Detection of Hepatitis B and C Viruses in Almost All Hepatocytes by Modified PCR-Based *In Situ* Hybridization[▽]

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Received 28 February 2010/Returned for modification 22 April 2010/Accepted 16 August 2010

Although PCR-based *in situ* hybridization (PCR-ISH) can be used to determine the distribution and localization of pathogens in tissues, this approach is hampered by its low specificity. Therefore, we used a highly specific and sensitive PCR-ISH method to reveal the lobular distribution and intracellular localization of hepatitis B virus (HBV) and HCV in chronic liver disease and to clarify the state of persistent HBV and HCV infection in the liver. HBV genomic DNA was detected in almost all hepatocytes, whereas HBV RNA or protein was differentially distributed only in a subset of the HBV DNA-positive region. Further, HCV genomic RNA was detected in almost all hepatocytes and was localized to the cytoplasm. HCV RNA was also detected in the epithelium of the large bile duct but not in endothelial cells, portal tracts, or sinusoidal lymphocytes. In patients with HBV and HCV coinfection, HCV RNA was localized to the noncancerous tissue, whereas HBV DNA was found only in the cancerous tissue. Using this novel PCR-ISH method, we could visualize the staining pattern of HBV and HCV in liver sections, and we obtained results consistent with those of real-time detection (RTD)-PCR analysis. In conclusion, almost all hepatocytes are infected with HBV or HCV in chronic liver disease; this finding implies that the viruses spreads throughout the liver in the chronic stage.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the primary causative agents of chronic liver disease (2, 9, 17). HBV infection remains a global health problem; it is estimated that 350 million individuals are persistently infected with the virus and that approximately 15% to 25% of these individuals will die due to the sequelae of the infection (23, 29). Further, more than 170 million people are infected with HCV worldwide (21). HCV has a single-stranded RNA genome (8, 19), does not have canonical oncogenes, and can easily establish chronic infection without integration into the host genome (3, 20), resulting in hepatic steatosis and hepatocellular carcinoma (HCC) (28). The viruses share a similar route of transmission, such as via the transfusion of infected blood or body fluids or use of contaminated needles.

Several studies have shown that 10% to 35% of the individuals infected with HBV also have HCV infection, although the prevalence varies depending on the population studied (4, 32, 34). The relationship between coinfection and acceleration of malignant transformation remains unclear, but HBV and HCV coinfection seems to alter the natural history of both HBV-related and HCV-related liver disease (2, 12). HCV has been shown to inhibit HBV gene expression (7, 15). The high prevalence of occult HBV infection may indicate that HCV also

inhibits HBV replication (34). Most epidemiological studies of HBV have been performed by using diagnostic serological assays (16). We recently used a novel, highly sensitive diagnostic PCR method to demonstrate that the HBV genome is detectable in the sera of a substantial proportion of patients with chronic HCV infection who are seronegative for the standard HBV-related markers (1, 34). Further, we reported the levels of HBV DNA and HCV RNA in cancerous and noncancerous liver tissue using real-time detection (RTD)-PCR (34). RTD-PCR is an accurate assay method, but it can determine the levels of genomic DNA and RNA only in homogenized tissue. In this study, we developed a PCR-based *in situ* hybridization (PCR-ISH) method for detecting and visualizing HBV DNA, HBV RNA, and HCV RNA and comparing their protein expression patterns, with the aim to reveal the lobular distribution and intracellular localization of HBV and HCV in chronic liver disease and to clarify the state of persistent HBV and HCV infection in the liver.

MATERIALS AND METHODS

Patients. Twenty-nine patients were admitted to Tokyo Metropolitan Komagome Hospital for the treatment of hepatic tumors. Of these patients, 14 were considered to have chronic HCV infection (persistently positive results for HCV antibody), 8 were diagnosed with chronic hepatitis B (persistently positive results for HBV surface antigen [HBsAg]), and 7 showed negative results for both viral markers but had metastatic liver cancer (6 with colonic cancer and 1 with gastric cancer). We used four samples from seven patients as controls for PCR-ISH and four samples from seven patients as controls for reverse transcriptase PCR (RT-PCR)-ISH (Table 1). Of the 14 patients with chronic hepatitis C, two showed positive results for HBV DNA by RTD-PCR. HBsAg and second-generation HCV antibody were measured by using enzyme-linked immunosor-

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[▽] Published ahead of print on 25 August 2010.

