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An Intramolecular Disulfide Bridge between Cys-7 and Cys61 Determines the Structure of the Secretory Core Gene Product (e Antigen) of Hepatitis B Virus

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Hepatitis B virus, the prototypic member of the *Hepadnaviridae*, is a small enveloped DNA virus that replicates via reverse transcription. Efficient usage of its compact 3.2-kb genome is exemplified by the *pre-C/C* gene from which two proteins with largely overlapping primary sequences but distinctly different properties are synthesized: the self-assembling core protein p21c (hepatitis B core antigen [HbcAg]) and the secretory, nonparticulate protein p17e (hepatitis B e antigen [HbeAg]). Mature p17e carries a 10-amino-acid N-terminal extension with a Cys residue (Cys-7). Using transient transfection of a human liver cell line with constructs expressing wild-type p17 or a series of Cys mutants of p17, we show that Cys-7 forms an intramolecular S-S bond to Cys61, which in assembly-competent core proteins is available for intermolecular disulfide bonds between two neighboring subunits. Removal of the Cys-7/Cys61 bond by mutating either residue has differential effects: in the absence of Cys-7, secretion is relatively efficient and independent of Cys61; however, the molecules are exported as homodimers exhibiting both HBe and HBc antigenicity. In the absence of Cys61, the nonpaired Cys-7 interferes with secretion efficiency. The amino acid sequence flanking Cys-7 also contributes to the formation of the proper intramolecular S-S bond. These results suggest that the Cys-7/Cys61 bond imposes on p17e a conformation that is critical for its secretion and distinct biophysical and antigenic properties. This mechanism adds selective disulfide formation to the repertoire of hepatitis B virus for efficient use of its tiny genome.

Hepatitis B virus (HBV) is the prototypic member of the *Hepadnaviridae*, small enveloped DNA viruses that replicate via reverse transcription of an RNA intermediate, the pregenome (see reference 25 for recent reviews on various aspects of hepadnavirus biology). Its tiny, circular 3.2-kb genome carries only four major open reading frames (Fig. 1A). However, a variety of mechanisms allow the virus to increase the number of possible gene products, among them the synthesis of mRNAs with staggered 5′ termini which bracket potential initiation codons. Thereby, proteins with common C termini but variously extended N-terminal regions can be synthesized, as exemplified by the *pre-C/C* gene products (Fig. 1): the RNA pregenome serves as mRNA for the 21-kDa viral core protein (p21c), which has the intrinsic capability to self-assemble into the 180-subunit capsid (reviewed in reference 35), and the replication enzyme (P). The 5′-extended pre-C RNAs encompass the upstream initiation codon of the pre-C region, translation from which results in the synthesis of a precore protein (p25) with an additional 29 N-terminal amino acids (aa). The first 19 residues act as a signal peptide which targets p25 into the cell's secretory pathway (36); after N- and C-terminal processing, the final product is found as a soluble 17-kDa protein (p17e) in the sera of infected individuals. It consists of 149 aa that are identical to the first 149 aa in p21c, preceded by 10 aa remaining from the pre-C sequence (45, 46).

Despite p17e and p21c sharing a large stretch of identical primary sequence, p17e is nonparticulate, whereas p21c self-assembles into core particles, and both proteins act as distinct antigens during HBV infection (26). Core particles (hepatitis B core antigen [HbcAg]) elicit a rapid and strong humoral immune response in almost all infected individuals with no apparent protective effect; p17e (hepatitis B e antigen [HBeAg]) (23) induces a distinct, weaker, and delayed response, which frequently correlates with virus elimination (13). The lack of cross-reactivity of anti-HBcAg and anti-HBeAg indicates that the two antigens have different structures. Part of this can be attributed to their different physical states, i.e., particulate and nonparticulate; however, although denaturation of HBcAg exposes HBeAg epitopes (22), denatured p21c is unlikely to represent true HBeAg, whose biosynthesis involves the passage of quality control compartments which would not allow efficient export of malfolded proteins (14). Therefore, the amino acid sequence specified by the *pre-C/C* gene conceivably might adopt different conformations, one characteristic for the subunits of the nucleocapsid and one characteristic for the nonassembling p17e.

p21c has a characteristic two-domain structure: the basic C-terminal region is involved in nucleic acid binding and proper virus replication (12, 32), and the sequence from the N terminus to about aa position 140 is required and sufficient for assembly (2, 9). Surprisingly, the entire assembly domain is also present in p17e (Fig. 1B), suggesting a structure-determining role for its unique N-terminal 10-aa extension. Using a series of mutants of the four Cys residues in the core protein (at positions 48, 61, 107, and 183), we have derived a topological model for the structure of p21c in the core particle (34) which showed a pronounced tendency of Cys61 to form an intermolecular S-S bond to Cys61 in a neighboring subunit, generating covalently stabilized dimers. Such dimers are also formed by assembly-competent, truncated core proteins (p16 in Fig. 1B) ending with the same amino

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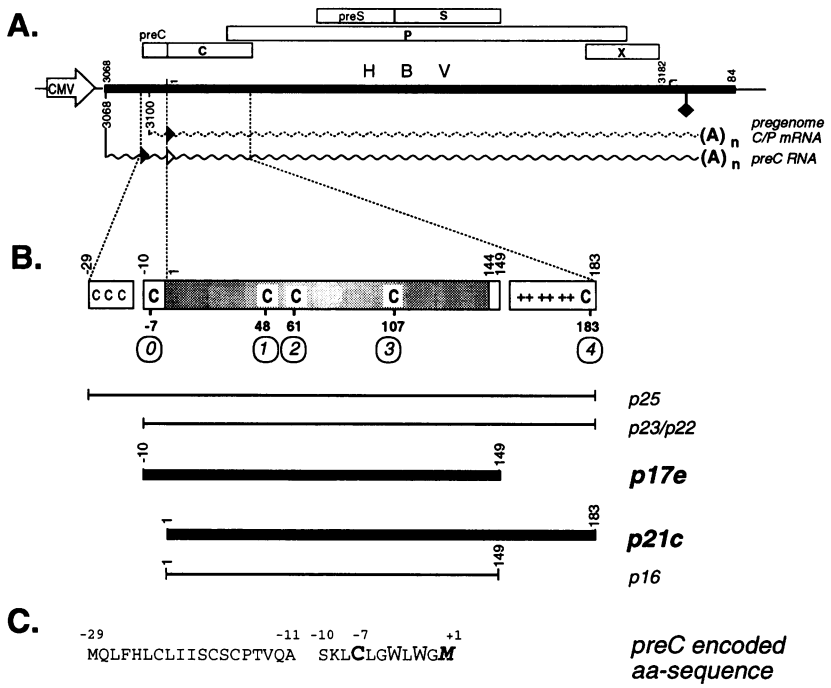

