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Structured HCV nucleocapsids composed of P21 core protein assemble primary in the nucleus of *Pichia pastoris* yeast

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Abstract

The relationship between HCV core protein (HCcAg) processing and the structural composition and morphogenesis of nucleocapsid-like particles (NLPs) produced in *Pichia pastoris* cells was studied. At early stages of heterologous expression, data suggest that HCcAg (in the P21 form) was transported soon after its synthesis in the cytoplasm into the nucleus. HCcAg assembly into nucleocapsid-like particles with 20–30 nm in diameter took place primary in the cell nucleus. However, at later stages, when P21 and P23 forms were co-detected, data suggest that new assembly of nucleocapsid particles containing P21 possibly occurs at ER membranes and in the cytoplasm. This is the first report showing that structured HCV NLPs composed of P21 core protein assemble primary in the nucleus of *P. pastoris* yeast.

Keywords: Hepatitis C; Core antigen; Nucleocapsid-like particles; *Pichia pastoris*

Hepatitis C virus (HCV) is a member of the genus Hepacivirus, within the family Flaviviridae [1,2]. The viral genome is a positive-stranded RNA in which a long open reading frame encodes for a large polyprotein. This is cotranslationally processed into the structural and non-structural proteins by cellular signal peptidase or viral proteases [3,4]. Among the viral structural proteins, the core protein (HCcAg) is located at the N-terminus of the polyprotein and is predicted to have a length of 191 aa (P23) [3]. Additional processing of P23 produces the mature core protein consisting of 173 aa (P21) [5–10]. In addition, P21 has shown to represent the native HCcAg found in viral particles present in the sera of HCV-infected patients [7]. Previous studies had shown that HCcAg proteolytic processing is linked to its function and subcellular localization [6,9]. However, the role of this processing in HCV nucleocapsid composition and morphogenesis has not been systematically studied.

HCV not only exists as enveloped virions but also as free, non-enveloped nucleocapsids in the sera of HCV-infected patients [11]. It is thought that HCcAg plays an important role in viral assembly. Particles containing HCcAg have been observed in vivo and by using bacterial, yeast, mammalian, and insect cell culture systems [12–19]. In addition, it has been shown that HCcAg self-assembles into nucleocapsids in cell-free assays [15,18]. A recent study, using a replicon-based construct expressing HCV structural proteins, demonstrated that HCcAg self-assembles to form a structured capsid [19]. However, early events on HCV nucleocapsid morphogenesis remain unknown.

Previously, it had been shown that the *Pichia pastoris* yeast could be an appropriate host for the analysis of HCV structural polyprotein processing and nucleocapsid assembly [8,13,17,20]. The relationship between

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HCcAg processing and HCV nucleocapsid composition and morphogenesis was studied in this report.

Materials and methods

Strains. The *P. pastoris* strain MP-36/C-E1.339.12, transformed with pNAO.COE1.339 plasmid coding for the entire HCcAg, and the first 148 aa of the HCV E1 protein have been previously described [13,20]. The MP-36 [20] strain was used as a negative control.

Antibodies. The following mouse monoclonal antibodies (mAbs) were used for immunoblotting and immunoelectron microscopy (IEM) studies: anti-HCcAg CBSS-HepC.1 mAb recognizing aa 5–35 and anti-E1 CBSS-HepC.2 mAb recognizing aa 190–219 [8].

Protein analysis. Samples were directly mixed with an equal volume of 2× Laemmli sample buffer [21] and heated for 10 min. Then, protein samples were separated in a 12.5% SDS–PAGE and stained with Coomassie brilliant blue R250 (CBB, Sigma, St. Louis, USA) [21].

Immunoblotting assay. For immunoblotting, the samples were either resolved by SDS–PAGE and the proteins transferred electrophoretically to nitrocellulose membrane (HYBOND C, Amersham, England) (Western blot) or directly applied to nitrocellulose membrane (Dot blot). Binding of IgG antibodies was detected as previously described [17].

