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Proteaselike Sequence in Hepatitis B Virus Core Antigen Is Not Required for e Antigen Generation and May Not Be Part of an Aspartic Acid-Type Protease

MICHAEL NASSAL,* PETER R. GALLE, AND HEINZ SCHALLER

Zentrum für Molekulare Biologie Heidelberg, Universität Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, Federal Republic of Germany

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The hepatitis B virus (HBV) *C* gene directs the synthesis of two major gene products: HBV core antigen (HBcAg[p21c]), which forms the nucleocapsid, and HBV e antigen (HBeAg [p17e]), a secreted antigen that is produced by several processing events during its maturation. These proteins contain an amino acid sequence similar to the active-site residues of aspartic acid and retroviral proteases. On the basis of this sequence similarity, which is highly conserved among mammalian hepadnaviruses, a model has been put forward according to which processing to HBeAg is due to self-cleavage of p21c involving the proteaselike sequence. Using site-directed mutagenesis in conjunction with transient expression of HBV proteins in the human hepatoma cell line HepG2, we tested this hypothesis. Our results with HBV mutants in which one or two of the conserved amino acids have been replaced by others suggest strongly that processing to HBeAg does not depend on the presence of an intact proteaselike sequence in the core protein. Attempts to detect an influence of this sequence on the processing of HBV *P* gene products into enzymatically active viral polymerase also gave no conclusive evidence for the existence of an HBV protease. Mutations replacing the putatively essential aspartic acid showed little effect on polymerase activity. Additional substitution of the likewise conserved threonine residue by alanine, in contrast, almost abolished the activity of the polymerase. We conclude that an HBV protease, if it exists, is functionally different from aspartic acid and retroviral proteases.

Retroviral proteases are attracting increasing attention, both for their important role in the viral life cycle, in which they are apparently required for processing of the viral Gag and Gag-Pol precursor proteins (14), and as potential targets for antiviral therapeutics. Few of these proteases have been isolated and characterized (14, 17), but all of the retroviral genomes with known sequences encode a characteristic stretch of amino acids which closely resembles the active-site residues of aspartic proteases (26). Its most prominent feature is the presence of the extremely conserved sequence DTG preceded by two hydrophobic amino acid residues. In generic aspartic proteases, this D residue represents the catalytically essential amino acid which gave the entire family of proteases its name. A second conserved sequence motif exists 50 to 60 residues further toward the C terminus (26). Recent studies suggest that retroviral proteases are closely related to aspartic proteases (9, 30).

Hepadnaviruses, with human hepatitis B virus (HBV) as their prototype, are the causal agents of type B hepatitis (for a recent review, see reference 4). They are enveloped DNA viruses with a partially double-stranded, circular genome about 3 kilobases long. Despite their DNA genome, they share with retroviruses at least two important characteristics. (i) Replication proceeds via reverse transcription (31), and (ii) their genomes are arranged in the same typical order of *C-P-S* and *gag-pol-env*, respectively. The HBV *C* gene encodes capsid protein p21c, serologically defined as HBV core antigen (HBcAg), and in addition, a nonparticulate, secreted, and proteolytically processed form called HBV e antigen (18) (HBeAg [p17e]), whose biological role has not been established.

Data published over the last 2 years indicate that an intact pre-*C* region, a short in-phase open reading frame (ORF) preceding the *C* gene, is required for secretion and that the joint pre-*C*–*C* gene product (p25) is the precursor of HBeAg (5, 24, 29, 33, 34). Although N-terminal cleavage of this product is therefore most likely due to the signal peptidase located in the endoplasmic reticulum membrane, the way in which C-terminal cleavage takes place is completely unknown.

Recently, an amino acid sequence in the core proteins of human and other mammalian HBVs has been reported (19) which contains a conserved motif very similar to the proteaselike sequences in retroviruses, i.e., LLDTAS for most HBV subtypes. On the basis of this sequence similarity, a specific model was proposed according to which the HBV *C* gene product, HBeAg, would be generated by self-cleavage of HBcAg by the protease activity inherent to the core protein, implying HBcAg processing analogous to Gag processing in retroviruses.

We tested this hypothesis by using site-directed mutagenesis in conjunction with expression of the mutant genes in human hepatoma cells. The retroviral and hepadnaviral proteaselike sequences contain an invariant Asp residue which in aspartic proteases is essential for function and is apparently also required for the activity of retroviral proteases (12, 16, 21). Accordingly, we replaced this aspartic acid by Asn, His, or Tyr; in addition, the likewise conserved Thr residue following the putative catalytic aspartate was substituted by alanine. The effects of these mutations on HBeAg secretion were studied by transient expression of the mutant HBV genomes in HepG2 cells (11).

