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Ubiquitous Presence of Cellular Proteins That Specifically Bind to the 3′ Terminal Region of Hepatitis C Virus

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The 3′ terminal region (3′-X tail) of hepatitis C virus (HCV) genomic RNA forms a stable stem-loop structure. The 3′-X tail consists of 98 nucleotides (nt) that are highly conserved among the HCV strains and supposed to function as a *cis*-acting region for replication of negative strand RNA and/or viral encapsidation. In the present study, by UV cross-linking assay we found two kinds of cellular proteins of approximately 87 and 130 kDa, which specifically bind to the full-length 3′-X tail (nt 1 to 98), but not the 3′- or 5′-truncated 3′-X tail, consisting of nt 1 to 50 or nt 51 to 98, respectively. These proteins were detected in human cell lines such as hepatic tumor cell lines and a T-lymphocyte cell line and also in a human embryonic lung fibroblast cell strain. In addition, human hepatocellular carcinoma tissues expressed these proteins regardless of infection or uninfection of HCV. Furthermore, these proteins were also detected in normal human tissues derived from the lung, heart, kidney, stomach, intestine, and colon. Thus, these cellular proteins, which are ubiquitously present in human tissues, might be involved in viral replication and/or encapsidation.

Hepatitis C virus (HCV) is the major agent of non-A, non-B hepatitis, and efficiently causes chronic hepatitis, which often gives rise to liver cirrhosis and then hepatocellular carcinoma (1, 2). HCV is classified into the genus within the *Flaviviridae* family (3). HCV is a 9.4 kb single-stranded positive strand RNA virus, consisting of 5′ untranslated region (5′ UTR), a long open reading frame encoding approximately 3,000 amino acids, 3′ untranslated region (3′ UTR) with a poly(U) or a poly(A) stretch (4, 5), and the 3′ terminal region (3′-X tail) discovered recently (6). The 3′-X tail of HCV genomic RNA has a novel sequence consisting of 98 nucleotides (nt), which is highly conserved among different HCV strains and has a potential to form stable secondary structure (7, 8). Since other *Flaviviridae* family members also form stable secondary structure which is thought to be necessary for viral replication, the 3′-X tail may also function as *cis*-acting element for the initiation of viral replication. Despite its importance, little is known about the details of the 3′-X tail function. Studies on other positive strand RNA viruses suggest that host-encoded proteins are often implicated in viral RNA replication (9, 10). The best-characterized RNA-dependent RNA polymerase (RdRp) is a protein of bacteriophage Qβ, which consists of one viral encoded polypeptide and three host polypeptides (9). In the study on cucumber mosaic virus, two virus-encoded polypeptides and one host polypeptide were detected in the replication complexes (10). In addition, some cellular proteins have been reported to bind to the 3′ UTR of various RNA viruses (11-13). The terminal 3′ UTRs of these viral RNAs also form stable stem-loop structures like the 3′ X-tail. Therefore, it is supposed that some cellular proteins may bind to the 3′ X-tail, and the interaction between host proteins and viral RNA may play an important role in the initiation of negative-strand RNA replication. Thus, using human cell lines and tissues we examined whether there would be any cellular proteins which bind to the 3′-X tail of HCV. Here we report that two kinds of cellular proteins of 87 and 130 kDa, which specifically bind to the full-length 3′-X tail, are ubiquitously present in various human cell lines and tissues.

MATERIALS AND METHODS

Cell culture. Human hepatic tumor cell lines (HuH-6, HuH-7, HLE, JHH-1, JHH-4, and JHH-6), a human embryonic lung fibroblast cell strain (TIG-7), and a human cervical carcinoma cell line

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(HeLa) were cultured at 37°C in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml kanamycin. A human T cell leukemia virus type I (HTLV-I)-infected T cell line, MT-2, was cultured at 37°C in RPMI 1640 (Nissui) supplemented with 10% FBS and 100 μg/ml kanamycin.

