarily localized the TP-DNA linkage on the basis of its alkali resistance as involving a phosphodiester bond to a tyrosine residue and on the basis of size analysis of peptide-linked DNA fragments as being located in a tryptic peptide displaying a mass equivalent to about 2.5 nucleotides on a DNA sequencing gel. These data suggested three tyrosine residues as preferential targets for a genetic mapping of the primer site. As outlined in Fig. 1, the TP domain of the DHBV P protein contains eight tyrosines, six of which are conserved between avian and mammalian hepadnaviruses. Of these, the ones most compatible with the above predictions (located at positions 96, 170, and 181 in the FP10 segment and in tryptic peptides of 8, 4, and 9 amino acids, respectively) were converted individually by site-directed mutagenesis into phenylalanines, and the mutant P genes were then subjected to functional analysis.

TABLE 1. Effects of P protein mutations on P protein functions[a]

Mutant	Amino acid change	% RNA encapsidation	% DNA priming	% DNA polymerase
P15	Y-96→F	>90	<1	<1
P16	Y-170→F	>90	≈75	≈10
P17	Y-181→F	>90	>90	>90
P18	D-513→H	>90	<1	<1

[a] Percentages, relative to the wild type, of encapsidated RNA genomes (Fig. 2B), of in vitro priming activity normalized to equal amounts of ^{35}S-labelled P protein (Fig. 3B), and of endogenous DNA polymerase activity normalized to equal core gene expression (Fig. 2C and D) (2).

For testing in transfected cultured cells, the Tyr→Phe mutations were transferred into plasmid pOL16, which contains an overlength DHBV genome directing the synthesis of replication-competent DHBV core particles (18). These were harvested from transfected HepG2 cells and analyzed for alterations in DNA synthesis or in RNA packaging in assays extensively used for characterizing P gene mutations. Figure 2C shows the results of an endogeneous polymerase assay, in which viral DNA genomes contained in the nucleocapsid are radiolabelled by the incorporation of deoxynucleotides and monitored by autoradiography after agarose gel electrophoresis (18). After normalizing the data to equal amounts of core protein (determined by Western blot; Fig. 2D) to correct for varying transfection efficiencies, the results were evaluated relative to those obtained with the wild-type construct. As shown in Table 1, mutant P17 (Y-181→F) showed no significant change; in contrast, a complete loss of activity was observed with mutant P15 (Y-96→F) and a reduction to about 10% was observed with mutant P16 (Y-170→F).

No such changes were detected with any of the three tyrosine mutants in an RNase protection assay (11) monitoring the amount of encapsidated pregenomic RNA relative to the total amount of viral RNA genomes produced in transfected cells (Fig. 2B and Table 1). This observation eliminates the possibility that the changes in activity observed in the endogenous polymerase reaction were caused by a reduced efficiency in packaging of RNA template into core particles. Thus, the genetic analysis presented above characterizes tyrosine 96 as being essential and tyrosine 170 as being important for a P protein function required for DNA polymerase activity in the replication-competent core particle.

Tyrosine 96 is essential for priming of DNA synthesis. Recently, a cell-free system in which priming of DHBV reverse transcription is detected by covalent binding of [α-^{32}P]deoxynucleotides to P protein molecules produced by in vitro translation has been described (21). This reaction thus provides a means to directly test for a role in the priming step of any one of the three tyrosine residues in the P protein we had mutated and tested before for their importance in core particle-associated P protein functions. For this purpose, DHBV fragments encoding the relevant Tyr→Phe sequence changes were transferred into plasmid pT7/AMV-pol16, an expression construct that allows efficient in vitro synthesis of the DHBV polymerase in a reticulocyte lysate from an RNA synthesized from a T7 promoter element (Fig. 3A). The DHBV P protein was synthesized in the presence of [^{35}S] methionine, and in the subsequent priming reaction it was ^{32}P labelled by incubation in a priming buffer containing

