Virology **296,** 84–93 (2002)
doi:10.1006/viro.2002.1371

Hepatitis C Virus Core Protein Inhibits Apoptosis via Enhanced Bcl-x_L Expression

Motoyuki Otsuka, Naoya Kato,[1] Hiroyoshi Taniguchi, Hideo Yoshida, Tadashi Goto, Yasushi Shiratori, and Masao Omata

Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Received September 24, 2001; returned to author for revision October 23, 2001; accepted January 17, 2002

Previous studies indicated that hepatitis C virus core protein influences cellular apoptosis. However, the precise mechanisms of the effects are not fully understood. Therefore, in this study, we examined the mechanisms of the effects on cell apoptosis by core protein, using transiently transfected and magnetically collected core-producing HepG2 cells. First, to elucidate the target site of core protein in the apoptotic pathway, we examined the activation of caspases after anti-Fas antibody stimulation. Core protein inhibited the apoptotic cascade downstream from caspase 8 and upstream from caspase 3. Next, to clarify more direct mechanisms of this effect, mRNA levels of several *bcl-2*-related genes were examined. An RNase protection assay showed that the mRNA of *bcl-xl* increased in the core-producing cells. We showed that this increase was mediated by the enhancement of *bcl-x* promoter activity by core protein through an extracellular-regulated kinase pathway. These results suggest that core protein inhibits apoptosis at the mitochondria level through augmentation of Bcl-x expression, resulting in an inhibition of caspase 3 activation.

Key Words: HCV core protein; apoptosis; Bcl-x; extracellular-regulated kinase.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus and a major causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Kuo *et al.,* 1989; Saito *et al.,* 1990; Shiratori *et al.,* 1995; Takano *et al.,* 1995). More than 170 million persons are chronically infected with HCV worldwide (WHO, 1998). The HCV genome contains a large open reading frame encoding a polyprotein precursor of 3010–3033 amino acids and an untranslated region at the 5′ and 3′ ends of the genome. The putative organization of the HCV genome includes the 5′ untranslated region, three or four structural proteins (core, E1, and E2/p7), six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the 3′ untranslated region (Choo *et al.,* 1991; Hijikata *et al.,* 1991; Grakoui *et al.,* 1993).

Apoptotic cell death with viral infection can be induced by the host immune response or by viral proteins, and apoptosis is considered to be a common pathway of virus clearance. On the other hand, viruses encode proteins that suppress apoptosis and escape this clearance mechanism (reviewed by White, 1996). CrmA, a cowpox virus genome product, encodes a protease inhibitor of the caspase family and prevents apoptosis by tumor necrosis factor α (TNF-α) (Beidler *et al.,* 1995), and adenovirus E1B and human papilloma virus E6 protein suppress p53-dependent apoptosis by binding and inactivating p53 function (Scheffner *et al.,* 1990). These alterations in cell survival contribute to the establishment of persistent infection and the pathogenesis of a number of diseases including viral oncogenesis (Thompson, 1995; Rudin and Thompson, 1997).

In the case of HCV infection, previous studies demonstrated controversial phenomena concerning apoptosis. There are several studies indicating that core protein suppresses apoptosis mediated by cisplatin, c-myc, TNF-α, or the Fas signaling pathway (Ray *et al.,* 1996, 1998; Marusawa *et al.,* 1999; Machida *et al.,* 2001), while other studies showed that core protein sensitizes Fas-, TNF-α-, or serum starvation-induced apoptosis (Ruggieri *et al.,* 1997; Zhu *et al.,* 1998, 2001; Honda *et al.,* 2000; Hahn *et al.,* 2000). However, the precise mechanisms of the effects of core protein on the apoptotic pathway are not fully understood.

Therefore, in this study, using a magnetic concentration system for transiently transfected cells, we investigated the effects of the core protein on the apoptotic pathway.

RESULTS

Caspase-3 activation was suppressed by core protein

The binding of Fas ligand to Fas results in the activation of the caspase cascade. To determine the target site where the core protein affects the apoptotic cascade, the activation of caspases, including procaspase-8, pro-

[1] To whom correspondence and reprint requests should be addressed at Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: +81-3-3814-0021. E-mail: kato-2im@h.u-tokyo.ac.jp.

0042-6822/02 $35.00

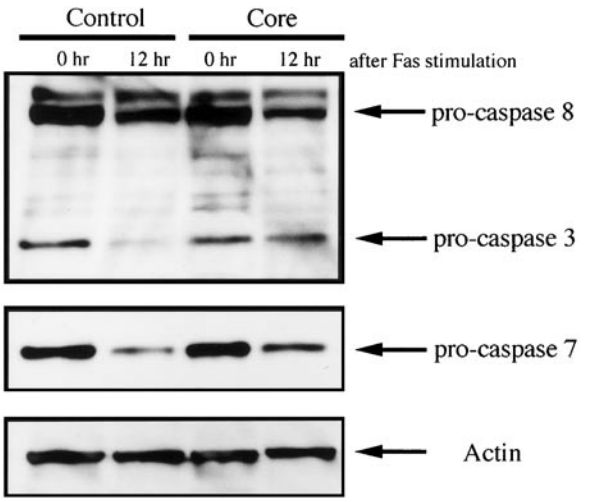

FIG. 1. Core protein suppresses procaspase-3 activation after Fas stimulation. HepG2 cells were transfected with pCXN2 or pCXN2-core with pMACS K^k for 24 h. After magnetic concentration, cells were treated with anti-Fas and CHX for 0 or 12 h. Then, the cells were collected and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies.

caspase-3, and procaspase-7, in the core-producing HepG2 cells after anti-agonistic Fas antibody treatment was examined by Western blotting. These forms are converted into active subunits during activation. Thus, decreases in the densities of the bands for procaspase-8, -3, or -7 on Western blotting indicate activation of the respective caspase. Activation of the effector caspases 3 and 7 was diminished in the core-producing HepG2 cells compared with activation in the control cells (Fig. 1). However, activation of the initiator caspase 8 was almost the same, or rather stronger, in the core-producing HepG2 cells as in the control cells (Fig. 1). Therefore, core protein was considered to inhibit apoptosis downstream from caspase 8 and upstream from caspase 3 in the apoptotic pathway.

Core protein enhances the expression of bcl-xl mRNA and Bcl-x_L protein

In hepatocytes, Bcl-2 members have been reported to act as selective links between specific upstream signals, such as caspase 8, and downstream death effectors, such as caspase 3 (Adams and Cory, 1998), and to protect the liver from Fas-induced apoptosis (Lacronique *et al.,* 1996). Therefore, we examined the amounts of mRNA of several Bcl-2-related genes, including *bcl-xl, bcl-xs, bfl1, bik, bak, bax, bcl2,* and *mcl1* in the core-producing HepG2 cells by using an RNase protection assay. In pCXN2-core-transfected HepG2 cells, the mRNA level of *bcl-xl,* which is known to inhibit apoptosis, was increased after transfection, and *mcl1,* which is also known to inhibit apoptosis, was also slightly increased 20 and 25 h after transfection (Fig. 2). However, none of the other members of the bcl family tested was significantly affected by core protein expression (Fig. 2). The mRNA levels of GAPDH and L32, which were investigated as internal controls at the same time, were also not changed. In addition, the transfection of the empty vector did not affect any of the genes investigated.

