

### Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Millville, NJ) and homogenized on ice by 30 strokes in 1.2 mL of nuclei buffer [300 mM sucrose in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 5 mM MgCl<sub>2</sub> and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500g for 5 min at 4°C. The resulting pellet was resuspended, layered over 2 mL of nuclei buffer containing 2 M sucrose, and centrifuged at 23,000g for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250  $\mu$ L of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.<sup>16</sup>

### Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA<sup>TM</sup> Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20  $\mu$ g protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.<sup>17</sup> For analysis of other proteins, hepatocyte nuclear fraction (100  $\mu$ g protein) or whole liver lysate (200–300  $\mu$ g protein) was subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. The origin of the primary rabbit polyclonal antibodies against fatty acid-metabolizing enzymes and protein was described earlier.<sup>17</sup> For immunoblot analysis of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), a polyclonal anti-mouse antibody<sup>18</sup> or commercial antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The antibodies against cell-cycle regulators and oncogene products were purchased commercially (Santa Cruz Biotech.).<sup>19</sup> Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprobing the membranes with an antibody against  $\beta$ -actin and histone H1, respectively. The band intensity of nuclear PPAR $\alpha$  was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

### mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit<sup>TM</sup> (Qiagen, Valencia, CA). Five microgram of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20 $\times$  SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with <sup>32</sup>P-labeled cDNA probes. The blots were exposed to a phosphorimager screen cassette and were analyzed using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, CA). The origin of the cDNA probes has been described elsewhere.<sup>17–19</sup> Northern blot of  $\beta$ -actin was used as the internal control. The blot intensity was quantified, normalized to that of  $\beta$ -actin and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

### Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated from transgenic and control mice by the modified *in situ* perfusion method.<sup>20</sup> After perfusion with 0.05% collagenase solution (Wako, Osaka, Japan), the isolated hepatocytes were washed thrice by means of differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed fetal bovine serum for 1 hr at 37°C. The medium

was replaced with the same medium containing 300  $\mu$ Ci/mL of [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech.). After 3-hr of incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8 or 16 hr. The labeled cells were washed, homogenized and centrifuged for preparation of the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the nontransgenic mice, suggesting that the [<sup>35</sup>S]methionine uptake capacity in the former hepatocytes is similar to that in the latter. The nuclear fraction was lysed in RIPA buffer [10 mM Tris-HCl, pH 7.4, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM PMSF, 10  $\mu$ g/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPAR $\alpha$  antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The nuclear fractions of the pulse-labeled preparations were also used for immunoblot analysis of PPAR $\alpha$ .

### Affinity chromatography for PPAR $\alpha$ complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a 4-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the PowerSonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000g for 20 min. The supernatant was applied to an immobilized anti-PPAR $\alpha$  IgG column (1.0  $\times$  4.0 cm<sup>2</sup>), prepared with the Affigel HZ Immunoaffinity kit<sup>R</sup> (Bio-Rad, Hercules, CA) and equilibrated with 10 mM potassium phosphate, pH 7.5, 20 mM NaCl and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least thrice. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM NaCl, in a total volume of 2 mL. The eluate was resolved by 10 and 15% SDS-polyacrylamide gel electrophoresis for PPAR $\alpha$  and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.<sup>21</sup> The monoclonal antibody against the core protein was purchased commercially (ViroGen, Watertown, MA).

### Cytochemical staining of peroxisomes

Liver peroxisome proliferation was evaluated by using 3,3'-diaminobenzidine (DAB) staining for catalase according to the method of Novikoff and Goldfischer with minor modifications.<sup>22</sup> Small pieces of liver were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, for 3 hr at 4°C, rinsed with sodium cacodylate buffer and cut into 100- $\mu$ m sections with a Lancer<sup>R</sup> Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hr at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H<sub>2</sub>O<sub>2</sub>) and postfixed with 1% OsO<sub>4</sub> in 100 mM sodium phosphate, pH 7.4 for 1 hr. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One micrometer sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1- $\mu$ m sections were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate and visualized with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

### Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron photomicrographs. For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of 4,000 $\times$ . At this magnification, peroxisomes smaller than 450 nm were clearly

identified. Peroxisomes were easily detected because of their high contrast because of the positive DAB reaction. In each frame, the number of peroxisomal profiles and the area of each individual profile were determined. The numerical density and volume density of peroxisomes were calculated using the following equations: numerical density (number/ $\mu\text{m}^2$ ) =  $N_p/(A_T - A_{\text{empty}})$ , and volume density (%) =  $A_{\text{TP}}/(A_T - A_{\text{empty}}) \times 100$ , where  $N_p$  is the peroxisome number in the test area,  $A_T$  is the test area,  $A_{\text{empty}}$  is the area of the vascular and biliary lumens and that of the hepatocyte nuclei and lipid droplets, and  $A_{\text{TP}}$  is the area of total peroxisomal profiles in the test area. The area was measured with a Luzex AP image analyzer (Nireco, Tokyo, Japan).

#### Immunofluorescence staining

Liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in Tissue-Tek O.C.T compound<sup>TM</sup> (Sakura Finetek, Torrance, CA) and frozen. Frozen liver 5- $\mu\text{m}$  sections were prepared, washed with PBS, blocked with bovine serum albumin for 1 hr and incubated overnight with rabbit polyclonal antibodies against cyclin D1 (1:50 dilution)<sup>19</sup> and PPAR $\alpha$  (1:100 dilution),<sup>18</sup> and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).<sup>19</sup> After 5 washes with PBS, these sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or donkey anti-mouse IgG (Dako). The sections were mounted and viewed with an Olympus Fluoview confocal laser scanning microscope (Olympus, Tokyo, Japan). Two-thousand hepatocyte nuclei were examined for each mouse, and the number of hepatocyte nuclei stained with the antibodies against cyclin D1, PPAR $\alpha$  and PCNA was counted and expressed as a percentage.

#### Assessment of apoptotic hepatocytes

Liver samples were cut into small pieces and then fixed in 4% paraformaldehyde in PBS. These samples were dehydrated, embedded in paraffin and cut into 4- $\mu\text{m}$  sections. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using a MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories, Nagoya, Japan). The number of apoptotic hepatocytes in 2,000 hepatocytes was counted for each mouse, and expressed as a percentage.

#### Other methods

Hepatic caspase 3 activity was measured as described elsewhere.<sup>23</sup> For analysis of the nuclear contents of nonesterified fatty acids (NEFAs), ~150  $\mu\text{L}$  of the hepatocyte nuclear fraction, containing 1–2 mg of protein, was treated with a microsonicator. Lipid extraction was performed according to a modification of the method developed by Folch *et al.*<sup>24</sup> and the nuclear content of NEFAs was measured with a NEFA C-test kit<sup>TM</sup> (Wako).

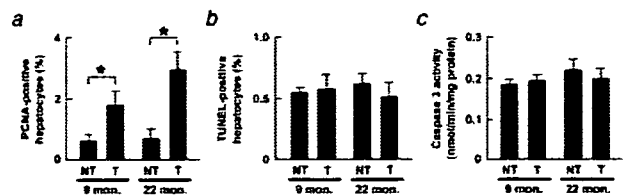
#### Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. The results are expressed as the mean  $\pm$  standard deviation. A probability value of less than 0.05 was considered to be statistically significant.

## Results

#### Accelerated hepatocyte proliferation in HCV core gene transgenic mice

To evaluate hepatocyte proliferative activity, PCNA-positive hepatocytes were counted in male transgenic mice and nontransgenic mice. Although hepatic inflammation and hepatocyte necrosis were not detected in either group, the numbers of PCNA-positive hepatocytes were significantly increased in the 9-month-old transgenic mice compared with the 9-month-old nontransgenic mice (Fig. 1a). The increase was more significant in the



**FIGURE 1** – Increase in hepatocyte proliferative activity. (a) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice. (b) The number of apoptotic hepatocytes. The number of TUNEL-positive hepatocytes in 2,000 hepatocytes was determined for each mouse. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ). (c) Caspase 3 activity. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ).

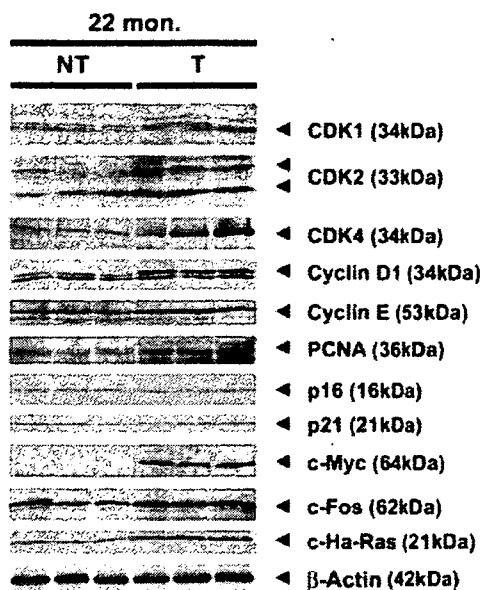
22-month-old transgenic mice (Fig. 1a). The numbers of PCNA-positive hepatocytes in the 22-month-old transgenic mice corresponded with those in HCV polyprotein-expressing transgenic mice with HCC.<sup>25</sup> On the other hand, the parameters of apoptosis, *i.e.*, the numbers of TUNEL-positive hepatocytes and hepatic caspase 3 activity, remained unchanged between the 2 groups at the same ages (Figs. 1b and 1c). These results suggest that spontaneous hepatocyte proliferation occurs as early as the age of 9 months and persists for a long time in HCV core gene transgenic mice.

#### Simultaneous induction of cell-cycle regulators and oncogene products in HCV core gene transgenic mouse livers

To examine the changes in the expression of proteins associated with hepatocyte division, the livers of the 9- and 22-month-old mice were subjected to immunoblot analysis. The levels of many proteins including cell-cycle regulators [cyclin-dependent kinase (CDK) 1, 2 and 4, cyclin D1 and E, and PCNA], and oncogene products (c-Myc, c-Fos and c-Ha-Ras) were significantly higher in the 22-month-old transgenic mice than in the control mice (Fig. 2). The levels of CDK inhibitors such as p16 and p21 were similar between the 2 groups. Similar results were obtained from the 9-month-old transgenic mice (data not shown). Time course changes in the expression of key G1-S checkpoint regulators, cyclin D1 and CDK4, are shown in Figure 3a. The simultaneous increase in the expression of cyclin D1 and CDK4 in the transgenic mice was continuous and more pronounced with age. Northern blot analysis revealed that the increase of these proteins occurred at the transcriptional level (Figs. 3b and 3c). Thus, these results reveal that various proteins which accelerate cell-cycle progression were induced simultaneously, persistently and age-dependently in the transgenic mice.

