Expression, assembly competence and antigenic properties of hepatitis B virus core gene deletion variants from infected liver cells

Petra Preikschat,1 Galina Borisova,2 Olga Borschukova,2 Andris Dislers,2 Guna Mezule,2 Elmar Grens,2 Detlev H. Krüger,1 Paul Pumpens2 and Helga Meisel1

1 Institut für Virologie, Universitäts-Klinikum Charité, Humboldt-Universität Berlin, Schumannstraße 20/21, D-10117 Berlin, Germany
2 Biomedical Research and Study Center, University of Latvia, Riga, Latvia

Previous studies have shown that the progression of hepatitis B virus-related liver disease in long-term immunosuppressed kidney transplant recipients is associated with the accumulation of virus variants carrying in-frame deletions in the central part of the core gene. A set of naturally occurring core protein variants was expressed in Escherichia coli in order to investigate their stability and assembly competence and to characterize their antigenic and immunogenic properties. In addition, a library of core gene variants generated in vitro with deletions including the major immunodominant region (MIR) of the core protein was investigated. The position and length of deletions determined the behaviour of mutant core proteins in E. coli and their assignment to one of the three groups: (i) assembly-competent, (ii) stable but assembly-incompetent and (iii) unstable proteins. In vivo core variants with MIR deletions between amino acids 77 and 93 belong to the first group. Only proteins with the shortest deletion (amino acids 86–93) showed stability and self-assembly at the same level as wild-type cores, and they showed reduced antigenicity and immunogenicity. Mutants with deletions extending N-terminally beyond residue G73 or C-terminally beyond G94 were found to be assembly-incompetent. We suggest that G73 and G94 are involved in the folding and the native assembly of core molecules, whereas the intervening sequence determines the antibody response. Depending on their ability to form stable proteins or to assemble into particles, core mutants could contribute to liver cell pathogenesis in different ways.

Introduction

Infection with hepatitis B virus (HBV) may lead to a broad spectrum of clinical manifestations, ranging from an asymptomatic, self-limited course to fulminant, acute hepatitis or chronic infection with possible development of cirrhosis or hepatocellular carcinoma. It is widely believed that the host response plays a critical role in HBV pathogenesis. Recently, it was shown in a transgenic mouse model of HBV infection that non-cytopathic, cytokine-dependent processes may also contribute to virus clearance without killing hepatocytes (Chisari & Ferrari, 1995; Guidotti & Chisari, 1996).

Although wild-type HBV is not directly cytopathic to liver cells (Chisari & Ferrari, 1995), HBV mutants carrying altered structural genes may lead directly to liver failure. Recently, we have shown that HBV mutants with deletions in the central part of the core (C) gene can appear and accumulate in long-term immunosuppressed renal transplant recipients. In most patients, persistence of these mutants for more than 1–2 years was associated with rapidly progressive liver disease (Günther et al., 1996a, b). Similar variants were also found in patients with long-term HBV infection [hepatitis B e-antigen (HBeAg)/HBV DNA positive] and varying severity of liver disease (Wakita et al., 1991; Akarca & Lok, 1995; Okumura et al., 1996; Zoulim et al., 1996; Guidotti & Chisari, 1996). C gene deletion variants were eliminated preferentially compared with wild-type virus during spontaneous seroconversion from HBeAg to anti-HBe or during interferon α treatment of patients with chronic active hepatitis B (Marinos et al., 1996). Thus, these variants seemed to be at a selective replication disadvantage under the pressure of an active immune response. Consistent
Table 1. Origins of naturally occurring HBV C gene deletion variants

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Clinical outcome</th>
<th>Clone</th>
<th>Deletion</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ESLD</td>
<td>Alive after antiviral therapy (lamivudine) in 1997</td>
<td>11</td>
<td>41–51</td>
<td>1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8b</td>
<td>86–93</td>
<td>1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8c</td>
<td>45–6/86–93</td>
<td>1993</td>
</tr>
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<td></td>
<td></td>
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<td>75–95</td>
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<tr>
<td>D</td>
<td>ESLD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CH</td>
<td>Mild hepatitis</td>
<td>12b</td>
<td>85–93/102–113</td>
<td>1997</td>
</tr>
</tbody>
</table>

Table 1. Origins of naturally occurring HBV C gene deletion variants

ESLD, End-stage liver disease; CH, chronic hepatitis. Clones were numbered according to the number of amino acids deleted; the deletions are shown as the amino acid positions that were deleted.

with this hypothesis, we observed a preferential elimination of the C gene mutants after cessation of immunosuppression or following superinfection with hepatitis C virus ( Günther et al., 1996a) and also under antiviral therapy with lamivudine (Meisel et al., 1999).

