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Hepatitis C virus core protein represses the p21 promoter through inhibition of a TGF-β pathway

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The increased proliferation rate of hepatocytes is one of the major risk factors for the development of hepatocellular carcinoma. In this study, we investigated the mechanism by which hepatitis C virus (HCV) core protein represses transcription of the universal cyclin-dependent kinase inhibitor p21 gene in murine fibroblast NIH 3T3 cells. From the transient reporter assays of p21 promoter, we found that the TGF-β-responsive element (TβRE) located between −83 and −74 of the p21 promoter is responsible for the effect. The TGF-β-induced p21 promoter activity was specifically decreased by HCV core protein and in the presence of the inhibitory Smad7 the repression effect was almost completely abolished. Furthermore, HCV core protein stimulated the growth rate of NIH 3T3 cells and could overcome growth arrest by TGF-β but not by butyrate, suggesting that HCV core protein stimulates cell cycle progression by repressing p21 transcription through a TGF-β pathway.

Introduction

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus that accounts for most cases of post-transfusion non-A, non-B hepatitis (Houghton, 1996). About half of acute hepatitis C cases are followed by chronic hepatitis, and 20% of the patients with chronic hepatitis may develop cirrhosis and hepatocellular carcinoma. The viral genome is 9·5 kb long and encodes a single large polyprotein of about 3000 amino acids, which is cleaved to yield at least 10 structural and nonstructural viral proteins (Houghton, 1996). Among these the viral nucleocapsid, core protein, exhibits pleiotropic features. It transcriptionally regulates, either positively or negatively, several cellular and viral genes, including c-*myc*, c-*fos* and hepatitis B virus (Shih *et al.*, 1995; Ray *et al.*, 1995, 1997). Also, it is reported to repress apoptosis induced by cisplatin, c-Myc, Fas antigen or tumour necrosis factor (TNF) (Ray *et al.*, 1996a; Marusawa *et al.*, 1999). Furthermore, in cooperation with the *ras* oncogene product, Core transforms rodent cells into a tumorigenic phenotype (Ray *et al.*, 1996b) and transgenic mice expressing Core in the liver tend to develop hepatocellular carcinoma (Moriya *et al.*, 1998). These properties suggest that HCV core protein is involved, either directly or indirectly, in HCV-mediated hepatocarcinogenesis.

HCV core protein promotes cell proliferation through several mechanisms, for example by upregulation of the cyclin E expression level (Cho *et al.*, 2001a), modulation of the Rb pathway through pRb downregulation and E2F-1 upregulation (Cho *et al.*, 2001b), or activation of the MAP kinase pathway (Tsuchihara *et al.*, 1999; Hayashi *et al.*, 2000). HCV core protein also represses transcription of the universal cyclin-dependent kinase (CDK) inhibitor p21 gene, as demonstrated by *in vitro* transient expression assays using murine fibroblasts (NIH 3T3), human hepatocellular carcinoma (HepG2) and human cervical carcinoma (HeLa) cells (Ray *et al.*, 1998; Jung *et al.*, 2001; Yoshida *et al.*, 2001). Considering the anti-proliferative function of p21, the effect might play an important role during HCV-mediated hepatocellular carcinogenesis. The effect might result from the inhibition of a major p21 upstream regulator, p53, either by protein–protein interactions or transcriptional repression. Otherwise, HCV core protein may repress the p21

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gene through a p53-independent pathway. In this study we performed a detailed mutational analysis of the p21 promoter to investigate the mechanism by which HCV core protein represses transcription of the p21 gene. Furthermore, we tested whether the repression effect actually stimulates cell cycle progression in Core-expressing cells.

Methods

■ **Plasmid used.** Plasmid pCI-neo-core K, which encodes the core region of the HCV-K isolate (genotype 1b) under control of the human cytomegalovirus immediate-early promoter, was described previously (Chang *et al.*, 1998). p21P, p21PΔp53 and other mutant luciferase constructs of the $p21^{waf1}$ promoter were described by Datto *et al.* (1995a). pGL2 T+I 4 × TβRE, which contains four copies of the TGF-β responsive element in pGL2-basic (Promega), was described previously (Datto *et al.*, 1995a).

■ **Transfection and luciferase assay.** NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Cells were seeded at 2×10^5 cells per 60 mm diameter plate and transfected the next day with a calcium phosphate–DNA precipitate containing 3 μg each of target and effector plasmid DNAs as previously described (Gorman *et al.*, 1982). To control for variation in transfection efficiency, 2 μg of plasmid pCH110 (Pharmacia) containing the *E. coli lacZ* gene under control of the SV40 promoter was cotransfected. After 48 h, the level of expression from the target gene (luciferase activity) was analysed and values obtained were normalized to the β-galactosidase activity measured in the corresponding cell extracts. Each experiment was repeated at least three times.

