Biochemical and Biophysical Research Communications **287,** 122–125 (2001)
doi:10.1006/bbrc.2001.5561, available online at http://www.idealibrary.com on IDEAL®

Characterization of the HCV Core Virus-like Particles Produced in the Methylotrophic Yeast *Pichia pastoris*

Nelson Acosta-Rivero,*[,1] Julio C. Aguilar,* Alexis Musacchio,* Viviana Falcón,† Ariel Viña,* María Cristina de la Rosa,† and Juan Morales*

**Division of Vaccines and †Physical Chemistry Department, Electron Microscopy Laboratory, Center for Genetic Engineering and Biotechnology, Ave. 31 e/158 y 190, P.O. Box 6162, C.P. 10600, C. Havana, Cuba*

Received July 16, 2001

Little is known about the mechanism of hepatitis C virion assembly. So the capacity of the entire Hepatitis C virus core protein (HCcAg) produced in *Pichia pastoris* to form particles either in its native soluble state or after detergent treatment of HCcAg associated to cell debris were studied. Size exclusion chromatography suggested that HCcAg assembled into high molecular weight structures. HCcAg was also specifically recognized by a serum from a chronic HCV carrier patient. This antigen migrated with buoyant density values similar to those obtained for native nucleocapsid particles from infected patients when analyzed using sucrose density gradient centrifugation. The analysis by electron microscopy of purified HCcAg showed aggregates resembling virus-like particles (VLPs) with an average diameter of 30 nm. These results indicated that the HCcAg obtained from *P. pastoris* assembled into VLPs resembling HCV nucleocapsid particles in a mature stage. Such HCcAg aggregates characterized here could be a valuable tool to elucidate the mechanisms of HCV nucleocapsid assembly. © 2001 Academic Press

Key Words: **Hepatitis C; core antigen; virus like particles; *P. pastoris*.**

Hepatitis C virus (HCV) is a major cause of parenterally acquired viral non-A, non-B hepatitis (1). At least 60% of infected individuals develop a chronic viremia that is usually associated with significant long-term morbidity (2). This virus contains a positive-stranded RNA encoding a single polyprotein precursor of about 3000 amino acids (aa) that is cleaved by both host and viral proteases to generate three putative structural proteins (Core, E1 and E2) and at least six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (3). The core protein (HCcAg) is cleaved out from the amino terminus of HCV polyprotein by host signal peptidase. This HCcAg undergoes post-translational modifications before it could be incorporated into mature virions (4). The HCcAg possesses numerous intriguing properties: these include in vitro RNA and ribosome binding activities (4), nucleocytoplasmic localization (5), a regulatory role on cellular and unrelated viral promoters (6, 7), an inhibitory role in programmed cell death (apoptosis) under certain conditions and interaction with cellular proteins (8). In this study, the HCV core-like particles (VLPs) obtained from *Pichia pastoris* cells were characterized, in order to obtain HCcAg suitable for structural as well as functional studies.

MATERIALS AND METHODS

Strains. The *P. pastoris* strain MP-36/C-E1.339, transformed with pNAO.COE1.339 plasmid (Fig. 1) coding for the entire HCcAg and the first 148 aa of the HCV E1 protein have been previously described (9). The MP-36 (9) strain was used as a negative control.

Antibodies. A mouse monoclonal antibody against the residues 5–35 of the HCV core protein (mAb SS-HepC.1) (9) was used to detected HCcAg from sucrose density gradient centrifugation in immunoblotting experiments. A human serum from a chronically HCV infected person (positive for HCcAg and negative for E1 antigen) (10) was used to detected HCcAg from CL-4B Gel filtration chromatography in immunoblotting experiments.

Growth conditions for P. pastoris strains. MP-36/C-E1.339 and MP-36 strains were grown using conditions already established (9). In brief, the MP36/CE1.339.5 was grown in minimal glycerol medium (MYG) (1.3% yeast nitrogen base, 1% glycerol and 0.4 μg/ml biotin) at 30°C for 48 h. The HCcAg expression from the methanol oxidase promoter was induced by replacing MYG with minimal methanol medium (MM) (1.3% yeast nitrogen base; 0.5% methanol, 0.4 μg/ml biotin) and further incubation at 30°C for 96 h. The strain MP-36 was used as a negative control under the same growth conditions. At the end of the yeast cell culture, the cells were harvested and washed twice in TEN buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 150 mM de NaCl).

