

## Proline-138 is essential for the assembly of hepatitis B virus core protein

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**In small RNA viruses, arm-like segments located at the N or C termini have been suggested as mediators in the assembly of the capsid proteins. In many cases the arms of several subunits converge at a common point (the symmetry axis). Recent advances in studies of the hepatitis B virus (HBV) core protein attest the convergence of the segments preceding the protamine region, around the symmetry axis, where five or six HBc protein subunits converge. We report a mutation study of the region that we have suggested forms an arm-like structure, which reveals that a single mutation, Pro-138 → Gly, prevents the full-length HBV core protein self-assembling into particles.**

In many viruses, extended conformations frequently found at the N terminus of the capsid protein are supposed to play an important role in the assembly of the viral particles (Harrison, 1992). In simian virus 40, for example, the capsid contains 72 pentamers of the VP1 protein. The pentamers are linked by the interaction of the C-terminal arms of the VP1 protein with five or six neighbouring pentamers (Liddington *et al.*, 1991). These interactions supplant the absence of complementary interfaces between pentamers.

The human hepatitis B virus (HBV) has been shown to form particles of two different sizes, one major population of capsids containing 240 copies of HBc protein ( $T = 4$  capsids, where  $T$  is the triangulation number) and a minor population of capsids containing 180 copies ( $T = 3$  capsids) (Crowthers *et al.*, 1994; Kenney *et al.*, 1995; Zlotnick *et al.*, 1996). In both cases, the capsids reveal tightly clustered dimers composed of a shell domain and a protruding spike. Two recent papers (Böttcher *et al.*, 1997; Conway *et al.*, 1997) have made an important contribution to the knowledge of the structure of the HBV capsid. It has been revealed that the previously reported

protruding spike of the HBV core capsid consists of a four-helix bundle, as proposed in a model developed by one of us (Bringas, 1997). This model suggests that the region preceding the protamine domain of the HBc protein converges at the point where five or six HBc dimers come together (fivefold and sixfold symmetry axes). This prediction was demonstrated in the cryomicroscopy reports of Böttcher *et al.* (1997) and Conway *et al.* (1997). In both cases, an arm-like conformation is observed around the fivefold and sixfold symmetry axes.

Mutation studies of HBc variants by Birnbaum & Nassal (1990) have shown that the assembly capability of the HBV core protein resides in its first 140 to 144 amino acids and that the entire basic C-terminal domain is not necessary for assembly. HBc variants containing the first 139 or less residues are not able to assemble into particles, while variants containing 144 residues or more are still capable of assembling. Additionally, Zlotnick *et al.* (1996) found that the region between residues 140 and 147 is important in determining whether HBc protein will produce predominantly  $T = 4$  or  $T = 3$  capsids. These results imply that these residues are implicated in inter-subunit interaction.

The model of the HBc protein developed by Bringas (1997) proposes the existence of an arm-like structure in the region preceding the protamine domain. This region is abundant in proline residues. As it happens in some plant viruses, this arm would separate the protamine domain from the shell domain (Harrison *et al.*, 1978; Abad-Zapatero *et al.*, 1980; Hogle *et al.*, 1986). In the HBV core protein, it has been suggested that this segment plays a key role in the virus assembly process, acting as hinge facilitating conformational changes that accompany nucleic acid and dimer packaging during particle assembly (Seifer & Standring, 1994). In order to test the possible role of prolines in such segments we present a mutational study of prolines preceding the protamine domain.

Vaccinia virus recombinants encoding the wild-type HBc and HBe proteins have been described by Schlicht & Schaller (1989). The other HBc mutants are based upon a construct that contains only the HBc gene sequences. Stop codons and Pro → Gly mutations were introduced by PCR mutagenesis.

Cultivation and infection of HepG2 cells were carried out as follows: HepG2 cells (Aden *et al.*, 1979) (ca.  $6 \times 10^6$  cells

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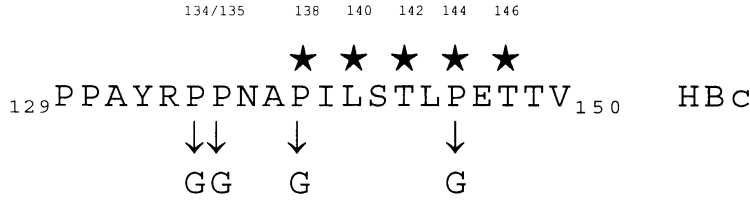


Fig. 1. The region preceding the protamine domain. The Pro → Gly exchanges were introduced at positions 134/135, 138 and 144 as indicated. Stop codons (indicated by stars) were introduced at positions 138, 140, 142, 144 and 146.

