Properties of modified hepatitis B virus surface antigen particles carrying preS epitopes

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The current hepatitis B virus (HBV) vaccines contain the small (S) and middle (M) viral envelope proteins in particulate form but lack the large (L) protein. Although these particles elicit protective immunity to HBV, inclusion of the immunogenic preS1 region of the L protein may enhance their efficacy. To present preS1-derived epitopes on secretable subviral particles we rearranged the HBV envelope ORF by fusing part or all of the preS1 region to either the N or C terminus of the S protein. Fusion of the first 42 residues of preS1 to either site allowed efficient secretion of the modified particles and rendered the linked sequence accessible at the surface of the particle. Conversely, fusion of preS1 sequences to the C terminus of the M protein completely blocked secretion. This block to secretion could be rescued by provision of a heterologous N-terminal signal sequence. All these particles displayed preS1, preS2 and S protein antigenicity. In mice, each construct elicited high titres of preS1-specific antibodies which recognized the authentic L protein. Particles composed of the modified M protein also induced a preS2-specific response. Unexpectedly, however, neither particle elicited S protein-specific antibodies. Nonetheless, the genetic approach employed here represents a strategy to incorporate preS1-derived epitopes both in high density and in highly immunogenic form into their authentic carrier matrix.

Introduction

The small (S) envelope protein of hepatitis B virus (HBV) carrying the major surface antigen (HBsAg) has the unique property of mobilizing cellular lipids into empty envelope particles which are secreted from infected liver and transfected cell lines (reviewed in Heermann & Gerlich, 1991). Such subviral HBsAg particles are non-infectious and are now widely and safely used worldwide for hepatitis B vaccination (Valenzuela et al., 1982; Harford et al., 1983; Michel et al., 1984). Because of its intrinsic adjuvant potential, particulate HBsAg has been developed as a carrier for the presentation of various foreign antigens, e.g. epitopes of herpes simplex virus type 1 glycoprotein D (Valenzuela et al., 1985), neutralization epitopes of the capsid protein VP1 of poliovirus (Delpeyroux et al., 1986), malarial parasite antigen (Rutgers et al., 1988; von Brunn et al., 1991) or selected human immunodeficiency virus type 1 determinants (Michel et al., 1988). In this respect, particulate HBsAg should also be useful for the preparation of multivalent hepatitis B immunogens containing additional protective epitopes of the HBV envelope.

The HBV envelope contains the middle (M) and large (L) protein in addition to the S protein. All three envelope proteins are encoded in a single ORF and initiate at separate in-phase ATG codons spaced at intervals of 108 (or 119, depending on serotype) and 55 codons. The segments downstream of the three initiation codons are called the preS1 and preS2 regions, and S gene, respectively (reviewed in Heermann & Gerlich, 1991).

The preS region carries various B and T cell epitopes which are highly immunogenic in mice and humans (Neurath et al., 1986a, 1989a; Milich et al., 1988). In congenic mice, inclusion of preS regions elicits a broader spectrum of protective antibodies which augment the anti-S response and circumvent non-responsiveness to the S protein (Neurath et al., 1986a, 1989a; Milich et al., 1988). In humans, the preS1 region has been shown to be a particularly efficient immunogen at the T and B cell level. A dominant T cell recognition site was identified in the N-terminal residues 21–28 (serotype adw) of the preS1 sequence (Jin et al., 1988; Ferrari et al., 1989), while B cell determinants were mapped to residues 12–32, 32–53 and 94–117 (serotype adw) (Milich, 1988; Neurath et al., 1989a). Moreover, the preS1 region carries the viral receptor for hepatocytes (Neurath et al., 1986b) and antibodies to a synthetic peptide which mimics the receptor binding site (residues 21–47, serotype adw) can protect chimpanzees from HBV infection.

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(Neurath et al., 1989a; Thornton et al., 1989). Neutralizing epitopes have also been demonstrated within the preS2 region (Neurath et al., 1986a; Thornton et al., 1989). Therefore, empty envelope particles which also carry the preS1 and preS2 sequences have been considered to be an improvement over the actual HBV vaccine. By broadening the immune response, such immunogens may also prevent the emergence of escape mutants and may protect against variant viruses (reviewed in Rutgers et al., 1993).

