Detection of Genomic and Intermediate Replicative Strands of Hepatitis C Virus in Liver Tissue by In Situ Hybridization

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Abstract

Nonisotopic in situ hybridization using a digoxigenin-labeled cDNA probe to the 3′ nonstructural region (NS5) of hepatitis C virus (HCV) was performed on liver tissue from 33 patients. The results were compared with PCR detection of HCV RNA performed on 24 of the biopsies. Nonisotopic in situ hybridization correlated well with PCR findings. Hybridization signals were detected, within the cytoplasm and nuclei/nucleoli of hepatocytes, mononuclear, and biliary epithelial cells. In patients with clinically and histologically defined chronic active hepatitis related to active HCV infection, HCV genome was frequently detected in biliary epithelium and correlated well with biliary damage, an otherwise uncommon finding in chronic active hepatitis due to other hepatotropic viruses. Further studies using sense and antisense probes synthesized from the 5′ noncoding region of the HCV genome confirmed the localization of positive strand of HCV in the above cell populations. The replicative intermediate strand was also present in all cells, although less frequently observed, apart from biliary epithelium, where negative strand of HCV was undetectable. The findings of HCV genome in liver biopsies of two patients with no significant histological abnormalities may suggest that the damage seen in chronic HCV infection is immune mediated, although the cytopathic effect of the virus may also be important. (*J. Clin. Invest.* 1993. 91:2226–2234.) Key words: In situ hybridization • polymerase chain reaction • hepatitis C virus • formalin-fixed tissue • liver disease

Introduction

Hepatitis C virus (HCV),[1] the causative agent of the majority of cases of posttransfusion hepatitis has recently been isolated, cloned, and sequenced (1, 2) and is a single stranded RNA virus of ~ 10 kb in length. Comparative sequencing studies of the HCV genome suggest that this virus is distantly related to flaviviruses and pestiviruses (3, 4). Although the availability of tests for antibody (anti-HCV) to nonstructural and structural antigens of HCV has greatly advanced knowledge of the epidemiology of non-A, non-B hepatitis (NANBH), a significant proportion of patients with autoimmune liver disease have false positive reactivity to anti-HCV (C100) (5) and the significance of a positive anti-HCV test in patients with liver disease is presently unknown. Furthermore, antibody testing cannot determine whether a patient with anti-HCV has recovered from the infection or is still viremic. During the early phase of primary HCV infection there is a period of several months of seronegativity in the presence of ongoing viremia during which time HCV RNA detected by PCR is the only evidence of infection (6, 7). Similarly, in the chronic state, the presence of antibody does not always correlate with the persistence of infection as defined by detection of HCV RNA in the serum (8). Recently there have been a number of reports using PCR and "nested" PCR to detect the viral genome in serum and liver tissue (9, 10). However, PCR of HCV RNA extracted from either serum or liver tissue cannot provide information regarding the site of infection and/or replication of HCV. In the present study we have applied nonisotopic in situ hybridization (NISH) to detect the HCV genome within liver tissue from patients with a variety of liver disorders and compared the results obtained with nested PCR performed on different sections obtained from the same tissue.

Methods

Patients. 33 formalin-fixed paraffin-embedded liver biopsy specimens from patients with both acute and chronic liver disease were studied (Table I); 20 of the 33 patients had chronic liver disease (mean age 40±11, 12 male: 8 female), 8 had acute but unresolved hepatitis (mean age 39±10, 4 male: 4 female), while biopsies from 5 other patients demonstrated no histological abnormality (mean age 41±25, 2 male: 3 female). 16 of the 20 patients with chronic disease had been diagnosed as NANBH after serological exclusion of hepatitis A, B, Epstein-Barr virus, and cytomegalovirus in patients with an antecedent history of blood transfusion (n = 10), drug abuse (n = 3), or origin from a high risk area (n = 3). Of the 4 remaining cases, 1 had primary biliary cirrhosis, 1 autoimmune chronic active hepatitis (CAH), and 2 had hepatitis B virus–related CAH. Both patients with autoimmune CAH and primary biliary cirrhosis had received multiple blood transfusions. 17 of patients with chronic liver disease were tested for anti-HCV (United Biomedical Inc., Lake Success, NY), and 12 were positive. Of the 8 cases with acute unresolved hepatitis, 7 were thought to have NANBH according to the above criteria and one had acute hepatitis B. All 7 with NANBH were tested for anti-HCV early in the course of their illness and 2 were positive. 3 of the 5 biopsies showing no significant histological abnormalities were obtained from patients biopsied during investigation for chronically abnormal liver function tests and the only one routinely tested for anti-HCV was negative. 2 samples were taken from normal, "cut-down" donor liver obtained at the time of orthoto-

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1. *Abbreviations used in this paper:* CAH, chronic active hepatitis; HCV, hepatitis C virus; NANBH, non-A, non-B hepatitis; NISH, nonisotopic in situ hybridization.

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Table I. Clinical, Serological, and Histological Data (Mean±SD) of Patients Studied

	Chronic liver disease (n = 20)	Acute hepatitis (n = 8)	Normal histology (n = 5)
Age	40±11	39±10	41±25
Male/Female	12/8	4/4	2/3
Aspartate aminotransferase	87±22	409±643	36±7
Alkaline phosphatase	176±111	201±108	126±49
Bilirubin	52±59	193±215	15±12

pic liver transplantation to act as negative control tissues. Nested HCV PCR performed on fresh samples of this tissue was negative. 2 additional biopsy specimens, 1 from skin and the second from duodenum were obtained from HCV negative patients and used as a negative control for NISH.