In a second series of experiments, we determined whether mutations in the proteaselike sequence would affect the synthesis of the HBV polymerase and its processing from a larger precursor. As an assay, we used the activity of the

* Corresponding author.

enzyme to repair the gap in the HBV genome contained in viral cores (endogenous polymerase reaction).

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH1 (6) was used for transformations. Plasmids pPLC4-1, pMH3091s2, and pMH3097s2 have been previously described (22). They contain a completely or partially synthetic HBV *C* gene. A *Bgl*II site at position 84 was introduced into pPLC4-1 by replacing the *Eco*RI [nucleotide (nt) 22]-to-*Pst*I (nt 141) fragment with the corresponding fragment from plasmid pLTC1-1 (22).

Mammalian cells. HepG2 cells (11) were grown in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum, 5 mM glutamine, and 100 μg of each of penicillin and streptomycin per ml.

Chemicals and enzymes. Diisopropylphosphoramidites for oligonucleotide synthesis were from Applied Biosystems (Foster City, Calif.). Reagents for polyacrylamide gel electrophoresis were obtained from Serva (Heidelberg, Federal Republic of Germany). [α-^{35}S]dATP (800 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]ATP (3,000 Ci/mmol), and ^{125}I-labeled protein A were from Amersham Buchler (Braunschweig, Federal Republic of Germany), and protein A-Sepharose and Sephadex G-100 were from Pharmacia (Freiburg, Federal Republic of Germany). All enzymes for recombinant DNA work were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) or New England BioLabs, Inc. (Schwalbach, Federal Republic of Germany) and used as recommended by the manufacturer.

Site-directed mutagenesis. Two oligodeoxynucleotides, GAT CTT TTA (A/C/T)AT (A/G)CC GCC TC and CGC GGA GGC GG(C/T) AT(A/G/T) TAA AA, constituting the upper and lower strands of a *Bgl*II (nt 84)-to-*Bss*HII (nt 104) restriction fragment, were synthesized on an Applied Biosystems 380A DNA synthesizer and purified as described before (23). They were annealed and ligated into plasmid pPLC4-1, which was modified to contain a *Bgl*II site at position 84 and had been digested partially with *Bgl*II and to completion with *Bss*HII. The presence of the desired mutations was verified by directly sequencing the double-stranded plasmids. For expression of HBcAg and HBeAg in HepG2 cells, the *Cla*I (nt 7)-to-*Hpa*I (nt 220) fragments harboring the mutations were transferred into pMH3/3091s2 and pMH3/3097s2 (22). Plasmids of the pMH-34/2922 series were obtained by three-factor ligation: the large *Xho*I-to-*Hin*dIII fragment of pMH-34/3091 (M. Junker-Niepmann and H. Schaller, unpublished data) carrying a 3′-terminally truncated metallothionein promoter, the small *Hin*dIII-to-*Taq*I fragment from plasmid pHTW/2922 (34), and the small *Cla*I = *Taq*I (nts 7 and 8 in HBV)-to-*Xho*I (nt 1409 in HBV) fragments from plasmid pMH3/3097 carrying the above mutations in the proteaselike sequence.

Transient expression of HBV gene products in HepG2 cells. Cells of about 50% confluency were transfected by the calcium phosphate procedure with 20 μg of CsCl gradient-purified plasmid DNA per 100-mm-diameter dish. In complementation experiments, both plasmids were used at this concentration. Proteins were harvested 3 days after transfection. HBV surface antigen, HBcAg, and HBeAg were determined by radioimmunoassay (RIA) with the Ausria and HBe-RIA from Abbott Laboratories (Wiesbaden, Federal Republic of Germany). Immunoprecipitation of *C* gene products was performed as previously described (7).

Western blotting (immunoblotting). Proteins were separated on 0.1% sodium dodecyl sulfate (SDS)–15 or 12.5% polyacrylamide gels as described by Laemmli (15). Western blotting was performed as previously described (22), with rabbit α-c/e antiserum.

Purification of core particles. Cleared lysates (1 ml) of HepG2 cells from a 100-mm-diameter dish, transfected with the appropriate plasmids, were loaded on sucrose step gradients consisting of 1 ml of 10% sucrose, 1 ml of 30% sucrose, and a cushion of 0.5 ml of 70% sucrose in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8). After centrifugation for 4 h at 4°C and 30,000 rpm in an SW60 rotor, fractions of 0.3 ml were collected from the bottom and HBcAg was detected by RIA. In all cases, the HBcAg peak was found in fraction 2, which, after the volume was made 1.5 ml with TNE, was subjected to isopycnic centrifugation in CsCl gradients consisting of 0.5 ml of CsCl at 1.2 g/ml, 1.0 ml at 1.25 g/ml, 1 ml at 1.35 g/ml, and 0.5 ml at 1.5 g/ml. After 17 h at 20°C at 35,000 RPM (SW60 rotor), the presence of HBcAg was again detected by RIA.