Cell disruption and HCcAg extraction from cell debris. Cells disruption was performed using glass beads in TEN buffer. The lysate was clarified by centrifugation at 12,000*g* for 20 min and supernatant and pellet fractions were obtained. After cell disruption, the yeast cellular debris was treated with 0.5% Sarkosyl in TEN buffer

[1] To whom correspondence should be addressed. Fax: (53 7) 33 6008 21 8070. E-mail: nelson.acosta@cigb.edu.cu.

0006-291X/01 $35.00

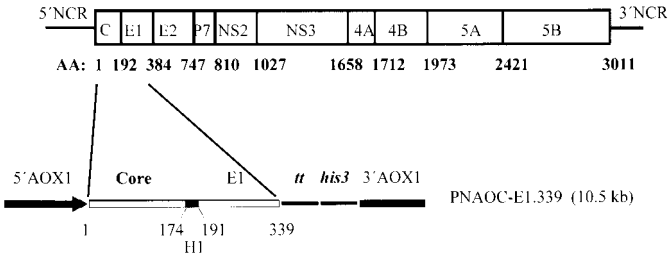

FIG. 1. Schematic representation of the expression vector pNAO.COE1.339 used for transforming the *P. pastoris* strain MP-36. 5′AOX1 for the expression of HCcAg. 5′AOX1, methanol oxidase promoter. ***tt,*** transcription terminator. AA, amino acid. 5′UTR and 3′UTR, 5′ and 3′ untranslated regions, respectively. C, E1, E2 and P7, HCV structural Core, Envelope 1, Envelope 2 and P7 proteins, respectively. NS2, NS3, 4A, 4B, 5A, 5B, HCV nonstructural proteins.

(a ratio of 10 ml for each g of cellular debris was used). The suspension was incubated for 120 min at room temperature with gentle agitation and then centrifuged at 12,000*g* for 20 min. The denaturalized protein sample (0.5 mg/ml) was renaturalized overnight at 4°C by dialysis against TEN buffer.

Protein analysis. The protein samples were separated in a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (11) and stained with Coomassie brilliant blue R250 (CBB, Sigma, St. Louis, MO). The purity was determined using the software 1-D Manager for Windows 95 (Ver. 2.0, TDI, sa, Madrid, Spain). HBsAg and HCcAg were quantitied using Dc Protein assay (Bio-Rad).

Immunoblotting assay. For immunoblotting the samples were applied to a nitrocellulose membrane and binding of antibodies was detected as previously described (12). In brief, the membrane was blocked for 1 h at room temperature with phosphate-buffered saline solution (PBS) (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4 pH 7.4) containing 5% skim milk, then incubated either with mAb SS-HepC.1 or human serum for 1 h at 37°C. After washing, the membrane was reacted with 1:1000 dilution of horseradish peroxidase-conjugated protein-A (Amersham, UK) for 1 h at 37°C. Immunoreactivity was detected using 200 μg/mL 9-amino-4-ethyl carbazole (Sigma) and 0.1% H_2O_2 in 50 mM NaAc, pH 5.3. The intensity of the resultant bands was quantitied by measuring the optical density with the Eagle Eye II still video system (Stratagene).

Sucrose density gradient centrifugation. Five hundred microliters of either the supernatant fraction (1 mg/ml) or the renaturalized HCcAg (0.1 mg/ml) were applied to a 5–50% (w/v) sucrose density gradient in TEN buffer. Centrifuged at 100,000*g* for 16 h and fractionated. A 500 μl aliquot of each fraction was collected from the bottom of the tube. The refractive index of each fraction was measured using an Abbe-3L refractometer (Milton Roy).

Analysis in CL-4B Gel filtration chromatography. One milliliter of either the supernatant fraction (1 mg/ml) or the renaturalized HCcAg (0.1 mg/ml), or the particulate hepatitis B surface antigen (HBsAg) (0.1 mg/ml) (13) were applied to a column (90 × 1.5 cm diameter) of Sepharose CL-4B (Pharmacia) equilibrated with TEN buffer. They were run at a flow rate of 0.5 mL/min.

Purification. The HCcAg extracted from the cell debris with 0.5% Sarkosyl was applied to a column (90 × 1.5 cm diameter) of Sepharose CL-4B (Pharmacia) as described above. Fractions containing HCcAg with more than 80% of purity were pooled.

Electron microscopy. A purified HCcAg was fixed in glutaraldehyde and negatively stained with uranyl acetate prior to analysis by transmission electron microscopy as previously described (9).

