

## Mosaic hepatitis B virus core particles allow insertion of extended foreign protein segments

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**Because of its particular immunological properties, the core protein of hepatitis B virus (HBcAg) has become one of the favoured 'virus-like particles' for use as a carrier of foreign epitopes. A new strategy to construct core particles presenting extended foreign protein segments was established based on the introduction of a linker containing a translational stop codon between sequences encoding a C-terminally truncated HBcAg (HBcAg $\Delta$ ) and a foreign protein sequence. Expression in an *Escherichia coli* suppressor strain allowed the simultaneous synthesis of both HBcAg $\Delta$  and a read-through fusion protein containing a part of the hantavirus nucleocapsid protein. After purification, the presence of core-like mosaic particles with HBc and hantavirus antigenicity was demonstrated by electron microscopy and immunological tests. This strategy of partial stop codon suppression should improve the use of HBcAg as a carrier of foreign epitopes by allowing insertion of long foreign sequences into particle-forming proteins. The resulting mosaic particles should be of general interest for further vaccine developments.**

In recent years, chimeric virus-like particles (VLPs), formed by modified virus capsid or envelope proteins containing additional foreign determinants, have been established as a promising basis for the construction of new vaccines (for reviews see Lomonosoff & Johnson, 1995; Ulrich *et al.*, 1996). VLPs are non-infectious particles which contain T helper cell epitopes and should present B cell epitopes at a high density. This results in strong immunogenicity of the inserted sequences, circumventing the need for adjuvant in immunization. Moreover, in various studies it turned out that VLPs based on the hepatitis B virus (HBV) core antigen (HBcAg) can

be produced in various heterologous systems, and even in attenuated *Salmonella* strains as a live vaccine (for review see Schödel *et al.*, 1996). As an additional advantage these core particles may allow the stimulation of the immune system in a T cell-independent way (Milich *et al.*, 1987). Thus, since their first description (Clarke *et al.*, 1987; Borisova *et al.*, 1989; Stahl & Murray, 1989; Schödel *et al.*, 1990) the core particles of HBV have become one of the most frequently studied systems for the exposure of foreign sequences (for reviews see Pumpens *et al.*, 1995; Ulrich *et al.*, 1997). The foreign epitopes presented to the immune system should be able to induce a strong humoral and cellular immune response, as measured by virus-neutralizing antibodies and cytotoxic T cells.

Although the three-dimensional structure of HBcAg has not been determined so far, potential insertion sites along the HBcAg molecule have been predicted at its N and C termini and in its central part by use of sequence-based prediction methods, epitope-mapping studies and empirical insertion approaches. The central position forms the preferable insertion site because foreign sequences placed here elicit high antigenicity and immunogenicity (Pumpens *et al.*, 1995; Schödel *et al.*, 1996). However, the C terminus of a C-terminally truncated HBcAg (HBcAg $\Delta$ ; deletion downstream of aa 144, 149 or 156) tolerates the largest inserts. Contradictory results about the maximum insertion capacity without loss of particle-forming ability have been reported: whereas not more than 90 aa of human immunodeficiency virus type 1 Gag protein were shown to be tolerated downstream of aa 144 (Ulrich *et al.*, 1992), one group (Yoshikawa *et al.*, 1993) reported HBV core particles carrying a 720 aa segment (four copies of an 180 aa sequence) derived from the hepatitis C virus core protein.

Apparently, the insertion capacity of the HBcAg molecule is a limiting factor in the generation of multivalent vaccines based on the coupling of different epitopes to one carrier molecule. Insertion of larger amino acid sequences should be advantageous not only for presentation of a larger number of epitopes but also to allow appropriate folding of conformational epitopes, which seems to be of special relevance for the presentation of virus structural proteins to the immune system.