To confirm the enhanced expression of the Bcl-x_L

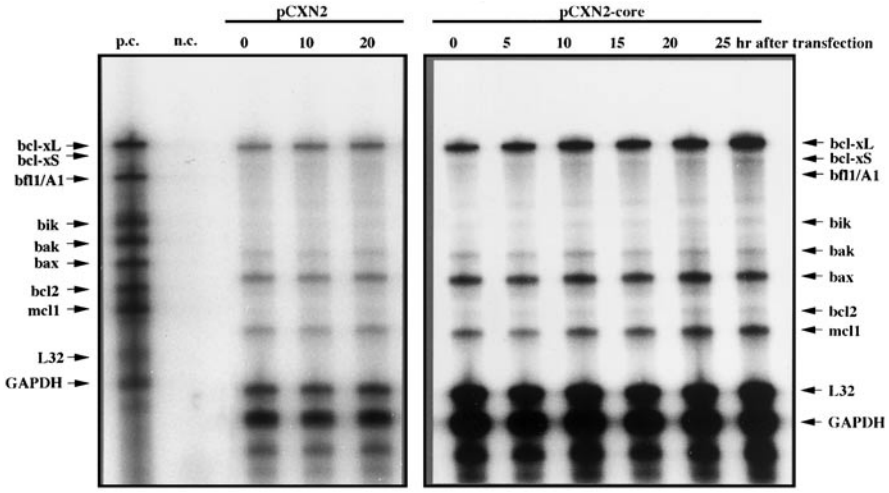

FIG. 2. Core protein enhances the expression of *bcl-xl* mRNA. RNase protection assays with specific probes for *bcl-xl, bcl-xs, bfl1, bik, bak, bax, bcl2,* and *mcl1* genes were performed by using 10 μg total RNA extracted from HepG2 cells after 0, 5, 10, 15, 20, and 25 h of transient transfection with 3 μg of pCXN2-core. As internal controls, the mRNA levels of a ribosomal protein (L32) and GAPDH were simultaneously analyzed. As a control, the RNA extracted from the cells transfected with the empty vector which were collected 0, 10, or 20 h after transfection was also examined. To identify the protected bands, the probe without RNase treatment was also applied as a positive control (p.c.). The nomenclature was from the supplier's kit. Each probe band migrates slower than its protected band due to the nonhomologous flanking sequences transcribed along with the probe that is not protected by mRNA. n.c. means negative control using yeast tRNAs which were not protected. Similar results were obtained in another set of independent experiments.

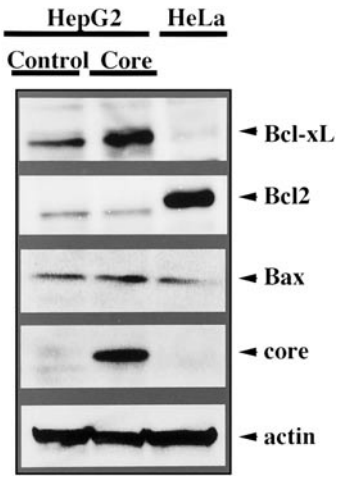

FIG. 3. Core protein enhances the expression of Bcl-x_L protein. Western blot analysis of Bcl-x_L, Bcl2, Bax, core, and actin expression was performed using magnetically concentrated HepG2 cell extracts transiently transfected with pCXN2 (control) or pCXN2-core (core). The cells were collected 36 h after transfection. HeLa cell extracts were used as a control. Specific protein bands are indicated by arrows. Similar results were obtained in another set of independent experiments.

protein by core protein, Western blotting was performed. Bcl-x_L expression was enhanced in the core-producing HepG2 cells compared with the control cells (Fig. 3). Bcl-2 expression was detected in HeLa cells used as a control, but not in HepG2 cells, consistent with previous reports (You *et al.,* 1999). Bax expression was not changed in the core-producing cells compared with that in the control cells (Fig. 3). Therefore, the increase of Bcl-x_L protein expression in the core-producing cells was considered to contribute to the inhibition of apoptosis by core protein.

Transactivation of bcl-x promoter by core protein

To determine the effect of core protein on *bcl-x* promoter activity, HepG2 cells were cotransfected with each of seven HCV protein expression plasmids and a reporter plasmid (pGL2 (848)) containing an 848-bp segment of the 5′ flanking region of the *bcl-x* gene. Of all the HCV proteins investigated, only core protein transactivated *bcl-x* promoter activity. The relative firefly luciferase activity of pCXN2-core transfected cell lysate was 2.4 ± 0.6 (mean ± SD) times higher than that of pCXN2 control vector transfected cell lysates (Fig. 5). This enhancement occurred in a dose-dependent manner (Fig. 4).

To confirm the similar effects of the core protein on the *bcl-x* promoter activity and Bcl-x_L protein expression in the presence of its downstream E1 and E2 envelope proteins, the HCV structural protein expression vector pCXN2-HCV-S was cloned and similar experiments were performed. As shown in Figs. 4C and 4D, core protein transactivated *bcl-x* promoter activity and increased the expression of the Bcl-x_L protein in proportion to the amount of core protein even in the presence of the envelope proteins.

NF-κB activation is not involved in the transactivation of the bcl-x promoter by core protein in HepG2 cells

Since core protein activates NF-κB-associated signaling (Marusawa *et al.,* 1999; You *et al.,* 1999; Kato *et al.,* 2000) and an up-regulated expression of Bcl-x was reported to be partly mediated by NF-κB signaling (Tsukahara *et al.,* 1999; Tamatani *et al.,* 1999; Lee *et al.,* 1999; Chen *et al.,* 2000), we investigated whether transactivation of the *bcl-x* promoter by core protein in HepG2 cells was also mediated by NF-κB signaling. A deletion construct, pGL2 (822), which lacked the NF-κB site compared with pGL2 (848), was used for the luciferase assay. However, this construct also responded to core protein significantly (Fig. 5A). Furthermore, a mutated NF-κB-luc plasmid, pGLκBM, which was the same length as pGL2 (848), but had a CC-to-GG mutation at positions −841 and −840 within the NF-κB motif, exhibited similar enhancement of core-induced transactivation as observed with pGL2 (848) (Fig. 5A).