Growth conditions for P. pastoris strain and cell disruption. The MP36/CE1.339.12 transformant and the MP-36 strain were grown using conditions already established [17]. MP36/CE1.339.12 recombinant cells were harvested at 0, 3, 6, 9, 12, and 25 h after methanol induction. The strain MP-36 was used as a negative control under the same growth conditions. At each time, the cells were harvested and washed twice in TEN buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 150 mM de NaCl). Cells disruption was performed using glass beads in TEN buffer containing 10% glycerol (TENG) to improve supernatant HCcAg yields. Lysates were clarified by centrifugation at 12,000*g* for 20 min. The supernatant was further centrifuged at 23,000*g* for 20 min, 4 °C. Particles from 10 ml of supernatant were subsequently pelleted (2.5 h, 180,000*g*, 20 °C, Beckmann SW41Ti rotor). The pellet was resuspended in TEN buffer.

Equilibrium sucrose density gradient centrifugation. Equilibrium sucrose density gradient centrifugation experiments were performed as previously described [17].

Electron microscopy. Samples of sucrose fractions containing HCcAg and yeast cells were fixed and analyzed by transmission electron microscopy (EM) as previously described [13,17]. For particle quantification, 50 recombinant *P. pastoris* cells were analyzed at each time point in triplicate. They were calculated by counting the total number of these structures from 50 recombinant *P. pastoris* cells in electron micrographs of different visual fields taken at random. Two thousand and one hundred microphotographs were analyzed in this study.

Immunoelectron microscopy. Samples of sucrose fractions containing HCcAg were placed onto a 400-mesh copper grid coated with formuar-carbon film. After 1 min contact time, excess sample was blotted off. The yeast cells were fixed and dehydrated as previously described [13]. Grids or ultrathin sections of yeast cells were incubated for an hour with the SS-HepC.1 or SS-HepC.2 mAbs and then incubated for 1 h at RT with gold-labeled anti-mouse IgG (Amersham, England) diluted 1:100 in BSA–PBS. As control the primary antibody was substituted by normal mouse serum. After washing with distillated water, the samples were stained and analyzed with a transmission electron microscope as mentioned above.

Results and discussion

To elucidate in the *P. pastoris* model some uncharacterized topics of HCV nucleocapsid morphogenesis, kinetics of HCcAg expression in MP36/CE1.339.12 recombinant cells was studied as described above.

At 6 h of induction with methanol, Western blot analysis revealed the P21-specific band coinciding with the lower HCcAg expression levels detected (Fig. 1). Afterwards, as it had been previously demonstrated [8], HCcAg was processed to produce two antigenic bands with 21 and 23 kDa (P21 and P23, respectively). The highest expression levels for P21 and P23 were obtained at 25 h. The present study suggests that at low HCcAg expression levels, P21 was completely processed from the polyprotein precursor. Possibly at this stage, proteolytic cleavage at aa 173–174 took place simultaneously with that at aa 191–192. But, when HCcAg expression levels increased, both P21 and P23 were detected. Presumably, at this point the cellular signal peptidase could no longer completely process P23, thus allowing P21 and P23 being co-detected.

In order to elucidate the structural role of P21 and P23 in NLPs composition, sufficient HCcAg was obtained for the characterization studies. HCcAg was analyzed using a 5–50% (w/v) sucrose density gradient centrifugation. At 6 h, this study evidenced a peak fraction containing HCcAg that migrated to a position in the gradient corresponding to a buoyant density of 1.27 g/ml (Fig. 2A, left). To analyze the protein composition of this fraction, proteins were separated by SDS–PAGE. The Western blot analyses showed that this HCcAg population exclusively contains P21 (Fig. 2A, right), which is the only form detected at early stages of HCcAg expression (Fig. 1).