In recent years, we have focused on the characterization of

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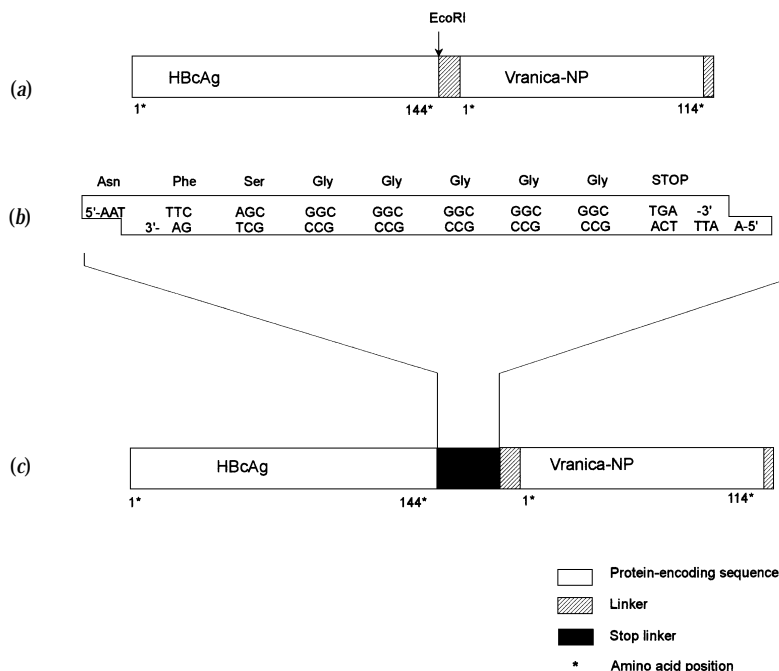


Fig. 1. Scheme of the 'readthrough' engineering strategy for the preparation of mosaic VLPs. (a) Primary structure of the HBcAg $\Delta$ -Vranica NP fusion protein encoded by pHBc-Msc1. (b) Nucleotide sequence of the inserted oligonucleotide duplex supplying a TGA stop codon and the coding information for an additional 5 aa. (c) Primary structure of the 'readthrough' protein encoded by pHBc-Msc-Stop126/127. The original sequence 5' GAATTC was substituted by 5' GAATTT due to the oligonucleotide duplex insertion, resulting in a loss of the *EcoRI* site.

the diagnostic and immunogenic potential of the hantavirus nucleocapsid protein (NP), especially the NP of Puumala hantavirus (subtype Vranica), the nucleotide sequence of which was recently determined (Reip *et al.*, 1995). Because of the medical significance of hantavirus infections and the need to develop protective vaccines (for reviews see Lundkvist & Niklasson, 1994; Schmaljohn, 1994), we have tried to construct an HBV core-based VLP containing a hantavirus NP sequence. As the N-terminal region of hantavirus NP (approximately 120 aa) was capable of inducing protective immunity against hantavirus infection (Lundkvist *et al.*, 1996; our unpublished data), we felt encouraged to construct HBV core-like particles presenting the N-terminal 114 aa of Vranica NP. However, we failed to detect self-assembly of the fusion protein consisting of HBcAg $\Delta$  and the N-terminal 114 aa of Vranica NP (named pHBc-Msc1; see Fig. 1a) by sucrose gradient centrifugation and subsequent Ouchterlony radial immunodiffusion or negative stain electron microscopy. These data, observed in both *Escherichia coli* K12 K802 cells and a suppressor strain derivative of K802 (see below), support our earlier observation about a size limitation of the insertable sequence (Ulrich *et al.*, 1992). Therefore, we have developed a new strategy to incorporate larger sequences into HBcAg $\Delta$ -derived particles based on a 'readthrough' mechanism in protein synthesis.

