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Hepatitis C Virus Nonstructural Protein NS3 Transforms NIH 3T3 Cells

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Clinical evidence suggests that hepatitis C virus (HCV) is etiologically involved in hepatic cancer and liver cirrhosis. To investigate whether the HCV nonstructural protein NS3 has oncogenic activity, NIH 3T3 cells were transfected with an expression vector containing cDNA for the 5′- or 3′-half sequence of the HCV genome segment encoding NS3. Only cells transfected with the 5′-half cDNA rapidly proliferated, lost contact inhibition, grew anchorage independently in soft agar, and formed tumors in nude mice. PCR analysis confirmed the presence of the 5′-half DNA in the transfectants. These results suggest that the 5′ region of the HCV genome segment encoding NS3 is involved in cell transformation.

Hepatitis C virus (HCV) is the major agent of non-A, non-B hepatitis. The HCV genome is a plus-stranded RNA about 10 kb in length, and its organization resembles that of members of the family *Flaviviridae* (8, 16, 23). The genome encodes a single polyprotein that includes structural and nonstructural proteins which are processed by cellular and virus-encoded proteinases (4, 16). On the basis of expression and functional analyses, the HCV nonstructural protein NS3 has been identified as a virus-encoded serine proteinase (1, 2, 4, 6, 7, 13, 26). NS5B is expected to be an RNA-dependent RNA polymerase on the basis of the presence of a Gly-Asp-Asp sequence (4, 8, 23). However, the biological functions of these proteins have not been sufficiently characterized.

Persistent infection with HCV leads to cirrhosis and hepatocellular carcinoma (HCC) (3, 5, 12, 14, 17, 27), and although the mechanism of transformation by HCV is unknown, the viral genome and replication have been detected in liver cells with those pathological changes. Because NS3 has not only proteinase but also helicase activity, including ATPase (21), and the antibody titer against NS3 was correlated with the severity of liver disease (24), we tried to transfect cells with the NS3 region to investigate cellular changes. We report here that NIH 3T3 mouse fibroblasts become transformed after transfection with HCV NS3 cDNA and are tumorigenic in nude mice.

The cDNAs for the 5′-half and 3′-half portions of the HCV genome segment encoding NS3 were the kind gift of M. Kohara (Tokyo Metropolitan Medical Research Institute) (10) and were used to construct expression vectors, pHCN3-N′ and pHCN3-C′, respectively, in *Escherichia coli* as described by Takegami and Hasumura (24). From plasmid pHCN3-N′, we isolated a 0.96-kb *Xba*I-*Eco*RI DNA fragment containing 87 bp of plasmid pET3a with an ATG initiation codon, a sense-oriented HCV NS3 5′-half (NS3-5′) region from nucleotides 3354 to 4210 (Ile-1020 to Thr-1295) (9), and 21 bp of pET3a with a TGA stop codon. The DNA fragment was ligated into the mammalian expression vector pRcCMV encoding neomycin and ampicillin resistance genes (Invitrogen Co., San Diego, Calif.), and the final expression vector was designated pRcHCNS3-5′. Similarly, we constructed plasmid pRcHCNS3-3′ containing the sense-oriented HCV NS3-3′ region from nucleotides 4116 to 5147 (Phe-1263 to Trp-1608). Expression of these inserted cDNAs was under the control of the human cytomegalovirus (CMV) major immediate-early promoter (Fig. 1).

NIH 3T3 (Japanese Cancer Research Resources Bank, Tokyo, Japan) is a nontumorigenic mouse fibroblast cell line and was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (CS) and 50 μg of gentamicin per ml. Fresh cells from the same lot stored in liquid nitrogen were passaged every 3 days at a constant split ratio of 1:8 and used within 1 month to avoid any spontaneous transformation. Transfection was performed by the liposome protocol (18). In brief, NIH 3T3 cells were seeded at a density of 2×10^5 cells per dish in a 35-mm-diameter culture dish. Next day, the cells were transfected with pRcHCNS3-5′, pRcHCNS3-3′, or pRcCMV DNA at 10 μg per dish in Lipofectin reagent (Gibco BRL, Gaithersburg, Md.) by using 1 ml of serum-free Opti-MEM-I medium (Gibco BRL). After 5 h, the culture medium containing the plasmid DNA-Lipofectin complex was removed and replaced with fresh DMEM containing 10% CS, and the incubation continued for 2 days. Cells were reseeded into a 150-mm-diameter tissue culture dish which contained 20 ml of a selection medium containing G418 (400 μg/ml) (Geneticin; Sigma). This medium was replaced with fresh selection medium every 3 days for 2 weeks. G418-resistant colonies were isolated with a cup cylinder and maintained in DMEM with 10% CS containing G418 (200 μg/ml) until analyzed.

