A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigens in *Xenopus* oocytes

(synthetic mRNA/SP6 polymerase/carboxyl-terminal processing)

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ABSTRACT Using synthetic hepatitis B virus (HBV) mRNAs, we have shown that expression of HBV core-antigen gene sequences in *Xenopus* oocytes leads to the stable accumulation of 21-kDa cytoplasmic core protein (P21). In contrast, expression of precore plus core sequences leads mainly to the secretion of a heterogeneous population of proteins ranging in size from 15 to 22 kDa that collectively display viral e antigen (HBeAg) activity. We demonstrate that the precore region contains a cleavable 19 amino acid signal peptide that targets the precore proteins to the secretory pathway. The initial product of translocation (P22) is further processed during migration through the secretory pathway, apparently by a series of cleavage events at the arginine-rich carboxyl terminus, to yield multiple proteins of 15–18 kDa (P15–P18) that are secreted along with some P22. Our results indicate that serum HBeAg is generated by a signal peptide-mediated secretion event dependent on precore sequences.

Hepatitis B virus (HBV) e antigen (HBeAg) is an important serological marker of acute HBV infection, indicative of the presence of infectious 42-nm HBV virion (Dane) particles (1, 2). HBeAg is related to the 21-kDa core (C) protein (P21), the major structural protein of the 27-nm viral nucleocapsid. P21 and HBeAg display antigenic crossreactivity (3) and HBeAg can be derived from recombinant C protein (4). HBeAg has also been characterized in serum as a 15.5-kDa polypeptide, which has been shown by carboxyl-terminal sequence analysis to lack the last 34–36 amino acid residues of P21 (5). Although HBeAg is released upon disruption of core or virion particles (3, 6), the mechanism behind the genesis of serum HBeAg has been unknown until recently.

The HBV C gene contains a pre-C region encoding 29 largely hydrophobic amino acids located between the first two in-frame ATG codons of the open reading frame (ORF). Since HBV produces multiple pregenomic transcripts with initiation sites that straddle the pre-C ATG (7, 8), both pre-C and C proteins can be generated *in vivo*. Transient expression of the pre-C/C locus in mammalian cells has shown that the pre-C region is required for the secretion of HBeAg (9–12). A signal peptide within pre-C was proposed to mediate HBeAg secretion (9). However, these studies provided no molecular characterization of secreted HBeAg or the pathway involved in its genesis. Previously, we showed (13) that *Xenopus* oocytes programmed with synthetic mRNAs provide a powerful means to express the three HBV surface antigens and to elucidate interactions between them. In the work described in this report, we have used the oocyte system to translate synthetic mRNAs containing the C ORF sequence with and without the pre-C sequence. Expression

of the C ORF without pre-C results in the accumulation of stable, cytoplasmic C protein (P21). Inclusion of pre-C sequence leads to secretion of a heterogeneous array of proteins that display HBeAg activity. A 19 amino acid signal peptide within pre-C mediates this secretion process. Removal of up to 36 amino acid residues from the carboxyl terminus of the pre-C/C protein accompanies HBeAg secretion.

MATERIALS AND METHODS

Plasmid Constructions. M13 phage-mediated site-directed mutagenesis was performed as described (14) to generate a unique *Hind*III site at nucleotide 1757 (sequence TAGGTT to AAGCTT for the pre-C construct) or nucleotide 1821 (sequence AACTTT to AAGCTT for the C construct) of the HBV genome (15). A separate mutagenesis experiment generated a unique *Sal* I site at location 1940 (sequence GTGAG to GTCGAC). The two different 1.7-kilobase *Hind*III–*Eco*RI fragments and the 1.8-kilobase *Eco*RI–*Sal* I fragment were ligated into *Hind*III/*Sal* I double-digested plasmid pSP64 (16). This generated two plasmids (pSP-PC and pSP-C) containing the entire HBV genome flanked by direct repeats of 180 or 120 base pairs, respectively. In pSP-PC the pre-C AUG abuts the SP6 promoter, vs. the C AUG for pSP-C. To construct the truncated mutant, the sequence AGACGA (nucleotides 2351–2356) was mutated to two in-phase termination codons (TGATGA), thus removing the arginine-rich tail from the pre-C protein sequence. The mutated sequence was introduced as described (17) into pSP-PC to generate the plasmid pSP-PC δ . All the mutations were verified by direct nucleotide sequencing.