The cDNA probe (designated pDM 415) used for NISH was 1,700 nucleotides in length and homologous to nucleotides 6,997–8,764 of the 3′ end of the HCV genome NS5 (JG2). (Byrne, J., B. Rodgers, and D. Parker, manuscript in preparation). This probe was constructed by insertion of a PCR fragment into pUC13 plasmid and cloning before excision of the insert and gel purification by electroelution. The probe was digoxigenin labeled by random priming (multi-prime kit; Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, 500 ng of the probe was denatured and mixed with hexanucleotide, dNTP digoxigenin labeling mixture, and Klenow enzyme, and incubated for 20 h at 37°C before purification of the probe by ethanol precipitation.

Sense and antisense RNA probes were used to detect the replicative intermediary and positive strands of HCV genome respectively. Two RNA probes were constructed in the following way: a PCR fragment corresponding to the 5′ noncoding region of the HCV genome was inserted into Bluescript plasmid (Stratagene, Cambridge, UK). The DNA was linearized using the restriction enzymes BamHI (to prepare negative strand HCV RNA nucleotides 1–390) or NheI (to prepare positive strand HCV RNA nucleotides 1–249). After extraction with phenol/chloroform and ethanol precipitation, the probes were labeled with digoxigenin (DIG-UTP; Boehringer Mannheim) according to the manufacturer's instruction. Briefly, 1 μg of linearized DNA was added to NTP labeling mixture, DTT, transcription buffer, and either SP6 or T7 RNA polymerase and RNase inhibitor. After incubation for 2 h at 37°C the template DNA was digested with RNase-free DNase and probes were purified by ethanol precipitation.

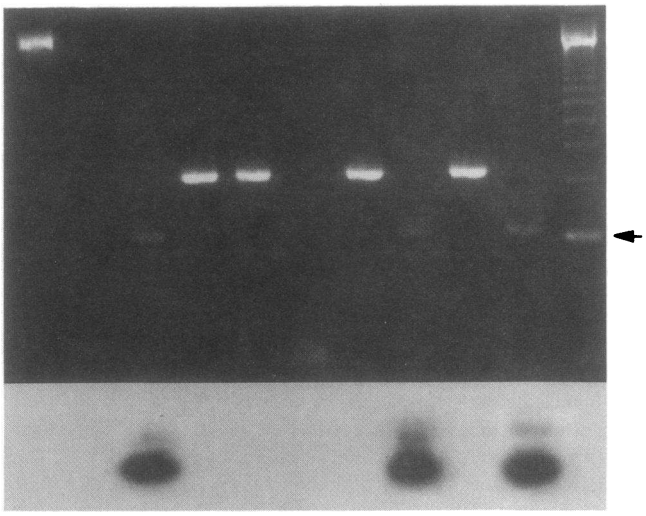
The sensitivity of detection using this digoxigenin-labeled probe and the digoxigenin detection system was determined by serial dilutions of target sequence fixed to nylon membranes via a dot-blot manifold; the target sequence was detectable in the range of 1–5 pg, corresponding to 50–250 copies of the cloned HCV cDNA.

Expression of HCV genome into insect cells. The NS5 or 5′ noncoding regions of HCV genome were inserted into a baculovirus vector and insect cells (*Spodoptera frugipedra,* BCH7 and BCH10, respectively) were infected with recombinant viruses (11) and used as positive controls. Uninfected cells and insect cells infected with wild-type baculovirus were used as negative controls. These cells were cultured in Tc 100 fluid (Flow Laboratories, Irvine, Scotland) for 72 h at room temperature, then fixed in formalin and processed in a similar fashion to liver biopsies.

In situ hybridization. In situ hybridization was performed as previously described (12). Briefly, after dewaxing and rehydration, the sections were treated with 0.2 M HCl, then digested with 15 μg/ml proteinase K (Sigma, Poole, UK) in 100 mM Tris/50 mM EDTA pH 8.0 for 30 min at 37°C, rinsed with 2 mg/ml glycine/PBS and then refixed in 4% para-formaldehyde/PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8). The tissue was then prehybridized with a mixture containing 50% formamide (Sigma), 10% dextran sulfate (Sigma), 1× Denhardt's solution (Sigma), 150 μg/ml sheared salmon sperm DNA (Sigma), 300 mM DTT (Sigma), Tris (0.05 M), EDTA (5 mM), 600 mM NaCl, and 0.1% sodium pyrophosphate. After incubation for 1 h at 37°C, 20 μl solution containing denatured labeled cDNA probe (6–10 ng) and 500 μg/ml transfer RNA (Sigma) in the above hybridization mixture was applied to each section which were then covered with siliconized coverslips and incubated for 16–20 h at 42°C. In situ hybridization for the detection of positive and intermediary replicative strands of HCV RNA was identical to the one described above with one exception; tissues were incubated at 95°C in an oven for 10 min before hybridization with RNA probes and the hybridization was carried out at 52°C. Control slides were treated with ribonucleases (RNases) A and T_1 and deoxyribonucleases (DNase) (Sigma) at 100 μg/ml before hybridization. A cDNA probe specific for IFN-γ, ~ 900 nucleotides in length, was labeled in a similar fashion and used as a control. After incubation the slides were rinsed in 4× SSC and then washed twice in 50% formamide/4× SSC for 45 min at 37°C. The sections were washed twice for 30 min in 2× SSC, once in 0.2× SSC at 65°C and once in 0.1× SSC for 15 min at room temperature. After posthybridization washing, digoxigenin-labeled probes were detected according to the manufacturer's instruction (digoxigenin detection kit; Boehringer Mannheim GmbH). Briefly after preincubation of sections for 30 min with 5% normal sheep serum, 0.3% Triton in Tris-buffered saline, the sections were incubated with 1:500 dilution of alkaline phosphatase conjugated antidigoxigenin mouse monoclonal antibody in the above buffer for 2 h at room temperature. The slides were then washed for 30 min and color reaction was developed by the addition of substrate containing 2.4 mg/ml levamisole (Sigma) and incubated for 16 h in the dark.

