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# Expression and replication of the hepatitis B virus genome under foreign promoter control

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ABSTRACT

A novel expression system was established that allows expression and propagation of the human hepatitis B virus (HBV) genome in cultured animal cells. An overlength HBV genome encoding the viral pregenomic RNA was put under transcriptional control of the human metallothionein $II_A$ promoter thereby replacing the endogenous HBV core gene promoter. Transient expression of this construct in hepatoma cells resulted in formation of particles indistinguishable from HBV (Dane particles). Uncoupling of the promoter from overlapping HBV genes facilitated a mutational analysis of HBV gene functions. For example, removal of the preC start codon completely prevented HBeAg synthesis whereas formation of HBV-like particles remained unaffected. In addition, overexpression of the core gene led to detection of minor and otherwise undetectable core gene products, including a core/pol fusion protein and larger precursor molecules of the secreted HBeAg.

## INTRODUCTION

Human hepatitis B virus (HBV) is the prototype of the hepadna viridae, a recently discovered new family of hepatotropic, enveloped animal viruses (for a recent review, see reference 1). Like other hepadnaviruses, HBV is characterized by containing a DNA genome of 3.2 kb which is circular but not covalently closed and only partially double-stranded, and to which a protein is covalently attached. This unconventional genome structure reflects the unconventional mode of its replication which involves reverse transcription of an RNA pregenome of 3.5 kb as a first step. The pregenomic RNA also serves as mRNA for the tissue-specific expression of the viral core protein and the viral DNA polymerase (reverse transcriptase). A second major mRNA of about 2.1 kb specifies the expression of the viral surface proteins in a less tissue-specific manner.

Because of narrow host range and tissue specificity and the

lack of an appropriate tissue culture system, the molecular analysis of the HBV life cycle has been limited to animal systems or to expression of cloned HBV genes in heterologous cultured cells. Only recently has work from several laboratories demonstrated that well differentiated human hepatoma cell lines are competent for expression of all HBV genes in cultured cells and for production of viral particles that possess all properties of authentic HBV (2, 3, 4).

In this study we describe a different approach towards production of HBV in tissue culture. Since the synthesis of the pregenomic RNA/core gene mRNA appears to be the crucial tissue-specific step in HBV replication we positioned the corresponding HBV DNA sequence under control of the human metallothionein $II_A$ promoter (MT promoter) which is known to be active in a variety of animal cells (5). In transient expression experiments, plasmids containing such constructs directed the synthesis of all known core gene products and of HBV-like particles independent of the cell type used. As core gene expression was high, the system allowed also to detect minor core gene products and to assess the functional role of the precore region in core gene expression and virus formation.

## MATERIALS AND METHODS

### Construction of plasmids

For construction of pMH3/3091 (Fig. 1) the vector pUC13 (6) was modified by elimination of the EcoRI site by cutting, repair with DNA polymerase I, and religation. Into this pUC13ΔR, a 3357 bp HBV DNA fragment comprising positions 3091 - 84 was inserted between the HindIII and BamHI sites in a two step procedure. First, the 2.3 kb BglII fragment 938-84 from pSHH2.1 (7) was cloned into the BamHI site placing HBV position 938 (8) near the HindIII site. Then, HBV sequences were completed by insertion of a HindIII - EcoRI fragment from plasmid pHTW3091 (9) including HBV positions 3091 - 1280. Next, a synthetic SalI linker with the sequence 5'-AGCTAGATCTGTCGACCATGGA-3' and in the complementary strand 5'-AGCTTCCATGGTCGACAGATCT-3' was inserted into the HindIII site that abrogated the upstream HindIII site but left the downstream one intact. The resulting plasmid was cut by SalI and