FIG. 1. HBV *pre-C/C* gene products. (A) General structure of the HBeAg expression plasmids used in this study. The basic construct pCHT-9/3068 contains a 1.1-unit-length HBV type 2 (8) genome (thick black line) with its own polyadenylation signal (diamond) under control of the human CMV-IE promoter (arrow marked "CMV"). The numbering system is that of Pasek et al. (38). The positions of the HBV open reading frames are indicated on the top. The wavy lines below depict genomic HBV RNAs; the RNA pregenome–C-P mRNA, not detectably produced from this construct, starts at nt position 3100. The CMV promoter drives transcription of an RNA starting at or close by position 3068, corresponding to one of the previously mapped start sites of the pre-C RNAs. The arrowheads indicate the positions of the pre-C and C initiator codons. (B) Map of the pre-C/C-encoded precore protein and its processing products. Boldface numbers are amino acid positions. The shaded area represents the assembly domain as present in p21c. Cys residues are indicated by "C," together with their positions; the circled numbers below refer to the nomenclature of the Cys mutants as described in the text. Pluses indicate the Arg residues in the clipped-off C-terminal part of the precore protein. The primary sequences of the HBcAg- and HBeAg-related proteins referred to in this report are indicated by bars. Cleavage of the signal peptide (positions −29 to −11) from p25 generates p23/22, which is then C-terminally processed to yield p17e. The core protein p21c consists of aa 1 to 183; p16 (aa 1 to 149) is an artificially truncated assembly-competent core protein variant. (C) Amino acid sequence encoded by the pre-C region. Cys-7 and the Trp residues W-4 and W-2, which were mutated in this study, are highlighted. The italic "M" represents the starting methionine of the core protein p21c.

acids as p17e; p17e, however, carries an extra Cys residue (Cys-7; Fig. 1B and C) in its N-terminal extension which, because of the oxidizing conditions in the secretory pathway (15), could influence structure by disulfide formation. We therefore used a similar mutational approach to investigate the role of the Cys residues in the secretion and structure of HBeAg. Our results show that the authenticity of HBeAg indeed depends critically upon Cys-7, which forms an intramolecular S-S bridge predominantly to Cys61. Mutation of either residue has drastic effects on the secretion competence, structure, and antigenicity of the variant HBeAgs; in particular, replacement of Cys-7 by Ala or Ser produces dimeric HBeAg with HBcAg-like properties. These results indicate that the intramolecular S-S bridge keeps p17e in a specific conformation that prevents assembly and that is responsible for its distinct antigenicity.

MATERIALS AND METHODS

Chemicals and enzymes. All enzymes for molecular cloning experiments were obtained from Boehringer (Mannheim, Germany) or New England Biolabs (Schwalbach, Germany) and were used as recommended by the manufacturer. Oligonucleotides were prepared on an Applied Biosystems 380B synthesizer and purified by electrophoresis on urea-containing polyacrylamide gels. Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were purchased from Serva (Heidelberg, Germany), and agarose and low-melting-point agarose were purchased from FMC Corp., Marine Colloids Division (Rockland, Maine). Protein A-Sepharose and Sephacryl S-200HR were obtained from Pharmacia (Freiburg, Germany).

Bacterial strains, plasmids, and construction of mutants. For all cloning experiments, *Escherichia coli* DH5α cells were used. The bacteria were grown in the presence of 50 μg of ampicillin per ml at 37°C in Standard I medium (Merck, Darmstadt, Germany). The parental HBeAg-expressing construct pCHT-9/3068 is a derivative of pCHT-9/3091 (32); in brief, it contains the human cytomegalovirus (CMV) IE1 promoter followed by a linker containing a *Hin*dIII recognition site and a slightly overlong HBV genome starting at nucleotide (nt) position 3068 (nomenclature according to reference 38), extending through one unit length, and ending with a terminal redundancy at nt position 88. Transcription was experimentally confirmed to initiate within plus or minus 2 nt of nt position 3068, and it ends shortly after the HBV polyadenylation signal at nt positions 16 to 21. This RNA is therefore essentially identical to one of the previously mapped pre-C RNAs (16), whereas no RNA prege-

nome (start site at nt position 3100) should be produced. Mutations at the *C* gene-encoded Cys codons were introduced by transferring suitable restriction fragments from the corresponding *E. coli* expression plasmids (34) into pCHT-9/3068; the sequence between nt positions 7 and 241 is therefore derived from a synthetic *C* gene (30) in which several silent nucleotide exchanges introduce new restriction sites. The codon for Cys-7 (TGC) was changed to Ala (GCC) or Ser (AGC) via polymerase chain reaction with mutagenic primers. Trp4 and Trp2 were replaced by Gly residues, individually and in combination, by replacing the TGG codons with GGG by the same procedure. All mutations were confirmed by sequencing with Sequenase (USB, Cleveland, Ohio). The nomenclature of the constructs and their gene products is as follows: the four *C* gene-encoded Cys residues at aa positions 48, 61, 107, and 183 are consecutively numbered 1, 2, 3, and 4, and the pre-C-encoded Cys-7 is designated 0 and further specified by A or S to indicate the amino acid replacing Cys-7. The construct name indicates which Cys residue(s) is mutated. For instance, mutant 134 is a mutant in which Cys48, Cys107, and Cys183 have been replaced, and so on. The Trp→Gly mutants in the pre-C region are designated −2, −4, and −4−2 to indicate which Trp residue has been replaced by glycine.

Cells and transfections. HuH7 cells (29) were transfected with 20 μg of the appropriate plasmid construct per 10-cm-diameter dish by the calcium phosphate method and were lysed 3 days posttransfection as previously described (33).

Immunological techniques. For Western immunoblotting, proteins were separated on 15% polyacrylamide gels containing 0.1% SDS by the Laemmli system (19); for reducing gels, 50 mM dithiothreitol was added to the sample buffer. Proteins were transferred to nitrocellulose membranes (BA85; Schleicher and Schuell, Dassel, Germany) and detected by incubation with a polyclonal rabbit antiserum that recognizes all known forms of core protein and HBeAg and ^{125}I-labeled protein A (Amersham, Braunschweig, Germany). For immunoprecipitations, the corresponding antiserum was immobilized to protein A-Sepharose. The sandwich enzyme-linked immunosorbent assay (ELISA) to detect HBcAg- or HBeAg-related antigens has been previously described (2); in brief, the polyclonal rabbit anti-HBc/HBe antiserum is used to immobilize the antigen to the ELISA plate, and various HBcAg- or HBeAg-specific monoclonal antibodies are used for detection.

Antibodies and antisera. The polyclonal rabbit antiserum was elicited against denatured core protein produced in *E. coli*. The monoclonal antibodies have been classified as HBcAg or HBeAg specific by competition with human anti-HBcAg or anti-HBeAg antisera (39). The monoclonal antibodies mc158 and mc03 are specific for HBeAg; mc158 recognizes a linear epitope (aa 128 to 133; HBe2 [41]), and mc03 presumably recognizes a discontinuous epitope (HBe1) that partially overlaps with the major HBcAg epitope (39). The monoclonal antibodies mc312 and mc275 are both HBcAg specific and recognize the major HBcAg epitope between aa 76 and 85; they differ in that mc312 recognizes aa 77 to 82 as a linear epitope and also reacts with core protein immobilized on nitrocellulose membranes (39, 40), whereas mc275 probably recognizes a discontinuous epitope in the same region which appears to be present only in particulate core protein. These antibodies were kindly provided by M. Noah, Behringwerke (Marburg, Germany).