RESULTS AND DISCUSSION

Characterization of the HCV Core Protein from the Supernatant Fraction

It had been demonstrated that the *P. pastoris* MP-36/C-E1.339 clone, transformed with pNAO.COE1.339 plasmid, coding for the first 339 aa of the HCV polyprotein (Fig. 1), formed intracellular VLPs (9). After cell disruption HCcAg was mainly found in the pellet fraction although a little portion was also located in the supernatant (9). In order to characterize the HCcAg in the soluble fraction after cell disruption, the supernatant was analyzed by gel filtration chromatography in Sepharose CL-4B matrix. As a control, the HBsAg was also analyzed at the same conditions. Figure 2 revealed that HCcAg was distributed close to the exclusion volume that corresponds to a molecular mass over 2×10^6 Da. This value is a slightly higher than that of particulate HBsAg (Fig. 2). This indicates that HCcAg is forming high molecular weight structures in the supernatant after cell disruption, which is in agreement with the VLPs previously found inside cells (9).

The soluble protein fraction was also analyzed using a 5–50% (w/v) sucrose density gradient centrifugation. The sucrose gradient was fractionated and the individual fractions assayed for the presence of HCcAg (Fig. 3). The immunoblot evidenced major peak fractions containing the HCcAg (B2 and B3) that migrated to a position in the gradient corresponding to a buoyant density of 1.19–1.20 g/cm^3. This accounted for more than 70% of the total HCcAg applied. In addition, higher density HCcAg fractions (A8 and A9) with 1.28–

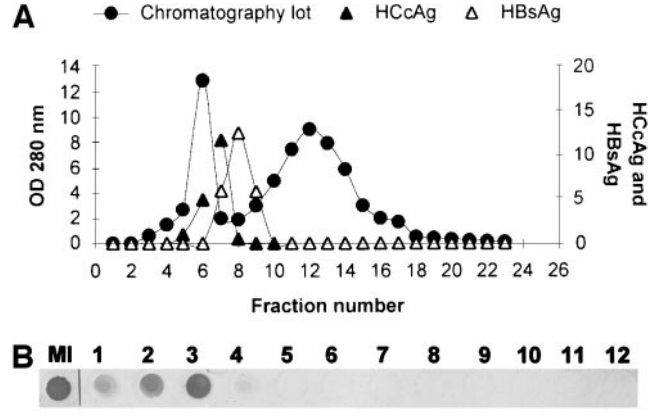

FIG. 2. Size exclusion chromatography on Sepharose CL-4B. (A) Chromatography lot of the soluble protein sample containing HCcAg. The HBsAg was analyzed at the same conditions. HCcAg in the chromatography fractions were analyzed by densitometry of dot blot. Values for HCcAg and HBsAg are in micrograms. (B) Dot blot of the HCcAg using a serum from a chronically infected patient. The leftmost lane (MI) contains the unfractionated material loaded onto the CL-4B column. Chromatography fractions from 5 to 15 were applied from 1 to 11. Spot 12 contains the supernatant obtained after cell disruption of *P. pastoris* MP-36 strain used as a negative control.

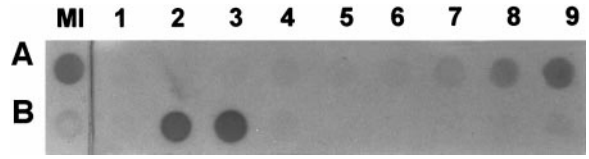

FIG. 3. Dot blot of the sucrose density gradient centrifugation of the soluble protein sample containing HCcAg using mAb SS-HepC.1. The leftmost lane (MI-A) contains 20 μl of the unfractionated material loaded directly onto the sucrose gradient and (MI-B) 1/20 dilution of the same sample. Forty microliters of the gradient fractions from bottom to top fractions were applied from A1 to B9. Detection of HCcAg was analyzed by densitometry.

1.29 g/cm^3 were also observed. This result is similar to values obtained for native nucleocapsid particles (14), indicating that the high molecular weight structures containing HCcAg analyzed here are similar in buoyant density to those found in sera from HCV infected patients.

Characterization of the HCV Core Protein from the Pellet Fraction

Afterwards, the capacity of the membrane associated HCcAg to assemble into high molecular weight structures was studied. After cell disruption, the HCcAg was extracted from the pellet fraction with the ionic detergent Sarkosyl and then purified by gel filtration in Sepharose CL-4B matrix (Fig. 4A). Fractions 6, 7 and 8 (lanes 2, 3 and 4) containing HCcAg, with purity higher than 80%, were pooled and renaturalized. When renaturalized HCcAg was analyzed by electron microscopy aggregates resembling VLPs were observed (Fig. 4B) with an average diameter of 30 nm. These results are consistent with those found in infected patients where 33 nm particles associated with nucleocapsid of HCV were observed in an aggregated form (15).

