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Abstract

To establish the most proper method of in situ hybridization in detection of HCV-RNA in the liver, various detailed procedures were examined using frozen as well as paraffin-embedded sections of tissue derived from patients. In frozen sections of the liver from hepatitis C patients obtained at autopsy or surgery, HCV-RNA was detectable by in situ hybridization using thymine-thymine dimerized oligonucleotide DNA probes when the sections were treated with ethanol-acetic acid at first, then 0.2 N hydrochloric acid, proteinase K (0.02 u/ml) and DNase. When the paraffin-embedded liver sections were used, more intense proteinase K treatment (0.2-2 u/ml) was required to expose viral RNA and even after that, the positive HCV-RNA signals were less than those in frozen sections, because the cytoplasmic RNA in the routine paraffin-embedded sections was preserved unevenly and less than in frozen sections. These findings indicate that in situ hybridization of HCV-RNA is useful for diagnosing HCV infection and should be a potent tool for monitoring the state of virus activities during therapy. However, the liver biopsy method should be modified so that RNA is retained properly to utilize biopsies more effectively for the routine diagnosis of HCV infection.

KEYWORDS: hepatitis C virus, RNA of hepatitis C virus, in situ hybridzation, thyminethymine dimer, oligonucleotide DNA probe

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Localization of Hepatitis C Virus RNA in Human Liver Biopsies by *In Situ* Hybridization Using Thymine-Thymine Dimerized Oligo DNA Probes: Improved Method

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To establish the most proper method of *in situ* hybridization in detection of HCV-RNA in the liver, various detailed procedures were examined using frozen as well as paraffin-embedded sections of tissue derived from patients. In frozen sections of the liver from hepatitis C patients obtained at autopsy or surgery, HCV-RNA was detectable by *in situ* hybridization using thymine-thymine dimerized oligonucleotide DNA probes when the sections were treated with ethanol-acetic acid at first, then 0.2 N hydrochloric acid, proteinase K (0.02 u/ml) and DNase. When the paraffin-embedded liver sections were used, more intense proteinase K treatment (0.2-2 u/ml) was required to expose viral RNA and even after that, the positive HCV-RNA signals were less than those in frozen sections, because the cytoplasmic RNA in the routine paraffin-embedded sections was preserved unevenly and less than in frozen sections. These findings indicate that *in situ* hybridization of HCV-RNA is useful for diagnosing HCV infection and should be a potent tool for monitoring the state of virus activities during therapy. However, the liver biopsy method should be modified so that RNA is retained properly to utilize biopsies more effectively for the routine diagnosis of HCV infection.

Key words : hepatitis C virus, RNA of hepatitis C virus, *in situ* hybridization, thymine-thymine dimer, oligonucleotide DNA probe

Currently hepatitis C virus (HCV) infection of the liver (1) is indirectly diagnosed, singularly or in combination, by the immunochemical detection of HCV-associated antibodies in sera (2, 3), detection of HCV-RNA in sera by reverse transcription-polymerase chain reaction (RT-PCR) (4), findings of HCV-associated pathological changes in liver biopsies, or more recently, an immunohistochemical detection of antigens associated with HCV in liver biopsies (5). However, none of the above unequivocally demonstrate the HCV infection at the cellular level of the liver. Only the *in situ* demonstration of sense as well as anti-sense HCV-RNA in cells of the liver proves an active infection of HCV (6).

In our previous studies on the *in situ* localiza-

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tion of HCV-RNA by using thymine-thymine (T-T) dimesized synthetic oligonucleotide DNA probes (7, 8), the optimization of conditions for *in situ* hybridization was performed mainly using frozen sections obtained at autopsy or surgical operation, and the original hybridization procedure was established (5, 9). However, with frozen sections of needle biopsies (10) and sections of paraffin-embedded tissues, it was found that background staining was high and the distinction between the positive signal and the nonspecific one was difficult. Nevertheless, most liver tissues available for histological examination are those fixed in neutral formalin for various durations, dehydrated and embedded in paraffin. Consequently, in order for *in situ* detection of the HCV-RNA to be a definitive diagnosis for HCV infection in routine clinical laboratories, one has no option other than to utilize paraffin-embedded liver tissues.

In this study, we further optimized the *in situ* hybridization of HCV-RNA and added various treatments to increase the specificity and sensitivity. In order to make this method applicable to routine clinical diagnosis, procedural conditions for diverse materials such as frozen sections of paraformaldehyde (PFA) fixed liver from autopsy, surgery and needle biopsy, and paraffin-embedded sections of formalin fixed liver from autopsy, surgery and needle biopsy were established.