The C open reading frame encodes two primary translation products, the core protein (p21) and the pre-C/C polypeptide (p25). The latter is processed to p17 and then to p17 or HBcAg (Standring et al., 1988). The HBV core protein p21 is the structural unit of virus core particles. It encapsidates the complex consisting of the viral DNA polymerase and the RNA pregenome into a nucleocapsid, where the cycle of HBV DNA replication is completed. By recognizing the appropriate S and pre-S1 targets specifically (Poisson et al., 1997), the virus cores bud through the endoplasmic reticulum membranes containing HBV surface proteins. Only mature cores containing a partially double-stranded viral DNA genome are enveloped and secreted (Gerelsaikhan et al., 1996; Wei et al., 1996). During the course of HBV infection, core protein induces the strongest B-cell, T-cell and CTL responses of all HBV polypeptides (Milich, 1988; Penna et al., 1996; Rehmann et al., 1996).

The ability of HBV core protein to undergo correct folding (i.e., dimerization) and self-assembly during heterologous expression in bacteria (Pasek et al., 1979), forming protein shells which are indistinguishable from those seen in the infected liver, is an important advantage for the investigation of the structural and immunological properties of HBV core particles (Cohen & Richmond, 1982). HBV core protein forms dimers, linked by disulphide bridges between the C61 residues in two monomers (Nassal et al., 1992). These dimers are the only detectable assembly intermediates (Zhou & Standring, 1992). Cryoelectron microscopic studies have shown that alternative icosahedral shells, containing 90 (T = 3) and 120 (T = 4) dimer subunits, are produced by the core protein (Crowther et al., 1994). The basic C-terminal region (up to 39 amino acid residues long), believed to interact with the nucleic acid, is dispensable for self-assembly, and C-terminally truncated HBV core proteins (truncated as far as amino acids 144 or 140) may mimic the structural and immunological properties of the full-length protein (for review see Pumpens et al., 1995). According to mapping data, the major immunodominant region (MIR) of HBV core protein is interposed between residues 74 and 89 (Salfeld et al., 1989; Sällberg et al., 1991; Pushko et al., 1994). Recent structural investigations allowed the MIR to be localized to the spikes of the core particle, within a loop comprising at least amino acids 78–82 at the tip of the hairpins, the most exposed region of the spikes (Böttcher et al., 1997).

In the present work, we studied the capacity of naturally occurring MIR-deleted core polypeptides to form particles. For this purpose, we expressed a set of naturally occurring HBV C gene deletion mutants in an E. coli system and compared it with a set of artificially generated C gene deletion mutants constructed by in vitro mutagenesis. The mutated HBV core products were expressed either as full-length (‘long’) or C-terminally truncated (‘short’) polypeptides. We could show that the amino acid residues within the MIR between positions G73 and G94 are not directly involved in capsid assembly. Only some of the naturally occurring core mutants were able to form particles, though these had altered immunological properties. We suggest that the accumulation of assembly-competent, as well as assembly-incompetent, mutated polypeptides, and their interference with wild-type core protein assembly, could lead to toxic effects on the liver cell.
Methods

Patients. HBV C gene deletion variants were found in the sera and liver samples of five patients under long-term immunosuppression after renal transplantation (Table 1). Appearance and accumulation of HBV C gene deletion variants were associated with the development of liver cirrhosis in four patients, three of whom died from end-stage liver disease (ESLD). One patient with ESLD survived under antiviral therapy (lamivudine), accompanied by the preferential loss of deletion variants. In the fifth patient, with mild hepatitis, the C gene deletion variant did not accumulate during the observation period of 6 years.