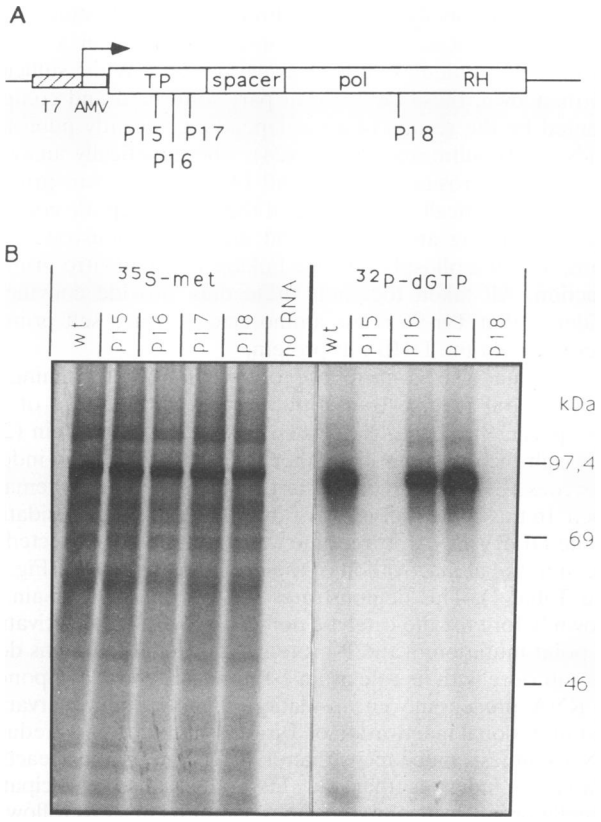

FIG. 3. Effects of tyrosine mutations in the DHBV P protein on the in vitro priming reaction. (A) Structural organization of promoter and leader elements in plasmid pT7/AMV-pol16 and the approximate localization of the mutations analyzed. Labelling is as in Fig. 1. (B) SDS-PAGE of DHBV P proteins radiolabelled by incorporation of [^{35}S]methionine during in vitro translation (left panel), or with [α-^{32}P]dGTP in the priming reaction (right panel).

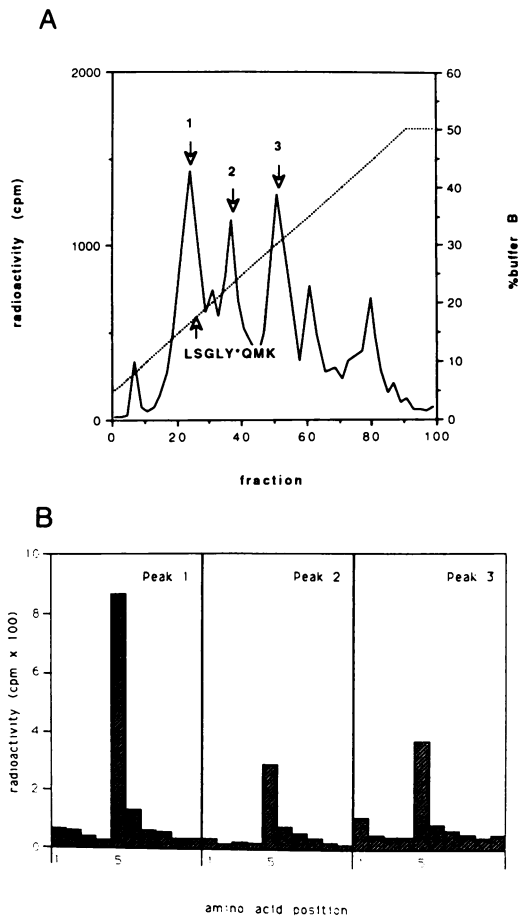

FIG. 4. HPLC separation and amino acid sequencing of tryptic cleavage products from DHBV polymerase primed by [α-^{32}P]dGTP. (A) Elution profile obtained by HPLC separation of ^{32}P-labelled tryptic peptides as determined by Cerenkov counting. The percentage of elution buffer B is indicated by the dotted line. The three major peaks of ^{32}P-labelled peptides that were further analyzed (marked 1 to 3) and the elution position of a tyrosine-phosphorylated reference peptide (see the text) are indicated by arrows. (B) Release of ^{32}P label during amino acid sequencing of the HPLC-purified peptides. Fractions 23 to 25 (peak 1), 37 (peak 2), and 50 to 52 (peak 3) were used.

