

# Nuclear Factor- $\kappa$ B in the Liver of Patients With Chronic Hepatitis C: Decreased RelA Expression Is Associated With Enhanced Fibrosis Progression

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The mechanisms of liver damage in chronic hepatitis C virus (HCV) infection are poorly understood. The transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B), regulates the expression of genes involved in apoptosis, inflammation, and anti-viral response. It plays a protective role in several forms of liver damage. In this study, we analyzed NF- $\kappa$ B by gel mobility shift assay and immunohistochemistry in liver biopsies from HCV-infected patients, and we have determined the hepatic levels of the components of the NF- $\kappa$ B system by semiquantitative polymerase chain reaction (PCR). We found that NF- $\kappa$ B was activated in the liver of patients with chronic hepatitis C. Neither NF- $\kappa$ B activity nor the RNA levels of NF- $\kappa$ B subunits showed correlation with liver inflammatory activity, viral load, or HCV genotype. By contrast, hepatic mRNA values of RelA, the main element of active NF- $\kappa$ B, correlated inversely with apoptosis ( $r = -.68$ ;  $P < .05$ ) and with the rate of fibrosis progression ( $r = -.51$ ;  $P < .04$ ). In intermediate/rapid fibrosers, RelA mRNA levels were significantly decreased as compared with slow fibrosers ( $P < .003$ ) and with normal livers ( $P < .03$ ). In conclusion, we found that NF- $\kappa$ B is activated in chronic HCV-infected livers, and that the expression of RelA is inversely correlated with liver cell apoptosis and with the rate of fibrosis progression. Our data thus suggest that RelA expression may protect against liver fibrosis and hepatocellular damage. (HEPATOLOGY 2001;34:1041-1048.)

Hepatitis C virus (HCV) infection leads to chronic liver damage in the majority of infected patients. Liver disease develops gradually, with a variable progression rate to cirrhosis in different subjects.<sup>1,2</sup> Men who consume alcohol and are

infected after the age of 40 develop cirrhosis in 12 to 15 years. By contrast, in women infected at younger ages, the disease may evolve with very little liver damage for more than 40 years after infection.<sup>1</sup> The molecular mechanisms determining the intensity and progression of the liver damage in different patients remain ill-defined.

The main histologic finding in chronic hepatitis C is the presence of liver inflammation in portal and periportal areas. In these areas and within the hepatic lobule, a variable proportion of hepatocytes undergoes apoptosis.<sup>3,4</sup> Fibrosis in the liver appears as a consequence of intense and persistent inflammation.<sup>1</sup> However, cirrhosis may occur in patients with very low levels of serum transaminases.<sup>5,6</sup> The relationship between the histologic grade of inflammation and fibrosis staging has not been clearly established. In chronic HCV infection, the production of cytokines in the liver plays an important role in determining the severity of liver damage.<sup>7-9</sup> Inflammatory cytokines such as interferon gamma and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) may activate antiviral intracellular mechanisms,<sup>10</sup> but they also participate in causing hepatocellular injury.<sup>11</sup> Intracellular nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation occurs in response to viral infection and to cytokines such as TNF- $\alpha$ .<sup>12</sup>

NF- $\kappa$ B is a ubiquitous factor that controls the expression of genes involved in immune response and inflammation.<sup>13</sup> This factor also plays a central role in protecting cells against apoptotic signals.<sup>14</sup> NF- $\kappa$ B activity comprises homo- or heterodimers formed by members of the Rel/NF- $\kappa$ B family of transcription factors. The functional specificity and selectivity of the NF- $\kappa$ B response is thought to arise primarily from the binding of Rel/NF- $\kappa$ B complexes to specific DNA regulatory sites ( $\kappa$ B sites) of target genes in different cell types.<sup>15,16</sup> In humans, there are 5 subunits of this family of proteins: RelA (also called p65), NFKB1 (also called p50), NFKB2 (also called p52), RelB, and c-Rel. NFKB1 and NFKB2 are originally synthesized as the inactive precursors p105 and p100 that have inhibitory function.<sup>17</sup> They require proteolytic processing to yield the p50 and the p52 subunits, respectively. In mammalian cells, the most common heterodimer is formed by RelA-p50 subunits.<sup>13,15</sup> NF- $\kappa$ B is kept inactive in the cytoplasm by binding to one of the inhibitory proteins called I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , p105, and p100.<sup>18</sup> As a result of the phosphorylation and subsequent degradation of the inhibitory subunits, NF- $\kappa$ B translocates to the nucleus, binds to  $\kappa$ B sites, and regulates target genes.

HCV core protein can influence NF- $\kappa$ B activation and cell apoptosis *in vitro*, but conflicting results have been obtained

Abbreviations: HCV, hepatitis C virus; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; NF- $\kappa$ B, nuclear factor- $\kappa$ B; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; DTT, dithiothreitol; TUNEL, TdT-mediated dUTP nick end labeling.

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in different studies. In experiments with cell lines expressing HCV core protein, variable data showing inhibition, no effect, and enhancement of TNF- $\alpha$ -induced NF- $\kappa$ B activation have been reported.<sup>19-21</sup> On the other hand, some articles have shown that NF- $\kappa$ B activity is increased in several unstimulated human cell lines expressing HCV core protein,<sup>19-21</sup> resulting in protection from apoptotic stimuli.<sup>19,20</sup> Only two studies<sup>20,22</sup> have addressed the status of NF- $\kappa$ B in livers from HCV-infected patients. Tai et al.<sup>20</sup> studied liver tissue obtained at surgery from patients with HCV-related hepatocellular carcinoma using electrophoretic mobility shift assay (EMSA) and immunohistochemistry. Gaweco et al.<sup>22</sup> analyzed by immunohistochemistry liver biopsies from transplanted patients who had recurrent HCV infection. Thus, available information concerning NF- $\kappa$ B in HCV infection is restricted to cases with advanced liver damage<sup>20</sup> and to transplanted patients,<sup>22</sup> the latter being a situation that does not reflect the course of natural infection and in which immunosuppressive therapy can influence the status of NF- $\kappa$ B activation.<sup>23</sup> To our knowledge, there are no clues in the literature on the role of the Rel/NF- $\kappa$ B family of transcription factors on the evolution of HCV-induced liver disease.