Endogenous polymerase reaction. Core particles were isolated by either immunoprecipitation or density gradient centrifugation and incubated with deoxynucleoside triphosphates (dNTPs) and ^{32}P-labeled dATP as previously described (7). After digestion of protein by proteinase K, the bulk of unincorporated dNTPs was removed by gel permeation through Sephadex G-100. Radioactively labeled nucleic acids were separated on 1.5% agarose gels containing 0.1% SDS and visualized by autoradiography.

RESULTS

Mutagenesis of the proteaselike sequence in HBV. We have recently prepared a completely synthetic HBV *C* gene which, when transferred into the cloned HBV genome and transfected into HepG2 cells, was shown to be competent for HBcAg and HBeAg synthesis (22). Since the activity or biosynthesis of the viral polymerase is greatly reduced in HBV genomes harboring a synthetic sequence in the *C-P* overlap region (22), constructs in which only the 5′-terminal half of the *C* gene is contributed by synthetic DNA were used in the present study. A short restriction fragment between the *Bgl*II (nt 84) and *Bss*HII (nt 104) restriction sites (Fig. 1) was exchanged for a chemically synthesized oligonucleotide duplex containing mixtures of bases at nts 94 and 97, which are part of the codons for the consensus amino acids Asp-32 and Thr-33 (Table 1). Mixtures were chosen such that six different mutants would be generated in a single cloning step. Asp-32 was replaced by Asn, His, or Tyr; Thr-33 was either kept or substituted by Ala (Table 1). Because of the high efficiency of the mutagenesis procedure used, we characterized the various mutants by direct sequencing. Among 10 isolates sequenced, all six of the expected mutants were detected. They were named after the amino acids (single-letter code) they encode at positions 32 and 33, replacing the original DT sequence.

The proteaselike sequence is not required for HBeAg generation. Previous studies (7) have shown that the pMH plasmids which contain a slightly overlong HBV genome under control of the human metallothionein promoter (Fig. 1), upon transfection into HepG2 cells, are suitable to direct the synthesis of HBcAg and HBeAg, as well as all other known HBV gene products. Constructs lacking the first nucleotide of the pre-*C* initiation codon (pMH3/3097) yield only the intracytoplasmically synthesized p21c, whereas those including the complete pre-*C* ORF (pMH3/3091 and pMH-34/2922) give rise to both p21c and secreted p17e. Core particles produced in this system contain viral nucleic acid

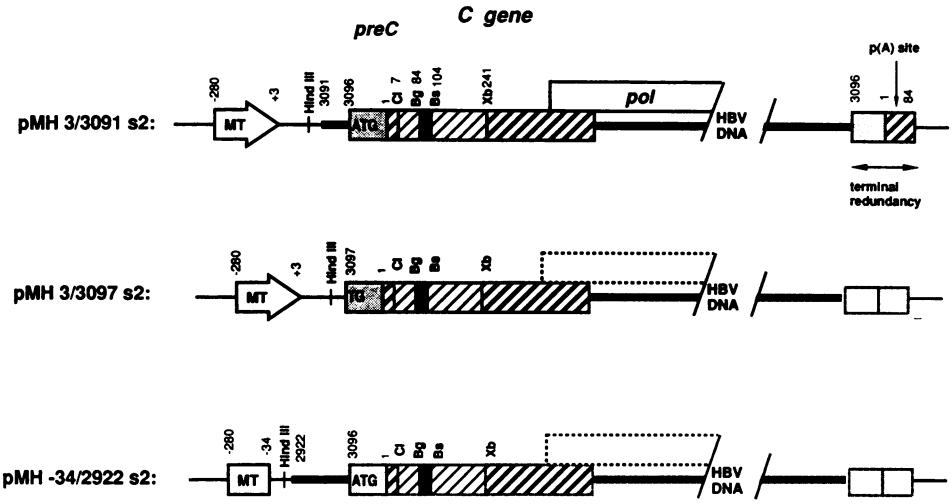

FIG. 1. Structures of the pMH expression plasmids (7) used in this study. All of the plasmids are derivatives of pUC in which a slightly overlong HBV genome was placed under control of the human metallothionein promoter (MT). The thick lines indicate the HBV sequence, and the boxes represent HBV genes. The numbering system, starting with the A of the *C* gene initiation codon, is that of Pasek et al. (25). The dotted box from nts 3096 to 1 represents the pre-*C* ORF, and the dashed box represents the *C* gene. Restriction sites: Cl, *Cla*I; Bg, *Bgl*II; Bs, *Bss*HII; Xb, *Xba*I. The numbers refer to the position of first nucleotide in each of the recognition sequences. In all of the constructs, the sequence between the *Cla*I and *Xba*I restriction sites was derived from a synthetic *C* gene (22). The black box between *Bgl*II and *Bss*HII indicates the restriction fragment that was replaced by a synthetic duplex generating the mutations in the proteaselike sequence at codons 32 and 33. All of the plasmids contained a terminal redundancy providing the polyadenylation signal [p(A)]. The constructs differ at their HBV 5′ ends. In pMH3/3091, the HBV sequence starts at nt 3091, thus including the pre-*C* ATG. pMH3/3097 starts at nt 3097 and therefore does not contain the pre-*C* initiation codon. The numbering in the metallothionein part is with regard to the expected RNA start site. In pMH-34/2922, the metallothionein promoter is 3′-terminally truncated to position −34 and therefore lacks the TATA box. The HBV sequence starts at nt 2922.