It was found that in frozen sections of the liver, HCV-RNA was most effectively detectable by *in situ* hybridization using T-T dimerized oligonucleotide DNA probes when the sections were treated with ethanol-acetic acid at first, then 0.2 N hydrochloric acid (HCl), proteinase K (0.02 u/ml) and DNase. When the paraffin-embedded liver sections were used, more intense proteinase K treatment (0.2–2 u/ml) was required to expose viral RNA, and even after that, the positive HCV-RNA signals were less than those in frozen sections. This was because the cytoplasmic RNA in the routine paraffin-embedded sections was less preserved than in frozen sections, and was retained unevenly.

Materials and Methods

DNA probes. According to the reported sequences of HCV cDNA (11), anti-sense probes to seven different regions and sense probes corresponding to five of the seven regions were synthesized with a model 391 PCR-MATE DNA synthesizer (Applied Biosystems Japan, Tokyo, Japan) (Fig. 1). We also synthesized an anti-sense DNA probe to rat prolactin cDNA (12) as a control probe (Fig. 1). At the time of synthesis, to each of the probes, repeated adenine-thymine-thymine (A-T-T) sequences were added (13). T-T dimers were introduced into the DNAs by ultraviolet (UV)-irradiation. From the irradiation doses of 5,000, 7,000, 10,000, 12,000, 15,000 J/m^2, the optimal dose was selected which yielded the strongest signal by immunohistochemical detection and dot blot hybridization as described previously (7, 8).

Tissues. Human liver tissues were obtained at the time of autopsy, at the time of surgery for hepatoma excision, and by needle biopsy (Table 1). Some of them were from patients with type C chronic liver disease whose sera was positive for anti-c100-3 by Ortho HCV antibody ELISA test (Ortho Diagnostic Systems, Japan) and/or for HCV-RNA by RT-PCR. Needle biopsy specimens from patients with chronic hepatitis B, and from patients whose sera were negative, both HBV and HCV makers were also used (Table 1). Each tissue was histologically diagnosed as to the disease state. At the time of sample collection, the tissues were divided into two parts. One segment was fixed at 4°C for 4h to overnight in 4% PFA dissolved in phosphate buffered saline (PBS), washed in 30% sucrose in distilled water containing 0.02% diethylpyrocarbonate, embedded in O. C. T. compound (Miles Inc., Elkhart, USA) and frozen. The other segment was fixed in neutral-buffered 10% formalin at room temperature for 4h to overnight, dehydrated, and embedded in paraffin. The frozen or paraffin-embedded tissues were sectioned at 6μm in thickness and placed on gelatin coated glass slides. The sections were air dried thoroughly to ensure a tight adherence to the slide. Before the paraffin-embedded tissues were used for *in situ* hybridization, the sections were deparaffinized, hydrated until in 70% ethanol, and were immersed and kept in PBS.

Estimation of RNA retention. In order to estimate the RNA retention in the sections, methylgreen-pyronin

staining (14) was performed prior to hybridization. The sections which proved to be poor in RNA preservation were omitted from this study. In some cases, the staining was carried out to confirm the effect of the treatments, such as DNase and proteinase K treatments.

Procedures for in situ hybridization. Original *in situ* hybridization was performed according to the following steps, and all procedures were carried out at room temperature unless otherwise specified: Step 1: Sections were rehydrated with PBS. Step 2: Sections were treated with 0.2 N HCl for 20 min. Step 3: Sections were digested with 0.02 unit/ml of proteinase K dissolved in PBS at 37°C for 10 min in order to expose viral RNA, and washed 3 times with PBS for 5 min each. Step 4: Specimens were fixed in 4% PFA in PBS for 5 min and rinsed with PBS. The remaining aldehyde was quenched twice by immersion in 2 mg/ml glycine in PBS for 15 min each. Step 5: Specimens were rinsed with PBS, immersed and kept in 40% (v/v)deionized formamide in 2 × SSC until hybridized. Step 6: Hybridization: Basically the hybridization mixture contained 10 mM Tris/HCl (pH7.4), 1 mM EDTA, 0.6 M NaCl, 1 × Denhardt's solution, 40% (v/v) deionized formamide, 250 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA and T-T dimerized DNA probes at total concentrations of 1.0 to 3.5 μg/ml. Mixtures of three to seven anti-sense probes or of sense probes were used as DNA probes. Twenty μl of the hybridization mixture was applied to each slide, mixed, and incubated in a moist chamber at 37°C overnight. The hybridization temperature and the concentrations of salts and formamide were set according to the estimated Tm (13). Step 7: The slides were

HCV ANTI-SENSE DNA PROBES

*a.(-36- -67)

5'-TTATTA-AAGCACCCTATCAGGCAGTACCACAAGGCCTT-ATTATTATT-3'

*b.(8- -36)

5'-TTATTA-GTGCTCATGGTGCACGGTCTACGAG

ACCTCCCGGGGCACTCGCA-ATTATTATT-3'

c.(5062-5030)

5'-TTATTA-AGACGACCCTGCCCACTATGACCACGCAGCCAG-ATTATTATT-3'

d.(5096-5066)