The novel constructs were based on the plasmid pHBc-Msc1 (Fig. 1a). We generated oligonucleotides possessing a TGA stop codon as well as the coding information for an additional 5 aa predicted to form a 'coil' secondary structure (see Fig. 1b). The oligonucleotides were annealed and the resulting duplex was inserted into *EcoRI*-linearized pHBc-Msc1 between the sequences encoding HBcAg $\Delta$  and the N-terminal

114 aa of Vranica NP. Recombinant clones were enriched by preparation of a DNA pool from approximately 250 colonies, followed by *EcoRI* cleavage and subsequent re-transformation into *E. coli* K12 XL-1 Blue cells. Plasmids which had lost the *EcoRI* site due to insertion of the oligonucleotide duplex were selected and sequenced. In *E. coli* K12 K802 cells, a plasmid with the intact insert in the correct orientation (named pHBc-Msc-Stop126/127; Fig. 1c) expressed HBcAg $\Delta$  exclusively. This plasmid was then re-transformed in a derivative of *E. coli* K12 K802 possessing an 'opal' TGA-Trp suppressor tRNA under *lac* repressor control (Smiley & Minion, 1993). Crude lysates of the transformed cells were analysed by SDS-PAGE and Western blotting, demonstrating the co-expression of HBcAg $\Delta$  and the readthrough HBcAg $\Delta$ -Vranica NP fusion protein (see Fig. 2).

To isolate the protein, *E. coli* cells were disrupted by lysozyme and subsequent sonication as described previously (Borisova *et al.*, 1993). Most of the HBcAg $\Delta$  as well as the HBcAg $\Delta$ -Vranica NP readthrough or fusion protein was found to be soluble and was quantitatively precipitated by ammonium sulphate. This precipitate was loaded onto a 20–50% discontinuous sucrose gradient and run for 22 h at 28 000 r.p.m. at 4 °C (Beckman L7–55, rotor SW28). After centrifugation, 2 ml fractions were collected and analysed in 15% SDS-PAGE gels (Fig. 2a) and Western blots (Fig. 2b, c). The recombinant HBcAg $\Delta$ -derived proteins expressed by pHBc-Msc-Stop126/127 were obtained exclusively in fractions 7–9 of a 20–50% linear sucrose gradient (data not shown here). The fractions cover a sucrose concentration range of approximately 36–40%, corresponding to a buoyant density of approximately 1.16 g/cm<sup>3</sup>. Similarly, the peak of HBcAg $\Delta$  (pHBc2-9) was