In the present work, we investigated the NF- $\kappa$ B activation status and the transcriptional expression of the elements of the Rel/NF- $\kappa$ B family of proteins in liver biopsies from patients with chronic hepatitis C. We show that the NF- $\kappa$ B signaling pathway is activated in HCV-infected livers, and that reduced levels of RelA mRNA are associated with a higher apoptotic rate and more rapid progression of fibrosis. Our data suggest that RelA expression may protect against liver fibrosis and hepatocellular damage in chronic HCV infection.

#### PATIENTS AND METHODS

**Patients and Controls.** Liver biopsy specimens were obtained from 41 patients with chronic hepatitis C. Diagnosis of chronic hepatitis C was based on elevation of serum transaminases for more than 6 months, positivity for anti-HCV antibody (ELISA second-generation, Ortho Diagnostic System, Raritan, NJ), presence of HCV RNA by reverse-transcription polymerase chain reaction (PCR) in serum, and histologic evidence of chronic hepatitis. Alcohol consumption

TABLE 1. Clinical Characteristics of the HCV-Positive Patients and Control Subjects Enrolled in the Study

| Characteristics                    | HCV+   | Controls                    |
|------------------------------------|--|-----------------------------|
| Number of subjects                 | 41   | 22                          |
| Male/female                        | 25/16  | 13/9                        |
| Mean age (range)                   | 45.5 (19-68)   | 55.7 (45-70)                |
| Serum biochemistry (mean $\pm$ SD) |  |                             |
| AST (IU/L)                         | 50.9 $\pm$ 40  | 19.3 $\pm$ 9.0<br>(n = 19)  |
| ALT (IU/L)                         | 83.5 $\pm$ 66.7  | 14.7 $\pm$ 9.0<br>(n = 19)  |
| GGTP (IU/L)                        | 38.1 $\pm$ 37.0  | 30.1 $\pm$ 16.3<br>(n = 19) |
| Albumin (g/dL)                     | 4.1 $\pm$ 0.3 (n = 33)   |                             |
| Median viremia (copies/mL) (range) | 3.1 $\times$ 10 <sup>8</sup> (10 <sup>6</sup> -10 <sup>9</sup> )<br>(n = 33) |                             |
| Genotype (n)                       |  |                             |
| 1b                                 | 26   |                             |
| Non-1b                             | 13   |                             |
| Not determined                     | 2  |                             |

Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; GGTP,  $\gamma$ -glutamyl transpeptidase.

TABLE 2. Histologic Inflammatory Activity (Grade) and Fibrosis Score (Stage) in the 3 Cohorts Studied

| Liver Histology | Gene Expression | EMSA | Immunohistochemistry |
|-----------------|-----------------|------|----------------------|
| Grade 1         | 7*              | 2    | 2                    |
| Grade 2         | 6               | 2    | 6                    |
| Grade 3         | 8               | 4    | 4                    |
| Stage 0-1       | 11              | 8    | 7                    |
| Stage 2, 3, 4   | 10              | 0    | 5                    |

\*Number of patients.

and other causes of liver disease were excluded in all cases. Serum HCV RNA was determined as previously described.<sup>24</sup> HCV genotyping was performed by PCR hybridization with specific probes for different genotypes as previously described.<sup>25</sup> Competitive PCR was used to quantify the serum viral load.<sup>24</sup> None of the patients had received treatment with interferon alfa in the 6-month period previous to the study. The main clinical, biochemical, virologic, and histologic features of the patients at the time of liver biopsy are described in Tables 1 and 2. Liver biopsies were obtained with a Tru-cut needle and were divided in two parts. The first was fixed in 10% formaldehyde and embedded in paraffin for histologic examination and apoptosis detection, and the second part was snap-frozen in liquid nitrogen-cooled isopentane and embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, the Netherlands), stored at  $-80^{\circ}\text{C}$ , and used for all the NF- $\kappa$ B determinations. Inflammatory liver damage and fibrosis stage were scored according to Bedossa et al.<sup>26</sup> The rate of fibrosis progression was calculated as described by Poynard et al.<sup>1</sup> In cases with known duration of the infection, the fibrosis score was divided by the number of years after infection. In patients who had two separated liver biopsies, the fibrosis score change was divided by the number of years between biopsies. Only patients with more than 8 years of disease duration were included. A patient was considered a slow fibroser if his fibrosis progression rate was lower than 0.07 units per year (more than 50 years to cirrhosis). Normal liver samples were obtained from 22 controls at laparotomy and stored at  $-80^{\circ}\text{C}$  in OCT. Intervention was performed in 18 cases because of cancer (11 colorectum, 7 stomach), 3 because of cholelithiasis, and 1 because of hydatidic cyst. In all these cases, histologic examination of the surgical biopsy showed normal liver architecture. Written consent was obtained in all cases.