[α-^{32}P]dGTP as the only nucleotide. As visualized with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, the ^{35}S-labelled 90-kDa P protein was produced in amounts comparable with those of the wild type, irrespective of any of the Tyr→Phe mutations (Fig. 3B, left part). In the priming reaction (Fig. 3B, right part), however, mutant P15 (Y-96→F) showed a complete loss of the capacity to accept the priming dGMP residue, as was observed with a negative control, mutant P18, in which the DNA polymerase function is inactivated by changing the conserved YMDD motif in the reverse transcriptase domain to YMHD (2, 8, 16). In contrast, only minor, if any, effects were observed with mutants P16 and P17. This result demonstrates that the Tyr residue located at position 96, in contrast to those at positions 170 and 181, is indispensable for the DHBV P protein to carry out the in vitro priming reaction. Furthermore, and in accord with data from others (21), the loss of priming activity by mutation P18 demonstrates that the priming reaction requires a functional DNA polymerase domain in the P protein molecule.

The priming dGMP is covalently linked to Tyr-96. In the cell-free system used as described above for a genetic analysis, priming of DNA synthesis allows the radiolabelling of the DHBV P protein at the primer site through covalent linkage to the initiating [α-^{32}P]deoxyguanosine nucleotide (21). To characterize this site in the polymerase polypeptide biochemically, the priming reaction was carried out on a preparative scale and the radiolabelled product was used to isolate and to sequence tryptic P protein fragments carrying the ^{32}P label. To facilitate the characterization of the proteolytic peptides, the P protein had been synthesized additionally in the presence of [^{35}S]methionine. The tryptic peptides generated were separated by HPLC (Fig. 4A) or, alternatively, by thin-layer chromatography. Several major ^{32}P-labelled peptides were obtained by either method. Of these, the species that was eluting first on HPLC or migrating fastest on thin-layer chromatography (not shown) closely resembled in these properties a reference octapeptide that had been chemically synthesized and ^{33}P labelled by in vitro phosphorylation with the *fyn* tyrosine kinase to represent the expected tryptic cleavage product containing Tyr-96; no correlation with a second phosphorylated reference nonapeptide (containing Tyr-181 and Tyr-175; see Fig. 1) was observed in the highly resolving HPLC (not shown). To determine the position of the [^{32}P]dGMP-linked amino acid, peak fractions from the first three HPLC peaks were subjected to stepwise chemical degradation progressing from the N

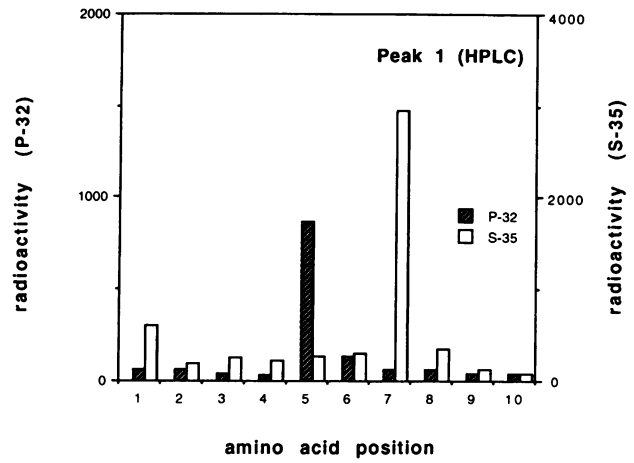

FIG. 5. Release of ^{32}P and ^{35}S radiolabel from a major tryptic cleavage product obtained from doubly radiolabelled DHBV polymerase (HPLC; Fig. 4A, peak 1). Radioactivities (shown in counts per minute) were differentiated by liquid scintillation counting.

terminus. A solid-phase method was employed to allow the isolation of the highly polar nucleotide-linked amino acid derivative also (1).