Construction of recombinant plasmid. HCV cDNA corresponding to full-length 3′-X tail (7) was synthesized by polymerase chain reaction (PCR) using a sense primer, 5′-GGTGGCTCCATCTTAGCCCTAGTCACGGCTAGCTGTGAAAGGTCCGTGAGCCGCATGAC-3′, and an antisense primer, 5′-ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCTCACGGACCT-3′. The PCR products were directly subcloned into plasmid pCR2.1 (Invitrogen, Carlsbad, CA), and the plasmid was further amplified by PCR using a sense primer, 5′-TTAGGGCCCGGTGGCTCCATCTTAG-3′, and an antisense primer, 5′-CG**GGATCC**ACATGATCTGCAGAGAGG-3′. The resulting PCR products were digested with *Apa*I (underlined) and *Bam*HI (boldface) and ligated into pCR2.1 vector to generate plasmid pHCV3′X1′-98′. Similarly, plasmids pHCV3′X1′-50′ and pHCV3′X51′-98′ containing from nt 1 to 50 or from nt 51 to 98 of the 3′-X tail cDNA were generated. The nucleotide sequence of the cloned 3′-X tail was confirmed by AmpliCycle sequencing kit (Perkin-Elmer, Foster, CA).

In vitro transcription. Plasmid DNA was purified by the CsCl density gradient centrifugation method (14) and linearized by treatment with *Bam*HI. A ^{32}P-labelled RNA probe was produced by *in vitro* transcription with T7 RNA polymerase (50 U; Gibco BRL, Rockville, MD) in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 500 mM ribonucleotides (ATP, CTP, and GTP), 100 μCi of [^{32}P]UTP (400 Ci/mmol; HAS, Budapest, Hungary), 1 μg of linearized DNA template, and RNase inhibitor (20 U; Takara, Tokyo, Japan). The reaction mixture was incubated at 37°C for 60 min. Then, RNase-free DNase I (5 μg; 5 PRIME→3 PRIME, Boulder, CO) was added, and the incubation further continued for 15 min. After ethanol precipitation, RNA was dissolved in diethylpyrocarbonate treated-distilled water.

Preparation of cytoplasmic extract. Cells were grown to confluence in 10-cm dishes, then washed with cold phosphate-buffered saline (PBS), scraped, and centrifuged at 1,500 × g at 4°C for 5 min. Cytoplasmic extracts were prepared by the method of Schreiber et al. (15) with slight modifications. Briefly, cell pellets were resuspended by gently pipetting in 400 μl of a cold extraction buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride] and allowed to swell on ice for 15 min. After addition of 25 μl of 10% Nonidet P-40, the mixture was vortexed vigorously for 10 sec and centrifuged for 1 min by a microcentrifuge. The supernatant was collected and adjusted to 100 mM KCl and 10% glycerol by addition of these stock solutions. Human tissues obtained were immediately washed with PBS, frozen at −80°C, and broken to pieces with hammer. The pieces were homogenized in the extraction buffer, and the extraction steps were carried out as described above. Protein concentration was determined by the Bio-Rad assay.

Gel shift assay. Gel shift assay was carried out by the method of Konarska and Sharp (16) with slight modifications. Briefly, cytoplasmic extracts were preincubated at 30°C for 10 min with yeast tRNA (10 μg) in 10 μl of a reaction mixture consisting of 5 mM HEPES (pH 7.9), 25 mM KCl, 2 mM $MgCl_2$, 3.8% glycerol, 2.5 mM DTT, and RNase inhibitor (10 U). ^{32}P-labelled RNA probe (approximately 3×10^5 cpm) was then added, and the mixture was incubated at 30°C for 10 min. For dephosphorylation experiments, prior to adding the probe, the reaction mixture was treated with calf intestine alkaline phosphatase (1∼2 U; Boehringer-Mannheim, Mannheim, Germany). For control, calf intestine alkaline phosphatase which was inactivated by heating at 100°C for 30 min was used. For competition