**RNA Extraction From Liver Biopsies: Nuclear Extracts Preparation.** Before RNA extraction, the liver tissue was homogenized in 1 mL Ultraspec (Biotex, Houston, TX) with an Ultraturrax Driver T.25 (Janke & Kunkel, Ika-Labortechnik, Germany), and total RNA was obtained following the Ultraspec protocol, which is based on the method described by Chomczynski and Sacchi.<sup>27</sup> Liver nuclear extracts were obtained following previously described methods.<sup>28</sup> Briefly, the tissue was homogenized in a small dounce homogenizer using pestle type B (Kontes Glass Company) in 300  $\mu\text{L}$  of cold buffer A (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol bis-( $\beta$ -aminoethyl ether)-N,N-tetraacetic acid, 1 mmol/L dithiothreitol [DTT], 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 1 mmol/L benzamide). After 15 minutes on ice, NP-40 at 0.5 wt/vol (Boehringer Mannheim, Mannheim, Germany) was added and disruption was favored by vortexing. Nuclei were collected by centrifugation, suspended in buffer C (20 mmol/L HEPES [pH 7.9], 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol bis-( $\beta$ -aminoethyl ether)-N,N-tetraacetic acid, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, 1 mmol/L benzamide), incubated on ice for 15 minutes, and spun at 14,000g for 5 minutes. Supernatants containing the protein nuclear extracts were collected and stored at  $-80^{\circ}\text{C}$ . One small aliquot of total and nuclear extracts was used to determine protein concentration with the Bradford reagent (Bradford Bio-Rad protein assay, Bio-Rad, Hercules, CA).

TABLE 3. Primer Sequences, PCR Amplification Product Length, and Number of Amplification Cycles for RelA, NF- $\kappa$ B1, and  $\beta$ -Actin

| Gene           | Upstream Primer*     | Downstream Primer    | Product Length (base pair) | Cycles |
|----------------|----------------------|----------------------|----------------------------|--------|
| RelA           | TGCTGTGCGGCTCTGCTTCC | AGGCTCGGGTCTGCGTAGGG | 321                        | 26     |
| NF $\kappa$ B1 | CTGCTCCTTCCAAAACACTT | TCCTTCTGCCATAATCAT   | 332                        | 24     |
| $\beta$ -Actin | TCTACAATGAGCTGCGTGTG | GGTGAGGATCTTCATGAGGT | 314                        | 22     |

\*Primer sequences for both primers are named (5'-3').

**Analysis of mRNA Expression by Reverse-Transcription PCR.** Total RNA was extracted from liver tissues, and NF $\kappa$ B1 (p50) and RelA (p65) mRNA were determined by semiquantitative RT-PCR in a Perkin-Elmer Gene Amp PCR System 2400 (Perkin Elmer, Foster City, CA). One and a half micrograms of total RNA was reverse-transcribed (60 minutes at 37°C) with 500 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) in 50  $\mu$ L volume of 5 $\times$  RT buffer (250 mmol/L Tris-HCl [pH 8.3], 375 mmol/L KCl, 15 mmol/L MgCl<sub>2</sub>), supplemented with 5 mmol/L DTT, 0.5 mmol/L deoxynucleoside triphosphate (Boehringer Mannheim), 60 U ribonuclease inhibitor (Promega Corporation, Madison, WI), and 500 ng random hexamers (Boehringer Mannheim). After heating (95°C, 1 minute) and quick-chilling on ice, an aliquot of 10  $\mu$ L (0.3  $\mu$ g) of the cDNA pool was used for PCR amplification, in 40  $\mu$ L of 10 $\times$  PCR buffer (160 mmol/L [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>, 670 mmol/L Tris-HCl [pH 8.8], 0.1% Tween-20), containing upstream and downstream primers (80 ng each for NF $\kappa$ B1 and RelA), 1.5 mmol/L MgCl<sub>2</sub>, and 2 U of Biotaq DNA polymerase (Biolone, London, UK).

As an internal control for each sample, PCR amplification of a fragment of  $\beta$ -actin cDNA (using a 10- $\mu$ L aliquot of the cDNA pool) was performed. Validation experiments of PCR assays using known quantities of total RNA showed linearity of the optical density of amplification bands from 0.125 to 1  $\mu$ g for all genes including  $\beta$ -actin. Thus, 0.3  $\mu$ g was chosen as the suitable amount of total RNA to quantify the mRNA levels. The primer's sequences, the PCR-product lengths, and the number of amplification cycles to avoid the plateau effect are shown in Table 3. All liver samples have been quantified on the linear amplification part of the curve. In the PCR conditions used, the amplification efficiency for the 3 genes studied range from 88% to 92% in the different liver samples. After PCR amplification, 20- $\mu$ L aliquots of the PCR reactions were electrophoresed in 2% agarose gel stained with ethidium bromide. To ensure accurate DNA band quantification, less than 50 ng of DNA was loaded in each case. The bands were visualized with a UV lamp and analyzed with commercial software (Molecular Analyst/PC, Bio-Rad). Finally, values corresponding to NF $\kappa$ B1 and RelA mRNA were normalized to those of  $\beta$ -actin mRNA. We previously reported<sup>24</sup> that  $\beta$ -actin mRNA expression does not change in liver or peripheral blood mononuclear cells from patients with chronic hepatitis C; thus, it is a reliable gene to normalize mRNA values. The identity of the PCR products was verified by automatic sequencing (ABI PRISM 310 Genetic Analyzer, Perkin Elmer).

**Determination of NF- $\kappa$ B Activity by EMSA.** NF- $\kappa$ B binding activity was determined by EMSA with a commercial oligonucleotide containing the  $\kappa$ B consensus site (Promega) labeled with [ $\gamma$ -<sup>32</sup>P]-adenosine triphosphate. Seven micrograms of liver nuclear extract was incubated with 100,000 cpm of the labeled probe in 10 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT, 4% glycerol, and 1  $\mu$ g poly dI-dC (Boehringer Mannheim) during 20 minutes at room temperature. For competition and supershift experiments, a molar excess of the unlabeled  $\kappa$ B oligonucleotide or 1  $\mu$ g of the specific antibodies against RelA and p50 were incubated on ice for 20 minutes before the addition of the labeled probe. Separation of the complexes was performed in a 5% nondenaturing polyacrylamide gel electrophoresis in 0.5 $\times$  Tris borate EDTA (TBE) during 2 hours at 150 V. The gel was dried and exposed overnight to x-ray film. Band intensities were analyzed with commercial software.