Isolation of naturally occurring HBV C gene deletion variants. HBV DNA was isolated from patients’ sera (30 µl) and liver samples by phenol–chloroform extraction and proteinase K digestion. The complete C gene was amplified by using primers 1 (nt 1757–1788) and 2 (nt 2516–2493) and then cloned into the pCR II plasmid (TA cloning kit, Invitrogen) as described previously (Günther et al., 1996a). The nucleotide sequences were analysed on an ALFExpress DNA sequencer by using the Cy5-AutoRead sequencing kit (Pharmacia).

Expression of naturally occurring C gene deletion variants in E. coli. Plasmids pHBC3 (Borisova et al., 1987) and pT31 (Borisova et al., 1996) were used to allow the expression of full-length core protein and its C-terminally truncated form (aa 1–144; HBcA), respectively (Fig. 1a).

Construction of a library of C gene deletion variants with artificially shortened MIR. For the construction of a library of HBV C gene deletion variants, purified p2-19 vector (Borisova et al., 1996) was used (Fig. 1b). This plasmid, containing a synthetic C-terminally shortened C gene (nt 1–429) with a central insertion of an oligonucleotide sequence encoding the DPAFR-preS1 epitope with unique Eco72I and Eco105I restriction sites at its borders, was cleaved either by Eco72I or Eco105I and subjected to Bal31 nuclease digestion (Fig. 2). Core deletion variants were screened for their capsid-forming ability as described previously (Borshukova et al., 1997).

Expression in E. coli and purification of deleted core proteins. E. coli strains K802, BL21, RR1 and JM109 were tested for expression capacity of hepatitis B core derivatives under different aeration conditions in LB broth and Casamino acids-enriched M9 medium at 30 °C and 37 °C for 20 h culture (not shown). E. coli strains K802 and BL21, grown at 37 °C overnight (14–16 h) with limited aeration in the Casamino acids-enriched M9 medium, were found to show the highest levels of synthesis. Therefore, in general, cells harbouring the appropriate plasmids were grown overnight on a rotary shaker (150 r.p.m.) at 37 °C in 500 ml flasks containing 150 ml M9 minimal medium supplemented with 1% Casamino acids (Difco) and 0·2% glucose to an OD260 of 3–5. Cells were pelleted and lysed by freeze–thawing in lysis buffer containing 50 mM Tris–HCl (pH 8·0), 5 mM EDTA, 100 µg/ml PMSF and 2 mg/ml lysozyme. Core particles were purified by ammonium sulphate precipitation and Sepharose CL4B chromatography as described previously (Crowther et al., 1994).

SDS–PAGE and immunoblotting. Bacteria were pelleted, suspended in SDS–PAGE sample buffer containing 2% SDS and 2% 2-mercaptoethanol and lysed by heating at 100 °C for 5 min. Proteins were separated by PAGE on a 12–18% gradient running gel and a 4% stacking gel. Western blotting was conducted as described by Towbin et al. (1979) and core proteins were detected with monoclonal anti-HBc/e antibodies 14E11 and C1-5, recognizing amino acids 134–140 and 78–83, respectively (Pushko et al., 1994), 148B6 (Sorin Biomedica) and antimouse IgG–peroxidase conjugate (Dako) as a secondary antibody.

Antigenic properties: ELISA. For the direct ELISA, purified HBV core variants (10 µg/ml) were coated overnight onto microtitre plates (Nunc). After post-coating at room temperature for 1 h with blocking buffer (0·1% Tween 20, 1% BSA in PBS), serial dilutions of the various antibodies were incubated for 1 h at room temperature, followed by incubation for 1 h at 37 °C with secondary antibodies conjugated to horseradish peroxidase (Sigma). The plates were washed six times with 0·1% Tween 20 in PBS. Absorbances of the reaction products were measured at 492 nm. For the competitive ELISA, serial dilutions of antigens were pre-incubated with an antibody dilution yielding 50% of the maximal A492 and the reaction was continued as described above.

Immunogenic properties of deleted core proteins: immunization of mice. Mice (five per group) were immunized intradermally with 10 µg purified core protein in 0·1 ml complete Freund’s adjuvant (Difco) followed by two booster immunizations of the same protein in Freund’s incomplete adjuvant (Difco) on days 10 and 24. Sera obtained on days 24 and 48 were analysed by ELISA for reactivity with wild-type HBV core protein and its deletion variants as described above.