HindIII and the MT promoter was inserted using a DNA fragment comprising MT nucleotides -280 to +3. This fragment had been prepared from plasmid pMTCATΔ5'-286 (10) by Bal31-deletion and addition of synthetic oligonucleotide linkers, creating a SalI site at its 5'-end and a HindIII site at its 3'-end (11). Plasmid pMH3/3097 was derived from pMH3/3091 by replacing the HindIII - EcoRI HBV DNA fragment with the corresponding DNA fragment from pHTW3097 comprising HBV position 3097 - 1280 (9). Plasmid pMH-9/3091 was derived from pMH3/3091 by oligonucleotide directed mutagenesis. A 1.6 kb PstI - EcoRI fragment from pMH3/3091 containing the MT promoter and half of the HBV insert was subcloned into the single-stranded vector M13mp18 (12) and mutated according to (13). The oligonucleotide used for this procedure had the sequence 5'-GCTATAAACACTGCTTGTCGACGCACCATGCAACTTTTTCACC-3' linking MT position -15 (via the sequence 5'-TCGAC-3') to HBV position 3091 thereby creating a SalI site in the junction sequence. The mutated PstI - EcoRI fragment was reintroduced together with the 2 kb EcoRI - SstI HBV fragment from pMH3/3091 into pUC13ΔR that had been cut with PstI and SstI.

Cells, immunoassays and protein analysis

Expression experiments were performed with HeLa cells and HepG2 cells. Cells were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 5 mM glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. Transfections were performed with 20 µg of DNA per 100 mm dish (14). Expression of HBc/eAg and HBsAg was assayed by the appropriate radioimmunoassays (RIA, Abbott Laboratories).

For immunoblot analysis, viral proteins from cell culture media and cell lysates were concentrated by immunoprecipitation. Media were clarified by centrifugation prior to immunoprecipitation. Cell lysates were prepared by washing plates with PBS (8mM $Na_2HPO_4$, 2 mM $NaH_2PO_4$, 140 mM NaCl) and lysing cells in PBS/1 % Triton X-100. Nuclei and cell debris were removed by centrifugation and the remaining cytoplasmic fraction subjected to immunoprecipitation. Preswollen protein A Sepharose was washed 3 times in RIPA-buffer (PBS, 1 % Nonidet P 40 (NP40), 0.5 % sodiumdeoxycholate, 0.05 % sodiumdodecylsulfate (SDS)). Antisera were adsorbed for 1 h, the Sepharose washed 3 times with RIPA-

buffer, samples added and immunoprecipitation carried out in RIPA-buffer for 16 h at 4° C. After immunoprecipitation the Sepharose was washed 5 times with RIPA-buffer. Proteins were analysed using SDS polyacrylamide gel electrophoresis (SDS-PAGE) (15) and "Western" blotting (16). Antisera used were directed against native recombinant HBcAg and amino acids 110 - 183 of the HBV core frame, respectively (17). The anti-pol antiserum was elicited against the amino acid sequence encoded by the P-ORF between HBV nucleotides 494 and 1006 (17).

Cesium chloride gradients

Particles positive for HBc/eAg or HBsAg were pelleted from cell culture medium through a 30 % sucrose cushion in a 45 Ti rotor at 106.000 x g for 16 h, resuspended in TNE (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 0.1 mM EDTA), loaded onto a preformed cesium chloride gradient with steps of 1 ml 1.15 g/ml, 2 ml 1.2 g/ml, 2.5 ml 1.25 g/ml, 3 ml 1.3 g/ml, 1.5 ml 1.4 g/ml, and centrifuged in a SW40 rotor at 160.000 x g for 36 h at 4°C. Fractions of 0.4 ml were collected and aliquots assayed by HBc/eAg and HBsAg RIA.

