

Fig. 3 Maximum likelihood estimates of the effective number of infections through time in Mongolia for HCV (a) and HDV (b) (black curves). The dotted stepwise plots represent corresponding nonparametric estimates. Y-axis value indicating the number of infections is presented in log<sub>10</sub>.

for HCV and HDV, respectively. Expansion time, when the number of infections for each of the viruses started its exponential growth was 1927 (95% CI; 1906–1935) and 1975 (1970–1980) for HCV and HDV, respectively. The growth rate of HDV was two times higher than that of HCV; 0.1961 (95% CI; 0.1314–0.2735) vs 0.0967 (0.0643–0.1255) (Table 1).

## DISCUSSION

As was demonstrated by previous reports, in Mongolian patients, HBV + HDV and HBV + HDV + HCV co-infection patterns have significantly stronger association with HCC when compared with HCV-monoinfection [6,7]. Nevertheless, HCV monoinfection is still frequently detected among HCC patients in this country (39%) [6]. As was previously demonstrated, there is an association among HCV seroprevalence, HCC mortality and time when HCV spread out in a given population [13]. In the present study, using similar approach based on the coalescence theory, we demonstrate different epidemic dynamics of HCV and HDV in Mongolia, with estimated 50 years time lag of HDV spread. The mutation rate of HDV [22] implemented in this report

Table 1. Host characteristics and maximum likelihood estimations of HCV and HDV in Mongolia

	HCV only (n = 52)	HBV + HDV (n = 32)
<b>HOST</b>		
Gender; Male, n(%)	17 (33)	13 (41)
Age; yrs (mean+SD)	52.9+13.5	48.6+11.4
Age; yrs (range)	27-86	21-70
Diagnosis; HCC/LC <sup>1</sup> , n(%)	27 (52)	16 (50)
<b>VIRUS</b>		
MRCA time <sup>2</sup>	1880	1955
Expansion time	1927	1975
95%CI	1906–1935	1970–1980
Growth rate	0.0967	0.1961
95%CI	0.0643–0.1255	0.1314–0.2735

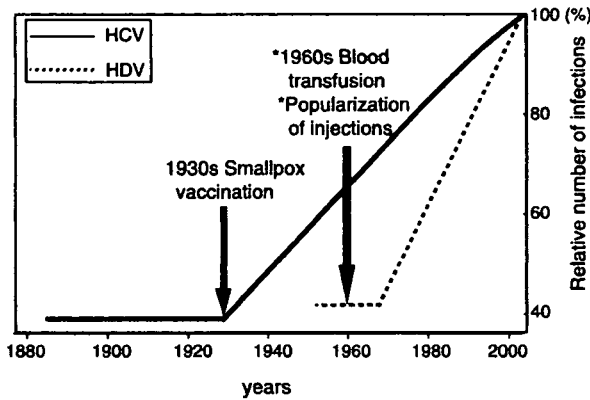
<sup>1</sup>HCC/LC – patients with hepatocellular carcinoma and/or liver cirrhosis

<sup>2</sup>MRCA time – time of divergence from most recent common ancestor

was relatively higher than that of HCV ( $1.1 \times 10^{-3}$  vs  $0.58 \times 10^{-3}$ ) [10]. However, other reports were also indicating still higher mutation rate of the virus [23,24]; therefore, further studies are required to reconfirm the evolution rate of HDV. As HDV infection requires the presence of HBV [25] and in Mongolia, most of the HBsAg carriers are coinfecting with HDV [4,5,26], it would be helpful to trace HBV population dynamics in this country. However, as no significant clustering was observed for Mongolian HBV strains deposited in the database, it was not possible to estimate the effective number of infections in this population [8,9]. Moreover, evaluation of the molecular evolution of HBV is associated with several difficulties; particularly, HBV genome represents overlapping reading frames; synonymous substitution in one of them might cause amino acid replacement in the other and thereby result in different mutation rates depending on the genome region [27]. Furthermore, the mutation rate of HBV may also vary significantly in association with HBeAg status [28]. And finally, frequently observed inter- and intra-genotypic recombination [29,30] must be taken into account when evaluating the evolutionary rate of HBV.

The relative dynamics of the infections represented as per cent of the current rate are graphically depicted in Fig. 4, which demonstrate the time lag between epidemic processes associated with each of the viruses.

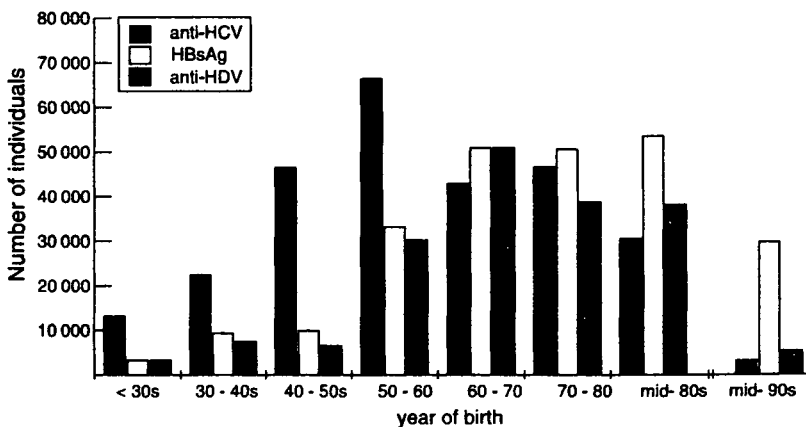
In total, about 4 million individuals were immunized against smallpox during 1933–1939, in the country. Further, in 1960, 120 000 children were vaccinated [31]. The association between national vaccination against Small Pox and the HCV epidemic has been already documented in Pakistan [32], suggesting that this factor can be a potential



**Fig. 4** Merged HCV and HDV dynamics of the relative number of infections through time expressed in per cent of the number of infections at present. Social events corresponding to the time of exponential expansion are indicated with black arrows (see Discussion for details).

trigger for the epidemic in Mongolia. In a previous study, we reported an association of HCV spread with a particular social environment, i.e. in Uzbekistan, intravenous drug use (IDU) caused an epidemic with distinct genotype (HCV/3a), and was relatively independent from the blood products-associated (HCV/1b) epidemic [33]. However, no published data are available on IDU in this country. The preparation of blood for Surgical Hospital in Mongolia was started in 1957, and in 1963 the National Blood Center was established (Fig. 4). In addition, a wide range of diagnostic kits became available since early 1960s and large blood screening programmes were implemented in this country. According to the local records, an increase in the epidemic of hepatitis shortly after that could be associated with HDV epidemic spread (T. Oyunsuren personal communication).