The result of such an analysis is presented in Fig. 4B. It shows that the three ^{32}P-labelled peptide species generated by tryptic cleavage each released its ^{32}P label together with amino acid 5. Furthermore, as shown in Fig. 5, the peptide from peak 1 released [^{35}S]methionine label at position 7. The same was found, although with lesser selectivity, with the peptide(s) from peak 2 (not shown), while no such preference was observed with those from peak 3, indicating that they were presumably contaminated by the many other tryptic peptides containing one or the other of the 16 methionines present in the DHBV P polypeptide. These data are interpreted as indicating that the ^{32}P-labelled tryptic peptides analyzed were all derived from the same amino acid sequence in the polymerase polypeptide, the two more slowly migrating species having been created by incomplete tryptic digestion. This interpretation is strongly supported by the fact that Tyr-96 is the only tyrosine occurring at position 5 in a tryptic peptide from the DHBV TP domain (another one occurs in the polymerase domain), while Met-98 is the only methionine at position 7 in any tryptic peptide that can be produced from the DHBV P protein sequence. Taken together, these data provide compelling evidence that Tyr-96 is indeed the site where the initiating nucleotide was linked to the P protein polypeptide in the in vitro priming reaction.

DISCUSSION

Previous work had indicated that hepadnavirus DNA minus strands were covalently linked at their 5′ terminus to the viral P gene product, suggesting that the hepadnaviral polymerase polypeptide functions also as a primer during the initiation of reverse transcription of the RNA pregenome. The analysis we now present confirms and extends this view by identifying the priming amino acid in the DHBV P protein as the tyrosine residue in position 96 in the center of the TP domain. The evidence for this assignment is severalfold. Functional analysis of Tyr→Phe mutations in the TP domain revealed that Tyr-96 was essential for DHBV DNA synthesis in transfected cells, while Tyr-170 and Tyr-181 were not. Essentially the same result was observed in the priming reaction in a cell-free system. Additionally and more directly, the nucleotide-linked tyrosine was located by amino acid sequencing in a unique position of a unique tryptic cleavage product. While sufficient on their own, these data are in part confirmed and complemented by the results of an independent, recently published study by Zhoulim and Seeger (24), who genetically analyzed the roles of tyrosines 96, 132, and 145 in the in vitro priming reaction, genetically characterized the tryptic peptide containing the priming amino acid, and directly demonstrated the formation of a phosphotyrosine linkage in the in vitro priming reaction. All taken together, these data provide convincing evidence that Tyr-96 is the amino acid at which self priming occurs within the DHBV P protein.

Our data also confirm the observation that priming of hepadnaviral reverse transcription can occur outside of the core particle in a cell-free system lacking capsid protein (21), although the question of whether the priming reaction indeed precedes RNA encapsidation in the infected cell still remains open. In this context, it is important to note that encapsidation of the DHBV RNA pregenome was not noticeably affected by the mutational inactivation of the priming amino acid (Fig. 2B and Table 1). This demonstrates for the priming domain, as shown before for the catalytic domains (3, 8), that inactivation by point mutation of the P protein's individual functions does not interfere with its role as an essential structural component in RNA pregenome encapsidation. Finally, the observation that mutational inactivation of Tyr-170 (mutation P16) reduces DNA synthesis much more than it affects the priming reaction (Table 1) indicates that the TP domain also participates, directly or indirectly, in viral DNA synthesis in steps following the priming reaction.