To determine the biological role of HCV NS3 in cell transformation, we compared the growth characteristics of duplicate representative NIH 3T3 mouse fibroblast cultures transfected with plasmid pRcCMV, plasmid pRcHCNS3-5′, or plasmid pRcHCNS3-3′; selected in G418-containing medium; and cloned. Nontreated NIH 3T3 cells were used as controls. Cells (10^4 cells per well) were seeded in 12-well plates (Corning, Corning, N.Y.) and counted daily. All measurements were made in triplicate. At 4 days of culture, the number of cells in the two cultures transfected with pRcHCNS3-5′, designated A and B, was approximately 5×10^4, or double the number for parental cells or mock-, pRcCMV-, or pRcHCNS3-3′-transfected cells (Table 1), and other cell lines transfected with NS3-5′ also showed a similar growth pattern.

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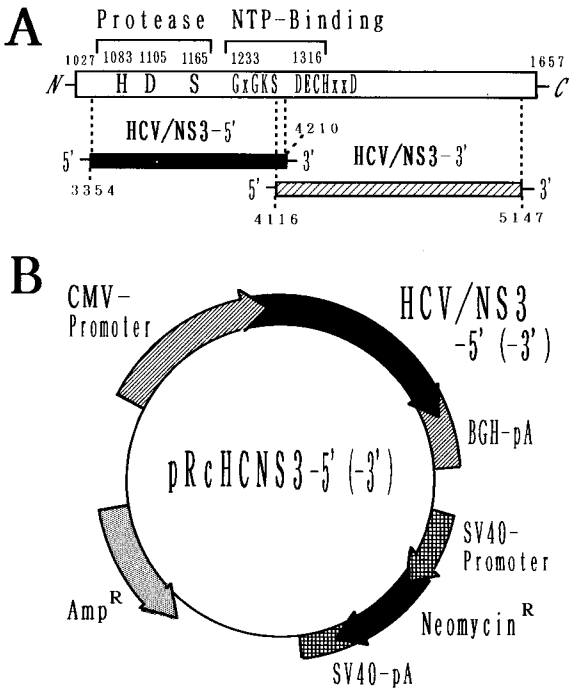

FIG. 1. Construction of pRcHCNS3 expression vector. (A) HCV NS3 genome. Amino acid numbers at the boundaries of the HCV NS3 protein and catalytic centers (serine proteinase and nucleoside triphosphate-binding sites) are indicated. The black and hatched boxes represent the cDNAs of HCV NS3-5′ (nucleotides 3354 to 4210) and -3′ (nucleotides 4116 to 5147) (9), respectively, which were inserted into the pRcCMV vector. (B) Expression vectors pRcHCNS3-5′ and -3′. The HCV NS3-5′ or -3′ cDNA was inserted downstream of the human CMV promoter of expression vector pRcCMV. The 3′ terminus of HCV NS3-5′ or -3′ was bounded by the transcription termination and polyadenylation signal of a human growth hormone gene (BGH-pA). These expression vectors encode neomycin and ampicillin resistance genes. SV40, simian virus 40.

