Biosynthesis of the Secretory Core Protein of Duck Hepatitis B Virus: Intracellular Transport, Proteolytic Processing, and Membrane Expression of the Precore Protein

HANS-JÜRGEN SCHLICHT
Department of Virology, University of Ulm, Albert Einstein Allee 11, W-7900 Ulm, Germany

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The biosynthesis of the secretory core gene product of the duck hepatitis B virus (DHBc protein) was examined. Recombinant vaccinia viruses were constructed encoding either the full-length or C-terminally truncated forms of the DHBc precursor protein (precore protein) and used to express these proteins in the human hepatoma cell line HepG2. Western immunoblot analysis of core gene products isolated from cells producing the full-length precore protein revealed the presence of DHBc precursor proteins containing the strongly basic C-terminal sequence which is lacking in the mature DHBc protein. These proteins were not secreted, suggesting that C-terminal proteolytic processing of the precore protein represents an obligatory step for DHBc biosynthesis. Pulse-chase experiments showed that this cleavage reaction occurs late during DHBc synthesis. Interestingly, when mutated precore proteins were expressed which lacked the basic C-terminal domain, proteins were produced which were glycosylated but not secreted. This shows that the transient presence of this region is essential for intracellular transport of the precore protein. Cell sorter analyses revealed that production of a cell surface-expressed variant of the secretory core protein is a feature conserved between the duck and the human hepatitis B viruses. Surprisingly, the C terminus of the membrane-expressed DHBc protein was accessible from the outside, showing that the topology of this interesting protein is more complicated than expected.

One feature of hepatitis B virus (HBV) gene expression is that at least two different core gene products are synthesized in infected cells (6). One of these proteins, referred to as the c protein, is a cytoplasmic protein which has a strong tendency to aggregate. It forms the viral capsid and is exported from the cells only as part of a virus particle. In contrast, the other protein, referred to as the e protein, is a secretory protein which does not form aggregates. It can be detected in large amounts in the serum and serves as a serologic marker for diagnosis of an HBV infection. Why HBVs produce a secreted core gene product is unknown. This protein is not essential for the production of infectious virus (5, 22); however, it is speculated that it might possess immunomodulatory properties which could be important during the development of chronic infections (13).

During the last few years, much has been learned about the mechanisms of c and e protein biosynthesis (7-9, 15, 16, 22, 25) (Fig. 1a). The e protein is derived from a larger precursor (the precore protein) whose translation starts at an AUG that is located slightly upstream from the AUG where c protein translation initiates. For this reason, the precore protein contains a short N-terminal extra sequence which is lacking in the c protein. This portion, which is called the pre-C sequence, functions as a signal sequence and mediates the translocation of the precore protein into the endoplasmic reticulum (ER). During in vitro translation of HBV precore mRNA in the presence of microsomal membranes, most of the precore protein generated is N-terminally processed (7, 25). Another proteolytic processing step occurring in vivo appears to be the removal of a strongly basic domain located at the C terminus of the precore protein, since this part is lacking in the secretory core protein which can be isolated from the serum of HBV-infected humans or ducks (22, 26). Thus, the processed precore protein, which for historic reasons is also called the e protein (12), differs from the c protein in that it contains a short extra sequence at its N terminus and lacks the basic domain at the C terminus.

While the principles of c and e protein biosynthesis are now clear, there are still many open questions. For instance, the time points and the intracellular locations of the processing steps and their consequences for the different physical and serological properties of the c and e proteins are unclear. Moreover, as has been shown recently (23), cells producing the HBV e protein also express a membrane-bound variant of this protein. How the e protein, which otherwise behaves like a bona fide secretory protein, can be incorporated into the cell membrane is enigmatic.

To examine the biosynthetic pathways leading to the production of the different core gene products, recombinant vaccinia viruses encoding wild-type or mutated duck HBV (DHBV) precore proteins were constructed and used to express these proteins in tissue culture cells. With this system, expression levels could be achieved which were high enough to detect intermediates of DHBV e protein (DHBc) biosynthesis.