***In Vitro* Transcription and *Xenopus* Oocyte Microinjection.** Procedures for *in vitro* transcription and oocyte microinjection were as described (13). In brief, plasmids were linearized with *Sal* I and transcribed *in vitro* by the SP6 polymerase in the presence of the methylated cap m⁷GpppG. Approximately 20–30 nl of the capped RNA (0.4 mg/ml in H₂O) was injected into each oocyte. After labeling of oocytes with [³⁵S]methionine and preparation of oocyte extracts (13), immunoprecipitations were performed as described (13) with 0.5–1 μ l of rabbit antiserum prepared against denatured C protein (gift of George Kuo, Chiron Corp.). Immunoprecipitated proteins were resolved in NaDodSO₄/15% polyacrylamide gels.

Proteinase K Protection Experiments. Oocytes were injected with either pre-C or C mRNA. Batches of 38–50 healthy oocytes were selected the next day. Oocyte proteins were labeled with [³⁵S]methionine. Labeled oocytes were

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Abbreviations: HBV, hepatitis B virus; HBeAg, HBV e antigen; C, core; pre-C, precore; ORF, open reading frame.

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homogenized in 0.8–1.0 ml of ice-cold buffer containing 10% sucrose, 150 mM NaCl, 2 mM KCl, 10 mM Mg(OAc)₂, 200 mM Tris chloride (pH 7.5), and 1 mM dithiothreitol. Proteinase K digestions were performed in a final volume of 170 μ l containing 160 μ l of oocyte homogenate and 10 mM CaCl₂. Proteinase K and Triton X-100 were added, as indicated, to final concentrations of 240 μ g/ml and 1% (vol/vol), respectively. Digestion was performed for 3–4 hr at 0°C and then stopped by rapid addition of 1 μ l of 1 M phenylmethanesulfonyl fluoride followed by transfer of the reaction mixture to a tube containing 20 μ l of 1 M Tris chloride (pH 9.5) and 10 μ l of 20% (wt/vol) NaDodSO₄, mixing, and heating to 100°C for 2 min. After brief cooling, proteins were immunoprecipitated with anti-C antibody prior to NaDodSO₄/polyacrylamide gel analysis.

Amino Acid Sequencing. Incubation medium from $\approx$ 100 pre-C mRNA-injected oocytes was pooled after 48 hr of labeling with either [³⁵S]methionine or [³H]leucine. HBeAg was immunoprecipitated with 7.5 μ l of rabbit antiserum. The immune complexes were adsorbed onto 100 μ l of prewashed 10% Pansorbin (Calbiochem), washed four times with wash solution and twice with 10 mM (NH₄)HCO₃, and finally boiled in 200 μ l of 10 mM (NH₄)HCO₃ for 10 min. After brief centrifugation in a microcentrifuge, supernatants containing 25,000 cpm ([³⁵S]methionine) or 75,000 cpm ([³H]leucine) of labeled HBeAg were subjected to sequencing by Edman degradation procedures. The radioactivity in each cycle fraction was determined by scintillation counting.

RESULTS

Translation of C and Pre-C mRNAs in Oocytes: Only Pre-C mRNA Gives Rise to Secreted HBeAg. The autoradiogram shown in Fig. 1 depicts the results of an experiment in which oocytes injected with C or pre-C mRNA were incubated for increasing lengths of time with [³⁵S]methionine. C mRNA (lanes 2–6) produced a predominant immunoprecipitable species of 21 kDa (P21) that comigrated with the product of *in vitro* translation of C mRNA (lower band, lane 7) and thus