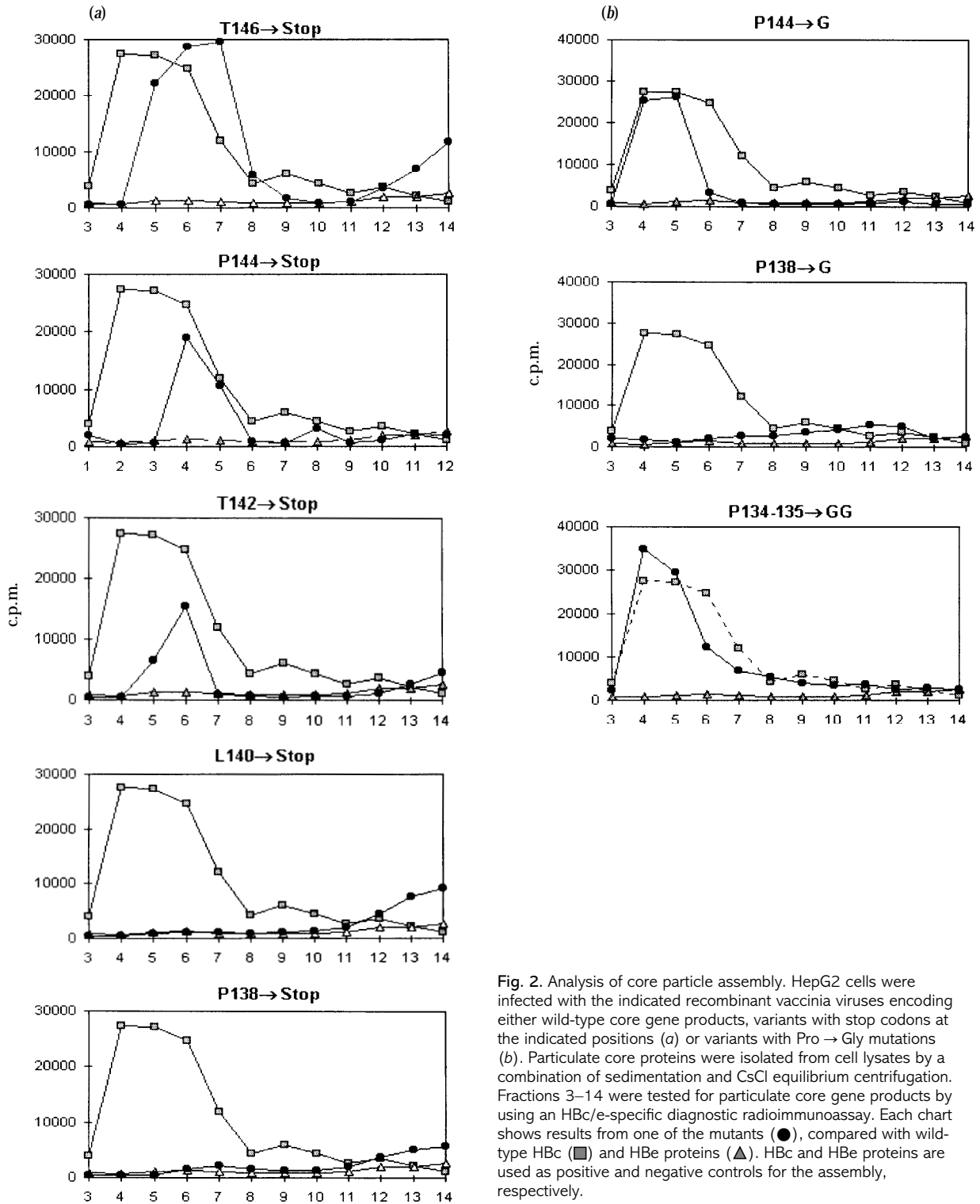


Fig. 2. Analysis of core particle assembly. HepG2 cells were infected with the indicated recombinant vaccinia viruses encoding either wild-type core gene products, variants with stop codons at the indicated positions (a) or variants with Pro → Gly mutations (b). Particulate core proteins were isolated from cell lysates by a combination of sedimentation and CsCl equilibrium centrifugation. Fractions 3–14 were tested for particulate core gene products by using an HBC/e-specific diagnostic radioimmunoassay. Each chart shows results from one of the mutants (●), compared with wild-type HBC (■) and HBe proteins (▲). HBC and HBe proteins are used as positive and negative controls for the assembly, respectively.

grown in a 55 cm<sup>2</sup> dish with Dulbecco's minimal essential medium containing 10% foetal calf serum at 5% CO<sub>2</sub>) were infected with recombinant vaccinia virus at an m.o.i. of 10 in 1 ml serum-free medium. After 90 min at 37 °C, the inoculum was removed and 2 ml fresh medium containing 10% foetal calf serum was added. Gradient purification of particulate core gene products was done 16–36 h after infection.

Cells from three 55 cm<sup>2</sup> dishes were lysed in 4.5 ml PBS containing 1% Triton X-100 and the insoluble material was pelleted by centrifugation. The cleared lysates were layered on a gradient consisting of (from top to bottom) 1.5 ml of 20% sucrose, 1.5 ml of 30% sucrose, 2 ml CsCl solution (density 1.2 g/ml), and 2 ml CsCl solution (density 1.5 g/ml) prepared in an SW41 centrifugation tube (Beckman). All solutions were made up in TNE (20 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA). Gradients were run for 20 h at 26000 r.p.m. (ca. 115000 g) at 20 °C in an SW41 rotor (Beckman) and 14 fractions (400 µl each) were collected from the bottom. For radioimmunoassays, 10 µl of the gradient fractions 3–14 were diluted 1:20 with water.