To confirm our estimates of spread time for HCV and HDV, we have analysed sero-epidemiological data published in the recent studies in Mongolia. A total of 1683 representatives of general population; including 249 apparently healthy adults [34], 289 blood donors [4] and 1145 children [35] were



**Fig. 5** Estimated number of infected individuals in Mongolia. Per cent value of the serological findings in different age groups of general population from previous independent studies [4,34,35] extrapolated into entire population of the country according to age related pyramid.

enrolled in the recent sero-surveys. The data on HCV, HBV and HDV sero-prevalence were transferred into the absolute numbers of infected individuals according to the age pyramid of Mongolian population (Source: US Census Bureau, International Data Base, <http://www.census.gov>). The result graphically presented in Fig. 5, indicates age-related difference in the HCV and HBV + HDV infections' pool, with the greatest estimated number of HCV infected subjects born in 1950s, whereas peak of HBV and HDV infections corresponded to population born in 1960s and 1970s (Fig. 5). Although the number of HCV-infected individuals born in 1930s and 1940s is smaller than those born in 1950s, the percentile ratio of infected in each of the category consisted 24%, 30% and 20%, respectively, which is in agreement with ML estimated spread time since the middle of 1930s. In respect of HDV history, as almost all HBV infected in Mongolia are co-infected with HDV, it is likely that the infection was acquired not vertically (at birth) but in the early childhood and adolescence. This could explain the great number of infected individuals born in 1950s–1960s whereas ML estimated the spread time that occurred in the middle of 1970s.

Thus, taken together, the results of coalescence theory-based tracing of the viral epidemic history, data of the sero-epidemiological surveys and the social-historical events in the background of the country were all in mutual agreement.

In conclusion, this study confirmed that the co-infection with HDV is associated with increasing risk of cancerogenesis and the comparative contribution of the HCV mono-infection in the high rate of HCC in this country is result of the 50 years' earlier spread time. In addition, this study demonstrated the value of viral gene sequences as a source of epidemiological and historical information.

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## Original Article

## Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

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People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretrovirus therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by dis-

tributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfecting with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfecting patients is expected.

**Key words:** coinfection, hepatitis C, HIV, liver disease

## INTRODUCTION

H E P A T T I S C V I R U S (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.<sup>1</sup> The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.<sup>2</sup> Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.<sup>3</sup> Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jirovecii*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have

declined. However, the reduction in mortality due to opportunistic infection has made patients coinfecting with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.<sup>4,5</sup>

Coinfection with HIV has been shown to increase the HCV load in HCV infection,<sup>6</sup> being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.<sup>7,8</sup> It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.<sup>9,10</sup> Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.<sup>11</sup> There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,<sup>12</sup> and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

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email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

## METHODS

**I**N THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: [http://www.acc.go.jp/mLhw/mLhw\\_frame.htm](http://www.acc.go.jp/mLhw/mLhw_frame.htm).

## RESULTS

**T**HE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.<sup>12</sup> However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but

**Table 1** Number of hospitals categorized by the number of patients infected with HIV and those coinfecting with HIV and HCV

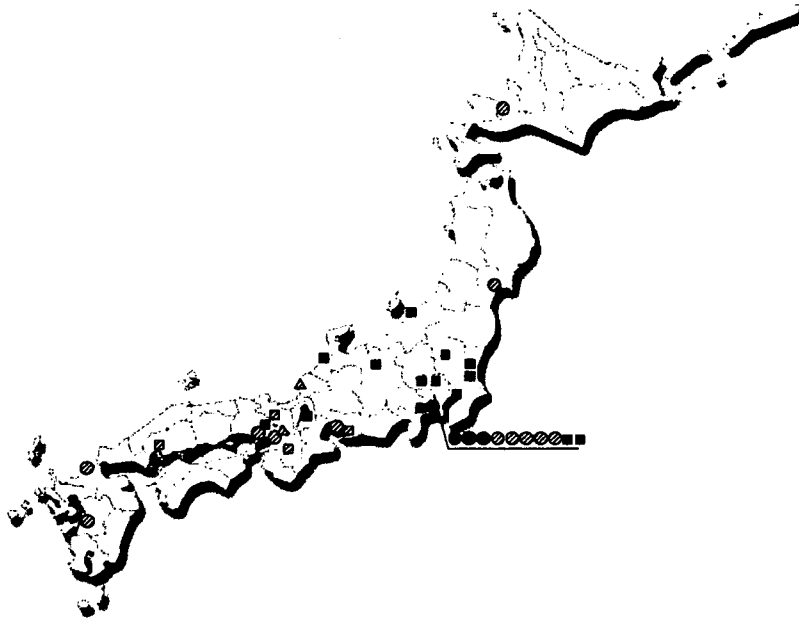
No. of HIV(+)/HCV(+)	No. of HIV(+)				Total
	0	1-19	20-49	50+	
0	43	52	5	1	101
1-9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

a majority of HIV-positive patients in Japan were enrolled in the study.

