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Expression of the Hepatitis B Virus Core Gene In Vitro and In Vivo

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The core gene of hepatitis B virus contains two in-phase AUG codons which may both be used in the viral life cycle. By in vitro translation of transcripts produced in vitro, we investigated the corresponding core gene products and their counterparts in vivo. Depending on the location of the 5′ end of the transcripts, two major core gene-derived proteins were obtained. In transcripts with both in-phase AUGs, only the first one was efficiently used and resulted in synthesis of a 25-kilodalton protein (precore). This protein contains a leader sequence and could be cotranslationally processed to a protein of 22.3 kilodaltons. Translation of transcripts lacking the first AUG of the core gene produced a core protein of 21.5 kilodaltons which comigrated with the core antigen expressed in infected livers. These data suggest that the major nucleocapsid protein expressed in vivo is initiated at the second ATG of the C gene and that a precore protein is probably synthesized as a precursor protein which is cotranslationally processed. Proteins consistent in size with processed and unprocessed precore proteins detected in woodchuck hepatitis virus-infected livers support this conclusion.

Hepatitis B virus (HBV) has a lipid envelope containing three virus-encoded envelope proteins (pre-S1, pre-S2, and hepatitis B surface antigen) (6, 25). The lipid envelope is wrapped around a core particle which is composed of hepatitis B core antigen (HBcAg), the viral DNA with a genome-linked protein, and a DNA polymerase (reverse transcriptase) (for a review, see reference 28). The envelope proteins are synthesized from one continuous open reading frame (divided into pre-S and S regions) by initiation of translation at different AUG codons. The major nucleocapsid protein (HBcAg) and a derivative thereof, hepatitis B e antigen (HBeAg), are encoded by the core gene (11, 20). Two conserved in-phase initiation codons divide the core gene into the precore and core regions. The translation initiation codon used for synthesis of the 21.5-kilodalton (kDa) HBcAg and the 15-kDa HBeAg is not known since no amino-terminal protein sequencing data are available. Processing of a precursor protein initiated at the precore AUG or initiation of translation at the core AUG codons are possible pathways of synthesis for both proteins. The precore sequence resembles a signal sequence and has been shown to play a role in HBeAg synthesis and secretion (15, 19, 22, 29). In addition, a cloned HBV DNA in which a point mutation created a translation termination codon in the precore sequence appeared to be noninfectious (30).

Several transcripts have been identified which may serve as templates for HBcAg and HBeAg synthesis in HBV and related viruses (4, 18, 21, 23, 31) of Eastern woodchucks (woodchuck hepatitis virus [WHV]) (26) and Beechey ground squirrels (ground squirrel hepatitis virus) (13). Since these transcripts initiate heterogeneously both upstream of and within the precore region, the initiation codon used for translation of core and e antigen cannot be identified by transcription analysis.

In this study, we tried to determine by in vitro translation of core gene transcripts which of the two in-phase AUG codons serves as the translation initiation codon. The data obtained suggest that the major core antigen expressed in vivo initiates at the second ATG of the C gene and that a precore protein is also expressed and cotranslationally processed to a protein slightly larger than the major core protein.

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MATERIALS AND METHODS

Antisera and liver extracts. The antisera used for immunoprecipitation and immunoblotting were raised in rabbits against a denatured *lac*-HBcAg fusion protein (24, 32) and an MS2-woodchuck hepatitis core antigen (WHcAg) fusion protein (containing the 99 amino-terminal amino acids of the MS2 polymerase protein and the 184 carboxy-terminal amino acids of WHcAg), both produced in *Escherichia coli*. Protein extracts from human and woodchuck livers were prepared by homogenization of liver tissue (taken from autopsy material of HBV-infected patients and chronically WHV-infected and noninfected woodchucks) in PBS-NP buffer (10 mM phosphate [pH 7.5], 140 mM NaCl, 0.1% Nonidet P-40).