5'-TTATTA-TCCCTGTCAGGTATGATGCTCGCCGAGCAGT-ATTATTATT-3'

*e.(5235-5191)

5'-TTATTA-AACCTCTGCCTGACGGGACGCGGTC

TGCAGGAGGCCGAGGGCCTT-ATTATTATT-3'

*f.(5701-5657)

5'-TTATTA-TTGCTGCACAGACCACGCCGACTAC

GAGGGCTCCGGGCGAGAGGA-ATTATTATT-3'

*n.(-223- -267)

5'-TTATTA-TCCTGGAGGCTGCACGACACTCATA

CTAACGCCATGGCTAGACGC-ATTATTATT-3'

RAT PROLACTIN ANTI-SENSE DNA PROBE

(329-296)

5'-TTATTA-ACATATCTGTATACAGGGTATGGATGTAGTGAGA-ATTATTATT-3'

Fig. 1 Sequences of oligonucleotide DNA for HCV anti-sense probes and a rat prolactin anti-sense probe. HCV sense probes corresponding to five of seven HCV anti-sense probes were also synthesized (*).

Table 1 Detection of HCV-RNA in the liver by *in situ* hybridization in paraformaldehyde fixed frozen tissues (A) and formalin fixed paraffin-embedded tissues (B)

Types of hepatitis	Method for obtaining tissues	Number of patients				
		Histological diagnosis				
		CPH	CAH	LC(HCC)	OTHER	TOTAL
(A)						
Type C chronic liver disease [a] n = 23	Autopsy	0(0)	0(0)	5(4)	0(0)	5(4)
	Surgery	0(0)	3(1)	1(0)	0(0)	4(1)
	Needle biopsy	7(2)	6(1)	1(0)	0(0)	14(3)
Chronic hepatitis B [b] n = 2	Needle biopsy	1(0)	1(0)	0(0)	0(0)	2(0)
Non-B, non-C [c] n = 1	Needle biopsy	0(0)	0(0)	0(0)	1 [d] (0)	1(0)
(B)						
Type C chronic liver disease [a] n = 16	Autopsy	0(0)	0(0)	2(0)	0(0)	2(0)
	Surgery	0(0)	2(0)	1(1)	0(0)	3(1)
	Needle biopsy	4(1)	6(1)	1(0)	0(0)	11(2)
Non-B, non-C [c] n = 1	Needle biopsy	0(0)	0(0)	0(0)	1 [d] (0)	1(0)

a: With sera positive for anti-c100-3 by ELISA and/or for HCV-RNA by RT-PCR. *b*: With sera positive for HBsAg by radioimmunoassay. *c*: With sera negative for both HCV and HBV markers. *d*: Showing histology of liver fibrosis. Figures in parentheses indicate the number of patients with HCV-RNA in the liver detected by *in situ* hybridization. CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

washed three times with 50 % (v/v) formamide in 2 × SSC at 37°C, twice with 50 % (v/v) formamide in 1 × SSC at 37°C for 1 h each, and twice in 1 × SSC for 15 min each. Step 8: Hybridized T-T dimerized DNA probes were immunohistochemically detected using the indirect horseradish peroxidase (HRP) labeled antibody method (15). The details were described previously (7, 8, 13).

Modification of in situ hybridization procedures. In order to reduce background staining and to increase signals, various modifications, individually or in combination, were experimented. Some of them are listed below: **1.** Before step 1, the air dried frozen sections or the paraffin-embedded sections which were deparaffinized and hydrated with PBS, were immersed in an ethanol-acetic acid (3:1) mixture for 20 or 30 min, acetone for 10 min at 4°C, 100 % ethanol for 30 min after acetone for 10 min at 4°C or immersed in PBS for control. **2.** Between steps 1 and 2, some of the sections were heated in PBS for 5 min at 90°C. The effect of the treatment on morphology was examined after proteinase K digestion by hematoxylin and eosin stain in some cases. **3.** In step 2, the concentration of HCl was increased to 1 N HCl, the duration of treatment was decreased to 15 min, and the temperature was increased to 60°C. **4.** Instead of steps 2 through 4, some rehydrated sections were immersed in a sodium dodecyl sulfate (SDS) solution containing 10 mM Tris/HCl and 0.6 M NaCl at the SDS concentration of 1.0, 0.1 or 0 % for 30 min at 80°C. The effect of the treatment was also observed by methylgreen-pyronin staining. **5.** In step 3, when using paraffin-embedded sections, the concentration of proteinase K in PBS was varied from 0.02 to 5.0 unit/ml and digested for 10 to 30 min at 37°C. **6.** Between steps 3 and 4, some of the sections were treated with DNase. The sections were washed in 0.1 M sodium acetate buffer (pH 5.0) for 5 min and treated with DNase I (30 unit/ml) in 0.1 M sodium acetate buffer (pH 5.0) containing 0.01 M