In addition the renaturalized sample was analyzed by gel filtration chromatography in Sepharose CL-4B matrix. As for the HCcAg present in the soluble fraction, this renaturalized HCcAg elutes predominantly close to the size corresponding to the particulate form of HBsAg (Fig. 5). The VLPs containing HCcAg are recognized as well by specific antibodies from a chronically HCV infected patient (Figs. 2B and 5B) suggesting that HCcAg studied here shares epitopes with the native HCcAg.

The renaturalized HCcAg was also analyzed by using a 5–50% (w/v) sucrose density gradient centrifugation. The study of gradient fractions by immunoblotting evidenced a buoyant density value of 1.28–1.29 g/cm^3 for HCcAg (A8 and A9) (Fig. 6) which is similar to that of minor population of the HCcAg observed in the soluble disruption fraction. However the major HCcAg fraction found in the soluble disruption fraction was not observed this time. This may reflect the lack (in the sample treated with detergent) of either DNA or

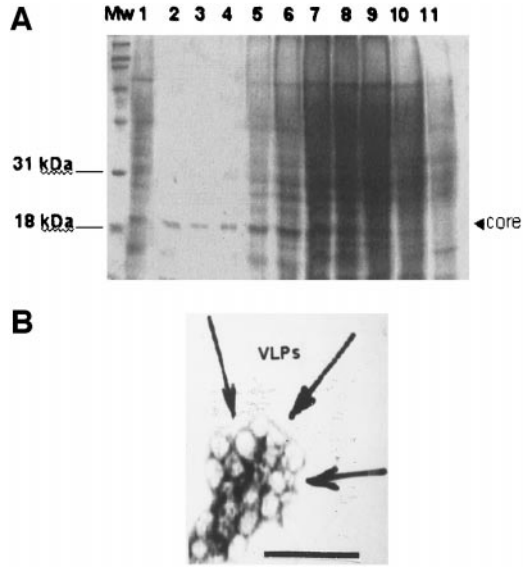

FIG. 4. Purification of HCcAg from the denaturalized protein sample by gel filtration in Sepharose CL-4B matrix. (A) Equal amounts from various fractions of the HCcAg purification procedure were separated by SDS-PAGE. The lanes contains size markers (MW), the unfractionated material loaded onto the CL-4B column (1), and chromatography fractions 6 to 15 (2–11). The arrow on the right indicate the position of HCcAg. (B) Electron micrograph of negatively stained VLPs separated by gel filtration. Particles from pooled fractions 6 to 8 are shown. Bar, 100 nm.

membrane components interacting with the HCcAg particles which were shown before to confer heterogeneity to the particles seen inside cells (9). These high buoyant density values are characteristic for non-enveloped HCV nucleocapsid particles. Nucleocapsid

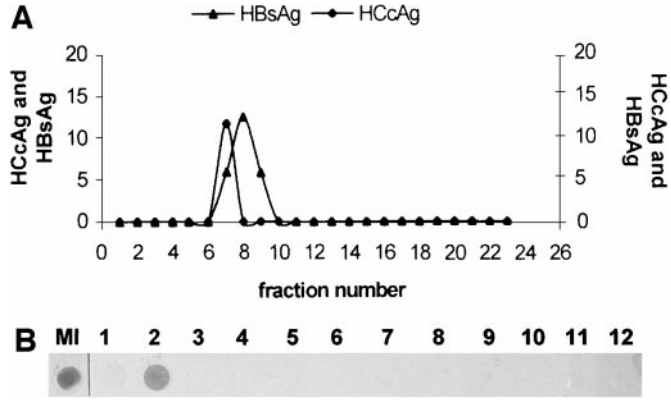

FIG. 5. Size exclusion chromatography on Sepharose CL-4B. (A) Chromatography lot of the purified HCcAg after renaturalization and HBsAg. HCcAg in the chromatography fractions were analyzed by densitometry of dot blot. Values for HCcAg and HBsAg are in micrograms. (B). Dot blot of the HCcAg using a human serum from a chronic patient. The leftmost lane (MI) contains the renaturalized HCcAg loaded onto the CL-4B column. Chromatography fractions from 6 to 16 were applied from 1 to 11. Spot 12 contains the HBsAg used as a negative control.