FIG. 1. Translation of pre-C and C mRNAs in frog oocytes. The autoradiogram depicts the ³⁵S-labeled intracellular products of translation of C mRNA (lanes 2–6) or pre-C mRNA (lanes 8–12), fractionated in a NaDodSO₄/15% polyacrylamide gel. The injection, labeling, and immunoprecipitation procedures are described in *Materials and Methods* and in ref. 13. Each lane depicts immunoprecipitated proteins derived from 3.5 oocytes labeled with [³⁵S]methionine for the following lengths of time postinjection: lanes 2 and 8, 1.5 hr; lanes 3 and 9, 2.5 hr; lanes 4 and 10, 5 hr; lanes 5 and 11, 9 hr; lanes 6 and 12, 25 hr. Lane 1 shows intracellular proteins immunoprecipitated from control (uninjected) oocytes labeled for 25 hr. The position of the C protein (P21) is shown at left, together with the positions of the protein molecular mass markers (in kDa); the positions and approximate sizes of the major pre-C proteins are shown at right. The corresponding secreted proteins generated during 25 hr of labeling are shown in lanes 13 (pre-C mRNA), 14 (C mRNA), and 15 (control oocytes). The products of *in vitro* translation of pre-C and C mRNAs are shown in lane 7 (marked by dots).

presumably represents authentic C protein. A minor 46-kDa protein (e.g., lane 6) may represent a dimer of C protein. P21 accumulated stably in the oocyte cytoplasm during longer labeling periods with only traces of degradation (lanes 2–6). No C-related species were secreted into the medium (lane 14). In contrast, translation of pre-C mRNA in oocytes resulted in a cluster of proteins (P15–P24) ranging from 15 to 24 kDa in size (lanes 8–12), whereas only a single species of 25 kDa was observed upon translation *in vitro* (upper band, lane 7). Upon brief labeling (1.5 hr; lane 8), only two species (P22 and P24), migrating in size between the *in vitro* C and pre-C proteins, were visible. After longer labeling (e.g., 9 hr; lane 11), at least four smaller species (P15–P18) were seen. After 25 hr of labeling (lane 12) the P24 band was substantially diminished, suggesting that this protein is unstable [a 25-hr labeling period constitutes a significant chase (18)]. Comparison of the profiles of P15–P18 in lanes 10–12 suggests that the smaller species (P15–P16) become more prominent with increasing incubation time. Analysis of the oocyte medium (lane 13) showed that P15–P18 were the major secreted species. P22 was secreted (although not efficiently with respect to its intracellular concentration); no P24 was found in the medium. It should be noted that P22 and P24 are actually not single species. In most experiments both P22 and P24 were resolved into two proteins.

By radioimmunoassay (data not shown) we detected significant HBeAg + HBeAg activity in the medium from pre-C-injected oocytes (P/N ratio = 8, see ref. 9 for methodology) but no HBeAg activity alone (P/N $\approx$ 1.0). Thus, the secreted proteins in Fig. 1 must contain or represent HBeAg.

Pulse-Chase Analysis of Pre-C Protein Processing. The relationship among the various pre-C proteins was examined by pulse-chase experiments. In two such experiments (Fig. 2 A and B), longer chase times with nonradioactive methionine resulted in a decrease in the intracellular levels of both P24 and P22 with a concomitant increase in both intracellular and secreted P15–P18 (Fig. 2 A, lanes 2–7, and B, lanes 2–5). From these data it appears that either P24 or P22 (or both) gives rise to intracellular P15–P18.

The radioactivity in intracellular P15–P18 species was also chased with time from higher to lower molecular mass. With no chase, P17–P18 predominated (Fig. 2A, lane 2); after a 7-hr chase (lane 6) P15 was the major species. The profile of secreted products showed a similar shift in molecular mass with increasing time of chase (Fig. 2A, lanes 12–15; Fig. 2B, lanes 10–12). At least in Fig. 2A the disappearance of intracellular P15–P18 correlates well with the appearance of these products in the medium.

It is apparent from Fig. 2 that the levels of the different precore proteins vary somewhat from experiment to experiment. P24 is particularly variable in quantity, whereas P22 consistently appears at high levels in all experiments. The levels and composition of both the intracellular and secreted HBeAg (P15–P18 and P22) also vary between experiments (cf. Fig. 2 A and B). These variations do not affect the conclusions drawn from our experiments.