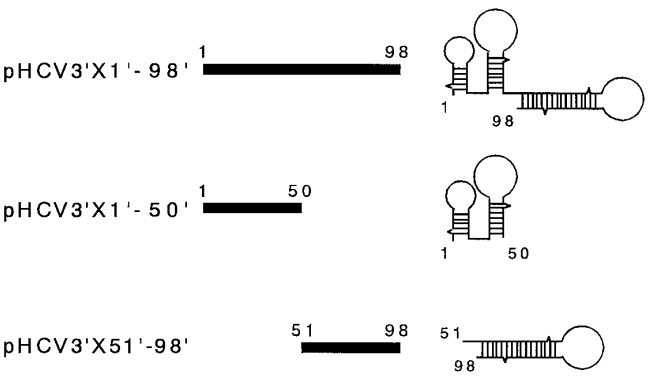

FIG. 1. Schematic diagram of *in vitro* transcripts representing the 3′-X tail. All the RNA transcripts were prepared as described in Materials and Methods. Plasmid pHCV3′X1′-98′ contains full-length cDNA of the 3′-X tail. Plasmids pHCV3′X1′-50′ and pHCV3′X51′-98′ contain 50 nt from nt 1 to 50 and 48 nt from nt 51 to 98 of the 3′-X tail, respectively. Predicted secondary structure of RNA transcripts are indicated in the right panel.

experiments, various amounts of unlabelled transcripts was added to the reaction mixture and incubated at 30°C for 10 min prior to the addition of ^{32}P-labelled RNA probe. The reaction mixtures were then loaded onto 5% polyacrylamide gel containing 0.5 X Tris-borate-EDTA and 2.5% glycerol and electrophoresed at 200 V for 3 hr at 4°C.

UV cross-linking assay. The RNA-protein binding reaction was first carried out as described for the gel shift assay. The reaction mixture was then irradiated with UV light for 30 min on ice. After irradiation, RNase T_1 (2 U; Ambion, Austin, TX) was added, and the reaction mixture was further incubated at 37°C for 15 min. For control, proteinase K (10 μg ; Boehringer-Mannheim) was added to the reaction mixture prior to the UV irradiation and incubated at 37°C for 15 min. A sample buffer was then added to the reaction mixtures to give final concentrations of 10% glycerol, 5% β-mercaptethanol, 3% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl (pH 6.8). The samples were boiled for 3 min and electrophoresed on a 10% SDS-polyacrylamide gel (17). Protein molecular mass was estimated by molecular mass standards (Bio-Rad, Hercules, CA).

Northwestern blot. Fifty μg of JHH-4 cytoplasmic extract was electrophoresed on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane (Amersham, London, UK). The membrane was washed with HYB100 [20 mM HEPES (pH 7.9), 100 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% Nonidet P-40] and then pre-hybridized with yeast tRNA(10 μg/ml) in HYB100 for 60 min. ^{32}P-labelled full-length 3′-X tail RNA in HYB100 (5×10^4 cpm/ml) was hybridized with membrane-bound proteins for 90 min. The membrane was then washed three times with HYB100, air dried, and subjected to autoradiography.

RESULTS

We carried out gel shift assay to detect cellular proteins that specifically bind to the 3′-X tail (3′X1′-98′) using its RNA probe prepared by *in vitro* transcription (Fig. 1). Cytoplasmic extracts from a human hepatocellular carcinoma cell line (JHH-4) were used in this experiment, because this cell line persists 5′ UTR of HCV genomic RNA (18). As a result, four kinds of RNA-