#### Correlative induction of PPAR $\alpha$ targets in HCV core gene transgenic mouse livers

As shown in Figure 2, the expression of many kinds of cell-cycle regulators and oncogene products is known to be induced by the functional activation of PPAR $\alpha$ .<sup>19,26–30</sup> To investigate whether PPAR $\alpha$  is activated in the livers of transgenic mice, the expression of representative PPAR $\alpha$  target genes,<sup>30</sup> acyl-CoA oxidase (AOX), peroxisomal thiolase (PT) and liver-type fatty acid-binding protein (L-FABP), was examined. As demonstrated in Figure 3a, the levels of AOX, PT, and L-FABP were increased in the 9-month-old transgenic mice compared with the nontransgenic mice, and the increase was more pronounced in the 22-month-old transgenic mice. Northern blot analysis demonstrated that the increase in these PPAR $\alpha$  targets was based on the increase in the transcriptional activity (Figs. 3b and 3c). The increase in the

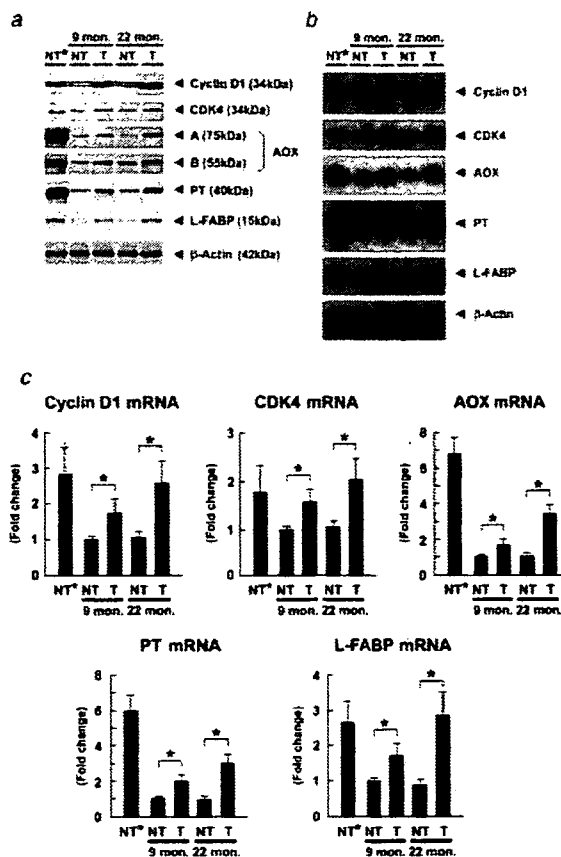


**FIGURE 2** – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200  $\mu$ g) was loaded in each lane. The band of  $\beta$ -actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice.

mRNA expression of AOX, PT and L-FABP corresponded exactly with that of cyclin D1 or CDK4 (Figs. 3b and 3c). Therefore, these results demonstrate the strong correlation between continuous and age-dependent induction of cell-cycle regulators and functional activation of PPAR $\alpha$  in these transgenic mice. Furthermore, the induction of these 5 proteins was also observed in wild-type mice treated with clofibrate, a potent PPAR $\alpha$  activator; however, the degree of the induction of AOX and PT in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Fig. 3), suggesting that the PPAR $\alpha$  activation found in the transgenic mice was not as intense as that in the mice treated with clofibrate.

#### Histological evaluation of PPAR $\alpha$ activation

An increase in the numbers of peroxisomes is associated with PPAR $\alpha$  activation.<sup>18</sup> To determine whether peroxisome proliferation occurs in the HCV core gene transgenic mice, cytochemical staining for peroxisomal catalase was performed. A scattered distribution of hepatocytes with numerous peroxisomes was observed in the 9-month-old transgenic mice (Fig. 4a). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Fig. 4a). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Fig. 4a). To quantitatively evaluate the degree of peroxisome proliferation, morphometric analysis of peroxisomes was conducted. The numerical density and volume density were significantly increased in the transgenic mice compared with those in the nontransgenic mice (Fig. 4b). The volume density, the most reliable parameter of peroxisome proliferation, was increased age-dependently in the transgenic mice, but the degree of the increase was not as prominent as that observed in mice with clofibrate administration (Fig. 4b). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Fig. 4a) is noteworthy, since it seems to correlate with the finding that intense expression of the core protein was observed only in particular hepatocytes.<sup>10</sup> These histological analyses reveal that spontaneous, continuous and age-dependent peroxisome proliferation and PPAR $\alpha$  activation occur heterogeneously in the transgenic mouse

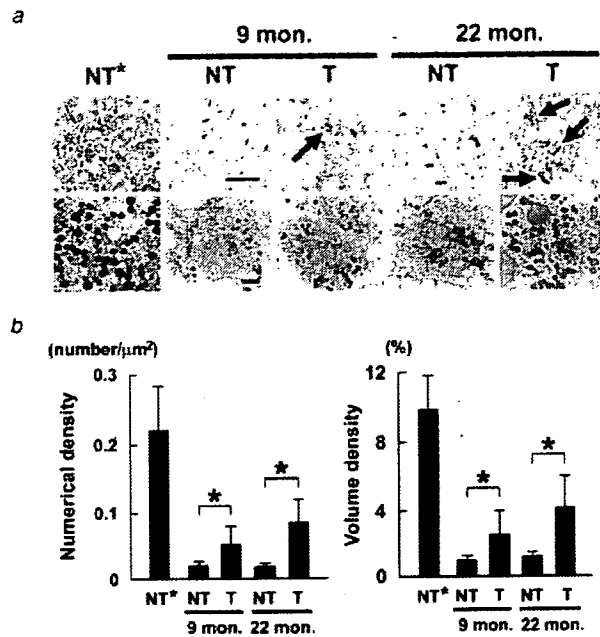


**FIGURE 3** – Analysis of PPAR $\alpha$ -regulated proteins. (a) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse ( $n = 8$ /group) was mixed and homogenized. Whole liver lysate (200  $\mu$ g for cyclin D1 and CDK4, and 20  $\mu$ g for others) was loaded in each lane. The band of  $\beta$ -actin was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT\*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks; A and B, full-length and truncated AOX, respectively. (b) Northern blot analysis concerning the proteins in (a). Ten milligram of liver pieces from each mouse ( $n = 8$ /group) was mixed and homogenized, and total liver RNA was extracted. Hepatic RNA (5  $\mu$ g) was separated on a denaturing gel, transferred to membranes and hybridized with the indicated <sup>32</sup>P-labeled cDNA probes. The blot of  $\beta$ -actin was used as the internal control. Results are representative of 4 independent experiments. (c) Quantification of hepatic mRNA levels. The mRNA level was quantified using a phosphorimager, normalized to that of  $\beta$ -actin, and subsequently normalized to that of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean  $\pm$  standard deviation. Abbreviations are identical with those in (b). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

livers, which is different from the response observed in the mice receiving clofibrate treatment.

#### Appearance of PPAR $\alpha$ - and cyclin D1-positive hepatocytes

We tried to detect abnormal hepatocytes to clarify the mechanism of hepatocarcinogenesis in the transgenic mice. On PPAR $\alpha$  immunofluorescence staining, PPAR $\alpha$  was primarily detected in the cytoplasm of the nontransgenic mice and the clofibrate-administered mice. Some hepatocytes having nuclei positively stained

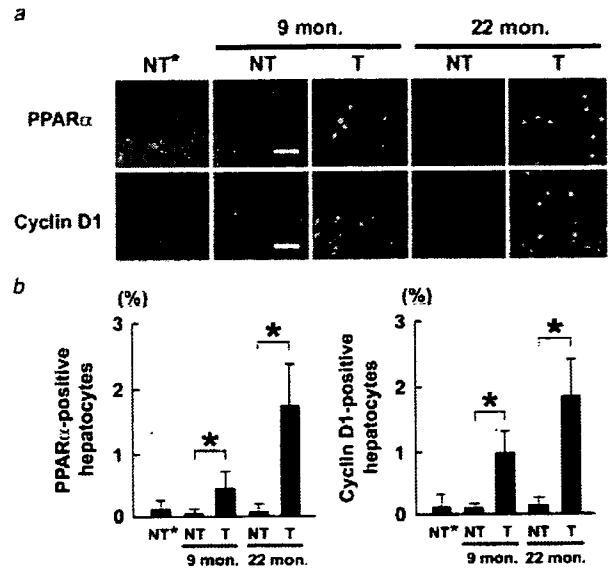


**FIGURE 4** – Cytochemical staining for hepatic peroxisomes. (a) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and electron photomicrographs of 9-month-old nontransgenic mice indicate 50 and 2  $\mu\text{m}$ , respectively. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT\*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (b) Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and the area of each individual peroxisome profile were measured in 10 photomicrographs for each mouse, and morphometric parameters such as numerical density and volume density were calculated. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ). Abbreviations are identical with those in (a). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

by anti-PPAR $\alpha$  antibody were detected only in the transgenic mice (Fig. 5a). Similar to the case of PPAR $\alpha$ , the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Fig. 5a). A few hepatocytes stained by anti-CDK4 antibody were also observed only in the transgenic mice (data not shown). The frequency of appearance of PPAR $\alpha$ -, or cyclin D1-positive hepatocytes was increased with age (Figs. 5a and 5b). Thus, the appearance of these specific hepatocytes in the transgenic mice seemed to be, at least in part, associated with sustained, age-dependent and heterogeneous PPAR $\alpha$  activation in the transgenic mice.

#### Changes in PPAR $\alpha$ levels

Since the expression of PPAR $\alpha$  is known to be enhanced by its activation,<sup>18,30</sup> the quantitative change in PPAR $\alpha$  was evaluated. The nuclear PPAR $\alpha$  level in the transgenic mice was increased age-dependently, as expected (Figs. 6a, upper panel and 6b), but the PPAR $\alpha$  level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPAR $\alpha$  in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figs. 6a, upper panel and 6b). Northern blot analysis revealed a higher PPAR $\alpha$  mRNA level in the clofibrate-treated mice than in the controls, although this parameter in the transgenic mouse groups of each age was similar to that in the controls (Figs. 6a, lower panel and 6b). These results indicate that the increase in



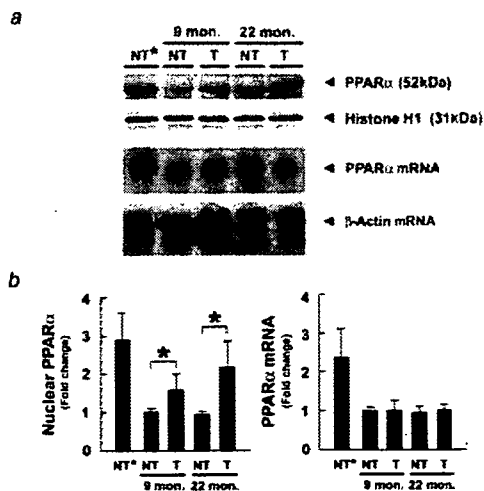
**FIGURE 5** – Immunofluorescence staining for PPAR $\alpha$  and cyclin D1. (a) Immunofluorescence staining using antibodies against PPAR $\alpha$  and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50  $\mu\text{m}$ . 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT\*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (b) The number of PPAR $\alpha$ -, or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPAR $\alpha$  or anti-cyclin D1 antibody was counted. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ). Abbreviations are identical with those of (a). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

nuclear PPAR $\alpha$  in the transgenic mice occurs mainly at the post-transcriptional level, which is distinct from that observed in the clofibrate-treated wild-type mice.

#### Stabilization of PPAR $\alpha$ through a possible interaction with HCV core protein in hepatocyte nuclei

The increased stability of PPAR $\alpha$  in hepatocyte nuclei is thought to be one of the possible causes of a disproportional increase in the nuclear PPAR $\alpha$  level. To examine this possibility, a pulse-chase experiment was performed using isolated hepatocytes. The half-life of nuclear PPAR $\alpha$  was  $\sim 7$  hr in the control mice and 12.5 hr in the transgenic mice (Fig. 7a). In addition, the intensity of the labeled PPAR $\alpha$  band (P in Fig. 7a, upper panels) in the control mice was similar to that in the transgenic mice. The finding that the [<sup>35</sup>S]methionine uptake in the hepatocytes from the control mice was similar to that from the transgenic mice suggests that the increase in nuclear PPAR $\alpha$  in the hepatocytes from the transgenic mice (Fig. 7a, lower right panel), as well as that *in vivo* (Fig. 6a, upper panel), is not because of the increased PPAR $\alpha$  transfer into the nucleus.