MATERIALS AND METHODS

Constructs. To obtain efficient expression of DHBc core gene products in tissue culture cells, recombinant vaccinia viruses were constructed encoding either the secretory or the cytoplasmic core protein (DHBc and DHBc protein, respectively; Fig. 1b). For production of a DHBc-expressing vaccinia virus (referred to as DHBc-VAC), an EcoRV-Kpnl fragment, corresponding to nucleotides (nt) 2653 to 1294 of the DHBV genome, was excised from plasmid pOL16, which contains a DHBV type 16 (DHBV-16) overlength genome (21). This fragment was then cloned into the HBV-vaccinia virus construct HBc-VAC described previously (23), from which all the HBV sequences except the HBV
ATG had been removed by cutting with EcoRV and KpnI. The construct Hbc-VAC contains a partially synthetic HBV core gene cloned downstream from the vaccinia virus 7,500-M, protein (7.5k protein) gene early/late promoter. In this gene, an EcoRV restriction site had been introduced immediately downstream from the HBV core gene AUG (14). Since this EcoRV site is in frame with the natural EcoRV site present in the DHBV-16 genome, it allowed the cloning of the DHBV core gene downstream of the vaccinia virus promoter starting precisely with the DHBc ATG. For production of a DHBc-encoding vaccinia virus (referred to as DHBc-VAC), a Tag1-KpnI fragment (nt 2490 to 1294 of the DHBV-16 genome) was cloned into a vaccinia virus expression vector downstream from the 7.5k protein gene early/late promoter. The first AUG on the mRNA expressed from this construct is the DHBc pre-C AUG.

To investigate the relevance of the basic C-terminal domain of the DHBc for DHBc biosynthesis, mutants lacking the last 12, 36, or 89 amino acids were generated (Fig. 1c). For construction of the 12- and 36-amino-acid deletion mutants, EcoRI-KpnI fragments were excised from two DHBc C terminus stop mutants described previously (19) and recloned into the DHBc-VAC construct. In these mutants, stop codons had been generated by single base exchanges at nucleotide positions 376 and 304. For generation of the 89-amino-acid deletion mutant, the DHBc-VAC construct was cleaved with Hincl and BglII, which cut only within the DHBV C gene sequence. After end-filling of the BglII site cut with Klenow polymerase, the plasmid was self-ligated. This leads to the deletion of nt 144 to 391 of the DHBV sequence. Translation terminates at a stop codon located within the polylinker of the plasmid, four codons downstream of the Hincl cleavage site. Thus, in this construct, the last 89 amino acids of the DHBc were deleted and four new amino acids were added (C-terminal sequence: T-W-L-S-i-s-l-a-stop; DHBV sequence in uppercase letters, plasmid sequence in lowercase letters). Generation of recombinant vaccinia viruses was done by standard methods (4, 10, 11). In the text, the viruses are referred to as DHBc-VAC12, DHBc-VAC36, and DHBc-VAC89.

Expression and analysis of DHBc and DHBc in tissue culture cells. Infection of HepG2 cells with recombinant vaccinia viruses, immunoprecipitation of DHBV core gene products, endoglycosidase F digestion, and subsequent Western immunoblot analyses were done essentially as described before (22, 23). In brief, HepG2 cells (1) (10² cells grown in a 10-cm² dish with 2 ml of Dulbecco's minimal essential medium containing 10% fetal calf serum [FCS] at 5% CO₂) were infected with recombinant or wild-type vaccinia virus at a multiplicity of infection of 10 in 1 ml of serum-free medium. After 90 min at 37°C, the inoculum was removed and 2 ml of fresh medium containing 10% FCS was added. Analyses were done 12 to 16 h after infection.

For biosynthetic labeling, cells infected for 16 h with DHBc-VAC were washed twice with phosphate-buffered saline (PBS), and 100 μCi of [%S]methionine (specific activity, >800 Ci/mmol; Amersham-Buchler, Braunschweig, Germany) in 1 ml of methionine-free medium was added. After 15 min at 37°C, the labeling medium was replaced by 2 ml of warmed complete medium. After various times, the medium was removed and the cells were lysed in 1 ml of PBS–1% Triton X-100. Insoluble material was removed by centrifugation, and core and gene products were isolated from the medium or the cleared lysates by immunoprecipitation with a rabbit antiserum raised against denatured DHBV core protein adsorbed to protein A-Sepharose (Pharmacia, Freiburg, Germany). For background reduction, the immunoprecipitate thus obtained was boiled for 3 min in 50 μl of PBS–2% sodium dodecyl sulfate (SDS)–1% 2-mercaptoethanol and diluted 1:20 with PBS–1% Triton X-100. Insoluble material was again removed by centrifugation, and the immunoprecipitation step was repeated. After being washed with PBS, precipitated proteins were solubilized in sample buffer and separated on a 12% polyacrylamide gel. The gels were treated with EnHance (Amersham-Buchler) and dried, and the isolated proteins were visualized by autoradiography at −70°C.