■ **Semi-quantitative RT–PCR and Western blotting analysis.** Total RNA was prepared from cells 48 h after transfection by the guanidinium isothiocyanate procedure (Chomczynski & Sacchi, 1987). For RT–PCR, 3 μg of RNA was reverse transcribed with the corresponding antisense primer. One-quarter of the reverse transcribed RNA was amplified with *Taq* polymerase (95 °C, 5 min; 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 30 s; final elongation step 72 °C, 5 min) using the appropriate primers. The primer pairs for p21 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were described previously (Ahn *et al.*, 2001). For the detection of HCV core transcripts, sense primer 5′ TCC GGA TCC CTG TCA TCT TCT GTC CCT 3′ and antisense primer 5′ TCG CTT AGT GGA TCC TGG GGG CAG 3′ were used.

For Western blotting analysis, cells were lysed in buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0·1% SDS, 1% NP-40) supplemented with protease inhibitors. Ten μg of cell extracts was separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Western blotting was performed with either anti-p53 monoclonal antibody, anti-p21 rabbit polyclonal IgG or anti-actin monoclonal IgG (all from Santa Cruz), and subsequently detected by chemiluminescent ECL (Amersham) as recommended by the manufacturer.

■ **Generation of stable cell lines and determination of cell growth rate.** NIH 3T3 cells transfected with HCV Core-expressing plasmid were selected and amplified to obtain stable cell lines as described previously (Kwun *et al.*, 2001). The expression level of transfected genes was checked by either RT–PCR or Western blotting analysis. For the determination of cell growth rate, 5×10^4 cells were plated in six-well plates (Nunc) and the total number of cells in each well was counted after incubation under the appropriate conditions.

Results and Discussion

Determination of the Core-responsive region in the p21 promoter

Initially, we tried to determine the regions of the p21 promoter responsible for the effect of HCV core protein. To this end, a series of progressive 5′ promoter deletion mutants of the p21 promoter were tested in NIH 3T3 cells (Fig. 1a). The full-length promoter construct, p21P, was repressed approximately 6-fold by co-expression of HCV core protein. Stepwise removal of the p53 binding sites from the p21 promoter hardly reduced the effect, as demonstrated with p21PΔp53 and p21PΔ1.9, suggesting that repression is not related to binding of p53 to the p21 promoter. Consistent with the previous report by Ray *et al.* (1998), deletion of the sequence between −460 and −113 reduced the effect approximately 2-fold, suggesting the presence of a Core-responsive element within this region. However, this region might be not essential for the effect of Core because the promoter in p21P 93-S, which contains 93 bp proximal to the transcriptional initiation site, was repressed 4·2-fold by Core. Moreover, internal deletion of the sequence between −93 and −61 in p21P SmaΔ2 almost completely abolished the effect. Therefore, we initially determined that the Core-responsive region lay between nucleotide positions −93 and −61.

Repression of the p21 promoter by HCV Core through a TGF-β pathway

To more precisely define the Core-responsive region in the p21 promoter, five mutant constructs (93-S mut#1 to 93-S mut#5) were tested, each containing 10 consecutive mutated bases between −93 and −44 in the promoter construct 93-S (Fig. 1b). The promoter in 93-S mut#1 was repressed by Core in a similar manner to that of the 93-S. However, the effect was significantly decreased in other mutant constructs and even completely lost in the case of 93-S mut#2. The Sp1 site can be considered a candidate for the effect because each of these mutant constructs contains at least one mutated Sp1 site. However, this cannot explain why only the mutation of Sp1-1 in p21P 93-S mut#2 completely abolished the Core-responsiveness of the promoter. Furthermore, a luciferase construct which contains two copies of Sp1 binding site in an heterologous promoter was not responsive to Core (data not shown), suggesting that the Sp1 binding itself is not responsible for the effect.

More direct evidence that the Sp1 binding site is not responsible for the loss of Core responsiveness was obtained with 93-S mut#2.3. This construct contains a mutation of bases −76 and −77 from CT to GG, and thus maintains the consensus Sp1 binding site, but showed a significantly reduced ability to be activated by TGF-β (Datto *et al.*, 1995a). Actually, the Core-responsive element in the p21 promoter defined above exactly overlapped the TGF-β responsive element