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20-49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfecting with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfecting patients in 18 (10.2%) hospitals. HIV/HCV coinfecting patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfecting patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfecting patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfecting patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission



**Figure 1** Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfecting patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfecting patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. ( $\Delta$ ) hospitals with 1-19 HIV-positive patients; ( $\square$ ) hospitals with 20-49 HIV-positive patients; ( $\circ$ ) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfecting patients. Closed figures: hospitals with less than 10 HIV/HCV coinfecting patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfecting patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfecting patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfecting patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

## DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.<sup>12</sup> The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfecting patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in

Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfecting patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.<sup>13</sup> In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.<sup>13</sup> Therefore, an HCV prevalence of 4% in MSM

**Table 2** Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfecting patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfecting patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfecting patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfecting with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

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## Involvement of the PA28 $\gamma$ -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein<sup>∇</sup>

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The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 $\gamma$ -dependent pathway. In this study, we examined whether PA28 $\gamma$  is required for HCV core-induced insulin resistance *in vivo*. HCV core gene-transgenic mice lacking the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$ CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$ CoreTg with PA28 $\gamma$ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28 $\gamma$  gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 $\gamma$ -dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2

diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic  $\beta$  cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF- $\alpha$ ) secretion (47) and that the HCV core protein is degraded through a PA28 $\gamma$ /REG $\gamma$  (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 $\gamma$  enhances latent proteasome activity, although the biological significance of PA28 $\gamma$  is largely unknown, with the exception that PA28 $\gamma$  is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28 $\gamma$  in the stability of IRS proteins and in the development of insulin resis-

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tance is not known. In this study, we examined the involvement of PA28 $\gamma$  in the induction of insulin resistance by the HCV core protein in vivo.

#### MATERIALS AND METHODS

**Preparation of PA28 $\gamma$ -knockout HCV core gene-transgenic mice.** C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 $\gamma^{+/+}$  CoreTg) line C49 and PA28 $\gamma^{-/-}$  mice have been described previously (35, 36). These two genotypes were crossed to create PA28 $\gamma^{+/-}$  CoreTg mice. PA28 $\gamma^{+/-}$  CoreTg mice were bred to generate PA28 $\gamma^{-/-}$  CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 $\gamma$  gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

**Glucose tolerance test.** The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

**Insulin tolerance test.** The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

**Histological analysis of pancreatic islets.** Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

**Estimation of tumor necrosis factor alpha and HCV core protein.** Mouse TNF- $\alpha$  was measured by using a mouse TNF- $\alpha$  ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

**In vivo insulin stimulation and immunoblot analysis.** Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28 $\gamma$ , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM NaF, 50 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate-2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

**Quantitative reverse transcription-PCR (RT-PCR).** Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF- $\alpha$ , IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5'-GGTACAACCCATCGGCTGGCA-3' (forward) and 5'-GCCACGTGGAAG-3' (reverse) for TNF- $\alpha$ , 5'-ATAG

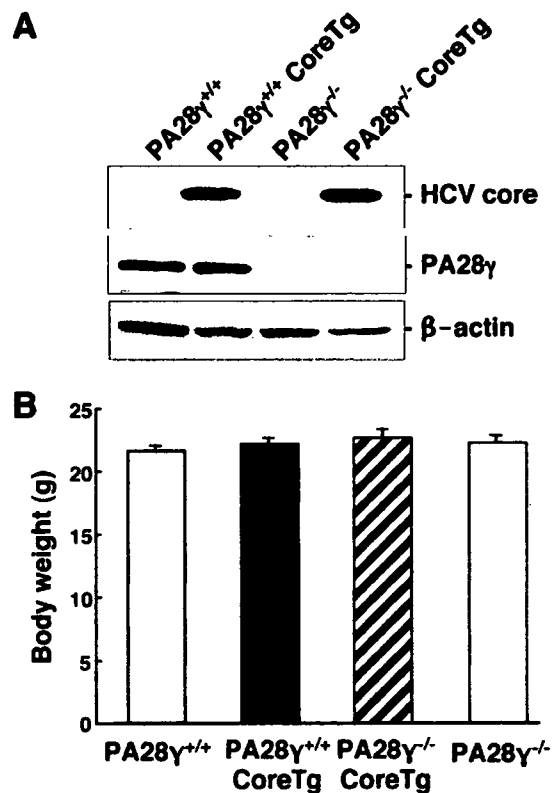


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene. (A) Expression of the HCV core protein and PA28 $\gamma$  in the livers of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$  CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$  CoreTg mice. Lysates obtained from liver tissues of the mice (100  $\mu$ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 $\gamma$ , and  $\beta$ -actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ( $n = 7$  in each group). There were no statistically significant differences in body weights among the mice ( $P > 0.05$ ).

CTCTGAGACCTTCTCAGCACCTAC-3' (forward) and 5'-GGAGTTGCCT CATTGCTGCCTAA-3' (reverse) for IRS1, 5'-AGCCTGGGATAATGGTG ACTATACCGA-3' (forward) and 5'-TTGTGGGCAAGGATGGGGACAC T-3' (reverse) for IRS2, and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' (forward) and 5'-GTAATGATCAGTCAACGGGGAC-3' (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

**Reporter assay for TNF- $\alpha$  promoter activity.** The promoter region of the TNF- $\alpha$  gene (located from residues -1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf- $\alpha$ Pro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase ( $n$ -fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28 $\gamma$ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