Nested PCR. RNA and DNA was extracted from four 10-μm thick formalin fixed paraffin embedded sections cut from pathological blocks made from either wedge or needle liver biopsy using minor modifications of the methods described by Jackson (13) as previously described (14). Briefly, the specimen was dewaxed in xylene at 90°C for 5–10 min, centrifuged at 12,000 *g* for 5 min, and xylene carefully aspirated from the resulting pellet. The remaining xylene was removed from the pellet with alcohol. The pellet was then dried before resuspension in 200 μl digestion buffer containing proteinase K at 100 U/ml, 0.5% SDS, 5 mM EDTA, and 10 mM Tris, pH 8, and vanadyl ribonucleoside complexes at 40 mM, overlaid with three drops of mineral oil (Sigma), and digested at 42°C for between 2 and 3 d, before phenol/chloroform extraction and overnight ethanol precipitation of nucleic acid at −20°C. Purified glycogen (20 μg; Boehringer Mannheim) was added to facilitate precipitation. Nucleic acid/glycogen was pelleted at 15,000 *g* for 15 min, washed in 70% alcohol, and resuspended in 20 μl water. 2 μl of the resulting RNA/DNA solution was then added to a reverse transcription mixture containing 5 U AMV reverse transcriptase (Promega, Southampton, UK), 1 mM DTT, 2.5 mM dNTPs (Pharmacia, Milton Keynes, UK), 2 μl of 10 × reverse transcription buffer containing 500 mM KCl, 200 mM Tris, pH 8.0, and 50 ng random hexamers in a total vol of 20 μl which was incubated at 42°C for 1 h. 2 μl of the resulting DNA/cDNA solution was then added to the first round PCR mix containing 60 ng of each the "outer" primers, 0.2 mM dNTPs, 2.5 μl of 10 × Taq buffer, 1.0 units of Taq polymerase (Biotech, Perth, Western Australia) in a total reaction vol of 50 μl. The reaction mixture was overlaid with three drops of oil before being subject to 30 rounds of amplification on a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using the following cycling parameters (file 4): 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min before a final extension step of 72°C for 7 min. 2 μl of the resulting product was then taken into a second round mixture which was identical to the first except that 120 ng of the inner primers were used. 10 μl of the resulting product was then electrophoresed on agarose gels and visualized using ethidium bromide staining under ultraviolet light.

The specificity of the PCR products was confirmed by Southern blotting using a probe internal to and not inclusive of the sequences of the internal primers (Fig. 1). Normal human liver taken from a cut-down donor organ was used as a negative control for liver tissue, while



1 2 3 4 5 6 7 8 9 10 11

Figure 1. Agarose gel electrophoresis (3% NuSieve GTG [FMC Corp., Rockland, ME] 1% agarose) and accompanying Southern blot of nested PCR products amplified as part of these experiments. (Lanes *1* and *11*) 123-bp molecular weight marker; (lane *2*) negative (reagent) control; (lane *3*) positive control (HCV, tissue); (lane *4*) positive control (albumin); (lanes *5* and *6*) sample positive for albumin (lane *5*) but negative for HCV (lane *6*); (lanes *7–10*) positive tissue samples. (Lanes *7* and *9*) Positive for albumin; (lanes *8* and *10*) positive for HCV. Arrow at level of HCV amplicon (~ 126 bp).

tissue known to be positive for HCV RNA by nested amplification from fresh tissue acted as a positive control. Using this protocol, HCV genome could reliably be detected in formalin-fixed tissues, but was about five times less sensitive than when applied to fresh tissue (14). As an internal control for tissue digestion, extraction, reverse transcription, and amplification primers directed at albumin mRNA were designed based on the nucleotide sequence described by Minghetti et al. (15). These primers flank a large exon and produce a DNA band of 822 bp and an RNA band of 273 bp, and initially were a generous gift of Dr. Kenneth Hillan, Royal Western Infirmary, Glasgow. The cycling parameters of the albumin primers were 94°C 10 s, 55°C 10 s, 72°C 1 min. For HCV amplification, primers described by Ulrich (16) were used. The sequences of all primers and the HCV probe are given in Table II. As well as negative and positive tissue controls, negative "contamination" controls were run with each experiment and to minimize problems of contamination the general measures suggested by Kwok and Higuchi (17) were adhered to. To minimize nonspecific amplifications, all PCR reactions were started "hot" by preliminary incubation of the reaction at 85°C before addition of Taq polymerase (18). In addition, to avoid cross contamination from the microtome blade, sections were cut using a fresh disposable blade for each sample and separate duplicate samples were cut on different days. Both cDNA preparation and HCV amplification were carried out in duplicate and during different days. PCR assays were carried out "blind" with respect to the in situ hybridization and serology results.