Computer analysis. The comparison of more than 200 natural HBV core sequences was done with the Basic Local Alignment Search
Results

Naturally occurring HBV C gene deletion variants

The C gene deletions in the HBV mutants that accumulated and persisted in long-term immunosuppressed patients with progressive liver disease (Table 1) were found to be predominantly in frame. In total, 92 of 110 clones tested (84%) carried in-frame C gene deletions. During the follow-up of the patients, we observed a relatively fast selection process, where shorter deletions were replaced by longer ones. With rare exceptions (variant #11 and secondary mutations in variants #8c and #12b; see Fig. 2a), C gene deletions affected the central part of the molecule within the MIR. Three independent clones, #8a, #8b and #8c, harboured the same deletion of amino acids 86–93; in one (#8b), an amino acid exchange (G94S) existed adjacent to the deletion.

Synthesis and self-assembly of naturally occurring deleted HBV core proteins in E. coli

The collection of mutated C genes (Fig. 2a) was subjected to expression in E. coli by using two bacterial vectors for the high-level synthesis of full-length and C-terminally truncated core protein. The central part of mutated genes, spanning nucleotides 85–505 or 85–429, was excised from the PCR-cloned HBV C gene fragments and inserted into the appropriate region of long and short variants of HBV C gene (Fig. 1a). Deleted core variants were screened for their ability (i) to be synthesized as stable proteins and (ii) to self-assemble in E. coli cells. For this purpose, different E. coli strains harbouring the plasmids were grown under optimized medium and aeration conditions (see Methods). Thereafter, SDS- or lysozyme-lysates of samples were analysed. The synthesis of
HBV C gene deletion variants

Fig. 3. Immunoblot analysis of the expression of naturally deleted HBV core proteins in E. coli cells with the monoclonal antibody 148B6. Samples were crude SDS lysates of a 4 ml cell culture (15 µl of a 250 µl lysate loaded per lane) (i) or aliquots of pellets (ii) and supernatants (iii) of the lysozyme lysate from a 150–200 ml culture (10–25 µl of a 4 ml lysate per lane). The numbers of the deletion mutants expressed as the full-length (long) or C-terminally truncated (short) form are indicated above each lane. Wild-type (wt) lysates in the short and the long form and mock lysates (E. coli K802 lysate after transformation with pUC18) are shown on the left. Arrows indicate the positions of the wt core proteins and the variants.

Fig. 4. Electron micrographs of purified core particles consisting of naturally occurring deleted core proteins synthesized in E. coli cells. Variants #8a (a) and #15 (b) are shown. Negative staining was performed with uranyl acetate. Bar, 50 nm.

Mutants were assigned to one of three groups, according to the location of the deletion: between the highly conserved glycine residues G73 and G94 (group I), extending N-terminally of G73 (group II) or C-terminally of G94 (group III) (Table 2). A comparison of more than 200 known natural HBV core sequences with the BLASTA program revealed full conservation of G94 and only a single G73V substitution, supporting the structural importance of both glycine residues. G94 is also conserved in all known woodchuck and ground squirrel hepatitis B virus sequences, whereas G73 is replaced in these sequences by serine. The conservation of G73 and G94 within amino acids 73–94 is especially remarkable. At other positions in this region, multiple substitutions by up to eight different amino acids were observed and only positions V86 and Y88 seem to be more conserved (single amino acid exchanges were found). From the analysis of protein databases, including PROSITE, one can conclude that G-to-S and G-to-V substitutions, as observed at positions 73 and 94 of the core protein, are frequently tolerated in various proteins without loss of structural and functional properties.