Flow cytometry. Cells infected as described above were put on ice and incubated for 45 min on ice with either a polyclonal rabbit antiserum raised against denatured core protein or a polyclonal rabbit antipeptide serum specific for the last 13 C-terminal amino acids of DHBe, diluted 1:500 in
TABLE 1. Detection of cell surface-bound DHBV core gene products by radioimmunoassay

<table>
<thead>
<tr>
<th>Virus</th>
<th>Antibody bound (cpm)</th>
<th>Anti-DHBc</th>
<th>Anti-DHBc CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHBV-VAC</td>
<td>509</td>
<td>1,008</td>
<td></td>
</tr>
<tr>
<td>DHB-VAC</td>
<td>17,348</td>
<td>9,628</td>
<td></td>
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<tr>
<td>DHB-VACΔ12</td>
<td>17,775</td>
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<tr>
<td>DHB-VACΔ36</td>
<td>21,156</td>
<td>362</td>
<td></td>
</tr>
<tr>
<td>DHB-VACΔ89</td>
<td>2,461</td>
<td>&lt;0</td>
<td></td>
</tr>
</tbody>
</table>

*HepG2 cells were infected either with wild-type vaccinia virus or with the recombinants DHBV-VAC, DHB-VAC, DHB-VACΔ12, DHB-VACΔ36, and DHB-VACΔ89. Membrane-bound core gene products were detected by incubating the cells first with antisera specific for either the whole precore protein (anti-DHBc) or its C terminus (anti-DHBc CT) and, after washing, with 125I-labeled protein A. Specifically bound antibody, as given in the table, corresponds to the difference between the values obtained for cells infected with the DHBc- or DHBc-expressing viruses and the wild-type virus-infected cells. The Western blot analysis shown in Fig. 3 was carried out with the lysates and culture supernatants from the cells used for the radioimmunoassay shown here.

PBS-2% bovine serum albumin (BSA)-0.1% NaN3. The anti-core protein serum binds to both the DBHe and the DHBc, whereas the antipeptide serum reacts only with the DHBc (22). After being washed with PBS-2% BSA-0.1% NaN3 and PBS, bound antibody was stained by incubating the cells for 30 min on ice with 0.7 ml of goat anti-rabbit immunoglobulin antiserum labeled with fluorescein isothiocyanate (FITC; Dianova GmbH, Hamburg, Germany) diluted 1:40 in PBS-2% BSA-0.1% NaN3. Washing was done as above. After staining, the cells were gently washed off the plate and propidium iodide (10 μg/ml) was added. Analysis was done with a Becton Dickinson FACScan flow cytometer. Computer gating was used on propidium iodide (red) fluorescence to exclude dead cells and on forward light scatter to exclude debris and aggregates.

**Cellular radioimmunoassay.** To further analyze the capacity of antiserum to bind to the membrane-expressed DBHe, a cellular radioimmunoassay was used. Human hepatoma cells (HepG2 cells) were infected either with wild-type vaccinia virus or with the recombinants DHBV-VAC, DHB-VAC, DHB-VACΔ12, DHB-VACΔ36, and DHB-VACΔ89. At 10 to 14 h after infection, the cells were chilled on ice, washed with PBS, and incubated for 45 min on ice with the antiserum described above for flow cytometry, diluted 1:500 in PBS-1% BSA-0.1% sodium azide (1 ml of diluted antiserum per 10-cm2 dish). To remove unbound antibodies, the cells were washed once with PBS and incubated for 20 min with 2 ml of PBS-1% BSA-0.1% sodium azide on ice. Specifically bound antibody was detected by incubating the cells for 30 min with 0.5 μCi of 125I-labeled protein A (Amersham) in 0.7 ml of PBS-1% BSA-0.1% sodium azide. Unbound protein A was removed by washing the cells with PBS and PBS-1% BSA-0.1% sodium azide as described above. The cells were then lysed in 1 ml of PBS with 1% Triton X-100, and bound radioactivity was determined in a gamma counter. All samples were tested in triplicate. Background binding of antiserum was tested on cells infected with wild-type vaccinia virus. Specifically bound antibody, as shown in Table 1, corresponds to the difference between the values obtained for cells infected with the DHBc- or DHBc-expressing viruses and the wild-type virus-infected cells. If a value of <0 is given for the bound antibody, the serum reacted more strongly with the wild-type vaccinia virus-infected cells than with the infected with the recombinant.