For radioimmunological determination of HBV core gene products in gradient fractions, the HBe rDNA diagnostic assay (Abbot Laboratories) was used, which detects both HBcAg and HBeAg. Gradient fractions were diluted 1:20 in water before use. The analysis was done in accordance with the manufacturer's instructions.

We designed a series of narrowly spaced C-terminal truncations of the HBV core protein between amino acids 137 and 145 and a series of Pro → Gly mutations in full-length HBc proteins (see Fig. 1). Stop codons were introduced at positions 138, 140, 142, 144 and 146, yielding mutants with lengths of 137, 139, 141, 143 and 145 residues, respectively. The Pro → Gly exchanges were introduced at positions 134/135, 138 and 144. In Fig. 2(a), we show the sedimentation profiles of the truncated HBc proteins, using as a positive control the full-length HBc protein and as a negative control the HBe antigen.

Despite deletion of 38 residues from the C terminus of the HBc protein (mutant Thr-146 → Stop), it remains assembly competent. Further deletions, however, begin to affect its assembly capability (see sedimentation profiles of mutants Pro-144 → Stop and Thr-142 → Stop), which is completely lost when 44 residues are deleted (mutant Leu-140 → Stop). The behaviour of all these mutants in SDS-PAGE under non-reducing conditions is similar to that of the HBc wild-type protein (data not shown). Thus, these proteins fold in a correct way, but the region which is responsible for inter-dimer contact is missing.

Proline residues were replaced by glycines at positions 134/135, 138 and 144. This change is considered conservative for some authors (Argos & Fuller, 1989). The double mutation Pro-134 → Gly/Pro-135 → Gly as well as the single mutation Pro-144 → Gly did not affect HBc aggregation capabilities (see Fig. 2b). However, the Pro-138 → Gly mutation completely abolished particle aggregation. The three mutants and the

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131  GG  G      G      150
HBc_human      AYRPPNAPILSTLPETT VVR R
HBc_woodchuck  PYRPPNAPILSTLPEHTVIR R
HBc_ground_squirrel  PYRPPNAPILSTLPEHTVIR R
HBc_heron      KYRGKDAPTIEAITRPIQVAQ
HBc_duck       KYGRDAPTIEAITRPIQVAQ
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Fig. 3. Partial sequence alignment of segment between residues 131 and 150 of the HBV core proteins of five different isolates of human, woodchuck, ground squirrel, heron and duck viruses. The alignment was performed with full HBc sequences using the Clustal W program (Thompson *et al.*, 1994). 'G' above the aligned sequences indicates where proline was substituted by glycine; \* indicates amino acids that are conserved in the five sequences; and dots show the positions of conservative substitutions of amino acids.

wild-type HBc protein show a similar behaviour in SDS-PAGE under non-reducing conditions (data not shown). This means that all three mutants still form dimers and indicates that the Pro-138 → Gly mutation does not affect the dimer interface. In the partial sequence alignment shown in Fig. 3, Pro-138 is conserved in all the sequences, including isolates from the less-related duck hepatitis virus (DHBV), while prolines at positions 134, 135 and 144 are not. These results suggest that residues following position 139 are implicated in interactions with neighbouring dimers around the symmetry axes. Replacing Pro-138 by Gly results in cessation of these interactions. This implies that Pro-138 is essential for keeping an assembly competent conformation.

Despite the recent achievements in determination of the structure of the HBV capsid, there are still many unanswered questions related to its structure. The involvement of Pro-138 in the proposed arm-like structure will be corroborated only with the solution of the three dimensional structure at high resolution, but our proposal and the interpretation of results presented here are consistent with the current knowledge of virus capsid structures. The proposed arms may provide the switching mechanism for the assembly of the two sizes of particles (Bringas, 1997), and Pro-138 may play a crucial role in this mechanism. Additional mutations, e.g. replacing Pro-138 by residues less flexible than glycine, may provide assembly competent mutants that would yield preferentially T = 3 or T = 4 capsids as in the case of C-terminal truncated mutants (Zlotnick *et al.*, 1996). The mutation of the proline residue in the core protein from avian isolates that is equivalent to Pro-138 may also provide additional information on the role of this conserved residue in hepadnaviruses.

Following submission of this manuscript, the X-ray structure of HBc protein was presented by Dr Crowthers during the 'Molecular Biology of Hepatitis B Virus' Meeting held in the Institute Pasteur, Paris, France. The existence of the arm-like structure and its convergence around the fivefold and sixfold symmetry axes was definitely confirmed. Proline-138 is located immediately before the arms from adjacent subunits get into contact around the symmetry axes.

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