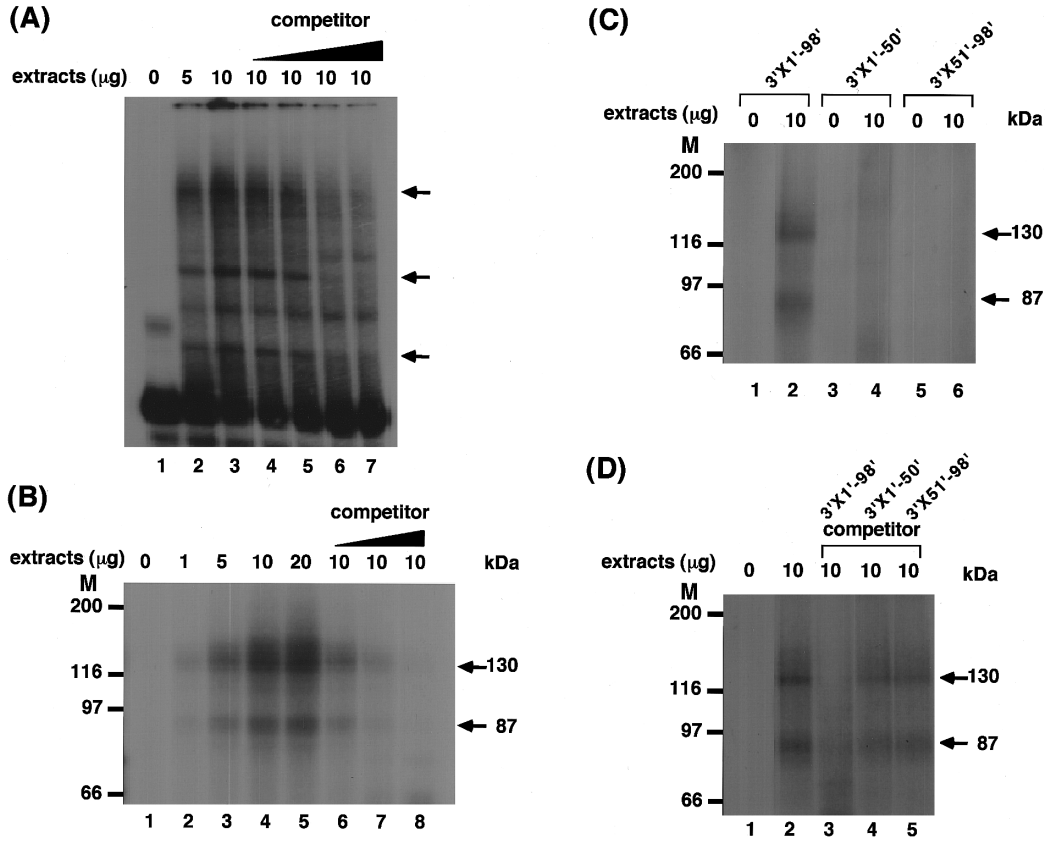

FIG. 2. Specific interaction of JHH-4 cell line-derived proteins with the full-length 3′-X tail. (A) Gel shift assay using JHH-4 cell extract. ^{32}P-labelled 3′X1′-98′ RNA was mixed without (lane 1) or with 5 or 10 μg of cytoplasmic extract (lanes 2 and 3). Ten μg of cytoplasmic extract was mixed with 5, 50, 500, or 1000 ng of the unlabelled 3′X1′-98′ RNA, prior to addition of ^{32}P-labelled 3′X1′-98′ RNA (lanes 4-7). (B) UV cross-linking assay using JHH-4 cell extract. ^{32}P-labelled 3′X1′-98′ RNA was mixed without (lane 1) or with 1, 5, 10, or 20 μg of cytoplasmic extract (lanes 2-5). The mixtures were irradiated with UV light, digested with RNase T_1, and separated on 10% SDS-polyacrylamide gel electrophoresis. In lanes 6-8, 10 μg of cytoplasmic extract was mixed with 50, 500, or 1000 ng of the unlabelled 3′X1′-98′ RNA prior to addition of ^{32}P-labelled 3′X1′-98′. (C) UV cross-linking assay with the labelled truncated 3′-X tail RNAs. UV cross-linking assay was carried out using ^{32}P-labelled 3′X1′-98′ RNA, 3′X1′-50′ RNA, and 3′X51′-98′ RNA without (lanes 1, 3, and 5) or with 10 μg of JHH4 cytoplasmic extract (lanes 2, 4, and 6). (D) Competition assay with the unlabelled truncated 3′-X tail RNAs. UV cross-linking assay was carried out using ^{32}P-labelled 3′X1′-98′ RNA without (lane 1) or with 10 μg of JHH-4 cytoplasmic extract (lanes 2-5). One thousand ng of the unlabelled 3′X1′-98′ (lane 3), 3′X1′-50′ (lane 4), or 3′X51′-98′ RNA (lane 5) was added to the reaction mixtures prior to addition of ^{32}P-labelled 3′X1′-98′ RNA. M, molecular mass standards (in kilodaltons).