Construction of deletion mutants. A genome-length *Eco*RI HBV DNA fragment (5) was inserted into the *Eco*RI site of vector pSP64 (16) in the sense orientation with respect to the SP6 promoter. The plasmid obtained was linearized with *Nco*I upstream of the X open reading frame (see Fig. 1) and incubated with nuclease BAL 31 at 32°C in 0.6 M NaCl–20 mM Tris hydrochloride (pH 8.0)–12 mM $MgCl_2$–12 mM $CaCl_2$–1 mM EDTA. Samples were drawn every minute, and the reactions were stopped with the same volume of 0.1 M ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. Twelve samples were pooled and digested with *Pst*I to remove the S gene sequences downstream of the SP6 promoter, including the adjacent *Eco*RI cleavage site. The DNA fragments were blunt ended with DNA polymerase I in the presence of 50 mM KCl and 25 μM deoxyribonucleoside triphosphates and isolated by electroelution from a preparative agarose gel as previously described (3). After blunt-end ligation, the plasmids were again digested with *Pst*I to reduce the number of background clones and transformed into *E. coli* by a $CaCl_2$ method (12). About one-third of the clones obtained were prescreened by hybridization with several HBV-specific oligonucleotides. Plasmid DNA was isolated as previously described (7) and directly used to determine the nucleotide sequence of the vector-HBV DNA junction. Sequencing was performed with a synthetic oligo-

nucleotide (21 bases long hybridizing 5 nucleotides upstream of the transcription initiation site) and the dideoxy sequencing method (14).

Transcription and translation in vitro. A modified protocol of Melton et al. (16) was used for in vitro transcription of deletion mutants (assay conditions, 40 mM Tris hydrochloride [pH 7.5], 6 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine, 0.5 to 1 μg of template DNA, 0.5 mM [each] ribonucleoside triphosphates, 30 U of RNasin, 5 U of SP6 polymerase; total volume, 40 μl; temperature, 40°C). The template DNA had been purified by cesium chloride-ethidium bromide equilibrium centrifugation and linearized at the unique *Eco*RI site. For quantification of the transcripts, the RNA was labeled in some experiments by addition of 6 μCi of [α-^{32}P]GTP.

In vitro translation was carried out with rabbit reticulocyte lysate N.90 (Amersham Buchler, Braunschweig, Federal Republic of Germany) at 30°C for 1 h. Approximately 0.2 μg of the in vitro-synthesized RNA was used for translation, and the proteins were labeled with 25 μCi of [^{35}S]methionine in a 25-μl assay. Proteins used for immunoblotting were translated in the absence of [^{35}S]methionine.

For protein processing experiments, the New England Nuclear protein processing-translation system (NEK 019) was used. A 25-μl assay contained 1 μl of RNA, 10 μl of reticulocyte lysate, 0 to 1 μl of pancreatic microsomal membranes, 1 to 0 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), and 13 μl of premix containing 30 μCi of [^{35}S]methionine, 225 mM potassium acetate, 1.3 mM magnesium acetate, and 5.5 μl of translation cocktail (including spermidine, creatine phosphate, dithiothreitol, and GTP in HEPES buffer). The assay was incubated at 37°C for 1 h, and samples were boiled in 2× sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris hydrochloride [pH 6.8], 10% β-mercaptoethanol, 10% glycerol, 0.02% bromphenol blue) for 15 min before SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 9) and fluorography (10).

Immunoprecipitation. The proteins synthesized in vitro were characterized by immunoprecipitation with the anti-HBcAg antiserum. Antiserum (5 μl) was incubated with 50 μl of preswollen protein A-Sepharose for 2 h at room temperature in an end volume of 200 μl of RIPA buffer (PBS-NP, 0.5% sodium desoxycholate, 0.1% SDS) and then washed three times with 1 ml of RIPA buffer each time. The antigen (10 μl of an in vitro translation reaction mixture) was added and incubated in a total volume of 600 μl of RIPA buffer for 2 h. After the pellet was washed twice with RIPA buffer, bound proteins were eluted by boiling for 5 min in 2× sample buffer and analyzed by SDS-PAGE.