and active HBV polymerase. The pMH plasmids, in conjunction with transient expression in a suitable hepatoma cell line, therefore, provide an experimental system to test the proposed importance of the HBV proteaselike sequence in HBeAg biosynthesis and its effects on other HBV gene products. Therefore, the above-described mutations in the homology region were transfected into HepG2 cells. At 3 days after transfection, HBcAg- and HBeAg-reactive proteins were isolated separately from the medium and from the lysed cells. *C* gene products were then detected by Western blotting. Immunoreactive proteins with the same mobility as wild-type p17e were observed with all six mutants (Fig. 2). The absolute amounts of the various secreted p17 proteins varied somewhat; note, however, that the intracellular p21 proteins were also present in somewhat smaller amounts. It

TABLE 1. Nucleotide sequences and resulting amino acid sequences of the HBV genomes mutated in the proteaselike sequence

HBV plasmid[a]	Nucleotide sequence at codons 32 and 33 of the *C* gene[b]		Amino acid sequence at the following position of the *C* gene[c]					
	94	97	30	31	32	33	34	35
Wild type[d]	GAT	ACC	L	L	D	T	A	S
Mutants								
NT	AAT	ACC	L	L	**N**	T	A	S
NA	AAT	GCC	L	L	**N**	**A**	A	S
HT	CAT	ACC	L	L	**H**	T	A	S
HA	CAT	GCC	L	L	**H**	**A**	A	S
YT	TAT	ACC	L	L	**Y**	T	A	S
YA	TAT	GCC	L	L	**Y**	**A**	A	S

[a] The general structures of the HBV expression plasmids used in this study are outlined in Fig. 1.

[b] Codons 32 and 33 correspond to nucleotides 94 to 99 in the HBV numbering system of Pasek et al. (25), starting with the *C* gene initiation codon.

[c] Exchanged amino acids (one-letter code) are shown in boldface.

[d] The nucleotide sequence of wild-type HBV is as determined by Galibert et al. (3).

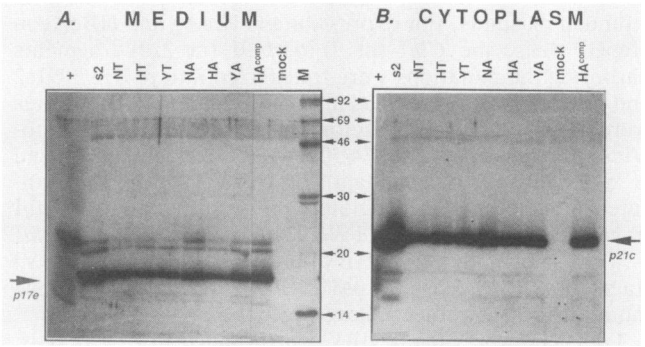

FIG. 2. Expression of HBcAg and HBeAg in HepG2 cells transfected with plasmid pMH-34/2922s2 (s2) carrying a partially synthetic *C* gene (22) encoding the authentic amino acid sequence and mutants NT, HT, YT, NA, HA, and YA (see Table 1 for abbreviations). *C* gene-related products were detected by immunoprecipitation followed by SDS–15% polyacrylamide gel electrophoresis and Western blotting with the same antiserum. +, Sample from an HBV-infected human liver; M, ^{14}C-labeled marker proteins of the indicated sizes (in kilodaltons), mock, HepG2 cells treated identically to the other samples except that no plasmid was present during the transfection procedure. HA^{comp} was from a cotransfection experiment (see Results for details). (A) Products from cell culture supernatants. (B) Products from cell lysates.