**Immunohistochemical Detection of Activated RelA.** A mouse monoclonal IgG3 Ab (Chemikon International, Temecula, CA) raised against

the nuclear localization signal was used to detect activated NF- $\kappa$ B. The antibody binds to the RelA subunit only after release from inhibitory I $\kappa$ B- $\alpha$ , and thus specifically recognizes activated RelA, allowing for the assessment of the NF- $\kappa$ B activation *in situ*. Tissues were sectioned in a cryostat at 4 to 6  $\mu$ m, air-dried, and fixed in acetone for 10 minutes. Endogenous peroxidase was inhibited for 15 minutes with 0.3% hydrogen peroxide in methanol. Sections were sequentially blocked with avidin-blocking solution, biotin-blocking solution (Vector Laboratories, Burlingame, CA), and normal goat serum (Vector Laboratories). Anti-NF- $\kappa$ B p65Mab was used at a dilution of 1:75 overnight at 4°C in a humid chamber. Secondary anti-goat biotinylated antibody was used at 1:200 dilution and incubated for 45 minutes. Tissues were then incubated with streptavidin-coupled peroxidase (Amersham), 1:200, and developed using a Dako Liquid DAB substrate chromogen system (Dako, Carpinteria,

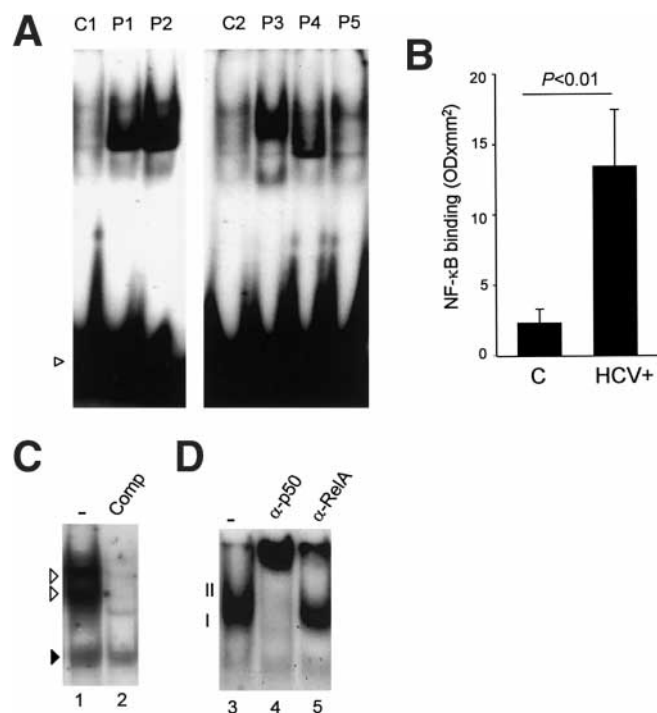


FIG. 1. NF- $\kappa$ B activation is observed in the liver of HCV-positive patients by EMSA. (A) Representative EMSA of 2 healthy liver samples (C1 and C2) and 5 patients with HCV infection (P1 to P5). Open arrowhead represents the free DNA probe. (B) Densitometric analysis of the blots showing increased NF- $\kappa$ B levels in patients with HCV infection in comparison with healthy livers (C). (C) Competition experiments demonstrate the specificity of the bands obtained. Lane 1, HCV-positive patient. Lane 2, the same sample as in lane 1 but including a 50-molar excess of unlabeled  $\kappa$ B probe. The  $\kappa$ B cold probe was incubated on ice for 20 minutes with the addition of the labeled  $\kappa$ B probe. Specific bands (open arrows) show decreased intensity in lane 2 with respect to lane 1. Unspecific band (closed arrow) shows similar intensity in both lanes. (D) Supershift assay with antibodies, anti-p50 and anti-RelA. Complex I and II showed in lane 3 are supershifted with anti-p50 antibody (lane 4), whereas only complex II is supershifted with anti-RelA antibody (lane 5), indicating that complex I is formed by p50 homodimers, whereas complex II is composed of p50/RelA heterodimers.