At 9 h, two different HCcAg populations were observed. Similar to that observed at 6 h, only P21 was found in the major peak fraction containing HCcAg that migrated with a buoyant density of 1.27 g/ml (Fig. 2B). Notwithstanding, the lower density HCcAg fraction (1.20 g/ml) contained both P21 and P23. At

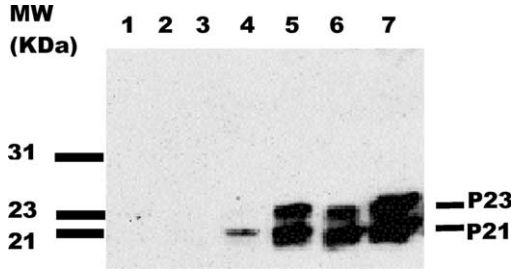

Fig. 1. Kinetics of HCcAg expressed from MP36/CE1.339.12 transformant. Proteins from total cell lysates were separated by SDS–PAGE and analyzed by Western blot with the specific SS-HepC.1 mAb. The same amount (20 μg/lane) of total cell protein was loaded in all lanes. (B) The lanes contain: *P. pastoris* strain MP-36, as negative control (1), MP36/CE1.339.12 transformant at 0 h (2), 3 h (3), 6 h (4), 9 h (5), 12 h (6), and 24 h (7). Bars on the right indicate the position of HCcAg and the position of molecular weight markers in kilodalton are indicated on the left (MW).

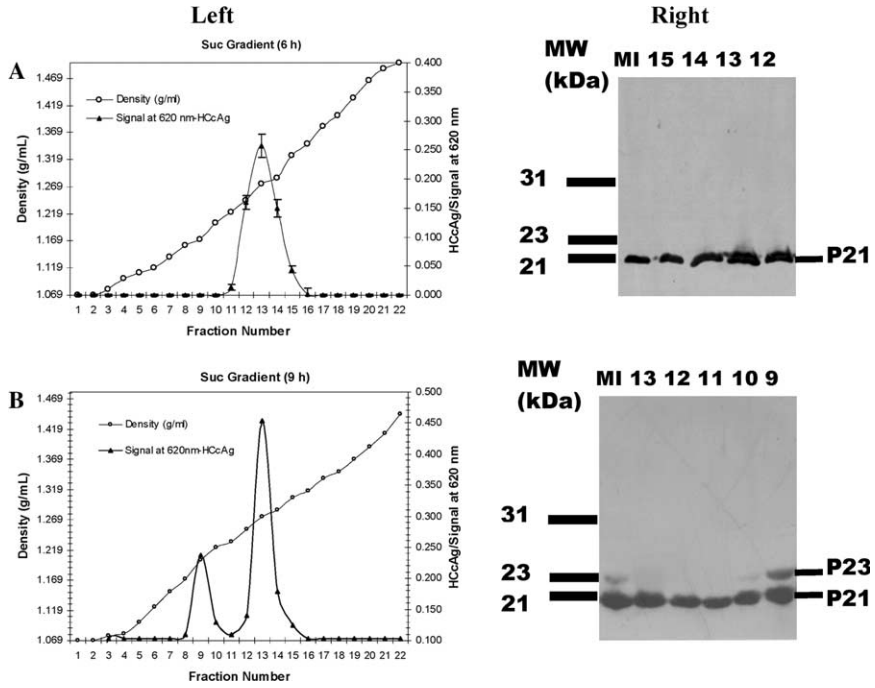

Fig. 2. Five to fifty percent (w/v) of sucrose density gradient centrifugation analysis. Left: The sucrose density of each fraction is shown (open circles; density (left ordinate) expressed in grams per milliliter). Detection of HCcAg was analyzed by densitometry of dot blot (closed triangles; HCcAg (right ordinate) expressed as signal (OD) at 620 nm). Right: Western blot of the peak fractions containing HCcAg. The same amount of HCcAg was loaded in all lanes. MI, protein sample containing HCcAg applied to the gradient. Bars on the right indicate the position of HCcAg and the position of molecular weight markers (MW) is indicated on the left. (A) Analysis at 6 h. (Left) the sample was applied at 2 mg/ml to the sucrose density gradient. (Right) The total loaded protein was 20, 29, 12.5, 7.5, and 12 μg in lanes containing MI and fractions 15, 14, 13, and 12, respectively. (B) Analysis at 9 h. (Left) The sample was applied at 2 mg/ml to the sucrose density gradient. (Right) The total loaded protein was 20, 5, 15, 17.5, 16, and 9.5 μg in lanes containing MI and fractions 13, 12, 11, 10, and 9, respectively.