Immunogens containing both S and preS2 sequences can easily be prepared using recombinant DNA technology (Michel et al., 1984) since the M protein is efficiently secreted from transfected cells together with the S protein. Attempts to obtain empty envelope particles carrying a high percentage of preS1 sequences, however, have been unsuccessful until now since the L protein is not secreted from transfected cells (Persing et al., 1986; Ou & Rutter, 1987).

In this work we have addressed this issue by rearranging the ORF of the HBV envelope proteins. Part or all of the preS1 region was fused to both the N and C termini of the S protein, and the preS2 region was optionally included. We analysed the properties of the modified envelope proteins with respect to assembly, secretion, antigenicity and immunogenicity using transient expression in COS-7 cells and immunization of animals.

Methods

Plasmid constructions. Rearrangements of the envelope (env) ORF of HBV (serotype ayw) were performed using plasmid pN12.S (Prange et al., 1991) which carries the S gene under the transcriptional control of the human metallothionein IIA promoter. For fusing preS1 encoding sequences to the C terminus of the S gene, the unique AccI restriction site, which cuts the S gene between codons 223 and 224 (nt 827 of the HBV genome), was first modified by addition of synthetic HincII-linker DNA. Flush-ended restriction fragments encoding either the entire preS1 sequence (BglII–EcoRI, nt 2839–3182) or its first 42 aa (BglII–Eael, nt 2839–2970), 62 aa (BglII–Stul, nt 2839–3034), 69 aa (BglII–Ssal, nt 2839–3052) or 94 aa (BglII–Fnu4HI, nt 2839–3126) were then inserted into the newly created HincII site of the S gene. These constructs were designated S.Le108, S.Le42, S.Le62, S.Le69 and S.Le94, respectively. Each construct carries five additional amino acids (E-S-I-Y-S) at the junction between the S and preS1 regions and terminates at the translational stop of the S gene. In order to incorporate the preS2 region as well, the shortest of these constructs (S.Le42) was used to replace the S region of the M gene encoded by plasmid pN12.M (Prange & Streeck, 1995) giving rise to construct M.Le42.

For fusing the first 41 aa of the preS1 region to the N terminus of the S protein, a genetically engineered Xhol restriction site was used, which is located 1 bp 5' to the methionine start codon of the S protein. A flush-ended BglII–BalI fragment (nt 2839–2972) was inserted into the filled-in Xhol site, thereby generating two additional aa (S-R) at the linkage between regions preS1 and S. To obtain this construct (S.Le41) a mNystaryl-defective mutant gene (L.Ala2) was used (Prange et al., 1991).

The introduction of the β-lactamase signal sequence into the M.Le42 construct was carried out in several steps. Briefly, an EcoRI restriction site was introduced at codon positions 29/30/31 of the β-lactamase gene located 17 bp 3' to the peptide cleavage site using oligonucleotide-directed mutagenesis. An SspI–EcoRI fragment encoding the first 29 aa of β-lactamase was then fused in frame to residue 4 of the M.Le42 construct by using its single EcoRI restriction site (nt 3182/0).

For cell-free transcription, translation and translocation, the S, M and L genes were cloned into plasmid pSP64Poly(A) (Promega), as described previously (Prange & Streeck, 1995).

For expression in bacteria, either the first 42 aa of the preS1 region or aa 4–49 of the preS2 region was fused in frame to the C terminus of the glutathione S-transferase (GST) encoded by plasmid pGEX-3X (Pharmacia). To achieve this, either a flush-ended BglII–BalI (nt 2839–2970) fragment or an EcoRI–Xhol (nt 3182/0–127) fragment, respectively, was inserted into the multiple cloning site of pGEX-3X.