Endogenous DNA polymerase assay

Samples from cesium chloride gradient fractions were diluted 3-fold with TNE and centrifuged at 109,000 x g for 16 h at 4°C. Pellets were dissolved in 38 µl pol-mix (50 mM Tris-Cl pH 7.6, 40 mM $NH_4Cl$, 1 % NP40, 0.3 % 2-mercaptoethanol, 12.5 µM of dTTP, dGTP and dCTP each and 0.5 µM of $\alpha$-($^{32}$P)-dATP (20 µCi, Amersham)). To assay for endogenous DNA polymerase (18), samples were incubated at 37°C for 2 h, followed by a 1 h chase by adding dATP to 12.5 µM. Exogenous DNA was digested with S. aureus nuclease (Boehringer Mannheim, Mannheim, final concentration 15 U/ml) for 20 min. at 37 °C and subsequently proteinase K was added (0.5 mg/ml final concentration) in presence of 1 % SDS for 2 h at 37 ° C. tRNA was added to 1 mg/ml, samples were extracted by an equal volume of phenol, desalted on a 2 ml Sephadex G-100 column in TE (10 mM Tris-Cl pH 7.5, 0.1 mM EDTA), and labelled DNA species were separated on 1,5 % agarose gels and autoradiographed.

RESULTS

The pMH expression plasmids

The general structure of the pMH plasmids is presented in Figure 1. A slightly overlength HBV genome was cloned into the vector pUC13 between the unique HindIII and BamHI sites. This terminal redundant genome, starting 5' with the preC/C region and ending 3' with the HBV polyadenylation signal, contains all HBV genes in the order present in the pregenomic RNA. The human metallothionein $II_A$ promoter was cloned in front of this genome, thereby replacing the endogenous HBV core gene promoter. Thus, the constructs are expected to synthesize RNA molecules equivalent to the HBV pregenome.

Three different pMH plasmids were constructed. Two (pMH3/3091 and pMH-9/3091) differ by location of the TATA box of the MT promoter relative to the start site of C gene translation and thus simulate the *in vivo* situation where heterogeneous transcripts are observed (4, 19, 20). In addition, the preC start was eliminated in the third plasmid pMH3/3097 by deleting its first nucleotide whereas plasmids pMH3/3091 and pMH-9/3091 contain the preC initiation codon. In pMH-9/3091 the MT promoter is fused to the HBV sequences in such a way that transcription is expected to start near position 3100 as it has been observed *in vivo* for the major C-mRNA in infected liver (19).

Transient expression in HeLa and HepG2 cells

In a first experiment, plasmid pMH3/3091 was introduced into HepG2 and HeLa cells and transient expression of viral proteins was investigated. Plasmid pSHH2.1, a plasmid containing a tandem repeat HBV genome and known to express all HBV genes (2), was used as a control. As shown in Fig. 2, both plasmids produced comparable amounts of HBsAg in each cell line. However, comparing the two cell lines with each other HBsAg expression was about 10-fold higher in HepG2 cells indicating that HeLa cells have only a limited potential to synthesize and to secrete the surface proteins. This is in contrast to the generally accepted assumption that synthesis of HBV surface protein is not correlated with the differentiated state of the host cell.