In the transgenic mice, HCV core protein accumulated in the nuclei, as evidenced by immunoelectron microscopy,<sup>11</sup> suggesting a possible interaction of the core protein with PPAR $\alpha$  in the nuclei. We therefore examined this possibility by anti-PPAR $\alpha$  IgG affinity chromatography. When proteins combining with PPAR $\alpha$  in hepatocyte nuclei were subjected to immunoblot analysis, the core protein was clearly detected (Fig. 7b). This result suggests the possibility of complex formation between the HCV core protein and PPAR $\alpha$ , which is consistent with an interaction of the core protein with retinoid X receptor (RXR)  $\alpha$ ,<sup>31</sup> an essential heterodimeric partner of PPAR $\alpha$ .<sup>32</sup> Thus, HCV core protein may



**FIGURE 6** – Analysis of PPAR $\alpha$ . (a) (Upper panels) Immunoblot analysis of nuclear PPAR $\alpha$ . Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse ( $n = 8$ /group) was mixed and homogenized to prepare the nuclear fraction. One-hundred microgram of nuclear protein was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and reacted with antibody against PPAR $\alpha$ . The band of histone H1 was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT\*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (Lower panels) Northern blot analysis of PPAR $\alpha$ . A sample used in Figure 3b was adopted. Hepatic RNA (5  $\mu$ g) was electrophoresed and hybridized with cDNAs for PPAR $\alpha$  and  $\beta$ -actin, respectively. Results are representative of 4 independent experiments. (b) Quantification of nuclear PPAR $\alpha$  levels and PPAR $\alpha$  mRNA levels. The nuclear PPAR $\alpha$  level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPAR $\alpha$  was quantified using a phosphorimager and normalized to that of  $\beta$ -actin. Values were subsequently normalized to those of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean  $\pm$  standard deviation. Abbreviations are identical with those in (a). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

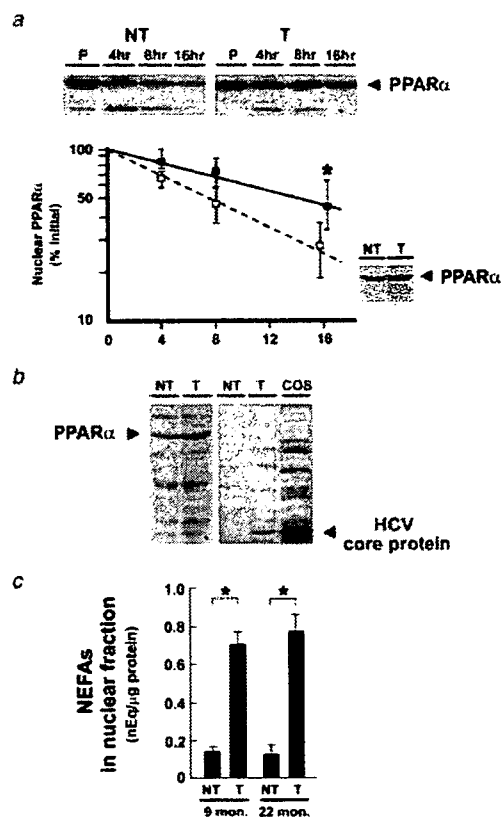
directly or indirectly affect the stability of PPAR $\alpha$  in hepatocyte nuclei.

#### Increase in PPAR $\alpha$ ligands

PPAR $\alpha$  is a ligand-activated transcription factor. Since the transgenic mice were fed a standard laboratory chow, endogenous substances such as NEFAs would serve as ligands of PPAR $\alpha$ <sup>33</sup>, therefore, the contents of NEFAs in hepatocyte nuclei were compared between the 2 groups. The levels of NEFAs in hepatocyte nuclei in the transgenic mice were  $\sim 5$  times higher than those in the control mice at the same age (Fig. 7c). This could account for the higher activation of PPAR $\alpha$  in the transgenic mice than in the controls.

#### Discussion

A large number of variables are involved in the induction of HCC by HCV core protein. While the precise mechanism underlying hepatocarcinogenesis in HCV core gene transgenic mice cannot be fully elucidated from this study, our results could provide some clues to explain this phenomenon. We found spontaneous, persistent, age-dependent and heterogeneous PPAR $\alpha$  activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association



**FIGURE 7** – Analyses of PPAR $\alpha$  stability, interaction between PPAR $\alpha$  with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (a) Pulse-label and pulse-chase experiments for nuclear PPAR $\alpha$  using isolated mouse hepatocytes. (Upper panels) Labeled PPAR $\alpha$  bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Material and methods. NT, nontransgenic mice; T, transgenic mice; P, pulse-label; 4, 8, 16 hr, pulse-chase for 4, 8, 16 hr, respectively. (Lower left panel) Intensity plot of PPAR $\alpha$  in 5 independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean  $\pm$  standard deviation. Open square, nontransgenic mice; black square, transgenic mice; \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice. (Lower right panel) Immunoblot analysis of an isolated hepatocyte nuclear fraction. NT, nontransgenic mice; T, transgenic mice. (b) Interaction between PPAR $\alpha$  and HCV core protein in the nucleus. (Left panel) Immunoblot analysis (PPAR $\alpha$ ) of the eluate on anti-PPAR $\alpha$  IgG affinity column chromatography. (Right panel) Immunoblot analysis (HCV core protein) of the same eluate. NT, nontransgenic mice; T, transgenic mice; COS, HCV core protein-overexpressing COS cell lysate. (c) Nuclear contents of NEFAs. The levels of NEFAs were measured using a hepatocyte nuclear fraction. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.

between HCV core protein-mediated hepatocarcinogenesis and persistent PPAR $\alpha$  activation.

Hepatocyte proliferation is influenced by various factors, such as mitogenic chemicals, cytokines, growth factors and transcription factors. It has been reported that various kinds of cell-cycle regulators and oncogene products are induced by PPAR $\alpha$  activation.<sup>19,26-30</sup> In particular, cyclin D1, CDK4, PCNA and c-Myc are potent and critical regulators of the G1-S checkpoint and cell-cycle progression,<sup>13,14</sup> and aberrant expression of these proteins is frequently detected in HCV-related HCC.<sup>34-37</sup> These key regulators are known to be induced in a PPAR $\alpha$ -dependent manner in mice<sup>19,30</sup>; the continuous induction of these proteins and the

resultant acceleration of hepatocyte proliferation found in the transgenic mice may be attributed to persistent PPAR $\alpha$  activation. In the current study, we demonstrated that there was a great variety of the intensity of PPAR $\alpha$  activation among different hepatocytes (Fig. 4). This persistent and heterogeneous PPAR $\alpha$  activation found especially in the transgenic mice may be linked with the age-dependent and multicentric hepatocarcinogenesis induced by the core protein.

It is well-known that the long-term administration of potent peroxisome proliferators such as fibrate drugs can induce hepatocarcinogenesis in rodents.<sup>29</sup> The findings observed in the transgenic mice markedly differ from those in mice with long-term treatment of peroxisome proliferators in several ways. Namely, the transgenic mice show no intense increase in AOX and PT (Fig. 3), no increase in PPAR $\alpha$  mRNA (Fig. 6), heterogeneous peroxisome proliferation (Fig. 4) and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPAR $\alpha$  or anti-cyclin D1 antibody (Fig. 5). Therefore, the mode of PPAR $\alpha$  activation and the mechanism of hepatocarcinogenesis caused by HCV core protein expression are indeed unique.

One of the mechanisms involved in the core protein-specific PPAR $\alpha$  activation in mice is stabilization of PPAR $\alpha$  in hepatocyte nuclei through a possible interaction with the core protein. In cultured cells expressing the core protein, it has been demonstrated that the core protein interacts with the PPAR $\alpha$ -RXR $\alpha$  heterodimer and enhances the transcriptional activation mediated by PPAR $\alpha$  regardless of the presence or absence of its ligands.<sup>31</sup> Since PPAR $\alpha$  is ubiquitinated and degraded *via* the proteasome pathway,<sup>38</sup> it may be postulated that HCV core protein directly or indirectly influences the degradation pathway. It has been reported that the core protein binds to the proteasome activator PA28 $\gamma$ <sup>39</sup> which is known to combine with steroid receptor coactivator-3 and to accelerate its degradation.<sup>40</sup> Another possible mechanism is an increase in NEFAs in hepatocyte nuclei. The PPAR $\alpha$  activation induced by the core protein enhances the expression of L-FABP,<sup>30</sup> which serves as a transporter of NEFAs into nuclei. Indeed, real-time confocal and multiphoton laser scanning microscopy has shown that L-FABP expression significantly increased the total uptake of medium- and long-chain fluorescent fatty acids into the nuclei of living cells.<sup>41</sup> Thus, increased L-FABP expression may facilitate the shuttling of NEFAs into hepatocyte nuclei for donating NEFAs to PPAR $\alpha$ , leading to PPAR $\alpha$  activation and further increase in L-FABP expression. Moreover, the binding of ligands

causes conformational alternation of PPAR $\alpha$ <sup>42</sup> and further stabilizes it in nuclei,<sup>32</sup> resulting in synergistic PPAR $\alpha$  activation. Therefore, these findings concerning spontaneous and persistent PPAR $\alpha$  activation induced by the core protein enable us to partially explain the precise molecular mechanism of hepatocarcinogenesis in HCV core gene transgenic mice.

The results obtained from the current study are consistent with the findings observed in chronically HCV-infected patients in several ways. That is, like the transgenic mice in the present study, chronically HCV-infected patients have been reported to show accelerated hepatocyte proliferation,<sup>43</sup> an increase in CDK4, cyclin D1 and E, PCNA, c-Myc and c-Fos,<sup>34-37</sup> and multicentric appearance of HCC.<sup>44</sup> Furthermore, it has been reported that a massive proliferation of peroxisomes was found in human nontumorous liver tissue adjacent to HCC.<sup>45</sup> Thus the earlier findings, including the unique function of HCV core protein *in vivo* and the diverse and significant roles of PPAR $\alpha$ , may help to partially understand the onset and development of HCC in patients with chronic HCV infection. It has been demonstrated that the function of hepatic PPAR $\alpha$  was impaired in patients with chronic HCV infection,<sup>46</sup> which is different from our results. Since HCC had not yet developed in the patients in the report, this discrepancy might derive from differences in the stage of the hepatocarcinogenic process.

The interpretation based on persistent activation of PPAR $\alpha$  pertains to only one possible mechanism of hepatocarcinogenesis induced by the effects of HCV core protein. We cannot rule out the presence of other mechanisms. The exact relationship between PPAR $\alpha$  activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPAR $\alpha$  activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPAR $\alpha$ -null mice bearing the core protein gene do not represent development of HCC.

In conclusion, we demonstrated for the first time that spontaneous, persistent, age-dependent and heterogeneous activation of PPAR $\alpha$  occurred in HCV core protein transgenic mice and caused continuous enhancement of hepatocyte proliferation, which may have contributed to the age-dependent and multicentric hepatocarcinogenesis observed in these mice. In addition, we observed nuclear stabilization of PPAR $\alpha$  and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPAR $\alpha$  activation.