Intracellular Pre-C Proteins Are Translocated into the Lumen of the Endoplasmic Reticulum. Fig. 3 shows the result of a protease protection experiment in which intact membrane preparations, prepared from oocytes injected with C mRNA (lane 8) or pre-C mRNA (lane 2), were treated with proteinase K to determine which proteins are translocated into the lumen of the endoplasmic reticulum (and are thus protease-resistant) and which are cytoplasmic (protease-sensitive). C protein (P21, lane 8) is cleaved, as expected, by protease (lane 9) to yield a fragment approximately the size of secreted P15. This fragment is intrinsically resistant to further protease digestion (17) even in the presence of 1 mg of proteinase K per ml (D.N.S., unpublished data). In contrast to P21, P22 is clearly protease-resistant (lane 3, note

FIG. 2. Pulse-chase analysis of pre-C proteins. Oocytes injected with pre-C mRNA were labeled with [35 S]methionine for 4.5 hr (A) or 5.5 hr (B) prior to the addition of nonradioactive methionine to the medium (final concentration, 20 mM) to initiate the chase. (A) Autoradiogram of the products immunoprecipitated from the intracellular (lanes 1–8) or secreted (lanes 9–16) oocyte proteins. Samples were prepared after the indicated times of chase: lanes 1 and 9 (control oocytes) and lanes 2 and 10, 0 hr; lanes 3 and 11, 1 hr; lanes 4 and 12, 2 hr; lanes 5 and 13, 4 hr; lanes 6 and 14, 7 hr; lanes 7 and 15 and lanes 8 and 16 (control oocytes), 20 hr. (B) Autoradiogram of immunoprecipitated intracellular (lanes 1–6) and secreted (lanes 8–13) oocyte proteins. Comparison with A demonstrates the variability of results obtained between different batches of oocytes. Chase times were as follows: lanes 1 and 8 (control oocytes) and lanes 2 and 9, 0 hr; lanes 3 and 10, 2 hr; lanes 4 and 11, 6 hr; lanes 5 and 12 and lanes 6 and 13 (control oocytes), 20 hr. *In vitro* translated pre-C and C protein markers are present in lane 7. Positions of the pre-C proteins P15–P18, P22, and P24 are indicated to the left of each panel; also given are the positions of the protein molecular weight markers (in kDa).

that the [35 S]protein background is removed by the protease, implying translocation into microsomal vesicles. Permeabilization of the membrane vesicles with Triton X-100 allows proteinase K to degrade the pre-C proteins (lane 4); Triton X-100 alone has no effect (data not shown). The translocation of P22 has been investigated by sedimentation of intact microsomal membranes prepared from oocytes injected with pre-C mRNA. P22 and P15–P18 cosedimented with the microsomes, consistent with the notion of translocation (D.N.S., unpublished data).

A 19 Amino Acid Signal Peptide Has Been Removed From the Amino Terminus of the Secreted Pre-C Proteins. Secretion of proteins into the cellular medium is often accompanied by removal of a signal peptide. To determine whether the secretion of HBeAg requires removal of a signal peptide, we sequenced the amino terminus of the secreted pre-C proteins. Oocytes injected with pre-C mRNA were labeled with either [35 S]methionine or [3 H]leucine. Secreted pre-C proteins immunoprecipitated from the incubation medium were subjected to protein sequencing. A discrete methionine peak was located at sequencing cycle 11 (Fig. 4A). A minor peak in cycle 1 was most likely caused by contaminating proteins. [3 H]Leucine-labeled HBeAg gave rise to three discrete peaks

at cycles 3, 5, and 8 (Fig. 4B). These data demonstrate a unique amino terminus for the secreted HBeAg proteins corresponding to the 20th amino acid of the pre-C sequence. This is consistent with *in vitro* experiments that show that the first 19 amino acids of the pre-C sequence function as a single peptide (17).

Heterogeneity of the Secreted Pre-C Proteins Resides at the Carboxyl Terminus. Since the secreted HBeAg products have a single amino terminus, their small size and heterogeneity might be a consequence of multiple processing events at the carboxyl terminus. Earlier characterizations of serum-

FIG. 3. Protease protection experiments demonstrating the translocation of pre-C proteins. Oocytes were injected with pre-C mRNA (lanes 2–4) or C mRNA (lanes 8–10). Control oocyte data are shown in lanes 5–7 and *in vitro* generated pre-C and C marker proteins in lane 1. After incubation of oocytes with [35 S]methionine, oocyte homogenates containing intact membranes were prepared and treated with the indicated reagents prior to immunoprecipitation: lanes 2, 5, and 8, no treatment; lanes 3, 6, and 9, proteinase K (240 μ g/ml); lanes 4, 7, and 10, proteinase K (240 μ g/ml) and Triton X-100 (1%, vol/vol). The positions of the major pre-C species are indicated at left.