25 h, the HCcAg sedimentation pattern was similar to that previously reported at later stages of HCcAg expression in *P. pastoris* cells [17].

In the material isolated at a density of around 1.20 g/ml, assembled protein aggregates were found in abundance (Fig. 3A). In contrast, structured spherical NLPs were observed in fractions banding at 1.27 g/ml (Fig. 3B). These structures showed a strong specific labeling with SS-HepC1 (Fig. 3C) and were not immunolabeled by using normal mouse serum as primary antibodies. Thus, it is possible judging from these findings that interaction between P21 and P23 may lead to HCcAg aggregates. On the other hand, these results suggest that P21 assemble into structured spherical nucleocapsids and therefore it may be considered as the main protein component of mature HCV nucleocapsids.

Finally, early events in HCV nucleocapsid assembly were analyzed by EM and IEM. Neither structured NLPs nor HCcAg were detected from non-recombinant *P. pastoris* cells used as negative control (Figs. 4 and 5A). Besides, using the SS-HepC.2 monoclonal antibody we failed to detect the E1 protein by IEM at any time point. At the time of methanol induction no HCcAg-specific immunostaining was seen in MP36/CE1.339.12 transformant (Fig. 4B). However, IEM showed that HCcAg was distributed on cytoplasm as early as 3 h post-induction reflecting nascent HCcAg polypeptides (Fig. 4C). It is interesting to note that at this time

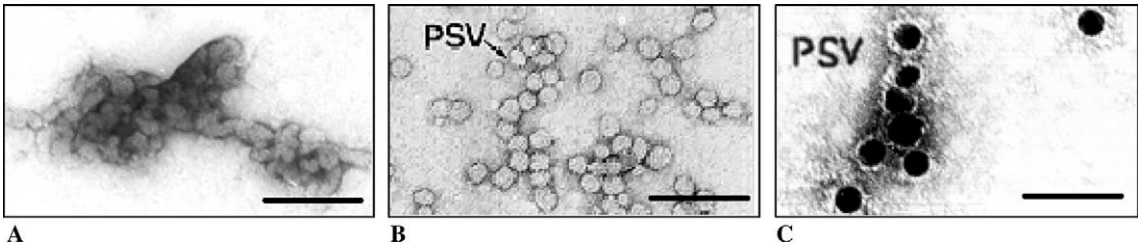

Fig. 3. Electron micrograph of negatively stained HCcAg populations separated by sucrose density gradient. At 9 h, HCcAg fractions banding either at 1.20 g/ml (A) or at 1.27 g/ml (B) were analyzed by EM. (C) IEM analysis of HCcAg fractions banding at 1.27 g/ml. Bar, 100 nm.

