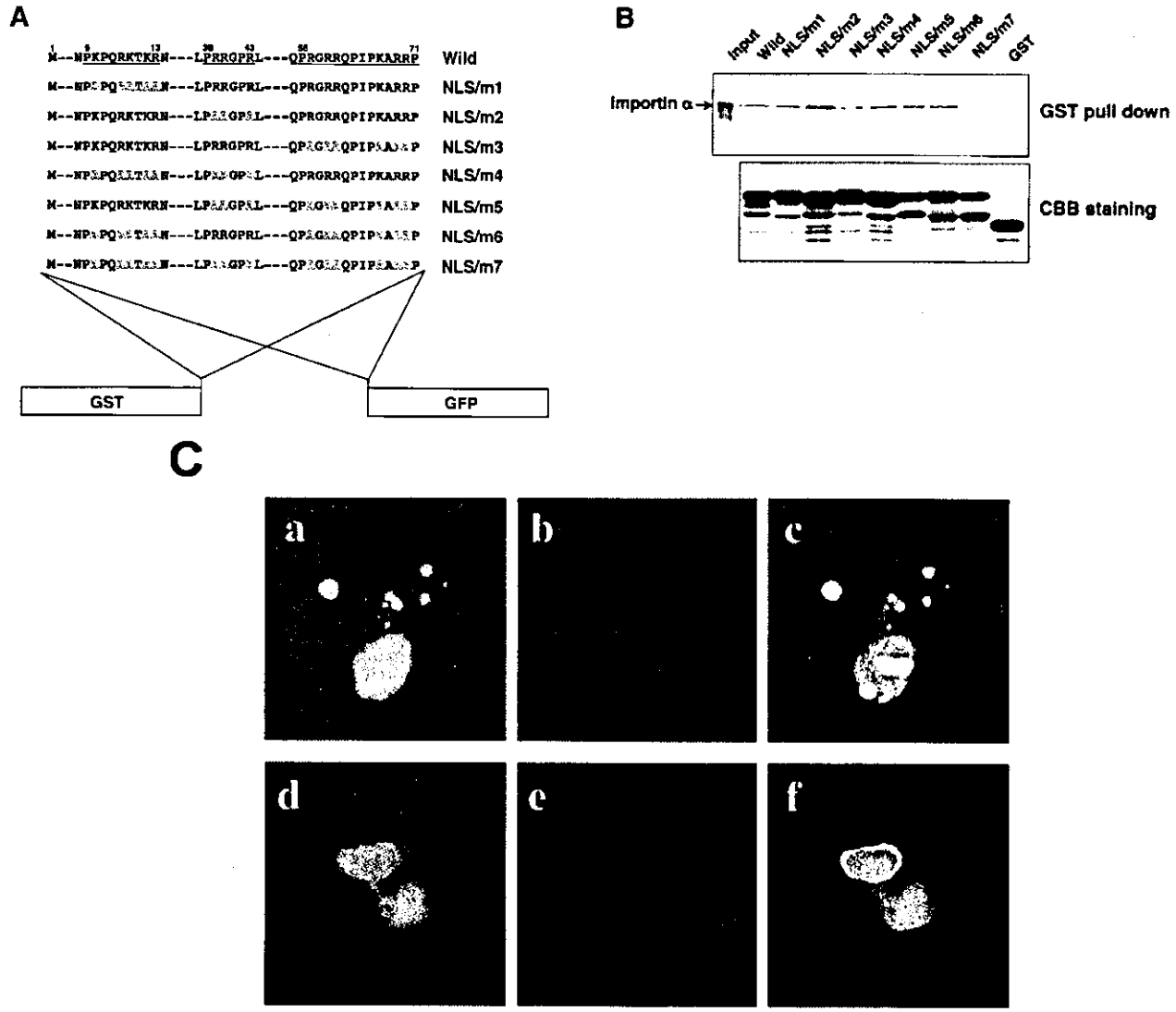

FIG. 7. Mutational analyses of NLS motifs in HCV core protein. (A) Schematic structures of fusion proteins and amino acid sequences corresponding to putative NLS motifs, three basic clusters (underlined) in the core protein. Two series of constructs fused with either GFP or GST were created. The mutated basic residues are indicated with outline letters. (B) GST pulldown assay. Equal amounts of GST fusions as described in A or GST alone was immobilized on glutathione-Sepharose 4B beads and incubated with in vitro-translated, [^{35}S]methionine-labeled importin-α. Bound material was separated by SDS-PAGE, and the amount of importin-α bound was detected by autoradiography. Direct electrophoretic separation of in vitro translation products served as a control (input). Coomassie brilliant blue staining of GST fusions and GST alone are shown in the bottom panel. (C) Confocal analysis of double staining for core-GFP fusion protein and HA-importin-α. 293T cells transfected with the wild-type core (1–71)-GFP (a to c) or NLS/m4 (d to f) expression plasmid and pCAG-HA-imp were allowed to express for 2 days. After the cells were fixed and permeabilized, they were incubated with a mouse anti-HA antibody. The red signals corresponding to HA-importin-α were obtained with a rhodamine-conjugated goat anti-mouse IgG secondary antibody (b and e). Overlay resulted in yellow signals indicative of colocalization (c and f). (D) Subcellular localization of GFP fusion proteins. GFP fusions with and without substitution mutations in the NLS motifs of the core protein as described in A were expressed in 293T cells. GFP images of the fixed cells were recorded.

tase (14), a predicted structure of an amphipathic α-helix present between amino acids 116 and 134 (Fig. 6B and C) possibly plays a role in directing the core protein to the mitochondrial outer membrane. Sequence comparisons demonstrate conservation of the amino acid sequence and secondary structure of the region, amino acids 112 to 152, among a variety of HCV isolates, including the infectious H77c clone (55), as well as a full-length adaptive replicon (3). To gain insight into the significance of the secondary structure of the region in targeting to the mitochondria, further structural and biochemical analyses are needed.

The association of HCV core protein with the mitochondrial membrane suggests that the core protein has the ability to modulate mitochondrial function, possibly by altering the permeability of the mitochondrial membrane. The core protein induces the production of cellular reactive oxygen species in

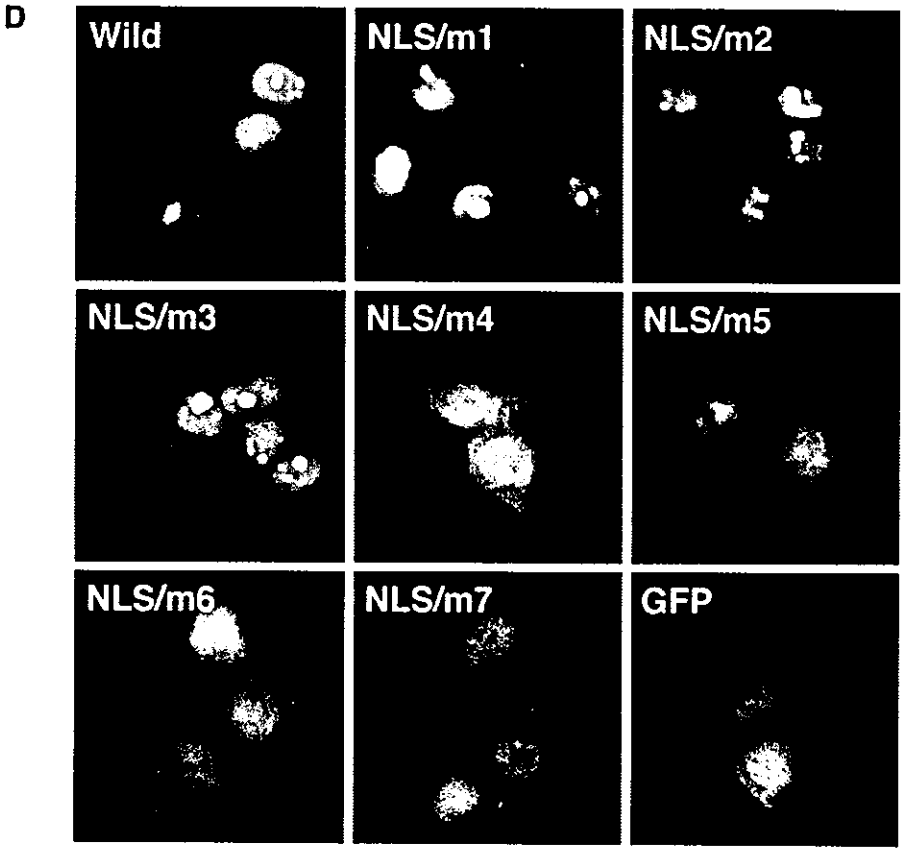

FIG. 7—*Continued.*

the livers of core-transgenic mice and in core-expressing cell lines (35). Reactive oxygen species, predominantly generated in mitochondria, induce genetic mutations and act as secondary messengers to regulate a variety of cellular functions, including gene expression and proliferation (1). Although the molecular mechanism by which core protein induces reactive oxygen species production is still unclear, HCV core protein is known to impair the mitochondrial electron transfer system (35). The core protein may also modulate apoptosis, since mitochondria play a major role in regulating programmed cell death. Expression of HCV proteins, including the core protein, suppresses the release of cytochrome *c* from mitochondria to the cytoplasm in HCV-transgenic mice, thus inhibiting Fas-mediated apoptosis (27).

Okamoto et al. recently reported that not only the C-terminal signal sequence but also amino acids 128 to 151 are required for ER retention of the core protein by using a series of N-terminally truncated core protein constructs (38). Here, in this study, we further showed that amino acids 112 to 152 mediate association of the core protein with the ER in the absence of the C-terminal signal sequence. Hope and McLauchlan demonstrated that the central domain of the core protein, amino acids 119 to 174, is important for association with lipid droplets (17). They also showed that this corresponding domain is shared with GB virus B, which is most closely related to HCV, but not with either pestiviruses or flaviviruses (18). It appears that the 41 residues identified as the sequence mediating association with the ER membrane in the present study are crucial for directing the core protein to lipid droplets, since the surface of lipid droplets must derive from the cytoplasmic side of the ER membrane.