$MnCl_2$ for 1 h at 37°C. In some cases, 10 mM $MgCl_2$ was substituted in place of $MnCl_2$ in the reaction buffer. Then sections were washed once in 0.1 M sodium acetate buffer (pH 5.0) containing 0.05 M EDTA and twice in PBS containing 0.05 M EDTA for 15 min each. The effects of DNase treatment and RNA retention were assessed by Feulgen reaction (16) and methylgreen-pyronin staining. **7.** Between steps 4 and 5, when paraffin-embedded sections were used, acetylation was performed to reduce non-specific staining. The sections were treated in freshly prepared 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min after quenching by glycine in PBS. **8.** Between steps 5 and 6, prehybridization was performed prior to hybridization with the hybridization mixture described above, containing 15 μg/ml oligo-DNA in place of T-T dimerized DNA probe for 15 h at 37°C. Oligonucleotide DNA with similar numbers of bases to the HCV probes but has little homology with both HCV-RNA and known genomic DNAs was used as a blocking DNA. **9.** In step 6, in the basic hybridization mixture, the concentration of deionized formamide was reduced to 30% (v/v). Three, five, or seven kinds of HCV anti-sense probes were mixed at the concentration of 0.5 μg/ml each to improve the detection efficiency. Each HCV anti-sense and sense probe was also used individually at the concentration of 1.0 μg/ml in the hybridization mixture, using paraffin-embedded sections from one case obtained at surgery. After applying and mixing the hybridization mixture, each slide was heated on a hot plate up to 80°C for 30 sec. **10.** In step 7, the SSC washing schedule after the 50% formamide in 1 × SSC washing was changed to 1 × SSC, 2 × SSC, 8 × SSC, 8 × SSC, 2 × SSC for 15, 15, 30, 30, and 15 min, respectively. **11.** In step 8, for the purpose of reducing non-specific binding of anti-T-T dimer antibodies, the sections were blocked by PBS containing normal sheep serum (× 10) instead of normal goat IgG before application of the first antibody, or washed after incubation of the first antibody with PBS containing 0.05% Brij five times for 15 min each. Alkaline phosphatase (ALP) conjugated anti-rabbit IgG antibody was used as the second antibody (17) or biotin labeled anti-rabbit IgG and HRP conjugated streptoavidin (ABC method) was used instead of HRP conjugated anti-rabbit IgG. Some sections were reacted with HRP conjugated goat anti-rabbit IgG for 1 h and were followed by an incubation with HRP conjugated rabbit anti-goat IgG for 1 h. The results were evaluated by visualization as usual.

Results

By immunohistochemical detection and dot blot hybridization, a dose of UV which generates maximally detectable T-T dimer among 5,000, 7,000, 10,000, 12,000, 15,000 J/m^2 was found to be 10,000 J/m^2 for the mixtures of HCV anti-sense as well as that of HCV sense oligonucleotide probes.

In most cases, the RNA was best retained in frozen sections of liver obtained at surgery, followed by paraffin sections of liver obtained at surgery and frozen sections of biopsy, and least retained in paraffin sections of needle biopsy by methylgreen-pyronin staining. The cytoplasmic RNA was lost in some specimens, but some specimens contained sufficient RNA to perform *in situ* hybridization. There was no fixed pattern on the retention of RNA, however, it was found that when needle biopsies were fixed in freshly made buffered paraformaldehyde solution immediately after resection, a good amount of RNA was retained. Although the degree of RNA retention, the conditions of *in situ* hybridization, and the additional procedures varied considerably among the specimens, it was found that in 8 of 23 frozen and 3 of 16 paraffin-embedded sections from patients with type C chronic liver disease, the positive signals with the HCV anti-sense probes were detected (Table 1).

With the original *in situ* hybridization protocol, a positive signal was observed mainly in the cytoplasm of hepatocytes in frozen sections obtained at autopsy or surgery, from hepatitis C patients, with HCV anti-sense probes as reported previously (5, 9). The positive cells were mainly scattered in the lobules, though in a few cases many positive cells were grouped in some lobules. With HCV sense probes, the distribution of positive cells was similar to that observed with the HCV anti-sense probes. With rat prolactin anti-sense probe, no hepatocyte was positive, but occasional infiltrating lymphatic cells appeared positive. No signal was also detected in the liver from patients without HCV markers. However,

when frozen or paraffin-embedded needle biopsies were used, strong non-specific staining was observed in the nuclei of hepatocytes as well as that of littoral cells with HCV anti-sense, HCV sense, and rat prolactin anti-sense probes. With the paraffin-embedded sections, there was particulated non-specific staining in addition to the general nuclear and background staining. In addition, with these specimens, cytoplasmic RNA was preserved unevenly when examined by methylgreen-pyronin staining. Generally, cytoplasmic RNA was well retained near the surface and less at the central region of the biopsy sections rather than in autopsy or surgical specimens. The positive signal with HCV anti-sense probes was detected in the region where RNAs were preserved whereas little or no signal was detected in areas where RNA was lost.