Among all naturally occurring HBV core mutants, only variants #8a and #8b, with the short deletion in the C-terminal half of the MIR (positions 86–93), showed highly efficient synthesis (#8a not shown, see Fig. 3 for #8b) and unimpaired self-assembly in E. coli, indistinguishable from the wild-type HBV core protein, in both the long and the short forms. Variant #17, with a more extended deletion (positions 77–93) within the MIR, showed reduced but detectable competence for particle formation in the short form, although the insufficient level of synthesis of the long form (Fig. 3) did not allow a final decision to be made concerning its ability to self-assemble. Variant #8c, differing from #8a and #8b by the deletion of two additional amino acids (positions 45–46), appeared as an insoluble protein when expressed in either form (Fig. 3). Variant #11, with a deletion of 11 amino acid residues from the same region of the HBV core molecule, expressed

core-like proteins in the cells was detected by silver staining and immunoblotting with the monoclonal anti-HBc/e antibodies 148B6 (Fig. 3) or 14E11 (data not shown), directed against amino acids 134–140, which remained intact in all core variants studied. The capsid-forming ability of the natural core variants was assessed by double radial immunodiffusion against polyclonal anti-HBc antibodies of human and rabbit origin (data not shown), as well as by electron microscopy of lysozyme lysates, before and after fractionation through a gel-filtration column (Fig. 4).
Table 2. Synthesis and self-assembly of long and short forms of the naturally occurring and artificially constructed deleted HBV core proteins in E. coli cells

Variants are designated according to the number of amino acids deleted. Naturally occurring variants are shown in bold. Levels of expression are shown as: + + +, strong, protein detected by Western blot of crude SDS lysate (4 ml cell culture), soluble after lysozyme lysis; + +, moderate, insoluble product after lysozyme lysis; +, low, not detectable by Western blot of a 4 ml SDS lysate, insoluble product after lysozyme lysis of 0.5 g cells (150–200 ml culture); −, no detectable synthesis. Self-assembly as determined by electron microscopy was scored as: + + +, maximal level compared with wild-type; +, traces of capsids; −, no detectable assembly. ND, Not determined.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Deletion</th>
<th>Full-length core (long)</th>
<th>C-terminally truncated core (short)</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>Expression</td>
<td>Self-assembly</td>
</tr>
<tr>
<td>Group I (deletion between G73 and G94)</td>
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<td></td>
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<tr>
<td>8d</td>
<td>75–82</td>
<td>ND</td>
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</tr>
<tr>
<td>17</td>
<td>77–93</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>79–81</td>
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</tr>
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<td>+ + +</td>
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<tr>
<td>8b</td>
<td>86–93*</td>
<td>+ + +</td>
<td>+ + +</td>
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<td>41–51</td>
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<td>Group III (deletion including and/or extending C-terminally beyond G94)</td>
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<tr>
<td>21</td>
<td>75–95</td>
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</tr>
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<td>27</td>
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<td>12b</td>
<td>85–93/102–113</td>
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<td>−</td>
</tr>
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</table>

*Variant 8b also contained a G94S substitution.

only as the long form, did not yield any detectable product (Table 2, group II).

For the naturally occurring HBV core mutants of group III, detectable levels of protein were observed in three of four cases (#30b, #39 and #12b) where the central loop amino acids D78P79 were still present. The core protein variants in which these amino acids were deleted (#21, #27 and #30c) were not detectable (Table 2).

Expression of HBV C gene deletion variants constructed in vitro

The data on the stability and self-assembly of naturally occurring core protein variants are in good agreement with the data on artificial MIR deletion variants (Table 2; Fig. 2b). The variants belonging to group I (Table 2), with deletions ranging from positions 75 to 93, showed high levels of expression and assembly competence. Variants from group II, with deletions including and/or extending N-terminally of position G73, demonstrated similar levels of expression but the core proteins were insoluble, without any trace of proper folding or self-assembly. By Western blot analysis, we were not able to select any variants that harboured deletions C-terminal of G94 (group III) after random Bal31 mutagenesis. This is an indirect indication of the folding-incompetence of such mutants. To prove the importance of G94 directly, a mutant was constructed with amino acids 86–94 deleted. Such a core variant was able to self-assemble (data not shown), perhaps due to a compensatory effect of the valine residue that in this case replaced G94. This may be analogous to the effect of the serine
Antigenicity of naturally occurring deleted HBV core proteins

The core variants that retained their capsid-forming ability were purified by gel filtration on Sepharose columns: #8a and #8b in both the long and the short form and #17 in the short form. Although variants #8a (Fig. 4) and #8b (not shown) of the HBV core protein formed core-like particles, as shown by electron microscopy, and were precipitable by human or rabbit polyclonal anti-HBc antibodies, the behaviour of mutant #8a in a competitive ELISA revealed that the organization of its capsid surface differed markedly from wild-type HBV core particles (Fig. 5a). Competition by the deleted core variant #8a for polyclonal human anti-HBc antibodies, as well as for the monoclonal anti-HBc antibody C1-5 targetted against amino acids 78–83, was decreased dramatically in comparison with the wild-type HBV core.