TABLE 1. Patient profiles and results of the present study

Patient no.	Age (yr)	Gender ^a	HCV antibody	HBs Ag	Liver histology	RT-PCR-ISH HCV	PCR-ISH HBV	Serum HBV DNA (copies/ml) ^b	Serum HCV RNA		IFN treatment	Note
									Copies/ml	KIU/ml ^d		
1	53	M	—	+	A2F3		+	1.1×10^2			—	
2	42	M	—	+	A2F2		+	4.0×10^5			—	Fig. 2A
3	43	M	—	+	A2F3		+	1.6×10^7			—	
4	53	M	—	+	A2F4		+	6.4×10^3			—	Fig. 1A
5	55	M	—	+	A2F3		+	1.0×10^3			—	Fig. 3A
6	54	M	—	+	A2F4	—	+	NT ^c			—	
7	31	M	—	+	A3F3		+	3.0×10^9			+	
8	61	F	—	+	A3F3		+	NT			—	
9	63	M	+	—	A3F4	+	—		5.6×10^6		—	Fig. 4D
10	56	M	+	—	A2F3	+	—		NT		+	
11	67	F	+	—	A3F4	—	—		NT		—	
12	73	M	+	—	A2F4	+			1.2×10^6		—	Fig. 1B
13	68	F	+	—	A3F4	+			1.1×10^6		—	
14	76	F	+	—	A2F4	+			9.5×10^5		—	Fig. 4A
15	62	M	+	—	A2F3	+			7.2×10^5		—	
16	65	M	+	—	A3F4	+			NT		—	
17	60	F	+	—	A3F4	+				41	—	
18	48	F	+	—	A2F1	+			7.6×10^6	>850	—	Fig. 4C
19	43	M	+	—	A2F3	+				389	—	
20	59	M	+	—	A2F3	+				>850	—	
21	72	F	+	—	A2F4	+	+	3.9×10^1	5.0×10^7			Fig. 5
22	69	M	+	—	A2F3	+	+	5.0×10^1	3.0×10^7			
23	69	M	—	—	Normal	—	—					Gastric cancer
24	58	M	—	—	Normal	—	—					Colon cancer
25	58	M	—	—	Normal	—	—					Colon cancer
26	59	F	—	—	Normal	—	—					Colon cancer
27	65	M	—	—	Normal	—	—					Colon cancer
28	47	M	—	—	Normal	—	—					Colon cancer
29	81	F	—	—	Normal	—	—					Colon cancer

^a M, male; F, female.^b Serum HBV DNA was positive in patients 21 and 22.^c NT, not tested.^d KIU, kilo international units.

bent assay (ELISA) kits (Abbott Laboratories, Chicago, IL, and International Reagent Corp., Kobe, Japan, respectively). All 29 patients underwent hepatic resection. Histological evaluation of the liver was carried out according to the METAVIR scoring system (3).

Ethical approval. The Institutional Review Board of Tokyo Metropolitan Komagome Hospital approved the study. Written informed consent was obtained from all the subjects.

Sample preparation. The liver tissue samples for HBV DNA detection were fixed in 10% buffered formalin (pH 7.4) for 18 h, embedded in paraffin, cut into 6- μ m-thick sections, and mounted on silane-coated glass slides for use with a GeneAmp *in situ* PCR system 1000 unit (Applied Biosystems, Foster City, CA). The slides were washed thrice in xylene for 8 min at each washing, rinsed thrice in 99.5% ethanol and 75% ethanol for 5 min at each rinsing, and rehydrated in distilled water for deparaffinization. For detecting HBV mRNA and HCV RNA, OCT-embedded frozen liver tissue samples were cut into 10- μ m-thick sections and mounted on silane-coated glass slides. They were then fixed in 10% buffered formalin (pH 7.4) for 17 to 21 h, rinsed twice in distilled water treated with 0.01% diethylpyrocarbonate (DEPC) for 2 min at each rinse, rinsed in 99.5% ethanol for 1 min, and then air dried and stored at -80°C until use. The tissue sections on the glass slides were digested with proteinase K (1 to 30 $\mu\text{g}/\text{ml}$ and 1 to 200 $\mu\text{g}/\text{ml}$ for the noncancerous and cancerous regions of the paraffin-embedded sections, respectively; 0.008 to 1.0 $\mu\text{g}/\text{ml}$ for the frozen sections) in 50 mM Tris (pH 7.5) at 37°C for 30 min in a humidified chamber. Subsequently, proteinase K was inactivated at 97°C for 10 min, and then the sections were rinsed with distilled water, dehydrated in 99.5% ethanol, and air dried.