RESULTS

Construction of HBeAg expression plasmids. The basic construct used was plasmid pCHT-9/3068, which contains a slightly overlong HBV genome under control of the CMV-IE promoter (Fig. 1A). Transcription initiates at one of the several authentic pre-C RNA start sites at nt position 3068, but no detectable amounts of the authentic RNA pregenome starting at nt position 3100 are made; hence, only p25 and no p21c should be produced from the construct. Preliminary experiments showed that, as expected, p17e was secreted into the medium. Intracellular fractions did, however, contain small amounts of a protein with the mobility expected for the core protein p21c that could originate from usage of the second AUG on the pre-C mRNA; however, no positive result was obtained when the formation of replication-competent core particles was tested with the endogenous polymerase reaction (17; data not shown), indicating that the construct is principally suited to study HBeAg biosynthesis without major interference by contaminating HBcAg.

From previous studies, we had available the whole set of *C* genes encoding core proteins in which from one to all four of the cysteines are replaced by Ser residues (34). These were transferred into the parental plasmid pCHT-9/3068 with suitable restriction fragments. Since Cys-7 in the N-terminal extension of HBeAg is surrounded by hydrophobic residues, we exchanged this residue not only for serine but also for alanine. For practical reasons, Cys-7 mutations were combined only with those Cys mutants in the core protein part that gave the most interesting phenotypes in the first round of analyses. Likewise, the three cysteines in the clipped-off part of the pre-C sequence were not included in the analysis.

In the presence of Cys-7, Cys61 is required for efficient secretion of HBeAg. In the first set of experiments, we analyzed by ELISA all variants in which the *C* gene-encoded cysteines had been replaced by Ser residues for the production of intracellular and secreted HBeAg. The assay uses an immobilized rabbit polyclonal antiserum which recognizes all known forms of core protein to bind the antigen and mouse monoclonal antibodies against HBcAg or HBeAg for detection. Strikingly, all variants lacking Cys61 (i.e., all variants with number 2 in their names) showed a dramatic decrease in signal intensity for secreted HBeAg. This could mean that the corresponding proteins are not produced efficiently; however, intracellular forms of HBeAg were clearly detectable. These results, summarized in Fig. 2, suggested a crucial role for Cys61 in the secretion of HBeAg. None of the other Cys residues is similarly important; e.g., mutant 134 lacks all core-derived cysteines but Cys61 was efficiently secreted.

A trivial explanation could have been that mutation of Cys61 alters an epitope recognized by the monoclonal antibodies, i.e., although the protein is secreted, it would not be detectable in the assay employed. This possibility was excluded by a different assay, i.e., immunoprecipitation followed by Western blotting, for both of which the polyclonal rabbit anti-HBe/HBc antiserum was used (Fig. 3). Again, all variants lacking Cys61 showed only very weak signals of the p17 proteins. Therefore, we conclude that, in the presence of Cys-7, Cys61 is required for efficient secretion of HBeAg.

Cys-7 in HBeAg is involved in intramolecular disulfide bridges. The gel in Fig. 3, which was run under nonreducing conditions, also reveals that Cys-7 is involved in intramolecular disulfide bonds. The last two lanes show the mobilities of two variants which do not contain any Cys residue

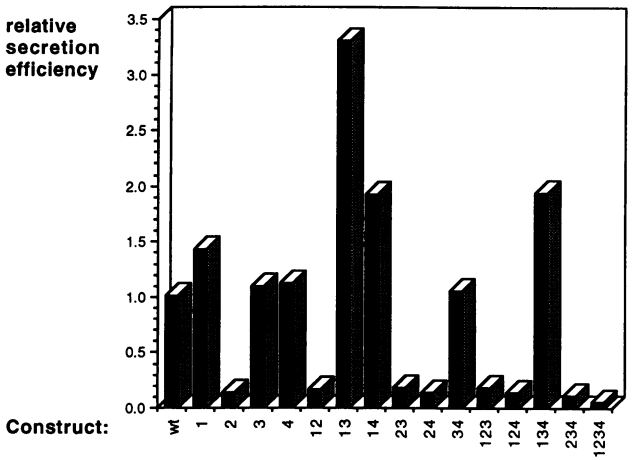

FIG. 2. Differential secretion competence of *C* gene-encoded Cys mutants. One percent (100 μl) of the culture media and 5% (50 μl) of the cytoplasmic lysates from HuH7 cells transfected with the constructs indicated on the *x* axis were separately analyzed for *pre-C/C* gene products by ELISA with monoclonal antibody mc03. Values on the *y* axis show the relative secretion efficiencies as measured by the ratio of optical density readings obtained with the medium samples versus those from the cell lysates; this accounts for differences in the transfection efficiencies and antigenicities of the individual samples. Note the low values for all variants lacking Cys61.

(mutants 0A1234 and 0S1234; see below) and hence cannot form any S-S linkages; they therefore serve as markers for the mobility of fully reduced p17e (the same results were obtained when the samples were run side by side under reducing and nonreducing conditions). Compared with these samples, all of the efficiently secreted p17 proteins showed an increased mobility; wild-type (wt) p17e appeared as two bands, both of which migrated faster than the reduced standard, suggesting that both products are intramolecularly disulfide bonded. Products comigrating with the fastest-migrating wt p17e band appeared in all other lanes of the well-secreted variants, i.e., those containing Cys61. Particularly revealing is variant 134, the only well-secreted triple mutant. Here, the only possible intrasubunit cross-link is between Cys-7 and Cys61. The secretory phenotype of this variant corresponds to that of wt p17e, suggesting that the crucial S-S bond in wt p17e occurs between Cys-7 and Cys61. The nature of the slightly slower second band of wt p17e is not clear at present; a band of similar mobility is only seen with mutant 3, which might suggest that here, and in wt p17e, an additional intramolecular S-S bridge involves Cys48. Interestingly, the band is absent from mutant 4, which has all of the cysteines that are present in wt p17e except that it lacks the C-terminal Cys residue of the p25 precursor, which is clipped off during export. It has been noted earlier that the deletion of the C-terminal region influences HBeAg secretion (42).