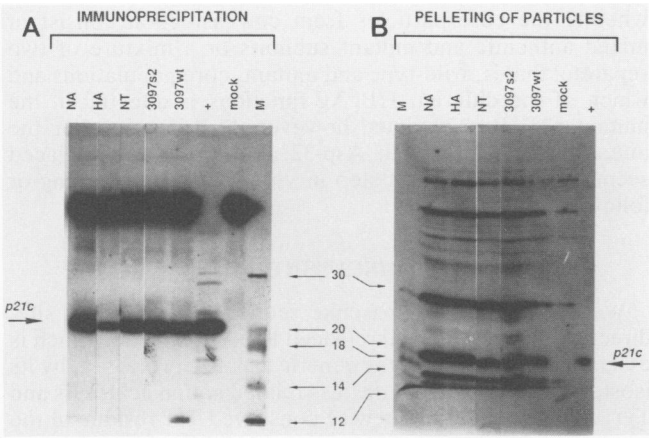

FIG. 3. Detection of p21c in HepG2 cells transfected with plasmids pMH3/3097 and pMH3/3097s2 (28) and mutants NT, NA, and HA. The abbreviations are as in Fig. 2. *C* gene-related products were detected as described in the legend to Fig. 2. (A) Products obtained by immunoprecipitation from cleared cell lysates and separated by SDS–15% polyacrylamide gel electrophoresis. (B) Pelleting of particulate products from cleared lysates. Lysates from HepG2 cells transfected with the indicated plasmids were centrifuged at 80,000 rpm for 35 min in a TLA-100 rotor. The pellets were suspended, and the proteins were separated on an SDS–12.5% polyacrylamide gel. Note that although a number of bands lit up unspecifically, the p21c band was not present in mock-transfected cells.

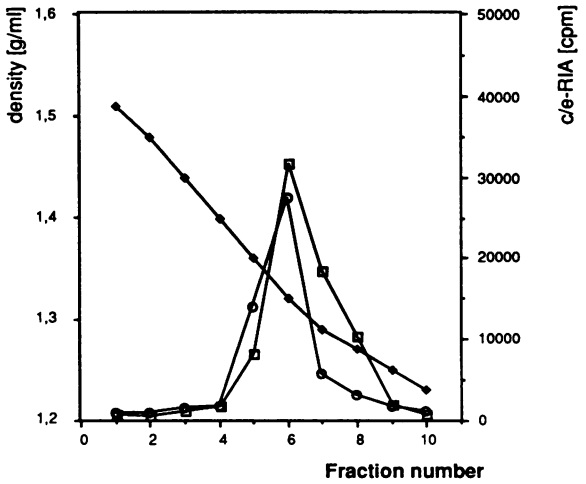

FIG. 4. Comparison of the buoyant densities of core particles from HepG2 cells transfected with plasmid pMH3/3097s2 and mutant HA. The HBcAg peak fraction from a sucrose gradient was subjected to isopycnic centrifugation in CsCl. HBcAg was detected by RIA. Symbols: ■, density of individual fractions; □, RIA values for pMH3/3097s2-transfected cells; ○, RIA values for pMH3/3097HA-transfected cells. The same results were obtained for the other mutants (data not shown).

is particularly noteworthy that no accumulation of incompletely processed, larger precursor proteins was observed which would have been expected if C-terminal HBeAg processing was impaired by the mutations in the proteaselike sequence.

Partial characterization of mutant HBcAgs. To test whether the mutations we introduced would grossly alter the structure of the mutant *C* gene products, the intracytoplasmically synthesized p21 molecules were studied for the ability to form core particles. For these experiments, plasmids of the pMH3/3097 series, i.e., without the pre-*C* ATG codon, were used, since they direct the synthesis of relatively large amounts of p21c (7). Mutant p21 proteins expressed from cells transfected with pMH3/3097NT, pMH3/3097NA, and pMH3/3097HA were pelleted by high-speed centrifugation under conditions which also pellet authentic core particles and detected by Western blotting (Fig. 3). For a more detailed characterization, cleared lysates from HepG2 cells transfected with the corresponding wild-type or mutant HBV plasmids were purified by sucrose density gradients followed by isopycnic centrifugation in CsCl gradients. For mutant HA, the p21 *C* gene product banded at the same density (about 1.35 g/ml) as that from the wild type (Fig. 4). Identical densities were determined for the other mutants (data not shown). These results demonstrate that the amino acid exchanges in the proteaselike sequence do not inhibit the ability of the mutant proteins to self-assemble into HBV-like core particles.

Influence of mutations in the proteaselike sequence on the HBV polymerase. Even if the above-described results strongly suggest that the proteaselike sequence in HBV is not required for HBeAg generation, HBV, like retroviruses, could still encode its own protease for the processing of a primary *P* gene product(s) into enzymatically active polymerase. The most reliable assay available to detect the presence of an HBV Pol protein(s) is by its enzymatic activity in viral core particles during the so-called "endogenous polymerase reaction" (8). When provided with dNTPs, the enzyme is able to repair the gap in the DNA plus strand. The reaction can be monitored by incorporation of a radioactively labeled dNTP, yielding a characteristic pattern of linear and relaxed circular genomic HBV DNA upon agarose gel electrophoresis.