That the priming tyrosine is located in the center of the DHBV TP domain can most likely be extrapolated to the corresponding sequences in other hepadnaviruses of known nucleotide sequence. Significant sequence conservation around DHBV Tyr-96 (Fig. 6A), as well as throughout the TP domain (16), also allows the identification of the potentially priming tyrosine in the P proteins of other hepadnaviruses. As noted before for HBV and rhinovirus 14 (12) (highlighted by asterisks in Fig. 6A), these phylogenetically conserved sequences show a low but significant similarity to picornavirus RNA-linked polypeptides (VPgs), which are also connected by a phosphotyrosine linkage to the 5′ ends of their respective genomes. This hint for a common origin of protein-primed DNA synthesis and RNA synthesis from a viral RNA template is seconded by the occurrence of additional shared sequence motifs and by the basically similar functional organization of replication-related viral proteins. As outlined in Fig. 6B, the catalytic polymerase domain is preceded in both multidomain polypeptides, the hepadnaviral P protein and the unprocessed picornaviral P3 precursor, by sequences that participate in similar steps of genome replication. As best understood for poliovirus (for a recent review, see reference 23), the picornavirus 3AB protein, and not only the 3B peptide (subsequently released as VPg), appears to carry a primer function in picornaviral replication, while sequences from 3C participate in the recognition of a signal of high secondary structure near the 5′ end of the viral RNA template. The hepadnavirus TP domain apparently carries all the equivalent functions in that it most likely determines P protein binding to the highly structured packaging signal E near the 5′ end of the RNA pregenome (9, 11, 15), which it also uses as a signal for self-priming reverse transcription (22). It thus seems to be conceivable that the presently fundamentally differing replication apparatuses

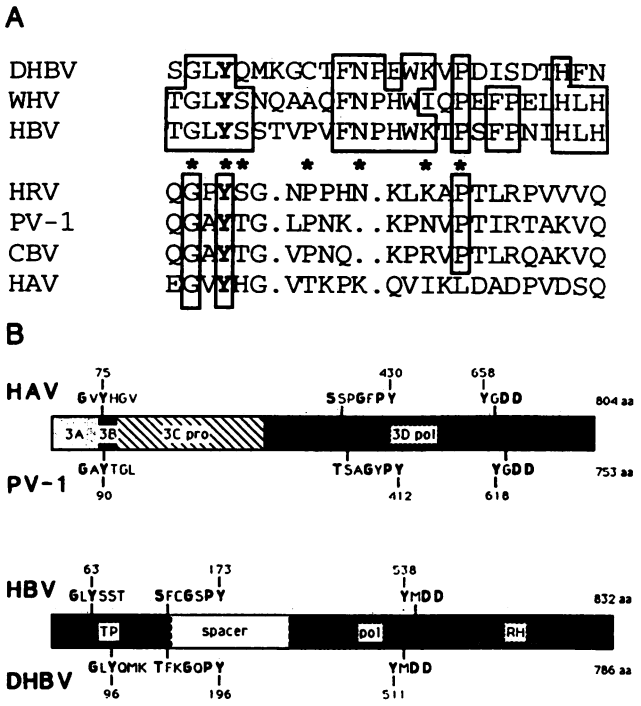

FIG. 6. Similarities in amino acid sequences and functional organization of replication-related gene products from hepadnaviruses and picornaviruses. (A) Amino acid sequences near the priming tyrosine from DHBV, woodchuck hepatitis B virus (WHV), and human hepatitis B virus (HBV). These are aligned to maximal similarity with the VPg amino acid sequences from human rhinovirus 14 (HRV), poliovirus type 1 (PV-1), coxsackie B virus (CBV), and hepatitis A virus (HAV). Amino acids identical between HBV and HRV (12) are marked by asterisks. (B) Comparison of domain organization of the picornavirus P3 precursor and the hepadnavirus P protein. Shared sequence motifs and their amino acid positions in the unprocessed proteins are presented for HBV and DHBV (labelling within the bar is as in Fig. 1) and for HAV and PV-1.

of hepadnaviruses and picornaviruses may have evolved from a primitive RNA replicase that used intramolecular protein priming for chain initiation.

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