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**Short Communication****Prevalence of hepatitis B virus infection in Japanese patients with HIV**

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998

patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

**Key words:** co-infection, hepatitis B, HIV, liver disease.

**INTRODUCTION**

**H**EPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.<sup>1</sup> The prevalence of HIV in the USA is also <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 transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the

prevalence of HBV co-infection is 6–14%.<sup>1,2</sup> Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,<sup>3,4</sup> in addition to HCV infection.<sup>5</sup>

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,<sup>1,6,7</sup> which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.<sup>8</sup> It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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Received 22 June 2007; revision 29 July 2007; accepted 31 July 2007.



inhibitory effects on the replication of HBV.<sup>9–12</sup> A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.<sup>11,12</sup> Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.<sup>9,10</sup>

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,<sup>13</sup> where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.<sup>14</sup> There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,<sup>14</sup> and approximately 0.8 million chronic HBV carriers.<sup>15</sup> However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

## PATIENTS AND METHODS

**I**N THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as “others”; (xiv) the number of HBsAg-positive patients among (xiii); and

(xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed 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 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.<sup>15</sup> It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.<sup>14</sup> However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).<sup>16</sup> Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as “others”, most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.<sup>15</sup> Among these, 77 (3.4%)

**Table 1** Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV-HBV co-infected patients. This shows a contrast to the status of HIV-HCV co-infection, in which the majority of HIV-HCV co-infected Japanese patients contracted both viruses from blood products.<sup>16</sup>

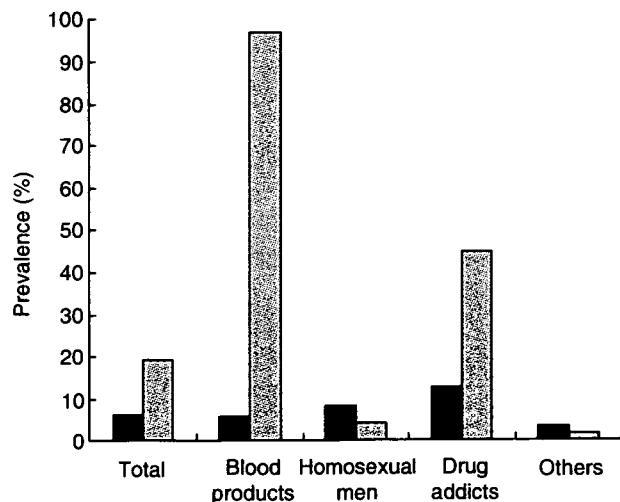
There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20-49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV-HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV-HBV co-infected

patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV-HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

## DISCUSSION

**A** LONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV-HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.<sup>14</sup> In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if



**Figure 1** Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (▨), anti-HCV, antibody to hepatitis C virus. \*Prevalence rates of anti-HCV are obtained from Koike K *et al.*<sup>16</sup>

**Table 2** Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				Total
	0	1-19	20-49	50+	
0	53	76	13	1	143
1-9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV-HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;<sup>16</sup> the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.<sup>16</sup> It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV<sup>17</sup> may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.<sup>15</sup>

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV-HBV co-infected patients in the current study. Nonetheless, one-third of HIV-HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV-HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV-HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV-HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV-HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV-HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

## ACKNOWLEDGMENTS

WE THANK MS. Ogawa for her assistance in the questionnaire inquiry. This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan (Research on HIV/AIDS). We thank the hospitals in the HIV/AIDS Network of Japan for the responses to the questionnaire. The list of hospitals can be view at [http://www.acc.go.jp/mLhw/mLhw\\_frame.htm](http://www.acc.go.jp/mLhw/mLhw_frame.htm).

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# PPAR $\alpha$ activation is essential for HCV core protein–induced hepatic steatosis and hepatocellular carcinoma in mice

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Transgenic mice expressing HCV core protein develop hepatic steatosis and hepatocellular carcinoma (HCC), but the mechanism underlying this process remains unclear. Because PPAR $\alpha$  is a central regulator of triglyceride homeostasis and mediates hepatocarcinogenesis in rodents, we determined whether PPAR $\alpha$  contributes to HCV core protein–induced diseases. We generated PPAR $\alpha$ -homozygous, -heterozygous, and -null mice with liver-specific transgenic expression of the core protein gene (*Ppara*<sup>+/+</sup>:HCVcpTg, *Ppara*<sup>+/-</sup>:HCVcpTg, and *Ppara*<sup>-/-</sup>:HCVcpTg mice. Severe steatosis was unexpectedly observed only in *Ppara*<sup>+/-</sup>:HCVcpTg mice, which resulted from enhanced fatty acid uptake and decreased mitochondrial  $\beta$ -oxidation due to breakdown of mitochondrial outer membranes. Interestingly, HCC developed in approximately 35% of 24-month-old *Ppara*<sup>+/-</sup>:HCVcpTg mice, but tumors were not observed in the other genotypes. These phenomena were found to be closely associated with sustained PPAR $\alpha$  activation. In *Ppara*<sup>-/-</sup>:HCVcpTg mice, PPAR $\alpha$  activation and the related changes did not occur despite the presence of a functional *Ppara* allele. However, long-term treatment of these mice with clofibrate, a PPAR $\alpha$  activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. Thus, our results indicate that persistent activation of PPAR $\alpha$  is essential for the pathogenesis of hepatic steatosis and HCC induced by HCV infection.

## Introduction

HCV is one of the major causes of chronic hepatitis, whereas patients with persistent HCV infection have a high incidence of hepatocellular carcinoma (HCC) (1, 2). Occurrence of HCC associated with chronic HCV infection has increased over the past 2 decades (3–5), and chronic HCV infection is recognized as a serious debilitating disease. However, the mechanism in which chronic HCV infection mediates hepatocarcinogenesis remains unclear.

HCV core protein was shown to have oncogenic potential (6). To examine how HCV core protein participates in HCV-related hepatocarcinogenesis, transgenic mouse lines were established in which HCV core protein is expressed constitutively in liver at cellular levels similar to those found in chronic HCV-infected patients (7). These mice exhibited hepatic steatosis (7) and insulin resistance (8) as early as 3 months of age; on further aging, these symptoms worsened and hepatic adenomas developed in approximately 30% of mice between 16 and 18 months of age (9). Finally, HCC was found within hepatic adenomas in a classic “nodule-in-nodule” pathology (9). Interestingly, no hepatic inflammation or fibrosis was found in these mice throughout

the course of HCC development (9), which suggested that the HCV core protein itself induces hepatic steatosis and HCC independently of hepatitis.

Several studies support the contention that hepatic steatosis promotes the development of HCC (10). Epidemiologic data have identified hepatic steatosis as a major accelerating factor of hepatocarcinogenesis in chronic HCV-infected patients (11). Moreover, increases in ROS production that can cause oxidative DNA damage, mitochondrial abnormalities, and accelerated hepatocyte proliferation were observed in the steatotic livers (12–14). Thus, an intriguing possibility has emerged that alteration of fatty acid metabolism in hepatocytes may be central to the pathogenesis of HCC induced by HCV core protein.

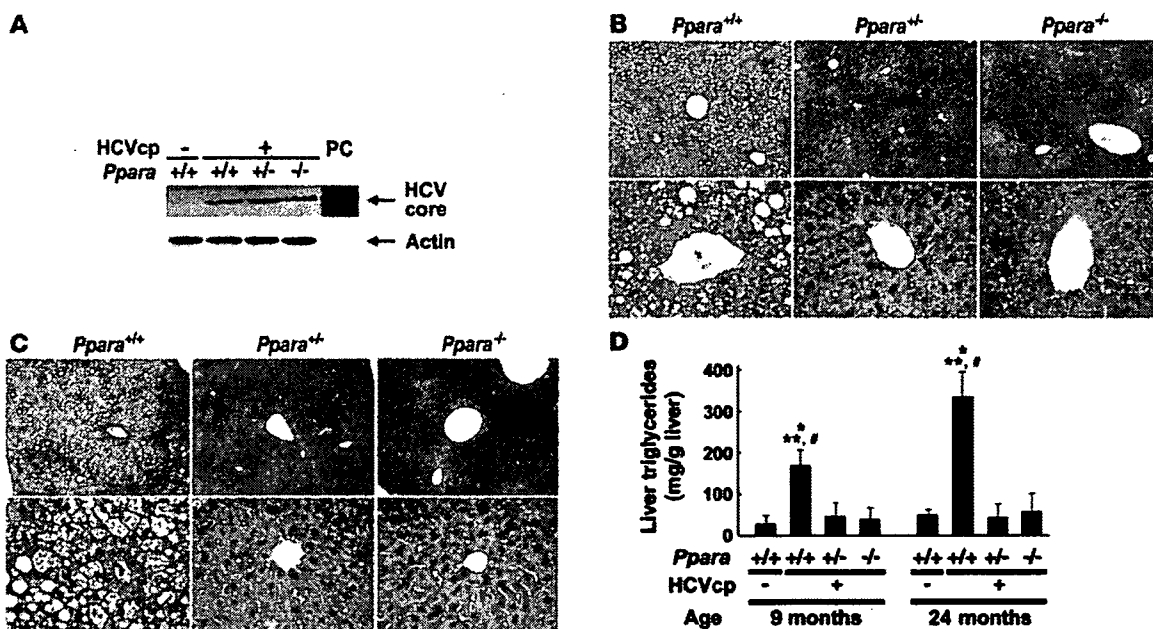
PPARs are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily; 3 isoforms designated as  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  exist, all of which are involved in lipid homeostasis (15). PPAR $\alpha$  regulates constitutive transcription of genes encoding fatty acid–metabolizing enzymes (16) and is associated with the maintenance of fatty acid transport and metabolism, primarily in liver, kidney, and heart. Administration of PPAR $\alpha$  agonists, such as the widely prescribed fibrate drugs clofibrate, gemfibrozil, and fenofibrate, ameliorate hyperlipidemia in humans (17) and hepatic steatosis in mice (18).

On the other hand, long-term administration of PPAR $\alpha$  ligands to rodents causes accelerated hepatocyte proliferation, increased ROS generation, and development of HCC (19, 20). Disruption of the PPAR $\alpha$  gene was shown to prevent the development of HCC caused by long-term exposure to PPAR $\alpha$  activators (21). Interestingly, accumulation of fatty acids/triglycerides in hepatocytes

**Nonstandard abbreviations used:** ACC, acetyl-CoA carboxylase; AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; CYP4A1, cytochrome P450 4A1; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; HCVcpTg, HCV core protein-expressing transgenic; L-FABP, liver fatty acid-binding protein; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, microsomal transfer protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCNA, proliferating cell nuclear antigen; RXR $\alpha$ , retinoid X receptor  $\alpha$ .

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Citation for this article:** *J. Clin. Invest.* 118:683–694 (2008). doi:10.1172/JCI33594.



**Figure 1**

Phenotype changes in transgenic mouse liver. (A) Immunoblot analysis of HCV core protein expression in livers of 9-month-old mice. Because no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver prepared from each mouse ( $n = 6$ /group) was mixed and homogenized. Whole-liver lysate (50  $\mu$ g protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 independent experiments. PC, lysate prepared from COS-1 cells overexpressing HCV core protein as a positive control. (B) Histological appearance of hematoxylin- and eosin-stained liver sections from 9-month-old HCVcpTg mice. Upper and lower rows show a lower ( $\times 40$ ) and higher ( $\times 400$ ) magnification, respectively. Microvesicular and macrovesicular steatosis was found only in  $Ppara^{+/+}$ :HCVcpTg mice. No inflammation or hepatocyte degeneration was evident in any of the genotypes. (C) Histological appearance of hematoxylin- and eosin-stained liver sections from 24-month-old HCVcpTg mice. Upper and lower rows show a lower ( $\times 40$ ) and higher ( $\times 400$ ) magnification, respectively. Hepatic steatosis was marked in  $Ppara^{+/+}$ :HCVcpTg mice, but not in other mice. Hepatic inflammation, fibrosis, and hepatocyte degeneration were not observed. In  $Ppara^{+/+}$ :HCVcpTg and  $Ppara^{-/-}$ :HCVcpTg mice, dysplastic hepatocytes and precancerous lesions were not detected throughout the entire liver. (D) Content of liver triglycerides. Results are expressed as the mean  $\pm$  SD ( $n = 6$ /group) and compared between genotypes at the same age. \* $P < 0.05$  compared with  $Ppara^{+/+}$  nontransgenic mice; \*\* $P < 0.05$  compared with  $Ppara^{+/-}$ :HCVcpTg mice; # $P < 0.05$  compared with  $Ppara^{-/-}$ :HCVcpTg mice.

could lead to continuous PPAR $\alpha$  activation because of the presence of fatty acid metabolites that serve as natural PPAR $\alpha$  ligands. For example, mice lacking expression of the peroxisomal acyl-CoA oxidase (AOX) gene showed massive accumulation of very-long-chain fatty acids in hepatocytes, severe microvesicular steatosis, chronic PPAR $\alpha$  activation, and development of hepatic adenoma and HCC by 15 months of age (22). These results suggest a strong contribution of activated PPAR $\alpha$  to liver tumorigenesis.