Primers and probes for PCR-ISH and RT-PCR-ISH. The primers used to amplify the S and X regions of HBV and the 5' untranslated region (5'-UTR) of HCV as well as the corresponding probes are listed in Table 2. We created a

digoxigenin (DIG)-dUTP tail at the 3' end of the 5'-DIG probe using a DNA tailing kit (Roche, Basel, Switzerland).

PCR-ISH for detecting HBV DNA. PCR was performed by using one of two sets of antisense and sense primers complementary to the sequences located in the S and X regions of HBV. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl_2 , 0.8 mM each primer, 197 mM deoxynucleoside triphosphates (dNTPs), and 10 U/50 μl *Taq* DNA polymerase (AmpliTaQ Gold; Applied Biosystems).

The tissue slides were warmed to 70°C , and 50 μl of the PCR mixture was overlaid onto the proteinase K-treated tissue specimens. An Ampli cover disc with Ampli cover clips (Applied Biosystems) was attached to each specimen. The slides were placed in the GeneAmp *in situ* PCR system 1000 unit at 70°C . PCR was performed at 95°C for 10 min, followed by 35 to 55 cycles at 95°C for 30 s and 60°C for 2 min and a final extension at 72°C for 10 min. Immediately after the PCR, the slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at 37°C , washed in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 min, rinsed with distilled water for 2 min, dehydrated in 99.5% ethanol for 1 min, and then air dried. ISH was performed by mixing the DIG-labeled probe (final concentration, 100 ng/ml) with 65 μl of hybridization buffer (50% deionized formamide, $4\times$ SSC, $1\times$ Denhardt's solution [0.2% bovine serum albumin {BSA}, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll 400], 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 100 $\mu\text{g}/\text{ml}$ yeast RNA, and 1 mM EDTA) and then adding the mixture to each section, heating to 97°C for 10 min, and cooling to 37°C in decrements of $1^\circ\text{C}/\text{min}$ (27). Hybridization was carried out overnight at 37°C . Stringency washes were conducted with the following: $2\times$ SSC twice for 10 min at 37°C , $0.03\times$ SSC for 10 min at 50°C , 0.1% Triton X-100 in TBS (0.1 M Tris [pH 7.5], 0.1 M NaCl) for 10 min at room temperature, and TBS for 5 min at room temperature. After incubation in blocking reagent (0.1 M Tris

TABLE 2. Primers and probes for PCR-ISH and RT-PCR

Technique	Virus or protein (region)	Type	Description, nucleotides	Primer or probe	
				Primer	Sequence
PCR-ISH	HBV (HBs)	Primer	Forward (HB-166-S21), 166-186	5'-CACATCAGGATTCTAGAGACC-3'	
		Reverse (HB-344-R20), 344-325	5'-AGGTGGTGAGTGGAGG-3'		
	HBV (HBx)	Probe	(HB-242-S45D), 242-286	5'-(DIG)-CAGAGTCTAGACTGCTGTGAGACTTCTCTCAATTTCTAGGGGGA-(DIG)n-3'	
		Primer	Forward (HB-1584-S21), 1584-1604	5'-CTTGCTCTACCTCTGACAGT-3'	
		Reverse (HB-1744-R23), 1744-1722	5'-CCCACTCTCCAGTCTTAAA-3'		
		Probe	(HB-1705-R45D), 1705-1661	5'-(DIG)-TATGCTCAAGGTCGCTGTTGACATTGCTGAGAGTCCAGAAGTTC-(DIG)n-3'	
RT-PCR-ISH	HCV (5'-UTR)	Primer	Forward (R6-129-S19), 129-147	5'-CCGGGAGAGCCATAGTGT-3'	
		Reverse (R6-290-R19), 290-272	5'-AGTACCAAGGCTTTTCG-3'		
		Probe	(R6-225-S45D), 225-269	5'-(DIG)-ATTGGGGCGTGGCCCCCGGAGACTGCTAGCCGAGTAGTGTGGGT-(DIG)n-3'	
TaqMan	HBV (S)	Probe	Forward (HB-242-S26FT), 242-267	5'-CAGAGTCTAGACTGCTGTGAGTTC-3'	
	HBV (X)	Probe	Forward (HB-1681-S25FT), 1681-1705	5'-TGTCAACGACCGACTTGGAGCAT-3'	
	HCV (5'-UTR)	Primer	Forward (R6-130-S17), 130-146	5'-CGGAGAGCCATATGATG-3'	
		Reverse (R6-290-R19), 290-272	5'-AGTACCAAGGCTTTTCG-3'		
		Probe	Forward (R6-148-S21FT), 148-168	5'-CTGGGGAACCGGTGAGTACAC-3'	
		Primer	Forward (β-ACT-1998-S20), 1998-2017	5'-CAGTGTGACATGGTGATCT-3'	
	β-Actin (genomic DNA)	Probe	Reverse (β-ACT-2246-R24), 2246-2223	5'-GTGAGGATCTTCATGAGGTATGCA-3'	
		Probe	Forward (β-ACT-2063-S22FT), 2063-2084	5'-ACGTGCTATCCAGGCTGTGCT-3'	
	GAPDH	Primer	Forward (GAPDH-514-S24), 514-537	5'-TGCACCAACCACTGCTAGACCC-3'	
		Reverse (GAPDH-837-R24), 837-814	5'-CTTGATGTCATCATATTTGGCAGG-3'		
		Probe	Forward (GAPDH-584-S25FT), 584-608	5'-TGACCACAGTCCATGCCATCTGC-3'	