In addition to HBsAg, either plasmid directed the production

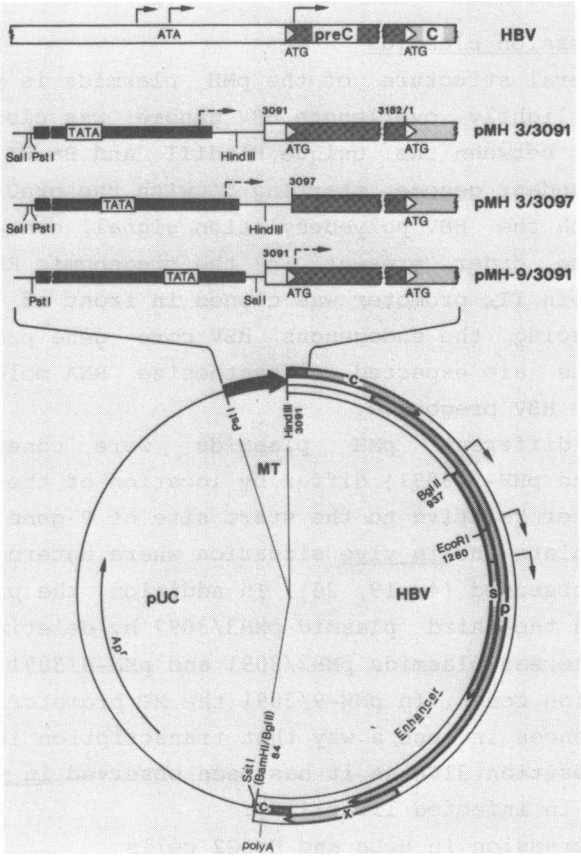


Figure 1: The pMH plasmids. The basic structure is presented for plasmid pMH3/3091, and the junctions between MT promoter and HBV preC/C region are shown in detail for all three pMH constructs in comparision to the corresponding HBV region. The MT promoter is shown in black, junction sequences as thin line, and HBV sequences as wide bar. Restriction sites used for plasmid construction are indicated. The joining sequence between MT nucleotide +3 and HBV in pMH3/3091 and pMH3/3097 is 5'-CCAAGCTTGGGC-3'; in pMH-9/3091 the sequence 5'-TCGAC-3' joins positions MT -15 and HBV 3091. Known RNA starts are shown as solid arrows, predicted ones as broken arrows. Abbreviations: C, core gene; preC, precore region; S, surface antigen gene; P, polymerase open reading frame (ORF); X, X-ORF; MT, human metallothionein $II_A$ promoter; $Ap^r$, β-lactamase gene; polyA, HBV polyadenylation signal.

of substantial amounts of HBc/eAg in HepG2 cells. In contrast, only transfection with pMH3/3091 resulted in significant HBc/eAg production in heterologous HeLa cells, and no C-gene products were detected with pSHH2.1 (Fig. 2). These results indicate that

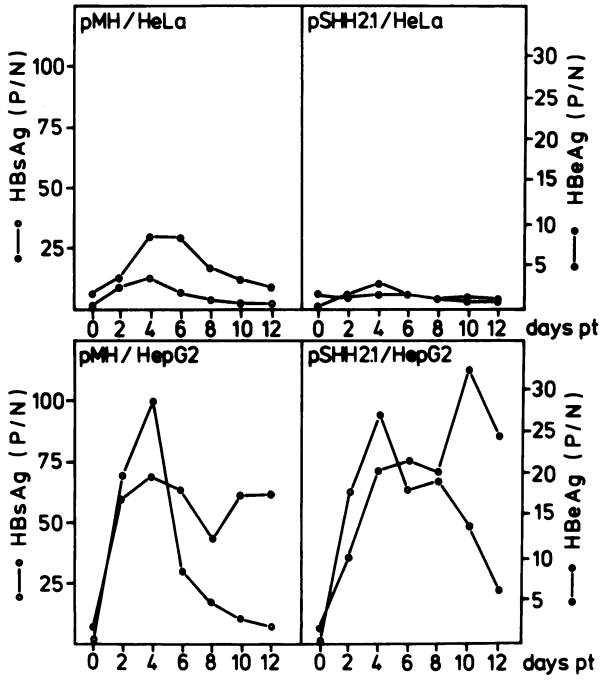


Figure 2: Time course of transient expression of plasmids pMH3/3091 and pSHH2.1 in HeLa and HepG cells. P/N values from cell culture medium are shown for HBsAg (open circles) and HBeAg (filled circles). Abbreviation: pt, post transfection.

expression of the preC/C ORF is no longer restricted to hepatocytes if it is placed under control of the MT promoter.

Since the Abbott RIA does not distinguish between HBcAg and HBeAg, the corresponding proteins were characterized by immunoprecipitation and Western blotting (Fig. 3A). HBeAg (p17e) could be detected in the culture medium as a single band of about 16 kd molecular weight (MW). The relative levels of HBeAg expression observed in these blots are in good agreement with the RIA data (Fig. 2). Therefore, preC/C gene products released into the medium from pMH3/3091 transfected cells consist almost exclusively of HBeAg. This is in accordance with the in vivo situation, where only HBeAg but not HBcAg can be detected in sera from HBV-infected patients.