With the frozen biopsy sections, the most effective treatment to reduce the non-specific staining among the four fixatives was ethanol-acetic acid (3:1) as reported previously (10). The treatments in ethanol alone and ethanol after acetone were somewhat effective, but acetone alone had little effect in reducing the non-specific background staining. Although these treatments reduced non-specific staining extensively, some in the nuclei remained. The treatment in 1 N HCl was no more effective than 0.2 N HCl in reducing background staining. The DNase treatment reduced the non-specific staining in the nuclei significantly, although some background staining remained as reported previously (10). The Feulgen reaction and methylgreen-pyronin staining before and after DNase treatment indicated that the digestion of DNase was effective in removing DNA without an effect on RNA. The insertion of a prehybridization step was not particularly effective in reducing background or nuclear staining. The most effective way to reduce non-specific nuclear and background staining was found to be ethanol-acetic acid fixation followed by DNase treatment when frozen needle biopsies were used. With these treatments, the sites of hybridization with HCV anti-sense as well as sense DNA probes in the cytoplasm of hepatocytes were clearly distinguished from the non-specific ones (Fig. 2a, b, d). No signal was observed with a rat prolactin anti-sense probe (Fig. 2c).

With paraffin-embedded biopsy or surgical specimens, DNase treatment was also found to be effective for the reduction of the non-specific staining in the nuclei, however, it had no effect on the particulated staining. The use of Mg instead of Mn in the DNase incubation buffer was ineffective. Acetylation was somewhat effective in reducing the general tissue background staining, but did not reduce the non-specific staining in the nuclei or the particulated staining. The change in the concentration of deionized formamide in the hybridization mixture from 40 % (v/v) to 30 % (v/v), and the change in the SSC washing schedule after the 50 % formamide in 1 × SSC were not particularly effective in reducing background and nuclear staining. Among various attempts to expose viral RNA, sections treated with SDS loosened the morphology, but the specific positive signal was not increased. It was found that higher concentrations of proteinase K were required to yield sufficient signals for paraffin-embedded tissue sections than what was required for frozen sections. For needle biopsies embedded in paraffin, a digestion with 0.5 unit/ml of proteinase K for 30 min at 37°C was required, whereas for some of the autopsy or surgical specimens embedded in paraffin, as much as 5.0 unit/ml was required. The effectiveness of proteinase K treatment on the sections which were warmed in PBS increased, however, the distortion of morphology was too severe and this procedure could not be used for *in situ* hybridization. Under the condition that the HCV-RNA was sufficiently exposed, the additional ethanol-acetic acid treatment followed by a DNase treatment was also the most effective way to reduce non-specific nuclear and background staining for paraffin-embedded sections, but the particulated staining could not be removed. With these treatments, the positive staining with HCV anti-sense

as well as sense DNA probes in the cytoplasm of hepatocytes was observed (Fig. 3a, b). No signal was observed with a rat prolactin anti-sense probe (Fig. 3c). Heating the tissue sections before hybridization slightly improved the detection level of the positive signal and was not so deteriorative to the morphology.

When HCV anti-sense and sense probes were used individually using paraffin-embedded surgery sections, the level of the positive HCV-RNA signal and the background staining were obrerved differently among them. However, when a mixture of the probes was used, more positive signals were detected than when the probes were used individually.

The procedure of blocking with normal sheep serum, washing after incubation of the first antibody with PBS containing 0.05% Brij or visualizing with ALP conjugated antibody did not have much beneficial effect, although the Brij reduced background staining somewhat. An attempt to increase the level of immunohistochemical detection of T-T dimers using the ABC method resulted with more background staining than the specific signal. In our preliminary study, when the second antibody was followed by HRP conjugated anti-goat IgG and visualized as usual, the signal was increased about three times. But in this study, there was no increase in positive signals, and it resulted with very high background staining.