[pH 7.5], 0.1 M NaCl, 10% sheep serum, 3% BSA) at room temperature for 15 min, the slides were covered with 100 μl anti-DIG antibody conjugated with alkaline phosphatase (Roche) and diluted at 1:900 with 1% BSA in TBS at 37°C for 60 min. After this reaction, the slides were washed twice (3 min each) with 0.1% Triton X-100 in TBS, then with TBS alone, and finally with APS (0.1 M Tris [pH 9.0], 0.1 M NaCl, 50 mM MgCl₂) at room temperature. The slides were incubated in 100 μl dye solution (338 μg/ml nitroblue tetrazolium chloride [NBT], 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt [BCIP], and 450 μM Levamisole [Vector Labs, Burlingame, CA] in APS) at 37°C in the dark. After sufficient color development, they were washed with deionized water for 1 min and then mounted with aqueous mounting medium.

RT-PCR-ISH for detecting HBV RNA. The OCT-embedded frozen sections were placed on glass slides. After proteinase K treatment, the tissue sections were digested with RNase-free DNase I (Roche; diluted to 3 U/μl in 0.1 M sodium acetate and 5 mM MgSO₄). The DNase I reaction mixture (66 μl) was overlaid onto the tissue sections, which were then enclosed in a frame. The slides were reacted in an aluminum box at 37°C for 20 min and inactivated at 97°C for 10 min. They were then washed in DEPC-treated water, dehydrated in 99.5% ethanol, and air dried.

Moloney murine leukemia virus (MMLV) reverse transcriptase (10 U/μl; Invitrogen, Carlsbad, CA) was used in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM MgCl₂, 1 μM antisense primer, 1 mM dNTPs, 2 U/μl RNase inhibitor (Takara, Otsu, Japan), and 10 mM dithiothreitol (DTT). The specimens were then overlaid with the mixture, reacted at 42°C for 60 min, washed with distilled water, dehydrated in 99.5% ethanol, and air dried. The subsequent procedures were the same as described for PCR-ISH.

Immunohistochemical staining for detecting HBV proteins. Deparaffinized formaldehyde-fixed sections or fixed frozen liver tissue sections on glass slides were soaked in distilled water, digested with 0.1% pronase (protease P8038 XXIV; Sigma-Aldrich, Tokyo, Japan) for 1 min, and washed with PBS at room temperature. After 30 min of incubation in blocking reagent (1% BSA and 2.5 mM EDTA in PBS) at room temperature, the slides were reacted with 100 μl of anti-HBs and anti-HBc polyclonal antibody solutions for 3 h at room temperature and then overnight at 4°C. The following polyclonal antibodies were used: anti-HBs rabbit polyclonal antibody anti-HBc rabbit polyclonal antibody (Novocastra Laboratories, Newcastle, United Kingdom), or normal rabbit serum diluted in blocking reagent. After the reaction, the slides were washed four times with PBS at room temperature and incubated for 60 min at room temperature in 100 μl anti-rabbit IgG conjugated with peroxidase (Amersham ECL; GE Healthcare, Piscataway, NJ) diluted to 1:100 in blocking reagent. The slides were then washed four times with PBS at room temperature and stained by using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Labs). Following counterstaining with Mayer's hematoxylin solution, the tissue specimens were dehydrated in 99.5% ethanol and 80% xylene. The slides were sealed by using Bioleil (Oken Shoji, Tokyo, Japan).

RT-PCR-ISH for detecting HCV RNA. HCV RNA was detected by using methods similar to those used for detecting HBV RNA except for the following steps: the DNase I step was omitted, and 1.5 mM MgCl₂ was used in the PCR mixture.