Next, we examined whether blocking of the NF-κB pathway affects core protein-induced transactivation of *bcl-xl* by using a superrepressor form of IκBα mutant (IκBαM). IκBαM, which specifically suppresses NF-κB activity, contains serine to alanine mutations at residues 32 and 36; these abolish sites of phosphorylation by IκB kinase. This IκBαM expression vector and pCXN2-core were cotransfected into HepG2 cells along with pGL2 (848) or pNFκB-luc, a synthetic promoter that contains tandem repeats of the binding sites for NF-κB linked with the luciferase gene. The elevated luciferase activity from pNFκB-luc in response to core protein was markedly suppressed by IκBαM, but not from pGL2 (848) (Fig. 5B). These results suggest that the NF-κB site located in the *bcl-x* promoter region is not involved in the core protein-induced transactivation of *bcl-x* in HepG2 cells.

Mapping of core-responsive regions of the bcl-x promoter

To examine the responsible sites for core-induced transactivation of *bcl-x* promoter, a series of luciferase reporter constructs with several fragments of promoters of different lengths was prepared and transiently transfected into HepG2 cells along with core expression plasmid or control plasmid. Both the basal and the core-response luciferase activities were markedly reduced when using pGL2 (197) compared with the results of other constructs (Fig. 6). These results suggested that the bases from −198 to −255 in the *bcl-x* promoter form one of the major responsive elements of the basal and core-induced transactivation of *bcl-x* promoter activity.

The ERK1/2-mediated pathway was involved in the bcl-x promoter activity

Since we previously showed that core protein activates not only the NF-κB pathway but also the serum

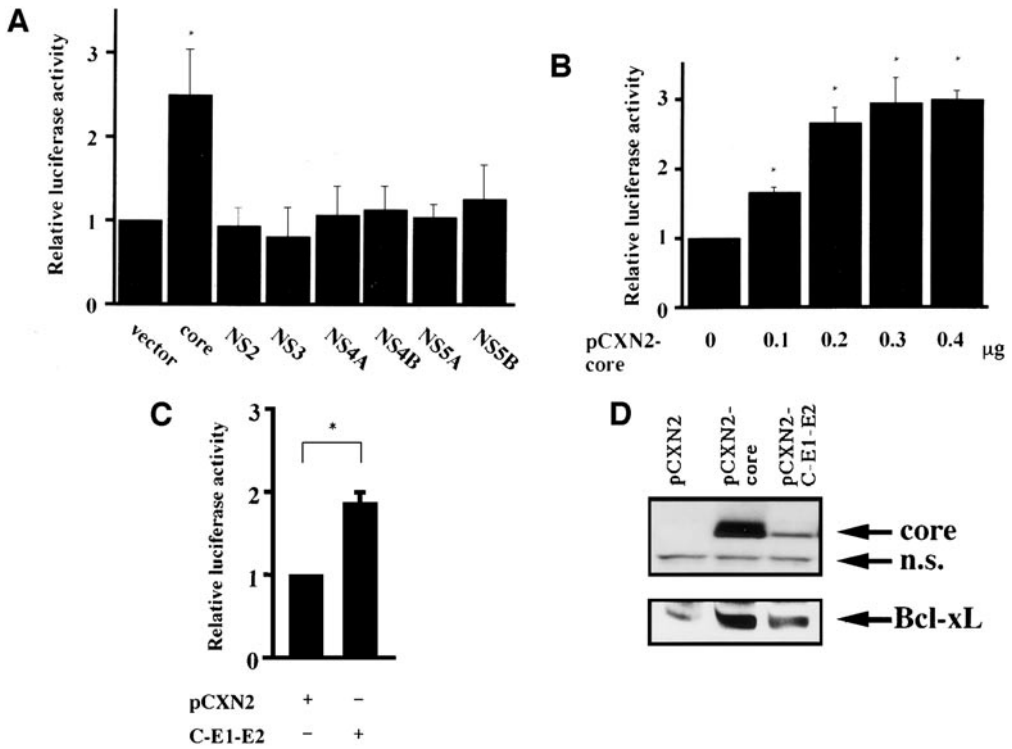

FIG. 4. Transactivation of the *bcl-x* promoter by core protein. (A) Approximately 4×10^5 HepG2 cells were cotransfected with 0.49 μg of pGL2 (848), 0.01 μg of pRL-TK, and 0.3 μg of pCXN2 or various types of the indicated expression plasmids. Thirty-six hours after transfection, luciferase assays were carried out with the PicaGene dual sea pansy system. Firefly luciferase activity and sea pansy luciferase activity were measured as relative light units. Firefly luciferase activity was normalized for transfection efficiency based on sea pansy luciferase activity. The results are expressed by taking the luciferase activity of pCXN2-transfected control cell lysate as 1. The data represent the mean ± the standard deviation of three independent experiments. * $P < 0.05$ vs pCXN2. (B) Approximately 4×10^5 HepG2 cells were cotransfected with 0.49 μg of pGL2 (848), 0.01 μg of pRL-TK, and indicated amounts of pCXN2-core. Total amounts of transfected DNA were adjusted up to 0.8 μg by adding pCXN2 control vector. Luciferase activities were measured as described for part (A). * $P < 0.05$ vs pCXN2. (C) 4×10^5 HepG2 cells were cotransfected with 0.49 μg of pGL2 (848), 0.01 μg of pRL-TK, and 0.3 μg of pCXN2 or pCXN2-HCV-S. Luciferase activities were measured as described for part (A). * $P < 0.05$. (D) Western blot analysis of Bcl-x_L and core expression was performed using magnetically concentrated HepG2 cell extracts transiently transfected with pCXN2, pCXN2-core, or pCXN2-HCV-S. The cells were collected 36 h after transfection. n.s. means nonspecific band to indicate that the same amount of protein was applied to each lane.

response element (SRE)-associated pathway (Tsuchihara *et al.,* 1999; Kato *et al.,* 2000; Aoki *et al.,* 2000) and the activity of transcription factors associated with the SRE pathway is regulated partly via extracellular signal-regulated kinases (ERK), we determined the effect of PD98059, an inhibitor of ERK1/ERK2 activation, on *bcl-x* promoter activity. This inhibitor significantly reduced both the basal level and enhancement of the activity of the promoter by core protein (Fig. 7). Therefore, the ERK1/2-mediated pathway was considered to be involved in the activation of the *bcl-x* promoter activity at least in hepatocytes.

Core protein inhibits DNA fragmentation after induction of apoptosis via bcl-xl

To determine whether core protein actually down-regulates apoptosis and whether Bcl-x_L expression is responsible for the effects, we performed a DNA fragmentation assay using the magnetically concentrated core-producing HepG2 cells and *bcl-x* antisense oligonucleotide targeted to the initiation codon of the *bcl-x* gene. As shown in Fig. 8A, core-producing cells reduced the DNA fragmentation compared with the control cells after induction of apoptosis. This effect was reversed by the existence of the *bcl-x* antisense oligonucleotide, but not the sense oligonucleotide. Western blotting revealed that antisense treatment actually reduced the Bcl-x_L expression. These results suggested that core protein reduced the apoptotic activities via the Bcl-x_L expression.