**RESULTS**

Detection of C-terminally uncleaved DHBV precore proteins. For analysis of DBHe synthesis, a full-length DHBV-16 core gene starting with the pre-C ATG was cloned downstream from the vaccinia virus 7.5K protein gene early/late promoter and used to produce a recombinant vaccinia virus clone (referred to as DHBV-VAC; Fig. 1b). HepG2 cells were then infected with the recombinant virus, and after 16 h, DHBV core gene products were isolated from cell lysates and tissue culture supernatants by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. As shown in Fig. 2 (left, lane 1), within the cells four proteins could be detected with apparent sizes of 27, 30, 32, and 34 kDa. Since the 30- and 34-kDa species were found to be glycosylated (see below), they are referred to as gp30e and gp34e, respectively. The two other proteins will be referred to as p27e and p32e. Only p27e and gp30e were secreted (Fig. 2, left, lane 2). These proteins have the same size as the DBHe proteins which, as described previously (22), can be isolated from the serum of infected ducks (Fig. 2, left, lane 3). After digestion with endoglycosidase F, only p32e and p27e could be detected (Fig. 2, right). Since the e protein of DHBV-16 contains one glycosylation site which is utilized with an efficiency of about 20 to 50% (22), gp30e and gp34e apparently represent the glycosylated variants of p27e and p32e, respectively.

To test whether the size difference between p27e and gp30e and between p32e and gp34e is due to differences in the C terminus, recombinant vaccinia viruses were constructed expressing precore proteins lacking the last 12, 36, or 89 C-terminal amino acids (Fig. 1c, referred to as DHBV-VACΔ12, -Δ36, and -Δ89; see also Materials and Methods).
As shown in the left panel of Fig. 3, the 12-amino-acid deletion had already shifted p32° and gp34° to smaller sizes, whereas p27° and gp30° were affected only after deletion of 89 amino acids. This shows that p32° and gp34° in fact represent C-terminally uncleaved precore proteins and that cleavage takes place either at or upstream of the position where the stop codon had been introduced in DHBe-VACΔ36. Moreover, the finding that p32° and gp34° were not secreted (compare lane 1 with lane 2 in Fig. 2) strongly suggests that C-terminal cleavage is an obligatory modification during DHBe biosynthesis.

**Intracellular transport of a precore protein mutant lacking the last 89 C-terminal amino acids is blocked.** Another interesting observation was made when tissue culture supernatants from cells infected either with DHBe-VAC or with viruses expressing the C-terminally truncated forms were examined for DHBe proteins. As shown in the center panel of Fig. 3, the 12- and 36-amino-acid deletions did not interfere with DHBe secretion, a finding already observed in transient expression assays (19). However, not even trace amounts of DHBe protein could be detected in the medium from cells producing the 89-amino-acid deletion variant, in spite of the fact that significant amounts of core gene products could be detected within the cells (Fig. 3, left panel). The same observation was made with several independent virus clones from different recombinations as well as with several variants in which the signal sequence had been altered (unpublished observation). Moreover, another mutant with a 137-amino-acid-long C-terminal deletion was also not secreted (unpublished observation). To test whether this defect in secretion was due to a block in translocation, core gene products from cells infected with the Δ89 mutant were subjected to endoglycosidase F digestion. As shown in the right panel of Fig. 3, the intracellular protein was clearly glycosylated and therefore must have been translocated into the ER. Thus, the C-terminal deletion resulted in the production of a precore protein that was translocated but which was defective in intracellular transport.

**C-terminal cleavage occurs late during DHBe synthesis.** To test whether C-terminal cleavage of the precore protein is an early or late processing event, a pulse-chase experiment was performed. HepG2 cells were infected with DHBe-VAC for 16 h and labeled with [35S]methionine for 15 min. After the indicated chase times, cells were harvested, and core gene products were isolated from cell lysates (upper panel) or medium (lower panel) by immunoprecipitation and visualized by autoradiography.