Particle formation

Formation and release of core particles and complete HBV

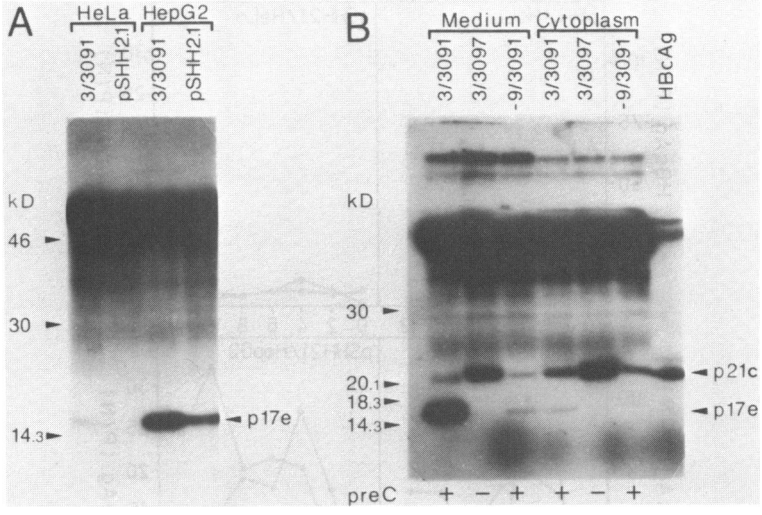


Figure 3: Western blots of core gene products from transient expression. A: Comparison of the amounts of HBeAg (p17e) in the medium of pMH3/3091 and pSHH2.1 transfected HeLa and HepG2 cells (day 2 pt). B: Different HBeAg (p17e) expression from pMH transfected HepG2 cells (day 3 pt). Presence or absence of the precore initiation codon is indicated (preC +/-). HBcAg from HBV infected liver was used as positive control.

Dane particles were assessed for all three pMH constructs by cesium chloride equilibrium centrifugation of cell culture supernatants, followed by endogenous polymerase reaction assay. Qualitatively similar results were obtained with all three pMH constructs. As shown in Fig. 4A for pMH-9/3091, two peaks were obtained, the higher density peak consisting of HBcAg, i.e. core particles, the lower density peak consisting of HBsAg. From the HBsAg peak, the denser fractions (expected to contain virus) were pooled and assayed for endogenous polymerase activity. The products of this reaction when characterized by agarose gel electrophoresis (Fig. 4B) gave the same pattern of circular and linear viral DNA as obtained with HBV from serum of an HBV-infected patient. With the other pMH constructs, the results were similar but viral DNA was less abundant and the circular DNA species was obtained in variable amounts (not shown). Furthermore, enveloped virus particles (of about 45 nm diameter) obtained from expression of all three pMH constructs were identified by electron microscopic examination of the same HBsAg-

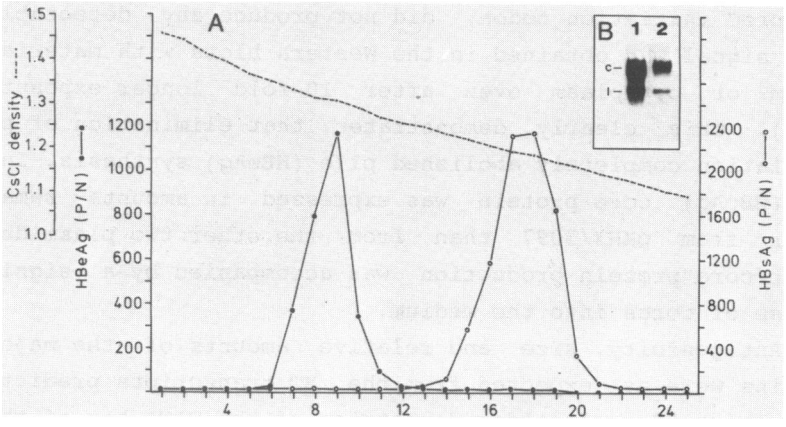


Figure 4: Detection of HBV-like particles. A: Cesium chloride equilibrium gradient profile of medium from pMH-9/3091 transfected HepG2 cells as assayed by HBeAg and HBsAg RIAs. B: Products of endogenous DNA polymerase assay separated on 1.5 % agarose gel. (1) HBV from human serum, (2) pooled fractions 13-16 from HBsAg peak of cesium chloride gradient. (c) relaxed circular HBV DNA. (l) linear HBV DNA.