DISCUSSION

In this study, we demonstrated that HCV core protein inhibits apoptosis downstream from caspase 8 and upstream from caspase 3. We also showed that core protein transactivates *bcl-x* promoter activity, and this may be one of the mechanisms inhibiting apoptosis mediated by Fas in HepG2 cells.

There are contradictory data about the effects of the core protein on apoptosis signals. As reported, the contradiction may be caused by the use of clonally selected,

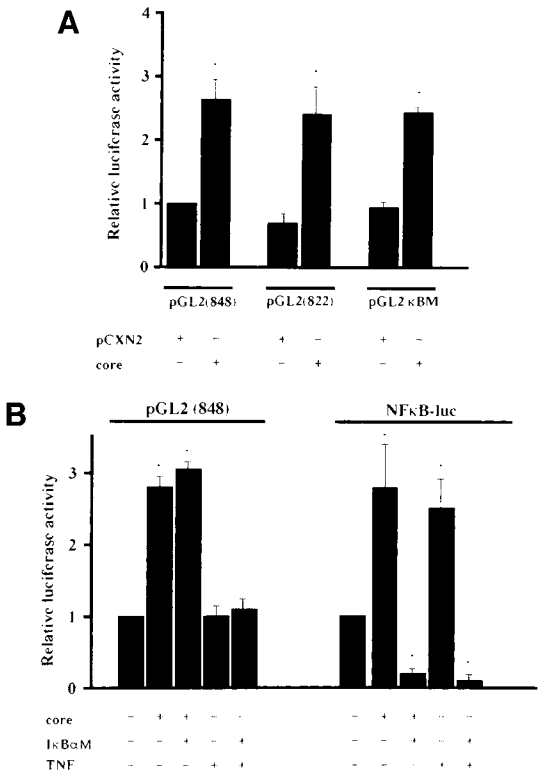

FIG. 5. NF-κB activation was not involved in transactivation of the *bcl-x* promoter by core protein in HepG2 cells. (A) HepG2 cells were transfected with 0.01 μg of pRL-TK, 0.3 μg of pCXN2-core or pCXN2, and 0.49 μg of the indicated reporter plasmids. pGL2 (822) deletes the NF-κB site. pGL2κBM contained mutations within the NF-κB site as described under Materials and Methods. Luciferase assays were measured as described in the legend for Fig. 4A. The results are expressed by taking the luciferase activity from pGL2 (848) of pCXN2-transfected control cell lysate as 1. Data represent the mean ± the standard deviation of three independent experiments. * $P < 0.05$ vs pCXN2. (B) HepG2 cells were transfected with 0.49 μg of pGL2 (848) or NFκB-luc, 0.01 μg of pRL-TK, and 0.3 μg of the indicated expression plasmids. Total DNA amounts were adjusted by adding pCXN2 to 1.1 μg. Thirty-six hours after transfection, luciferase assays were carried out as described in the legend for Fig. 4A. TNF-α (10 ng/ml) was added 24 h before the luciferase assays if necessary. The data represent the mean ± the standard deviation of three independent experiments. * $P < 0.05$ vs pCXN2.

permanent transfectant cells, which differ in their characteristic responses from parental cell lines (Marusawa *et al.,* 1999). Thus, to exclude the chance of selecting particular cells, we used magnetically concentrated cells after transient transfection. In this study, we showed that in magnetically concentrated core-producing HepG2 cells, activation of procaspase-3 mediated by Fas, but not procaspase-8, was inhibited compared with activation in the control cells. Therefore, core protein inhibited apoptosis downstream from caspase 8 and upstream from caspase 3 in the apoptotic pathway.

Although diverse effects of core protein on apoptosis have been reported, the underlying mechanisms are not fully understood. Previous studies showed that hepatocytes resemble type II cell lines, in which Fas-induced death is dependent on mitochondria (Scaffidi *et al.,* 1998), and that bcl-2 members act as a selective linkers between upstream signals and downstream death effectors (Lacronique *et al.,* 1996; Adams and Cory, 1998). In this study, using an RNase protection assay and Western blotting, we showed that not only *bcl-xl* mRNA, but also Bcl-x_L protein, was increased in the core-producing HepG2 cells. As Bcl-x_L is known to efficiently block the cleavage-mediated activation of procaspase-3, a critical downstream effector caspase in the apoptotic pathway (Chinnaiyan *et al.,* 1996; Erhardt and Cooper, 1996; Shimizu *et al.,* 1996), the core-producing HepG2 cells were considered to inhibit cleavage-mediated activation of procaspase-3, through enhanced expression of Bcl-x_L protein.

Recently, NF-κB was reported to be an inducer of *bcl-xl* in lymphocytes and primary neurons stimulated by CD40, TNF, or human T-cell leukemia virus type 1 Tax protein (Tsukahara *et al.,* 1999; Lee *et al.,* 1999; Tamatani *et al.,* 1999; Chen *et al.,* 2000). However, although core protein activates the NF-κB pathway (Marusawa *et al.,* 1999; You *et al.,* 1999; Kato *et al.,* 2000; Yoshida *et al.,* 2001), the enhanced expression of Bcl-x_L by core protein was not dependent on the NF-κB site in the *bcl-xl* promoter in this study (Fig. 4). The difference in the data may be explained by the fact that the regulation of *bcl-xl* expression appears to be quite complex and the contribution of NF-κB to this process may depend on cell type or activating stimuli. In fact, other studies failed to reveal alterations in *bcl-xl* transcript levels by NF-κB activation (Wang *et al.,* 1998; Wu *et al.,* 1998; Grumont *et al.,* 1999). On the contrary, it was reported that NF-κB activation was a negative regulator of Bcl-x_L in double-positive thymocytes (Hettmann *et al.,* 1999). Thus, further study is needed to clarify the fine regulation of Bcl-x_L expression by NF-κB.

Other transcription factors, such as Stat 1, Stat 5, and Ets 2, are also implicated in the regulation of *bcl-xl* expression (Fujio *et al.,* 1997; Sevilla *et al.,* 1999; Socolovsky *et al.,* 1999), and multiple binding sites for transcriptional factors were identified in the *bcl-x* promoter (Grillot *et al.,* 1997; Grad *et al.,* 2000). In this study, we found that the bases from −198 to −255 in the *bcl-x* promoter form one of the responsive elements of the basal- and core-induced transactivation of *bcl-x* promoter activity in hepatocytes (Fig. 6). Although an electrophoretic mobility shift assay revealed that nuclear protein actually binds to this region (data not shown), no serum response element was located within this region by our computer analysis. However, the present studies showed that PD98059, an inhibitor of ERK1/ERK2 activation, significantly reduced both the basal and the core-mediated activation of the *bcl-x* promoter (Fig. 7). These results were consistent with the recent report that the ERK signaling pathway regulates Bcl-x_L expression in