B cell immunogenicity of naturally occurring deleted HBV core proteins

Variant #8a was able to induce a significant anti-HBc immune response after immunization of mice, despite its markedly different antigenicity compared with wild-type core particles (see above). In Fig. 5(b), the reactivities of antibodies induced in parallel immunizations of mice with wild-type HBV core particles and with particles representing long and short forms of variant #8a are shown; titres of antibodies were determined against all the relevant antigens. Antibodies against the wild-type HBV core recognized HBV core deletion variants on the capsid surface at a lower titer than antibodies against the wild-type core.
poorly, especially the #8a short form. On the other hand, antibodies against the HBV deletion variants #8a long and #8a short possessed a novel specificity, with better recognition of the autologous antigens than of the wild-type HBV core.

Discussion

Elucidation of protein-folding and assembly competence in E. coli

We have shown recently that the progression of liver disease and liver failure in immunosuppressed renal transplant patients were correlated with the appearance and enrichment of deletion variants of the HBV C gene (Günther et al., 1996a, b). The predominant selection of in-frame mutants, carrying deletions primarily within the MIR, allowed us to propose that the emergence and accumulation of aberrant core peptides, unable to undergo correct folding and self-assembly or possessing novel properties altering their stability, epitope structure, presentation on and recognition by immunocompetent cells, could be directly responsible for liver failure (Günther et al., 1996a). Moreover, histological data suggested a direct causality between the accumulation of viral proteins and liver failure. The non-cytopathic character of the HBV infection might be transformed into a cytopathic one, with serious damage to infected hepatocytes, as a direct consequence of the accumulation of surface protein (Lau & Wright, 1993; Lenhoff & Summers, 1994; Chisari & Ferrari, 1995) or core protein (Reinke, 1989) or via the interference of mutant proteins with virus and/or cellular regulatory processes.

The E. coli expression system has been widely accepted as capable of the correct reproduction of specific HBV core properties. These include the highly efficient self-assembly of core molecules (Cohen & Richmond, 1982) and the native proportion of $T = 4$ to $T = 3$ particles characteristic of infected liver cells (Crowther et al., 1994; Böttcher et al., 1997). The E. coli system allows the synthesis of core proteins in the forms of full-length and C-terminally truncated molecules (Gallina et al., 1989). The advantage of the latter is their enhanced synthesis in bacteria (Borisova et al., 1988), associated with folding and assembly competence. The C-terminally truncated variants generate a spatial structure comparable to that of native wild-type cores (Crowther et al., 1994; Böttcher et al., 1997). Nevertheless, the C-terminally truncated variants are less stable than complete particles, probably as a consequence of the absence of encapsidated RNA (Birnbaum & Nasal, 1990; Maaßen et al., 1994). By including the deletion variants constructed in vitro in the study, it was possible to compare a greater variety of mutants, allowing a more precise definition of the borders of the regions responsible for folding and assembly.

In general, the stability of recombinant HBV core proteins in E. coli correlates with their ability to undergo subsequent correct folding and self-assembly. Since misfolded proteins should be degraded rapidly by E. coli proteases, their accumulation is usually not detectable or extremely low. We found that the appearance of deleted core proteins in E. coli and their capacity for self-assembly were, in fact, strongly dependent on the location and length of the deletion. In the case of long deletions, only traces of HBV core proteins, expressed by naturally occurring as well as artificial variants, were detectable by immunoblotting with monoclonal anti-HBc/HBe antibodies (Fig. 3).

HBV core variants deleted between glycine residues 73 and 94 (Table 2, group I) were completely folding- and assembly-competent. The data suggest that amino acid residues from at least 75 to 93 are not directly involved in the folding processes or stability of the shell. Naturally occurring mutants with an α amino acid deletion (variants #8a and #8b) belong to this group. Moreover, variant #17, with the deletion of amino acids 77–93, was also found to be assembly-competent. Considering the remarkable length of this deletion, it was not surprising that the formation of HBV cores of this variant was impaired, especially in the long form, where the level of synthesis is much lower (Fig. 3).