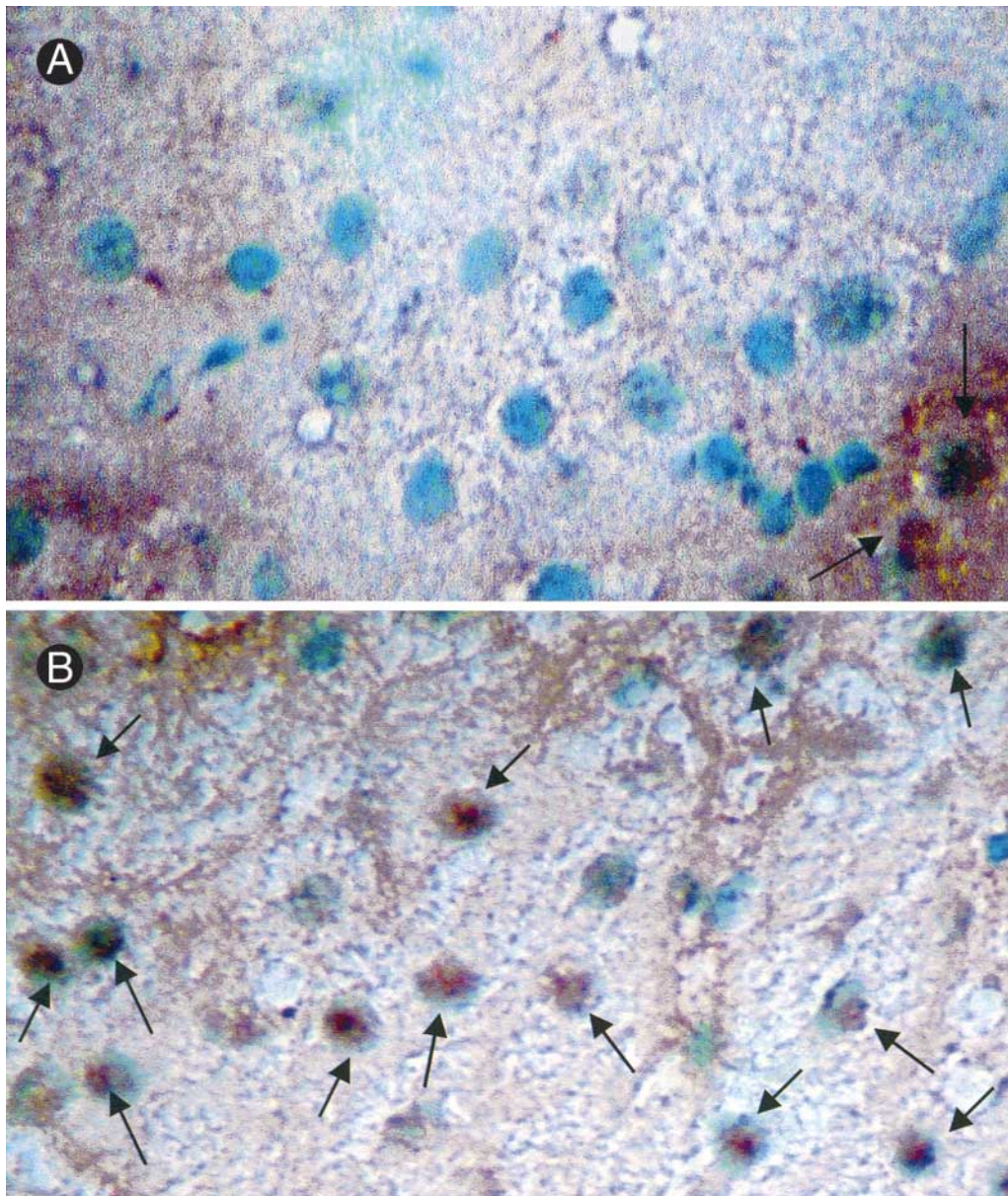


FIG. 2. Immunohistochemical detection of activated RelA in a healthy liver (A) and in the liver biopsy from an HCV-positive patient (B). Histologic sections were counterstained with methyl green. Hepatocyte nuclei stained in green indicate absence of nuclear NF- $\kappa$ B. Arrows indicate hepatocyte nuclei positive for NF- $\kappa$ B (brown staining).

CA). Control reactions were performed without primary antibody in all patients and controls. All preparations were counterstained with methyl green.

**In Situ Detection of Apoptosis.** Sections from the liver biopsies were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of alcohol. After rehydration, the slides were incubated with 20 mg/mL of proteinase K in phosphate-buffered saline. Endogenous peroxidase was inactivated by 3% hydrogen peroxide. Tissue sections were stained with an ApopTag system (Oncor, Purchase, NY) that identifies cells with internucleosomal fragmentation of DNA. The procedure was performed according to the manufacturer's instructions. The method is based on the preferential binding of terminal deoxynucleotidyl transferase to the 3'-hydroxyl ends of DNA.<sup>29</sup> ApopTag staining was validated with a tissue known to exhibit a high rate of programmed cell death (*i.e.*, human infarcted myocardium).<sup>30</sup>

Tissue sections from each specimen were examined microscopically at 300 $\times$  magnification, and all brown hepatocyte nuclei were counted. The apoptotic index was calculated by dividing the number of positive-staining hepatocytes by the total surface of the specimen expressed in square micrometers. The pathologist who analyzed the

specimens was unaware of the clinical and experimental data for all the biopsies examined.

**Quantitative Analysis of Liver Fibrosis.** Morphometric analysis (Olympus MicroImage 2.0, Hamburg, Germany) was performed in a manner similar to that described previously<sup>31</sup> using specimens stained with Sirius red.<sup>32</sup> A magnified image (40 $\times$ ) of a specimen captured by the camera mounted on the microscope was processed by the image-analysis computer. The collagen fibers stained red and the entire area of the liver tissue were automatically calculated and expressed in square micrometers. Collagen fibers that normally exist in the portal tract or central vein were also included in the measurement.

**Statistical Analysis.** Results are given as mean  $\pm$  SEM unless otherwise stated. Normality was assessed with the Shapiro-Wilks test. Statistical analyses were performed using parametric (Student *t*) and nonparametric (Mann-Whitney *U*) tests. Associations between quantitative variables were studied with the Spearman or Pearson's correlation coefficients. All *P* values were 2-tailed and were considered significant if the associated value was less than 0.05. SPSS 6.0 for Windows was used for statistical analysis.

TABLE 4. NF- $\kappa$ B Subunits in the Liver of HCV-Infected Patients and in Healthy Livers

|             | Controls        | n† | HCV (+)          | n  |
|-------------|-----------------|----|------------------|----|
| NFKB1 mRNA* | 0.95 $\pm$ 0.08 | 11 | 0.67 $\pm$ 0.06‡ | 21 |
| RelA mRNA   | 2.02 $\pm$ 0.19 | 11 | 1.81 $\pm$ 0.01  | 21 |

NOTE. Levels of mRNA were determined by quantitative PCR in whole-liver extracts.

\*Data are expressed as mean values  $\pm$  SEM.

†Number of subjects.

‡ $P < .005$ , HCV+ patients vs. healthy liver controls.