HepG2 cells were transfected with pMH-34/2922 plasmids carrying the mutant HBV genomes. Core particles were isolated from the cell lysates by immunoprecipitation and subjected to the endogenous polymerase reaction. The radioactively labeled products were separated by electrophoresis on an agarose gel and visualized by autoradiography (Fig. 5). Mutants NT, HT, and YT produced bands of the same mobility as the parental DT sequence and somewhat lower intensity, indicating synthesis of linear and circular viral genomes. Mutants NA, HA, and YA gave only very weak bands (Fig. 5A), which became visible only upon prolonged exposure (Fig. 5B). For semiquantitative determination, the radioactivity in the gel areas containing linear-plus-relaxed circular HBV DNA was measured (Table 2). Although exact quantitation was not possible, a clear trend was evident; all mutations led to a reduction in polymerase activity. However, the single mutants in which the postulated essential Asp residue was replaced still showed considerable activity; a massive drop down to a few percent of the wild-type level was observed only in mutants in which Thr-33 was also replaced. These results were confirmed in several experiments from independent transfections, including endogenous polymerase assays performed with cores isolated by sucrose and CsCl gradient centrifugations (data not shown). To confirm that this influence was due to the mutations in the core gene and not to adventitious alterations in the *pol* ORF, we transferred a 210-base-pair *Cla*I-*Hpa*I segment (nt 7 to 220) from pMH-34/2922HA into the parental plasmid and vice versa. The results obtained with these control constructs were completely in accord with those previously observed. Direct evidence for an intact *pol* ORF in the HA mutant comes from a complementation experi-

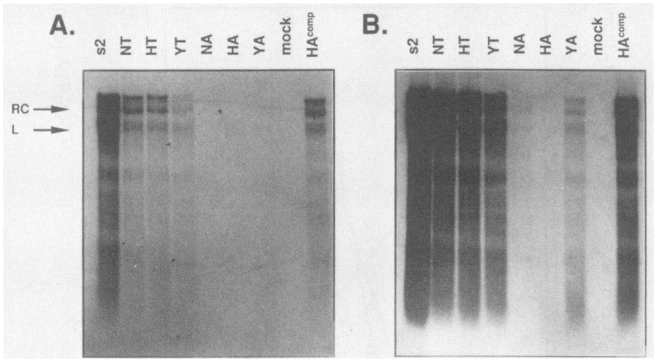

FIG. 5. Endogenous polymerase reaction products from HepG2 cells transfected with plasmid pMH-34/2922s2 (s2) and its mutant derivatives. Viral cores were immunoprecipitated from the cell lysates. After digestion with proteinase K, the bulk of unincorporated dNTPs was removed by gel filtration and the material from the exclusion peak was separated on a 1.5% agarose gel. L, Linear HBV DNA; RC, relaxed circular HBV DNA. The abbreviations are as in Fig. 2. Sample HA^{comp} was from cells cotransfected with mutant HA and a mutant with a defective *pol* gene and an intact *C* gene. (A) Autoradiograph after 10 h of exposure. (B) Same autoradiograph as in panel A but after 4 days of exposure. Very similar results were obtained with density gradient-purified cores (data not shown).

ment in which the mutant was cotransfected with a pMH plasmid harboring a complete HBV genome with a mutation in an essential part of the *pol* ORF (G. Radziwill and H. Schaller, manuscript in preparation) which by itself was negative in the endogenous polymerase reaction. Complementation worked efficiently, leading to a signal of almost normal intensity (Fig. 5, lane HA^{comp}). This experiment showed that the *pol* ORF in mutant HA was intact and that the mutation in the core protein could be complemented by wild-type p21c in *trans*. This is additional strong evidence that no core-Pol fusion protein (35) is required to generate functional HBV polymerase (28). We cannot differentiate whether the core particles from cotransfection consist of mixed authentic and mutant subunits or a mixture of two separate, that is, wild-type and mutant, core populations and which of the different HBcAg functions is affected in the mutant p21. It is obvious, however, that in particular the mutations in which both Asp-32 and Thr-33 are replaced seem to interfere with a step in virus maturation during or following core assembly.