Primers and probe sets in RTD-PCR for quantifying HBV DNA, HCV RNA, β-actin DNA, and GAPDH mRNA. The primer sets to quantify the S and X regions of HBV were the same as those used for PCR-ISH. The TaqMan probes for these regions, the primers and probe designed to quantify the 5'-UTR of HCV (33), and those used to quantify β-actin genomic DNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA (internal control) are shown in Table 2. Each PCR comprised 50 cycles (95°C for 30 s, 60°C for 40 s, and 72°C for 30 s) in a real-time PCR system (ABI Prism 7700 sequence detector system; Applied Biosystems).

Amplicor monitor assays. The Amplicor monitor assays were performed as described previously (22, 24, 33, 36).

HE staining. HBV- or HCV-infected deparaffinized formaldehyde-fixed sections or fixed frozen liver tissue sections were stained with hematoxylin and eosin (HE).

LCM of liver tissue. A frozen liver tissue sample was sectioned by using a cryostat and fixed in acetone, followed by HE staining. Laser capture microdissection (LCM) was performed by using an LM 200 system (Olympus, Tokyo, Japan) as described previously (6, 11). This procedure produced approximately 30 hepatocytes from each of three areas (perivenular, intermediate, and periportal) in the section. Total RNA was extracted from the LCM samples, and HCV RNA and GAPDH mRNA were quantified by RTD-PCR.

FIG. 1. (A) Panels a and b, HBV DNA detected by PCR-ISH and immunohistochemical staining in noncancerous (Non-Ca) (panel a) and cancerous (Ca) (panel b) liver tissues obtained from a patient infected with HBV. The numbers of PCR cycles were 37 and 42, respectively. Panels c and d, serial sections were stained with HE. Magnification, $\times 400$. S+S, primers and probe targeting the S region of HBV DNA. (B) Panels a and b, HCV RNA detected by RT-PCR-ISH (45 cycles of PCR) in noncancerous (panel a) and cancerous (panel b) tissues obtained from a patient infected with HCV. Panels c and d, no HCV RNA was detected in the RT-negative controls. Panels e and f, serial sections were stained with HE. Magnification, $\times 400$.

RESULTS

Sensitivity and specificity of PCR-ISH versus RTD-PCR for detecting HBV DNA and HCV RNA. PCR-ISH showed positive results for HBV DNA in 10 tissue specimens from eight HBsAg-seropositive patients and two patients whose serum HBV DNA was barely detected by RTD-PCR despite being their HBsAg negative (patients 21 and 22) (Table 1). PCR-ISH yielded negative results for four patients who were negative for serum HBsAg and HCV antibody (patients 23, 24, 28, and 29) and three patients who were negative for serum HBsAg but positive for HCV antibody (patients 9 to 11).

Thirteen of the 14 tissue specimens from the patients with serum HCV antibody had a positive result for HCV RNA by RT-PCR-ISH (sensitivity, 92.9%). In contrast, HCV RNA was not detected by RT-PCR-ISH in four tissue specimens from the patients negative for both HCV antibody and HBsAg (patients 23, 25, 26, and 27) or in the sample from an HBsAg-positive and HCV antibody-negative patient (patient 6).

We performed PCR-ISH and RT-PCR-ISH on the same HBV- or HCV-infected samples from noncancerous and cancerous regions in which we had previously quantitated viral genomic DNA or RNA by RTD-PCR (34). The noncancerous tissue contained 6.1×10^5 copies of HBV DNA/μg total DNA, and the cancerous regions included 4.5×10^5 copies/μg total DNA. Equivalent numbers of cells in the noncancerous and cancerous tissues stained positive for HBV on PCR-ISH (patient 4; Fig. 1A). The PCR-ISH results were consistent with the HBV DNA copy number previously determined by RTD-PCR (34).

Noncancerous tissue from an HCV-positive patient contained 2.0×10^5 copies HCV RNA/μg total RNA, whereas cancerous tissue contained 2.0×10^2 copies/μg total RNA

(patient 12; Fig. 1B). HCV RNA was observed by RT-PCR-ISH in hepatocytes of the liver tissue sections from an HCV-infected patient (Fig. 1B). In the noncancerous tissue, an intense hybridization signal was found at the perinuclear sites of almost all the hepatocytes in the section (Fig. 1B, panel a). In contrast, in the cancerous tissue, there was only a weak HCV RNA hybridization signal in the hepatocytes (Fig. 1B, panel b). When the RT step was omitted (control), no HCV RNA was detected in the noncancerous or cancerous tissue sections (Fig. 1B, panels c and d). These results were consistent with the previous quantitation of HCV RNA copy number by RTD-PCR (34).