On the basis of several lines of evidence, we hypothesized that PPAR $\alpha$  might contribute to hepatocarcinogenesis in HCV core protein-expressing transgenic (HCVcpTg) mice. To explore this possibility, PPAR $\alpha$ -homozygous ( $Ppara^{+/+}$ ), PPAR $\alpha$ -heterozygous ( $Ppara^{+/-}$ ), and PPAR $\alpha$ -null ( $Ppara^{-/-}$ ) mice bearing the HCV core protein gene, designated  $Ppara^{+/+}$ :HCVcpTg,  $Ppara^{+/-}$ :HCVcpTg, and  $Ppara^{-/-}$ :HCVcpTg mice, were generated, and phenotypic changes were examined. Surprisingly, we found that severe hepatic steatosis and HCC induced by HCV core protein developed only in  $Ppara^{+/+}$  mice, which were related to persistent PPAR $\alpha$  activation.

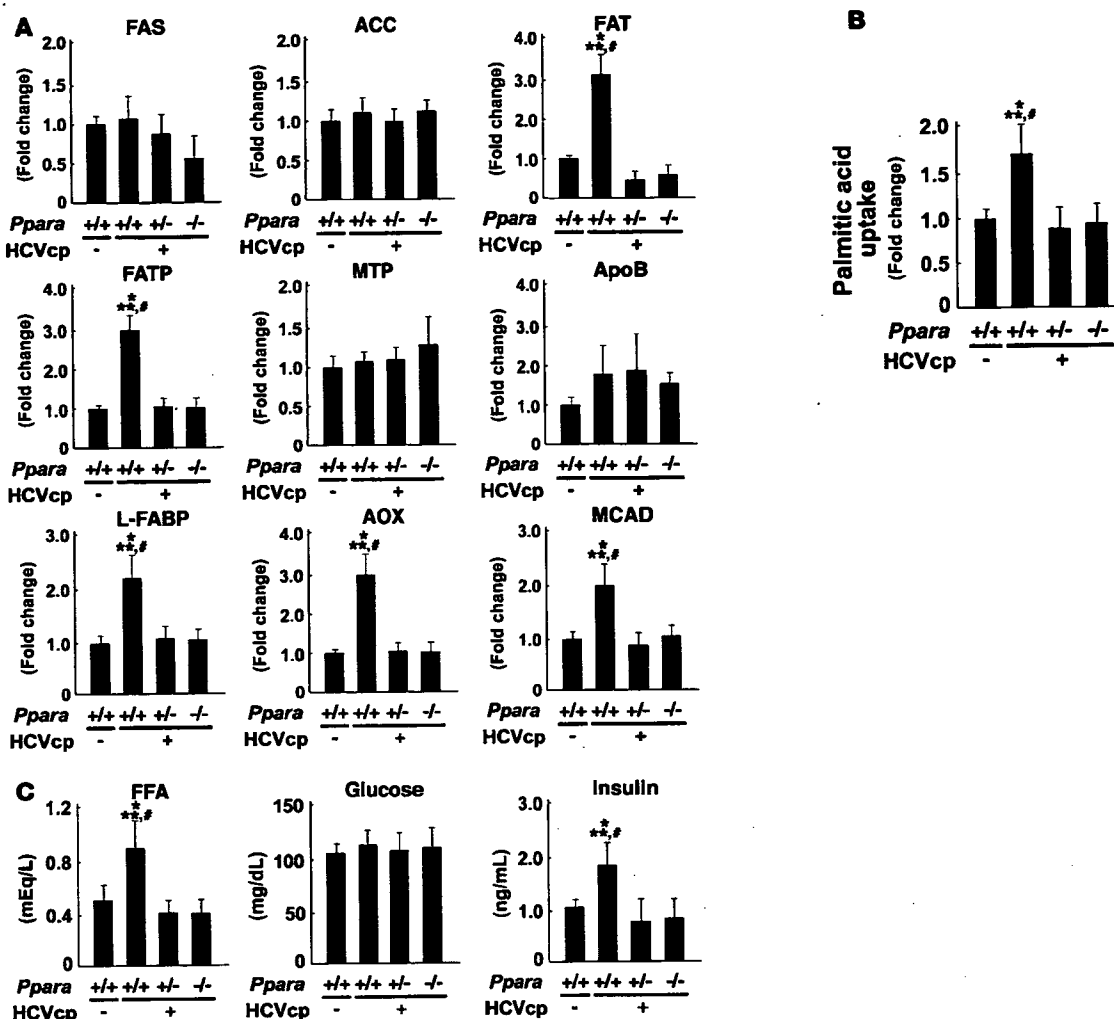
**Results**

*Expression of HCV core protein in transgenic mice.*  $Ppara^{-/-}$ :HCVcpTg and  $Ppara^{+/-}$ :HCVcpTg mice appeared healthy, and body weight in both genotypes was similar to that of  $Ppara^{+/+}$ :HCVcpTg and  $Ppara^{+/+}$  mice

without the transgene. When hepatic expression of HCV core protein in 9-month-old transgenic mice was examined by immunoblot analysis, it was similar among  $Ppara^{+/+}$ :HCVcpTg,  $Ppara^{+/-}$ :HCVcpTg, and  $Ppara^{-/-}$ :HCVcpTg mice (Figure 1A) and was also similar to expression in HCVcpTg mice reported previously (7, 9). Age and sex had only a minor influence on the hepatic expression of HCV core protein.

*Requirement of homozygous PPAR $\alpha$  for the development of hepatic steatosis in transgenic mice.* Livers of 9-month-old male HCVcpTg mice with or without the  $Ppara$  allele were examined. Those of  $Ppara^{+/+}$ :HCVcpTg mice were soft, slightly enlarged, and light in color and histologically showed macrovesicular and microvesicular steatosis with no apparent inflammation or hepatocyte necrosis (Figure 1B), in agreement with previous reports (7, 9). Biochemical analysis of liver extracts showed marked hepatic accumulation of triglycerides (Figure 1D). In contrast, livers of 9-month-old  $Ppara^{+/-}$ :HCVcpTg and  $Ppara^{-/-}$ :HCVcpTg mice showed neither histological abnormalities nor accumulation of triglycerides (Figure 1, B and D). Hepatic levels of free fatty acids in  $Ppara^{+/+}$ :HCVcpTg mice were approximately 3 times those in  $Ppara^{+/-}$ :HCVcpTg and  $Ppara^{-/-}$ :HCVcpTg mice or  $Ppara^{+/+}$  mice not expressing the HCV core protein.

In 24-month-old  $Ppara^{+/+}$ :HCVcpTg mice, hepatic steatosis was found (Figure 1C), and the hepatic levels of triglycerides were further increased (Figure 1D). Apparent inflammation, hepatocyte

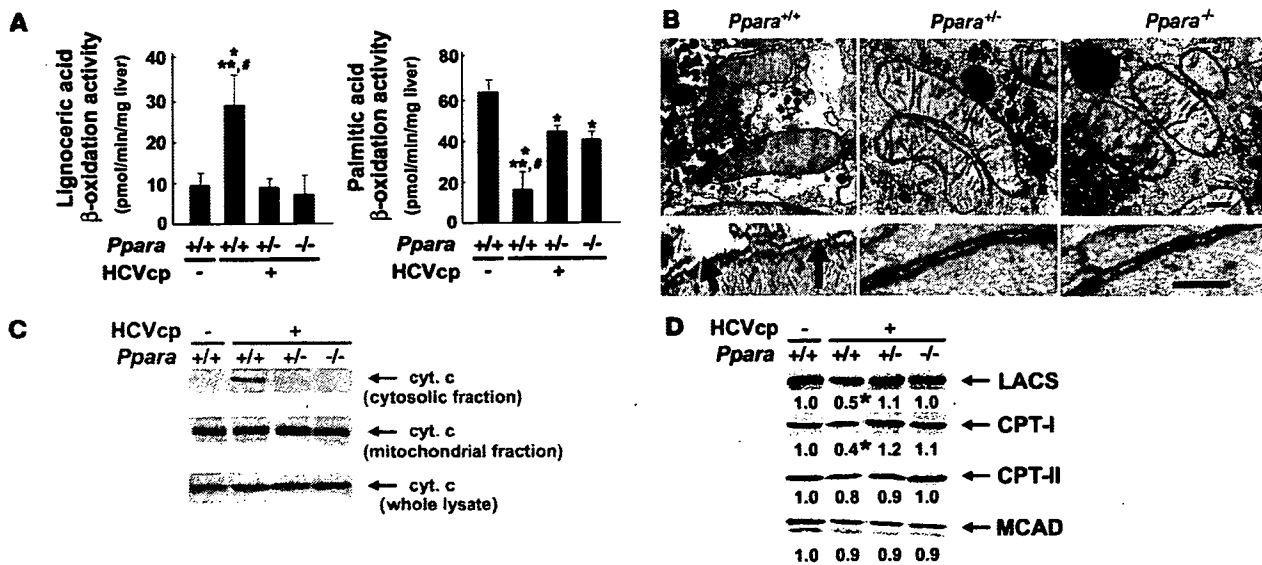


**Figure 2**  
 Analyses of factors associated with hepatic fatty acid and triglyceride metabolism. (A) Expression of genes associated with fatty acid and triglyceride metabolism in 9-month-old mouse livers. Total RNA was extracted from each mouse liver, and mRNA levels were determined by RT-PCR. mRNA levels were normalized by those of GAPDH and subsequently normalized by those in *Ppara*<sup>+/+</sup> nontransgenic mice. Results are expressed as the mean  $\pm$  SD ( $n = 6$ /group). \* $P < 0.05$  compared with *Ppara*<sup>+/+</sup> nontransgenic mice; \*\* $P < 0.05$  compared with *Ppara*<sup>+/-</sup>:HCVcpTg mice; # $P < 0.05$  compared with *Ppara*<sup>+/+</sup>:HCVcpTg mice. (B) Uptake of fatty acids in 9-month-old mouse livers. Liver slices obtained from 3 mice in each group were incubated in medium containing 0.8 mM [<sup>14</sup>C]palmitic acid for 7 h. Fatty acid uptake ability was estimated by the sum of palmitic acid converted to CO<sub>2</sub> and ketone bodies with that incorporated into total cellular lipids after incubation. The experiment was repeated 3 times. Results were normalized by those of *Ppara*<sup>+/+</sup> nontransgenic mice and expressed as the mean  $\pm$  SD. (C) Plasma concentrations of free fatty acids, glucose, and insulin. After an overnight fast, blood was obtained from each mouse and the above variables were determined. Results are expressed as the mean  $\pm$  SD ( $n = 6$ /group).

degeneration and necrosis, and fibrosis were not detected. On the other hand, *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice showed no steatosis (Figure 1, C and D). These results indicate that hepatic steatosis develops in *Ppara*<sup>+/-</sup>:HCVcpTg mice, but not in *Ppara*<sup>-/-</sup>:HCVcpTg and *Ppara*<sup>+/-</sup>:HCVcpTg mice.