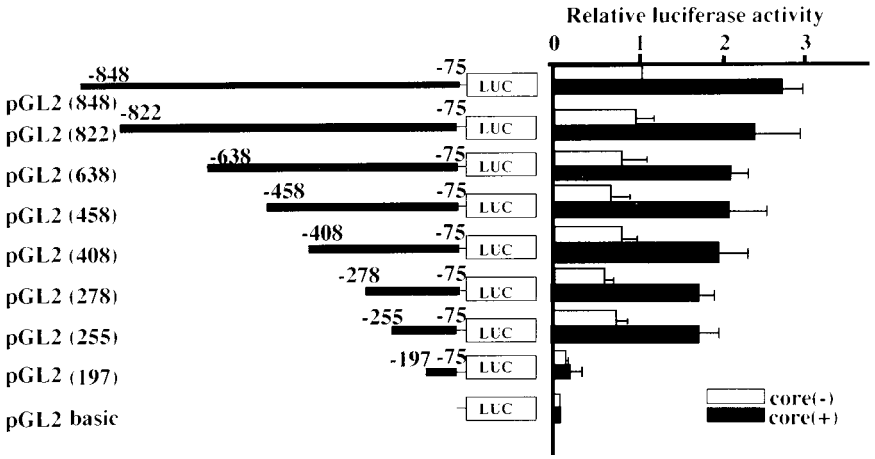

FIG. 6. Mapping of core-responsive regions in the *bcl-x* promoter. (A) HepG2 cells were transfected with 0.01 μg of pRL-TK, 0.3 μg of pCXN2-core or pCXN2, and 0.49 μg of a series of reporter plasmids with several fragments of different lengths of 5′-deleted *bcl-x* promoters. Luciferase activities were measured as described in the legend for Fig. 4A. The results are expressed by taking the luciferase activity from pGL2 (848) and pCXN2-transfected control cell lysate as 1. The data represent the mean ± the standard deviation of three independent experiments. Constructs of the reporter plasmids used are schematically illustrated in the left panel. Fragments of the 5′ flanking region of the *bcl-x* gene are represented by heavy lines, and the number at the 5′ end of each construct indicates the 5′-most end base positions from the translation initiation codon of the *bcl-x* gene.

pancreatic cancer cells (Boucher *et al.,* 2000). Therefore, to clarify precise mechanisms of the activation of this promoter activity, protein(s) binding to this region must be identified in the future.

Our findings do not exclude the possibility that the activation of NF-κB by core protein may lead to suppression of apoptosis, since NF-κB is known to induce expression of IAP families that inhibit apoptosis at various points in the apoptotic cascade (Deveraux and Reed, 1999). Although we could not detect the expression of c-IAP-2 protein in HepG2 cells (data not shown), there remains a possibility that other IAP families may also be involved in the inhibition of apoptosis by core protein through NF-κB activation.

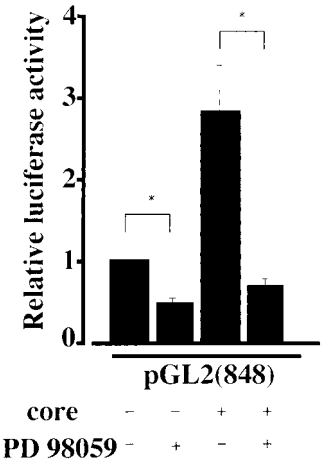

FIG. 7. The ERK1/2-mediated pathway was involved in the activation of *bcl-x* promoter activity by core protein. HepG2 cells were transfected with 0.49 μg of pGL2 (848), 0.01 μg of pRL-TK, and 0.3 μg of the indicated expression plasmids. Thirty-six hours after transfection, luciferase assays were carried out as described in the legend to Fig. 4A. PD98059, an inhibitor of ERK1/ERK2 activation (50 μmol/L), was added 24 h before the luciferase assays if necessary. Data represent the mean ± the standard deviation of at least three independent experiments. * $P < 0.05$.

The Fas system is known to play an important role in liver cell injury by HCV and in the clearance of viruses (Cerny and Chisari, 1999). The ability of core protein to inhibit the Fas-mediated apoptotic pathway by up-regulation of Bcl-x_L expression may provide a selective advantage for evasion of host antiviral defense mechanisms. Previous immunohistochemical analysis actually revealed that Bcl-x_L expression in hepatocytes was elevated in livers with HCV-related cirrhosis (Frommel *et al.,* 1999). Recently, it was reported that the expression of Bcl-x_L is high in hepatocellular carcinoma (Takehara *et al.,* 2001). In addition, core protein was reported to induce hepatocellular carcinoma in transgenic mice (Moriya *et al.,* 1998). Therefore, it is possible that the selection of Bcl-x_L-expressing cells by their capacity to inhibit apoptosis could account for the development of hepatocellular carcinoma in hepatitis C patients.

In conclusion, we demonstrated that HCV core protein enhanced the expression of Bcl-x_L, and this was associated with the inhibition of procaspase-3 activation. Core protein-mediated Bcl-x_L induction may be one of the mechanisms underlying its inhibition of apoptosis, which might contribute to the pathogenesis of HCV.

MATERIALS AND METHODS

Cell lines

Human hepatoblastoma cells (HepG2) and human cervical carcinoma cells (HeLa) were obtained from the Riken Cell Bank (Tsukuba Science City, Japan) and main-

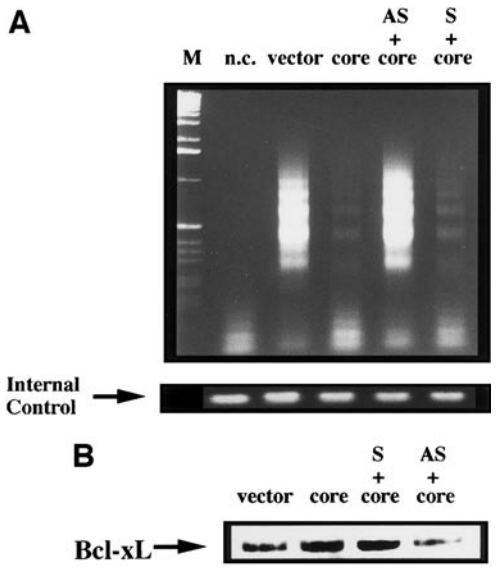

FIG. 8. Core protein inhibits DNA fragmentation after induction of apoptosis via *bcl-x*. (A) HepG2 cells were transfected with pCXN2 or pCXN2-core with pMACS K^k for 24 h, with or without *bcl-x* antisense or sense oligonucleotide transfection. After magnetic concentration, cells were treated with anti-Fas and CHX for 12 h. Then genomic DNA was extracted and DNA fragments were amplified from equal amounts of DNA as described under Materials and Methods. As an internal control, the En-2 gene product was also examined. The DNA products were loaded on a 1.2% agarose/EtBr gel. M means 1-kb ladder. n.c. means negative control derived from HepG2 cells without induction of apoptosis. (B) Western blot analysis of Bcl-x_L expression was performed using magnetically concentrated HepG2 cell extracts transiently transfected with pCXN2 (vector) or pCXN2-core (core) with or without bcl-x antisense or sense oligonucleotide transfection. The cells were collected 36 h after transfection. Cell extracts were normalized for protein concentration, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted.

tained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum.