Cell culture, transfection and immunoassays. Transient transfection of COS-7 cells was carried out as described previously (Prange et al., 1992) using the calcium phosphate precipitation technique. Three days after transfection, culture supernatants were harvested and analysed by ELISA. HbsAg was measured using the Auszyme II diagnostic kit (Abbott Laboratories). The preS1- and preS2-specific ELISAs were performed as described (Gerken et al., 1987), using the preS1-specific MAb 18/7 (Heermann et al., 1984) or preS2-specific MAB 1 in the solid phase and peroxidase-labelled HbsAg-specific MAbs (Auszyme II) in the detection phase. Alternatively, a poliovirus-specific MAB C3 (1:500 dilution) was used for coating (Delpeyroux et al., 1990) and peroxidase-labelled HbsAg-specific MAbs (Auszyme II) were used for detection of hybrid subviral particles carrying a poliovirus epitope.

Metabolic labelling and immunoprecipitation. For isotopic labelling, cells were washed 48 h after transfection and starved for 40 min in 0.6 ml of methionine-free minimal essential medium supplemented with 1% fetal calf serum (FCS). After addition of 250 μCi [35S]methionine/cysteine (1000 Ci/mmol; Dupont), cells were labelled for 3 h. The medium was then replaced with 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, and the cells were incubated further for 18 h. Lysis of cells, immunoprecipitation, and digestion with endoglycosidase H (endo H) were carried out as described previously (Prange et al., 1992), except that polycyonal antiserum was used which was raised in rabbits by immunization with yeast-derived HbsAg particles (Engerix-B; SmithKline Beecham).

Purification of subviral particles. Empty envelope particles were isolated from the culture supernatants of transfected cells. Supernatants were collected, clarified and precipitated with 50% ammonium sulphate at pH 7.5. After centrifugation for 30 min at 11000 r.p.m. and 4°C (HB4 rotor; Sorvall) pellets were dissolved in 10 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 1 mM-EDTA (TNE) supplemented with 2 mM-phenylmethylsulphonyl fluoride and 2 μg/ml aprotinin (Boehringer Mannheim) and dialysed against the same buffer. Samples were layered on a CsCl step gradient [10–50% (w/w) CsCl in TNE] and two successive runs of isopycnic centrifugation were performed for 16 h at 35000 r.p.m. and 10°C (SW40 rotor; Beckman). Fractions were collected from the top and were screened for HBsAg by ELISA. Peak fractions were pooled and dialysed at 4°C against TNE.

Immunization and measurement of antibody production. Groups of two or three BALB/c mice were immunized by intraperitoneal injections of 3 μg of the purified subviral particles (as measured by the Auszyme II ELISA) in complete Freund’s adjuvant, followed by two injections of the same dose of antigen in incomplete Freund’s adjuvant at intervals of 3–4 weeks. Sera were collected 14 days after the last boost. Alternatively, the immunogens were administered adsorbed to preformed aluminium hydroxide.

Antisera were evaluated for antibodies in ELISAs using solid-phase plasma-derived HbsAg particles (Ausab; Abbott Laboratories) or
bacterial GST-preS fusion proteins purified from the supernatants of bacterial lysates by affinity chromatography on glutathione–Sepharose 4B (Pharmacia) (Smith & Johnson, 1988). The fusion proteins were diluted to 2 μg/ml in 50 mM-sodium carbonate pH 9.5 and were bound to microtitre plates (Falcon). After washing and blocking with PBS containing 0.1% Tween 20, the plates were incubated with various antisera dilutions for 90 min at 37 °C. Immunoreactions were observed using horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:5000 dilution) and 1,2-phenyldiamine dihydrochloride (Sigma) as a substrate.

Cell-free transcription, translation and immunoprecipitation. In vitro synthesis of the env proteins was carried out with a coupled transcription/translation system (TNT Coupled Reticulocyte Lysate Systems; Promega) using rabbit reticulocyte lysate and dog pancreas microsomes, as described previously (Prange & Streeck, 1995). For immunoprecipitation, samples were diluted in 50 mM-Tris–HCl pH 7.5, 150 mM-NaCl supplemented with 0.5% Nonidet P-40, 0.05% sodium deoxycholate and 0.01% SDS, and were incubated with the indicated antisera (1:100 dilutions). Immune complexes were isolated with a 10% (w/v) suspension of protein A-Sepharose (1:10 dilution; Pharmacia).