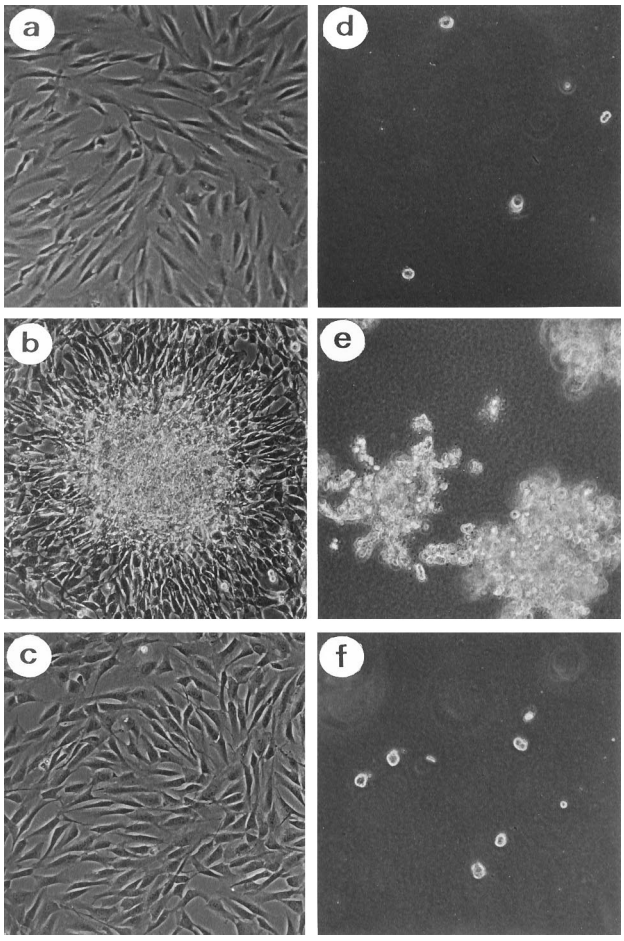

FIG. 2. Morphology of NIH 3T3 cells transfected with HCV NS3 cDNA constructs. NIH 3T3 cells transfected with pRcCMV (a and d), pRcHCNS3-5′ (b and e), and pRcHCNS3-3′ (c and f) were selected and grown in the presence of G418. Morphology (a, b, and c) and colony formation in soft agar (d, e, and f) were assessed by phase-contrast microscopy. Colony formation in soft agar was observed at 14 days of culture.

To examine the ability of G418-resistant cells to grow anchor-age independently, 10^4 cells were suspended in 0.25% agarose (FMC BioProduct, Rockland, Maine) containing DMEM and 10% CS and overlaid onto a bottom layer of 0.75% agarose in 6-well plates (Nunc, Roskilde, Denmark). After 10 days of culture, colonies with more than 20 cells were scored and the efficiency of colony formation was determined. Microscopic analysis of the morphology of the transfected cells (Fig. 2) revealed colony formation at 14 days of culture in soft agar (panel e) and the absence of contact inhibition (panel b) in the NS3-5′ transfectants, whereas the pRcCMV (panels a and d) and pRcHCNS3-3′ (panels c and f) transfectants, as well as the parental cells, showed contact inhibition under the same conditions.

TABLE 1. Tumorigenicity of NIH 3T3 cells transfected with HCV NS3-5′ and -3′

Cell line	Contact inhibition	Doubling time (h)	Cloning[a] efficiency (%)	Tumor/inoculum[b]	
				2 wk	6 wk
NIH 3T3	+	21.2	0.02	ND[c]	ND
pRcCMV-A	+	22.6	0.05	0/5	0/5
pRcCMV-B	+	19.8	ND	ND	ND
pRcHCNS3-5′A	−	12.7	47	2/10	10/10[d]
pRcHCNS3-5′B	−	11.3	39	ND	ND
pRcHCNS3-3′A	+	19.7	0.12	0/5	1/5
pRcHCNS3-3′B	+	21.1	ND	ND	ND

[a] Cells (10^3) were plated in 0.25% agar layered over 0.75% agar.
[b] Cells (10^7) were injected subcutaneously into the right flank of nude mice. Data are the number of mice with tumors/total number of mice inoculated.
[c] ND, not determined.
[d] Average tumor size, >3 cm^2 of sectional area.

To detect cDNA in stable transfectants, total genomic DNA was extracted according to standard methods (18) and subjected to PCR and Southern blot analysis. The primers for amplification of the 5′-half sequence of the HCV NS3 region were 5′-CGGGCACGTTGTAGGCATC-3′ (sense) and 5′-AACGGACGGCTTTAGGACGA-3′ (antisense) and were based on published sequences (9). PCR conditions were 35 cycles of three steps (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 μM each deoxyribonucleoside triphosphate, 0.01% gelatin, 0.5 μg of total DNA, and 2 U of *Tth* DNA polymerase (Toyobo, Tokyo, Japan). PCR products were subjected to electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining, and confirmed by Southern blot hybridization using an internal probe, 5′-GGTTGCGAAGGCGGTGGACT-3′, chosen from the sequences in the HCV NS3-5′ region.