The HCV core protein contains NLS sequences which are composed of three stretches of sequences rich in basic residues. These sequences were originally identified by experiments with fused forms of wild-type and mutated core proteins with β-galactosidase (6, 48). C-terminally truncated versions of the core protein localize exclusively to the nucleus (48). A fraction of the core protein is detected in the nucleus even when full-length HCV core gene is expressed (Fig. 2) and as described (34, 56). However, it is difficult to demonstrate clearly the nuclear localization of the core protein by immunofluorescence, presumably because of the instability of nuclearly localized core protein (49, 33). We only observed a nuclear staining pattern of the matured core protein after adding proteasome inhibitors to the culture (33).

Generally, NLS sequences fall into two categories; (i) monopartite NLSs, which contain a single cluster of basic residues, and (ii) bipartite NLSs, which contain two clusters of basic residues separated by an unconserved linker sequence of variable length (reviewed in reference 12). Nuclear translocation of an NLS-containing cargo protein is initiated when the soluble import receptor (importin) recognizes the NLS-contain-

ing protein within the cytoplasm. Importin-α contains an NLS-binding site(s), and importin-β docks importin-cargo complexes to the cytoplasmic filaments of a nuclear pore complex, after which translocation occurs through the nuclear pore. Thus, importin-α functions as an adaptor between the bona fide import receptor and the NLS-containing protein.

We further characterized the NLS of the core protein and found that each of the NLS motifs of the core protein is able to bind to importin-α and that at least two NLS motifs are required for efficient nuclear distribution of the core protein in cells. It appears that double mutations among three NLS motifs decrease the ability of the core protein to bind importin-α. These observations suggest that the binding of the double mutants with importin-α leads to no or little active translocation of the core protein into the nucleus. The double mutations may also block subsequent interactions with importin-β1, GTPase Ran, and/or NTF2/p10, which are required for translocation through the nuclear pore complexes.

The findings obtained in this study suggest that HCV core protein NLS motifs have a bipartite function. Crystallographic studies of monopartite (e.g., simian virus 40 large T antigen) and bipartite (e.g., nucleoplasmin) NLSs show that the basic residue clusters of bipartite NLSs occupy separate binding sites on importin-α. In contrast, while monopartite NLSs can bind to the same sites as bipartate NLSs on importin-α, they mainly bind to the N-terminal binding site, which is referred to as the major binding site on importin-α (9, 11). A recent report describes an importin-α variant with a mutation in the major site which results in decreased ability to bind both monopartite and bipartite NLSs. Another variant with a mutation in the minor site exhibits decreased binding only to bipartite NLS-containing proteins, making importin-α nonfunctional in vivo (22). Thus, we favor a model in which the core protein bipartite NLS, composed of any two of the three basic clusters, occupies both major and minor binding sites on importin-α, resulting in efficient nuclear translocation. Importin-α may be equally accessible to all clusters, given their close proximity to one another, as well as the distinct conformational flexibility of the ≈70-residue N-terminal region of the core protein.

With regard to the molecular mechanisms participating in nuclear localization of the core protein, Moriishi et al. found that PA28γ is involved in nuclear localization of the core protein. Interaction of the core protein with PA28γ plays an important role in retention of the core protein in the nucleus (33). Furthermore, in yeast cells, nuclear transport of the core protein requires the activity of the small GTPase Ran/Gsp1p and is mediated by Kap123p, but neither importin-α nor importin-β is involved (20). Differences in nucleocytoplasmic transport between yeast and mammalian cells might explain the inconsistencies observed in the present study. Further experiments are required to characterize the exact nature of the interaction between the core protein and components of the nuclear import machinery, particularly in cells where HCV is replicating.

In conclusion, the mature HCV core protein has an internal 41-amino-acid sequence mediating association of the viral protein with the ER and mitochondria. We also provide evidence for a novel class of bipartite NLS contained within the core protein, which comprises two of three basic motifs, thus enabling efficient nuclear targeting. Multiple functional domains influence the subcellular localization of the core protein, which ultimately depends on the balance of the respective signals.

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Identification of Basal Promoter and Enhancer Elements in an Untranslated Region of the TT Virus Genome

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The regulation of TT virus (TTV) gene expression was characterized. Transient-transfection assays using reporter constructs revealed that a 113-nucleotide (nt) sequence within the untranslated region, proximal to the transcription initiation site and containing a TATA box motif, has a basal promoter activity. This sequence is well conserved among different TTV genotypes. Upstream stimulating factor bound to a consensus binding motif within this region and positively regulates TTV transcription. Furthermore, a 488-nt region upstream of the basal promoter exhibited enhancer activity, presumably in a cell type-specific manner. This study illustrates some of the mechanisms involved in the transcriptional regulation of TTV.

TT virus (TTV), which was discovered in a patient with acute hepatitis, is an unenveloped, single-stranded, circular DNA virus, with a genome of approximately 3.8 kb (6). TTV is thought to be a new member of the *Circoviridae* family of viruses, and it was recently proposed that the virus be named Torque Teno virus (6). The TTV genome includes an untranslated region (UTR) of approximately 1.2 kb and a coding region of approximately 2.6 kb, including two major open reading frames which are sandwiched by the TATA box and polyadenylation signal motifs (11, 13, 15). Analyses of TTV transcripts have revealed three spliced mRNA species of 3.0, 1.2, and 1.0 kb with common 5′ and 3′ termini (9, 14). However, the molecular mechanisms controlling TTV transcription are still unknown. In this study, the basal promoter and enhancer of a TTV isolate, SANBAN of genogroup 3 (5, 18), were identified and functionally characterized.

First, we determined the transcription initiation sites of the TTV genome by 5′ rapid amplification of cDNA end (5′-RACE) analysis (Marathon cDNA amplification kit; Clontech) using poly(A)-rich RNA from a human hepatocellular carcinoma cell line, HepG2, transfected with a cloned TTV genome. The 5′-RACE PCR products were cloned and sequenced. We observed two potential transcription initiation sites, which map at nucleotides (nt) 121 and 110 (numbered according to the sequence deposited in DDBJ/GenBank/EMBL databases under accession number AB025946). Although transcription may be initiated at both sites, the upper site was designated position +1 in this study.

The UTR of the TTV genome contains a TATA box element between positions −40 and −35, as well as a number of putative transcription factor-binding motifs (Fig. 1A). Despite considerable genetic diversity throughout the whole genome, the UTR sequence was relatively conserved among the different TTV genotypes, presumably reflecting its functional constraints (15, 16). Thus, we analyzed transcriptional regulation of the UTR sequence.

To characterize TTV promoter activity, a firefly luciferase reporter plasmid, p(−890/+115), was constructed by subcloning the TTV sequence from positions −890 to +115, which was amplified by PCR using appropriate primers with restriction sites at the 5′ ends, into the promoterless pGL3-Basic (Promega). Eleven different cell lines were transfected with p(−890/+115), along with a *Renilla* luciferase expression vector, pRL-TK, as an internal standard for determining transfection efficiency. Luciferase activities in cell lysates prepared after 16 h of transfection were determined (2). It is of interest that the 1.0-kb fragment demonstrated a pronounced promoter activity in all the hepatocellular carcinoma cell lines tested. Human (Huh7, HepG2, and FLC4 [1, 2]) and mouse (Hepa1-clc7) hepatocellular carcinoma cells were tested (Fig. 1B). This fragment demonstrated the greatest activity in Huh7 cells (~10-fold greater than in other cells). We observed substantial promoter activity in GL37 (African green monkey kidney) and CHO (Chinese hamster ovary) cells, whereas limited activity was observed in Caco2 (human colon carcinoma), MOLT4 (human T-cell leukemia), CV1 (African green monkey kidney fibroblast), 3T3 Swiss (mouse fibroblast), and CMT93 (mouse rectal carcinoma) cells. These results indicate that the UTR of the TTV genome functions as a promoter in a cell type-specific manner.

To assess basal, proximal promoter activity in the UTR, a series of 5′ deletions fused to the luciferase gene were constructed and transfected into Huh7 and HepG2 cells (Fig. 2). A deletion extending to nt −601 [p(−601/+115)] enhanced promoter activity in both cell lines (by ~1.5-fold), while deletion of another 274 nt [p(−327/+115)] decreased promoter activity by more than 80%, suggesting that there is a negative regula-

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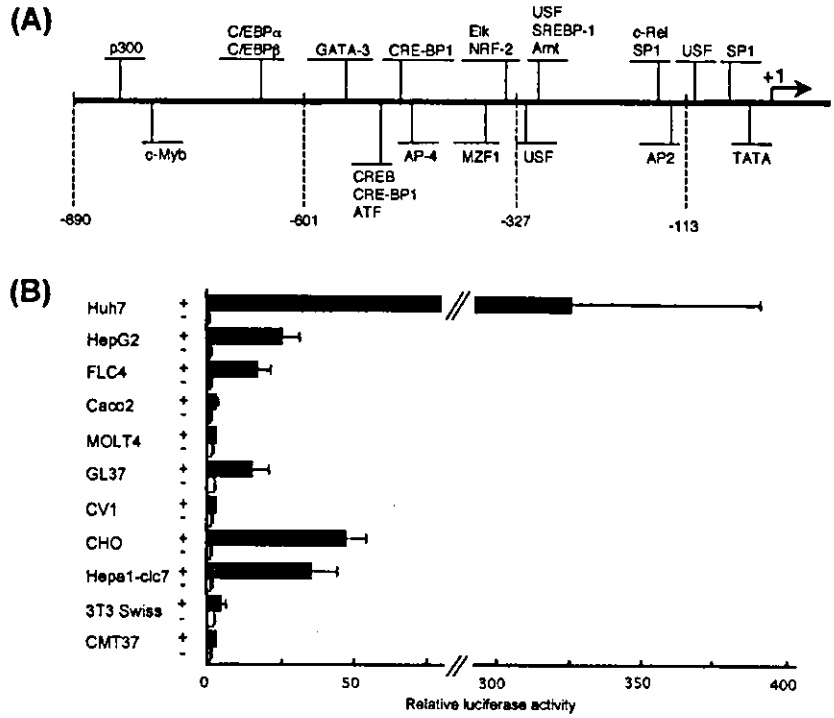