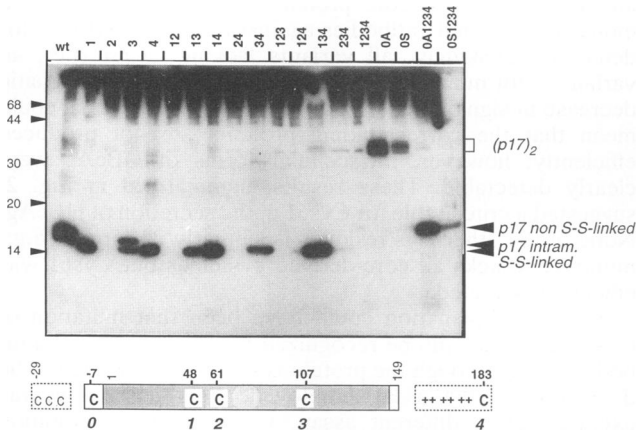

FIG. 3. Western blot of HBeAg-related proteins secreted into the medium. Pre-C/C proteins were immunoprecipitated from the culture media of cells transfected with the constructs indicated on the top of the figure with polyclonal rabbit anti-HBe/HBc antiserum, separated on a nonreducing 15% polyacrylamide gel, and detected with the same antiserum followed by ^{125}I-labeled protein A. Arrowheads on the left show the positions of molecular size markers with their apparent molecular sizes in kilodaltons. Arrowheads on the right indicate the positions of completely reduced and intramolecularly S-S-linked p17 and dimers of p17. For reference, a schematic representation of the precore protein with its Cys residues appears below.

Of the faint bands from the poorly secreted variants lacking Cys61, all but those from mutants 123 and 1234 also showed an increased mobility, indicating that they too have intramolecular S-S bonds. This suggests that in the absence of Cys61, alternative S-S bonds can form between Cys-7 and one of the remaining core-derived Cys residues (however, less efficiently). p17 from mutants 123 and 1234 has only Cys-7 left; therefore, the small amounts of p17 detectable have the same slow mobility as the Cys-free mutants on the right side of the gel.

Mutation of Cys-7 to Ala or Ser restores secretion efficiency but alters the quaternary structure of HBeAg. From the above data, we concluded that efficient secretion requires Cys-7 to be S-S linked to another Cys residue in the same molecule, preferentially to Cys61. We therefore directly tested the influence on secretion of mutants lacking Cys-7. As Cys-7 is surrounded by relatively hydrophobic residues, we constructed mutants in which this cysteine is replaced by alanine or by serine and combined these with the wt sequence and with variants lacking all core-derived cysteines (mutants 0A, 0S, 0A1234, and 0S1234). Surprisingly, all mutants were relatively efficiently secreted, as shown in the last four lanes of Fig. 3. In the Cys$^-$ context, both the Cys→Ala and Cys→Ser mutants showed one major band with a mobility of about 17 kDa (lanes 0A1234 and 0S1234) that was unchanged in the presence of reducing agent (not shown) and hence represents the mobility of fully reduced HBeAg (see above). More dramatically, in the wt context both mutants showed almost no signal in the expected 17-kDa region but instead gave a number of bands with mobilities of around 35 kDa, indicating the formation of intermolecularly S-S-linked dimers (lanes 0A and 0S). This behavior is strikingly similar to what we have previously observed for oxidatively induced cross-links in particulate core proteins (34). Further confirmation for a similar structure of the mutant dimeric p17 and dimers from core particles comes from additional variants which showed that the principal S-S bond linking the p17 dimers is also between two Cys61 moieties (Fig. 4); secreted p17 from mutant 0A2 having all of the core-derived cysteines except Cys61 remained essentially monomeric, whereas variant 0A134 lacking all cysteines but Cys61 was efficiently dimerized (lanes M under −DTT). Under reducing conditions (all lanes under +DTT), the patterns observed for both variants were very similar, confirming that the different mobilities observed

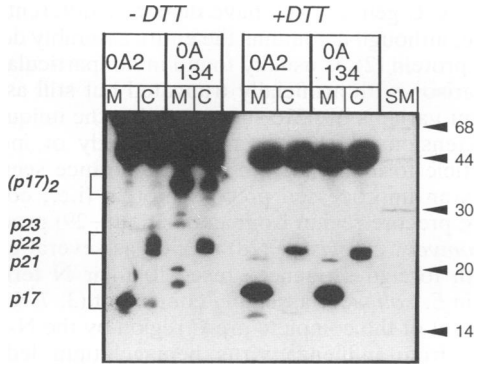

FIG. 4. Cys61/Cys61 is the major intermolecular S-S bond in dimeric p17. Intracellular (lanes C) and secreted (lanes M) forms of p17 from cells transfected with constructs 0A2 and 0A134 were subjected to Western blotting after separation under nonreducing (lanes under −DTT) or reducing (lanes under +DTT) conditions. Molecular size standards (in kilodaltons) are shown on the right (lane SM). Both variants lack Cys-7; 0A2 has all other cysteines except Cys61 and, under nonreducing conditions, is detected mainly as monomeric p17. Construct 0A134 has only Cys61 and is mainly detected as dimeric $(p17)_2$, whereas both variants show similar patterns under reducing conditions. DTT, dithiothreitol.

under nonreducing conditions are due to different disulfide linkages.

The differences between various mutants in secretion efficiencies were confirmed by analyzing intracellular and secreted forms of *pre-C/C* gene products by Western blotting after they were separated under reducing conditions (Fig. 5). Lanes C in Fig. 5 show the material immunoprecipitated from the cell lysates (which in this particular experiment, in addition to p23/22/21 and p17, contained smaller breakdown products). Relatively strong signals for secreted p17 (lanes M) in comparison to those for the intracellular forms (lanes C) were visible for the wt and variant 0A and, to a lesser extent, for variant 0A1234; variants 2 and 1234 gave very weak signals, confirmed by the relative intensities of p17 to contaminating bands at about 22 and 15 kDa (e.g., lanes M for variants 2 and 0A). Mock precipitations in which protein A-Sepharose without antiserum was used (lanes m) gave no specific signals, confirming that the bands observed in the other lanes are not due to an unspecific binding of the HBV proteins to the protein A-Sepharose matrix.

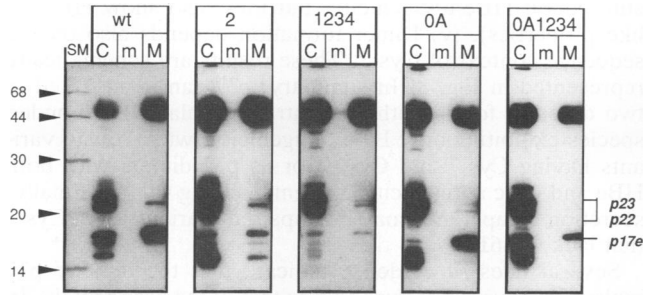

FIG. 5. Differential secretion efficiencies of *pre-C/C* gene products from selected variants. *Pre-C/C* gene products in the culture media (lanes M) or cytoplasmic lysates (lanes C) from cells transfected with the constructs indicated at the top were immunoprecipitated with a polyclonal anti-HBcAg/HBeAg serum or were mock-precipitated (lanes m) without antiserum, separated on a reducing 15% polyacrylamide gel, and detected by Western blotting with the same antiserum followed by ^{125}I-labeled protein A. The positions of the size marker proteins (lane SM) (in kilodaltons) are indicated on the left. The heavy band above the 44-kDa marker originates from the immunoglobulins in the antiserum. The 15- to 17-kDa bands present in the lysates (lanes C) in addition to p23/22/21 were not reproducibly seen in all experiments, and these presumably represent proteolytic breakdown products. Note the different amounts of secreted p17 in lanes M compared with those of the intracellular forms and the contaminating bands (p15 and p22) in the media.