Table II. Sequence and Position of Primers Used for PCR in This Work

Primer	Position*	Sequences	Product size
HCV JR12	Sense nt 1–20	5′-GGCGACACTC CACCATAGAT-3′	216 bp
HCV JR19	Antisense nt 197–216	5′-CGCCCAAATC TCCAGGCATT-3′	
HCV JR13	Sense nt 35–53	5′GAACTACTGT CTTCACGCA-3′	126 bp
HCV JR14	Antisense nt 140–161	5′-GGCAATTCCG GTGTACTCAC C-3′	
Probe	115–135	5′-GAGAGCCATA GTGGTCTGCG-3′	
Albumin	Sense	5′-TGAAATGGCT GACTGCTGTG-3′	
Albumin	Antisense	5′GCAGCTTTAT CAGCAGCTTG-3′	

* HCV primers numbered according to Okamoto et al. (3). nt, nucleotide.

Results

In situ hybridization

cDNA probes. The specificity of the digoxigenin-labeled probe (pDM 415) was confirmed using insect cells infected with recombinant baculovirus containing NS5 region of HCV genome. The hybridization signal was only detected in insect cells infected with recombinant baculovirus. Uninfected insect cells or cells infected with the wild type of baculovirus did not show hybridization. (Fig. 2, *a* and *b*).

In our original experiments, the nuclear HCV staining was more intense than the cytoplasmic signal (19), which was often masked by counterstaining. In subsequent work, performed without counterstaining, the cytoplasmic signal became more prominent, and the cytoplasmic signal was seen in a greater proportion of cells. When NISH was performed on human tissue using pDM 415 as probe, the HCV genome, when present was found in both the cytoplasm and nuclei (in some cases also in nucleoli) of hepatocytes and mononuclear cells, but also in bile duct epithelial and sinusoidal cells (Fig. 3, *a–d*). Hepatitis C virus genome was detected by NISH in 14 of 20 patients with chronic liver disease, 7 of 8 patients with acute hepatitis, and in both patients with chronically abnormal liver function despite normal histology (Tables III and IV). The HCV genome was not detected in either of the two cut-down donor livers or the two other nonliver tissues (skin and duodenal biopsies). The proportion of cells positive for HCV genome in liver tissues generally varied from 1 to 5%. However, in one exceptional patient, with autoimmune chronic active hepatitis on long-term treatment with corticosteroids, a positive NISH signal was seen in ~ 20% of hepatocytes. Two patients who did not have NANBH as the primary diagnosis had HCV genome demonstrated in their liver by NISH; one patient had autoimmune CAH and one primary biliary cirrhosis; both were HCV RNA positive by reverse transcription PCR and had been multiply transfused as a consequence of their disease. HCV genome was not detected in the two HBV-related liver biopsies tested using either PCR or in situ hybridization.

RNase treatment of liver biopsies before hybridization reduced the HCV genome signals in the tissues by ~ 75–80%. Although DNase treatment also reduced the proportion of cells expressing HCV genome in the nuclei, the reduction was negligible and was probably the result of minor RNase contamination. The cDNA IFN-γ probe was used as a control probe for 23 of the liver biopsies and a positive signal was found in only 4 of 23 liver biopsies tested and was confined to mononuclear cells infiltrated into the portal areas.

When the results were analyzed as a whole, there was no correlation between the intensity of expression of the HCV