**Hepatic fatty acid and triglyceride metabolism in transgenic mice.** To investigate the mechanism responsible for the development of severe steatosis in *Ppara*<sup>+/-</sup>:HCVcpTg mice, the expression of genes associated with fatty acid and triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As shown in Figure 2A, the mRNA levels of genes related

to de novo lipogenesis (fatty acid synthase [FAS] and acetyl-CoA carboxylase [ACC]) and secretion of VLDL (microsomal transfer protein [MTP] and apoB) were constant in all groups. The mRNA levels of fatty acid translocase (FAT) and fatty acid transport protein (FATP), which are associated with the uptake of fatty acids into hepatocytes, were significantly increased only in *Ppara*<sup>+/-</sup>:HCVcpTg mice, but the mRNA levels of hepatic triglyceride lipase, another contributor to fatty acid uptake, remained unchanged (data not shown). The mRNA levels of liver fatty acid binding protein (L-FABP) were also elevated only in *Ppara*<sup>+/-</sup>:HCVcpTg mice. Surprisingly, the mRNA levels of AOX and medium-chain acyl-CoA



**Figure 3**

Analyses of mitochondrial abnormalities. (A) Lignoceric and palmitic acid  $\beta$ -oxidation activities in 9-month-old mice. Results are expressed as the mean  $\pm$  SD ( $n = 6$ /group). \* $P < 0.05$  compared with *Ppara*<sup>+/+</sup> nontransgenic mice; \*\* $P < 0.05$  compared with *Ppara*<sup>+/+</sup>:HCVcpTg mice; # $P < 0.05$  compared with *Ppara*<sup>-/-</sup>:HCVcpTg mice. (B) Electron microscopic features of hepatic mitochondria of 9-month-old HCVcpTg mice. Upper and lower rows show a lower and higher magnification, respectively. In *Ppara*<sup>+/+</sup>:HCVcpTg mice, some mitochondria showing discontinuance of outer membranes (arrows) and amorphous inner structures were observed. In *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice, mitochondria appeared normal; the scale bars represent 200 nm (top) and 30 nm (bottom), respectively. (C) Immunoblot analysis of cytochrome *c* in 9-month-old mice. Whole-liver lysate, mitochondrial fraction, or cytosolic fraction (50  $\mu$ g protein) was loaded in each well. Results are representative of 4 independent experiments. (D) Immunoblot analysis of representative mitochondrial  $\beta$ -oxidation enzymes using a mitochondrial fraction prepared from 9-month-old mouse livers. The mitochondrial fraction (20  $\mu$ g protein) was loaded in each well. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically and normalized by that in *Ppara*<sup>+/+</sup> nontransgenic mouse. The mean value of the fold changes is shown under the representative band. LACS, long-chain acyl-CoA synthase; CPT, carnitine palmitoyl-CoA transferase.

dehydrogenase (MCAD), a rate-limiting enzymes in the peroxisomal and mitochondrial  $\beta$ -oxidation pathways, respectively, were significantly increased in *Ppara*<sup>+/+</sup>:HCVcpTg mice. When fatty acid uptake ability was measured in fresh liver slices, it was significantly enhanced only in *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 2B). Additionally, plasma free fatty acid levels were higher in these mice than in mice in the other groups. Although there were no differences in fasting plasma glucose levels between the groups, hyperinsulinemia was observed only in *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 2C), in agreement with the previous observation that significant insulin resistance developed in these mice (8). Similar results were obtained from 24-month-old mice (data not shown). These results combined show that the increased plasma fatty acid levels, which were likely due to enhanced peripheral fatty acid release caused by insulin resistance, and the increase in fatty acid uptake ability are consistent with steatogenesis in *Ppara*<sup>+/+</sup>:HCVcpTg mice.

**Decreased mitochondrial  $\beta$ -oxidation in transgenic mice.** Although the transcriptional activities of major  $\beta$ -oxidation enzymes were markedly increased, *Ppara*<sup>+/+</sup>:HCVcpTg mice had severe steatosis. To explore this discrepant result, peroxisomal and mitochondrial  $\beta$ -oxidation activities were measured using lignoceric and palmitic acids as substrates, respectively. The lignoceric acid-degrading capacity was increased only in *Ppara*<sup>+/+</sup>:HCVcpTg mice, where it corresponded to an increase in AOX expression. However, the capacity for palmitic acid degradation, which occurs particularly in mitochondria, was significantly lower in *Ppara*<sup>+/+</sup>:HCVcpTg mice than in *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice (Figure 3A).

Thus, decreased mitochondrial  $\beta$ -oxidation ability was considered to be another important mechanism for the development of steatosis induced by the core protein.

We further evaluated mitochondrial abnormalities. In electron microscopic examination, discontinuous outer membranes (Figure 3B, arrows) and lack of an internal structure were observed in some mitochondria of *Ppara*<sup>+/+</sup>:HCVcpTg mouse livers, in agreement with the previous report (9). However, these abnormalities were not seen in *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice (Figure 3B). Immunoblot analysis showed that cytochrome *c*, which is usually localized in the mitochondrial intermembrane space, was present in the cytosolic fractions of *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 3C). Moreover, immunoblot analysis using mitochondrial fractions showed that the expression levels of long-chain acyl-CoA synthase and carnitine palmitoyl-CoA transferase-I, which are enzymes indispensable to the initial step of mitochondrial  $\beta$ -oxidation and are localized mainly in mitochondrial outer membranes, were significantly decreased only in *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 3D).

Overall, these results suggest that homozygous PPAR $\alpha$  is essential to the pathogenesis of hepatic steatosis induced by the HCV core protein, which results from a decrease in mitochondrial fatty acid degradation capacity caused by the breakdown of mitochondrial outer membranes and a disproportionate increase in the uptake of fatty acids. Interestingly, steatosis and the related changes did not occur in *Ppara*<sup>+/-</sup> and *Ppara*<sup>-/-</sup> mice expressing the HCV core protein, which suggested that these changes were not caused by the core protein itself.





**Table 1**  
Incidence of HCC in 24-month-old mice

HCV core protein	<i>Ppara</i>	Mice (n)	Mice with HCC (n)	Incidence (%)
-	+/+	20	0	0
-	+/-	18	0	0
-	-/-	20	0	0
+	+/+	17	6	35.3 <sup>A</sup>
+	+/-	16	0	0
+	-/-	14	0	0

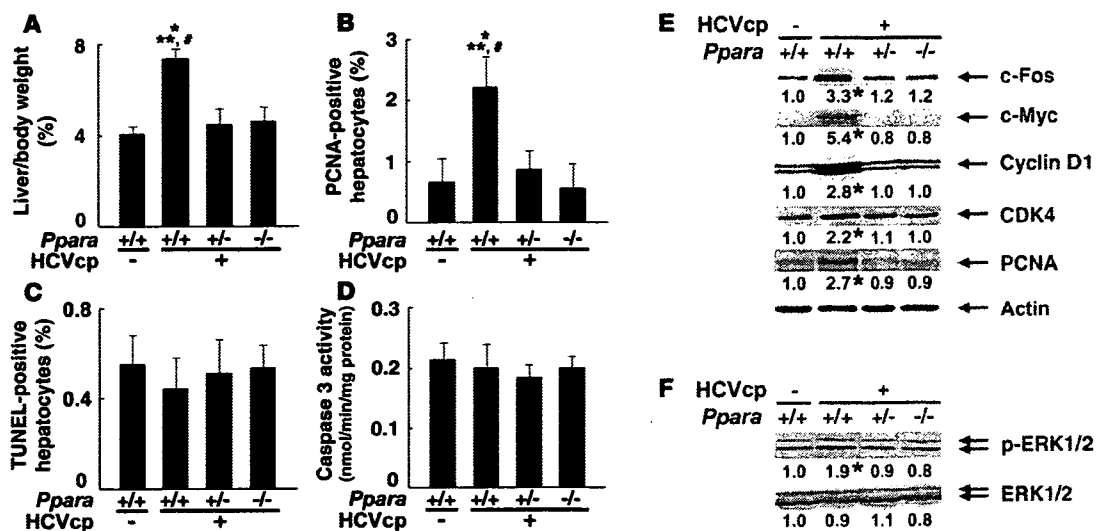
Mice were killed at 24 months of age for analysis. HCC was diagnosed according to histological findings. <sup>A</sup>*P* < 0.05 compared with *Ppara*<sup>+/+</sup> nontransgenic mice, *P* < 0.05 compared with *Ppara*<sup>+/+</sup>:HCVcpTg mice, *P* < 0.05 compared with *Ppara*<sup>-/-</sup>:HCVcpTg mice.

**Requirement of homozygous PPAR $\alpha$  for hepatic tumor development in transgenic mice.** At 9 months of age, hepatic nodules were not observed at all in transgenic mice, whereas, at 24 months, approximately 35% of *Ppara*<sup>+/+</sup>:HCVcpTg mice had macroscopically evident hepatic nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, *Ppara*<sup>-/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice of the same ages developed no evidence of hepatic tumors, despite the expression of HCV core protein at similar levels to those found in *Ppara*<sup>+/+</sup>:HCVcpTg mice (Table 1). Microscopic examination showed that there were no dysplastic cells

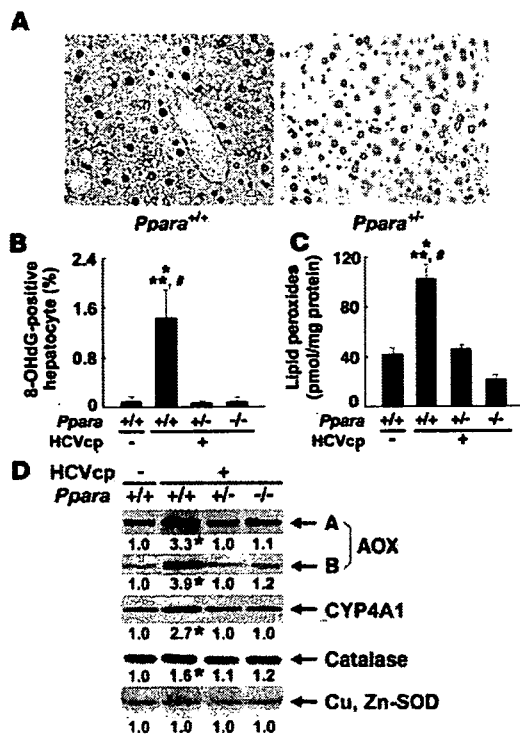
or precancerous lesions throughout the livers in *Ppara*<sup>-/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice (Figure 1C). These results provide strong evidence that homozygous PPAR $\alpha$  is essential for hepatic tumorigenesis induced by HCV core protein.

**Increased hepatocyte proliferation only in *Ppara*<sup>+/+</sup>:HCVcpTg mice.** Because sustained acceleration of hepatocyte proliferation relative to apoptosis may promote the development of HCC, these opposing processes were quantified in the livers of 24-month-old mice. Both the liver-to-body weight ratio and the number of hepatocytes expressing proliferating cell nuclear antigen (PCNA) were increased only in *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 4, A and B). In contrast, the number of TUNEL-positive hepatocytes and the hepatic caspase 3 activity, indicators of hepatocyte apoptosis, remained similar among the 3 mouse strains (Figure 4, C and D). Interestingly, despite the presence of HCV core protein, the amounts of these proliferative and apoptotic markers in *Ppara*<sup>-/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice were similar to those in *Ppara*<sup>+/+</sup> nontransgenic mice. Expression levels of several proteins, such as protooncogenes (c-Fos and c-Myc), cell-cycle regulators (cyclin D1, cyclin-dependent kinase [CDK] 4, and PCNA), and phosphorylated ERK 1 and 2, all of which are associated with hepatocyte proliferation, were elevated in *Ppara*<sup>+/+</sup>:HCVcpTg mice but not in other genotypes (Figure 4, E and F).