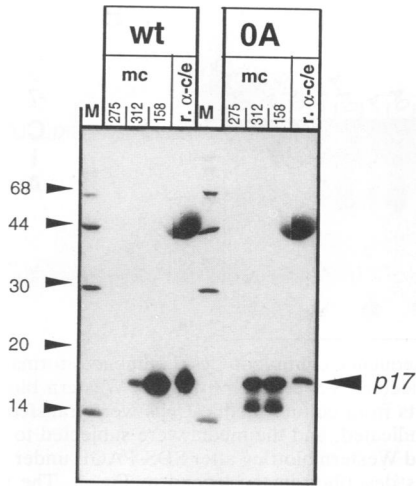

FIG. 6. Dimeric p17 from variant 0A exhibits HBc antigenicity. Equal aliquots of media from cells transfected with the wt construct, or mutant 0A, were subjected to immunoprecipitation with the monoclonal antibodies (lanes mc) mc275 and mc312 (both HBcAg specific), mc158 (HBeAg specific). The polyclonal rabbit anti-HBcAg/HBeAg antiserum (lanes r.α-c/e) was used as the positive control. Proteins were detected by Western blotting after separation by reducing SDS-PAGE as described in the legend to Fig. 3. The positions of marker proteins (in kilodaltons) are indicated on the left. The wt p17e was efficiently recognized by mc158 and the polyclonal rabbit antiserum; mc312 gave only a weak signal. Variant p17 from mutant 0A was equally well recognized by mc158 and mc312.

Finally, the structures of wt p17e and its dimer-forming variant 0A were probed by immunoprecipitation with different antibodies (Fig. 6). A rabbit antiserum recognizing all forms of core gene products (r.α-c/e) was used as a control. Monoclonal antibody mc158 is HBeAg specific, and mc312 and mc275 are HBcAg specific; they differ in that mc275 requires particles for recognition, whereas mc312 recognizes a linear epitope. The wt p17e reacted strongly with the α-c/e serum and mc158, weakly with mc312, and not detectably with mc275. By contrast, the 0A variant was recognized equally well by mc312 and mc158. While the lack of reaction with mc275 indicates that no particles were formed, the result with mc312 suggests that the variant p17 has both HBe and HBc antigenicity and hence a structure similar to that of the core protein p21c.

From the above results, we conclude that (i) for efficient secretion, Cys-7 must be S-S bonded, preferentially to Cys61 in the same molecule, and (ii) that in the absence of Cys-7, p17 secretion works efficiently whether Cys61 is present or absent; however, if Cys61 is present, p17 is secreted as an S-S-linked dimeric species which resembles particulate core protein in its properties.

Flanking sequences influence the ability of Cys-7 to form intramolecular S-S bridges. Cys-7 in the HBeAg-specific N-terminal extension is surrounded by a number of hydro-

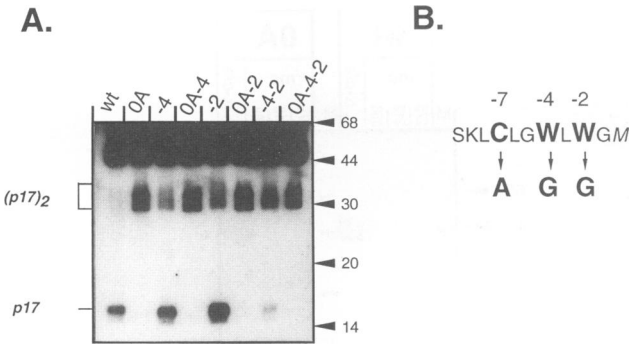

FIG. 7. Sequence context of Cys-7 influences formation of intra- and intermolecular S-S bonds in p17. (A) Western blot of *pre-C/C* gene products from culture media. Cells were transfected with the constructs indicated, and the media were subjected to immunoprecipitation and Western blotting after SDS-PAGE under nonreducing conditions as described in the legend to Fig. 3. The positions (in kilodaltons) of marker proteins are indicated on the right, and those of monomeric and dimeric HBeAg are indicated on the left. (B) Sequence of the pre-C-encoded N-terminal extension of p17 and mutated amino acid residues. The italic M represents the starting Met residue of p21c. The Trp residues at positions −4 and −2 were replaced by glycines, in either the presence (constructs −4, −2, and −4-2) or absence (constructs 0A-4, 0A-2, and 0A-4-2) of Cys-7.

phobic residues (SKLCLGWLWG in Fig. 1B) which might be important for keeping Cys-7 in spatial proximity to Cys61 to allow formation of the Cys-7/Cys61 bond. To test for such a potential influence, we replaced the large, hydrophobic Trp residues at positions −2 and −4 by the polar, small Gly residues, in the context of both Cys-7 (variants −2, −4, and −4-2) and its Ala-7 derivative (variants 0-2, 0-4, and 0-4-2). p17 secreted into the medium from cells transfected with the corresponding constructs was isolated by immunoprecipitation and analyzed for dimer formation by Western blotting after being separated on nonreducing gels. The result is shown in Fig. 7. As expected, predominantly dimers were detected from the mutants lacking Cys-7 (lanes 0A, 0A-4, 0A-2, and 0A-4-2); however, also in the presence of Cys-7 a significant increase in the amount of dimeric p17 compared with that of the wt is seen for the single Trp→Gly mutants (lanes −4 and −2) and, in particular, for the double mutant (lane −4-2). This result suggests that the ability of Cys-7 to form intramolecular S-S bridges is, at least in part, dependent on its sequence context. Gel filtration of the S-S-linked dimeric p17 molecules produced from variants 0A, −4-2, and 0A-4-2 showed that these proteins are dimeric not only under the denaturing conditions of SDS-PAGE but also under native conditions (data not shown). This indicates that preventing the intrasubunit S-S link, either directly by mutating Cys-7 or indirectly by mutating its flanking sequence, has a similar effect on the tertiary and quaternary structure of p17.

DISCUSSION

Hepadnaviruses have evolved a number of unconventional mechanisms to efficiently use their tiny genome. One of these is to use a gene both by itself and in combination with in-phase upstream open reading frames to produce C-terminally colinear proteins with differing N-terminal extensions. While this simple type of economical usage of protein domains appears to hold for the three HBV envelope proteins, the situation is more complex for the two products of the *pre-C/C* gene, which have distinctly different properties. p17e, although it contains the entire assembly domain of the core protein (2), is usually found in nonparticulate form. A comparison with artificially truncated but still assembly-competent variants of p21c suggested that the unique N-terminal extension of p17e interferes, directly or indirectly, with particle formation. Mere steric hindrance seemed unlikely, as an unprocessed precore protein (i.e., containing the entire precore region from aa position −29) produced in *Saccharomyces cerevisiae* (28) as well as several core proteins with foreign sequences fused to their N termini expressed in *E. coli* were assembly competent (3, 7, 43). Also, replacement of the complete pre-C region by the N-terminal sequence from influenza virus hemagglutinin led to the production, in mammalian cells infected with recombinant vaccinia viruses (42), of p17 molecules differing from authentic HBeAg. These results implied that it is the specific sequence encoded by the pre-C region that influences the structure of the p17e protein and that is responsible for its distinct biophysical and antigenic properties.