FIG. 4. The amino-terminal sequence of secreted HBeAg. Edman degradation profiles are shown for HBeAg labeled with [35 S]methionine (A) or [3 H]leucine (B). The radioactivity released in each cycle is given in cpm for 35 S and dpm for 3 H. The sequence of the first 30 amino acids of the pre-C/C ORF is depicted beneath the figure. The 19 amino acid signal peptide is highlighted with a box. The cleavage site between Ala-19 and Ser-20 is marked by an arrow. The positions of the leucine residues (solid arrowheads) and the methionine residue (unfilled arrowhead) are indicated above the sequence along with their corresponding positions with respect to the amino terminus of secreted HBeAg. This particular grouping of leucine and methionine residues is not found elsewhere within the pre-C/C ORF.

FIG. 5. Secretion of a mutant HBeAg. The autoradiogram shows the proteins immunoprecipitated from intracellular (lanes 2–4) or secreted (lanes 5–7) oocyte fractions. The oocytes were injected with wild-type (lanes 2 and 5) or mutant (PC δ , lanes 3 and 6) pre-C mRNA or were uninjected (control, lanes 4 and 7). Each lane represents the proteins expressed by 4 oocytes (wild-type) or 6 oocytes (mutant and control). The positions of the various oocyte pre-C species and the *in vitro* pre-C and C markers (lane 1) are indicated at left; molecular mass markers (in kDa) are presented at right.

derived HBeAg support the idea of carboxyl-terminal processing (5) that removes the arginine-rich tail (the protamine-like domain) of the C protein. To investigate the processing at the carboxyl terminus, we created a mutant in which two consecutive in-phase termination codons in the pre-C gene precisely remove the 37 amino acid arginine-rich tail (17 out of the 37 residues are arginines).

A protein of about 15 kDa was immunoprecipitated from oocytes injected with mutant pre-C δ RNA (Fig. 5, lane 3). The same protein, without further processing, was secreted (lane 6). Under these conditions the major intracellular wild-type pre-C product was P22 (lane 2) and the major secreted pre-C proteins were 17–18 kDa (lane 5) with traces of 16- and even 15-kDa proteins visible upon longer exposure of the autoradiogram. These data suggest that the heterogeneity of secreted HBeAg is indeed caused by cleavage within the arginine-rich carboxyl terminus, and they further support the idea of a unique amino terminus. The mobility of the mutant protein in NaDodSO₄/polyacrylamide gels is essentially equal to that of the fastest migrating species of secreted HBeAg, suggesting that the carboxyl-terminal processing of pre-C ultimately results in an almost precise removal of the arginine-rich region. Although the presence of multiple cleavage sites within the carboxyl terminus of HBeAg satisfactorily accounts for the species visible in our gels, we have not ruled out the possibility that unknown modifications may contribute to the heterogeneity.

DISCUSSION

Using *Xenopus* oocytes programmed with synthetic HBV mRNAs, we have shown that C mRNA directs synthesis of mature C protein, P21. This antigen is found exclusively in the oocyte cytoplasm. In contrast, pre-C mRNA, which includes the 29 amino acid pre-C region along with the C coding sequence, does not generate significant quantities of P21. Instead, pre-C mRNA directs the synthesis and secretion of a heterogeneous set of proteins that display HBeAg activity. We have demonstrated that the secretion of HBeAg is mediated by the first 19 amino acids of the pre-C region, which constitute a signal peptide. The HBeAg proteins are exported from the oocyte via the normal cellular secretory pathway.

We propose the following model of HBeAg secretion: on translation the 19 amino acid signal peptide directs the pre-C protein to the endoplasmic reticulum membranes, where the signal peptide is cleaved, probably concomitantly with the translocation of P22 (17). Protein sequence analysis of gel-

purified P22 has verified that the signal peptide has been removed (data not shown). P22 in turn is further cleaved by an activity that removes part or all of the 37 amino acid arginine-rich carboxyl-terminal tail. The resultant four to five proteins (P15–P18), together with small amounts of P22, are secreted into the medium. These proteins collectively display HBeAg activity. Thus, HBeAg is essentially a C protein with an extra 10 amino acids at the amino terminus and a segment of up to 37 amino acids (comprising the putative DNA-binding domain) removed from the carboxyl terminus.