Results

Fusion of preS1 sequences to the C terminus of the S protein

Subviral HBsAg particles which have a high content of the immunogenic preS1 sequence cannot be obtained from transfected cells since the L protein inhibits secretion of such particles. The intracellular retention of the L protein is due to both N-terminal myristylation and to internal preS1 sequences (Kuroki et al., 1989; Prange et al., 1991). Since the myristylation signal is only operative at N-terminal positions (Towler et al., 1988), we analysed whether translocation of the preS1 region from the N terminus of the L protein to the C terminus of the S protein allows secretion and assembly of subviral particles carrying preS1 epitopes.

A series of mutants was constructed by fusing either the entire preS1 region or its first 42, 62, 69 or 94 residues to the C terminus of the S protein (Fig. 1). The mutants were transiently expressed in COS-7 cells and the env proteins were analysed by immunoprecipitation with polyclonal antiserum to HBsAg particles and SDS-PAGE. Lysates of transfected cells contained the 24 kDa non-glycosylated and 27 kDa glycosylated forms of the S protein (Fig. 2a, lane 1) and at least two forms of each mutant protein (Fig. 2a, lanes 2–6) although synthesis and/or stability of mutant S.Lc69 were reduced (Fig. 2a, lane 4). The more slowly migrating polypeptides correspond to glycosylated forms, which was confirmed by treatment with endo H (Fig. 2b, lanes 2 and 4). This indicates that mutants S.Lc42 and S.Lc62 were once-
Fig. 2. Synthesis, glycosylation and secretion of wild-type and mutant S proteins carrying preS1 sequences at the C terminus. (a) Transfected COS-7 cells were metabolically labelled, and equal portions of cellular lysates or supernatants were precipitated with rabbit antiserum to HBsAg particles and subjected to SDS–PAGE. (b) Analysis of N-linked oligosaccharides on wild-type S and mutant S.Lc42 proteins. Cellular lysates were divided into two portions and were either mock-treated (lanes 1 and 3) or digested with endo H (lanes 2 and 4).

and twice-glycosylated (Fig. 2a, lanes 2 and 3; Fig. 2b, lane 3), probably at the usual carbohydrate attachment site, Asn146 of the S region, and, most likely, at the potential glycosylation site, Asn4, of the fused preS1 region.

When the secretory phenotype of the mutants was analysed, only mutant S.Lc42 was efficiently secreted (Fig. 2a, lane 8). Further extension of the fused preS1 sequence significantly impaired secretion (Fig. 2a, lanes 9–12). Nonetheless, mutant S.Lc42 was secreted with high efficiency and was chosen for further analysis.

Fusion of preS1 sequences to the N terminus of the S protein

In a parallel approach to incorporate preS1 sequences into secretable subviral particles, the first 41 aa of the preS1 region were placed in front of the S protein-encoding region (i.e. aa 42–163 of the L protein were removed; see Fig. 1). To avoid N-terminal myristylation of this construct (S.Ln41), a myristylation-defective preS1 mutant was used in which the myristyl-acceptor glycine at position 2 was changed to alanine (Prange et al., 1991). Mutant S.Ln41 was predominantly synthesized in mono- and diglycosylated form together with minor amounts of its non-glycosylated derivative (Fig. 3, lane 2), as evidenced by digestion with endo H (Fig. 3, lane 1). Importantly, mutant S.Ln41 was extracellularly released (Fig. 3, lane 3).