Cysteines have the ability to form covalent bonds between amino acid side chains; Cys-7, unique to p17e, was therefore a prime candidate to exhibit such a structural influence. For p21c in the reducing cytoplasm, the Cys residues are not essential (31). The precore protein, by contrast, is translocated from the cytoplasm into the more oxidizing environment of the endoplasmic reticulum, where disulfide formation in most exported proteins takes place.

Our analysis of Cys variants of p17 transiently expressed in a human liver cell line revealed the following. (i) Efficient secretion of authentic HBeAg critically depends on Cys61 forming an intramolecular S-S bridge to Cys-7 (the other core-derived cysteines can only inefficiently substitute for Cys61). (ii) Secretion of mutants lacking Cys61 can be restored by replacing Cys-7 with Ala or Ser. (iii) Mutants lacking Cys-7 are exported as native, S-S-linked dimers (covalent dimerization is due to formation of an intermolecular disulfide bridge between two residues of Cys61. (iv) Such p17 dimers are structurally similar to isolated core protein dimers but are distinct from authentic HBeAg (they still exhibit HBe antigenicity, but they also show HBcAg-like properties). (v) Dimer formation depends also on the sequence context of Cys-7. These results are schematically represented in Fig. 8. In summary, p17 can be secreted in two different forms: either as intramolecularly S-S-bonded species exhibiting only HBe antigenicity (wt p17e and variants having Cys-7 and Cys61) or as p17 dimers with both HBe and HBc antigenicity (variants lacking Cys-7). Finally, secretion competence may be impaired (variants with Cys-7 that lack Cys61).

Several lines of evidence indicate that the dimeric p17 molecules have a structure akin to that of the core protein. In the presence of Cys61, they form S-S-linked dimers, as revealed by nonreducing SDS-PAGE. Cys61 is required and sufficient for covalent dimerization; hence, Cys61/Cys61 represents the major cross-link in these molecules. Moreover, the variant p17 proteins are also homodimeric under native conditions. These properties closely resemble characteristic features of the core protein as present in viral capsids (34, 49, 50). Whether p17 dimers are indeed assembly competent is presently not established; our preliminary attempts to isolate $(p17)_2$ in sufficient quantities and concentrations have been hampered by the huge excess of serum proteins present in the culture media. However, p17 dimers react with HBcAg-specific antibodies, a finding complemented by similar results obtained with variants of p17

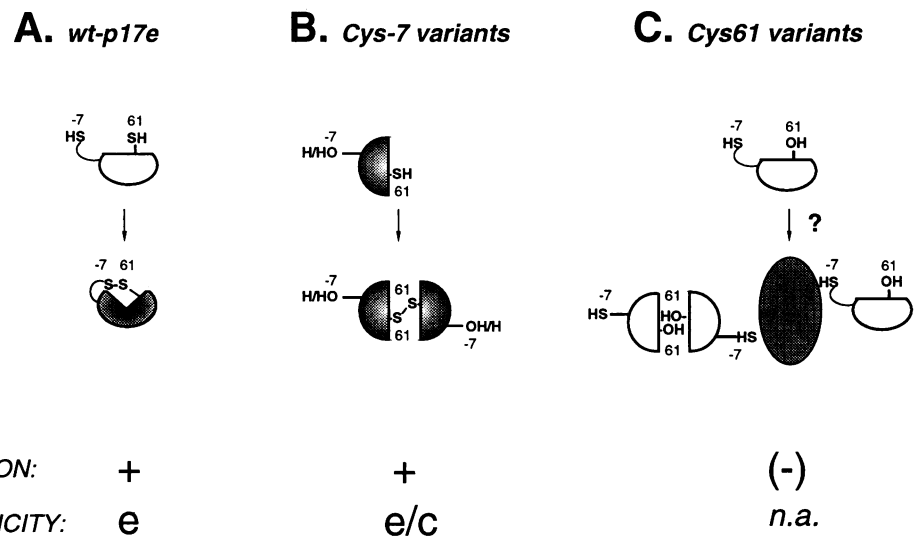

FIG. 8. Model for secretion of wt p17e and variants lacking Cys61 and/or Cys-7. (A) In wt p17e, an intramolecular S-S bond is formed between Cys-7 and Cys61, either co- or posttranslationally. Secretion is efficient, and the molecules display only HBe antigenicity. (B) Variants with Ala or Ser instead of Cys-7 form homodimers that are secreted as such and exhibit both HBe and HBc antigenicity. In the presence of Cys61, these dimers are covalently linked by Cys61/Cys61 bonds. (C) Variants having Cys-7 but lacking Cys61 are poorly secreted. The secretion defect is probably mediated by a free Cys-7, either on monomeric or on dimeric p17, which may induce association with resident proteins of the secretory pathway (shaded oval). Alternatively, the absence of Cys61 may lead to the formation of improper S-S bonds between Cys-7 and Cys48 or Cys107 (not shown). *n.a.*, not applicable (the amounts of secreted p17 were too small for a detailed analysis).

produced by using a high-efficiency vaccinia virus expression system (47). Together, these data strongly suggest that the intramolecular disulfide bridge between Cys-7 and Cys61 prevents HBeAg from adopting an HBcAg-like tertiary structure. Thus, authentic HBeAg is neither a denatured HBcAg nor merely a nonassembled nonparticulate core protein (27) but has its own distinct structure.

The presence of Cys-7 is one obvious prerequisite for the formation of the intramolecular S-S bridge, but the sequence context is also important. A mutant in which two of the large hydrophobic Trp residues neighboring Cys-7 were replaced by glycines showed predominantly S-S-linked dimeric p17. A similar although less pronounced dimerization has recently been described for p17 variants with the sequence Trp-Leu-Trp replaced by Asp-Asn-Asn or Ala-Asp-Leu (47). These data suggest that the ability of Cys-7 to bind to Cys61 is, at least in part, controlled by its sequence context, which argues for a specific interaction between the N-terminal extension of p17e and the region flanking Cys61 in the same molecule. In particulate core protein, Cys61 resides at the interface between two subunits (34). Hence, in p17e, formation of an intramolecular S-S bridge from Cys-7 to Cys61 is likely to alter this interface (Fig. 8) and to inhibit dimerization and, consequently, particle formation, thus explaining the nonparticulate nature of HBeAg.