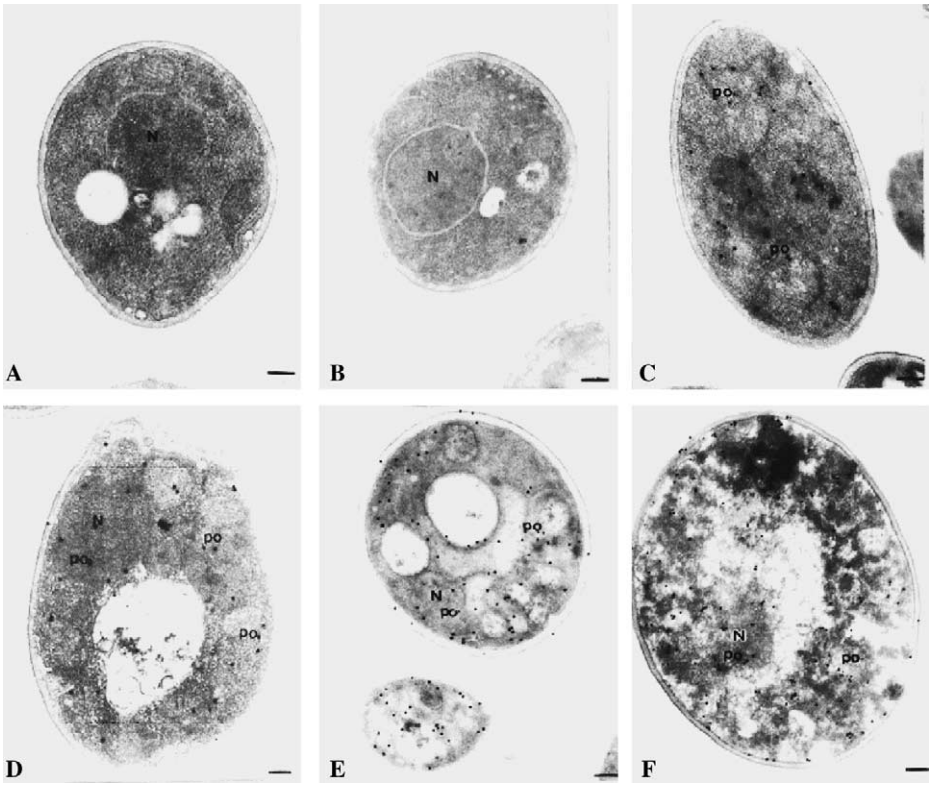

Fig. 4. Immunoelectron micrograph of MP-36/C-E1.339.12 transformant. (A) *P. pastoris* MP-36 strain used as a negative control. No immunogold labeling (PO) of HCcAg can be seen using the SS-HepC.1 mAb. Nucleus (N). (B) MP-36/C-E1.339.12 at 0 h. No immunogold labeling of HCcAg can be seen using the SS-HepC.1 mAb. Nucleus (N). (C) MP-36/C-E1.339.12 at 3 h. Immunostaining of HCcAg with SS-HepC.1 mAb was confined to the cytoplasm. Gold particles (PO). (D) MP-36/C-E1.339.12 at 6 h. HCcAg-specific immunostaining was observed in the cytoplasm as well as in the Nucleus (N); Gold particles (PO). (E) MP-36/C-E1.339.12 at 9 h. Immunostaining of HCcAg was observed in the cytoplasm as well as in the Nucleus (N); Gold particles (PO). (F) MP-36/C-E1.339.12 at 12 h. HCcAg-specific immunostaining was observed in the cytoplasm, Nucleus (N), and vacuoles. (Bar, 200 nm in A–F).

HCcAg could not be detected by Western blot, illustrating its very low expression levels.

On the other hand, up to 3 h after induction no NLPs were seen in MP36/CE1.339.12 transformant by EM (Figs. 5B and C). Interestingly after 6 h, the EM study revealed structured particles resembling nucleocapsids, which appeared almost exclusively in the nucleus with an average size of 30 nm (Fig. 5D). In order to quantify the HCcAg assembly into NLPs, an average of 297* NLPs were counted exclusively in the cell nucleus of 50 recombinant *P. pastoris* cells (Fig. 6). At this time HCcAg-specific immunolabeling was also detected in the nucleus (Fig. 4D). In addition, not NLPs but HCcAg-specific immunolabeling was also detected in the cytoplasm (Figs. 4 and 5D).