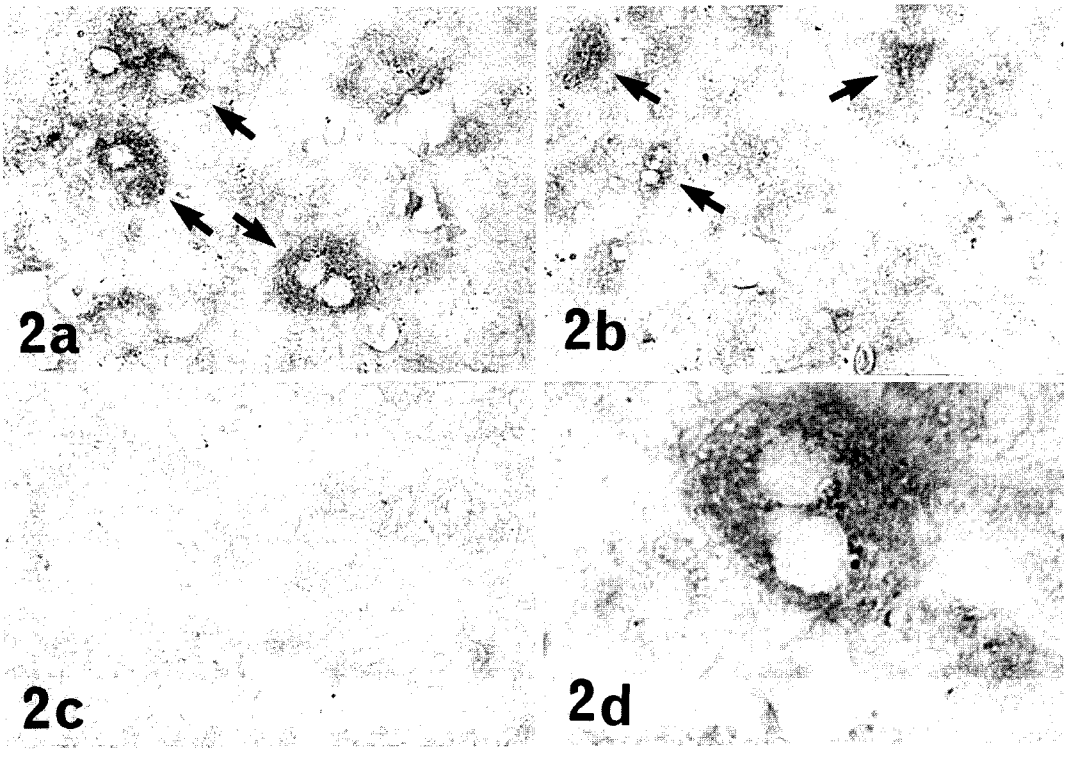

Fig. 2 *In situ* hybridization of HCV-RNA using paraformaldehyde fixed frozen liver biopsy specimens of patients with chronic hepatitis C. (a) The positive signal with HCV anti-sense probes was observed in the cytoplasm of hepatocytes, and the positive cells were scattered in lobules (shown by arrows). (× 440) (b) The positive signal with HCV sense probes was also observed similarly as with HCV anti-sense probes (shown by arrows). (× 440) (c) No signal was detected with the rat prolactin anti-sense probe. (× 440) (d) Higher magnification of positive hepatocytes for HCV-RNA shown by an arrow in Fig. 2a. (× 1100)

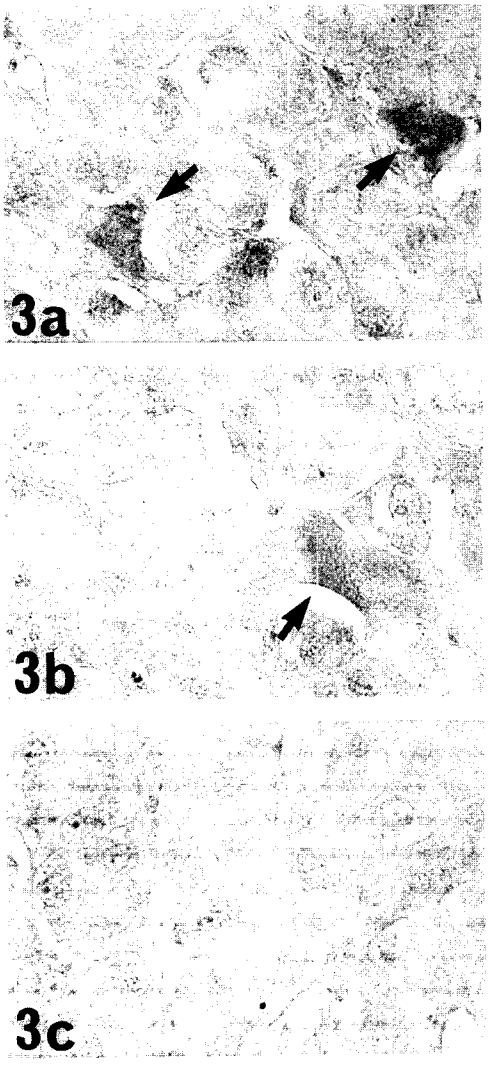

Fig. 3 *In situ* hybridization of HCV-RNA using the formalin fixed paraffin-embedded liver tissue of a liver cirrhosis type C patient obtained at surgery. (a) The positive signal with HCV anti-sense was observed in the cytoplasm of hepatocytes after proteinase K treatment (0.2 u/ml) (shown by arrows). (× 400) (b) The positive signal with HCV sense probes was weakly observed (shown by an arrow). (× 400) (c) No signal was detected with the rat prolactin anti-sense probe. (× 400)