The data described above indicate that the amino acid sequence common to p17e and p21c can adopt two different conformations. Since replacement of a single SH group in p17e by OH or H (i.e., substituting Cys-7 with Ser or Ala) yields dimeric p17 with HBcAg-like properties, this suggests that folding of the primary sequence into the HBcAg-like structure is energetically favored. Thus, in p17e the specific intramolecular interaction of two regions in the peptide chain together with the special redox conditions in the secretory compartments appears to lock the molecule in its distinct, characteristic conformation. The mechanism for formation of the HBeAg-specific intrasubunit S-S link could be either cotranslational (i.e., the HBeAg-specific structure can form only so long as translocation of the peptide chain is incomplete) or posttranslational (reflecting a greater stability of the HBeAg-like conformation under the specific conditions inside the secretory compartment [4]). We are currently establishing stable precore-producing cell lines which should allow us to address this mechanistic question.

Note that Garcia et al. (10) have reported a puzzling observation for in vitro translated-translocated precore protein. The N-terminal signal sequence was clipped off from most of the molecules, indicating successful translocation; however, a substantial fraction of the molecules slipped back into the outside compartment. If translocation were delayed in the in vitro system, the peptide chain may prematurely adopt the stable core-like structure and hence become translocation incompetent. If a similar slip-back mechanism existed in vivo, it could explain the previously reported, partially nuclear localization of precore-derived protein(s) (37, 48) and also provide clues as to why expression of the precore protein can suppress HBV replication (20).

The third phenotype, observed for p17 variants lacking Cys61 but having Cys-7, was a severe secretion defect, the exact nature of which is presently unclear. Apparently, these molecules cannot adopt the structure of authentic or dimeric p17. The small amounts of secreted p17 observed with these variants were intramolecularly S-S linked so long as at least either Cys48 or Cys107 was present. This could indicate either that most of the molecules have an unpaired Cys-7 and only the small fraction with the intrasubunit bond is secretion competent or that formation of such incorrect S-S bonds is efficient but impairs secretion and only a small fraction escapes the quality control mechanisms of the endoplasmic reticulum. Malfolded proteins are usually not exported, because they either aggregate, are degraded (18), or are retained by interaction with resident endoplasmic reticulum

proteins such as BiP or protein disulfide isomerase (6). The amounts of intracellular forms of precore protein were not significantly different for the variants with Ser or Cys at position 61, arguing against a rapid degradation of such variants. In fact, from the large number of variants analyzed, we consider it not very likely that poor secretion is simply due to malfolding: a severe secretion defect was observed for mutant 2 in which only Cys61 is replaced. In contrast, mutant 134 with three amino acid exchanges was efficiently exported. Likewise, Ser61 is tolerated when Cys-7 is absent, as shown, for instance, by mutant 0A1234 which lacks all cysteines. Hence, Ser61 does not seem to by itself induce misfolding. We therefore favor the notion that it is a free SH group at position −7 that appears to interfere with efficient secretion. A secretion defect due to the presence of an unpaired Cys residue that was overcome by mutation or by the addition of a reducing agent to the cell culture medium has been previously demonstrated for immunoglobulin M assembly intermediates (1, 44). In wt p17e, the intramolecular S-S bond to Cys61 prevents the inhibitory effect of the Cys-7 SH group and concomitantly establishes the protein's characteristic structure; in the Cys-7 variants, interference by the SH group is circumvented by replacing it with OH or H, but these molecules adopt, likely by default, the core-like structure.

In summary, HBV uses selective disulfide formation to generate two distinct antigens from basically one gene, adding another elaborate mechanism to its repertoire for efficient usage of its tiny genome. The biological significance of this mechanism is most likely related to the different immune responses produced by HBcAg and HBeAg. HBV infection almost invariably elicits a strong anti-HBcAg response with no apparent protective effect, whereas seroconversion to anti-HBeAg occurs less frequently, particularly in chronic B hepatitis, and is delayed and weaker, but it is strongly correlated with lowered viremia and often precedes virus elimination (13, 21). Chronic B hepatitis despite the presence of anti-HBeAg may in fact be explained, at least in part, by the selection of HBV variants with defects in their pre-C regions which do not secrete HBeAg anymore (5, 11). Plausibly, active secretion of an HBcAg-like p17 might recruit the anti-HBcAg response to substitute for anti-HBeAg in mediating virus elimination. The different tertiary structure imposed on the p17e polypeptide by the intramolecular S-S bond keeps its quaternary structure and its antigenicity distinct from those of p21c and allows the virus to escape such a potentially deleterious situation. Finally, a significant accumulation of pre-C mutations leading to substitution of Cys-7 has recently been reported in HBV sequences from hepatocellular carcinoma but not from the peritumoral tissue (24). Whether this reflects a particular pathogenic phenotype associated with the ability or inability to produce authentic HBeAg remains to be determined.

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REFERENCES

1. **Alberini, C. M., P. Bet, C. Milstein, and R. Sitia.** 1990. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. Nature (London) **347**:485–487.
2. **Birnbaum, F., and M. Nassal.** 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. J. Virol. **64**:3319–3330.
3. **Borisova, G. P., I. Berzins, P. M. Pushko, P. Pumpen, E. J. Gren, V. V. Tsibinogin, V. Loseva, V. Ose, R. Ulrich, H. Siakkou, and H. A. Rosenthal.** 1989. Recombinant core particles of hepatitis B virus exposing foreign antigenic determinants on their surface. FEBS Lett. **259**:121–124.
4. **Braakman, I., J. Helenius, and A. Helenius.** 1992. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. EMBO J. **11**:1717–1722.
5. **Brown, J. L., W. F. Carman, and H. C. Thomas.** 1992. The clinical significance of molecular variation within the hepatitis B virus genome. Hepatology **15**:144–148.
6. **Chessler, S. D., and P. H. Byers.** 1992. Defective folding and stable association with protein disulfide isomerase/prolyl hydroxylase of type I procollagen with a deletion in the Proα2(I) chain that preserves the Gly-X-Y repeat pattern. J. Biol. Chem. **267**:7751–7757.
7. **Dalseg, R.** 1990. Ph.D. thesis. University of Heidelberg, Heidelberg, Germany.
8. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature (London) **281**:646–650.
9. **Gallina, A., F. Bonelli, L. Zentilin, G. Rindi, M. Muttini, and G. Milanesi.** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acid. J. Virol. **63**:4645–4652.
10. **Garcia, P. D., J.-H. Ou, W. L. Rutter, and P. Walter.** 1988. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J. Cell Biol. **106**:1093–1104.
11. **Günther, S., H. Meisel, A. Reip, S. Miska, D. H. Krüger, and H. Will.** 1992. Frequent and rapid emergence of mutated preC sequences in HBV from e-antigen positive carriers who seroconvert to anti-HBe during interferon treatment. Virology **187**:271–279.
12. **Hatton, T., S. Zhou, and D. N. Standring.** 1992. RNA- and DNA-binding activites in hepatitis B virus capsid protein: a model for their roles in viral replication. J. Virol. **66**:5232–5241.
13. **Hoofnagle, J. H., G. M. Dusheiko, L. B. Seeff, E. A. Jones, J. G. Waggoner, and Z. B. Bales.** 1981. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. Ann. Intern. Med. **94**:744–748.
14. **Hurtley, S. M., and A. Helenius.** 1989. Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5**:277–307.
15. **Hwang, C., A. J. Sinskey, and H. F. Lodish.** 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science **257**:1496–1502.
16. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9**:3389–3396.
17. **Kaplan, P., R. Greenman, J. Gerin, R. Purcell, and W. Robinson.** 1973. DNA polymerase associated with human hepatitis B virus antigen. J. Virol. **12**:995–1005.
18. **Klausner, R. D., and R. Sitia.** 1990. Protein degradation in the endoplasmic reticulum. Cell **62**:611–614.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lamberts, C., M. Nassal, I. Velhagen, H. Zentgraf, and C. H. Schröder.** Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol. **67**:3756–3762.
21. **Loriot, M. A., P. Marcellin, E. Bismuth, M. Martinot-Peignoux, N. Boyer, C. Degott, S. Erlinger, and J. P. Benhamou.** 1992. Demonstration of hepatitis B virus DNA by polymerase chain reaction in the serum and the liver after spontaneous or therapeutically induced HBeAg to anti-HBeAg or HBsAg to anti-HBsAg seroconversion in patients with chronic hepatitis B. Hepatology **15**:32–36.
22. **MacKay, P., J. Lees, and K. Murray.** 1981. The conversion of