protein complexes were detected (Fig. 2A). The amount of RNA-protein complexes increased in parallel with amount of the applied cytoplasmic extracts. To examine specificity of the binding, the unlabelled 3′X1′-98′ RNA was used as a competitor for protein binding. The complex formations by the three kinds of cellular proteins reduced inversely with increase in the unlabelled 3′X1′-98′ RNA. These results indicate that three kinds of cellular proteins in JHH-4 cells specifically interact with 3′X1′-98′ RNA.

To determine the molecular masses of these cellular proteins interacting with 3′X1′-98′ RNA, we irradiated the complexes with UV light to form covalent cross-link between the labelled 3′X1′-98′ RNA and the cytoplasmic proteins. After treatment with RNase T_1 to digest the unprotected probe, the cross-linked complexes were analyzed on SDS-polyacrylamide gel. Although three kinds of specific protein complexes were detected by gel shift assay, only two bands of approximately 87 and 130 kDa were detected by UV cross-linking assay (Fig. 2B). The other complex may be unstable and dissociated under these conditions. The amount of the cross-linked complexes increased in parallel with amount of the applied cytoplasmic extracts. No cross-linked complexes were detected with proteinase K-treated cytoplasmic extracts (data not shown). These results clearly show that 87 and 130 kDa proteins in the cytoplasmic extracts have activity to bind to 3′X1′-98′ RNA. To further demonstrate specificity of the binding, the unlabelled 3′X1′-98′ RNA was used as a competitor for UV cross-linking assay. As a result, the intensities of 87 and 130 kDa bands diminished

inversely proportion to amount of the applied unlabelled 3′X1′-98′ RNA. Although we performed Northwestern blot to determine whether 87 kDa and/or 130 kDa proteins consist of each single polypeptide, we could not clarify it because of the high background (data not shown).

Next, to identify the binding site of these cellular proteins to the 3′-X tail, two kinds of truncated RNAs corresponding to 3′X1′-50′ (nt 1 to 50) and 3′X51′-98′ (nt 51 to 98) of the 3′-X tail were prepared as described in Materials and Methods (Fig. 1). As shown in Fig. 2C, neither 87 nor 130 kDa proteins cross-linked to these truncated RNAs. Furthermore, when both the truncated RNAs were used as competitors for protein binding, these RNAs did not inhibit the complex formation by the labelled 3′X1′-98′ RNA and 87 and 130 kDa proteins (Fig. 2D). These results indicate that the two proteins specifically bind to the full-length 3′-X tail, i.e., the 3′X1′-98′ RNA, but not the 3′X1′-50′ and 3′X51′-98′ RNAs.

Although post-translational phosphorylation of protein is thought to be important for interaction with RNA (19) and binding activity of protein to RNA is abrogated by treatment with alkaline phosphatase (13), no change was observed in binding activity of these proteins to 3′X1′-98′ RNA after treatment with alkaline phosphatase (data not shown).