Discussion

There are several prerequisites in order for *in situ* hybridization to be practical and useful for the diagnosis of viral infection of the liver in routine histology laboratories. These include that the method should be, first of all, reliable, safe, yield results quickly, be performed with ease by laboratory technicians, and be applicable to the majority of fresh, fixed and paraffin-embedded liver sections. In major medical centers, specimens are usually delivered either as fresh tissue or in buffered formalin, whereas those from peripheral hospitals and clinics for referral are often already embedded in paraffin or as tissue sections mounted on glass slides. Hence, our attentions were first focused on the condition of paraffin blocks on the state of cytoplasmic RNA. It was found that the amount of RNA retained varied considerably from tissue to tissue and from facility to facility as judged by pyronin staining (14). In general, the RNA was best retained in frozen sections of liver obtained at surgery, followed by paraffin sections of liver obtained at surgery and frozen sections of biopsy, in that order, when the in-house specimens were compared. Those that retained the least RNA were paraffin sections of needle biopsy. Some of the biopsies were essentially void of RNA but some contained sufficient RNA to perform *in situ* hybridization. There was no discernible rule on the retention of RNA, and even among those needle biopsies which were fixed in freshly made buffered paraformaldehyde solution immediately after resection, some contained a good amount of RNA and others did not. The reason why RNA was preserved less in the needle biopsy is not clear, but from the zonations observed on the degree of RNA preservation, it is perhaps partially the result of compression and squeezing which were caused by the biopsy needle which prevented even penetration of fixatives into the specimens. In this regard, the procedure for needle biopsy requires some modification so that maximum amount of RNA can be retained. It also points to a difficulty of performing the procedure on specimens which have been stored for retrospective studies, since the amount of RNA retained may vary among biopsies. Conse-

quently, most of the remaining experiments were carried out on specimens which were proven to contain a sufficient amount of RNA by pyronin staining.

For the successful implementation of *in situ* hybridization, it is essential that the viral RNA which are embedded in masses of proteins in hepatocytes are made accessible to the probes. The removal of proteins is usually done either by solubilizing proteins in acidic solution or by digesting with pronases. From our previous studies of formalin fixed tissues, the acid treatment alone is insufficient, and digestion with pronase is required for the exposure of cytoplasmic RNAs (18). The concentration and duration of proteinase K treatment varied from specimen to specimen, but those liver tissues embedded in paraffin required about 10 to 100 times more extensive digestion than those of frozen sections. To expose further the viral RNA to the probes, the sections were heated after the application of the hybridization mixture. We found this maneuver was effective for increasing the signals. Even after extensive proteinase K treatment and heating, less positive signals with HCV anti-sense and sense probes were detected in paraffin-embedded sections than in frozen sections. The tendency was more evident for paraffin-embedded needle biopsy specimens even when cytoplasmic RNA was preserved well.

As was with frozen sections, we suspect that the entire viral RNA was not exposed even after the extensive proteinase K digestion and heating, and we opted for the use of a mixture of probes so as not to miss the exposed stretches. The use of a mixture of probes also helped to avoid the stretches known to be rich in mutation and non-specific hybridization. Several anti-sense and sense oligonucleotide DNA probes were synthesized from different regions of HCV cDNA which are known either to be well conserved or used for the synthesis of antigenic peptides for the detection of antibodies. Those sequences were from the 5' non-coding region to the core portion of the structural region or from a non-structural (NS) 4 region. The former region was reported as highly conserved (19), and the latter corresponds to c100 polypeptide (2). The sequences were also selected as not having had adjacent thymine residues in order to avoid introducing mismatches when the probes were UV irradiated later on. In addition, the base sequences were selected to avoid intra and inter hybridization. In a preliminary experiment, we found indeed when a mixture of probes was used, more signals were generated than when probes were used individually. All of the probes were designed to be detectable by non-radioactive means by adding repeated sequences of A-T-T on the 5'-and 3'-ends (12). This offered the advantage of being able to perform *in situ* hybridization in a routine histology laboratory (7) and avoided the need of having an expertise in molecular technology (13).

When paraffin embedded tissue sections were used, we found extremely strong non-specific nuclear staining, particularly in the case of needle biopsy. We reasoned that those tissues embedded in paraffin were exposed to organic solvents and some contaminants in the solvents adhered to nuclei, possibly to DNA, and induced the non-specific affinity to the probes. It was found that some of the non-specific staining was reduced by washing the section with ethanol or an ethanol-acetic acid (3:1) mixture, and reduced further when the sections were treated with DNase. With addition of these modifications, the positive HCV-RNA signals could be effectively detected.

At the level of immunohistochemical detection of T-T dimers, several other immunohistochemical procedures such as the ABC method, and the use of HRP-labeled third antibodies were tried, but they did not result with benficial effects although extensive studies were not carried out.