Detection of HBV DNA by PCR-ISH. HBV DNA was detected by PCR-ISH in the tissue sections obtained from an HBV DNA-seropositive patient. Amplified PCR products were detected by using a probe for either the S or the X region (Fig. 2A, panels a to d) but were not detected by using a heterologous probe (Fig. 2A, panels e and f). Amplification of either the S or the X region of HBV DNA gave the same pattern of hybridization (Fig. 2A, panels a to d). HBV DNA was detected by PCR-ISH in almost all hepatocytes (Fig. 2A, panels a to d) and was very obvious even under low magnification (Fig. 2A, panels a and c). An intense hybridization signal was observed predominantly at the perinuclear site under high magnification (Fig. 2A, panels b and d). In contrast, HBV DNA was not detected by using HBs- and HBx-matched primer and probe combinations in sections obtained from an HBV DNA-seronegative patient (data not shown). DNA fragments amplified by using the S and X region primer sets were 179 bp and 161 bp, respectively (Fig. 2B). Sections from an HBV DNA-seronegative patient were negative in the PCR analysis (data not shown).

FIG. 2. (A) Panels a to f, HBV DNA detected in liver tissue sections from a patient with chronic hepatitis B by PCR-ISH (42 cycles of PCR). Panels g and h, serial sections were stained with HE. Magnifications, $\times 100$ (panels a, c, e, and g) and $\times 400$ (b, d, f, and h). S+S, primers and probe targeting the S region; X+X, primers and probe targeting the X region; S+X, primers and probe targeting the S and X regions, respectively. (B) Amplified DNA fragments in the PCR mixture of the section in panel A visualized by 3% agarose gel electrophoresis. The PCR product of the X region was 161 bp, and that of the S region was 179 bp.

Localization of HBV DNA, HBV RNA, HBsAg, and HBcAg in liver tissue. HBV DNA, HBV RNA, HBsAg, and HBcAg were detected by PCR-ISH, RT-PCR-ISH, and immunohistochemical staining of serial sections from an HBV-infected patient (patient 5; Fig. 3A, panels a to h). HBV DNA was detected in almost all the hepatocytes by PCR-ISH, although there was wide variation in the hybridization signal intensity between different areas of the section (Fig. 3A, panels a and b). The staining pattern of HBV RNA was similar to that of HBV DNA (Fig. 3A, panels c and d). Intracytoplasmic and intranuclear staining for HBsAg and HBcAg, respectively, was found in some hepatocytes (Fig. 3A, panels f and h). PCR and RT-PCR results were confirmed by gel electrophoresis of the amplified products in the supernatant from the tissue section (Fig. 3B, panels a and b).

Detection of HCV RNA by RT-PCR-ISH. HCV RNA could be detected by RT-PCR-ISH in almost all the hepatocytes in the liver sections obtained from an HCV RNA-seropositive patient (Fig. 4A, panels a and b). Under high magnification, a strong HCV RNA signal was detected in the perinuclear area (Fig. 4A, panel b). A negative-control test (no RT) did not detect any HCV RNA (Fig. 4A, panels c and d). The expected 162-bp DNA fragment amplified by RT-PCR in the supernatant from the tissue section was detected (Fig. 4B, lane 2). In

contrast, HCV RNA was not detected in the liver section obtained from an HCV RNA-seronegative patient, regardless of whether RT was used (data not shown).

Isolation of HCV RNA in hepatocytes by LCM. Hepatocyte groups were captured from the perivenular, intermediate, and periportal areas by LCM (Fig. 4C, panel a). HCV RNA was quantified by RTD-PCR in approximately 30 hepatocytes captured by LCM and normalized against the picogram weight of GAPDH mRNA (Fig. 4C, panels b to d); the HCV RNA levels were equivalent in all three regions (Fig. 4C, panel d).

Detection of HCV RNA in the epithelium of the large bile duct. HCV RNA was detected by RT-PCR-ISH in the epithelium of the large bile duct, which was surrounded by dense fibrous and elastic tissue (Fig. 4D, panels a and b). In contrast, no HCV RNA was detected in the epithelium of the small bile duct. Further, HCV RNA was not detected in the portal vein or its branches (Fig. 4D, panel a).