Fusion of preS2 sequences to the N terminus of S.Lc42

Like preS1, the preS2 region of the M and L proteins carries epitopes which have been shown to induce protective immune responses (Neurath et al., 1986a; Thornton et al., 1989). Therefore, we constructed a modified env gene carrying both preS2 and preS1 antigenic determinants by fusing the entire preS2 region to the N terminus of the S.Lc42 construct. As shown in Fig. 1, this construct (M.Lc42) resembles a C-terminally extended M protein. When synthesized in transfected cells mutant M.Lc42 appeared in several forms (Fig. 4a, lane 5), as does the M protein (Fig. 4a, lane 1) and mutant S.Lc42 (Fig. 4a, lane 3). The wild-type M
protein was expressed in 33 kDa mono- and 36 kDa diglycosylated form (Heermann & Gerlich, 1991). In addition, the 24 kDa and 27 kDa forms of the S protein were obtained as a consequence of internal translational initiation (Fig. 4a, lane 1). Similarly, mutant M.Lc42 appeared as a 38 kDa mono- and 41 kDa diglycosylated polypeptide (see also below) together with the three different forms of the internally initiated S.Lc42 protein (Fig. 4a, lane 5; also compare lanes 3 and 5). Since the M and S.Lc42 proteins were efficiently secreted from transfected cells (Fig. 4a, lanes 2 and 4), mutant M.Lc42 was reasoned to be extracellularly released as well. Rather unexpectedly, however, secretion of mutant M.Lc42 was completely blocked (Fig. 4a, lane 6).

While analysing the transmembrane structure of the env proteins, we and other workers (Ostapchuk et al., 1994; Bruss et al., 1994; Prange & Streeck, 1995) have recently demonstrated that the preS1 sequences critically affect the location of the preS2 region: while the preS2 region of the M protein faces the luminal side of the endoplasmic reticulum (ER) membrane, the preS2 region of the L protein is predominantly cytoplasmically disposed during or soon after biosynthesis. We therefore speculated that the block to secretion of mutant M.Lc42 might be due to inefficient co-translational translocation of its preS2 region across the ER membrane mediated by preS1 sequences fused downstream. In order to address this issue, we fused a heterologous signal sequence of β-lactamase in phase to the N terminus of mutant M.Lc42 (see Fig. 1). Mutant βM.Lc42 displayed a similar pattern of glycosylation and additional internal initiation of translation (Fig. 4b, lane 1) to M.Lc42 lacking a signal sequence. N-linked glycosylation was confirmed by treatment with endo H; under these conditions only the non-glycosylated forms of βM.Lc42 and S.Lc42 were found (Fig. 4b, lane 2). When separated on the same polyacrylamide gel the mono- and diglycosylated forms of M.Lc42 and βM.Lc42 present in cellular lysates displayed an almost identical electrophoretic mobility (data not shown), thus indicating that the signal sequence (i.e. its first 23 residues) of βM.Lc42 was cleaved off. Most importantly, mutant βM.Lc42 was secreted from transfected cells along with the internally initiated S.Lc42 protein (Fig. 4b, lane 3). Like wild-type M protein, the secreted forms of mutant βM.Lc42 appeared in diffuse bands. Such bands could not be identified in supernatants of non-transfected cells (Fig. 4b, lane 4), which we included as a control.

Characterization and antigenicity of modified S.Lc42, S.Ln41 and βM.Lc42 particles

To determine whether the secreted S.Lc42, S.Ln41 and βM.Lc42 proteins resemble authentic HBsAg particles, we fractionated culture supernatants of transfected cells by isopycnic CsCl gradient centrifugation and analysed the fractions by HBsAg-specific ELISA. As shown in Fig. 5 all three modified proteins banded at a buoyant density of about 1.2 g/cm³, characteristic for wild-type HBsAg lipoprotein particles. In addition, when analysed by sucrose gradient velocity centrifugation, the sedimentation profiles of the three modified proteins and wild-type S protein were nearly coincident. All four proteins banded at a buoyant density of about 1.140 g/cm³ in sucrose, indicating that the modified particles closely resembled wild-type HBsAg spherical
Table 1. HBsAg-, preS1- and preS2-specific antigenicity of wild-type and modified particles