FIG. 1. Functional analysis of the TTV promoter in the viral UTR. (A) Schematic representation of the 1.0-kb UTR sequence. The TATA box element and putative transcription factor-binding sites are shown. Transcription factor-binding sites were identified using the TRASFAC database and a search program (http://motif.genome.ad.jp/). The transcription initiation site (+1) is indicated and corresponds to nt 121 (AB025946). The numbers at the bottom of vertical dotted lines indicate the start points of the full-length promoter construct and deletion mutants of the promoter constructs used in Fig. 1B and 2. (B) Cell type specificity of the TTV promoter activities. Cells were transfected with p(−890/+115) (+) or promoter-less pGL3-Basic (−) together with pRL-TK (*Renilla* luciferase). Cell extracts were prepared 16 h after transfection, and luciferase activities in the extracts were determined using a dual-luciferase reporter assay system (Promega) with the Lumat LB9501 luminometer (Berthold). All values were normalized to *Renilla* luciferase activities and are shown as means ± standard deviations (error bars) of three independent samples.

tory element between nt −890 and −601. A deletion extending to nt −113 [p(−113/+115)] resulted in a slight to moderate reduction in activity, but promoter activity still remained greater than that observed with p(+15/+113), in which the TATA box and the transcription start sites were deleted. These findings suggest that the 113 nt immediately upstream of the transcription initiation site contains a basal promoter region critical for TTV gene expression.

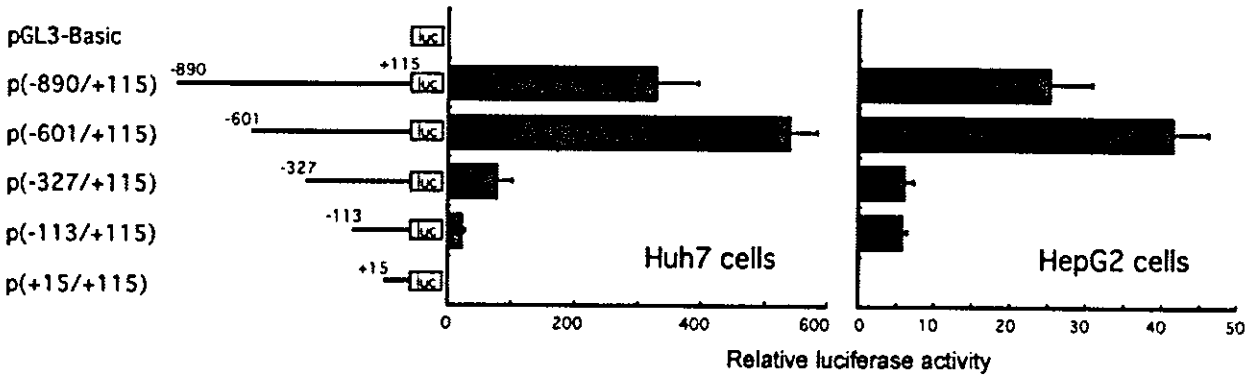

FIG. 2. Deletion analysis of the TTV promoter in Huh7 and HepG2 cells. The structures of the luciferase reporter constructs containing various lengths of the TTV UTR sequence are shown to the left. A series of DNA fragments with 5′ deletions of the TTV promoter were amplified by PCR using the full-length TTV DNA of SANBAN isolate as a template with the same reverse primer and various forward primers. The fragments were cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples.

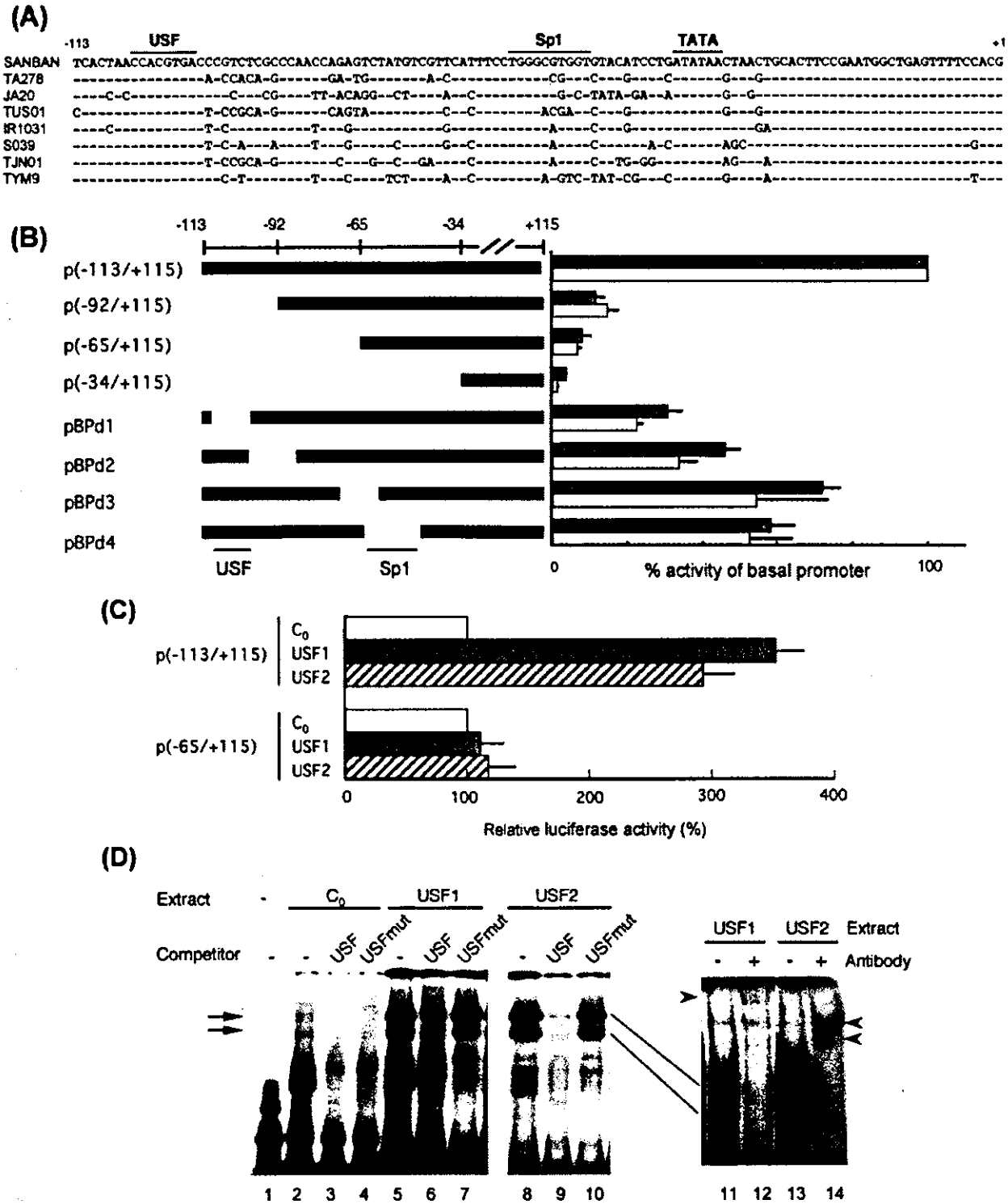

FIG. 3. Basal promoter activity of TTV regulated by USF. (A) Alignment of the putative basal promoter regions from TTV isolates SANBAN (DDBJ/GenBank/EMBL accession number AB025946), TA278 (AB017911), JA20 (AF122914), TUS01 (AB017613), IR1031 (AB038619), S039 (AB038620), TJN01 (AB028668), and TYM9 (AB050448). The transcription initiation site is numbered +1. The TATA box and positions of putative binding sites for USF and SP1 are indicated. Nucleotides that are identical to those in the SANBAN isolate (−) are indicated. (B) Effect of deleting DNA from the basal promoter region on the TTV basal promoter activity. A series of DNA fragments with 5′ or internal deletions of the basal promoter region were amplified by PCR and cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells (gray bars) or HepG2 cells (white bars). Relative luciferase activity in each transfectant was determined as described in the legend

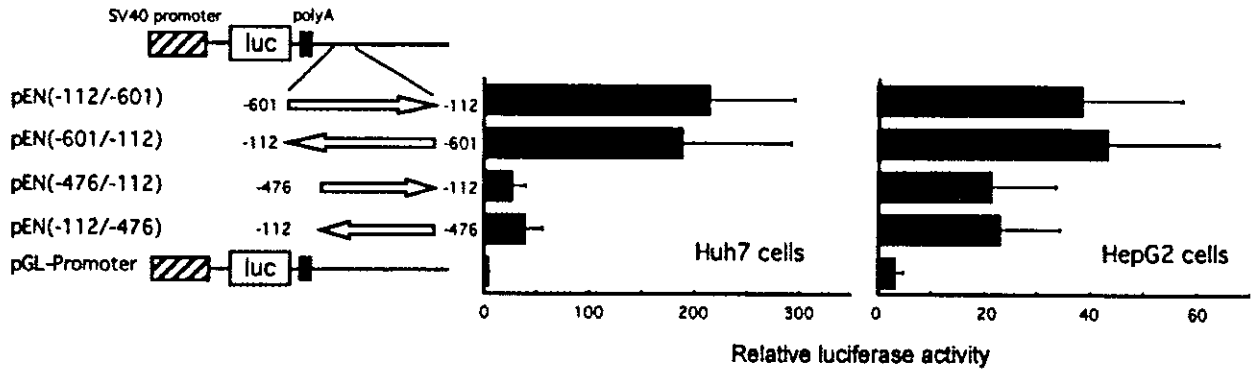

FIG. 4. Enhancer activity of the 488-nt fragment (−601/−114) of the TTV UTR. The 488-nt (−614/−114) and 363-nt (−476/−114) fragments were PCR amplified using primers with 5′ overhangs containing BglII (sense) and BamHI (antisense) sites. The fragments were then cloned into pGL3-Promoter at the BamHI site. The indicated constructs (left panel) were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples. SV40, simian virus 40.