Immunoblotting. Proteins were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose filter (1). The filter was saturated with bovine serum albumin (1.5% in phosphate-buffered saline) and incubated with antiserum (dilution 1:1,500) in 1.5% bovine serum albumin-phosphate-buffered saline overnight. After intensive washing with PBS-NP, the filter was incubated for 3 h with 2.5 μCi of ^{125}I-labeled protein A in 1.5% bovine serum albumin-phosphate-buffered saline, washed several times in PBS-NP and H_2O, dried, and exposed to an X-ray film.

RESULTS

To study mechanisms of HBV core gene expression at the translational and posttranslational levels, several SP6 HBV plasmids were constructed. The plasmids were used for synthesis of transcripts with 5' ends upstream of and within the core gene. The gene products obtained by in vitro translation were analyzed by immunoprecipitation and immunoblotting and compared with core proteins expressed in vivo.

Translation of HBV transcripts in vitro. The 5' ends of the HBV transcripts translated in vitro are indicated in Fig. 1. The core gene products were immunoprecipitated with an anti-HBcAg antiserum (Fig. 1). From all RNAs with HBV sequences up to 173 nucleotides upstream of the first AUG of the C gene at position 3096 (clones pHTW 2922 to 3095), a major protein of about 25 kDa and a minor protein of 21.5 kDa were translated. After deletion of the first AUG (plasmids pHTW 3097 to 3182), the 25-kDa protein was no longer synthesized; instead, the 21.5-kDa protein became the major species. This indicates that the 25-kDa protein is initiated at AUG 1 of the C gene (precore protein), whereas the 21.5-kDa protein is initiated at AUG 2 at nucleotide position 1. Faint signals corresponding to proteins of molecular masses of 19.5, 18.5, 15.5, and 13 kDa were observed with RNAs with 5' ends downstream of AUG 2 (Fig. 1). These proteins could be initiated at other codons further downstream or could result from premature termination of translation. To determine the efficiency of translation initiation at the different codons, in vitro translation was performed with standardized amounts of transcripts (see Materials and Methods for quantification). The efficiency of ribosome recognition of AUGs 1 and 2 of the C gene was dependent on the length of the 5' untranslated sequence (Fig. 2). The longer this sequence, the less efficiently was the corresponding protein expressed. This effect was not due to competition for ribosome-binding sites because it was still seen after removal of the only two AUG codons of the 5' untranslated sequence (Fig. 1).

Processing of the precore protein in vitro. Indirect evidence suggests that the precore protein is expressed in vivo and may contain a signal sequence (19, 22, 29). Therefore, we tested whether the precore protein can be cotranslationally processed in vitro. Translation of a transcript initiating upstream of the precore sequence derived from plasmid pHTW 3095 was performed with and without pancreatic microsomal membranes, and the translation products were analyzed by SDS-PAGE (Fig. 3). A human pituitary mRNA was used as a positive control (Fig. 3, control lanes). The control protein was efficiently expressed and completely processed in the presence of membranes. The precore protein was efficiently synthesized without membranes. Using high concentrations of pancreatic membranes, translation efficiency decreased by a factor of about 3 to 4. At intermediate concentrations, approximately 25% of the precore protein was processed to a protein of 22.3 kDa, and at low concentrations, precore expression was virtually unaffected (Fig. 3, lanes 3095). Addition of membranes after completion of the translation reaction did not lead to precore processing (data not shown), suggesting cotranslational rather than posttranslational processing. The protein initiated at the second ATG of the core gene (Fig. 3, lane 3177) exhibited a slightly higher electrophoretic mobility than the processed precore protein, suggesting its cleavage within the precore sequence.