To make sure whether JHH-4 cells uniquely express such proteins or not, we first carried out UV cross-linking assay using five other human hepatic tumor cell lines (HuH-6, HuH-7, HLE, JHH-1, and JHH-6), a human cervical carcinoma cell line (HeLa), a HTLV-infected human T cell line (MT-2), and a human embryonic lung fibroblast strain (TIG-7). The reasons for using these cells are as follows: [1] major target of HCV is hepatocyte, [2] several kinds of proteins from HeLa cells bind to the 5′ UTR of HCV (20), and [3] MT-2 cells efficiently support HCV replication for a long period of time *in vitro* (21). All the cell lines tested expressed both 87 kDa and 130 kDa proteins, and the expression levels of these proteins did not greatly differ among these cell lines (Fig. 3A). In addition, We could also detect these two proteins in both HCV-positive and -negative hepatocellular carcinoma (HCC) tissues (Fig. 3B). There was no significant difference in the expression levels of these proteins between HCV-positive and -negative HCCs. Furthermore, these two proteins were also detected in fetal tissues (lung, heart, kidney, and colon) and in adult tissues (liver, stomach, and intestine) (Fig. 3C). The results indicate that the 3′-X tail binding proteins of 87 and 130 kDa are ubiquitously present in human tissues.

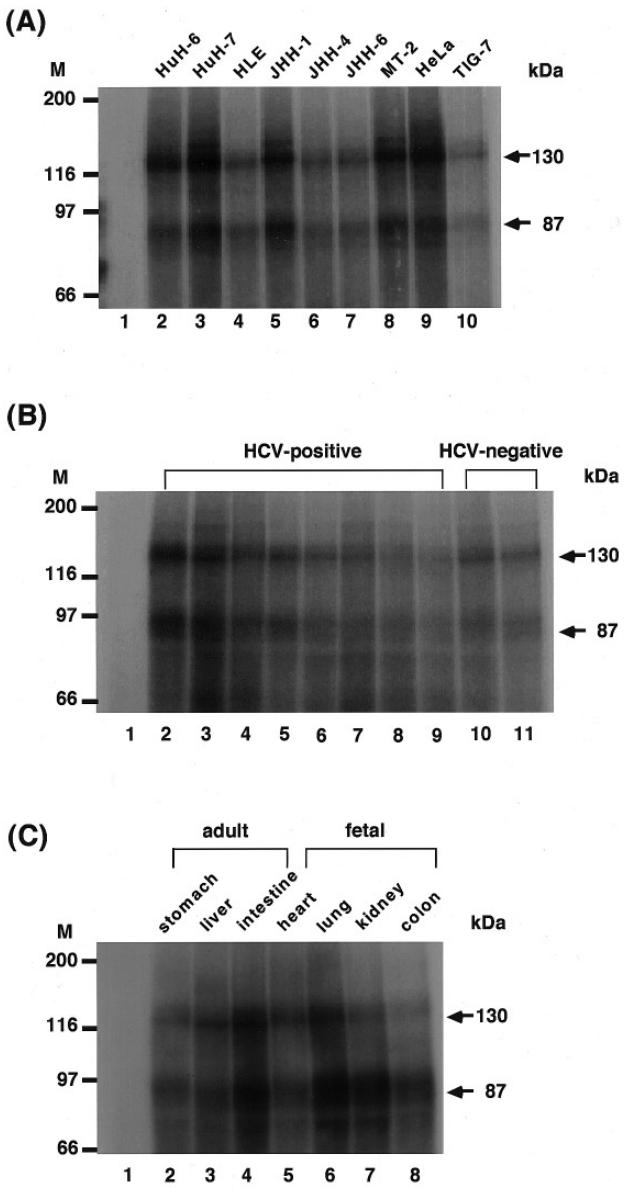

FIG. 3. Specific interaction of proteins from various human cell lines and tissues with the full-length 3′-X tail. (A) ^{32}P-labelled 3′X1′-98′ RNA was mixed without (lane 1) or with 10 μg of cytoplasmic extracts from HuH-6, HuH-7, HLE, JHH-1, JHH-4, JHH-6, MT-2, HeLa, and TIG-7 cells (lanes 2-10). (B) ^{32}P-labelled 3′X1′-98′ RNA was mixed without (lane 1) or with cytoplasmic extracts from HCV-infected HCCs (lanes 2-9) or HCV-uninfected HCCs (lanes 10-11). (C) ^{32}P-labelled 3′X1′-98′ RNA was mixed without (lane 1) or with cytoplasmic extracts from various normal human tissues, such as adult stomach, liver, and intestine (lanes 2-4) and fetal heart, lung, kidney, and colon (lanes 5-8). M, molecular mass standards (in kilodaltons).