Recent data from several groups provide strong support for the above model for the mechanism of serum HBeAg biogenesis. Expression studies on the pre-C/C locus in mammalian cells first suggested that the pre-C region is required for the efficient secretion of HBeAg (9–12, 19). Introduction of a stop codon into the pre-C region of duck HBV prevents the secretion of duck HBeAg in tissue culture cells (20). Pre-C sequences have been shown to target pre-C proteins to cell membranes (9, 21). When pre-C mRNA is translated *in vitro* in the presence of microsomal membranes, cotranslational processing and segregation of a 22-kDa pre-C protein is observed (17, 22, 23). These studies confirmed that a 19 amino acid signal peptide is removed during production of P22 (17, 22). Thus the role of the pre-C signal peptide in HBeAg secretion is clear. In the present work we found that secretion of C proteins does not occur if the pre-C region is absent, although others have claimed (10, 11) that low-level secretion of C antigen (HBeAg) or even HBeAg can be achieved in the absence of pre-C sequences. Such reports could also be attributable to cell lysis (12). Processing of HBeAg by removal of carboxyl-terminal residues is also well established. Takahashi *et al.* (5) showed that serum HBeAg is colinear with C antigen but lacks the last 34–36 amino acids of P21 (5). More recently, Schlicht *et al.* (20) provided an immunological demonstration that the carboxyl terminus of the duck HBV C protein is missing from secreted duck HBeAg. The only remaining point of contention concerns the extent of heterogeneity of the HBeAg species. Some studies (5, 19) suggest that HBeAg is a single species of 15–16 kDa; this study and others (20, 22) find evidence for HBeAg heterogeneity. The last 36 amino acids of the C sequence contain four Arg-Arg sequences. We propose that cleavage by an enzyme or enzymes at one or more of these sites could satisfactorily account for the heterogeneity of HBeAg observed in the oocyte system. Since P22 is stable for several hours following translocation (see Fig. 1), we propose that the cleavage of HBeAg is likely to occur in a distal part of the secretory pathway—e.g., the Golgi apparatus. Variations in the level or efficiency of this cleavage activity might explain the different extents of HBeAg processing seen in different systems. Our data do not provide support for the recent idea (24) that HBeAg may be generated by self-cleavage of C or pre-C proteins. Although we cannot completely rule out the latter possibility, it is clear from the data in Fig. 1 that P21 is stable for long periods of time without giving rise to HBeAg products.

Although the basic details of HBeAg secretion are outlined here, the secretion process may be more complex than our simple model suggests. *In vitro* translation of pre-C mRNA in the presence of microsomal membranes yields two species that comigrate with the two proteins designated P22 in this study (see Fig. 2B, lane 5). Careful *in vitro* translation studies indicate a precursor-product relationship between the lower and higher molecular mass species of P22, respectively (17), consistent with an unknown modification event. Surprisingly, the higher molecular mass species appears to be quantitatively released into the cytoplasm despite removal of the signal peptide. It is not clear whether such an unprecedented release mechanism operates *in vivo*. We have not seen evidence for this in the oocyte system, although protein

degradation could easily obscure such an event. These observations, together with the unexplained nature of the P24 product observed in oocytes, demonstrate that the process of secretion of HBeAg is not completely understood.

Finally, these observations raise questions concerning the role of HBeAg in HBV biology (for reviews of HBV see refs. 25 and 26). The most definitive data on this point come from elegant studies involving the infection of ducks with duck HBV in which the pre-C region has been removed by mutation. These studies reveal that the pre-C region plays no obvious role in the assembly, replication, or transmission of the duck virus (26, 27). Although the distant homology between the duck and human viruses and the different structures of the pre-C/C regions (for instance, the duck C protein is 35 kDa vs. 21 kDa for the human HBV C protein) make it dangerous to extrapolate this result to the human virus, it now appears that the pre-C region is dispensable at least for the formation of HBV virions (19). Nevertheless, the conservation of the pre-C region among all the hepatitis viruses throughout evolution, together with the observation that a pre-gag region may mediate the secretion of gag-related proteins in Moloney leukemia virus (28), argue for some function, albeit perhaps a subtle one, for the pre-C region. Perhaps HBeAg modulates the host immune response to HBV infection. Additional studies using animal models will be required to elucidate this point.