**DHBc-producing cells express a membrane-bound DHBe protein.** Recently, evidence has been provided that cells expressing the e protein of the human HBV also express a membrane-bound variant of this protein (23). To test whether this property is shared by the duck and the human HBVs, HepG2 cells infected with DHBe-VAC were examined for cell surface-expressed DHBe proteins by flow cytometry. As controls, HepG2 cells were used which had been infected either with a vaccinia virus recombinant expressing the cytoplasmic core protein (DHBe-VAC; see Fig. 1b) or with wild-type vaccinia virus. In the first experiment, the result of which is shown in the left part of Fig. 5, cell surface-expressed core gene products were detected with a rabbit antiserum raised against denatured DHBe protein (anti-DHBe). This serum binds to both the DHBe and the DHDe. Apparently, the DHBe-VAC-infected cells were stained very strongly by this antiserum, whereas no significant reaction could be observed with the control cells.
FIG. 5. Flow cytometry analysis of HepG2 cells infected either with wild-type (WT) vaccinia virus or with recombinants encoding the DHBV c or e protein. Cell surface-expressed core gene products were detected by using rabbit antisera raised against the whole DHBV c protein (anti-DHBV core; left) or a peptide comprising the last 13 C-terminal amino acids (anti-DHBV core C-terminus; right). The data are presented as contour plots. x axis, forward scatter (linear scale); y axis, FITC fluorescence (log scale). The mean fluorescence of the cells analyzed is also given.

To obtain information about the topology of the membrane-bound DHBe protein, the cell sorter analysis was repeated with an antiserum raised against a peptide comprising the last 13 amino acids of the DHBV core protein (anti-DHBe CT [22]). As is demonstrated in the right panels of Fig. 5, this antiserum also bound strongly to the DHBe-producing cells, showing that the C terminus of this protein is accessible from the outside. In addition, since DHBe proteins containing this region are not secreted, this finding also provides a strong argument against the assumption that antibody binding might be due to adsorbed DHBe proteins.

To examine this point further, cellular radioimmunoassays were performed with cells infected either with wild-type vaccinia virus or with the recombinants DHBe-VAC, DHBe-VAC, DHBe-VACA12, DHBe-VACA36, and DHBe-VACA89. As shown in Table 1, in this assay the anti-DHBe serum also bound strongly to the DHBe-producing cells. Deletion of the last 12 or 36 amino acids of the precore protein did not influence binding, showing that most of the basic C-terminal domain of the precore protein is dispensable for precore membrane expression. Since only a very weak reaction was observed with cells producing the 89-amino-acid deletion mutant, secretion appears to be essential for membrane transport.

The antipeptide serum also reacted with the DHBe-producing cells, a finding consistent with the observations made during the cell sorter analysis. Interestingly, this serum also reacted with the cells infected with the 12-amino-acid deletion mutant, that is, with cells producing a precore protein lacking 12 of the 13 amino acids against which this serum had been raised. Several independent analyses showed the same result. Inspection of the DHBV precore amino acid sequence then revealed that the C-terminal sequence of the full-length precore protein (last six amino acids, S-P-S-P-R-K) is very similar to the C-terminal sequence of the 12-amino-acid deletion mutant (last six amino acids, S-P-L-P-R-S). In fact, four of the last six amino acids are identical. Thus, it appears quite likely that the reactivity of the antipeptide serum with the cells producing the 12-amino-acid deletion mutant is due to this high degree of amino acid identity. In any case, deletion of the last 36 amino acids completely abolished reactivity with the antipeptide serum, whereas binding of the anti-DHBe serum was not affected. Therefore, at least part of the membrane-expressed precore protein must be arranged in such a way that the C-terminal sequence is extracytoplasmic.

DISCUSSION

Biosynthesis of the secretory core gene product of DHBV was examined by using recombinant vaccinia viruses. Compared with other methods which have been used previously for the study of HBV gene expression in tissue culture cells, this system has the advantage that protein quantities can be expressed that are large enough to detect biosynthetic intermediates. One possible drawback is that the influence of other viral proteins on precore processing can only be studied if the cells are infected with more than one recombinant. However, as has been shown in Fig. 2, the DHBe protein synthesized by the DHBe-VAC-infected cells did not differ from the DHBc which can be isolated from duck sera. Thus, it appears that other viral proteins are not important for precore processing. In particular, this finding demonstrates that the C-terminal cleavage of the precore protein either is autocatalytic or, as seems more likely, is mediated by a cellular protease.

When lysates from cells infected with a recombinant encoding the full-length precore protein were analyzed for core gene products, C-terminally uncleaved precore proteins could be readily detected. These proteins were located exclusively intracellularly. Thus, the uncleaved precore proteins either are actively retained within the cells or, alternatively, are not released. In fact, it appears possible that precore membrane expression is due to molecules which escaped from C-terminal processing.