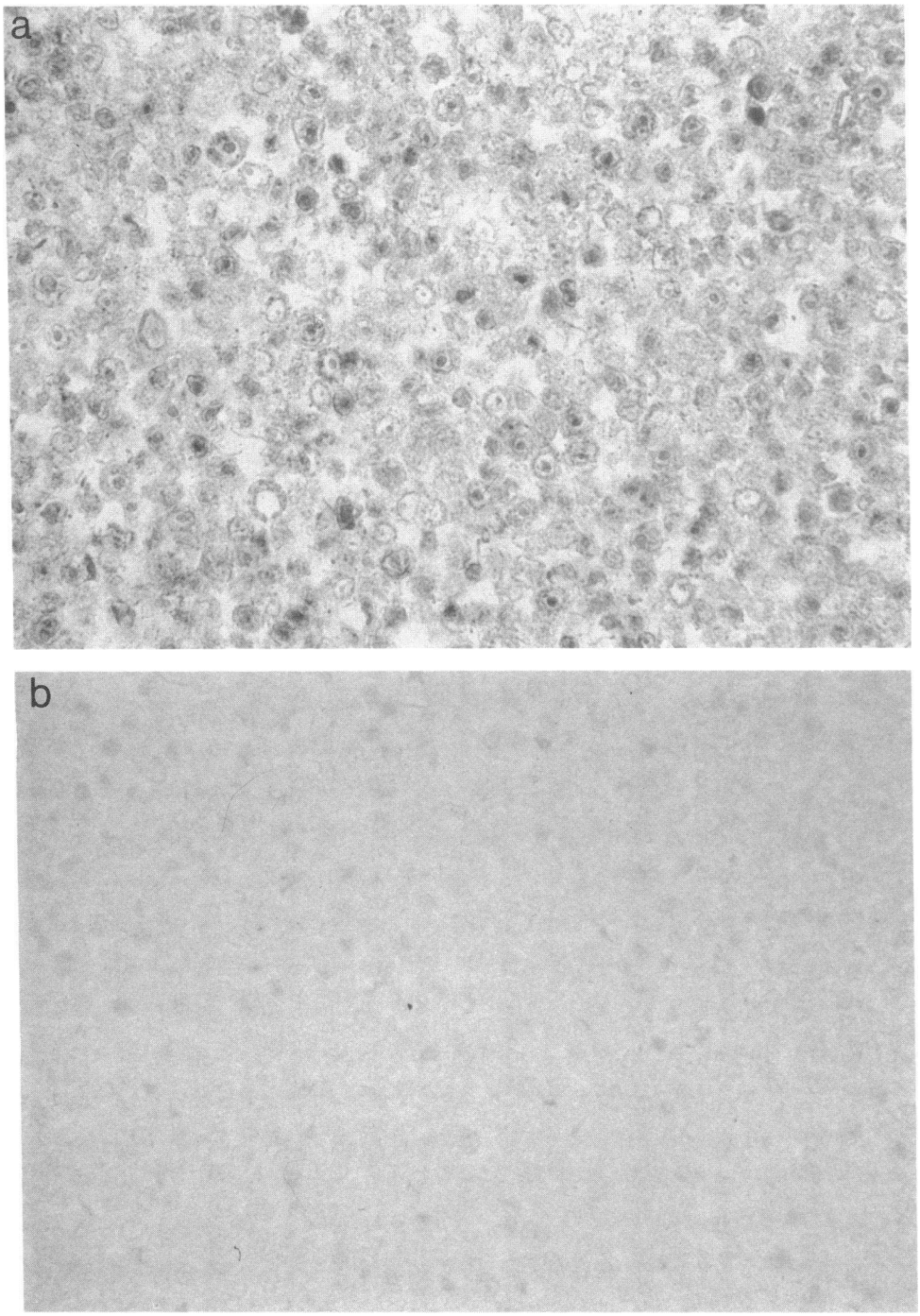


Figure 2. NISH applied to insect cells infected with recombinant baculovirus expressing NS5 region of HCV genome (*a*) uninfected insect cells or cells infected with wild type baculovirus did not show hybridization signal (*b*). ×250.

genome (scored 0–5) in the liver tissue using the cDNA probe and the disease activity as judged by lymphocytic infiltration in the periportal or centrilobular areas (scored 0–3). Nor was there a significant correlation between the presence of HCV genome in the liver and either serological or biochemical parameters such as aspartate aminotransferase. The serum anti-HCV result and the presence of HCV genome in liver tissue showed a close correlation in patients with chronic liver disease (13 either both positive or both negative), whereas only one patient with acute hepatitis was positive in both tests. The liver tissue from patients with acute hepatitis generally demonstrated a higher proportion of cells expressing the HCV genome when compared with biopsy specimens from chronic cases, however the difference was not statistically significant.

Sense and antisense RNA probes. The specificity of the digoxigenin-labeled RNA probes was also confirmed using NISH on insect cells infected with recombinant baculovirus expressing the 5′ noncoding region of HCV genome (data not shown). The hybridization signal was not detected when insect cells infected with baculovirus expressing NS5 region of HCV genome or wild type baculovirus.

Using RNA probes, NISH was performed on 10 of the above liver tissues known to be positive with PCR and NISH using the cDNA probe. Both positive and replicative interme-