<table>
<thead>
<tr>
<th>Secreted particles</th>
<th>HBsAg</th>
<th>preS1</th>
<th>preS2</th>
<th>polio</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>1.954</td>
<td>0.167</td>
<td>0.132</td>
<td>NT</td>
</tr>
<tr>
<td>S.Lc42</td>
<td>1.223</td>
<td>1.929</td>
<td>0.111</td>
<td>NT</td>
</tr>
<tr>
<td>βM.Lc42</td>
<td>1.196</td>
<td>1.940</td>
<td>0.736</td>
<td>NT</td>
</tr>
<tr>
<td>Control*</td>
<td>0.055</td>
<td>0.208</td>
<td>0.103</td>
<td>NT</td>
</tr>
<tr>
<td>S.Polio minus detergent</td>
<td>1.865</td>
<td>NT</td>
<td>NT</td>
<td>0.176</td>
</tr>
<tr>
<td>S.Polio plus detergent</td>
<td>1.923</td>
<td>NT</td>
<td>NT</td>
<td>1.916</td>
</tr>
<tr>
<td>Control minus detergent</td>
<td>0.052</td>
<td>NT</td>
<td>NT</td>
<td>0.125</td>
</tr>
<tr>
<td>Control plus detergent</td>
<td>0.051</td>
<td>NT</td>
<td>NT</td>
<td>0.144</td>
</tr>
</tbody>
</table>

* Concentrated cell culture media of non-transfected cells.
NT, Not tested.

particles rather than wild-type HBsAg filaments which were shown to have a higher sedimentation coefficient (Marquardt et al., 1987). These data demonstrate that neither N- nor C-terminal linkage of preS regions to the S protein grossly affected proper assembly or size of the HBsAg particles.

The antigenicity of the modified particles was evaluated by ELISA. The accessibility of HBsAg and preS1 epitopes on the surface of both S.Lc42 and S.Ln41 particles was confirmed using an HBsAg- and a preS1-specific immunoassay (Table 1). The βM.Lc42 particles displayed in addition preS2-reactivity, thus indicating that preS1-, preS2- and HBsAg-specific epitopes are exposed on their surface (Table 1). To exclude the possibility, however, that the observed preS1- and preS2-specific reactivities were due to distortion of the particles during the course of the experiment, particle integrity was analysed using a hybrid S.Polio protein. This protein carries a poliovirus epitope inserted at residue 50 of the S protein (Delpeyroux et al., 1990), a site which is proposed to be internally located in the mature particle (reviewed in Heermann & Gerlich, 1991). As shown in Table 1, native S.Polio particles failed to react in a polio-specific ELISA unless these particles were disrupted with detergent (i.e. 0.5% Nonidet P-40). The inaccessibility of the internally located poliovirus epitope in the absence of detergent strongly supports the integrity of the modified particles analysed.

Immunogenicity of modified S.Lc42, S.Ln41 and βM.Lc42 particles

The immunogenicity of modified particles was examined by immunization of BALB/c mice with purified S.Lc42, S.Ln41 and βM.Lc42 particles. To discriminate between anti-preS1, anti-preS2 and anti-S immune responses, both the preS1 and preS2 regions had to be physically uncoupled from the S-encoding region. To this end, either the first 42 aa of the preS1 region or aa 4-49 of the preS2 region were expressed in bacteria as C-terminal fusions to GST. Purified GST-preS1 and GST-preS2 fusion proteins were then used as coating antigens to evaluate the mice sera for antibodies to the preS1 or preS2 region, respectively. The anti-S immune response was determined using solid-phase coated HBsAg from human sera.

Modified S.Lc42 and S.Ln41 particles administered with Freund’s adjuvant elicited high titres of preS1-specific antibodies (Fig. 6a), indicating that preS1
epitopes were highly immunogenic at either location. Similarly, βM.Lc42 particles given in combination with either Freund’s adjuvant or alum induced a high titre of preS1-specific antibodies, irrespective of the adjuvant used (Fig. 6a). In addition, the βM.Lc42 particles conjugated to either adjuvant elicited preS2-specific antibodies although at a lower level than antibodies to preS1 (Fig. 6b). However, all the modified particles failed to induce an appreciable antibody response to HBsAg despite repeated boosting and irrespective of the adjuvant used (Fig. 6c). Conversely, control mice which were immunized with equal amounts of yeast-derived recombinant HBsAg particles lacking preS sequences developed an antibody response to HBsAg when the particles were administered with alum (Fig. 6c).