At 9 h, while increasing numbers of NLPs were shown by EM in the cytoplasm (350,3*), a proportion of them still remained in the nucleus (155,4*) (Figs. 5E and 6). In addition, some of these particles were observed in close proximity to the nuclear membrane (Fig. 5E). Moreover, IEM clearly showed the presence of HCcAg in the cytoplasm as well as in the nucleus (Fig. 4E).

Even though the total particles quantification was similar at 9 and 12 h (505,7* and 563*, respectively), their distribution changed significantly. Thus, while little amounts of NLPs remained in the nucleus (73,6*), growing numbers of them were observed in the vacuoles at 12 h (242,4*). In addition, 247* NLPs were quantified in the cytoplasm (Figs. 5F and 6). This distribution coincided with that observed for HCcAg by IEM (Fig. 4F). At 25 h, a similar pattern to that previously reported was observed (not shown) [16]. A notable increase (five to sixfold of the total particle levels obtained at 12 h) in NLPs assembly was observed (2888*) (Fig. 6). They appeared predominantly forming crystalloid bodies in the cytoplasm (representing 80% of the total particles observed, 2270*) [13].

We failed to detect E1.148 protein by IEM using the SS-HepC2 mAb. However, the same anti-E1 mAb immunolabeled the ER and the cytoplasm of hepatocytes from chronically HCV-infected patients [22]. Previously, it had been reported that this truncated E1.148 protein was barely detected by Western blot analysis [8]. Instability of E1.148 protein due to the lack of its C-terminal sequence was suggested. Therefore, the low E1.340 levels