DISCUSSION

In this study, by UV cross-linking assay we found two kinds of cellular proteins of 87 and 130 kDa which could specifically bind to the full-length 3′-X tail of HCV. These proteins are ubiquitously present in human tissues, such as the liver, lung, heart, kidney, stomach, intestine, and colon.

The 3′-X tail is highly conserved among different HCV strains and is thought to form a stable secondary structure (6-8). The genus flaviviruses includes Japanese encephalitis virus and yellow fever virus as well as HCV, of which the 3′ ends of genomic RNAs are similar each other and form a similar secondary structure (22, 23). The 3′ end of West Nile virus (another flavivirus) consisting of 83 nucleotides also forms the stable secondary structure, and three cellular proteins specifically bind to the 3′ terminal structure. One of these proteins is translation elongation factor-1α, EF-1α (24). The bacterial EF-1α homolog, EF-Tu, is a component of the bacteriophage Qβ replicase (9). These findings indicate that the stable secondary structures at the 3′ ends as well as the 3′-X tail of positive strand RNA viruses may play an important role for replication of negative strand RNA. Thus, the present 3′-X tail-binding cellular proteins of 87 and 130 kDa might be included in viral replication.

The HCV-encoded RdRp shows replication activity in the presence of non-viral RNA as well as HCV 3′ UTR (25). Thus, the action of RdRp is non-specific. Furthermore, it has been reported that *in vitro* transcribed HCV RNA without the 3′-X tail is infectious to HuH-7 human hepatic tumor cells (26). These studies suggested that the 3′-X tail had little relation with virus replication. Virus titers in the system were, however, low in comparison to those in patients. In addition, introduction of the full-length RNA containing 3′-X tail markedly increased the viral genome titer (27, 28). These findings indicate that the 3′-X tail may be necessary for efficient and specific viral replication.

Recently it has been shown that polypyrimidine tract-binding protein (PTB) in PH5CH cells has high affinity to 26 nt sequence containing 5′-19 nt of the 3′-X tail and 3′-7 nt of poly(U) stretch.(29). PTB has a molecular mass of 57 kDa and is ubiquitously present. However, we could not detect about 57 kDa protein by UV cross-linking assay under our experimental conditions. Since PTB is a nuclear and perinuclear protein (30), its concentration may be so low in cytoplasmic extracts in the present study that it can not be detected by UV cross-linking assay.

PTB also binds specifically to the 5′ UTR of HCV RNA and plays an important role in the internal initiation of translation (31). Interaction between the 5′ and 3′ UTRs is required for viral replication and encapsidation (32), but no cross-link between the 5′ UTR and 3′-X tail is detected (33). PTB may make the 5′ and 3′ UTRs interact with each other through binding to the both UTRs and can further make other cellular proteins associate with the complex. Therefore, binding of other cellular proteins, e.g., 87 and 130 kDa proteins might cause a specific signal of initiation of viral replication.

The present study demonstrates that two kinds of cellular proteins, 87 and 130 kDa, bind specifically to the 3′ terminal region of HCV. However, we are not yet certain whether these proteins consist of each single polypeptide and are actually involved in HCV replication. Further identification and biological functions of these proteins as well as development of construction of *in vitro* HCV specific replication system are very important to answer this question.