## RESULTS

**Liver NF- $\kappa$ B Activity and Rel/NF- $\kappa$ B mRNA Levels in HCV Infection.** We studied NF- $\kappa$ B binding activity in 8 HCV-positive and 5 control livers by EMSA. We detected the NF- $\kappa$ B binding signal in 6 of 8 patients and in 1 of 5 controls ( $P < .05$ ). A representative EMSA from 2 healthy livers and 5 HCV-positive cases is shown in Fig. 1A. Figure 1B shows NF- $\kappa$ B activity values (as estimated by densitometric analysis of the autoradiograms) in patients with chronic hepatitis C and healthy controls. As shown in this figure, NF- $\kappa$ B activation is significantly higher in patients than in controls ( $12.1 \pm 3.6$  vs.  $2.1 \pm 0.86$ ;  $P < .01$ ). Competition experiments with cold probe confirmed the specificity of the bands obtained (Fig. 1C). To identify the NF- $\kappa$ B subunits present in the complexes, we performed supershift analysis, which revealed that the active NF- $\kappa$ B present in cell nuclei from HCV-positive patients was composed of p50/p50 homodimers and p50/RelA heterodimers (Fig. 1D). RelB and c-Rel were not encountered when supershift analysis was performed with the appropriate antibodies (data not shown).

To ascertain by other methods the presence of active NF- $\kappa$ B in HCV-infected livers, we studied NF- $\kappa$ B by immunohistochemistry in frozen liver sections using a monoclonal antibody that recognizes activated RelA. We studied 12 HCV-positive patients and 6 healthy livers (as negative controls). Two controls showed faint nuclear staining, whereas the other 4 showed no positive signal. By contrast, all patients with chronic hepatitis C showed nuclear staining in liver cells (5 faint and 7 intense nuclear staining; HCV-positive cases vs. negative controls:  $P < .01$ ) (Fig. 2).

We also analyzed the transcriptional expression of Rel A and NFKB1 (the gene from which p50 is transcribed) in the liver specimens. We found that hepatic RelA mRNA values were not significantly different in patients and controls, whereas NFKB1 mRNA levels were lower in the liver from subjects with chronic hepatitis C than in healthy livers ( $P < .005$ ) (Table 4).

**NF- $\kappa$ B and Liver Damage.** Despite the important role of NF- $\kappa$ B in inflammation, we did not find any correlation between NF- $\kappa$ B activity or Rel/NF- $\kappa$ B mRNA values in the liver and the histologic inflammatory activity or serum transaminases. Interestingly, however, we found a relationship between NF- $\kappa$ B and fibrogenesis. Thus, patients with liver fibrosis (stage 2 or higher, as determined by METAVIR score;  $n = 10$ ) had lower values of RelA mRNA than patients with absent or minimal fibrosis (stage 0 or 1;  $n = 11$ ) ( $1.59 \pm 0.05$  vs.  $1.96 \pm 0.17$ ;  $P = .05$ ). Similarly, 3 of 5 patients with minimal RelA nuclear staining had fibrosis stage 3, whereas 5 of 7 patients with intense positive nuclear staining had absent or minimal fibrosis (stage 0 or 1). Although these immunohisto-

chemical data indicate a tendency for higher fibrosis in patients with lower RelA nuclear staining, the differences were not significant.

To further investigate the relationship between NF- $\kappa$ B expression and fibrosis, we measured collagen content of the liver by Sirius red staining of the samples. We found a significant inverse correlation between liver collagen content and RelA mRNA expression ( $r = -.57$ ;  $n = 17$ ;  $P < .05$ ). In 17 patients, we were able to calculate the rate of fibrosis progression per year either because we had two separated liver biopsies (5 cases; time between biopsies ranged from 6 to 14 years), or because the duration of the infection could be precisely established (3 had received blood transfusions, 3 had been drug abusers, 5 had had major surgical procedures or parenteral exposures, and 1 was infected by vertical transmission). In these 17 patients, RelA was inversely correlated with the yearly rate of fibrosis progression ( $r = -.51$ ;  $n = 17$ ;  $P < .04$ ). The group of intermediate/rapid fibrosers (*i.e.*, fibrosis progression rate higher than 0.07 units/year) had significantly lower RelA mRNA than slow fibrosers ( $P < .003$ ) and than normal controls ( $P < .03$ ). No differences were detected between slow fibrosers and controls (Fig. 3).

To investigate whether expression of NF- $\kappa$ B subunits were related to the degree of apoptotic cell death in hepatocytes, we determined by TdT-mediated dUTP nick end labeling (TUNEL) staining the apoptotic index (number of apoptotic hepatocytes per square micrometer) in 18 patients and 5 normal controls. The apoptotic index in normal livers ranged from 2.31 to 3.79 (mean  $\pm$  SD:  $2.80 \pm 0.27$ ). Apoptosis was markedly variable among HCV-infected patients, ranging from 0.66 to 9.29 (mean  $\pm$  SD:  $4.09 \pm 0.58$ ). Figure 4 shows TUNEL staining in a healthy liver and in a HCV-positive liver. We found that in patients with evidence of increased programmed cell death rate (mean apoptotic index of controls plus 2 SD), the apoptotic index was inversely correlated with RelA-mRNA values ( $r = -.68$ ;  $n = 9$ ;  $P < .05$ ) (Fig. 5). Apoptosis was not correlated with the extent of liver fibrosis, nor with the inflammatory activity.

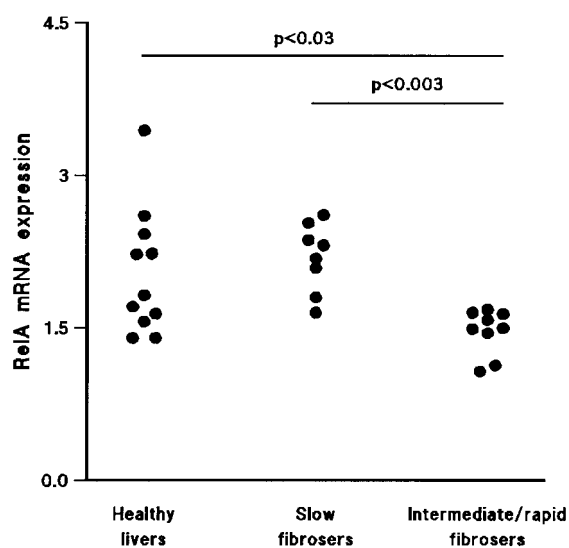


FIG. 3. RelA mRNA expression in the liver of HCV-positive patients with a known duration of infection divided into slow fibrosers (fibrosis progression rate lower than 0.07 units/year;  $n = 8$ ) and intermediate/rapid fibrosers ( $n = 9$ ).