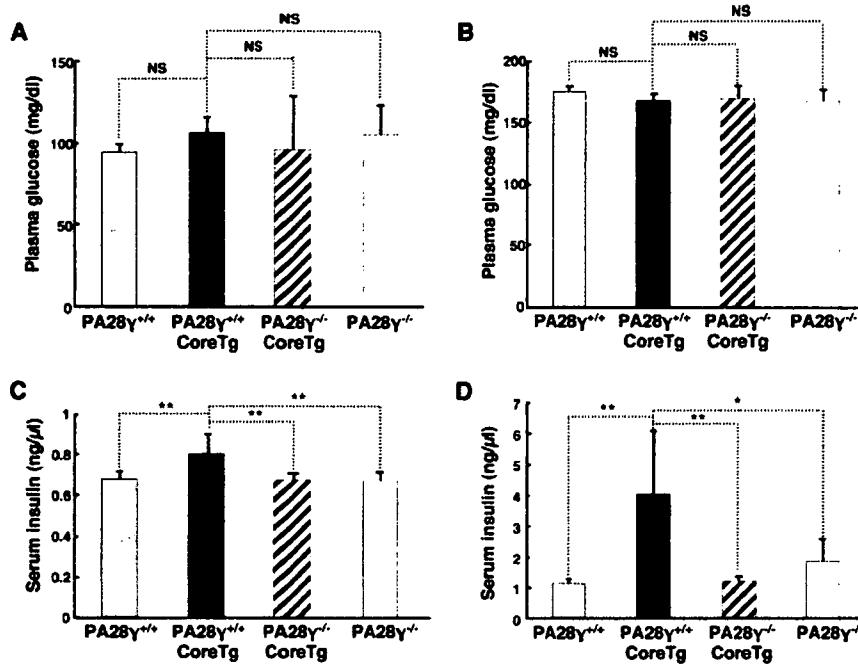


FIG. 2. Knockout of the PA28 $\gamma$  gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$  CoreTg, PA28 $\gamma^{-/-}$  CoreTg, and PA28 $\gamma^{-/-}$  mice under fasting (A) or fed (B) conditions ( $n = 7$  in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice ( $n = 7$  in each group) are also shown. Values are represented as means  $\pm$  standard deviations. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant.

**Statistical analysis.** The results are presented as means  $\pm$  standard deviations. The significance of the differences was determined by Student's  $t$  test.  $P$  values of  $< 0.05$  were considered statistically significant.

**RESULTS**

**HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene.** To investigate the role of PA28 $\gamma$  in the development of insulin resistance in HCV core gene-transgenic (PA28 $\gamma^{+/+}$  CoreTg)

mice, we generated HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$  CoreTg). A PA28 $\gamma^{+/+}$  CoreTg mouse expressing an amount of PA28 $\gamma$  equal to that of its normal littermates (Fig. 1A) was crossedbred with a PA28 $\gamma^{-/-}$  mouse to generate a PA28 $\gamma^{+/-}$  CoreTg mouse. PA28 $\gamma^{+/-}$  CoreTg mice were bred with each other, and a PA28 $\gamma^{-/-}$  CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 $\gamma^{+/+}$  CoreTg and PA28 $\gamma^{-/-}$  CoreTg

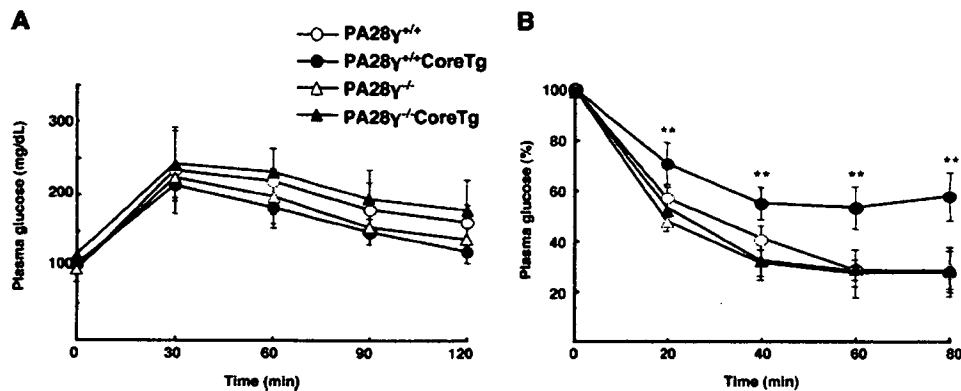


FIG. 3. Knockout of the PA28 $\gamma$  gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ( $n = 5$  in each group). There were no significant differences in glucose levels among the mice ( $P > 0.05$ ). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration ( $n = 5$  in each group). The values for the PA28 $\gamma^{+/+}$  (open circles), PA28 $\gamma^{+/+}$  CoreTg (closed circles), PA28 $\gamma^{-/-}$  (open triangles), and PA28 $\gamma^{-/-}$  CoreTg (closed triangles) mice are represented as means and  $\pm$  standard deviations. Significant differences in insulin sensitivity ( $P < 0.01$ ) in PA28 $\gamma^{+/+}$  CoreTg mice compared to that in PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$  CoreTg mice are indicated by double asterisks (\*\*). There were no significant differences among PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$  CoreTg mice ( $P > 0.05$ ).

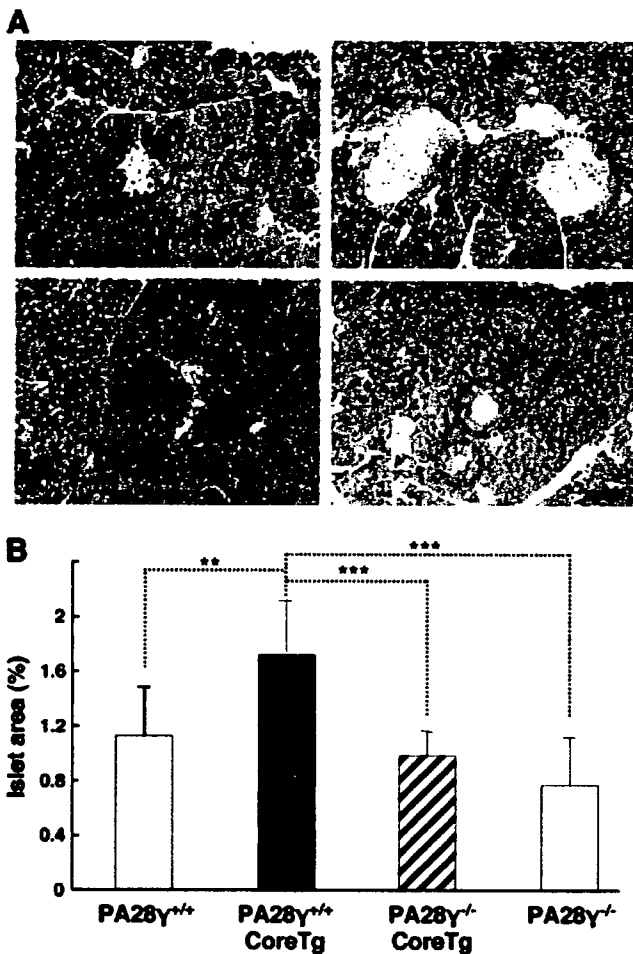


FIG. 4. PA28 $\gamma$  participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . The scale bar indicates 100  $\mu\text{m}$ .