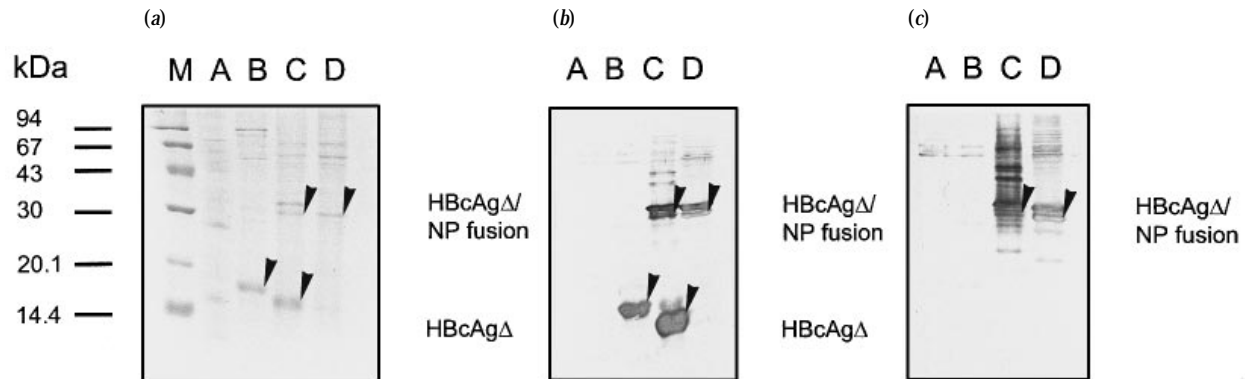


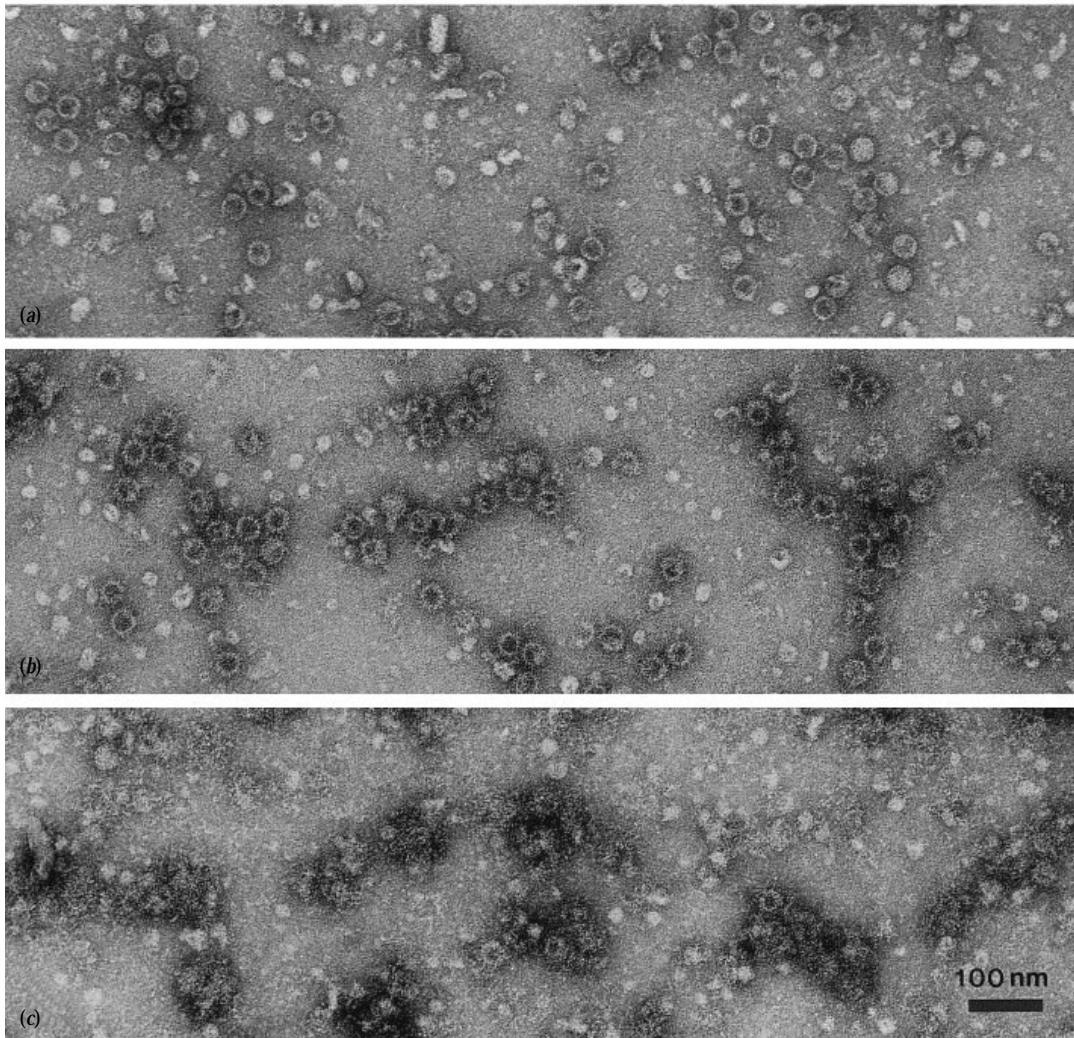
Fig. 2. Detection of recombinant HBcAg $\Delta$ -derived proteins in lysates of suppressor-containing cells transformed with pHBc2-9 (HBcAg $\Delta$  from fraction 8; lane B), pHBc-Msc-Stop126/127 (particulate mosaic HBcAg $\Delta$  and HBcAg $\Delta$ -Vranica NP from fraction 8; lane C) or pHBc-Msc1 (non-particulate HBcAg $\Delta$ -Vranica NP fusion protein from fraction 4; lane D) after sucrose density gradient fractionation. Lysate of pQE40-transformed M15pREP4 cells (lane A) was applied as a control. (a) Coomassie blue-stained 15% SDS-PAGE gel. (b) Western blot analysis using an anti-HBe-specific monoclonal antibody (129C9). (c) Western blot analysis applying a pool of hantavirus NP-specific monoclonal antibodies, 3G5, 5E1, 4C3 and 2E12 (Lundkvist *et al.*, 1991). The HBcAg $\Delta$ -derived proteins are marked by arrowheads. The larger protein bands are probably caused by an additional readthrough on a pHBc2-9-derived TGA codon.