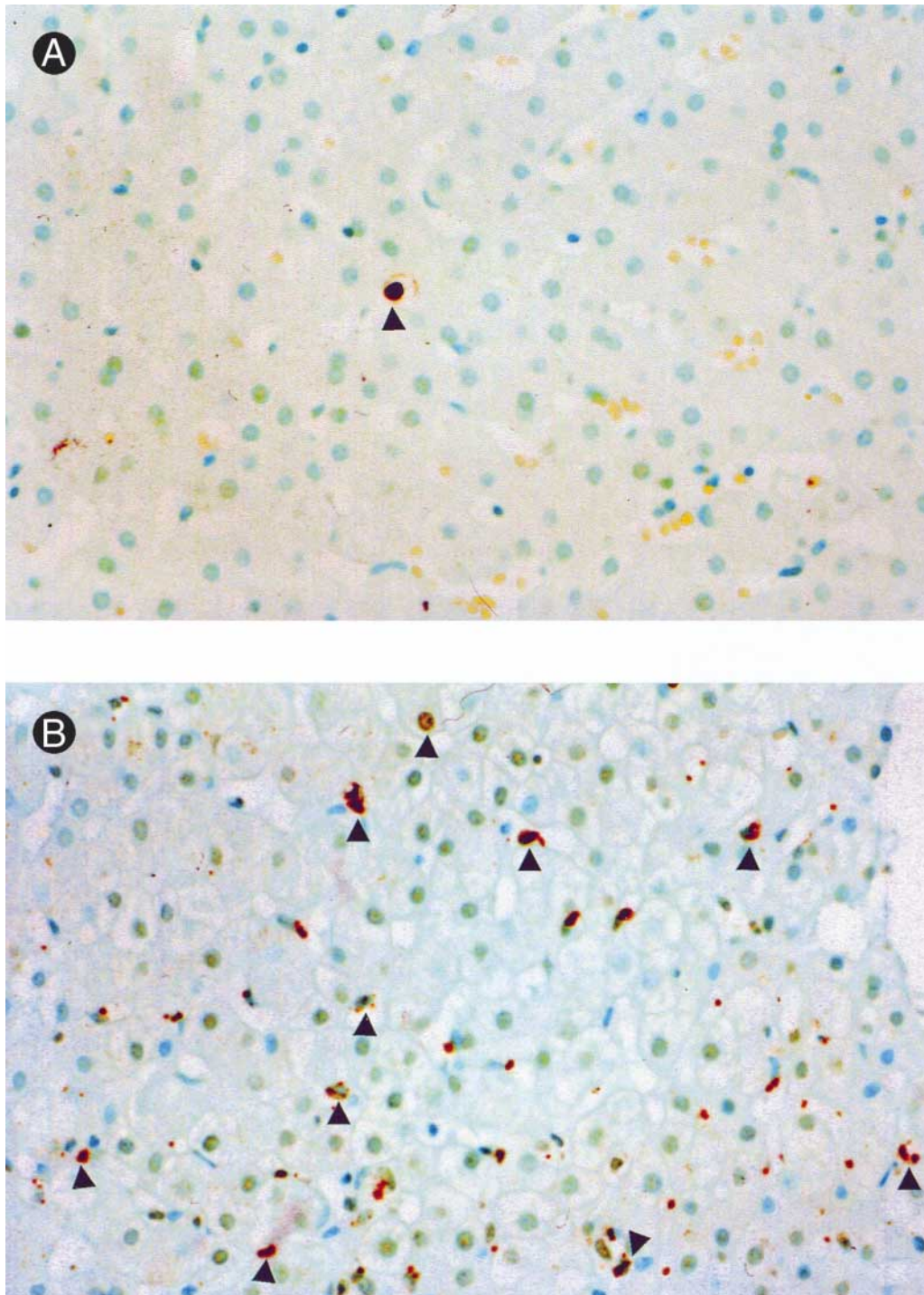


FIG. 4. TUNEL staining in a healthy liver (A) and in an HCV-positive liver (B). Arrows show hepatocytes with evidence of apoptosis.

Hepatic NFKB1-mRNA levels did not show any correlation with fibrosis or apoptosis.

**Hepatic NF- $\kappa$ B Expression and Viral Replication.** NF- $\kappa$ B is activated by viral proteins. HCV core protein has been reported as a plausible NF- $\kappa$ B activator.<sup>19-21</sup> However, no relationship was found between the expression of NF- $\kappa$ B and serum viral load or viral genotype.

#### DISCUSSION

In the present study, we analyzed, in the liver from patients with chronic hepatitis C, the status of Rel/NF- $\kappa$ B family of transcription factors to determine whether there is a relation-

ship between these parameters and liver damage or disease progression. Our EMSA data show that HCV infection is associated with nuclear translocation of p50/p65 heterodimers and p50/p50 homodimers. In agreement with these findings, the immunohistochemistry study also revealed the presence of active RelA in the nuclei of hepatocytes. Increased NF- $\kappa$ B function is consistent with the enhanced expression of several NF- $\kappa$ B-dependent genes such as interferon beta,<sup>8</sup> TNF- $\alpha$ ,<sup>24,33,34</sup> inducible NO-synthase,<sup>35</sup> and intercellular adhesion molecule-1<sup>36</sup> found in the liver of chronic hepatitis C patients. NF- $\kappa$ B is an important mediator of the inflammatory response, and persistent NF- $\kappa$ B activation has been detected