Computer-assisted analysis of this basal promoter region identified potential binding sites recognized by upstream stimulating factor (USF) and Sp1, which are conserved among TTV genotypes (Fig. 3A). To determine whether these sequences contribute to TTV promoter activity, 5′ or internal deletion mutations were introduced into p(−113/+115), which was then examined for dual luciferase activity. Deletions, those found in p(−92/+115) and pBPd1, reduced promoter activity by 70 to 85%, suggesting that the USF-binding sequence is crucial for TTV promoter activity (Fig. 3B). Deletion of a Sp1-binding sequence (pBPd4) also conferred a decrease in promoter activity, although their effects were relatively moderate, suggesting that the Sp1-binding motif and/or its encompassing sequence may play a role in regulating TTV promoter activity by maintaining the structural integrity of the transcriptional machinery.

In genes where USF regulates transcription, cotransfection of USF expression vectors with reporter genes stimulates reporter activities. To further investigate the effect of USF on TTV promoter activity, we cotransfected USF1 or USF2 expression vectors (pCMV-USF1 and pCMV-USF2) (7) with p(−113/+115) into HepG2 cells. The cotransfection significantly increased promoter activity (by threefold), suggesting that USF proteins regulate TTV transcription (Fig. 3C).

USF is a family of basic-helix-loop-helix-leucine zipper transcription factors, initially identified by their ability to bind to the 5′-CACGTG-3′ sequence within the adenovirus major late promoter (3, 4, 10). USF1 and USF2 have been subsequently shown to bind to the promoters of various cellular and viral genes. To determine whether the TTV basal promoter was capable of USF binding, gel mobility shift assays were performed on an end-labeled oligonucleotide (nt −113 to −84) containing the putative USF-binding motif (Fig. 3D). DNA-protein-binding complexes were observed in nuclear extracts from cells transfected with pCMV-USF1 (Fig. 3D, lane 5), pCMV-USF2 (lane 8), and the empty vector (lane 2). An excess of unlabeled homologous probe competed with the protein binding (lanes 3, 6, and 10), whereas a mutated USF sequence failed to compete (lanes 4, 7, and 10). The addition of anti-USF antibodies to the binding reaction mixture supershifted the DNA-protein complexes (lanes 12 and 14). The combined data demonstrate that USF binds to its binding motif within the TTV basal promoter to up-regulate viral transcription.

On the basis of the results of the luciferase assays using 5′ deletions of the TTV UTR (Fig. 2), the positive regulatory element appears to be located immediately upstream of the basal promoter. To ascertain whether the 488-bp fragment between nt −601 and −113 functions as the enhancer region, this fragment or a 5′ deletion of this fragment was placed downstream of the polyadenylation signal in pGL3-Promoter (Promega), driven by the simian virus 40 promoter, in either

to Fig. 1B. Results are shown as a percentage of the activity in cells transfected with p(−113/+115); values are shown as means ± standard deviations (error bars) (n = 3 per group). (C) Effects of USF overexpression on basal promoter activity. HepG2 cells were cotransfected with each reporter construct with pCMV-USF1 (USF1), pCMV-USF2 (USF2), or empty pC_0 vector (C_0). Luciferase activity was determined 48 h after transfection. For each reporter construct, relative luciferase activity is presented as a percentage of the activity in pC_0-transfected cells. (D) Binding of USF proteins to the region from nt −113 to −84 in the TTV basal promoter. The electrophoretic mobility shift assays were performed as described previously (19). A double-stranded oligonucleotide corresponding to the TTV sequence from nt −113 to −84 was used as a probe. Nuclear extracts from the cells transiently transfected with pCMV-USF1 (USF1; lanes 5, 6, 7, 11, and 12), pCMV-USF2 (USF2; lanes 8, 9, 10, 13, and 14), or pC_0 (C_0; lanes 2 to 4) or no extract (lane 1) were mixed with ^{32}P-labeled probe for the binding reaction mixtures. Competitors, unlabeled probe (USF), and a mutant with the USF-binding motif (USFmut) were added at a 25-fold molar excess. The sense sequence (5′-TCACTAAC CAATTGACCCGTCTCGCCCAAC [the mutated nucleotides are underlined]) and complementary sequence of the mutant with the USF-binding motif were added. A supershift experiment was also performed by incubating antibody against USF1 (lane 12) or USF2 (lane 14) (+) with the nuclear extracts before the probe was added. The positions of specific binding complexes (arrows) and supershifted complexes (arrowheads) are indicated.

the sense or antisense orientation. Luciferase activity of constructs containing the 488-bp fragment [pEN(−601/−114) and pEN(−114/−601)] led to 50- and 10-fold stimulation in Huh7 and HepG2 cells, respectively. The 5′ deletion extending to nt −476 [pEN(−476/−114) and pEN(−114/−476)] reduced enhancer activity (Fig. 4). No enhancement was observed by transfection of MOLT4 cells with pEN(−601/−114) and pEN(−114/−601) (data not shown). These results demonstrate that the 488-bp region upstream of the basal promoter contains an enhancer element, suggesting cell-specific transcription of the TTV genome. It is noteworthy that the enhancer element is conserved among TTV genotypes. For example, 72% homology has been observed between clones SANBAN and TA278, and the database search has revealed at least 20 potential transcription factor-binding sites within this element, including CREB and CRB, which are activated upon cyclic AMP signaling-dependent phosphorylation (12, 17).

While the manuscript was being prepared, Kamada et al. reported the promoter and enhancer activities in the UTR of TTV, clone VT416 whose genome is 98% similar to that of TA278, and its cell tropism (8). However, they did not identify transcription factors that bind to the region and regulate TTV transcription. In summary, the findings reported by Kamada et al. and the findings of our present study emphasize the important role of the UTR as a basal promoter and enhancer within the UTR. Other areas of interest for further study include the identification of additional factors involved in tissue-specific TTV transcription and determining the significance of polymorphism of the regulatory elements.

We are grateful to M. Sawadogo (University of Texas M. D. Anderson Cancer Center, Houston, Tex.) for providing pCMV-USF1, pCMV-USF2, and pC_0. We thank M. Yahata and S. Yoshizaki for technical assistance and T. Mizoguchi for preparation of the manuscript.

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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Hepatitis C Virus Infection and Diabetes: Direct Involvement of the Virus in the Development of Insulin Resistance

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See editorial on page 917.

Background & Aims: Epidemiological studies have suggested a linkage between type 2 diabetes and chronic hepatitis C virus (HCV) infection. However, the presence of additional factors such as obesity, aging, or cirrhosis prevents the establishment of a definite relationship between these 2 conditions. *Methods:* A mouse model transgenic for the HCV core gene was used. *Results:* In the glucose tolerance test, plasma glucose levels were higher at all time points including in the fasting state in the core gene transgenic mice than in control mice, although the difference was not statistically significant. In contrast, the transgenic mice exhibited a marked insulin resistance as revealed by the insulin tolerance test, as well as significantly higher basal serum insulin levels. Feeding with a high-fat diet led to the development of overt diabetes in the transgenic mice but not in control mice. A high level of tumor necrosis factor-α, which has been also observed in human chronic hepatitis C patients, was considered to be one of the bases of insulin resistance in the transgenic mice, which acts by disturbing tyrosine phosphorylation of insulin receptor substrate-1. Moreover, administration of an anti-tumor necrosis factor-α antibody restored insulin sensitivity. *Conclusions:* The ability of insulin to lower the plasma glucose level in the HCV transgenic mice was impaired, as observed in chronic hepatitis C patients. These results provide a direct experimental evidence for the contribution of HCV in the development of insulin resistance in human HCV infection, which finally leads to the development of type 2 diabetes.

Approximately 200 million people are chronically infected with hepatitis C virus (HCV) in the world. Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma, thereby being a worldwide problem both in medical and socioeconomical aspects.[1,2] In addition, chronic HCV infection is a multifaceted disease, which is associated with numerous clinical manifestations, such as essential mixed cryoglobulinemia, porphyria cutanea tarda, and membranoproliferative glomerulonephritis.[3] Recent epidemiological studies have added another clinical condition, type 2 diabetes, to a spectrum of HCV-associated diseases.[4–7] However, the establishment of a definite causative relationship between HCV infection and diabetes is hampered by the presence of other factors such as obesity, aging, or liver injury in patients with chronic HCV infection.