mice but not in PA28 $\gamma^{+/+}$  (normal littermates) or PA28 $\gamma^{-/-}$  mice. PA28 $\gamma$  was found at a similar level in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$  mice but was not present in either PA28 $\gamma^{-/-}$  or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28 $\gamma^{-/-}$ CoreTg ( $1.36 \pm 0.44$  ng/mg of total protein;  $n = 7$ ) than in PA28 $\gamma^{+/+}$ CoreTg ( $1.23 \pm 0.22$  ng/mg of total protein;  $n = 7$ ) mice, but these values were not significantly different ( $P > 0.05$ ). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28 $\gamma^{-/-}$  mice were slightly smaller than their normal littermates (PA28 $\gamma^{+/+}$ ) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were

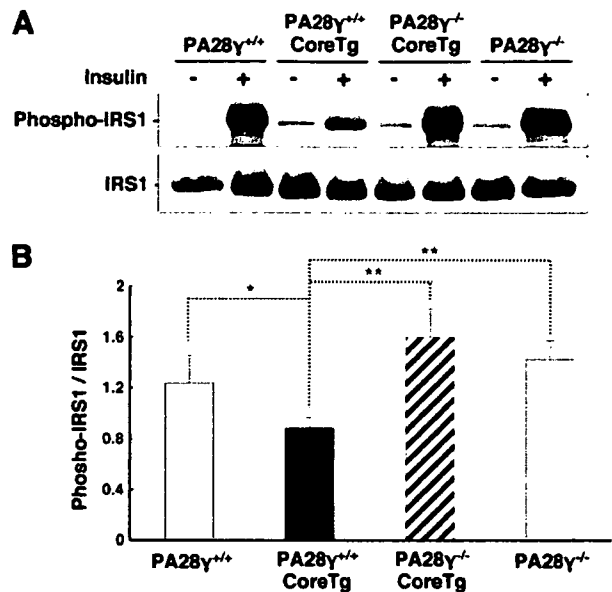


FIG. 5. PA28 $\gamma$  participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (-). The samples (100  $\mu\text{g}$  of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ( $n = 5$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{+/+}$  mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28 $\gamma$  is involved in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28 $\gamma^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28 $\gamma$  in the development of insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28 $\gamma^{+/+}$ CoreTg mice than in PA28 $\gamma^{+/+}$  mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level similar to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28 $\gamma^{+/+}$ CoreTg mice. In our previ-

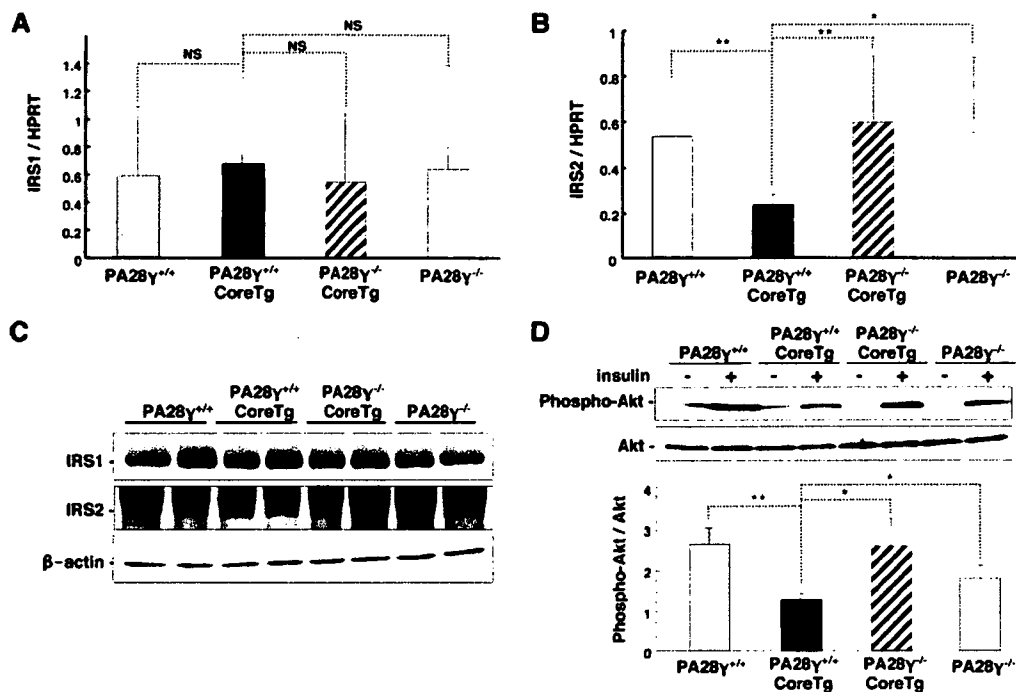


FIG. 6. PA28 $\gamma$  participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ( $n = 5$  in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ( $n = 3$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28 $\gamma^{+/+}$  CoreTg mice (47). In this study, PA28 $\gamma^{-/-}$  CoreTg mice exhibited a normal insulin level comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice by an insulin tolerance test, in contrast to PA28 $\gamma^{+/+}$  CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28 $\gamma^{+/+}$  CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 $\gamma$  participates in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$  CoreTg mice.

**Morphology of pancreatic islets.** Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28 $\gamma^{+/+}$  CoreTg mice. To clarify whether a knockout of the PA28 $\gamma$  gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28 $\gamma^{-/-}$  CoreTg mice was smaller than that of PA28 $\gamma^{+/+}$  CoreTg mice and comparable to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28 $\gamma$  also participates in the enlargement of pancreatic islets induced in PA28 $\gamma^{+/+}$  CoreTg mice.

**PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2.** Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice and that the administration of anti-TNF- $\alpha$  antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28 $\gamma$  gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28 $\gamma^{-/-}$  CoreTg mice to levels comparable to those in PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28 $\gamma^{+/+}$  CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice

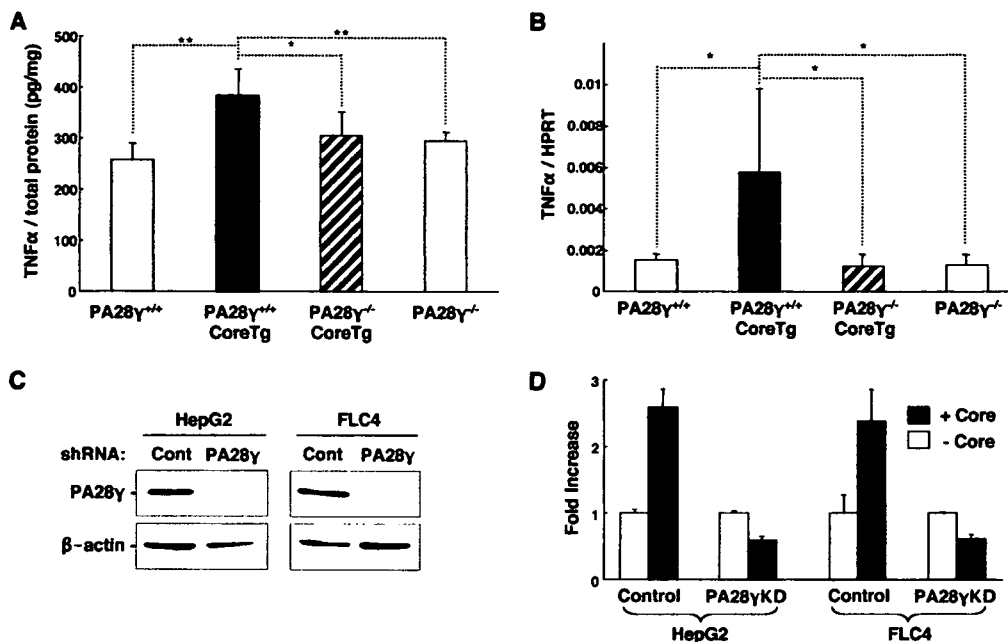


FIG. 7. PA28 $\gamma$  was required for activation of the TNF- $\alpha$  promoter by the HCV core protein. (A) Expression of TNF- $\alpha$  in the livers of mice was determined by ELISA ( $n = 5$  in each group). (B) TNF- $\alpha$  mRNA in the livers of mice was examined by quantitative RT-PCR ( $n = 5$  in each group). (C) Knockdown of the expression of PA28 $\gamma$  in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 $\gamma$  gene. The expression levels of PA28 $\gamma$  and  $\beta$ -actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF- $\alpha$  in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28 $\gamma$ -knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28 $\gamma^{+/+}$ CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but not in those of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

**PA28 $\gamma$  is required for activation of the TNF- $\alpha$  promoter by HCV core protein.** TNF- $\alpha$  is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF- $\alpha$  level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF- $\alpha$  levels have also been demonstrated in the livers of PA28 $\gamma^{+/+}$ CoreTg mice (47). To determine the involvement of PA28 $\gamma$  in the enhancement of TNF- $\alpha$  expression, the expression of TNF- $\alpha$  in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF- $\alpha$  were increased in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice. To determine the effect of PA28 $\gamma$  expression on the promoter activity of TNF- $\alpha$  in human liver cells, PA28 $\gamma$ -knockdown human hepatoma cell lines HepG2 and FLC4 were

established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 $\gamma$  gene in the cell lines. The expression of PA28 $\gamma$  was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF- $\alpha$  promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28 $\gamma$ -knockdown cell lines (Fig. 7D). These results suggest that PA28 $\gamma$  is required for the activation of the TNF- $\alpha$  promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

## DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lipolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-trisphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28 $\gamma^{+/+}$  CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 $\gamma$  gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 $\gamma^{+/+}$  CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 $\gamma$ -dependent pathway.

Our previous study suggested that the induction of TNF- $\alpha$  by the HCV core protein plays a role in insulin resistance (47). An increase in TNF- $\alpha$  levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF- $\alpha$  induces insulin resistance is not completely known. The expression of TNF- $\alpha$  has been shown to be increased in PA28 $\gamma^{+/+}$  CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 $\gamma^{+/+}$  CoreTg was improved by the administration of an anti-TNF- $\alpha$  antibody (47). In the present study, the expression level of TNF- $\alpha$  in PA28 $\gamma^{-/-}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF- $\alpha$  gene in human liver cell lines but not in those with a knockdown of the PA28 $\gamma$  gene by RNA interference (Fig. 7D). These data suggest that PA28 $\gamma$  plays a crucial role in HCV core-induced expression of TNF- $\alpha$ . Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF- $\alpha$  (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28 $\gamma$  to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF- $\alpha$  or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polyprotein have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine

phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  CoreTg mice (data not shown). TNF- $\alpha$  signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice.

How does the HCV core protein induce TNF- $\alpha$  production? Our previous report suggests that the HCV core protein is degraded through a PA28 $\gamma$ -dependent pathway (32). Recently, PA28 $\gamma$  has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28 $\gamma$ -dependent pathway may regulate the promoter activity of the TNF- $\alpha$  gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28 $\gamma$ -dependent pathway activates the promoter of the TNF- $\alpha$  gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF- $\alpha$  and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28 $\gamma$ -dependent pathway, thereby leading to insulin resistance. PA28 $\gamma$  may be a novel target for the treatment of HCV-induced diabetes.

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# Critical role of PA28 $\gamma$ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

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Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28 $\gamma$ /REG $\gamma$  regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28 $\gamma$  gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and *srebp-1c* promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28 $\gamma$ -dependent manner. Heterodimer composed of liver X receptor  $\alpha$  (LXR $\alpha$ ) and retinoid X receptor  $\alpha$  (RXR $\alpha$ ) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXR $\alpha$ /RXR $\alpha$  to LXR-response element in the presence but not the absence of PA28 $\gamma$ . These findings suggest that PA28 $\gamma$  plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXR $\alpha$  | LXR $\alpha$

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of  $\approx 3,000$  aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects >170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average  $\approx 50\%$  (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NS5A suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of steroid regulatory element-binding proteins (SREBPs), which activate the expression of >30 genes involved in

the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR)  $\alpha$  and retinoid X receptor (RXR)  $\alpha$ , which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXR $\alpha$  is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the *srebp-1c* promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28 $\gamma$ /REG $\gamma$  in the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. PA28 $\gamma$  is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28 $\gamma$ s are identical (19). The homologous proteins, PA28 $\alpha$  and PA28 $\beta$ , form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28 $\gamma$  forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28 $\gamma$  binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28 $\gamma$  are largely unknown *in vivo*. In this work, we examine whether PA28 $\gamma$  is required for liver pathology induced by HCV core protein *in vivo*.