REFERENCES

1. Aach, R. D., Stevens, C. E., Hollinger, F. B., Mosley, J. W., Peterson, D. A., Taylor, P. E., Johnson, R. G., Barbosa, L. H., and Nemo, G. J. (1991) *N. Eng. J. Med.* **325,** 1325–1329.
2. Omata, M., Yokosuka, O., Takano, S., Kato, N., Hosoda, K., Imazeki, F., Tada, M., Ito, Y., and Ohto, M. (1991) *Lancet* **338,** 914–915.
3. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9524–9528.
4. Choo, Q.-L., Richmam, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2451–2455.
5. Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993) *J. Virol.* **67,** 1385–1395.
6. Tanaka, T., Kato, N., Cho, M.-J., and Shimotohno, K. (1995) *Biochem. Biophys. Res. Commun.* **215,** 744–749.
7. Tanaka, T., Kato, N., Cho, M.-J., Sugiyama, K., and Shimotohno, K. (1996) *J. Virol.* **70,** 3307–3312.
8. Kolykhalov, A. A., Feinstone, S. M., and Rice, C. M. (1996) *J. Virol.* **70,** 3363–3371.
9. Blumenthal, T., and Carmichael, G. G. (1979) *Ann. Rev. Biochem.* **48,** 525–548.
10. Hayes, R. J., and Buck, K. W. (1990) *Cell* **63,** 363–368.
11. Furuya, T., and Lai, M. M. C. (1993) *J. Virol.* **67,** 7215–7222.
12. Leopardi, R., Hukkanen, V., Vainionpää, R., and Salmi, A. A. (1993) *J. Virol.* **67,** 785–790.
13. Nakhasi, H. L., Rouault, T. A., Haile, D. J., Liu, T.-Y., and Klausner, R. D. (1990) *New Biol.* **2,** 255–264.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., pp. 1.42–1.46, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
16. Konarska, M. M., and Sharp, P. A. (1986) *Cell* **46,** 845–855.
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
18. Tsuboi, S., Miyazaki, M., Mihara, K., Fukaya, K., Teruya, K., Kosaka, T., Tsuji, T., and Namba, M. (1996) *J. Med. Virol.* **48,** 133–140.
19. Mohr, I. J., Stillman, B., and Gluzman, Y. (1987) *EMBO J.* **6,** 153–160.
20. Yen, J.-H., Chang, S. C., Hu, C.-R., Chu, S.-C., Lin, S.-S., Hsieh, Y.-S., and Chang, M.-F. (1995) *Virology* **208,** 723–732.
21. Kato, N., Nakazawa, T., Mizutani, T., and Shimotohno, K. (1995) *Biochem. Biophys. Res. Commun.* **206,** 863–869.
22. Takegami, T., Washizu, M., and Yasui, K. (1986) *Virology* **152,** 483–486.

23. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H. (1985) *Science* **229,** 726–733.
24. Blackwell, J. L., and Brinton, M. A. (1997) *J. Virol.* **71,** 6433–6444.
25. Behrens, S.-E., Tomei, L., and Francesco, R. De (1996) *EMBO J.* **15,** 12–22.
26. Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H.-S., Houghton, M., and Han, J. H. (1995) *J. Virol.* **69,** 32–38.
27. Yanagi, M., Purcell, R. H., Emerson, S. U., and Bukh, J. (1997) *Pro. Natl. Acad. Sci. USA* **94,** 8738–8743.
28. Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M., and Rice, C. M. (1997) *Science* **277,** 570–574.
29. Tsuchihara, K., Tanaka, T., Hijikata, M., Kuge, S., Toyada, H., Nomoto, A., Yamamoto, N., and Shimotohno, K. (1997) *J. Virol.* **71,** 6720–6726.
30. Ghetti, A., Piñol-Roma, S., Michael, W. M., Morandi, C., and Dreyfuss, G. (1992) *Nucleic Acids Res.* **20,** 3671–3678.
31. Ali, N., and Siddiqui, A. (1995) *J. Virol.* **69,** 6367–6375.
32. Pattnaik, A. K., Ball, L. A., Legrone, A., and Wertz, G. W. (1995) *Virology* **206,** 760–764.
33. Blight, K. J., and Rice, C. M. (1997) *J. Virol.* **71,** 7345–7352.