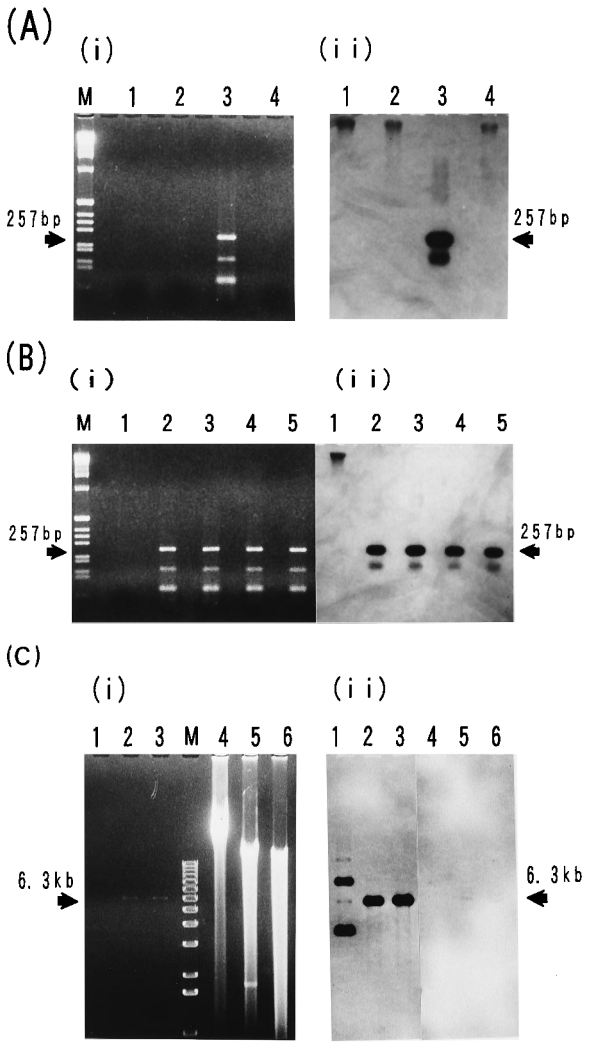

FIG. 3. PCR and Southern blot analysis of DNA from transfected cells. (A) Specific amplification of HCV NS3-5′ sequence by PCR. DNAs were prepared from the transfected cells, amplified by PCR, resolved by agarose gel electrophoresis, and stained with ethidium bromide (i). Southern blot analysis of PCR products using an internal oligonucleotide as described in the text was performed (ii). Lanes 1, parental NIH 3T3 cells; lanes 2, pRcCMV-transfected cells; lanes 3 and 4, pRcHCNS3-5′- and -3′-transfected cells, respectively; lane M, 1-kb ladder as size marker (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). (B) Presence of HCV NS3-5′ cDNA in stable G418-resistant transfectants. PCR and hybridization with an internal oligonucleotide were carried out. Parts i and ii are as described for panel A. Lanes 1, DNA from parental NIH 3T3 cells; lanes 2 to 5, DNA from the stable pRcHCNS3-5′ transfectants at passages 3, 6, 9, and 12, respectively. (C) Southern blot of genomic DNA from the transfectants. Plasmid pRcHCNS3-5′ (0.5 ng) and genomic DNA from the transfectants (10 μg) were digested with *Eco*RV (lanes 2 and 5, respectively) or *Pst*I (lanes 3 and 6, respectively) and then Southern blotted by using HCV NS3-5′ DNA as a probe. Parts i and ii are as described for panel A. Arrows indicate the size of plasmid pRcHCNS3-5′ (6.3 kb). Lanes 1 and 4 indicate undigested plasmid and genomic DNAs, respectively.

As shown in panel i of Fig. 3A, products were amplified from DNA of cells transfected with pRcHCNS3-5′. Southern blot analysis using the internal probe revealed the major 257-bp fragment expected from the HCV NS3 sequence (Fig. 3A, panel ii), whereas no DNA amplification was detected in cells transfected with pRcCMV or pRcHCNS3-3′. The stability of HCV DNA during cell passage of HCV NS3-5′-transfected cells was confirmed at passages 3, 6, 9, and 12 by PCR and Southern blot (Fig. 3B, lanes 2 to 5 in both panels). The presence of HCV DNA was also confirmed by direct Southern blot with genomic DNA from the transfected cells (Fig. 3C, panel ii, lanes 5 and 6).