TABLE 2. Expression of HBV antigens in HepG2 cells transfected with pMH-34/2922 plasmids carrying different HBV genomes and activities of the viral polymerase as detected in the endogenous polymerase reaction

HBV plasmid[a]	Expression (kcpm) of [b]:			
	Hepatitis B surface antigen in medium	HBcAg and HBeAg in cytoplasm	HBcAg and HBeAg in medium	*pol* activity [cpm][c]
s2	13.1	6.8	10.1	2,605
NT	10.6	1.7	4.2	592
HT	10.1	1.5	4.2	476
YT	11.6	1.5	3.0	228
NA	9.7	1.6	3.5	43
HA	8.6	0.9	2.4	37
YA	11.7	0.9	2.1	85
HA^{comp} [d]	15.2	5.1	13.1	517
Mock				22

[a] For abbreviations, refer to Table 1.
[b] RIA values were obtained after subtraction of the counts obtained from mock-transfected cells. The medium and cytoplasm were 1/50 of the supernatant, 1/25 of the cell lysate, respectively, from a 100-mm-diameter dish.
[c] The radioactivity contained in linear-plus-relaxed circular HBV DNA from the gel shown in Fig. 5 is shown.
[d] pMH-34/2922HA was cotransfected with a pMH plasmid carrying an HBV genome mutated in an essential part of the *pol* ORF. See Results for details.

DISCUSSION

We mutated the proteaselike sequence in HBV by site-directed mutagenesis and replaced the Asp residue, which is essential for catalysis in the generic aspartic proteases, by its isosteric analog Asn and the less related amino acids His and Tyr. Additionally, the likewise conserved Thr residue of the aspartic protease consensus sequence DTG was exchanged for the less polar Ala residue. Similar mutations in the proteaselike sequences of human immunodeficiency virus and avian sarcoma leukosis virus have recently been reported to abolish the activities of these viral enzymes (12, 13). Using the above mutations in conjunction with transient expression of HBV proteins in a human hepatoma cell line, we determined whether the proteaselike sequence in the HBV core protein would be involved (i) in the generation of the *C* gene product p17e, as previously suggested (19), and (ii) whether it would affect the activity of the HBV polymerase. Our findings are separately discussed below.

The proteaselike sequence of HBV is not essential for HBeAg generation. We monitored the maturation of HBeAg, a secreted and multiply processed product of the HBV *C* gene, by transient expression in HepG2 cells. Our data strongly suggest that the proteaselike sequence in the HBV core protein is not required for the generation of HBeAg. Mutants in which one or two of the absolutely conserved amino acids in the DTG consensus sequence were replaced by a sterically similar amino acid or by very different amino acids still produced a protein of about 17 kilodaltons with mobility identical to that of wild-type p17e. Also, we could detect no accumulation of larger, incompletely processed precursor proteins which should be expected if the protease activity proposed to act on these precursors was abolished in our mutant constructs. We cannot rule out completely the possibility that in the HBV mutants a cellular protease could substitute for the suggested inherent protease activity in cleaving the C terminus from the precursor *C* gene product. However, this cellular protease would then be expected to act also on the wild-type HBeAg precursor and an HBV protease, if it existed, would not appear to be relevant for HBV with regard to HBeAg generation. Therefore, the proposed model for HBeAg biosynthesis is very likely to be incorrect.

This also holds true for the actual HBeAg precursor, which is not the capsid protein p21c, as would be inferred from the proposed model. Mutational analysis in different systems (5, 29, 34) has shown that for HBeAg to be secreted the pre-*C* ORF must be intact, indicating that the pre-*C*–*C* fusion protein p25, so far detected only in vitro, is the precursor of HBeAg. The in vitro studies established that the first 19 of the 29 pre-*C* amino acids form a signal peptide which is most likely clipped off by a signal peptidase in the endoplasmic reticulum. A second, C-terminal cleavage has been mapped to the bond between Val-149 and Arg-150 (32), removing the arginine-rich nucleic acid-binding domain of the primary *C* gene product. Since this second processing step, which is apparently dependent on the primary se-

quence of the corresponding HBeAg precursor (27), is not due to self-cleavage in the way proposed, the nature of the protease responsible and its cellular location remain to be determined.

Is the proteaselike sequence in HBV involved in processing *P* gene products? Whereas in retroviruses a protease is required for maturation of the various Gag and Gag-Pol proteins (the precursors of the active capsid and polymerase proteins), similar processing of the corresponding HBV core and Pol proteins has not been observed. We have attempted to correlate the mutations in the proteaselike sequence with the activities of the *P* gene products in the mutant viruses. Our experiments do not allow clear-cut conclusions. Of the six mutants tested, those with only Asp-32 exchanged gave endogenous polymerase reactions which were close to normal; the others, in which Thr-33 was also replaced by Ala, showed largely diminished signals in the endogenous polymerase reaction. For one mutant, the possibility of unwanted extra mutations in other regions of the genome, which could give rise to the observed inhibition of Pol activity, was ruled out by mutual exchange of a small cassette of DNA harboring the mutant or wild-type proteaselike sequence. Furthermore, *trans* complementation could be obtained by cotransfecting the HA core mutant with a plasmid harboring an intact *C* gene but with a mutation in an essential part of the *pol* gene. The dramatic effects of some of the core mutations on polymerase activity in the endogenous polymerase reaction might be interpreted, at first glance, as being indicative of the existence of a core protease required for Pol processing. However, this seems highly unlikely since the most drastic effects were seen not with mutations affecting the Asp residue essential for retroviral proteases but rather with those in which the conserved T-33 residue was replaced. Furthermore, it should be recalled that the endogenous polymerase assay requires that a whole series of events has correctly taken place. The core protein must assemble into particles, and the correct pregenomic RNA and the polymerase must be packaged and correctly start synthesis of the minus strand DNA by its reverse transcriptase activity. Although the mutant core proteins apparently assemble into particles, the mutations in a region as highly conserved as the proteaselike sequence could inhibit any one of these steps besides the maturation of the Pol enzyme by proteolysis. The basic obstacle to unambiguous proof of the existence of an HBV protease is that the HBV *P* gene products have not been isolated; therefore, there is no way of monitoring the fate of a putative Pol precursor processing by simple sizing on gels. There are also no enzymatically active Pol proteins from heterologous expression systems available which could otherwise serve as substrates for a potential HBV protease.