The positive signal of the hybridization with HCV anti-sense as well as sense probes in the hepatocytes was observed in the liver of patients with hepatitis C. On the other hand, no signal was detected in the liver of patients without HCV markers. With either of the specimens, no signal was generated with a rat prolactin anti-sense

probe. These findings indicate that the sites of positive signals depicted the specific sites of HCV-RNA. Being able to denote the positive as well as the negative strands of HCV-RNA in sections of liver by *in situ* hybridization not only demonstrates virus infection but also alludes to the presence of a replicating virus (6, 20). Moreover, correlating the distribution of HCV-RNA with other histological, immunohistochemical and serological findings (5) will define more precisely the state of HCV infection by such interferons before, during, and after therapy.

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References

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW and Houghton M: Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science (1989) **244**, 359-362.
2. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee WS, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW and Houghton M: An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science (1989) **244**, 362-364.
3. Okamoto H, Munekata E, Tsuda F, Takahashi K, Yotsumoto S, Tanaka T, Tachibana K, Akahane Y, Sugai Y, Miyakawa Y and Mayumi M: Enzyme-linked immunosorbent assay for antibodies against the capsid protein of hepatitis C virus with a synthetic oligopeptide. Jpn J Exp Med (1990) **60**, 223-233.
4. Weiner AJ, Kuo G, Bradley DW, Bonino F, Saracco G, Lee C, Rosenblatt J, Choo QL and Houghton M: Detection of hepatitis C viral sequences in non-A, non-B hepatitis. Lancet (1990) **335**, 1-3.
5. Yamada G, Nishimoto H, Endo H, Doi T, Takahashi M, Tsuji T, Yoshizawa H, Nozawa M, Koji T and Nakane PK: Localization of hepatitis C viral RNA and capsid protein in human liver. Dig Dis Sci (1992) (in press).
6. Fong TL, Shindo M, Feinstone SM, Hoofnagle JH and Bisceglie AM: Detection of replicative intermediates of hepatitis C viral RNA in liver and serum of patients with chronic hepatitis C. J Clin Invest (1991) **88**, 1058-1060.
7. Nakane PK, Moriuchi T, Koji T, Tanno M and Abe K: *In situ* localization of mRNA using thymine-thymine dimerized cDNA. Acta Histochem Cytochem (1987) **20**, 229-243.
8. Koji T, Sugawara I, Kimura M and Nakane PK: *In situ* localization of c-myc mRNA in HL-60 cells using non-radioactive synthetic oligodeoxynucleotide probes. Acta Histochem Cytochem (1989) **22**, 295-307.
9. Yamada G, Takahashi M, Endo H, Takaguchi K, Shimomura H and Tsuji T: Pathogenesis and interferon treatment in chronic hepatitis C; in Frontiers of Mucosal Immunology, Tsuchiya, Nagura, Hibi and Moro eds, Vol 2, Excerpta Medica, Amsterdam (1991) pp 19-22.
10. Endo H, Yamada G, Nishimoto H, Tsuji T and Nakane PK: Localization of hepatitis C virus RNA in human liver by *in situ* hybridization using thymine-thymine dimerized DNA probes. Acta Histochem Cytochem (1991) **24**, 511 (abstract).
11. Houghton M, Choo QL, Kuo G, Han J, Urdea MS, Weiner AJ and Monroy GH: International patent application (1990) 355, 961.
12. Maurer RA, Erwin CR and Donelson JE: Analysis of 5' flanking sequences and intron-exon boundaries of the rat prolactin gene. J Biol Chem (1981) **256**, 10524-10528.
13. Koji T and Nakane PK: Localization *in situ* of specific mRNA using thymine-thymine dimerized DNA probes: Sensitive and reliable non-radioactive *in situ* hybridization. Acta Pathol Jpn (1990) **40**, 793-807.
14. Taft EB: The problem of a standardized technic methylgreen-pyronin stain. Stain Technol (1951) **26**, 205-212.
15. Nakane PK and Pierce GB: Enzyme-labeled antibodies: preparation and application for localization of antigens. J Histochem Cytochem (1966) **14**, 929-931.
16. Feulgen R: Von der nuclearfaarbung zum plasmalogen. Schrift der Univ Giesen H (1939) 1, 445.
17. Harlow E and Lane D: Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988) pp 505.
18. Koji T, Moriuchi T and Nakane PK: Improved tissue preparation for *in situ* hybridization of specific mRNA using non-radioactive DNA probes: Effects of protease digestion and probe size on signal detection in frozen and paraffin section of rat pituitary glands. Acta Histochem Cytochem (1988) **21**, 187-200.
19. Okamoto H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, Tsuda F, Miyakawa Y and Mayumi M: The 5'-terminal sequence of the hepatitis C virus genome. Jpn J Exp Med (1990) **60**, 167-177.
20. Cleaves GR, Ryan TE and Schlesinger RW: Identification and characterization of type 2 Dengue virus replicative intermediate and replicative form RNAs. Virology (1981) **111**, 73-83.

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