**Increased oxidative stress and DNA damage only in *Ppara*<sup>+/+</sup>:HCVcpTg mice.** HCV core protein is associated with increased production of ROS (23). Enhanced ROS production induces nuclear DNA damage, which results in the initiation of hepatocarcinogenesis, and can also injure organelles, which can result in disorders in their



**Figure 4** Increased hepatocyte proliferation in *Ppara*<sup>+/+</sup>:HCVcpTg mice at 24 months of age. (A) Liver-to-body-weight ratio. Results are expressed as the mean  $\pm$  SD (*n* = 6/group). (B) Numbers of proliferating hepatocytes. Two thousand hepatocytes were examined in each mouse, and hepatocyte nuclei positive for anti-PCNA antibody were counted. Results are expressed as the mean  $\pm$  SD (*n* = 6/group). For A and B, comparisons are designated as follows: \**P* < 0.05 compared with *Ppara*<sup>+/+</sup> nontransgenic mice; \*\**P* < 0.05 compared with *Ppara*<sup>+/+</sup>:HCVcpTg mice; #*P* < 0.05 compared with *Ppara*<sup>-/-</sup>:HCVcpTg mice. (C) Numbers of apoptotic hepatocytes. Liver sections were subjected to TUNEL staining, and TUNEL-positive hepatocyte nuclei were counted in 2,000 hepatocytes from each mouse. Results are expressed as the mean  $\pm$  SD (*n* = 6/group). (D) Caspase 3 activity. Results are expressed as the mean  $\pm$  SD (*n* = 6/group). (E) Immunoblot analysis of oncogene products and cell cycle regulators. The same sample used in Figure 1A (whole-liver lysate, 50  $\mu$ g protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*<sup>+/+</sup> nontransgenic mice. The mean value of the fold changes is expressed under each band. (F) Immunoblot analysis of phosphorylated ERK1/2 and total ERK1/2. The same samples in Figure 4E (50  $\mu$ g protein) were used.



**Figure 5**

Increased oxidative stress and DNA damage in *Ppara*<sup>+/+</sup>:HCVcpTg mice at 24 months of age. (A) Immunohistochemical staining using antibody against 8-OHdG. In *Ppara*<sup>+/+</sup>:HCVcpTg mice, some steatotic hepatocytes were positive for 8-OHdG. Original magnification, ×400. (B) Numbers of 8-OHdG-positive hepatocytes. Hepatocyte nuclei stained with anti-8-OHdG antibody were counted in 2,000 hepatocytes of each mouse. Results are expressed as the mean ± SD (*n* = 6/group). (C) Hepatic content of lipid peroxides. Results are expressed as the mean ± SD (*n* = 6/group). \**P* < 0.05 compared with *Ppara*<sup>+/+</sup> nontransgenic mice; \*\**P* < 0.05 compared with *Ppara*<sup>+/-</sup>:HCVcpTg mice; #*P* < 0.05 compared with *Ppara*<sup>+/-</sup>:HCVcpTg mice. (D) Immunoblot analysis of AOX, CYP4A1, catalase, and Cu, Zn-SOD. The whole-liver lysate used in the experiment in Figure 4E (20 μg protein for AOX and CYP4A1 and 50 μg for others) was loaded in each lane. The band of actin was used as the loading control. Results are representative of 4 independent experiments. A and B indicate full-length and truncated AOX, respectively. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*<sup>+/+</sup> nontransgenic mice. The mean value of the fold changes is expressed under each band. \**P* < 0.05 compared with *Ppara*<sup>+/+</sup> nontransgenic mice.

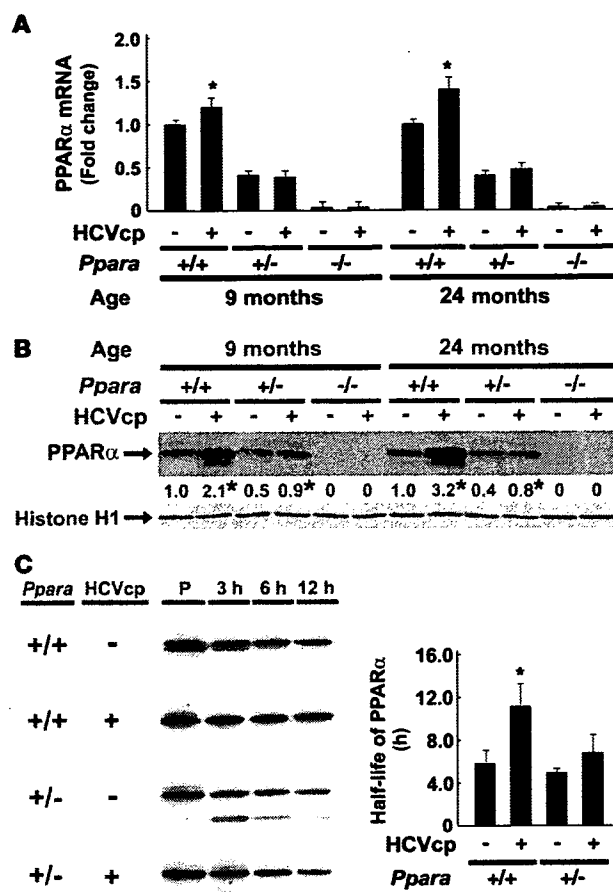
function. The number of hepatocytes positive for 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indicator of oxidative damage to nuclear DNA, was increased only in 24-month-old *Ppara*<sup>+/+</sup>:HCVcpTg mice (Figure 5, A and B). Lipid peroxides were slightly increased in the livers of 9-month-old *Ppara*<sup>+/+</sup>:HCVcpTg mice (data not shown) and were more abundant in the livers of 24-month-old *Ppara*<sup>+/+</sup>:HCVcpTg mice than in those of *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup>:HCVcpTg mice or *Ppara*<sup>+/+</sup> nontransgenic mice (Figure 5C). Expression of typical ROS-generating enzymes (AOX and cytochrome P450 4A1 [CYP4A1]) and ROS-eliminating enzymes (catalase and Cu, Zn-SOD) was examined. Immunoblot analysis showed marked increases in the expression of AOX and CYP4A1 and mild increases in that of catalase only in *Ppara*<sup>+/+</sup>:HCVcpTg mice. No changes in Cu, Zn-SOD were found in the subgroups of transgenic mice (Figure 5D). These results suggest that enhanced oxidative stress causes damage in nuclear DNA and probably in mitochondria in the *Ppara*<sup>+/+</sup>:HCVcpTg mice.

**Persistent and spontaneous PPARα activation in *Ppara*<sup>+/-</sup>:HCVcpTg mice.** Liver tumorigenesis induced by long-term exposure to peroxisome proliferators and the related changes, such as sustained hepatocyte proliferation and increased oxidative stress, are associated with persistent PPARα activation (19–21). To examine the activation of PPARα, we quantified the level of PPARα mRNA, which is induced by PPARα activation (24, 25). PPARα mRNA levels were higher in 9-month-old *Ppara*<sup>+/-</sup>:HCVcpTg mice than in *Ppara*<sup>-/-</sup> nontransgenic mice (Figure 6A). These increases were more pronounced at 24 months of age. However, there were no differences in PPARα mRNA levels between *Ppara*<sup>+/-</sup>:HCVcpTg and *Ppara*<sup>-/-</sup> nontransgenic mice at either 9 or 24 months of age. The expression levels of typical PPARα target genes (16, 25, 26) – such as FAT, FATP, L-FABP, AOX, and MCAD (Figure 2); c-Myc, cyclin D1, CDK4, and PCNA (Figure 4); and CYP4A1 (Figure 5)

– were simultaneously and synchronously increased in *Ppara*<sup>+/-</sup>:HCVcpTg mice, but not in *Ppara*<sup>-/-</sup>:HCVcpTg or *Ppara*<sup>+/-</sup>:HCVcpTg mice. These results confirm that persistent activation of PPARα occurs only in *Ppara*<sup>+/-</sup>:HCVcpTg mice. Various changes observed in *Ppara*<sup>+/-</sup>:HCVcpTg mice, i.e., increased fatty acid uptake, mitochondrial abnormalities, steatosis, ROS overproduction, accelerated hepatocyte proliferation, and hepatocarcinogenesis, were considered to be closely linked with sustained PPARα activation.

**Nuclear PPARα content.** The results described above suggest that persistent PPARα activation is critical to the steatogenesis and hepatocarcinogenesis induced by the HCV core protein. A question arises as to why *Ppara*<sup>+/-</sup>:HCVcpTg mice with an active *Ppara* allele do not exhibit the hallmarks of PPARα activation and do not develop HCC. To address this issue, the nuclear PPARα content was analyzed. Immunoblot analysis for PPARα showed that the amount of nuclear PPARα protein in *Ppara*<sup>+/-</sup>:HCVcpTg mice was approximately 2- to 3-fold that of *Ppara*<sup>+/+</sup> nontransgenic mice, which was disproportionate to the higher PPARα mRNA levels (approximately 1.2- to 1.6-fold) (Figure 6, A and B). The level of nuclear PPARα in *Ppara*<sup>+/-</sup>:HCVcpTg mice was significantly lower than that in *Ppara*<sup>+/+</sup>:HCVcpTg mice and was similar to that in *Ppara*<sup>-/-</sup> nontransgenic mice (Figure 6B). Thus, the lower amount of nuclear PPARα in *Ppara*<sup>+/-</sup>:HCVcpTg mice than in *Ppara*<sup>+/+</sup>:HCVcpTg mice might have heightened the threshold of expression required for long-term spontaneous PPARα activation.

The degree of an increase in nuclear PPARα levels was evidently higher than the degree of an increase in PPARα mRNA levels in HCVcpTg mice (Figure 6, A and B). To investigate this disparity, the stability of nuclear PPARα was evaluated by pulse-chase experiments using isolated hepatocytes obtained from these mice. The half-life of nuclear PPARα was significantly longer (*P* < 0.05) in *Ppara*<sup>+/-</sup>:HCVcpTg mice (11.5 ± 2.3 h) than in *Ppara*<sup>+/+</sup> nontransgenic mice (5.8 ± 1.4 h) (Figure 6C). The half-life of nuclear PPARα in *Ppara*<sup>+/-</sup>:HCVcpTg mice tended to be prolonged compared with that in *Ppara*<sup>-/-</sup> nontransgenic mice (Figure 6C). These results suggest that the stability of nuclear PPARα was increased as a result of HCV core protein expression. Because it is known that the core protein interacts with retinoid X receptor α (RXRα) (27) and that



**Figure 6**

Persistent PPAR $\alpha$  activation in *Ppara*<sup>+/-</sup>:HCVcpTg mice. (A) PPAR $\alpha$  mRNA levels. Total RNA was prepared from each mouse, and PPAR $\alpha$  mRNA levels were determined by RT-PCR, normalized by those of GAPDH, and subsequently normalized by those of 9-month-old *Ppara*<sup>+/-</sup> nontransgenic mice. Results are expressed as the mean  $\pm$  SD ( $n = 6$ /group). (B) Immunoblot analysis of nuclear PPAR $\alpha$ . The nuclear fraction obtained from each mouse (100  $\mu$ g protein) was loaded in each well. The band of histone H1 was used as the loading control. Results are representative of 6 independent experiments. The band intensity was quantified densitometrically, normalized by that of histone H1, and subsequently normalized by that in 9-month-old *Ppara*<sup>+/-</sup> nontransgenic mice. The mean value is expressed under each band. \* $P < 0.05$  compared with nontransgenic mice of the same age and *Ppara* genotype. (C) Pulse-chase experiments for 3, 6, and 12 h and pulse-label (P) experiments for nuclear PPAR $\alpha$  using isolated mouse hepatocytes. Left: labeled PPAR $\alpha$  bands on x-ray film. Pulse-label and pulse-chase experiments were performed as described in Methods. Results are representative of 4 independent experiments. Right: half-life of PPAR $\alpha$ . The band intensity was measured densitometrically and subsequently normalized by that of the pulse-label experiments. The percentage of the band intensity was plotted, and the half-life of PPAR $\alpha$  was calculated. Results obtained from 4 independent experiments are expressed as the mean  $\pm$  SD. \* $P < 0.05$  compared with nontransgenic mice in the same *Ppara* genotype.