The HBV core monomer is built of four $\alpha$-helices (Conway et al., 1997), two of which, amino acids 51–78 and 82–110, are responsible for the formation of the spike structure (Fig. 6; Böttcher et al., 1997). On the basis of our experimental data, the left border of the ‘dispensable’ region can be located close to the C terminus of $\alpha$-helix 51–78 and the right border can be

Fig. 6. Location of natural and artificial deletions on a diagram of the HBC monomer polypeptide fold (printed in grey) according to Böttcher et al. (1997). Cylinders represent $\alpha$-helices, the N and C termini are marked and an approximate numbering scheme for the amino acids is given. Representative regions of the HBC molecule found to be dispensable for assembly are shown by thick solid lines. Amino acid residues in squares mark the borders of these deletions. The two conserved glycine residues are circled. Regions demonstrated to be dispensable for folding are shown by the black dashed line and regions essential for folding are shown by the black dotted lines.
located at the centre of \( \alpha \)-helix 82–110 (Fig. 6), coinciding with the kink within the latter (Böttcher et al., 1997). Good candidates for the putative borders of the ‘dispensable’ region are the highly conserved glycine residues 73 and 94. Deletions that extended N-terminally of G73 or C-terminally of G94 led to a loss of folding and self-assembly competence (Table 2, groups II and III). G73 seems to be an essential component for correct assembly, since assembly-competent C gene deletions never included G73 or extended N-terminally of it (compare assembly-incompetent mutant #10b, Table 2). Deletions of amino acids around residue C48, involved in monomer contacts within the core dimer (Nassal et al., 1992), led to the loss of (#11), or a strong decrease in (#8c), synthesis of these polypeptides. In contrast, short insertions at positions 44 and 46 did not influence the level of core protein synthesis (Beames & Lanford, 1995). In accordance with the structural map of the shell (Böttcher et al., 1997), the presence of amino acids P45 and E46 seems to be important for correct protein folding and dimerization.

Concerning the naturally occurring deletions including and/or extending beyond G94 (Table 2, group III), amino acids 75–79, located at the dimer interface (Böttcher et al., 1997), appear to determine the fate of mutated polypeptides. Whereas the presence of these amino acids in variants #39, #30b and #12b ensured some residual synthesis of polypeptides in insoluble form, their absence was associated with the complete loss of detectable expression products in the cell.

The data presented are in agreement with earlier results that showed that the deletion of amino acids 85–92 did not prevent the synthesis of the polypeptide \textit{in vitro} in rabbit reticulocyte lysates (Fiordalisi et al., 1994) and that deletions in the loop region, amino acids 43–51, prevented self-assembly (Koschel, 1996). On the other hand, we have shown clearly that at least amino acids 77–93 within the regions 87–123 and 78–117, which were recently found to be necessary for the dimerization of core polypeptides (Koschel et al., 1999; König et al., 1998), are not essential for self-assembly.

**Immunological peculiarities of assembly-competent, MIR-deleted HBV core proteins**

The MIR forms the most exposed part of the core surface, on the tips of the spikes (Böttcher et al., 1997), determining the unusually high immunogenicity of HBV C gene products in experimental animals (Borschukova et al., 1997) and inducing the major B cell response in hepatitis B patients (Tordjeman et al., 1993). At the same time, the MIR sequence, being maximally exposed and sufficiently flexible, seems to be one of the rare regions within the HBV core molecule that can accommodate marked deletions/insertions without influencing folding and self-assembly (for reviews see Pumpens et al., 1995; Ulrich et al., 1998). From the point of view of viral genome structure, this region is also one of the rare areas that does not encode more than one HBV protein. Nevertheless, it is well conserved in wild-type genomes.