Expression plasmids and reporter plasmids

The expression plasmids pCXN2-core, NS2, NS3, NS4A, NS4B, NS5A, and NS5B containing the respective HCV genomic regions, driven by a β-actin-based CAG promoter, were constructed as described previously (Kato *et al.,* 2000). A control plasmid (pCXN2) was also used. The expression plasmid pCXN2-HCV-S, containing the HCV structural regions (core, E1, E2, and p7), was newly constructed by site-specific recombination-based cloning technology (Gateway Cloning Technology; Life Technologies, Rockville, MD) according to the manufacturer's instructions. Briefly, pCV-J4L6S, containing a full-length infectious HCV clone, was kindly provided by Professor Bukh (Yanagi *et al.,* 1998). Using this clone as a template, structural regions were amplified with the primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACCATGAGCACGAATCCTAAAC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGGCGTAAGCTCGTGGTGGTAACG-3′). Primers contained extra bases for recombination (underlined). The PCR products were cloned into pDONR201 (Life Technologies) and then cloned into pCXN2 destination vector by recombination protein. The pCXN2 destination vector was constructed by inserting a Gateway conversion cassette into the cloning site of the pCXN2 vector.

An expression vector of the superrepressor form of IκBαM containing serine to alanine mutations at residues 32 and 36, which specifically suppress NF-κB activity, was kindly provided by Dr. Hiroshi Suzuki (Suzuki *et al.,* 1999).

The following vectors containing the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element (TATA box) plus an inducible *cis*-enhancer element were utilized as reporter plasmids. The *bcl-x* promoter-driven luciferase reporter plasmids pGL2 (848) and pGL2 (822), which contained DNA fragments of the *bcl-x* promoter at the base positions of −848 to −75 and −822 to −75 from the translation initiation ATG of the mouse *bcl-x* gene, respectively, were kindly provided by Dr. Mari Kannagi (Tsukahara *et al.,* 1999), who also provided pGLkBM, a mutant of pGL2 (848) carrying CC-to-GG mutations at positions −841 and −840 within the NF-κB-binding site. pGL2 (638), pGL2 (458), pGL2 (408), pGL2 (278), pGL2 (255), and pGL2 (197) were newly generated by inserting DNA fragments of the *bcl-x* promoter at the base positions −638, −458, −408, −278, −255, and −75 from the translational initiation ATG of the *bcl-x* gene, respectively, into the pGL2 basic vector containing a luciferase reporter gene (Promega, Madison, WI). These DNA fragments were amplified by PCR using pGL2 (848) as a template and six sense primers (5′-CCGCTCGAGTGGTCCATGGAGGAACCAGG-3′, 5′-CCGCTCGAGCTATGATACAAAAGACCTTCC-3′, 5′-CCGCTCGAGAGATTTGAATGTAGGTGG-3′, 5′-CCGCTCGAGTGGTCCTTTCAATTTGACTTC-3′, 5′-CCGCTCGAGTGAAGTCCCTTAGAACCCG-3′, 5′-CCGCTCGAGGGTACCTGGAGGAATATTG-3′) and one antisense primer (5′-CCGCTCGAGGCGATTCTCTTCCAGGATC-3′). Gene integration was confirmed by DNA sequencing. A synthetic promoter that contains tandem repeats of the binding sites for NF-κB (pNFκB-Luc) was purchased from Stratagene (La Jolla, CA). To check transfection efficiency, pRL-TK, a control plasmid that expresses *Renilla reniformis* (sea pansy) luciferase driven by herpes simplex virus thymidine kinase (Toyo Ink, Tokyo, Japan), was used.

Concentration of transiently transfected cells and immunoblotting

We used the MACSelect system (Myltenyi Biotec, Bergisch Gladbach, Germany) for specific concentration of transiently DNA-transfected cells from the heterogeneous cell population as described previously (Goto *et al.,* 2001). Plasmid-transfected cells were concentrated

by magnetic isolation using a specific surface marker, a truncated mouse H-2K^k molecule, which was expressed from the cotransfected plasmid, pMACS K^k. Approximately 10^7 HepG2 cells were transfected with 3 μg of pCXN2-core or pCXN2 control plasmid, together with 1 μg of pMACS K^k. After 24 h, cells were trypsinized and dispersed by pipetting into single-cell suspensions. The cells were resuspended with 80 μl of micromagnetic beads conjugated with a monoclonal antibody against mouse H-2K^k and incubated for 15 min at room temperature. Magnetically labeled cells were recovered by the magnetic separation column. The transfected cells collected magnetically were treated with anti-agonistic Fas antibody (CH-11; MBL, Nagoya, Japan) at a final concentration of 10 ng/ml. A minimum of 500 ng/ml of cycloheximide (CHX) was required for anti-Fas-induced apoptosis in HepG2 cells, as described previously (Marusawa *et al.,* 1999). Both the detached and attached cells were collected 0 or 12 h after the start of anti-Fas treatment. Cell extracts were normalized for protein concentration with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted as described previously (Kato *et al.,* 2000). To evaluate caspase activation, anti-procaspase-8 (MBL), anti-procaspase-3 (Transduction Laboratories, Lexington, KY), and anti-procaspase-7 (Transduction Laboratories) were used in this experiment. To evaluate the expression levels in the cells transfected with pCXN2-core, pCXN2-HCV-S, or pCXN2, anti-Bcl-x_L (Transduction Laboratories), an anti-inhibitor of apoptosis protein (IAP) -2 (Genzyme, Cambridge, MA), anti-Bcl-2 (Santa Cruz, CA), anti-actin (Chemicon, Temecula, CA), and anti-HCV core (Austral Biologicals, San Ramon, CA) were used. The bound antigen was detected using the ECL-plus Western blotting detection system (Amersham, Pharmacia, Buckinghamshire, UK).

RNase protection assay

We analyzed the amounts of *bcl-xl, bcl-xs, bfl1, bik, bak, bax, bcl2,* and *mcl1* gene mRNAs in HepG2 cells transiently transfected with pCXN2-core by RNase protection assay (RPA) with the RPA III kit (Ambion, Austin, TX) as described previously (Kiefer *et al.,* 1995). Approximately 10^7 HepG2 cells were transiently transfected with 3 μg of pCXN2-core. After 0, 5, 10, 15, or 20 h of incubation, all the cells were lysed with Isogen solution (Nippon Gene, Toyama, Japan), and total cellular RNA was extracted by an acid guanidinium thiocyanate–phenol–chloroform method according to the manufacturer's instructions. RNA probes used for this assay were synthesized by *in vitro* transcription with templates included in an hAPO-2 human apoptosis template set (PharMingen, San Diego, CA) according to the supplier's instructions. The total RNA (10 μg) was hybridized with a mixture of ^{32}P-labeled antisense RNA probes (3×10^5 cpm) overnight at 56°C in a hybridization buffer and then treated with RNase A/T1. The resultant protected RNAs were separated on 5% acrylamide gels with 8 M urea and visualized by autoradiography. As internal controls, the mRNA levels of a ribosomal protein (L32) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed. As a control, the RNA extracted from the cells transfected with the empty vector which were collected 0, 10, or 20 h after transfection was also examined. To identify the protected bands, the probe without RNase treatment was also applied. The nomenclature was from the supplier's kit.