PPAR $\alpha$  influences the stability of RXR $\alpha$  (28), it is plausible that the core protein would affect its action in nuclei through an interaction with the PPAR $\alpha$ -RXR $\alpha$  heterodimer and stabilization of PPAR $\alpha$ .

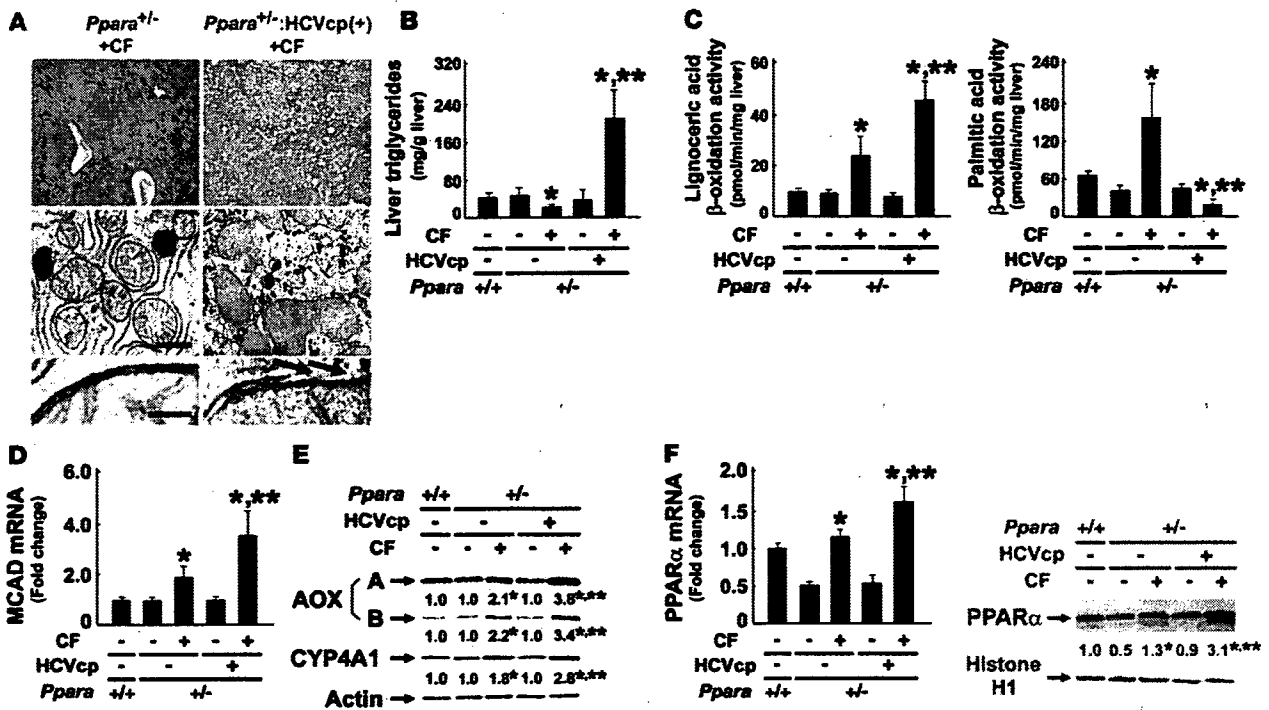
*Development of hepatic steatosis and HCC with long-term clofibrate treatment in Ppara*<sup>+/-</sup>:HCVcpTg mice. To further confirm the significance of persistent PPAR $\alpha$  activation on core protein-induced pathological changes, *Ppara*<sup>+/-</sup> and *Ppara*<sup>+/-</sup>:HCVcpTg mice were fed a standard diet containing 0.05% clofibrate for 24 months. Interestingly, hepatic steatosis appeared in the clofibrate-treated *Ppara*<sup>+/-</sup>:HCVcpTg mice, but not in the *Ppara*<sup>+/-</sup> mice under the same treatment conditions (Figure 7, A and B). Similar to our observations in *Ppara*<sup>+/-</sup>:HCVcpTg mice not treated with clofibrate, aberrant mitochondria with discontinuous outer membranes and decreased palmitic acid  $\beta$ -oxidation activity were found only in the clofibrate-treated *Ppara*<sup>+/-</sup>:HCVcpTg mice (Figure 7, A and C). In addition, levels of MCAD mRNA; AOX, and CYP4A1 proteins; PPAR $\alpha$  mRNA; and nuclear PPAR $\alpha$  protein were higher in the clofibrate-treated *Ppara*<sup>+/-</sup>:HCVcpTg mice than in the clofibrate-treated *Ppara*<sup>+/-</sup> mice (Figure 7, D-F), which suggests that the degree of PPAR $\alpha$  activation in the former group was greater than that in the latter group and similar to that in *Ppara*<sup>+/-</sup>:HCVcpTg mice not treated with clofibrate. Finally, the incidence of HCC after clofibrate treatment was higher in *Ppara*<sup>+/-</sup>:HCVcpTg mice (25%; 5 in 20 mice) than in *Ppara*<sup>+/-</sup> mice (5%; 1 in 20 mice). Therefore, these results corroborate the importance of constant PPAR $\alpha$  activation to the pathogenesis of hepatic steatosis and HCC in the transgenic mice.

## Discussion

A novel and striking finding in this study is the absolute requirement of persistent PPAR $\alpha$  activation for the development of HCV core protein-induced steatosis and HCC. Our data also show that the HCV core protein alone cannot induce steatosis and HCC in transgenic mice.

Mechanisms of development of steatosis in HCVcpTg mice were previously explained as an enhancement of de novo synthesis of fatty acids (29) and a decrease in MTP expression, the latter of which results in insufficient VLDL secretion from hepatocytes (30). In the present study, we revealed 2 novel mechanisms of steatogenesis in the transgenic mice, i.e., an impairment of mitochondrial  $\beta$ -oxidation due to the breakdown of mitochondrial outer membranes and an increase in fatty acid uptake into hepatocytes, associated with PPAR $\alpha$  activation. PPAR $\alpha$  activation, mitochondrial dysfunction, and hepatic steatosis appeared in 9-month-old *Ppara*<sup>+/-</sup>:HCVcpTg mice and continued until 24 months of age, clearly preceding development of HCC. These findings thereby indicate a correlation between PPAR $\alpha$  activation, hepatic steatosis, and HCC.

We obtained the novel and rather paradoxical finding that significant PPAR $\alpha$  activation, which generally is expected to reduce hepatic triglyceride levels, is essential for the development of severe steatosis induced by HCV core protein. According to the results of this study, the following hypothesis concerning the development of steatosis in *Ppara*<sup>+/-</sup>:HCVcpTg mice is proposed. First, the HCV core protein localizes partly in mitochondria (9). A recent study



**Figure 7**

Development of hepatic steatosis by long-term treatment of clofibrate in *Ppara*<sup>+/-</sup>:HCVcpTg mice. (A) Histological examination of *Ppara*<sup>+/-</sup> and *Ppara*<sup>+/-</sup>:HCVcpTg mice treated with diet containing 0.05% (w/w) clofibrate for 24 months (CF). Top: Histological appearance of H&E-stained liver sections. Magnification, ×40. Microvesicular and macrovesicular steatosis were detected only in clofibrate-treated *Ppara*<sup>+/-</sup>:HCVcpTg mice. Middle and bottom: Electron microscopic features of hepatic mitochondria. Some C-shaped mitochondria showing discontinuance of outer membranes (arrows) were found in clofibrate-treated *Ppara*<sup>+/-</sup>:HCVcpTg mice. Scale bars: 400 nm (middle), 30 nm (bottom). (B and C) Content of liver triglycerides and lignoceric and palmitic acid β-oxidation activities. (D) MCAD mRNA levels. mRNA levels were normalized to those of GAPDH and subsequently normalized to those in *Ppara*<sup>+/+</sup> nontransgenic mice. (E) Immunoblot analysis of AOX and CYP4A1. Whole-liver lysate (20 μg protein) was loaded in each lane. Actin was used as a loading control. Results are representative of 6 independent experiments. (F) PPARα mRNA levels and nuclear PPARα contents. Left: PPARα mRNA levels. The same samples used in D were adopted. Right: Immunoblot analysis of nuclear PPARα. Nuclear fraction obtained from each mouse (100 μg protein) was loaded in each well. Histone H1 was used as a loading control. In E and F, the mean value of the fold changes is shown under each band. Results are representative of 6 independent experiments. Band intensity was quantified densitometrically, normalized to that of the loading control, and subsequently normalized to that in *Ppara*<sup>+/+</sup> nontransgenic mice. \**P* < 0.05 compared with untreated mice of the same genotype; \*\**P* < 0.05 compared with clofibrate-treated *Ppara*<sup>+/-</sup> mice without core protein gene. Results are expressed as mean ± SD (*n* = 6/group).

showed that, in isolated mitochondria, the core protein directly increased Ca<sup>2+</sup> influx, inhibited electron transport complex I activity, and induced ROS production (31), all of which can increase the fragility of mitochondria and depress mitochondrial function. In addition, the HCV core protein also localizes in nuclei (9) and can coexist in PPARα-RXRα heterodimer through a direct interaction with the DNA-binding domain of RXRα, which enhances the transcriptional activity of PPARα target genes, such as AOX, despite the absence of PPARα ligands in cultured cells (27). The HCV core protein can also be involved in the PPARα-RXRα complex through a direct interaction with cyclic-AMP responsive element binding protein-binding protein (32), which is able to bind to PPARα (33). Thus, the core protein probably serves as a coactivator and stabilizer of PPARα in vivo, which was further confirmed in this study. Moreover, because it is also known that the core protein itself activates ERK1/2 and p38 mitogen-activated protein kinase (34), these activations might phosphorylate PPARα and thereby transactivate it (35). The core protein-induced PPARα activation enhances the basal expression of AOX and CYP4A1, which leads to increased

production of ROS and dicarboxylic acids. These toxic compounds can damage mitochondrial outer membranes, which impairs the mitochondrial β-oxidation system. These damages directly induce the accumulation of long-chain fatty acids in hepatocytes. Furthermore, PPARα activation increases the expression of FAT and FATP, which promotes the influx of fatty acids from blood. Long-chain fatty acids and their CoA esters accumulated in hepatocytes are likely to act as potent detergents, which further damages the outer membranes of mitochondria. Fatty acids and their derivatives function as natural ligands of PPARα, which results in the activation of PPARα and the induction of FAT, FATP, AOX, and CYP4A1, which further accelerates mitochondrial damage, the reduction of mitochondrial β-oxidation activity, and the accumulation of fatty acids in a vicious cycle.

Persistent PPARα activation increases oxidative DNA damage because of a disproportionate increase in ROS-generating enzymes relative to the levels of degrading enzymes such as catalase and SOD, which can predispose hepatocytes to malignant transformation. In addition, persistent PPARα activation leads to increased