Comparative analysis of core proteins synthesized in vivo and in vitro. The core proteins of three livers acutely infected with HBV were analyzed by immunoblotting (Fig. 4A, lanes 1 to 3) and compared with the precore and core proteins synthesized in vitro (Fig. 4A, lanes P and C). In two of the infected livers (Fig. 4A, lanes 2 and 3), one major core

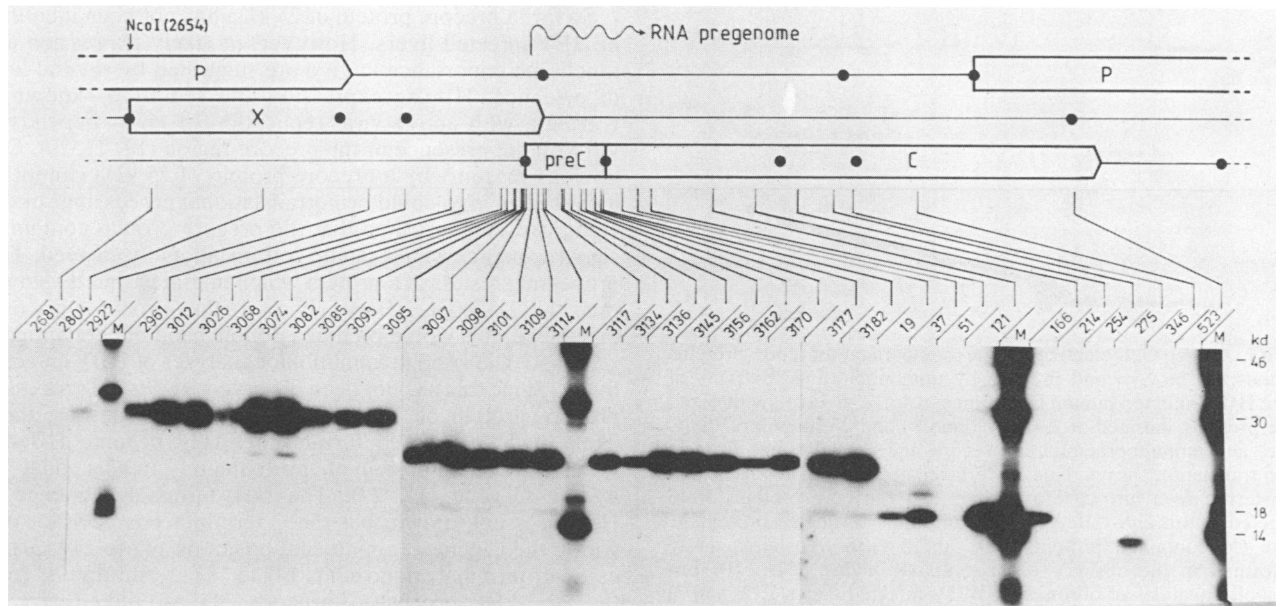

FIG. 1. Gel electrophoretic analysis of core gene-encoded proteins translated in vitro. The 5′ ends of the RNAs used for translation, their nucleotide positions (for nomenclature, see reference 20) on the HBV genome, and the 5′ end of C mRNA as identified in vivo at position 3100 ±1 are shown in the upper part of the figure. The black dots represent ATG codons. P, putative polymerase gene; X, open reading frame X. The lower part shows SDS-PAGE analysis of immunoprecipitated core proteins obtained by in vitro translation from the RNAs indicated above. M, ^{14}C-labeled molecular size markers; kd, kilodaltons.

protein of 21.5 kDa was detected, but none corresponding to the unprocessed form of the precore protein was found. This suggests that the major core protein of the liver is initiated at ATG 2 of the C gene. With a third HBV-infected liver containing higher concentrations of core proteins (Fig. 4A, lane 1), again no signal corresponding to the size of the unprocessed precore protein was detected. However, a minor band of 38 kDa and a smear in the molecular mass range of 24 kDa down to 14 kDa became visible. The 38-kDa protein has previously been identified tentatively as a core-*pol* fusion protein (32). The smear may be due to degradation of the core protein but may also include minor core proteins as observed in the in vitro system.