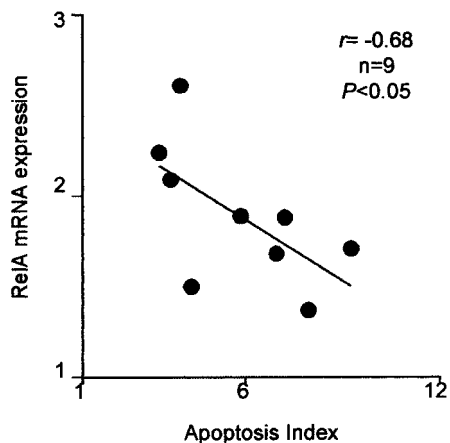


FIG. 5. Association between mRNA expression of RelA and apoptotic index (defined as the number of positive hepatocytes per square micrometer) in HCV-positive patients with evidence of an increased programmed cell death rate.

in several inflammatory diseases such as asthma,<sup>37</sup> rheumatoid arthritis,<sup>38</sup> and inflammatory bowel disease.<sup>39</sup> However, our data show that, in the setting of chronic hepatitis C, neither the hepatic DNA binding activity of NF- $\kappa$ B (as estimated by EMSA and immunohistochemistry) nor the transcriptional expression of RelA or NFKB1 in the liver were associated with the histologic grade of inflammation.

Gaweco et al.<sup>22</sup> assessed by immunohistochemistry the NF- $\kappa$ B status in liver allografts from patients with recurrent hepatitis C. These authors found that NF- $\kappa$ B staining correlated positively with the histologic grade of inflammation and with the stage of fibrosis.<sup>22</sup> The discrepancy between results from these authors and our data might be the result of the different clinical setting of the two studies: transplanted patients in the study by Gaweco et al. and nontransplanted patients in ours. It should be noted that immunosuppressive drugs can significantly influence NF- $\kappa$ B activity.<sup>23</sup>

Interestingly, in the present study, we found an inverse correlation between RelA expression and apoptotic index in those cases with chronic hepatitis C manifesting increased apoptotic rate. This is in agreement with the role of NF- $\kappa$ B as a stress-response regulator within the cell. It has been shown that NF- $\kappa$ B activation protects against TNF- $\alpha$ <sup>40-41</sup> and transforming growth factor  $\beta$ -induced<sup>42</sup> apoptosis. In particular, RelA, the main component of the active NF- $\kappa$ B heterodimer, displays antiapoptotic functions in embryonic liver<sup>43</sup> and in remnant liver posthepatectomy.<sup>44</sup> It has also been shown that NF- $\kappa$ B activation in hepatocytes is protective against cytokine-induced damage.<sup>45</sup> Our data in patients with chronic hepatitis C suggest that RelA might be implicated in defense against cell death in this viral infection.

The main event with prognostic consequences in the evolution of chronic hepatitis is the development of cirrhosis. Thus, progression of the disease would be better estimated by the fibrosis stage than by the grade of histologic activity.<sup>1</sup> Progression to cirrhosis varies widely from patient to patient, and subjects with HCV infection can be classified as slow fibrosers (time from infection to cirrhosis, more than 50 years), and intermediate/rapid fibrosers (with a shorter time of evolution to cirrhosis). It has been convincingly shown that individual factors such as sex and age at infection, together with alcohol consumption rather than viral parameters (viral load, genotype), determine the speed of progression to cir-

rhosis in chronic HCV infection.<sup>1</sup> The molecular mechanisms determining the individual proneness or resistance to develop cirrhosis have not been characterized. Our results show that patients with substantial fibrosis had lower RelA mRNA levels than those with absent or minimal fibrosis. Also, a significant negative correlation was found between liver collagen content and transcriptional RelA expression. In addition, we observed a significant, inverse correlation between progression of fibrosis and RelA mRNA levels. As shown in Fig. 3, intermediate/rapid fibrosers showed RelA values significantly lower than slow fibrosers and than normal livers. Our findings are in agreement with observations showing that RelA is a negative regulator of collagen gene expression in human stellate cells.<sup>46</sup>

On the other hand, by protecting liver cells against mitochondrial damage and apoptosis, RelA might reduce liver oxidative stress and subsequent fibrogenesis. Although the set of genes that NF- $\kappa$ B regulates in livers from patients with hepatitis C should still be determined, our data suggest that in chronic HCV infection, RelA-regulated genes might exert antiapoptotic and antifibrogenic effects, which may delay the development of cirrhosis.

HCV core has been found to activate NF- $\kappa$ B when expressed in selected cell lines,<sup>19-21</sup> but not in others.<sup>47-49</sup> In this study, we did not find any relationship between viral load and NF- $\kappa$ B status. It seems that other factors including cytokines that are up-regulated in chronic hepatitis C, such as TNF- $\alpha$ ,<sup>24,33,34</sup> could influence NF- $\kappa$ B activity in this condition.

Our finding of increased activity of NF- $\kappa$ B with decreased expression of RelA in a significant number of cases suggests a rather complex regulation of RelA mRNA. On the other hand, NF- $\kappa$ B is retained in the cytoplasm by binding to one of the inhibitory proteins called I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , p105, or p100.<sup>18</sup> As a result of activating signals, proteolytic degradation of the inhibitor releases NF- $\kappa$ B, which translocates to the nucleus regulating target genes. In our study, activation of NF- $\kappa$ B is associated with normal values of both total and phosphorylated I $\kappa$ B- $\alpha$  (data not shown), but with reduced levels of NFKB1 mRNA. Because the NFKB1 gene codifies a 105-kd protein, which functions as an inhibitory subunit by retaining NF- $\kappa$ B components in the cytosol,<sup>15,17</sup> it could be suggested that reduced NFKB1 expression may favor NF- $\kappa$ B nuclear translocation in HCV-infected livers.

In conclusion, NF- $\kappa$ B is activated in the liver of subjects with chronic hepatitis C, and expression of RelA in the liver of these patients is associated with lower apoptosis, less fibrosis, and slower progression to cirrhosis. Our data point to a protective role of hepatic RelA against cirrhosis development in chronic HCV infection.

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