We wondered if the preS1- and preS2-specific antibodies raised in the immunized mice, which had been identified using bacterially expressed GST fusion proteins, would recognize authentic env proteins. To this end, the env proteins were synthesized in vitro using a coupled transcription/translation/translocation system and were subjected to immunoprecipitation. Synthesis of env proteins in vitro closely resembles the in vivo situation, as the S, M, and L proteins appear in non-glycosylated and glycosylated forms (Fig. 7a, lanes 1–3). All three env proteins were specifically immunoprecipitated with rabbit antiserum to yeast-derived HBsAg particles (Fig. 7b, lanes 1–3). When mouse antiserum raised against βM.Lc42 particles was used for immunoprecipitation the M and L proteins could be specifically identified (Fig. 7c, lanes 2 and 3). However, consistent with the ELISA data, the S protein was not immunoprecipitated (Fig. 7c, lane 1). Almost identical results were obtained when native env proteins synthesized in COS-7 cells were immunoprecipitated with the βM.Lc42 antiserum (data not shown).

Discussion
The currently licensed recombinant hepatitis B vaccines obtained by synthesis of the S protein alone or the S and M proteins together in either yeast or mammalian cells (Valenzuela et al., 1982; Harford et al., 1983; Michel et al., 1984) are both safe and efficacious (reviewed in Rutgers et al., 1993). However, several lines of evidence indicate that inclusion of preS1 sequences may represent a feasible strategy to increase the efficacy of the HBV vaccines (Neurath et al., 1986a, 1989a; Milich, 1988; Thornton et al., 1989). In an attempt to present preS1-, preS2- and S-specific epitopes on subviral particles, we have reversed the natural order of the env ORF in this study and analysed the structural and immunological features of the modified env proteins.

Assembly and secretion of modified env proteins
The enhanced immunogenic character of particulate HBsAg has previously lent support for its use as a carrier for foreign epitopes (Valenzuela et al., 1985; Delpeyroux et al., 1986; Rutgers et al., 1988; Michel et al., 1988; von Brunn et al., 1991). The heterologous sequences were predominantly fused to the N terminus of the S protein (with or without preS2 sequences), as this site proved to be rather permissive for insertions (Valenzuela et al., 1985; Rutgers et al., 1988; Michel et al., 1988). Upon synthesis in transfected cells, insertions of up to 84 residues in the preS2 region were compatible with assembly and release of the modified HBsAg particles (Michel et al., 1988). Conversely, insertions within the S encoding region were only poorly tolerated and have been shown to be size-limited to about 20 residues (Delpeyroux et al., 1987; Bruss & Ganem, 1991). In this study we identified a novel site suitable for insertions: the C terminus of the S protein. We demonstrated that this site can accommodate at least the first 42 residues of the preS1 region of the L protein without serious effects on secretion. Moreover, this seems to be a favourable site for insertion of epitopes since the fused preS1 sequence is exposed on the surface of secreted particles, a prerequisite for efficient immunogenicity. Xu et al. (1994) have recently demonstrated that this site likewise accommodates residues 21–47 of preS1 (HBV, serotype adw). However, the capacity for incorporation of sequences at the C terminus seems to be restricted, since progressive extension of the fused preS1 sequence significantly impaired secretion. Whether the block to secretion of the
larger fusions was due to specific preS1 sequences or to non-specific conformational restraints needs to be analysed further.

Fusion of the first 41 residues of the preS1 region to the N terminus of the S protein (S. Lc42) yielded a similar secretory phenotype and surface accessibility of the linked preS1 sequence was similar to the corresponding C-terminal fusion (S. Lc42). However, secretion of the N-terminal fusion required mutational inactivation of the preS1 myristic acid attachment site (this work: R. Hillrich & R. E. Streeck, unpublished results). The secretion of both S. Lc42 and S. Ln41 fusion proteins supports our conclusion drawn from previous results (Prange et al., 1991) that myristylation plays a major role in intracellular retention of wild-type and mutant HBV (serotype ayw) env proteins. Accordingly, the data presented here again do not support the notion that an ER retention signal predicted to map to the preS1 sequence 6–19 of HBV serotype adw (Kuroki et al., 1989) mediates retention of the ayw serotype.