Core gene-derived proteins in chronically infected woodchuck livers. At the 5′ terminus of the core gene of WHV there are three in-phase initiation codons which could serve as initiation codons for synthesis of two precore proteins of 25.7 and 25.2 kDa and a WHcAg protein of 21.6 kDa as predicted from the sequence. Two major 5′ ends of putative C mRNAs in chronically infected liver were mapped upstream of AUGs 2 and 3 of the C frame (18). To test which of these AUGs is used in core gene expression, an anti-WHcAg antiserum was raised against the carboxy-terminal 184 amino acids of the core protein and used for immuno-

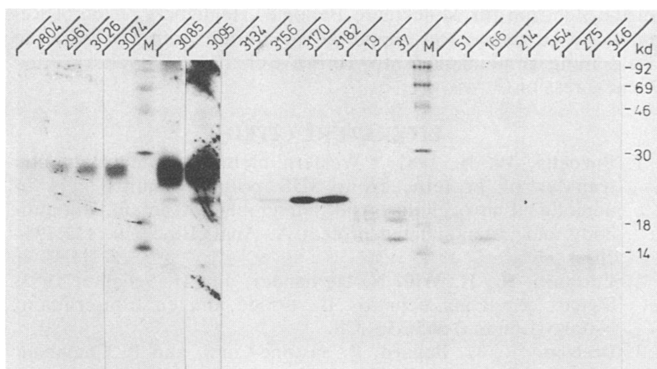

FIG. 2. Quantitative analysis of C mRNA translation in vitro. Identical amounts of mRNAs (see Materials and Methods for quantification) with different 5′ ends on the HBV genome (positions indicated on top) were translated in vitro, immunoprecipitated, and analyzed on an SDS–15% polyacrylamide gel. kd, Kilodaltons.

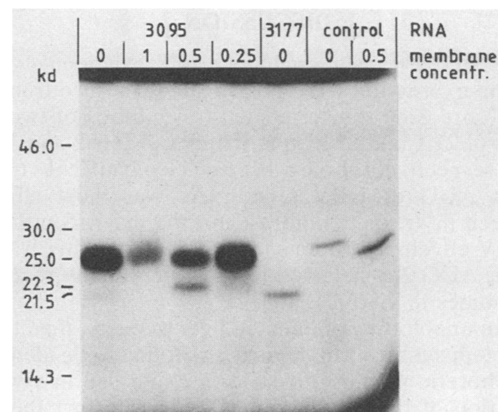

FIG. 3. Cotranslational processing of the precore protein in vitro. A pre-C mRNA from plasmid pHTW 3095 was translated in vitro in the presence or absence of pancreatic microsomal membranes (lanes 3095; the amounts of membranes added are given in microliters). Only with a certain amount (0.5 μl) of membranes did substantial processing of the precore protein occur (lane 3095, 0.5). The processed precore protein (22.3 kDa) had a slightly lower electrophoretic mobility than a core protein initiated at AUG 2 of the C gene synthesized from plasmid pHTW 3177 (lane 3177, 0). Human pituitary mRNA was used as a control for efficiency of processing (lanes control, 0 and 0.5). ^{14}C-labeled proteins (14.3, 30, and 46 kDa) were used as molecular size markers. kd, Kilodaltons; concentr., concentration.

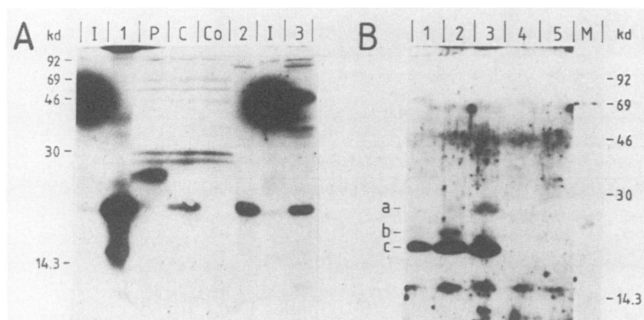

FIG. 4. (A) Gel electrophoretic comparison of core proteins synthesized in vivo and in vitro by immunoblotting. Extracts of three HBV-infected human livers (lanes 1 to 3), in vitro-synthesized core proteins initiated at ATGs 1 (lane P) and 2 (lane C) of the C gene, and immunoprecipitated precore and core proteins from in vitro translation assays (lane I) were separated by SDS-PAGE and transferred to a nitrocellulose filter, and the core proteins were detected by an anti-HBcAg antiserum and ^{125}I-labeled protein A. Lane Co contained proteins from an in vitro translation assay performed in the absence of exogenously added RNA. (B) Immunoblot analysis of chronically WHV-infected (lanes 1, 2, and 3) and uninfected (lanes 4 and 5) woodchuck livers with an anti-WHcAg antiserum. ^{14}C-labeled proteins were used as molecular size markers (M). kd, Kilodaltons.