positive cesium chloride gradient fractions as used for endogenous polymerase assay (data not shown).

Taken together, these results demonstrate that particles indistinguishable from Dane particles with respect to DNA species, biologically active endogenous DNA polymerase and electron microscopic image have been formed from expression of the pMH plasmids in HepG2 cells. Similar results have been obtained from transient expression of pMH3/3091 in Hela cells or in a stably transformed HeLa cell line, although yields of polymerase containing particles were about 10-fold reduced (not shown).

Characterization of core gene products from preC variants

Cell culture supernatants and cytoplasm of HepG2 cells transfected with each of the pMH plasmids were harvested, viral core gene products concentrated by immunoprecipitation with HBcAg/HBeAg specific antisera and analysed by SDS-PAGE and Western blotting. The results of this analysis (Fig. 3B) demonstrate that cells transfected with plasmids pMH3/3091 or pMH-9/3091 secreted p17e (HBeAg) into the medium and also contained p21c (core antigen) in their cytoplasm, but in different quantities. In contrast, cells transfected with pMH3/3097, which lacks an in-

tact preC initiation codon, did not produce any detectable p17e as no signal was obtained in the Western blots with material from medium or cytoplasm even after 10-fold longer exposure (not shown). This clearly demonstrates that elimination of precore translation completely abolished p17e (HBeAg) synthesis. Instead, p21c (HBcAg) core protein was expressed in amounts remarkably higher from pMH3/3097 than from the other two plasmids. This higher core protein production was accompanied by a significant release of cores into the medium.

Antigenicity, size and relative amounts of the major core proteins were as expected from the MT transcripts predicted to start about 30 nucleotides downstream of the TATA box of the MT-promoter (Fig. 1, upper part). Preliminary mapping of 5'-ends by primer extension analysis confirmed the presence of these transcripts, but additional 5'-ends were also detected further upstream within the MT promoter region. These could be translated to a minor extent and explain the low HBeAg expression from pMH-9/3091 whose normal MT transcript starts inside the precore sequence.

Detection of minor core gene products

In experiments with very high HBcAg/HBeAg expression, the cytoplasm of pMH3/3091 transfected cells contained in addition to proteins p21c and p17e other core gene-related proteins of approximately 25 kd, 23 kd and 22 kd MW (Fig. 5). These proteins may represent precursors for p17e derived from translation of the preC/C gene which were not fully processed and secreted under these particular conditions. Similar protein species have also been detected in cell free translation/secretion of preC/C mRNA synthesized *in vitro* (Garcia,P.D., Gerlich,W.H., personal communications).

In HBV-related liver carcinomas, proteins containing both core and pol sequences have been detected (17), which in analogy to similar fusions in other retroelements are believed to be related to yet undetected core/pol fusion proteins occuring in the normal HBV replication cycle. As production of intracellular p21c was the highest with plasmid pMH3/3097, HepG2 cells transfected with this plasmid were investigated for the presence of fused core and pol products. C-specific proteins were harvested by