To identify the expressed HCV NS3 protein in the transfectant, Western blotting (immunoblotting) with HCV patient sera was performed, as previously described (24, 25). The expressed HCV NS3-N′ was detected in the cell extract by diaminobenzidine staining, although only a faint band was observed (data not shown).

To examine the tumorigenic potential of the transfectants, nude mice (BALB/c, *nu*/*nu* females, 5 weeks old; Sankyo Labo, Tokyo, Japan) were inoculated subcutaneously in the right flank with pRcHCNS3-5′- or pRcHCNS3-3′-transfected cells (10^7 cells in 0.2 ml of phosphate-buffered saline) and monitored for tumor development (Table 1). Tumor size and animal weight were measured weekly, and tumors were removed before they reached a cross-sectional area of 3 cm^2. At 2 weeks after inoculation, tumors developed in 2 mice inoculated with the pRcHCNS3-5′ transfectants, and at 6 weeks, all 10 mice had tumors of considerable size around the inoculation site. One of five mice inoculated with pRcHCNS3-3′ transfectants developed a tumor after 6 weeks, but its size (1 cm in diameter) was much smaller than that (average diameter, >2 cm) of tumors in pRcHCNS3-5′-injected mice.

Cells were prepared by trypsinization from the tumors of mice inoculated with pRcHCN3-5′ and were cultured in the presence of G418. DNA from the established cell lines was subjected to PCR as described before, and the HCV-specific 257-bp fragment was amplified (data not shown).

We show here that an HCV NS3-5′ cDNA can transform NIH 3T3 mouse fibroblasts, although the mechanism of this transformation remains unknown. Clinical evidence indicates that HCV causes a persistent infection in liver cells and leads to HCC after about a 20-year incubation (3, 12, 17, 27). This is likely to reflect repeated replication of HCV and production of nonstructural proteins during persistent infection in vivo, factors which exert some effect on cellular function. The finding that NIH 3T3 cells transfected with HCV NS3-5′, which contains the sequences encoding proteinase activity, show transforming and tumorigenic ability (Fig. 2 and Table 1) suggests the involvement of proteinase activity in cellular transformation. To confirm this, further experiments, including the substitution of amino acids in the active site of the proteinase, are needed.

The association of HCC with HBV has been well documented over the years (19). However, the mechanism remains unknown, although it has been suggested that the activation of some oncogenes may be common to the process of hepatocarcinogenesis. Recent reports concerning the X protein of HBV have described its activity as a proteinase inhibitor and its apparently important role in transformation (19, 22). A correlation between the inhibitory effect of the HBV X protein on cellular proteinases and cellular transformation would implicate the cellular proteinase system in the regulation of cell growth. In fact, it has been reported that some proteinases seem to be essential for the cell cycle (15) and transformation (11).

Recent characterization of the HCV NS3 proteinase by using an in vitro translation assay and a bacterial or eukaryotic expression system (1, 4, 6, 26) showed that the cleavage site of HCV NS3 differed from that of flavivirus NS3 (16). It is possible that HCV NS3 cleaves cellular proteins, including oncogene products that regulate cellular function. One candidate may be the c-*raf* product, which becomes an active enzyme after cleavage at the amino-terminal region (20). Elucidation

of this notion of the function of HCV NS3 in vitro and in vivo requires further analysis.

Other possible etiologic factors in HCC are not excluded, because patients with HCC may be exposed simultaneously to multiple etiologic factors. Unsal et al. (28) have indicated the genetic heterogeneity of HCC by means of p53 gene analysis. Recently, Mangia et al. (14) have reported that HCV infection in patients with HCC in the United States is relatively infrequent, accounting for less than 30% of such cases. In these patients, some host factors appear to be involved in the carcinogenesis of liver cells.

In our study, injection of pRcHCNS3-5′-transfected mouse cells led to fibrosarcoma formation in nude mice, suggesting tumorigenic capability associated with the HCV NS3-5′ region. To our knowledge, this is the first experimental demonstration of transformation and tumorigenicity upon transfection with HCV NS3 DNA. The greater frequency of tumors induced by pRcHCNS3-5′ than by the 3′ construct points especially to the function of the amino terminus in cellular transformation. Elucidation of the precise mechanism involved and the possible role of other regions encoding HCV nonstructural proteins awaits further analysis.

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