Our experimental data relating to the antigenicity and immunogenicity of the deleted HBV core variant #8a show unambiguously that the external surface of mutant core particles differed from the surface of wild-type HBV cores. Competitive mapping of deleted HBV core variants with the monoclonal anti-HBc antibody C1-5, which recognizes the tip of the spike (positions 78–83), revealed directly the altered spatial organization of the spike tip in mutant cores (Fig. 5a). Such particles induced an anti-HBc response only with greatly reduced efficiency and their reactivity with murine anti-HBc antibodies was significantly lower than that of wild-type cores (Fig. 5b). In the light of these data, assembly-competent HBV core deletion variants may act as a form of immune escape, since patients under immunosuppression may still possess normal, pre-existing serum anti-HBc levels (Günther et al., 1996a).

Virus-like particles formed on the basis of HBV core protein can be exploited as carriers of foreign peptide sequences (for review see Ulrich et al., 1998). The MIR has been demonstrated to tolerate large foreign insertions (of more than 100 amino acids) that allow the induction of a strong B cell response against the foreign sequence (D. Koletzki, unpublished data). Therefore, naturally occurring, MIR-deleted HBV C gene variants that demonstrate diminished HBc antigenicity and immunogenicity have the potential to act as improved carriers for foreign immunodominant epitopes.

**The possible contribution of different core protein mutants to liver cell pathogenesis**

On the assumption that the \textit{E. coli} expression system is adequate as a model of the intracellular behaviour of mutated cores, our data should allow the investigation of several aspects of the pathogenic potential of the appearance of (i) assembly-competent, (ii) synthesis- but not assembly-competent and (iii) unstable variant core proteins in infected hepatocytes. It is likely that the mechanisms exerted by different core variants interact in the infected cell, leading to a rather complex pathobiological situation in the case of \textit{in vivo} infection.

Firstly, it is noteworthy that dimerization- and assembly-competent mutants were found to predominate in only a few immunosuppressed kidney transplant patients; however, their accumulation was always accompanied by rapid progress of liver disease and failure (Günther et al., 1996a; data not shown). It is not clear whether assembly-competent core variants can be enveloped and whether they are replication-competent. If not, their cytotoxic effect could be a direct consequence of their intracellular retention, in accordance with previous observations (Roingeard et al., 1990).

Secondly, the deleted core polypeptides may interact with wild-type core protein and interfere with capsid assembly. Such dominant-negative inference has been shown previously for artificial and natural core proteins (Scaglioni et al., 1994, 1996; von Weizsäcker et al., 1996; Yuan et al., 1998a) and for
the precore protein (Scaglioni et al., 1996). We now have evidence that mixed core particles may be formed by wild-type cores and the assembly-competent mutants #8a long, #8a short and #17 short, as well as the assembly-incompetent mutant #17 long (data not shown). It cannot be excluded that the pathogenic potential of such mutants is realized by a reduction of the capsid-forming ability of wild-type core protein and its segregation to insoluble or cell membrane-sequestered fractions.

The majority of the C gene deletion variants that accumulated and persisted in our ESLD patients encoded core proteins with deletions extending beyond G94 that were folding-incompetent, at least in E. coli cells. Although the half-lives of proteins and their degradation pathways might be distinct in prokaryotes and eukaryotes, the inability to detect the synthesis of unfolded polypeptides in E. coli should reflect the rapid degradation of such translation products in hepatocytes. This assumption agrees with recent investigations of Yuan et al. (1998b); after transfection of human hepatoma cell lines (HuH7 or HepG2) with HBV genomes encoding core variants with deletions of amino acids 87–136 and 81–123, no corresponding core polypeptides could be detected.

It was shown recently that eukaryotic cytosolic chaperonins, e.g. a 60 kDa protein related to the chaperonin t-complex polypeptide I, may play a distinctive role in HBV core monomer folding and assembly (Lingappa et al., 1994). Our preliminary experiments on the expression of C gene deletion variants in the presence of overexpressed E. coli chaperonins (DnaK, DnaJ, GrpE) have demonstrated enhanced accumulation of naturally occurring HBV core proteins with large deletions (#17 and #21) (data not shown). In order to overcome the possible disadvantages of the bacterial model, we have initiated work in the Semliki Forest virus expression system, which allows highly efficient synthesis of target proteins in a broad spectrum of cell lines (Liljestro¨m & Goroff, 1991; Sioberg et al., 1994, Torresi et al., 1997) including liver-derived cell cultures.

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References
HBV C gene deletion variants


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