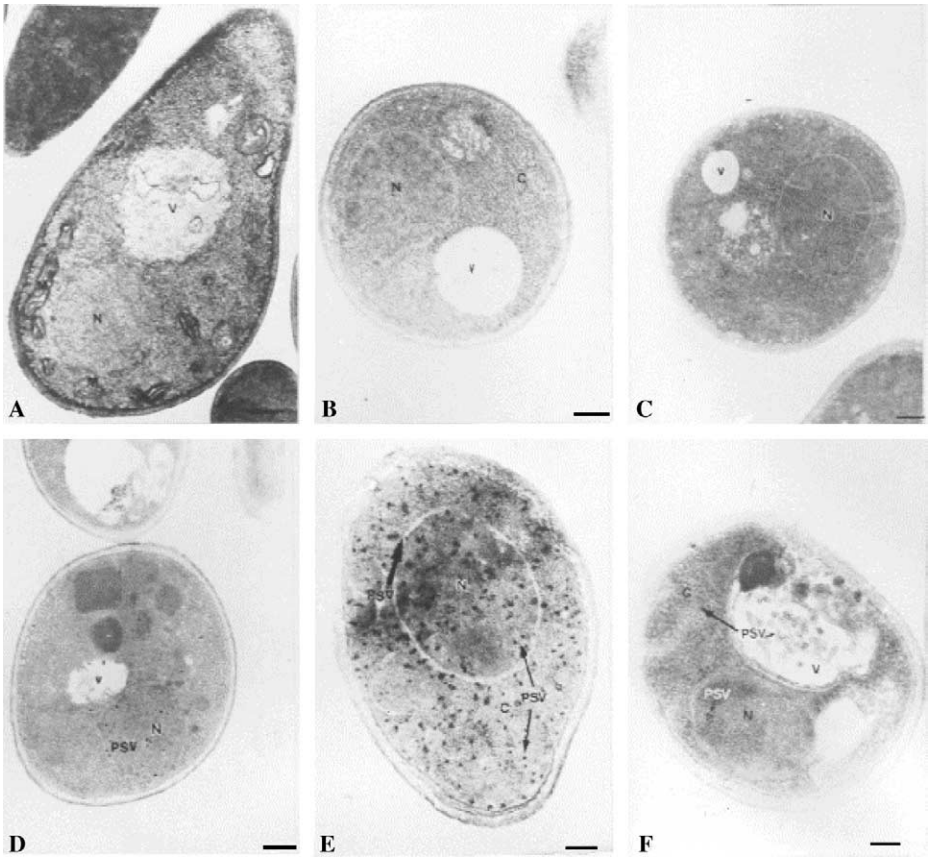

Fig. 5. Electron microscopy of nucleocapsid-like particles in MP-36/C-E1.339.12 transformant. (A) *P. pastoris* MP-36 strain used as a negative control at 24 h after methanol induction. No nucleocapsid-like particles (PSV) can be seen. Nucleus (N), vacuoles (V), mitochondria (M), and cytoplasm (C). (B) MP-36/C-E1.339.12 at 0 h. No PSV can be seen in the N, C, and V. (C) MP-36/C-E1.339.12 at 3 h. No PSV can be seen in the N, C, and V. (D) MP-36/C-E1.339.12 at 6 h. EM demonstrated PSV formation confined to the N. (E) MP-36/C-E1.339.12 at 9 h. EM demonstrated PSV formation with 20–30 nm in diameter in the N and C (solid arrows). Note some PSV in close proximity to the nuclear membrane. (F) MP-36/C-E1.339.12 at 12 h. EM demonstrated PSV formation with 20–30 nm in diameter in the N, C, and V (solid arrows). (Bar, 200 nm).

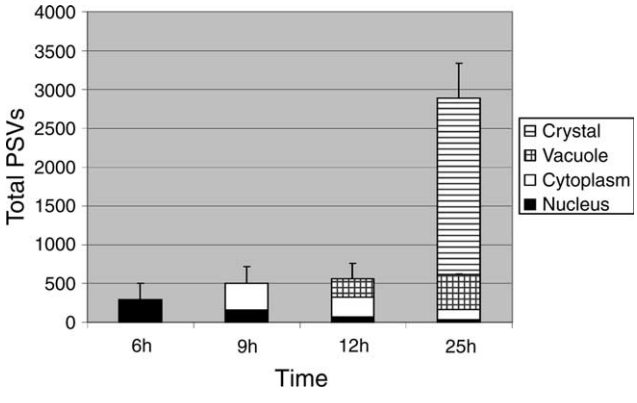

Fig. 6. Quantitative analysis of NLPs in nucleus (N), cytoplasm (C), vacuole (V), and crystaloid-bodies (CB). At each time point the presence of NLPs was quantified from 50 cells in triplicate. They were calculated by counting the total number of these structures from 50 recombinant *P. pastoris* cells in electron micrographs of different visual fields taken at random. While CB were detected in the C, they were calculated apart from the other particles present in the C.

in recombinant cells may partially explain its lack of staining by IEM.

Data suggest that HCcAg (in the P21 form) was transported soon after its synthesis in the cytoplasm into the nucleus. During the process of nucleocapsid morphogenesis, HCcAg assembly into nucleocapsid-like particles took place primary in the cell nucleus. Similarly, previous works had demonstrated that P21 translocates into the nucleus just in the absence of P23 [6,9]. Besides, P21 is the only form detected in the HCV virions from HCV-infected patients [7]. Later on when P21 and P23 were co-detected, data suggest that new assembly of nucleocapsid particles containing P21 possibly occurred at ER membranes and in the cytoplasm. We hypothesized that at later stages once a threshold amount of cytoplasmic P21 was reached, crystal-associated particles assembled. On the other hand, P23 may not be directly involved in nucleocapsid assembly but in its regulation or in other functions such as HCV replication.

It could be argued that the nuclear HCcAg assembly shown in this report may be an experimental artifact of

the expression system used due to nuclear expression of the mRNA encoding the core region. Interactions between HCcAg and the core encoding mRNA may play a critical role in this assembly [23]. Nevertheless, the recent finding of nucleocapsid-like particles in the nucleus of hepatocytes from a chronically HCV-infected patient raises the possibility of nuclear HCV nucleocapsid assembly in vivo [24]. The relevance of the various HCcAg populations and particulate structures found for HCV morphogenesis and pathogenesis in vivo remains to be demonstrated.

Acknowledgments

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