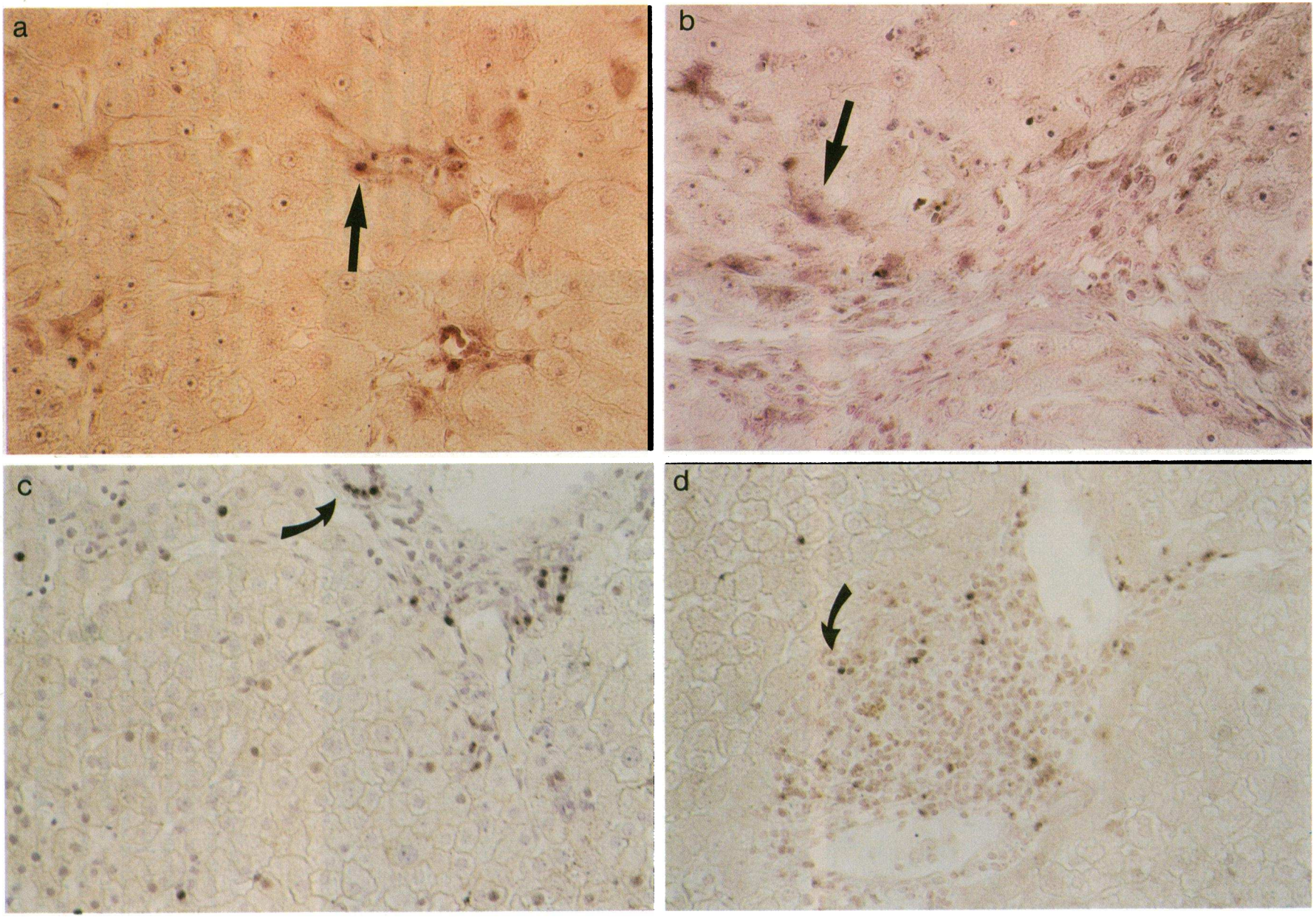


Figure 3. NISH performed on human liver tissues using pDM415 as a probe. The HCV genome was found in cytoplasm, nuclei, and nucleoli (*a* and *b*) of hepatocytes as well as bile duct epithelia cells (*c*) and mononuclear cells (*d*). Arrows demonstrate hybridization signals. ×500.

Table III. Results of In Situ Hybridization and PCR Performed on Liver Biopsies

	In situ hybridization			
	Hepatocytes	Bile ducts	Mononuclear cells/Sinusoidal cells	PCR
Chronic liver disease (n = 20)	14:20	8:20	9:20	8:11
Acute hepatitis (n = 8)	7:8	4:8	3:8	5:8
Normal histology (n = 5)	3:5	1:5	2:5	2:5

diary strands of the HCV genome were present in the cytoplasm and nuclei (perinuclei and nucleoli in some cases) of hepatocytes and mononuclear cells, albeit the proportion of cells expressing the negative strand was significantly less than the ones expressing positive strand. Although positive strand of HCV RNA was also found in the bile duct epithelium, we have been unable to detect the negative strand of HCV in the latter cells (Table V). Using RNA probes, there exists a trend between the cells expressing the replicative intermediary strand of HCV RNA and lymphocytic infiltration, particularly at the periphery of fibrous septa (Fig. 4, *a* and *b*). Once again RNase treatment of liver tissues before hybridization reduced the hybridization signals by ~ 90%. Bile duct damage was present in 12 of 20 (60%) patients with chronic liver disease, and in 6 of these 12, HCV genome was detectable by NISH using cDNA probe in biliary epithelial cells as well as hepatocytes. In contrast, only 2 of the 8 (25%) patients with no biliary damage showed HCV genome in biliary epithelium.

Polymerase chain reaction

Albumin mRNA was amplified and detected in all 24 liver biopsies tested (Fig. 1). The HCV genome products were detected in 8 of 11 biopsies from patients with chronic liver disease, 5 of 8 with acute NANB hepatitis and in both patients with chronically abnormal liver function tests without histological abnormality, but not in either of the biopsies with no significant histological changes or in the two normal control cutdown livers. When the PCR results were compared with the data obtained from the in situ studies a good correlation was observed; thus 20 biopsies showed concordant findings (either both positive or both negative), three were in situ positive, PCR negative, and one was in situ negative, PCR positive (Tables III and IV). Since two of three biopsies with no significant histological abnormalities were positive for HCV genome by both NISH and PCR, HCV RNA was sought in the sera of two

Table IV. Comparison between the Results of In Situ Hybridization and Nested Polymerase Chain Reaction

	PCR +ve	PCR −ve	Total
In situ +ve	15	3	18
In situ −ve	1	5	6
Total	16	8	24

Table V. NISH Performed Using Sense and Antisense RNA Probes on 10 Liver Sections Positive for HCV RNA Using PCR

	Hepatocyte nuclei/nucleoli	Hepatocyte cytoplasm	Mononuclear cells	Bile duct epithelium
+ve Strand	7	9	6	3
−ve Strand	4	6	4	0

of these patients by nested PCR (serum was not available for the third patient), which confirmed that HCV RNA was also present in the sera.

Discussion

Using NISH, we have demonstrated the presence of HCV genome in hepatocytes, mononuclear, bile duct epithelial cells, and sinusoidal cells within the liver tissue, and have shown that the NISH signal was present in both the nuclei/nucleoli and cytoplasm of hepatocytes, indicating a wide range of cellular tropism of HCV. Further studies, using sense and antisense probes confirmed these findings, except for the presence of the replicative intermediary strand of HCV RNA, which was detectable in all cell populations within the liver, except bile duct epithelium. These findings suggest that HCV may infect cells other than hepatocytes. These observations are buttressed by other studies; Lamas et al. in a recent publication have demonstrated HCV genomic and antigenomic RNA to be present in mononuclear cells infiltrated in liver tissues as well as hepatocytes from patients with chronic HCV infection using NISH (20), while a number of other investigators have detected both positive and replicative intermediate forms of HCV RNA in isolated peripheral blood lymphocytes using PCR (21–23). Similar findings have been reported for members of the flaviviridae family, thus dengue virus 2, dengue virus 4, and Japanese encephalitis virus are shown to infect and propagate in human hematopoietic cells (24–25). Although these findings support the hypothesis and are in favor of replication of HCV in mononuclear cells, they do not necessarily prove that replication takes place in these cell populations and may simply represent the uptake of viral material from the neighboring infected cells or plasma. On the other hand, in recently published work involving short-term experimental HCV infection in chimpanzees, the HCV genome was only detectable within the hepatocytes, and not in mononuclear cells, biliary, or Kupffer cells (26). Although chimpanzees are unquestionably a good animal model for HCV infection the extent to which these findings are applicable to human disease is uncertain.