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1. Nordenfelt, E. & Kjellern, L. (1975) *Intervirology* **5**, 225–232.
2. Takahashi, K., Imai, M., Tsuda, F., Takahashi, T., Miyakawa, Y. & Mayumi, M. (1976) *J. Immunol.* **117**, 102–105.
3. Takahashi, K., Akahane, Y., Gotanda, T., Mishiro, T., Imai, M., Miyakawa, Y. & Mayumi, M. (1979) *J. Immunol.* **122**, 275–279.
4. MacKay, P., Lees, J. & Murray, K. (1981) *J. Med. Virol.* **8**, 237–243.
5. Takahashi, K., Machida, A., Funatsu, G., Nomura, M., Usuda, S., Aoyagi, S., Tachibana, K., Miyamoto, H., Imai, M., Nakamura, T., Miyakawa, Y. & Mayumi, M. (1983) *J. Immunol.* **130**, 2903–2907.
6. Ohori, H., Yamaki, M., Onodera, S., Yamada, E. & Ishida, N. (1980) *Intervirology* **13**, 74–82.
7. Enders, G. H., Ganem, D. & Varmus, H. E. (1985) *Cell* **42**, 297–302.
8. Buscher, M., Reiser, W., Will, H. & Schaller, H. (1985) *Cell* **40**, 717–724.
9. Ou, J.-H., Laub, O. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1578–1582.
10. Roossinck, M. J., Janeel, S., Loukin, S. H. & Siddiqui, A. (1986) *Mol. Cell. Biol.* **6**, 1393–1400.
11. McLachlan, A., Milich, D. R., Raney, A. K., Riggs, M. G., Hughes, J. L., Sorge, J. & Chisari, F. V. (1987) *J. Virol.* **61**, 683–692.
12. Roossinck, M. J. & Siddiqui, A. (1987) *J. Virol.* **61**, 955–961.
13. Standring, D. N., Ou, J.-H. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9338–9342.
14. Craik, C. S., Largman, C., Fletcher, T., Rocznik, S., Barr, P. J., Fletterick, R. & Rutter, W. J. (1985) *Science* **228**, 291–297.
15. Valenzuela, P., Quiroga, M., Zaldivar, J., Gray, P. & Rutter, W. J. (1980) *ICN-UCLA Symp. Mol. Cell. Biol.* **18**, 57–70.
16. Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
17. Garcia, P. D., Ou, J.-H., Rutter, W. J. & Walter, P. (1988) *J. Cell Biol.* **106**, 1093–1104.
18. Colman, A. (1984) in *Transcription and Translation: A Practical Approach*, eds. Hames, D. & Higgins, S. (IRL, Oxford), pp. 271–301.
19. Junker, M., Galle, P. & Schaller, H. (1987) *Nucleic Acids Res.* **15**, 10117–10132.
20. Schlicht, H. J., Salfeld, J. & Schaller, H. (1987) *J. Virol.* **61**, 3701–3709.
21. Uy, A., Bruss, V., Gerlich, W. H., Kochel, H. G. & Thomsen, R. (1986) *Virology* **155**, 89–96.
22. Bruss, V. & Gerlich, W. H. (1988) *Virology* **163**, 268–275.
23. Weimer, T., Salfeld, J. & Will, H. (1987) *J. Virol.* **61**, 3109–3113.
24. Miller, R. H. (1987) *Science* **236**, 722–725.
25. Standring, D. N. & Rutter, W. J. (1986) *Prog. Liver Dis.* **8**, 311–333.
26. Ganem, D. & Varmus, H. E. (1987) *Annu. Rev. Biochem.* **56**, 651–693.
27. Chang, C., Enders, G., Sprengel, R., Peters, N., Varmus, H. E. & Ganem, D. (1987) *J. Virol.* **61**, 3322–3325.
28. Edwards, S. & Fan, H. (1980) *J. Virol.* **35**, 41–51.