It should be noted here that in duck HBV the *P* gene-encoded DNA-linked protein (terminal protein) appears to be heterogenous (2). While this heterogeneity is possibly indicative of processing of the primary *P* gene product, there are arguments favoring the notion that this primary product(s) does not have to be cleaved for activation. Since core-Pol fusion proteins have been ruled out as polymerase precursors (28), continued linkage of the terminal protein thought to prime reverse transcription in hepadnaviruses with the polymerase could provide an alternative mechanism of directing the enzyme to the RNA pregenome during core assembly (1).

Is the HBV proteaselike sequence at all part of an aspartic protease? Taken together, our results argue against the theory that the proteaselike sequence in the HBV core protein forms part of an active viral protease akin to aspartic acid proteases. It is doubtlessly not required for HBeAg generation, but we cannot completely rule out its involvement in polymerase maturation. However, we consider this possibility unlikely since mutants NT, HT, and YT gave positive endogenous polymerase reactions, although Asn, His, and Tyr should not easily substitute for the presumably essential Asp residue.

There is additional circumstantial evidence against the view that an HBV protease involving the proteaselike sequence, if it exists at all, is akin to the retroviral and aspartic acid enzymes. (i) Duck HBV, which is much more closely related to HBV than are the retroviruses, does not contain a similar proteaselike sequence in its core protein (although the sequence FQDTG occurs in its *S* gene products). Duck HBV still produces a protein related to HBeAg (DHBeAg), which argues against a role of this sequence in HBeAg biosynthesis (see above) or polymerase processing. (ii) In aspartic proteases and in all retroviral proteaselike sequences whose proteolytic activities have been established biochemically, the consensus sequence contains Gly at the second position after the essential Asp residue, whereas the mammalian hepadnaviruses have Ala at this position. Although Gly and Ala have similar side chains, on the basis of the three-dimensional structure of penicillopepsin as determined by X-ray crystallography and model building with other aspartic and retroviral proteases (26), it appears that only Gly would be suited to accommodate the sharp bend observed or predicted in the polypeptide chain directly following the catalytic Asp residue. Interestingly, replacement of glycine by alanine in the proteaselike sequence of human immunodeficiency virus type 1 protease destroys the catalytic activity of the enzyme (R. Swanstrom, personal communication). (iii) Generic aspartic and putative retroviral proteases contain a second, highly conserved sequence motif, an invariant Gly residue preceded by two hydrophobic amino acids which is located 50 to 60 amino acids downstream of the first homology region (26). There are several Gly residues encoded by the *C* gene, but only Gly-94, Gly-111, and Gly-123 are conserved between the human and the closely related woodchuck and ground squirrel HBV core proteins. Of these, none is preceded by two typically hydrophobic residues.

A second proteaselike sequence proposed to be encoded by the 5′-terminal part of the HBV *P* gene does not contain an aspartic acid (19, 20) and therefore cannot be part of an HBV protease that is closely related to the known retroviral and/or aspartic proteases.

In summary, our data show that intactness of the proteaselike sequence is not required for HBeAg biosynthesis and it does not seem to be essential for the generation of enzymatically active polymerase. Since no processing of *P* gene products in hepadnaviruses has been unequivocally demonstrated, an answer to the question of the type and specificity of a putative HBV protease will have to await the availability of the HBV polymerase precursor protein. Indirect evidence for the existence of an HBV protease and participation of the proteaselike sequence in its activity may come from infection experiments. For Moloney murine leukemia virus and human immunodeficiency virus, it has been demonstrated that mutants in the proteaselike sequence yield complete but noninfectious virions (10, 12).

The results of this study, together with the recent finding that duck HBV polymerase is not synthesized as a core-Pol fusion protein and the profoundly distinct priming mechanism during reverse transcription, point to a more distant

relationship between hepadnaviruses and retroviruses than was previously assumed.

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