Type 2 diabetes is a complex, multisystem disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin, all of which contribute to the development of overt hyperglycemia.[8,9] Although the precise mechanisms whereby these factors interact to produce glucose intolerance and diabetes are uncertain, it has been suggested that the final common pathway responsible for the development of type 2 diabetes is the failure of the pancreatic β-cells to compensate for the insulin resistance. Hyperinsulinemia in the fasting state is observed relatively early in type 2 diabetes, but it is considered to be a secondary response that compensates for the insulin resistance.[8,9] Overt diabetes occurs over time when pancreatic β-cells bearing the burden of increased insulin secretion fail to compensate for the insulin resistance.

In this study, to elucidate the role of HCV in a possible association between diabetes and HCV infection, transgenic mice that carry the core gene of HCV[10,11] were analyzed. We found that these mice developed insulin resistance. An addition of a high-calorie diet led to the development of type 2 diabetes by dis-

Abbreviations used in this paper: EDL, extensor digitorum longus; ELISA, enzyme-linked immunosorbent assay; FPG, fasting plasma glucose; HCV, hepatitis C virus; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α.

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rupting the balance between insulin resistance and secretion.

Materials and Methods

Transgenic Mice

The production of HCV core gene transgenic mice has been described previously.[11] Briefly, the core gene from HCV of genotype 1b, which is placed downstream of a transcriptional regulatory region from the hepatitis B virus, was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). The mice were cared for according to institutional guidelines, fed an ordinary chow diet (Funabashi Farms, Funabashi, Japan), and maintained in a specific pathogen-free state. At an indicated time, the mice were fed a high-fat diet (Oriental Yeast Co., Ltd., Tokyo, Japan) for up to 2 months. Caloric content of food was 4.70 kcal/g for high-fat diet and 3.56 kcal/g for ordinary diet. The high-fat diet contains 18.5% protein, 22.1% fat (4.7% vegetable fat and 17.4% animal fat), 5.4% ash, 2.5% fiber, 6.5% moisture, and 45.0% carbohydrate, and the ordinary diet contains 22.4% protein, 5.7% fat, 6.6% ash, 3.1% fiber, 7.7% moisture, and 54.5% carbohydrate. Because there is a sex preference in the development of liver lesion in the transgenic mice, we used only male mice that were heterozygously transgenic for the core gene, and as controls we used nontransgenic litter mates of the transgenic mice. Transgenic mice carrying the HCV envelope genes under the same regulatory region as that in the core gene transgenic mice were also used as controls.[12] At least 5 mice were used in each experiment and the data were subjected to statistical analysis.

Glucose Tolerance Test

The mice were fasted for >16 hours before the study. D-Glucose (1g/kg body weight) was administered by intraperitoneally (IP) injection to conscious mice. Blood was drawn at different time points from the orbital sinus, and plasma glucose concentrations were measured by using an automatic biochemical analyzer DRI-CHEM 3000V (Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (BIOTRAK; Amersham Pharmacia Biotech, Piscataway, NJ) with rat insulin as a standard.

Insulin Tolerance Test

The mice were fed freely and then fasted during the study period. Human insulin (1 U/kg body weight) (Humulin; Novo Nordisk, Denmark) was administered by IP injection to fasted conscious mice, and glucose concentrations were determined at the time points indicated. Values were normalized to the baseline glucose concentration at the administration of insulin.

Morphometric Analysis

Sections of the pancreas were prepared and evaluated for morphometry after H&E staining or immunostaining. Relative islet area and islet number were determined with an image analyzer (QUE-2; Olympus Optical Co., Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

ELISA for mouse tumor necrosis factor (TNF)-α was performed using a commercially available mouse TNF-α ELISA kit (BioSource International, Camarillo, CA). Samples were prepared as reported previously.[13] Briefly, the liver of transgenic and control mice were lysed with a buffer containing 1% Tween 80, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 0.05% sodium azide, 2 mmol/L PMSF, and the Protease Inhibitor Cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN) and homogenized on ice for 20 seconds. The homogenates were centrifuged at 11,000 × g for 10 minutes at 4° C, and the supernatants were collected and assayed. ELISA was performed in triplicate for each sample. The concentrations of the cytokines in the liver were normalized by determining the amount of total protein in each sample using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Immunoprecipitation and Western Blotting

For immunoprecipitation studies, liver tissues were homogenized in lysis buffer (10 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 1.0 mmol/L β-glycerophosphate, 1.0 mmol/L sodium orthovanadate [Na_3VO_4], 50 mmol/L sodium fluoride [NaF], the Protease Inhibitor Cocktail [Complete, Roche Molecular Biochemicals], and 1.0% Triton X-100), and homogenates were precipitated with an anti–insulin receptor substrate (IRS)-1 or anti–IRS-2 rabbit polyclonal antibody (UBI, Lake Placid, NY) and then with Sepharose 4B beads (Amersham Biosciences). Resulting pellets were washed 3 times and then subjected to Western blotting. Pellets were resuspended in Western sample buffer (5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCl, 1 mmol/L EDTA, 10% glycerol), and then subjected to 2%–15% gradient sodium dodecyl sulfate/PAGE (PAG Mini "DAIICHI" 2/15 (13W), Daiichi Diagnostics, Tokyo, Japan), and electrotransferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The filter was then reacted with antiphosphorylated tyrosine (Santa Cruz Biotechnology Inc., Santa Cruz, CA), antiphosphorylated serine (Cell Signaling Technology, Inc., Beverly, MA), anti–IRS-1 or anti–IRS-2 mouse monoclonal antibody (BD Biosciences, Lexington, KY), followed by immunostaining with secondary biotinylated IgG (Vector Labs, Inc., Burlingame, CA) and visualization using an ECL kit (Amersham Intl., Buckinghamshire, UK).[14]

Hyperinsulinemic-Euglycemic Clamp

Mice underwent a hyperinsulinemic-euglycemic clamp using D-[3-^{3}H]glucose (NEN Life Science, Boston, MA) to measure the rate of glucose appearance and hepatic glucose production (HGP) as described previously.[15] Three days after jugular catheter placement, a hyperinsulinemic-euglycemic clamp was conducted with a continuous infusion of human

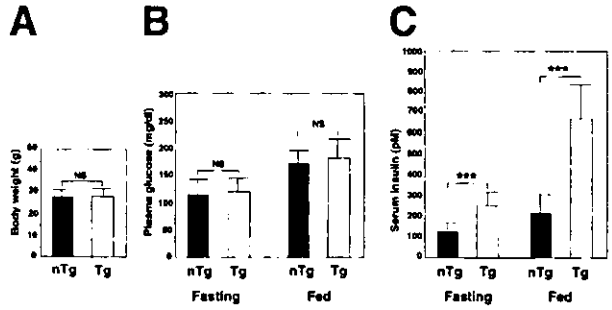

Figure 1. Altered glucose homeostasis in hepatitis C virus core gene transgenic mice. (*A*) Body weight of 2-month-old mice (n = 10 in each group). (*B*) Plasma glucose levels in fasting or fed mice (n = 10 in each group). (*C*) Serum insulin levels in fasting or fed mice (n = 10 in each group). The insulin level was significantly higher in the core gene transgenic mice than in control mice. Values are mean ± standard error; ***$P < 0.001$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

insulin to raise serum insulin within a physiological range. Blood samples were drawn at intervals for the immediate measurement of blood glucose concentration, and 20% glucose was infused at variable rates to maintain blood glucose at ca. 125 mg/dL. All infusions were done using microdialysis pumps (KD Scientific Inc., Boston, MA). The rate of glucose appearance (mg/kg per minute), which equals the rate of total body glucose utilization during steady state, was calculated as the ratio of the rate of infusion of [3-^{3}H]glucose and the steady state plasma [^{3}H-]glucose specific activity. HGP (mg/kg/min) during clamps was determined by subtracting the glucose infusion rate from the rate of glucose appearance.

Glucose Uptake by Skeletal Muscle

The extensor digitorum longus (EDL) or soleus muscle was excised from 2-month-old mice and exposed to insulin at the indicated concentrations. 2-Deoxyglucose uptake was determined as described previously.[16]

Treatment With Anti-TNF-α Antibody

To suppress TNF-α, a dose of 200 μg/mouse of neutralizing hamster monoclonal antibody (TN3-19.12, Santa Cruz Biotechnology Inc.) was administered by IP injection on days 1 and 4, and plasma glucose and insulin levels were determined at day 5.[17]

Statistical Analysis

The results are expressed as means ± standard error. The significance of the difference in means was determined by Student *t* test or Mann–Whitney *U* test whenever appropriate. $P < 0.05$ was considered significant.

Results

Hyperinsulinemia and Insulin Resistance in Transgenic Mice

At the age between 1 and 12 months, there was no significant difference in body weight between the core gene transgenic mice and control mice. Figure 1*A* shows body weight of 2-month-old mice. Fasting plasma glucose (FPG) levels were slightly elevated in the core gene transgenic mice compared with control mice, but the difference was not significant ($P = 0.79$, Figure 1*B*). In contrast, there was a marked increase in the level of serum insulin in the core gene transgenic mice than control mice ($P < 0.001$, Figure 1*C*). Hyperinsulinemia was observed in the core gene transgenic mice as early as 1 month old. These findings suggest that decreased responsiveness to the hormone may have resulted in compensatory hyperinsulinemia. Administration of glucose to 2-month-old core gene transgenic mice revealed mild glucose intolerance compared with control mice of the same age, but the difference was not statistically significant at any time points measured (Figure 2*A*). HCV envelope gene transgenic mice of the same age, in which the envelope genes were expressed under the same transcriptional regulatory region as the core gene transgenic mice, did not manifest hyperinsulinemia or elevated FPG levels, indicating that not the transcriptional regulatory region used but the expressed gene itself is essential in this phenotype.