Bile duct damage is a frequent finding in chronic NANBH but is uncommon in other forms of chronic viral hepatitis (27–29) and the etiology of this damage has yet to be explained. In the present study, although we have been unable to detect the replicative intermediary strand of HCV RNA in bile ducts epithelium, the bile duct damage seen in chronic HCV infection correlated with the presence of HCV genome as detected using a cDNA probe in these cells. One possible explanation for the apparent absence of replicative intermediary strand of HCV RNA in bile duct epithelium is that there has been passive uptake of HCV genome by these cells. On the other hand, antigenomic HCV RNA has been reported to be present in the

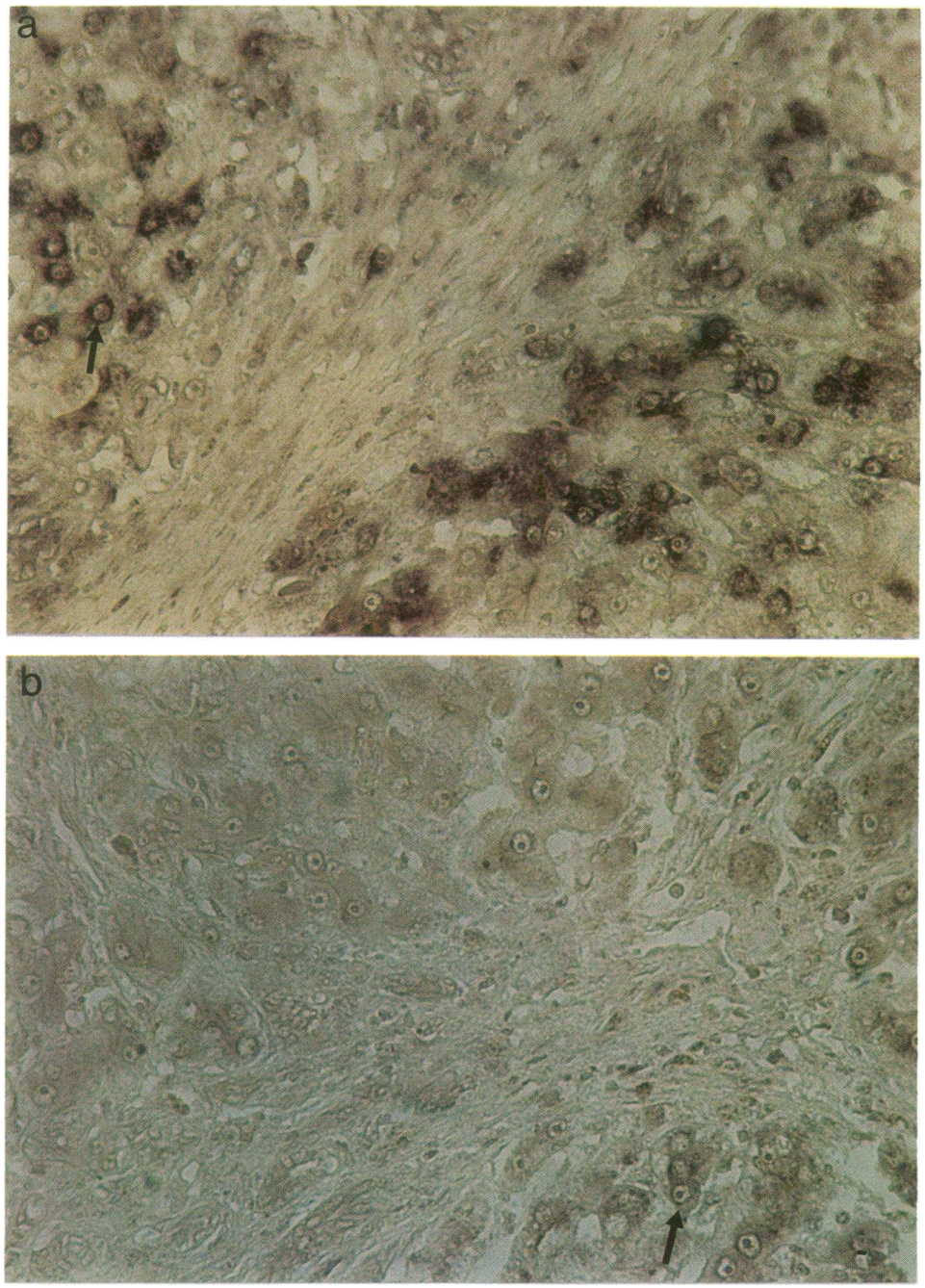


Figure 4. NISH performed on human liver tissues using sense and antisense probes. Positive (*a*) and intermediary replicative strands (*b*) of HCV RNA were found in cytoplasm/perinuclei of hepatocytes. Arrows demonstrate hybridization signals. ×500.

order of 10^2–10^3 times lesser concentration than the positive strand in serum, mononuclear cells, and liver tissue (22, 30), and this level may be beyond the sensitivity of NISH. Another explanation for the lack of detection of antigenomic HCV RNA in bile duct epithelium may be that these cells act as a "reservoir" for HCV, as it has been suggested for mononuclear cells (21). Furthermore, it is uncertain whether the bile duct damage seen in NANBH is a result of chronic liver disease or a direct consequence of infection of bile duct epithelium by HCV per se or whether other mechanism(s) (such as immunologically mediated cell killing) are important. However, the absence of this finding in chronic HBV infection would suggest the injury is either directly or indirectly related to HCV.