detected in fractions 6–9. These buoyant density values correspond well with those of earlier published data for chimeric core particles (von Brunn *et al.*, 1993; Isagulians *et al.*, 1996). In contrast, the majority of bacterial proteins were found in fractions 1–5 and 10–14. As expected, the HBcAg $\Delta$ -Vranica NP fusion protein expressed by pHBc-Msc1 (without the stop codon insert) was recovered in fractions 4–6 because of its inability to form particles (see below). The purified particles were further analysed by centrifugation in an analytical 20–50% sucrose gradient (Beckman L7–55, rotor SW40; 35 000 r.p.m., 4 °C, 12 h) using 30S, 50S and 70S ribosomal subunits as markers. The data obtained demonstrate sedimentation values of approximately 80S for both core and mosaic particles (data not shown here). This value was found by Gallina *et al.* (1989) for particles formed by core protein of similar size.

The purified proteins were then analysed in stained gels (Fig. 2a). The sizes of the proteins expressed in suppressor cells transformed by pHBc-Msc-Stop126/127 (Fig. 2a, lane C; predicted molecular masses of 16.9 and 30.7 kDa) corresponded to those of HBcAg $\Delta$  and the HBcAg $\Delta$ -Vranica NP fusion protein, respectively (lanes B and D). The apparent small difference in the molecular mass of HBcAg $\Delta$  synthesized by pHBc2-9 and pHBc-Msc-Stop126/127 is due to the linker insertion in the latter. Western blot analysis using HBe-specific monoclonal antibodies 129C9 (Fig. 2b) and 87-141/03 (data not shown) demonstrated HBe antigenicity of HBcAg $\Delta$  and the HBcAg $\Delta$ -derived fusion proteins as expected. Recombinant proteins containing the hantavirus NP sequence were detected by applying a pool of NP-specific monoclonal antibodies (Fig. 2c) and an NP-specific rabbit serum (data not shown). The yield as determined by Bradford analysis was approximately 7 and 5.6 mg/l bacterial culture (3.5 and 2.8 mg/g wet material) for core particles and mosaic particles, respectively. The relative

amount of the readthrough protein compared to HBcAg $\Delta$  was calculated by densitometric analysis to approach 30% at maximum. Fractions 8 of the sucrose gradients (cp. lanes B and C in Fig. 2a) likely to contain chimeric VLPs were further investigated by Ouchterlony radial immunodiffusion using a human anti-HBc-positive immune serum. A typical HBcAg precipitation line was revealed and HBe antigenicity of the particles was further confirmed by standard HBcAg ELISA. Likewise, hantavirus NP reactivity was demonstrated by ELISA, corroborating at least partial surface exposure of the Vranica NP sequence (data not shown).