The insulin tolerance test conducted at the age of 2 months revealed that the reduction in plasma glucose concentration after IP insulin injection was impaired in the core gene transgenic mice, displaying higher plasma glucose levels than those in control mice at all time points measured (Figure 2*B*). At 40 and 60 minutes, the difference was statistically significant between transgenic

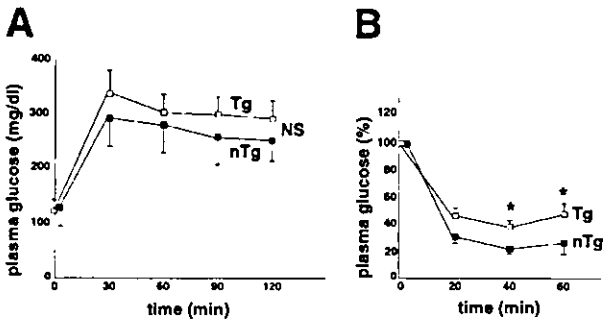

Figure 2. Insulin resistance in the core gene transgenic mice. (*A*) Glucose tolerance test (n = 5 in each group). Animals were fasted overnight (>16 hours). D-Glucose (1 g/kg body weight) was administered by IP injection to conscious mice, and plasma glucose levels were determined at the time points indicated. (*B*) Insulin tolerance test (n = 5 in each group). Human insulin (1 U/kg body weight) was administered by IP injection to fasted conscious mice and glucose concentrations were determined. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± standard error; *$P < 0.05$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

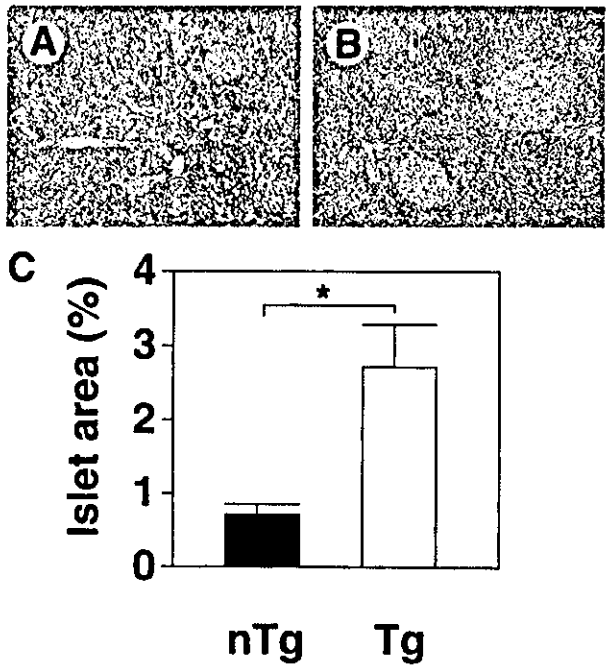

Figure 3. Analysis of pancreatic islet mass in the core gene transgenic and control mice. (*A* and *B*) Morphology of representative islets (H&E staining) from normal control mice (*A*) or the core gene transgenic mice (*B*). (*C*) Relative islet area, expressed as a percentage of the total stained pancreatic section, for control mice (nTg) and the core gene transgenic mice (Tg) (n = 10 in each group). Values are mean ± standard error; $*P < 0.05$.

and control mice (39.6 ± 1.3 vs. 24.4 ± 1.1 and 43.7 ± 2.1 vs. 26.4 ± 2.3, $P < 0.05$). These data are consistent with a defect in the actions of insulin on glucose disposal and/or production in the core gene transgenic mice.

Morphology of Pancreatic Islet Cells

Because a critical factor contributing to whether insulin resistance progresses to diabetes is the capacity of the pancreatic β-cells to respond to increased demands for insulin secretion, we evaluated the morphology of pancreatic islet cells by histologic examination. In the pancreas of HCV core gene transgenic mice, an approximately 3-fold increase in islet mass was observed (Figure 3, $P < 0.05$), which is consistent with β-cell compensation to insulin resistance. There was no infiltration of inflammatory cells within or surrounding the islets.

Feeding Transgenic Mice a High-Fat Diet Leads to Overt Diabetes

Thus, an insulin resistance is present but no apparent glucose intolerance (overt diabetes) in the HCV core gene transgenic mice. This is probably because of the genetic background of C57BL/6 mice, which has been shown to maintain either normal or mildly elevated glucose levels despite insulin resistance.[18] To determine whether a high-fat diet exacerbates the prediabetic phenotype, 2-month-old HCV core gene transgenic mice were fed a high-fat diet for up to 8 weeks. Both the transgenic and control mice showed a similar increase (about 30%) in body weight (Figure 4*A*). After 8 weeks on this diet, 100% (10 out of 10) of the transgenic mice exhibited casual (fed) plasma glucose levels >250 mg/dL, whereas none of the 10 control mice fed the same diet exhibited levels >250 mg/dL (325.0 ± 66.6 vs. 179.0 ± 17.4 mg/dL, $P < 0.01$, Figure 4*B*). Insulin levels were significantly higher in the core gene transgenic mice than in control mice both at fasting and fed state (Figure 4*C*, $P < 0.01$ and $P < 0.001$). In control mice, serum insulin levels in high-fat diet state were significantly higher than those in normal diet state at fed state (Figures 1*C* and 4*C*, $P < 0.01$). Although FPG levels were not significantly different between the transgenic and control mice, these results indicate that feeding a high-fat diet leads to the development of overt diabetes in a mouse model for HCV infection. Body weight gain, particularly with high levels of lipid, may trigger the process leading to overt diabetes in an insulin resistance model mouse with compensatory hyperplasia of islet cells.

Insulin Resistance in the Core Gene Transgenic Mice Is Chiefly Caused by Hepatic Insulin Resistance

We then investigated the mechanism of insulin resistance in the core gene transgenic mice. There was no

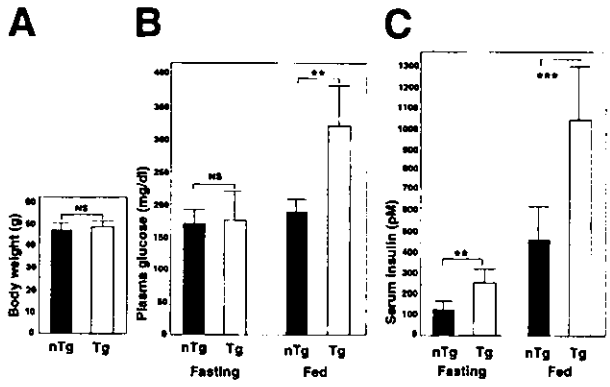

Figure 4. Body weight and glucose homeostasis after a high-fat diet. Control and transgenic mice were fed a high-fat diet for 8 weeks; thereafter, body weight and blood parameters were determined. (*A*) Body weight at the end of the high-fat diet (n = 10 in each group). (*B*) Plasma glucose levels determined in a fasting or fed state (n = 10 in each group). (*C*) Serum insulin levels in a fasting or fed state (n = 10 in each group). Values are mean ± standard error; NS, statistically not significant; $**P < 0.01$; $***P < 0.001$; nTg, nontransgenic mice; Tg, transgenic mice.

significant difference in body weight between the transgenic and control mice as already shown in Figure 1*A*. After the age of 3 months, the core gene transgenic mice developed hepatic steatosis, which is known to be one of the causes of insulin resistance in humans.[19] However, in 1-month-old mouse livers that were used in the analysis of insulin resistance, no hepatic steatosis was noted. No difference was observed in the levels of free fatty acids in the sera between the transgenic and control mice (0.56 ± 0.33 vs. 0.50 ± 0.21 mmol/L, n = 7 in each group, P = 0.65).

Then, we explored the role of the liver in pathogenesis of insulin resistance in the core gene transgenic mice. To directly measure HGP, the hyperinsulinemic-euglycemic clamp technique was conducted as described in Materials and Methods. The core gene transgenic mice showed a normal or slightly lower rate of HGP during the basal period as compared with control mice (Figure 5*A*). Although insulin infusion during the clamp suppressed HGP by 60% in the control mice, insulin induced little effect on HGP of the core gene mice (Figure 5*A*). This is consistent with the notion that insulin resistance in the core gene transgenic mice is chiefly depending on the shortage of insulin action on the liver.

To study the involvement of muscles in the development of insulin resistance in the core gene transgenic mice, we then examined whether or not insulin-stimulated glucose uptake is impaired in the skeletal muscles. The extensor digitorum longus muscle (EDL) from 2-month-old core gene transgenic and control mice were excised and exposed to insulin at the intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. There was no significant difference in 2-deoxyglucose uptake in the EDL muscle between the core gene transgenic mice and control mice at either insulin concentration (Figure 5*B*, at 0.30 nmol/L, P = 0.23 and at 10.0 nmol/L, P = 0.76). As another representative muscle that differs from EDL in metabolic properties, the soleus muscle was examined in the same manner as EDL. 2-Deoxyglucose uptake by the soleus muscle was not significantly different between the core gene transgenic and control mice (Figure 5*C*, at 0.30 nmol/L, P = 0.49 and at 10.0 nmol/L, P = 0.49). Thus, in the core gene transgenic mice, contribution of the peripheral skeletal muscle in the development of insulin resistance is negligible. This is in agreement with the observation that the core protein was exclusively present in the liver as detected by Western blotting,[20] which was confirmed by a sensitive enzyme immunoassay (Tsutsumi T. et al., unpublished data, December 2002).[21]