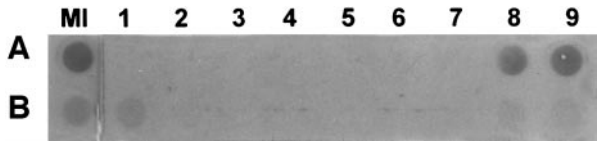

FIG. 6. Dot blot of the sucrose density gradient centrifugation of the purified HCcAg after renaturalization using mAb SS-HepC.1. The leftmost lane (MI-A) contains 20 μl of the renaturalized HCcAg loaded directly onto the sucrose gradient and (MI-B) 1/20 dilution of the same sample. Forty microliters of the gradient fractions from bottom to top fractions were applied from A1 to B9. Detection of HCcAg was analyzed by densitometry.

particles obtained after detergent treatment from sera of chimpanzees and humans with chronic non-A, non-B hepatitis, have shown similar buoyant density values (14). In that work the buoyant density of the intact HCV virions shifted from 1.08 g/cm^3 to 1.25 g/cm^3 after detergent treatment (14). Also we had been shown previously that a truncated HCV core protein expressed in *Escherichia coli* forms VLPs with a buoyant density of 1.28 g/cm^3 (16). The fact that structures resembling HCV nucleocapsid particles in a mature stage assembled in *P. pastoris* cells, could facilitate studies concerning structural composition and assembly mechanisms.

In conclusion, the results presented in this work showed that HCcAg, obtained from *P. pastoris* cells, forms VLPs with similar characteristics to those found in sera from HCV infected patients. Therefore, these VLPs would be important to elucidate the mechanisms behind the ability of HCcAg to assemble into a nucleocapsid structure.

REFERENCES

1. Kuo, G., Choo, Q. L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W. S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **244,** 362–364.
2. Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q. L., Houghton, M., and Kuo, G. (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87,** 6547–6549.
3. Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993) Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* **67,** 1385–1395.
4. Santolini, E., Migliaccio, G., and La Monica, N. (1994) Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J. Virol.* **68,** 3631–3641.
5. Lo, S., Masiarz, F., Hwang, S. B., Lai, M. M. C., and Ou, J. (1995) Different subcellular localization of hepatitis C virus core gene products. *Virology* **213,** 455–461.
6. Ray, R. B., Steele, R., Meyer, K., and Ranjit, R. (1997)Transcriptional repression of p53 promoter by hepatitis C virus core protein. *J. Biol. Chem.* **272,** 10983–10986.
7. Srinivas, R. V., Ray, R. B., Meyer, K., and Ray, R. (1996) Hepatitis C virus core protein inhibits human immunodeficiency virus type 1 replication. *Virus Res.* **45,** 87–92.
8. Ray, R. B., Meyer, K., and Ray, R. (1996) Suppression of apoptotic cell death by hepatitis C virus core protein. *Virology* **226,** 176–182.
9. Falcon, V., Garcia, C., de la Rosa, M. C., Menendez, I., Seoane, J., and Grillo, J. M. (1999) Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1). *Tissue Cell* **31,** 117–125.
10. Morales, J., Viña, A., Garcia, C., Acosta-Rivero, N., Dueñas-Carrera, S., Garcia, O., Guerra, I., and Falcon, V. (1998) Sequences derived from the genome of the hepatitis C virus, and use thereof. WO 98/25960.
11. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227,** 680–685.
12. Lorenzo, L. J., García, O., Acosta-Rivero, N., Dueñas-Carreara, S., Martínez, G., Alvarez-Obregón, J. C, Pichardo, D., Ramos, A., Guerra, I.., and Morales, J. (2000) Expression and immunological evaluation of the *Escherichia Coli*-derived hepatitis C virus envelope E1 protein. *Biotechnol. Appl. Biochem.* **32,** 137–143.
13. Hardy, E., Martínez, E., Diago, D., Díaz, R., González, D., and Herrera, L. (2000) Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris. J. Biotechnol.* **77,** 157–167.
14. Miyamoto, H., Okamoto, H., Sato, K., Tanaka, T., and Mishiro, S. (1992) Extraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and the polymerase chain reaction. *J. Gen. Virol.* **73,** 715–718.
15. Takahashi, K., Kishimoto, S., Yoshizawa, H., Okamoto, H., Yoshikawa, A., and Mishiro, S. (1992) p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected host. *Virology* **191,** 431–434.
16. Lorenzo, L. J., Dueñas-Carrera, S., Falcon, V., Acosta-Rivero, N., Gonzalez, E., de la Rosa, M. C., Menendez, I,, and Morales, J. (2001) Assembly of truncated HCV core antigen into virus-like particles in *Escherichia coli. Biochem. Biophys. Res. Commun.* **281,** 962–965.