Transient transfection and luciferase assay

To evaluate the ability of core protein to activate the transcription of *bcl-x,* transient transfection and a luciferase assay were performed. Approximately 4×10^5 cells were plated into wells of 6-well tissue culture plates 24 h before transfection. Transfection was performed using the FuGene6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Unless otherwise specified, transfection complexes containing 0.8 μg of plasmids (0.49 μg of firefly luciferase reporter plasmid, 0.01 μg of pRL-TK, and 0.3 μg of pCXN2 or various expression plasmids) were added to each well of the 6-well plates. Human recombinant TNF-α (10 ng/ml; Pharma Biotechnologie Hanover, Hanover, Germany) or PD98059 (50 μmol/L; Promega), an inhibitor of extracellular signal-regulated kinases (ERK1/ERK2), was added 24 h before the luciferase assays if necessary. Cells were harvested 36 h after transfection, and luciferase assays were carried out with the PicaGene dual sea pansy system (Toyo Ink). Firefly luciferase activity and sea pansy luciferase activity were measured as relative light units with a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). Firefly luciferase activity was normalized for transfection efficiency based on sea pansy luciferase activity. All assays were performed at least in triplicate.

Antisense oligonucleotide and transfection

Oligonucleotides with a phosphorothioate backbone were synthesized (Sawady Co., Tokyo, Japan). The antisense oligonucleotide encodes a sequence complementary to the human *bcl-x* translation initiation site and extending 3′ downstream for a total of 18 bases (Takehara *et al.,* 2001). A sense oligonucleotide was used as a control. The sequence of these oligonucleotides was as follows: antisense sequence, 5′-CCGGTTGCTCTGAGACAT-3′; sense sequence, 5′-ATGTCTCAGAGCAACCGG-3′.

To enhance cellular uptake, oligonucleotides were delivered in the form of complexes with FuGENE6 Trans-

fection Reagent. Each oligonucleotide was added to the cellular medium at the concentration of 400 nmol/L.

DNA fragmentation assay

For the semiquantitative analysis of apoptosis, a DNA fragmentation assay was performed using ApoAlert LM-PCR Ladder Assay kit (Clontech, Palo Alto, CA) according to the manufacturers' instructions. Briefly, using magnetically concentrated HepG2 cells transfected with pCXN2 or pCXN2-core with or without *bcl-x* antisense or sense oligonucleotides, apoptosis was induced by Fas stimulation as described. Both the detached and attached cells were collected 12 h after the start of anti-Fas treatment. From the collected cells, genomic DNA was extracted. After the adaptor ligation, 100 ng of each adaptor-ligated DNA was used as a template for PCR to amplify the DNA fragments. The PCR conditions were as follows: incubation for 8 min at 72°C, followed by 24 cycles of 94°C for 1 min and 72°C for 3 min. Then 10 μl of the each reaction was loaded directly on a 1.2% agarose/EtBr gel. To confirm that equal amounts of DNA were used for PCR, 100 ng of adaptor-ligated DNA and 1μm of human En-2 primers provided in the kit for an internal control was used.

Statistical analysis

The results of assays of luciferase activity were analyzed using analysis of variance (ANOVA) with a *post hoc* Scheffe test (StatView J, Abacus Concepts Inc., Berkeley, CA). The data represent the mean $\pm$ SD calculated from three independent experiments. Differences with a value of $P < 0.05$ were considered significant.

ACKNOWLEDGMENTS

We thank Dr. T. Tsukahara, Dr. M. Kannagi, and Dr. J. Bukh for providing us with plasmids. We are grateful to many colleagues for helpful discussions during the course of this work. This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan.

REFERENCES

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival. *Science* **281,** 1322–1326.

Aoki, H., Hayashi, J., Moriyama, M., Arakawa, Y., and Hino, O. (2000). Hepatitis C virus core protein interacts with 14-3-3 protein and activates the kinase Raf-1. *J. Virol.* **74,** 1736–1741.

Beidler, D. R., Tewari, M., Friesen, P. D., Poirier, G., and Dixit, V. M. (1995). The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* **270,** 16526–16528.

Boucher, M. J., Morisset, J., Vachon, P. H., Reed, J. C., Laine, J., and Rivard, N. (2000). MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. *J. Cell. Biochem.* **79,** 355–369.

Cerny, A., and Chisari, F. V. (1999). Pathogenesis of chronic hepatitis C: Immunological features of hepatic injury and viral persistence. *Hepatology* **30,** 595–601.

Chen, C., Edelstein, L. C., and Gelinas, C. (2000). The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol. Cell. Biol.* **20,** 2687–2695.

Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G., and Dixit, V. M. (1996). Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.* **271,** 4573–4576.

Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coito, D., Medina-Selby, R., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991). Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88,** 2451–2455.

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins—Suppressors of apoptosis. *Genes Dev.* **13,** 239–252.

Erhardt, P., and Cooper, G. M. (1996). Activation of the CPP32 apoptotic protease by distinct signaling pathways with differential sensitivity to Bcl-xL. *J. Biol. Chem.* **271,** 17601–17604.

Frommel, T. O., Yong, S., and Zarling, E. J. (1999). Immunohistochemical evaluation of Bcl-2 gene family expression in liver of hepatitis C and cirrhotic patients: A novel mechanism to explain the high incidence of hepatocarcinoma in cirrhotics. *Am. J. Gastroenterol.* **94,** 178–182.

Fujio, Y., Kunisada, K., Hirota, H., Yamauchi-Takihara, K., and Kishimoto, T. (1997). Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. *J. Clin. Invest.* **99,** 2898–2905.

Goto, T., Kato, N., Ono-Nita, S. K., Yoshida, H., Otsuka, M., Shiratori, Y., and Omata, M. (2001). Large isoform of hepatitis delta antigen activates serum response factor-associated transcription. *J. Biol. Chem.* **275,** 37311–37316.

Grad, J. M., Zeng, X. R., and Boise, L. H. (2000). Regulation of Bcl-xL: A little bit of this and a little bit of STAT. *Curr. Opin. Oncol.* **12,** 543–549.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* **67,** 1385–1395.

Grillot, D. A., Gonzalez-Garcia, M., Ekhterae, D., Duan, L., Inohara, N., Ohta, S., Seldin, M. F., and Nunez, G. (1997). Genomic organization, promoter region analysis, and chromosome localization of the mouse bcl-x gene. *J. Immunol.* **158,** 4750–4757.