The process of replication of HCV has not been studied in detail due to lack of an appropriate cell line or the ability to sustain infected hepatocytes in long-term culture. The observation of nuclear and in some cases also nucleolar localization of the HCV genome in infected cells was at first surprising. However, the expression of HCV genome in both the nucleus and cytoplasm of hepatocyte had been reported before (31). Further support for the observation of nuclear localization of the HCV genome comes from Miyamura et al. who was only able to detect truncated nucleocapsid protein within the nucleus of COS cells transfected with the whole HCV structural region, while the intact protein was detected in both cytoplasm and cell surface, suggesting either active transport of this protein to the nucleus or nuclear protein synthesis, which would require genomic HCV RNA (31a). Recent studies have also reported both nuclear and nucleolar localization of the nonstructural proteins (NS5) of the related flavivirus, yellow fever virus (32),

which is also consistent with previous studies showing identification of other flaviviruses (west Nile virus, yellow fever virus, and Japanese encephalitis virus) antigens (envelope, core, and NS1) within nuclei of infected cells (33–35). Nuclear involvement in the replicative stages of these viruses has been postulated in these studies. Our observation of both positive and in particular negative strands of HCV RNA located in nuclei/perinuclei of hepatocytes, in the present study, may further support this hypothesis and suggest that HCV may use the host genome or nuclear apparatus for replication or that parts of the viral particles are stored in the nuclei of infected cells in a similar fashion to hepatitis B virus. In a recently published work, using short-term experimental HCV infection in chimpanzees, Negro et al. found exclusively cytoplasmic expression of HCV in a high proportion of hepatocytes within 22 wk of infection, whereas NISH performed subsequently was negative despite confirmation of viraemia by PCR (26). In contrast, in our chronically infected patients, we found a low proportion of hepatocytes expressing HCV in both cytoplasm and nucleus. While differences in species may contribute to some of these discrepancies, an alternative explanation may be that after an acute phase during which time widespread expression of the genome within hepatocytes is seen, HCV enters into a latency which corresponds to a reduction in serum transaminase levels and reduced cellular replicative activity, manifested by reduced levels of detection within hepatocytes.

The discrepancies between NISH and PCR found in this study might relate to the differences in sensitivity of the two techniques, as well as to the effect sequence variation has on each; cases positive by NISH but negative by PCR might arise through minor sequence variation (especially critical at the 3′ end of each PCR primer) which would be insufficient to prevent the NISH probe hybridizing. Alternatively, the extensive tissue processing required to permit RNA PCR from formalin-fixed tissue may have resulted in some loss of viral genome and false negativity; conversely, the PCR-positive, in situ–negative case may be attributable to low concentration of the virus within the tissue which could effect NISH more than PCR.

While it is conceivable that the pDM 415 probe used in NISH may have nonspecifically hybridized to some cells resulting in false positive signals, the absence of hybridization signal after application of this probe to either normal or HCV negative nonliver tissue (skin and duodenum), and also the low proportion of positive cells per specimen argues against this possibility. Furthermore, the specificity of sense or antisense RNA probes used for the detection of HCV genome in the present study was confirmed when NISH was performed on vero cells (African green monkey kidney) infected with a flavivirus, yellow fever virus vaccine (strain 17D) (a generous gift from Dr. A. Barrett, Surrey University, UK) and no hybridization signal was detected (unpublished observations). Similarly, using IFN-γ probe, the low proportion of mononuclear cells expressing IFN-γ mRNA further support the specificity of NISH signals in our study.

The lack of correlation between the degree of hepatocellular damage as assessed histologically or serologically (e.g., aspartate aminotransferase) and the prevalence of HCV genome in liver tissue as detected by the cDNA probe might suggest that HCV is not directly cytopathic. This observation is supported by a recent report (36), where chronic hepatitis was diagnosed histologically in 9 of 16 individuals with normal levels of alanine aminotransferase, who were anti-HCV and HCV RNA positive using PCR (36). On the other hand, the topographical relationship between cells expressing the replicative intermediary strand of HCV RNA and the severity of liver damage as judged by lymphocytic infiltration using NISH and RNA probes may suggest a possible cytopathic effects associated with viral replication, although an additional immune-mediated liver damage in this condition cannot be excluded. The involvement of immune mediated damage in chronic HCV infection is supported by studies demonstrating that in patients with NANBH a significant proportion of the lymphocyte destroying autologous hepatocytes in in vitro cell-mediated cytolysis are T lymphocytes (37). Furthermore, T cell clones obtained from this group of patients showed high cytotoxic activity against hepatocytes (38). In a recent study Fong et al. (30) reported that the severity of liver damage, as assessed histologically or biochemically, was unrelated to the presence or concentration of either genomic or antigenomic (replicative intermediary) HCV RNA in either serum or liver tissue. These findings and that of HCV genome in healthy carriage of HCV (39) and the findings of HCV genome in liver biopsies of two patients with no significant histological abnormalities in the present study, using both in situ hybridization and PCR, all suggest that chronic liver damage due to HCV infection is immune mediated. However, our own findings using RNA probes show that direct cytopathic effects of the virus may also be important.

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