Electron microscopy of the sucrose gradient fractions 8 after negative staining (Gelderblom *et al.*, 1967) revealed the presence of particles. No apparent morphological differences between the pHBc2-9-derived HBcAg $\Delta$  and the pHBc-Msc-Stop126/127-derived HBcAg $\Delta$ -Vranica NP fusion protein particles could be found (data not shown). The sizes of core particles were determined by measuring the diameters of the uranyl acetate negatively contrasted samples. They were found to be approximately 33 and 36 nm for HBcAg $\Delta$ -derived particles and mosaic particles, respectively. To remove the contaminating debris originating from lysed bacteria, the materials were further purified by using CL4B-Sepharose (Pharmacia) molecular sieve chromatography. Thereafter, immunoelectron microscopy using a pool of hantavirus NP-specific monoclonal antibodies (data not shown) and a rabbit anti-hantavirus NP hyperimmune serum showed immunoaggregation of the HBcAg $\Delta$ -Vranica NP fusion protein particles and decoration of the cores by a fringe of antibodies (Fig. 3c), thus proving the surface exposure of the hantavirus Vranica NP sequence. The control incubation using an unrelated yellow fever-specific monoclonal antibody did not induce any immunoaggregation of cores nor any particle decoration pointing to the specificity of the immune reaction



**Fig. 3.** Demonstration of HBCAg and hantavirus NP determinants on the surface of the mosaic VLPs by direct immunoelectron microscopy using unlabelled antibodies. (a) The negative control using yellow fever-specific monoclonal antibody shows monodisperse core particles exhibiting clearly delineated capsid profiles and no particle aggregation. (b) After incubation with a polyclonal anti-HBc-specific IgG, most of the core particles are loosely immunoadgregated. The antibody-bound and antibody-decorated particles show defined distances. (c) Application of the polyclonal anti-hantavirus NP-specific rabbit serum results in particle aggregation and in heavy coating by a fringe of antibodies.

(Fig. 3a), but anti-HBc immune serum did react with the particles, demonstrating the appearance of HBc epitopes on the particle surface as well (Fig. 3b).

In summary, our strategy to increase the insertion capacity of particle-forming proteins was based on a co-expression of HBcAg $\Delta$ , with its high intrinsic capacity to self-assemble, and a certain number of HBcAg $\Delta$ -derived fusion protein molecules in the same *E. coli* cell exploiting a suppressor tRNA-mediated readthrough translation. A similar mechanism, namely leaky termination at an opal UGA codon, is used in the native replication of phage Q $\beta$ , resulting in the co-expression of the coat and the AI (readthrough) protein which are both essential structural components of the Q $\beta$  virion (for references see Hirashima *et al.*, 1979).

For these experiments, a hantavirus sequence was selected because of the importance of this virus for vaccine development. The introduction of a linker sequence with a TGA stop codon between the sequences encoding HBcAg $\Delta$  and the hantavirus NP allowed co-expression of HBcAg $\Delta$  and read-through protein in a UGA suppressor strain. We would like to propose that the interaction of unmodified particle-forming HBcAg $\Delta$  molecules and fused HBcAg $\Delta$ -Vranica NP molecules allows the incorporation of a limited number of fused proteins into the particles. The mosaic particles could be immunoadgregated and decorated using hantavirus NP-specific antibodies. ELISA tests have verified the exposure of antigenic determinants encoded by the inserted hantavirus NP fragment.

We have demonstrated the applicability of a readthrough

system to increase the insertion capacity of HBV core particles for a given foreign sequence. This approach will probably allow even longer or 'problematic', e.g. hydrophobic, sequences to be tolerated. It seems conceivable that due to a gradually inducible suppressor tRNA synthesis the ratio between HBcAg $\Delta$  and readthrough protein expression could be optimized for each foreign sequence. This approach could form the basis for the construction of multivalent HBcAg-derived vaccines. Finally, it might be rewarding to evaluate the described expression strategy for the development of chimeric VLPs based on other carrier systems.

**Note added in proof.** Considering its close genetic relationship with strains from Sweden hantavirus Vranica was renamed as Vranica/Hällnäs according to a recent agreement between the groups from Umeå, Stockholm, Helsinki and Berlin.

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