hepatitis B core antigen synthesized in E. coli into e antigen. J. Med. Virol. **8**:237–243.
23. **Magnius, L. O., and J. A. Espmark.** 1972. New specificities in Australia antigen positive sera distinct from Le Bouvier determinants. J. Immunol. **109**:1017–1021.
24. **Manzin, A., S. Menzo, P. Bagnarelli, P. E. Varaldo, I. Bearzi, G. Carloni, F. Galibert, and M. Clementi.** 1992. Sequence analysis of the hepatitis B virus pre-C region in hepatocellular carcinoma (HCC) and nontumoral liver tissues from HCC patients. Virology **188**:890–895.
25. **Mason, W. S., and C. Seeger (ed.).** 1991. Hepadnaviruses—molecular biology and pathogenesis. Curr. Top. Microbiol. Immunol., vol. 168.
26. **Milich, D. R.** 1988. T and B cell recognition of hepatitis B viral antigens. Immunol. Today **9**:380–386.
27. **Milich, D. R., A. McLachlan, S. Stahl, P. Wingfield, G. B. Thornton, J. L. Hughes, and J. E. Joyce.** 1988. Comparative immunogenicity of hepatitis B virus core and e antigens. J. Immunol. **141**:3617–3624.
28. **Miyanohara, A., T. Imamura, M. Araki, K. Sugawara, N. Ohtomo, and K. Matsubara.** 1986. Expression of hepatitis B virus core antigen gene in *Saccharomyces cerevisiae*: synthesis of two different polypeptides translated from different initiation codons. J. Virol. **59**:176–180.
29. **Nakabayashi, H., K. Taketa, K. Miyame, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated function in chemically defined medium. Cancer Res. **42**:3858–3863.
30. **Nassal, M.** 1988. Total chemical synthesis of a gene for hepatitis B virus core protein and its functional characterization. Gene **66**:279–294.
31. **Nassal, M.** 1992. Conserved cysteines of the hepatitis B virus core protein are not required for assembly of replication-competent core particles nor for their envelopment. Virology **190**:499–505.
32. **Nassal, M.** 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. J. Virol. **66**:4107–4116.
33. **Nassal, M., M. Junker-Niepmann, and H. Schaller.** 1990. Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell **63**:1357–1363.
34. **Nassal, M., A. Rieger, and O. Steinau.** 1992. Topological analysis of the hepatitis B virus core particle by cysteine-cysteine cross-linking. J. Mol. Biol. **225**:1013–1025.
35. **Nassal, M., and H. Schaller.** 1993. Hepatitis B virus nucleocapsid assembly. *In* W. Doerfler and P. Boehm (ed.), Virus strategies, in press. VCH, Weinheim, Germany.
36. **Ou, J.-H., O. Laub, and W. J. Rutter.** 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA **83**:1578–1582.
37. **Ou, J.-H., C.-T. Yeh, and T. S. B. Yen.** 1989. Transport of the hepatitis B virus precore protein into the nucleus after cleavage of its signal peptide. J. Virol. **63**:5238–5243.
38. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in E. coli. Nature (London) **282**:575–579.
39. **Salfeld, J., E. Pfaff, M. Noah, and H. Schaller.** 1989. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J. Virol. **63**:798–808.
40. **Sällberg, M., U. Rudén, L. O. Magnius, H. P. Harthus, M. Noah, and B. Wahren.** 1991. Characterisation of a linear binding site for a monoclonal antibody to hepatitis B core antigen. J. Med. Virol. **33**:248–252.
41. **Sällberg, M., U. Rudén, B. Wahren, M. Noah, and L. O. Magnius.** 1991. Human and murine B-cells recognize the HBeAg/beta (or HBe2) epitope as a linear determinant. Mol. Immunol. **28**:719–726.
42. **Schlicht, H.-J., and G. Wasenauer.** 1991. The quaternary structure, antigenicity, and aggregational behavior of the secretory core protein of human hepatitis B virus are determined by its signal sequence. J. Virol. **65**:6817–6825.
43. **Schoedel, F., A. M. Moriarty, D. L. Peterson, J. Zheng, J. L. Hughes, H. Will, D. J. Leturcq, J. S. McGee, and D. R. Milich.** 1992. The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. J. Virol. **66**:106–114.
44. **Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein.** 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. Cell **60**:781–790.
45. **Takahashi, K., S. Kishimoto, K. Ohori, H. Yoshizawa, A. Machida, H. Ohnuma, F. Tsuda, E. Munekata, Y. Miyakawa, and M. Mayumi.** 1991. Molecular heterogeneity of e antigen polypeptides in sera from carriers of hepatitis B virus. J. Immunol. **147**:3156–3160.
46. **Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aoyagi, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1983. Immunochemical structure of hepatitis e-antigen in the serum. J. Immunol. **130**:2903–2907.
47. **Wasenauer, G., J. Köck, and H.-J. Schlicht.** 1992. A cysteine and a hydrophobic sequence in the noncleaved portion of the pre-C leader peptide determine biophysical properties of the secretory core protein (HBe protein) of human hepatitis B virus. J. Virol. **66**:5338–5346.
48. **Yang, S. Q., M. Walter, and D. N. Standring.** 1992. Hepatitis B virus p25 precore protein accumulates in *Xenopus* oocytes as an untranslocated phosphoprotein with an uncleaved signal peptide. J. Virol. **66**:37–45.
49. **Zheng, J., F. Schoedel, and D. L. Peterson.** 1992. The structure of hepadnaviral core antigens. J. Biol. Chem. **267**:9422–9429.
50. **Zhou, S., and D. N. Standring.** 1992. Hepatitis B virus capsids are assembled from core protein dimers. Proc. Natl. Acad. Sci. USA **89**:10046–10050.