In order to include preS2 epitopes in the preS1-carrying particles, we designed a modified M protein carrying the preS2 region at its authentic position and the first 42 residues of preS1 at its C terminus. Rather unexpectedly, secretion of this M. Lc42 fusion was found to be completely blocked although both the M and the S. Lc42 protein were efficiently released. The block to secretion of the M. Lc42 protein may be due to inefficient translocation of the preS2 region across the ER membrane mediated by the C-terminal (preS1-specific) extension. In support of this notion, the proper transmembrane configuration and secretion of the M. Lc42 protein could be rescued by provision of a heterologous N-terminal signal sequence. This modified M protein (βM. Lc42) and the S. Lc42 and S. Ln41 proteins were secreted in particulate form closely resembling authentic HBsAg particles.

Antigenicity and immunogenicity of modified env proteins

The envelope particles obtained from the modified S. Lc42, S. Ln41 and βM. Lc42 proteins exposed preS and S antigenic determinants on their surface, as assayed by accessibility to antibody using immunoprecipitation and ELISA. As for preS1 and preS2, their surface accessibility correlated with their immunogenicity evaluated in mice: each of the modified particles induced a strong preS1-specific immune response and the βM. Lc42 particles also induced a weak though significant preS2-specific response. The weakness of the preS2-specific response most likely reflects the low density of preS2 epitopes on the modified βM. Lc42 particles which are composed of both full-length βM. Lc42 protein and internally initiated S. Lc42 protein lacking the preS2 sequence. Conversely, however, the surface accessibility of HBsAg was not reflected in a correspondingly high immunogenicity: the anti-S response was only negligible.

To account for the lack of response to the S antigen, mutual influences of preS1, preS2 and S epitopes might be considered. Such an interdependence of distinct epitopes of the env proteins has previously been suggested by studies analysing the immunogenicity of (i) a recombinant vaccinia virus encoding the L protein (Cheng et al., 1986), (ii) engineered subviral particles composed of the S and M proteins and chemically linked synthetic peptides from the preS1 region (Neurath et al., 1989b) and (iii) yeast-derived particles consisting of the S protein and a truncated L protein (Petre et al., 1992). Whereas these immunogens induced a strong preS1- and S-specific immune response in animals, the preS2 response was significantly reduced or completely absent. We do not know if the peculiar rearrangement of the env proteins analysed in this work, i.e. the binding of preS1 epitopes to each S or M molecule present in the particles suppresses rather than augments the anti-S response thereby leaving the anti-preS2 response largely unaffected.

Nonetheless, the genetic approach employed here represents a novel strategy to incorporate preS1-derived B and T cell epitopes both in high density and in highly immunogenic form into their authentic carrier matrix: particulate HBsAg, one of the few recombinant vaccines licensed today (reviewed in Rutgers et al., 1993). Related studies have exploited different strategies and systems. Since synthetic preS1 peptides are not immunogenic unless linked to liposomes, Neurath et al. (1989b) utilized authentic HBsAg particles as a lipoprotein carrier for preS1 peptides which were attached via hydrophobic tails. Alternatively, particulate HBV core antigen (HBcAg) has been used as a carrier for preS1 epitopes (Schödel et al., 1992). While this approach proved to be immunogenically promising, the development of an HBcAg-based carrier concept for human vaccine application is hampered due to the affinity of the core protein for nucleic acids (reviewed in Standring, 1991). For the development of yeast-derived multivalent HBV vaccines, Petre et al. (1992) designed a truncated L protein devoid of sequences affecting retention, stability and assembly but retaining immunologically important B and T cell epitopes of the preS region. Interestingly, the portion of the preS1 region chosen perfectly matches the preS1 sequence selected in this work. The truncated L protein, however, has to be co-expressed with the S protein to assemble into particles consisting of 30% L and 70% S (Petre et al., 1992). In contrast, the approach described in this work yielded particles in which each S
(or M) protein exposed preS1 epitopes when linked to the C terminus. Such particles, when mixed with traditional HBsAg particles, may therefore lead to a full immune response to both S and preS antigens and may induce protection in those persons who respond poorly or not at all to the current HBV vaccines.

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References


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