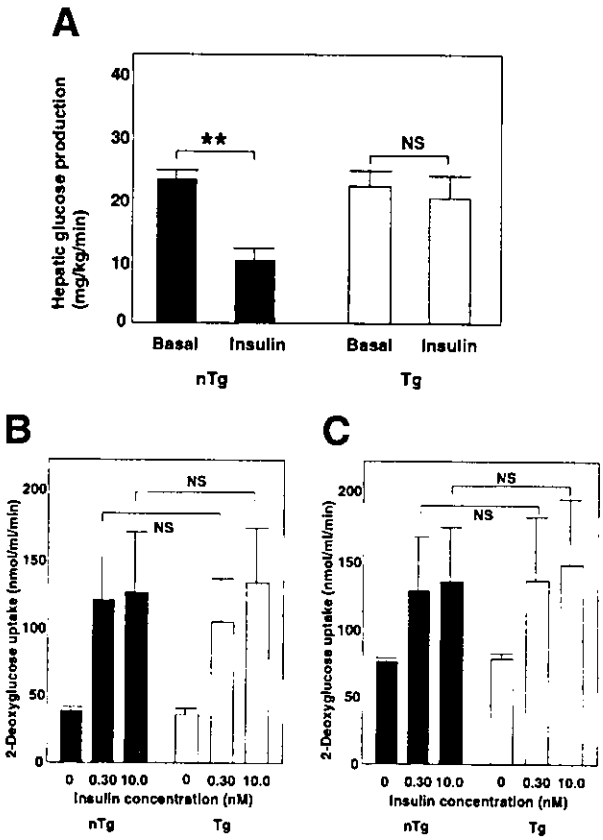

Figure 5. Characterization of glucose metabolism in the core gene transgenic mice. (*A*) Hyperinsulinemic-euglycemic clamp. Hepatic glucose production was calculated using hyperinsulinemic-euglycemic clamp. There was a failure of insulin to suppress hepatic glucose production in the core gene transgenic mice (n = 5 in each group). (*B* and *C*) Glucose uptake by the muscle after insulin stimulation. The extensor digitorum longus muscle (*A*) or soleus muscle (*B*) of 2-month-old mice were excised and exposed to insulin at intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. 2-Deoxyglucose uptake was determined as described in the Materials and Methods section (n = 8 in each group). Values are mean ± standard error; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

Elevated TNF-α Level and Altered Tyrosine Phosphorylation of Insulin Receptor Substrate-1 in the Liver and Insulin Resistance

We have noted an increase in TNF-α levels in the liver of HCV core gene transgenic mice,[13] which has also been documented in the sera of human hepatitis C patients.[22–25] On the other hand, TNF-α has been shown to induce insulin resistance in experimental animals and cultured cells.[26–29] Therefore, we next determined the protein expression level of TNF-α by ELISA in the liver of these mice that were used in the current study. The TNF-α levels in the liver of 2-month-old transgenic mice were 702.2 ± 283.3 pg/mg protein and 313.5 ±

113.6 pg/mg protein in that of 2-month-old control mice (n = 10 in each group, $P < 0.001$). Thus, the levels of TNF-α exhibited a more than 2-fold increase in the HCV core gene transgenic mice compared with the control mice, which may be associated with insulin resistance.

Suppression of tyrosine phosphorylation of IRS-1 and -2 is one of the mechanisms by which a high level of TNF-α causes insulin resistance.[29–31] We, therefore, examined the suppression of tyrosine phosphorylation of IRS-1 in response to insulin action in the core gene transgenic mice. Twenty minutes after the administration of human insulin (1 U/kg body weight), when the plasma glucose levels decreased (Figure 2*B*), IRS-1 in the liver of control mice exhibited a marked phosphorylation of its tyrosine. In contrast, phosphorylation level of tyrosine in IRS-1 in the liver of core gene transgenic mice manifested apparently no increase compared with the basal level after the administration of insulin (Figure 6). In contrast, there was no difference in the time course of tyrosine phosphorylation of IRS-2 between the core gene transgenic and control mice (data not shown). These results indicate that a suppression of tyrosine phosphorylation of IRS-1, that is, a suppression of the insulin action in the liver, is at least one of the mechanisms of insulin resistance in HCV transgenic mice, whereas pathways other than IRS-1 may also be involved.

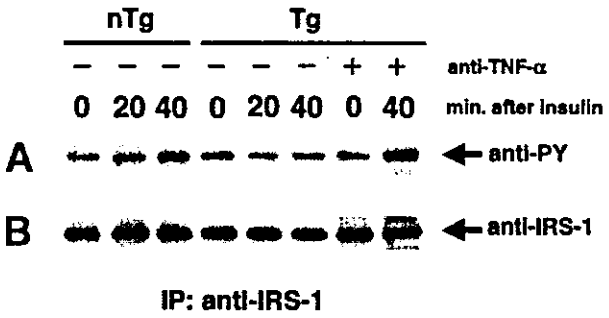

Figure 6. Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice and core gene transgenic mice with or without anti–TNF-α antibody treatment were analyzed before and 20 and 40 minutes after insulin administration. The samples were subjected to immunoprecipitation with anti-IRS-1 antibody and subsequently immunoblotted with antibodies as indicated. Experiments were performed in triplicate, and a representative picture is shown. (*A*) Immunoblot with antiphosphotyrosine antibody. There was no augmentation of phosphorylation of tyrosine in IRS-1 after insulin stimulation in the core gene transgenic mice, whereas tyrosine phosphorylation was markedly enhanced in control mice. Insulin-stimulated tyrosine phosphorylation was restored 40 minutes after anti–TNF-α antibody treatment. (*B*) Immunoblotting with anti-IRS-1 antibody as a control of IRS-1 load. nTg, nontransgenic mice; Tg, transgenic mice; anti-PY, antiphosphotyrosine antibody; anti-PS, antiphosphoserine antibody. IP, immunoprecipitation.

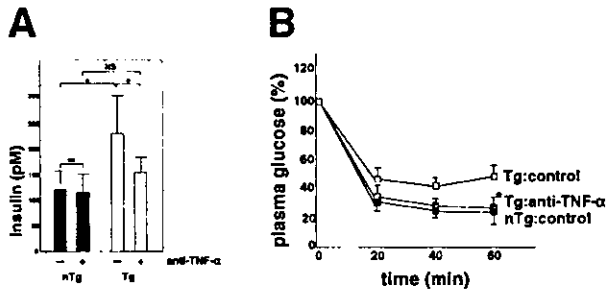

Figure 7. Serum insulin levels and insulin tolerance test after anti–TNF-α antibody treatment. (*A*) Serum insulin levels were determined in the fasting state with or without anti–TNF-α antibody treatment as described in the Materials and Methods section. Insulin levels decreased significantly after anti–TNF-α antibody treatment in the core gene transgenic mice (n = 5 in each group). (*B*) Insulin tolerance test (n = 5 in each group). Human insulin was administered by IP injection to fasted conscious mice and glucose concentrations were determined 24 hours after the second administration of anti–TNF-α antibody. As control, mice were injected with hamster IgG instead of anti–TNF-α antibody. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± standard error; $*P < 0.05$ when compared with Tg control; nTg, nontransgenic mice; Tg, transgenic mice.

The c-Jun N-terminal kinase (JNK) pathway has been shown to mediate the inhibitory effect of TNF-α on insulin action through the phosphorylation of serine in IRS-1.[32,33] Because an activation of the JNK pathway was observed in the liver of core gene transgenic mice,[13] phosphorylation of serine residues in IRS-1 was examined using antiphosphorylated serine monoclonal antibodies (Ser^{307} and Ser^{612}). However, there was no difference in the time course of serine phosphorylation after insulin stimulation between the core gene transgenic and control mice (data not shown).

Blockade of TNF-α Action Restores Insulin Sensitivity

Then the anti–TNF-α antibody was administered to block the in vivo activity of TNF-α in mice as described in the Materials and Methods section.[17] Twenty-four hours after the second administration of the anti–TNF-α antibody (200 μg/mouse), serum insulin levels in transgenic mice became significantly lower than the baseline (Figure 7*A*, 230.8 ± 70.7 vs. 153.6 ± 17.4 pmol/L, $P < 0.05$). Serum insulin levels in control mice also decreased, but there was no significant difference from the baseline (123.3 ± 36.1 vs. 112.0 ± 39.7 pmol/L, $P = 0.25$). Levels of FPG also decreased, but the difference from the baseline was not statistically significant. The insulin tolerance test conducted 24 hours after the second administration of anti–TNF-α antibody is shown in Figure 7*B*. As expected from serum insulin levels, anti–TNF-α antibody treatment restored the sen-

sitivity of the core gene transgenic mice to insulin activity. At this time point, phosphorylation of tyrosine in IRS-1 in the liver of transgenic mice in response to insulin action was restored to a similar level to that in control mice (Figure 6*A*, 40 minutes after insulin administration). These results strongly support the notion that the increased level of TNF-α is one of the bases for insulin resistance in the HCV core gene transgenic mice.

Taken together, these data indicate that the presence of the HCV core protein in the liver, at a level similar to that in human chronic hepatitis C patients,[21] confers insulin resistance to the mice by affecting the liver, by disturbing the insulin-induced suppression of hepatic glucose production.[34,35]

Discussion

Since Allison et al.[4] reported an association between HCV infection and diabetes, evidence has been accumulating connecting these 2 conditions. In such studies, HCV infection has a significantly stronger association with diabetes than hepatitis B viral infection.[4–7] The variables other than HCV infection that are associated with diabetes are cirrhosis, male sex,[5] and aging.[6] In addition to these clinic-based, case-control studies, Mehta et al.[7] have reported the result of investigation at population level. In this cross-sectional national survey, persons 40 years or older with HCV infection were more than 3 times more likely to have type 2 diabetes than those without HCV infection. Thus, the association of HCV infection with diabetes has become closer as shown by epidemiological studies. However, there are some difficulties in establishing a definite relationship between HCV infection and diabetes on the basis of epidemiological studies; in patients, there are other numerous factors perturbing the verification of the definite relationship, such as obesity, aging, or particularly advanced liver injuries. Moreover, the biological mechanism underlying diabetes or insulin resistance in HCV infection is unknown. In vitro or cultured cell studies have a very limited utility for the study of insulin resistance or diabetes because insulin resistance is a condition that involves multiple organs, such as the skeletal muscles and liver. Thus, the use of good experimental animal model systems may be useful both in establishing a definite relationship between diabetes and HCV infection and in elucidating the role of HCV in the development of insulin resistance.