Detection of HBV DNA and HCV RNA in noncancerous and cancerous liver tissue sections obtained from a patient with HBV and HCV coinfection. Figure 5 shows the results for HBV DNA and HCV RNA in liver samples from a patient with HCC having HCV and HBV coinfection. The amounts of serum HBV DNA and HCV RNA were 39 copies/ml and 5×10^7 copies/ml, respectively (patient 21) (34). In the noncancerous

FIG. 3. (A) Panels a to h, HBV DNA, HBV RNA, HBsAg, and HBcAg detected in OCT-embedded frozen liver tissue from a patient with chronic hepatitis B by PCR-ISH (panels a and b), RT-PCR-ISH (panels c and d), and immunohistochemical staining (panels e to h). Panels i and j, HE staining of serial sections. The primers and probe targeted the S region to detect HBV DNA and HBV RNA. Antibodies to the envelope and core proteins were used to detect HBsAg and HbcAg, respectively. Magnifications, $\times 50$ (panels a, c, e, g, and i) and $\times 400$ (panels b, d, f, h, and j). The number of PCR cycles was 42. (B) Amplified DNA fragments in the PCR mixture of the section in panel A. Panel a, the DNA fragments amplified by 42 cycles of PCR were visualized by 3% agarose gel electrophoresis. Panel b, RT-PCR after DNase I treatment (3 U/ μ l) and negative controls (no RT).

tissue from the patient with HBV and HCV coinfection, there was an intense hybridization signal for HCV RNA on RT-PCR-ISH in almost all the hepatocytes (Fig. 5A, panel b). There was also a positive but weak RT-PCR-ISH signal for HCV RNA in the tumor hepatocytes (Fig. 5B, panel b). Few hepatocytes in the cancerous tissue were positive for HBV DNA by PCR-ISH (Fig. 5B, panel a), and no HBV DNA hybridization signal was detected in the noncancerous tissue (Fig. 5A, panel a).

DISCUSSION

The standard assay for detecting replication of HBV and HCV in tissue is ISH, but results are often inconsistent and sometimes difficult to reproduce. The specificity of ISH is high but its sensitivity low, and it is difficult to detect low copy numbers of the HBV or HCV genome in tissue. PCR technology has been adapted to *in situ* amplification of viral genomes or their replicative intermediates in liver tissue sections, but sensitivity and specificity remain major challenges to the application of this approach (13, 18, 23, 25, 26, 30, 31). Here, we describe the use of a novel, highly specific and sensitive PCR-ISH method to determine the distribution and localization of HBV DNA, HBV RNA, and HCV RNA in both normal and cancerous liver tissues.

PCR-ISH is the most sensitive technology currently available for the detection of viral genomes, but a major potential limitation of this approach is the low specificity. We were able to improve the specificity of PCR-ISH by careful optimization of certain steps. PCR was performed using sets of antisense and sense primers that were complementary to the sequences located in the S and X regions of HBV and the 5'-UTR upstream of the core region of HCV. We added PCR templates to the PCR mixture and then added the PCR mixture to the HBV- or HCV-negative tissue sections. The slides were placed in the GeneAmp *in situ* PCR system 1000 unit, and PCR-ISH was performed as described in Materials and Methods. Following these results, we selected the primer and probe set that did not stain the HBV- or HCV-negative tissue sections by PCR-ISH. Second, the type and concentration of protease and the treatment time were adjusted to optimize permeabilization of membranes and release of protein-nucleic acid cross-linking while avoiding overdigestion. Third, to improve the specificity for detecting viral genomes, we limited the number of PCR cycles and fixed the liver tissue sections in 4% paraformaldehyde immediately after PCR amplification. This step is essential to avoid diffusion of PCR products into neighboring cells, a phenomenon known as the diffusion artifact. Limiting the number of PCR cycles was also important for eliminating the background staining, as too many cycles resulted in high background staining and loss of tissue morphology. Fourth, we added a DIG-dUTP tail at the 3' ends of the probes for PCR-ISH and RT-PCR-ISH. These 45-mer probes were optimized to improve their sensitivity without impairing the specificity.