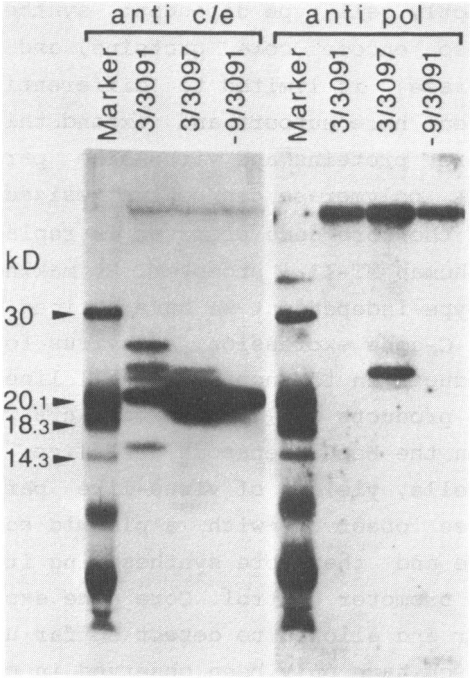


Figure 5: Western blot of minor core proteins. Core gene products from cytoplasm of pMH transfected HepG2 cells were collected by immunoprecipitation with HBc/eAg specific antisera. Proteins were analysed by Western blotting with HBc/eAg specific antisera (left, autoradiographed for 5 days) and a pol-frame specific antiserum (right, autoradiographed for 14 days). Marker, $^{14}C$ labelled protein size marker.

immunoprecipitation with a mixture of HBcAg-specific antisera and analysed in a Western blot using the same antisera or an antiserum elicited against N-terminal pol frame amino acid sequences (17 and Materials and Methods). As shown in Fig. 5, a protein of 23 kd MW was detected with both antisera and, therefore, appears to represent a c-pol fusion protein. No other larger core/pol fusion proteins or pol gene products as predicted from the HBV gene structure were detected.

## DISCUSSION

Circumstantial evidence from various systems indicates that synthesis of pregenomic transcripts is the crucial and tissue-specific step in HBV replication. While transcription of the S-

gene is not strictly cell type dependent, synthesis of pregenomic RNAs (which also encode core proteins, and probably also the viral DNA polymerase) is limited to differentiated hepatocytes. The data presented here support and extend this view in demonstrating that core proteins and virus-like particles containing viral DNA and DNA polymerase can be synthesized irrespective of the cell type if the core gene promoter is replaced by the ubiquitously active human MT-$II_A$ promoter. By making pregenomic RNA synthesis cell type-independent we have noticed however, that S-gene expression, C-gene expression, and virus formation were all substantially reduced in the non-liver cell line HeLa. Therefore, analysis of gene products and of preC variants was carried out in greater detail in the HepG2 hepatoma cell line.

In HepG2 cells, yields of virus-like particles were comparable to those observed with a plasmid containing a tandem repeat HBV genome and therefore synthesizing its pregenomic RNA under endogenous promoter control. Core gene expression was sometimes even higher and allowed to detect so far unknown minor core gene products which have only been observed in cell free translation systems (9) or in HBV-related tumor tissue (17). The detection of these products resulting from transient expression of the HBV genome supports the current concepts of biosynthesis of secreted HBeAg, as well as of viral DNA polymerase by ribosomal frame shifting (1).

Core gene transcripts appear to initiate heterogeneously around the preC start *in vivo* and *in vitro* (4, 19, 20) and thus lead to translation products with or without the preC region. As the MT-$II_A$ promoter is known to direct a single transcript, variants possessing different transcription initiation sites were constructed to simulate this situation. Ratios of core proteins synthesized during transient expression were in general agreement with their predicted transcripts (Fig. 1). Thus, plasmid pMH3/3091 expected to encode preC mRNA directed mainly HBeAg secretion and synthesis of only low amounts of intracellular cores. In contrast, intracellular 21 kd core antigen was the major product produced by pMH-9/3091, and plasmid pMH3/3097 lacking a functional preC initiation codon gave exclusively rise to large amounts of cores.