Grumont, R. J., Rourke, I. J., and Gerondakis, S. (1999). Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev.* **13,** 400–411.

Hahn, C. S., Cho, Y. G., Kang, B. S., Lester, I. M., and Hahm, Y. S. (2000). The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. *Virology* **276,** 127–137, doi:10.1006/viro.2000.0541.

Hettmann, T., DiDonato, J., Karin, M., and Leiden, J. M. (1999). An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. *J. Exp. Med.* **189,** 145–158.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. USA* **88,** 5547–5551.

Honda, M., Kaneko, S., Shimazaki, T., Matsushita, E., Kobayashi, K., Ping, Lh., Zhang, Hc., and Lemon, S. M. (2000). Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells. *Hepatology* **31,** 1351–1359.

Kato, N., Yoshida, H., Ono-Nita, S. K., Kato, J., Goto, T., Otsuka, M., Lan, K.-H., Matsushima, K., Shiratori, Y., and Omata, M. (2000). Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. *Hepatology* **32,** 405–412.

Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D., and Barr, P. J. (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature* **374,** 736–739.

Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W.-S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M.

(1989). An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **344,** 362–364.

Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., Joulin, V., and Kahn, A. (1996). Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nat. Med.* **2,** 80–86.

Lee, H. H., Dadgostar, H., Cheng, Q., Shu, J., and Cheng, G. (1999). NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc. Natl. Acad. Sci. USA* **96,** 9136–9141.

Machida, K., Tsukiyama-Kohara, K., Seike, E., Tone, S., Shibasaki, F., Shimizu, M., Takahashi, H., Hayashi, Y., Funata, N., Taya, C., Yonekawa, H., and Kohara, M. (2001). Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. *J. Biol. Chem.* **276,** 12140–12146.

Marusawa, H., Hijikata, M., Chiba, T., and Shimotohno, K. (1999). Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. *J. Virol.* **73,** 4713–4720.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* **4,** 1065–1067.

Ray, R. B., Meyer, K., and Ray, R. (1996). Suppression of apoptotic cell death by hepatitis C virus core protein. *Virology* **226,** 176–182, doi:10.1006/viro.1996.0644.

Ray, R. B., Meyer, K., Steele, R., Shrivastava, A., Aggarwal, B. B., and Ray, R. (1998). Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. *J. Biol. Chem.* **273,** 2256–2259.

Rudin, C. M., and Thompson, C. B. (1997). Apoptosis and disease: Regulation and clinical relevance of programmed cell death. *Annu. Rev. Med.* **48,** 267–281.

Ruggieri, A., Harada, T., Matsuura, Y., and Miyamura, T. (1997). Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. *Virology* **229,** 68–76, doi:10.1006/viro.1996.8420.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M., and Kuo, G. (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87,** 6547–6549.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* **17,** 1675–1687.

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63,** 1129–1136.

Sevilla, L., Aperlo, C., Dulic, V., Chambard, J. C., Boutonnet, C., Pasquier, O., Pognonec, P., and Boulukos, K. E. (1999). The Ets2 transcription factor inhibits apoptosis induced by colony-stimulating factor 1 deprivation of macrophages through a Bcl-xL-dependent mechanism. *Mol. Cell. Biol.* **19,** 2624–2634.

Shiratori, Y., Shiina, S., Imamura, M., Kato, N., Kanai, F., Okudaira, T., Teratani, T., Tohgo, G., Toda, N., Ohashi, M., Ogura, K., Niwa, Y., Kawabe, T., and Omata, M. (1995). Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology* **22,** 1027–1033.

Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1996). Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* **13,** 21–29.

Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C., and Lodish, H. F. (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: A direct role for Stat5 in Bcl-X(L) induction. *Cell* **98,** 181–191.

Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Furuichi, K., and Tanaka, K. (1999). IkappaBalpha ubiquitination is catalyzed by an SCF-like complex containing Skp1, cullin-1, and two F-box/WD40-repeat proteins, betaTrCP1 and betaTrCP2. *Biochem. Biophys. Res. Commun.* **256,** 127–132, doi:10.1006/bbrc.1999.0289.

Takano, S., Yokosuka, O., Imazeki, F., Tagawa, M., and Omata, M. (1995). Incidence of hepatocellular carcinoma in chronic hepatitis B and C: A prospective study of 251 patients. *Hepatology* **21,** 650–655.

Takehara, T., Liu, X., Fujimoto, J., Friedman, S. L., and Takahashi, H. (2001). Expression and role of Bcl-xL in human hepatocellular carcinoma. *Hepatology* **34,** 55–61.

Tamatani, M., Che, Y. H., Matsuzaki, H., Ogawa, S., Okado, H., Miyake, S., Mizuno, T., and Tohyama, M. (1999). Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. *J. Biol. Chem.* **274,** 8531–8538.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456–1462.

Tsuchihara, K., Hijikata, M., Fukuda, K., Kuroki, T., Yamamoto, N., and Shimotohno, K. (1999). Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. *Virology* **258,** 100–107, doi:10.1006/viro.1999.9694.

Tsukahara, T., Kannagi, M., Ohashi, T., Kato, H., Arai, M., Nunez, G., Iwanaga, Y., Yamamoto, N., Ohtani, K., Nakamura, M., and Fujii, M. (1999). Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax. *J. Virol.* **73,** 7981–7987.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998). NF-kappaB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281,** 1680–1683.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes Dev.* **10,** 1–15.

World Health Organization (WHO) (1998). Press releases. Available at http://www.who.int/inf-pr-1998/en/pr98–36.html.

Wu, M. X., Ao, Z., Prasad, K. V., Wu, R., and Schlossman, S. F. (1998). IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. *Science* **281,** 998–1001.

Yanagi, M., St. Claire, M., Shapiro, M., Emerson, S. U., Purcell, R. H., and Bukh, J. (1998). Transcripts of a chimeric cDNA clone of hepatitis c virus genotype 1b are infectious *in vivo*. *Virology* **244,** 161–172.

Yoshida, H., Kato, N., Shiratori, Y., Otsuka, M., Maeda, S., Kato, J., and Omata, M. (2001). Hepatitis C virus core protein activates nuclear factor kappa B-dependent signaling through tumor necrosis factor receptor-associated factor. *J. Biol. Chem.* **276,** 16399–16405.

You, L. R., Chen, C. M., and Lee, Y. H. W. (1999). Hepatitis C virus core protein enhances NF-kappaB signal pathway triggering by lymphotoxin-beta receptor ligand and tumor necrosis factor alpha. *J. Virol.* **73,** 1672–1681.

Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. M. (1998). Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J. Virol.* **72,** 3691–3697.

Zhu, N., Ware, C. F., and Lai, M. M. (2001). Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. *Virology* **283,** 178–187, doi:10.1006/viro.2001.0896.