Pulse-chase experiments showed that C-terminal proteolytic processing of the precore protein occurs relatively late during e protein biosynthesis. With respect to secretion efficiency, the DHBe protein behaved like a standard secretory protein. Secretion of pulse-labeled DHBe protein started about 45 min after labeling, and after 8 h, only small amounts remained within the cells. This is in contrast to findings reported for the HBV e antigen, suggesting that this protein is only poorly secreted (25).

Interestingly, no secreted e proteins could be detected after expression of precore mutants which lacked the basic C-terminal domain. Since the intracellular forms were glycosylated, truncation did not affect the translocation of the precore protein into the ER but apparently blocked its intracellular transport. This finding was unexpected, since it is generally assumed that the C-terminal domain is only important for nucleocapsid production. Because of its high content of basic amino acids, it is believed that this region
interacts with the packaged nucleic acid and thereby influences both core particle stability and genome replication. In fact, direct evidence supporting these assumptions has been provided recently for both DHBV and HBV (2, 19). With respect to e protein biosynthesis, it was thought that this part of the precore protein was unimportant or even detrimental, because, as shown by in vitro translation experiments (3), it may represent an obstacle for the translocation process. It thus appeared reasonable that this domain had to be cleaved to allow e protein secretion. Yet, as is shown here, the C-terminal domain is by no means useless; rather, it is crucial for e protein production. What the exact role of this sequence is remains to be determined. Although it is possible that it is directly involved in the transport process, it appears more likely that it is needed for proper folding of the precore protein. As has been shown for several viral and cellular proteins, mutations can result in the production of aberrant proteins which, in their misfolded form, bind to certain resident ER proteins. As a consequence of this binding, these proteins are efficiently sorted back to the ER immediately after they have reached a specialized pre-Golgi compartment ("salvage compartment") whose function appears to be to prevent the export of improperly synthesized secretory proteins (17, 18).

Recently, it has been found that HepG2 cells infected with a vaccinia virus recombinant encoding the e protein of human HBV produce a variant of this protein which is transported to the cell surface (23). Most importantly, this cell surface-expressed HBV e protein is recognized by human antibodies whose appearance usually correlates with virus elimination (24). Thus, it appears possible that termination of HBV infection might be due at least in part to an antibody-mediated attack on the infected hepatocytes. Owing to the narrow host range of HBV, this hypothesis can hardly be tested with HBV; therefore, whether production of a cell surface-expressed e protein is a feature conserved between HBV and DHBV was examined. As shown here, this is in fact the case. HepG2 cells infected with DHBV-VAC showed a strong surface binding of DHBV- and e-specific antibodies, whereas DHBV-producing cells and control cells did not. Thus, the DHBV system is currently being used to examine the in vivo relevance of this interesting viral protein. The mechanism by which the e protein is incorporated into the cell membrane is still unclear. To date, attempts to isolate the membrane-bound DHB e protein either directly or after cell surface iodination failed, most likely because of the scarcity of this protein (unpublished observations). However, in the case of HBV, development of an HBV e variant which expressed about 10-fold more of the membrane-expressed HBV e protein than the wild type allowed the detection of this protein, which appears to represent a C-terminally uncleaved precore protein (18a). That antibody binding is due to peptides which might be transported to the cell surface in association with histocompatibility antigens is unlikely, since cells infected with the secretion-deficient 89-amino-acid deletion mutant described here did not react with the antisera. Thus, it appears that cell surface expression requires secretion. Moreover, the fact that the surface-expressed DHB e protein was efficiently recognized by the C-terminus-specific antipeptide serum strongly argues against the possibility that binding is due to adsorption of secreted e protein since, as shown here, DHB e variants containing this sequence were not secreted. This finding also raises questions about the topology of the membrane-bound e protein. As has been discussed above, from in vitro translation studies, it has been proposed that the basic C-terminal domain of the precore protein might represent an obstacle for ER translocation (3). This view, however, had already been challenged by the finding that transmembraneous e proteins were not always observed after in vitro translation (7). Moreover, as is shown here, deletion of most of the basic C-terminal domain of the DHBV precore protein did not influence e protein membrane expression. In addition, the fact that the C-terminal domain of the membrane-bound DHB e protein is accessible from the outside cannot be reconciled with a topology which requires this domain to be located within the cytoplasm.

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