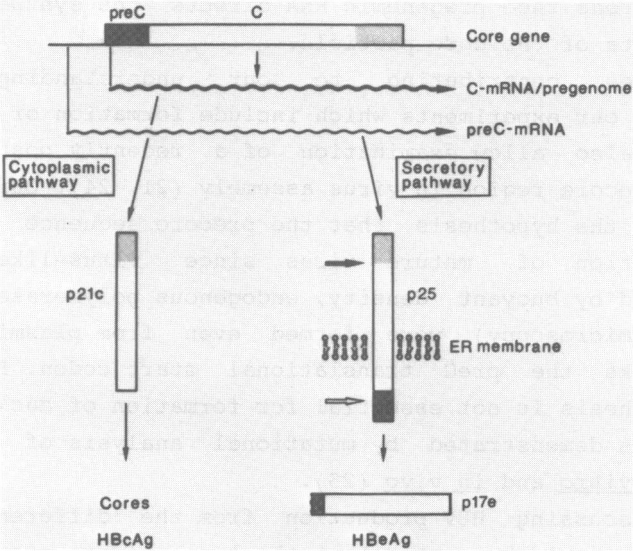


Figure 6: Schematic representation of the two alternate expression pathways for HBV core gene products. The preC region is dark shaded and the C-terminal part missing in HBeAg is light shaded. Messenger RNAs are shown as wavy lines, the C-mRNA as thick, and the less abundant (hypothetical) preC-mRNA as thin wavy line.

Essentially similar results have been obtained by others in tissue culture experiments in which the isolated C-gene was expressed under foreign promoter control and where deletion of most of the precore region was reported to largely abolish HBeAg synthesis (21, 22, 23). Our immunoblot analysis allows to go beyond this general interpretation. First, it shows that the HBeAg produced and secreted from the transfected cells corresponds to a single and homogeneous protein species. Second and more importantly, it demonstrates that translation of an intact preC region is an absolute prerequisite for HBeAg synthesis by secretion and processing of a preC/C precursor protein with a predicted MW of 25 kd because the precore region is maintained intact in pMH3/3091 and pMH-9/3091 and only lacking a single nucleotide in pMH3/3097. Thus, by directing heterogeneous transcriptional starts the HBV C-gene promoter can induce alternate pathways of core gene expression (Fig. 6): transcripts starting in front of the precore ATG lead to a primary core gene product containing a signal sequence for secretion and processing to

HBeAg, whereas the pregenomic RNA directs the synthesis of the constituents of the core particle.

Besides contributing to our understanding of HBeAg synthesis, our experiments which include formation of virus-like particles also allow examination of a recently postulated role for the precore region in virus assembly (21, 24). Our data disagree with the hypothesis that the precore sequence is required for formation of mature virus since virus-like particles (identified by buoyant density, endogenous polymerase assay and electron microscopy) were formed even from plasmid pMH3/3097 which lacks the preC translational start codon. Furthermore, HBeAg synthesis is not essential for formation of duck hepatitis B virus as demonstrated by mutational analysis of the precore region in vitro and in vivo (25).

In discussing HBV production from the different pMH constructs it should be noted that the transcripts produced, packaged, and reverse transcribed into HBV genomes differ with respect to sequence context and length at their 5'-ends and are different from the in vivo pregenomic RNA (Fig. 1). This situation may be reflected by the different amounts and ratios of linear and circular genomes observed after endogenous polymerase reaction. It will be interesting to learn whether all RNA sequences produced function equally well in packaging and reverse transcription or whether some are preferentially utilized as pregenome.

In conclusion, we have established an alternative cell culture system for the production of HBV from cloned viral DNA. First, by placing a foreign promoter into the appropriate position we have avoided the requirement for the ample redundancy (or circularisation) needed with HBV DNA alone to provide the fully functional HBV core gene promoter. This promoter encompasses with its tissue-specific upstream elements about one third of the HBV genome including the X-gene and part of the pol-gene (Fig. 1) (26). Second, by variation of the precore sequences close to the foreign promoter we have shown that the precore sequence is not essential for virus formation. In addition, uncoupling of genomic promoter and HBV genome as achieved in the pMH plasmids should facilitate a mutational analysis of these genes and avoid a loss

of mutations by circularisation of viral DNA templates during transient expression.

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