## Results

**PA28 $\gamma$ -Knockout HCV Core Gene Transgenic Mice.** To determine the role of PA28 $\gamma$  in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28 $\gamma$ -knockout core gene transgenic mice. The PA28 $\gamma$ -deficient, PA28 $\gamma^{-/-}$  mice were born without

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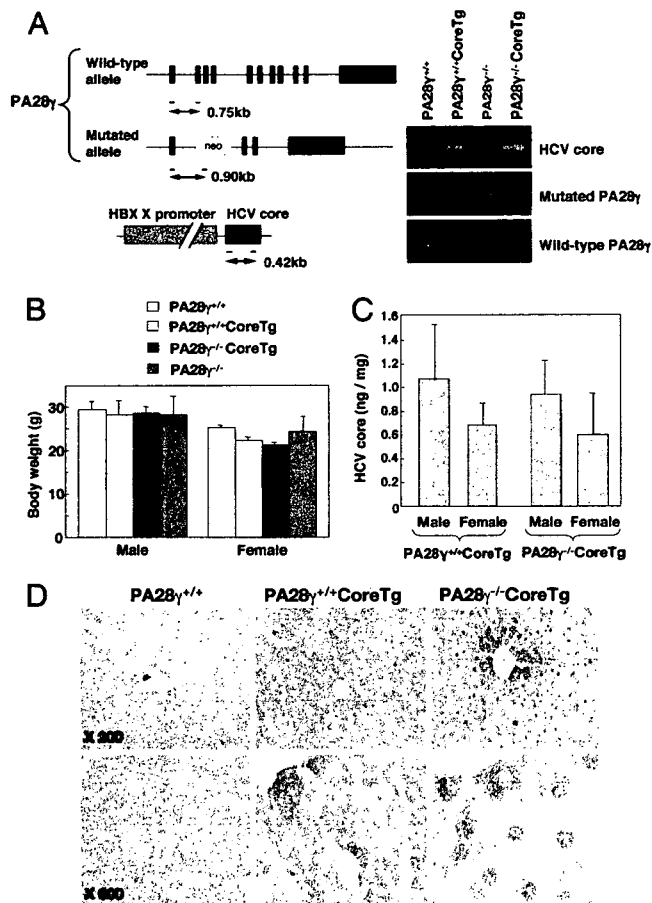
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Abbreviations: CoreTg, HCV core gene-transgenic; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LXR, liver X receptor; LXRE, liver X receptor-response element; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; RXR, retinoid X receptor; SRC-3, steroid receptor coactivator-3; SREBP, steroid regulatory element-binding protein.

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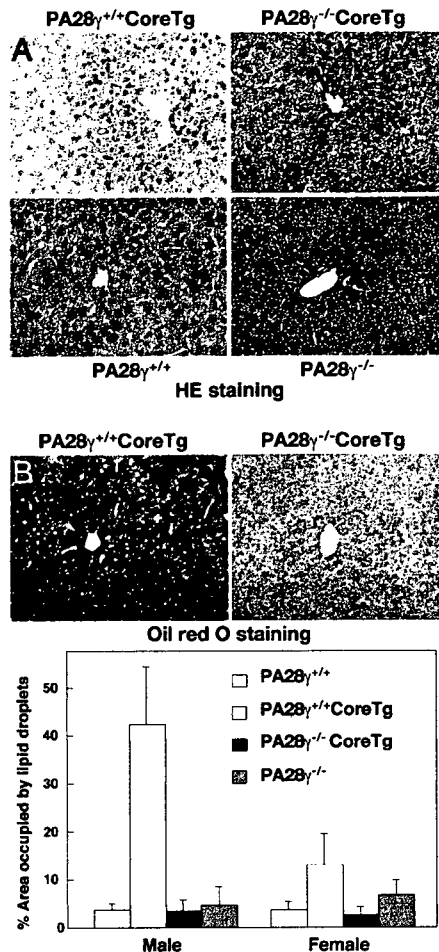
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**Fig. 1.** Preparation and characterization of PA28 $\gamma$ -knockout HCV core-transgenic mice. (A) The structures of the wild-type and mutated PA28 $\gamma$  genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28 $\gamma$  alleles were amplified from the genomic DNAs of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice. (B) Body weights of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$  mice at the age of 6 months. (C) HCV core protein levels in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice were determined by ELISA (mean  $\pm$  SD,  $n = 10$ ). (D) Localization of HCV core protein in the liver. Liver sections of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice were bred with PA28 $\gamma^{-/-}$  mice to create PA28 $\gamma^{+/-}$ CoreTg mice. The PA28 $\gamma^{+/-}$ CoreTg offspring were bred with each other, and PA28 $\gamma^{-/-}$ CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1A). No significant differences in body weight were observed among the 6-month-old mice, although PA28 $\gamma^{-/-}$  mice exhibited a slight retardation of growth (Fig. 1B). A similar level of PA28 $\gamma$  expression was detected in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$  mice (see Fig. 5B). The expression levels and molecular size of HCV core protein were similar in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1C; see also Fig. 5B).