HBV DNA was detected by PCR-ISH in a large number of hepatocytes in tissue sections from an HBV DNA-seropositive patient (Fig. 1A, panel a). HBV DNA was also observed by PCR-ISH in tumor hepatocytes in a section of cancerous tissue from the same patient (Fig. 1A, panel b). As shown in Fig. 2A,

FIG. 4. (A) Panels a and b, HCV RNA detected in liver tissue samples from a patient with chronic hepatitis C by RT-PCR-ISH. Panels c and d, HCV RNA was not detected in a negative control (no RT). The number of PCR cycles was 45. Panels e and f, serial sections were stained with HE. Magnifications, $\times 40$ (panels a, c, and e) and $\times 400$ (panels b, d, and f). (B) DNA fragments in the PCR mixture of the section in panel A were amplified with the RT step and detected by 3% agarose gel electrophoresis. Amplification without the RT step resulted in no detection of DNA fragments. (C) Panel a, after LCM of nine areas, sections of liver tissue obtained from a patient with chronic hepatitis C were stained with HE. Panels b and c, HCV RNA and GAPDH mRNA in each of the areas were quantified by RTD-PCR, and the results are expressed as copy number per LCM area. Panel d, the copy number of HCV RNA was corrected by using the picogram weight of GAPDH. (D) Panels a and b, HCV RNA detected in the epithelium of the large bile duct by RT-PCR-ISH. The number of PCR cycles was 45. Panels c and d, serial sections were stained with HE. Magnifications, $\times 60$ (a and c) and $\times 600$ (b and d).

we obtained clear and reproducible patterns of distribution or localization of the viral genomes in the tissue sections. The visual patterns of HBV DNA distribution were similar, irrespective of the primer sets and probes (Fig. 2A, panels a to d). These data indicate that our technique is highly specific and reproducible for the detection of HBV DNA.

The staining pattern of HBV RNA was similar to that of

HBV DNA (Fig. 3A, panels c and d). HBV DNA was also observed by PCR-ISH in tumor hepatocytes in a section of cancerous tissue from an HBV DNA-seropositive patient (Fig. 1A, panel b), but neither HBsAg nor HBcAg was detected in this section (data not shown). As shown in Fig. 3, the intensity of HBV DNA by PCR-ISH was almost the same as that of HBV RNA by RT-PCR-ISH but did not coincide with the

FIG. 5. Panels a and b, HBV DNA (panels a) and HCV RNA (panels b) in noncancerous (non-Ca) (A) and cancerous (Ca) (B) liver tissue obtained from a patient coinfecting with HBV and HCV were detected by PCR-ISH (55 cycles of PCR) and RT-PCR-ISH (45 cycles of PCR), respectively. Panels c, serial sections were stained with HE. Magnification, $\times 160$.

intensity of HBV protein expression. HBV has four overlapping open reading frames regulated by two enhancer elements and four promoters (14).

We had reported previously that HCV RNA and HCV core protein levels are relatively stable irrespective of fibrosis (33, 35). In the present study, HCV RNA was detected in almost all hepatocytes in all sections. Furthermore, we emphasize the similar staining pattern of almost all hepatocytes at the cellular level. HCV RNA was detected in the cytoplasm but not in the nucleus. The detection of an intense signal at the perinuclear site may reflect the replication process of HCV (10). HCV RNA was also detected in the epithelium of the large bile duct but not in the endothelial cells, portal tracts, or sinusoidal lymphocytes, which is consistent with the results of previous studies (5, 8). Thus, the epithelium of the large bile duct, but not that of the small duct, can support HCV replication.

We confirmed the specificity for detecting the HCV RNA copy number by quantification in a small area of an LCM section (Fig. 4C); similar amounts of HCV RNA were detected in each area. The detection of HCV RNA in each area by LCM and RTD-PCR excluded the possibility of diffusion of the PCR product by RT-PCR-ISH.

Recent molecular biological techniques have demonstrated low-level HBV viremia in some patients with chronic hepatitis C who were negative for all serological HBV markers. In these cases, HBV DNA not only is integrated in the human chromosomes but also replicates in hepatocytes (34). In the previous study, we measured the levels of HBV DNA and HCV RNA using RTD-PCR with singly infected or coinfecting noncancerous and cancerous liver tissues (34). In the case of coinfection, HCV replication was dominant in the noncancerous

tissue whereas HBV replication was dominant in the cancerous tissue. Some studies have shown that HCV inhibits HBV gene expression and replication (7, 15).

Using this novel, highly specific and sensitive PCR-ISH method, we could visualize the tissue staining patterns of HBV and HCV, which were consistent with those seen by RTD-PCR. This revealed the novel finding that almost all hepatocytes are infected with HBV or HCV in patients with chronic liver disease, suggesting that the viruses spread throughout the liver in the chronic stage. However, further study with a large number of samples from each stage of infection is needed to clarify the mechanism of persistent infection via our assay method.

ACKNOWLEDGMENTS

We are grateful to Yuichi Hirata of the Tokyo Metropolitan Institute of Medical Science and Kyoko Kohara of Kumamoto University for their critical comments and helpful discussions.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency of Japan, and the Ministry of Health, Labor and Welfare of Japan.

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