In the current study, the HCV core gene transgenic mice exhibited insulin resistance as early as 1-month old, despite an apparent absence of glucose intolerance. Development of insulin resistance without any liver injury[10,11] or excessive body weight gain, as shown in the current study, clearly indicates that infection of HCV per se is a cause of the development of insulin resistance. Although only the core protein is expressed in these mice instead of HCV replication in humans, the fact that the intrahepatic core protein levels are similar between the core gene transgenic mice and chronic hepatitis C patients[20] warrants extrapolating the result into hepatitis C patients. Certainly, dispersion in the intrahepatic core protein levels in human chronic hepatitis C patients compared with the constant amount of the core protein must be taken into account. The occurrence of insulin resistance in the core gene transgenic mice as early as 1-month old also excluded the possibility that aging is a cause of insulin resistance. Nonetheless, aging could be an aggravating factor for insulin resistance. Thus, the current analysis shows a definite causal relationship between HCV infection and the development of insulin resistance.

Our earlier studies have shown the development of hepatic steatosis in these HCV core gene transgenic mice after the age of 3 months.[11] However, insulin resistance invariably preceded the occurrence of hepatic steatosis, indicating that insulin resistance is not a consequence of hepatic steatosis in these mice. Certainly, it is possible that insulin resistance in the core gene transgenic mice may be affected or aggravated after the occurrence of hepatic steatosis. On the other hand, insulin resistance may be one of the factors that cause hepatic steatosis,[19] whereas the impairment of very-low-density lipoprotein (VLDL) secretion from the liver and hypo-β-oxidation of fatty acids are considered to be the bases of development of hepatic steatosis in the core gene transgenic mice.[21,36]

The general mechanism underlying insulin resistance is not precisely understood and is considered to be multifactorial.[8,9,37,38] Chiefly, it involves glucose consumption by the skeletal muscle and glucose production in the liver. Our current analysis revealed a failure of insulin in the suppression of HPG in the liver and an absence of suppression of glucose uptake by the muscles in the core gene transgenic mice. Combined, these results indicate the insulin resistance in the core gene transgenic mice is chiefly due to hepatic insulin resistance. An elevated intrahepatic TNF-α level plays one of the roles in causing insulin resistance through suppressing insulin-induced tyrosine phosphorylation of IRS-1. It should be noted that TNF-α levels are invariably elevated in the sera of patients with HCV infection.[22] Moreover, restoration of insulin sensitivity after anti–TNF-α antibody administration strongly supports the notion that TNF-α

is, at least in this animal model, a major factor for the development of insulin resistance in HCV infection. Taken together, insulin resistance in the core gene transgenic mice mainly depends on suppression of the inhibitory effect of insulin on hepatic glucose production. This is consistent with the observation that the core protein is present only in the liver but absent in the skeletal muscle of the core gene transgenic mice (Tsutsumi T., unpublished data, December 2002).[21] Impairment in other undetermined pathways may also be responsible for the development of insulin resistance in HCV infection.

Insulin resistance alone does not always lead to the development of overt diabetes in humans or murine models. Particularly, in the models with the C57/BL6 strain,[18] hyperplasia of the islets of Langerhans in the pancreas compensates for insulin resistance by secreting higher amounts of insulin. Along with a gain in body weight by being fed a high-calorie diet, the core gene transgenic mice but no control mice developed overt diabetes, showing that obesity is a risk factor for diabetes as observed in patients or as shown in animal models for diabetes unrelated to HCV infection.[37,38] This observation would suggest that HCV infection confers insulin resistance and additional factors such as obesity, aging, or possibly inflammation may contribute to the complete development of overt diabetes. The effect of high-fat diet on control C57BL/6 mice may be milder in the current study compared with a previous study.[39] However, there was a substantial increase in FPG levels in high-fat-diet-fed control mice compared with normal-diet-fed control mice (Figures 1*B* and 4*B*). In addition, at fed-state, serum insulin levels in high-fat-diet-fed control mice were significantly increased compared with those in normal-diet-fed control mice (Figures 1*B* and 4*B*). It is unclear why plasma glucose levels were not very high at fed-state in control mice, but one possible explanation is the lower calorie content in the current study than those in the previous report: 4.70 kcal/g for our high-fat diet vs. 5.55 kcal/g for high-calorie diet in the previous study. A shorter duration of high-fat diet than the previous study (2 months vs. 6 months) may be another possible explanation.[39] Such a mild elevation in plasma glucose levels in high-fat-diet-fed C57BL/6 mice as the one observed in our study has also been described in previous studies.[40]

In conclusion, the HCV core protein induces insulin resistance in transgenic mice without gain in body weight at young age. These results indicate a direct involvement of HCV per se in the pathogenesis of diabetes in patients with HCV infection and provide a molecular basis for insulin resistance in such a condition.

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Despite their results, even if they seem strongly suggest an important role for TNF-α, is our opinion that the responsibility of this cytokine should be considered carefully.

In our previous study (Int J Immunopathol and Pharmacol [in press]) we assayed the TNF-α serum levels in patients with and without DM during CHC, not finding any statistical significant differences in its concentrations. In our groups, all the patients did not present obesity or hypertrigliceridemia or else hypercholesterolemia.

Taking into consideration that insulin resistance and DM type 2 are correlated to peripheral alteration of glucose metabolism TNF related[7] and on the light of the authors evidences on transgenic mice model how we could take to mean our results?

These evidences taken together seem suggest the presence of 2 different network in CHC; hepatic and peripheral.

In the liver the likely source of TNF-α production should be the Kupffer cells,[8] so have the authors evaluated these cells at a liver biopsy or TNF-α concentrations in serum?

Otherwise, how they explain the increased levels found, considering also that the transgenic mice do not present the same inflammation of an HCV infected patient?

The significance of TNF-α augmented serum levels during CHC natural history, and its possible effects on metabolism seem to be not so clear. Consequently, considering that other cytokines are been suggested in diabetes mellitus as IL-6[9]; how we can judge as guilty the TNF-α?

Moreover, making an allowance for the infective and immune adverse effects,[10] which should be the rational of an eventual anti-TNF treatment in infected patients?

In conclusion, although the interesting work of Shintani et al., on transgenic mice, focuses new attention on HCV direct role on insulin resistance, the fascinating connection with a possible cytokine environments in this metabolic disorder is really still unclear. A wider cytokine network evaluation both in liver and periphery is required to understand the intricate inflammatory network in DM pathogenesis in humans during chronic hepatitis C.

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Reply. We appreciate the comments of Perrella et al.[1] on our recent study published in GASTROENTEROLOGY. We realized afresh that the association of diabetes and hepatitis C virus (HCV) infection is a major concern to gastroenterologists and researchers in related fields. We also appreciate their comment that our study has focused a new attention on the direct role of HCV on insulin resistance. Their key point is that it is not clear yet whether TNF-α is a central mediator for insulin resistance in chronic HCV infection according to their data and the considerations on the cytokine network.

We agree to their comment in the point that we should be careful in assessing the role of TNF-α in the genesis of insulin resistance in HCV infection, in particular, in human patients, where numerous other factors than those in an animal model would play roles in glucose homeostasis. In our article, we have stated that the increased level of TNF-α is one of the bases for insulin resistance in the mouse model but impairment in other undetermined pathways may also be responsible for the development of insulin resistance in HCV infection.[1] Insulin resistance in our mouse model is chiefly the central one, i.e., derived from the liver: suppression of insulin action on the hepatic glucose production is inhibited. Considering this specific involvement of the liver, the levels of TNF-α not in the serum but in the liver would be essential in development of insulin resistance in this mouse model. Unfortunately, we cannot access the data by Perrella et al. that there was no significant difference in serum TNF-α levels between the hepatitis C patients with and without diabetes, but we suppose the number of patients analyzed was large enough to verify the absence of difference. Nonetheless, the levels of TNF-α in the liver, where HCV replicates, may be critical in development of the central insulin resistance.

Naturally, it is possible that the core protein operates directly to inhibit insulin action of tyrosine-phosphorylation of insulin receptor sustrate-1,[1] which inhibition was, interestingly, also observed in the liver of chronic hepatitis C patients.[2] On the other hand, because the expression of the core protein was virtually limited to the liver in the mice,[3] the contribution of peripheral factor to insulin resistance,[4] which may be present in human hepatitis C patients, could not be evaluated in our study. Mitochondrial dysfunction, suggested to have

a role in insulin resistance in the elderly,[5] might also have a contribution in insulin resistance in HCV infection.

In our mouse model, we have not identified the type of cells that produce TNF-α in the liver, but determination of other cytokines including IL-6 was already done: only TNF-α and IL-1β levels were increased in the liver among numerous cytokines.[6] Serum levels of TNF-α were determined as well, resulting to be below the detection limit of sensitive Elisa assays (Moriya K, et al. unpublished data). As described previously, there was no histopathological inflammation in the liver of HCV core gene transgenic mice.[3] However, such increases in intrahepatic proinflammatory cytokine levels, combined with the overproduction of reactive oxygen species (ROS),[7] allow us to hypothesize that HCV core protein *per se* induces "biochemical inflammation" in the mouse liver in the absence of apparent inflammation.

In summary, from our data, an impairment of intracellular insulin signaling pathway in the liver is the basis for insulin resistance in HCV infection, in which an elevated intrahepatic TNF-α level would be one of the key factors. Additional factors associated with insulin resistance must be explored, in particular, associated with peripheral insulin resistance.

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