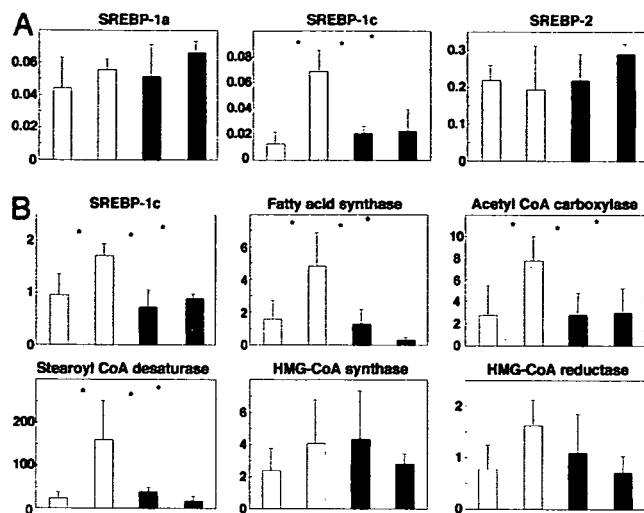
**PA28 $\gamma$  Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis.** HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28 $\gamma^{+/+}$ CoreTg mice and hepatitis C patients



**Fig. 2.** Accumulation of lipid droplets by expression of HCV core protein. (A) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (B) (Upper) Liver sections of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 6 months were stained with oil red O. (Lower) The area occupied by lipid droplets of PA28 $\gamma^{+/+}$  (white), PA28 $\gamma^{+/+}$ CoreTg (gray), PA28 $\gamma^{-/-}$ CoreTg (black), and PA28 $\gamma^{-/-}$  (dark gray) mice was calculated by Image-Pro software (MediaCybernetics, Silver Spring, MD) (mean  $\pm$  SD,  $n = 10$ ).

(6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28 $\gamma^{+/+}$ CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1D). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28 $\gamma^{+/+}$ CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe vacuolating lesions (Fig. 2A), which were clearly stained with oil red O (Fig. 2B Upper), whereas no such lesions were detected in the livers of PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{+/+}$ , or PA28 $\gamma^{-/-}$  mice at the same age. The areas occupied by the lipid droplets in the PA28 $\gamma^{+/+}$ CoreTg mouse livers were  $\approx 10$  and 2–4 times larger than those of male and female of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice, respectively (Fig. 2B Lower). These results suggest that PA28 $\gamma$  is required for the induction of liver steatosis by HCV core protein in mice.

**PA28 $\gamma$  Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver.** To clarify the effects of a knockout of the PA28 $\gamma$  gene in PA28 $\gamma^{+/+}$ CoreTg mice on lipid



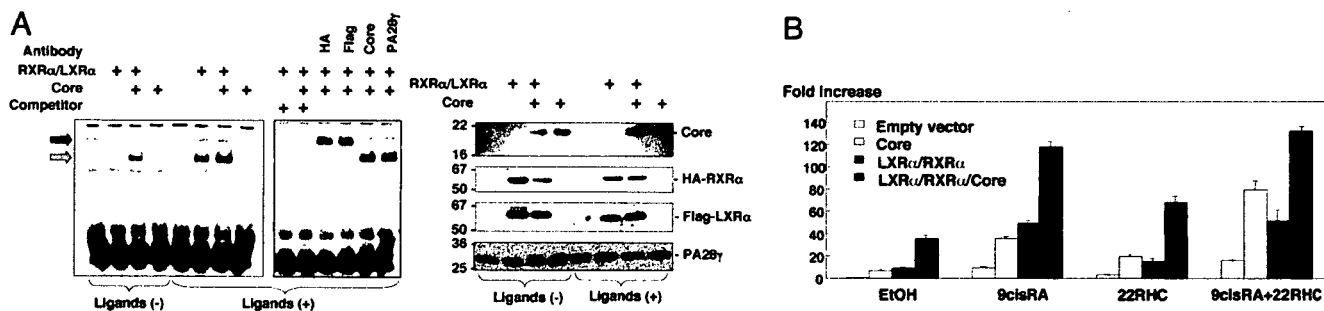
**Fig. 3.** Transcription of genes regulating lipid biosynthesis in the mouse liver. (A) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (B) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ( $n = 5$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). The transcription of each gene in PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$  mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of PA28 $\gamma^{+/+}$ CoreTg mice than in those of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3A). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, was also enhanced in the livers of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice compared with the levels in the livers of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3B). These results suggest the

following: (i) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28 $\gamma$ ; and (ii) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28 $\gamma$ -dependent proteasome pathway, does not activate the *srebp-1c* promoter.

**HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXR $\alpha$ /RXR $\alpha$ -Dependent Manner.** LXR $\alpha$ , which is primarily expressed in the liver, forms a complex with RXR $\alpha$  and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXR $\alpha$  by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and PA28 $\gamma$  on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXR $\alpha$ /RXR $\alpha$  complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4A). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXR $\alpha$  and HA-tagged RXR $\alpha$ , a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxylcholesterol, ligands for LXR $\alpha$  and RXR $\alpha$ , respectively. In contrast, coexpression of HCV core protein with LXR $\alpha$  and RXR $\alpha$  potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coinubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28 $\gamma$  caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXR $\alpha$ /RXR $\alpha$ -LXRE complex but indirectly enhances the binding of LXR $\alpha$ /RXR $\alpha$  to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of LXR $\alpha$ /RXR $\alpha$  (Fig. 4B). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXR $\alpha$ /RXR $\alpha$  was further potentiated by treatment with the ligands for LXR $\alpha$  and RXR $\alpha$ . The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxylcholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-



**Fig. 4.** Activation of the *srebp-1c* promoter by HCV core protein. (A) FLAG-LXR $\alpha$  and HA-RXR $\alpha$  were expressed in 293T cells together with or without HCV core protein. Ligands for LXR $\alpha$  and RXR $\alpha$  dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (-)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or PA28 $\gamma$ , or nonlabeled LXRE probe (Competitor). (Left) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (Right) Expression of HCV core, HA-RXR $\alpha$ , FLAG-LXR $\alpha$ , and PA28 $\gamma$  in cells was detected by immunoblotting. (B) Effects of ligands for RXR $\alpha$ , 9-*cis*-retinoic acid (9cisRA), and for LXR $\alpha$ , 22(*R*)-hydroxylcholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXR $\alpha$ , LXR $\alpha$ , and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5  $\mu$ M, and the cells were harvested after 24 h of incubation.