blotting of protein extracts prepared from chronically WHV-infected and noninfected woodchuck livers. In addition to the major WHcAg migrating at a position of 20.5 kDa (Fig. 4B, band c), we detected two proteins migrating at 22.5 and 27 kDa (Fig. 4B, bands b and a) which reacted specifically with the antiserum. The electrophoretic mobility of the WHV core proteins a and b (Fig. 4B) was very similar to that of the unprocessed and processed HBV precore proteins synthesized in vitro (Fig. 3). Therefore, they probably represent the corresponding WHV precore gene products.

DISCUSSION

In this study, translational and posttranslational events of core gene expression were investigated. In vitro translation of HBV core gene transcripts led to synthesis of two major proteins of 21.5 and 25 kDa initiated at ATGs 2 and 1 of the C gene, respectively. Like most eucaryotic mRNAs (8), only the first AUG of HBV C mRNAs was most efficiently recognized in vitro. In contrast, on the major S mRNA for two HBV envelope proteins (pre-S2 and hepatitis B surface antigen), AUG 2 is believed to be preferentially recognized by ribosomes in vivo (2).

By immunoblotting of infected liver extracts, the 21.5-kDa core protein synthesized in vitro was found to be identical in electrophoretic mobility to HBcAg expressed in vivo. The comigration of these two proteins suggests that the major HBV nucleocapsid protein expressed in vivo is initiated at AUG 2 of the C gene. Similar conclusions have been drawn from expression studies in *Saccharomyces cerevisiae* (17). Our interpretation is also consistent with the location of the 5′ end of the putative C mRNA of HBV within the precore region as identified in vivo (31). However, minor transcripts initiating upstream of the precore region (pre-C mRNAs) seem to exist for HBV both in vivo and in vitro (21, 23, 31) and are likely to serve as templates for precore protein expression (see below). Moreover, our data suggest that the 21.5-kDa HBcAg would not be translated efficiently from transcripts containing both in-phase AUGs.

So far, a precore protein of 25 kDa has not been identified in HBV-infected livers. However, its likely expression and functional importance in vivo are suggested by several lines of evidence. HBeAg expression and secretion—known to correlate with active viral replication in vivo—depends in vitro on the presence of the precore region (19, 21, 29). One obvious reason why a precore protein of 25 kDa cannot be identified in vivo could be cotranslational processing. In this study, we demonstrated that the precore protein contains a signal sequence which can be cotranslationally cleaved. This processing results in a protein which migrates slightly slower than the 21.5-kDa HBcAg. If no further processing would take place, this would predict a double band of core antigens (21.5 and 22.3 kDa) in immunoblot analyses of HBV-infected livers. Since this has not been observed, either the processed precore protein of 22.3 kDa is a minor species or, more likely, it is processed further to finally become HBeAg. HBeAg is a small protein of approximately 16 kDa coded for by the C gene (11, 27). The NH_2-terminal sequence of HBeAg is not known, but the C terminus has been located within the C gene at amino acid positions 147 to 149 (27). If carboxy-terminal amino acids 147 to 183 are subtracted from the 22.3-kDa processed precore, the predicted size of HBeAg would be approximately 18 kDa. This is slightly larger than the molecular weight of HBeAg as determined by SDS-PAGE (15, 27). HBeAg synthesis may, therefore, involve additional processing of the precore protein or posttranslational modification of HBeAg. Alternatively, HBeAg may have aberrant electrophoretic mobility.

Using WHV-infected livers, we observed core proteins consistent in size with processed and unprocessed precore proteins. The detectability of these putative precore proteins in WHV-infected livers and not in HBV-infected livers could be related to the higher levels of precore transcripts described for WHV (18, 31). This could result in higher levels of precore protein and in its partial escape from proteolytic processing. Further in vitro and in vivo experiments are in progress to test these hypotheses.

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