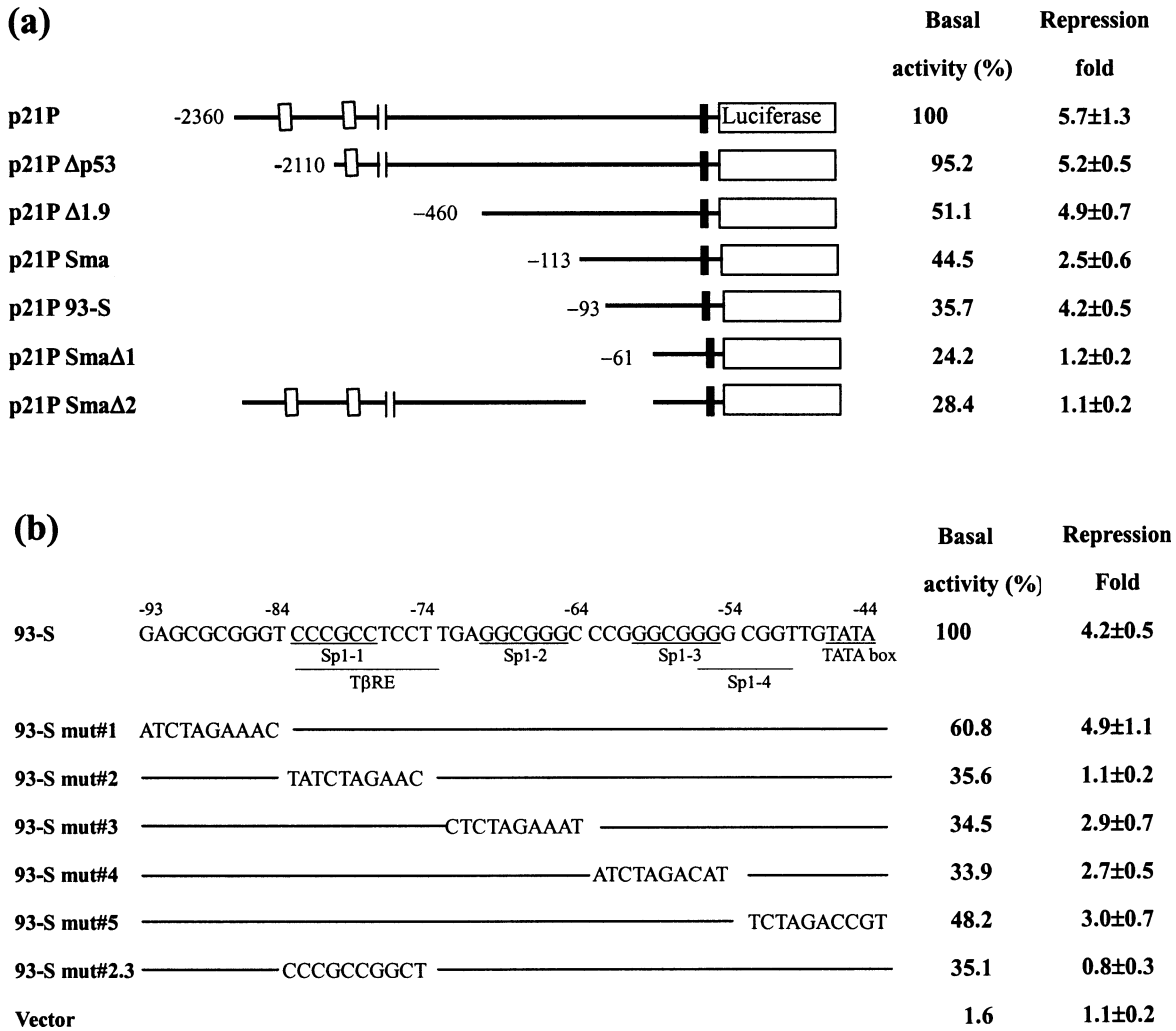

Fig. 1. Identification of the Core-responsive element in the p21 promoter. (a) Full-length and deletion p21 promoter constructs (Datto *et al*., 1995a) were cotransfected with either an empty vector or Core-expressing plasmid into NIH 3T3 cells. The basal luciferase activity of the full-length p21 promoter (p21P) was designated as 100%. Repression (-fold) was calculated by comparing the luciferase activity of Core-expressing cells with the basal activity of the control. The p53 binding sites located at −2285 and −1394 are indicated by open boxes. (b) 93-S mutant constructs (Datto *et al*., 1995a), identical to the wild-type p21P 93-S sequence with the exception of the sequences shown, were cotransfected with an effector plasmid into NIH 3T3 cells. The positions of transcription factor binding sites (Datto *et al*., 1995a) are underlined.

(TβRE), which is known to mediate transcriptional activation of the p21 gene by TGF-β (Datto *et al*., 1995a). The promoter activity in 93-S mut#2.3 was not repressed by Core at all (Fig. 1b), suggesting that destruction of the TβRE in both 93-S mut#2 and 93-S mut#2.3 is responsible for the loss of Core responsiveness. Moreover, pGL2 T+I 4 × TβRE, which contains four copies of the TGF-β responsive element inserted 5′ of the TATA box (Datto *et al*., 1995a), was responsive to core protein in a dose-dependent manner (Fig. 2a), suggesting that the TβRE is sufficient for the effect of Core. Therefore, from the above studies we concluded that the TβRE between −83 and −74 of the p21 promoter is the Core-responsive element. The decrease in repression observed with other 93-S mutants was probably due to their low basal activities resulting from the loss of Sp1 sites.

To provide further evidence that Core represses the p21 promoter through a TGF-β pathway, we tested whether Core specifically represses the TGF-β signalling pathway. In the presence of TGF-β, the promoter activity of pGL2 T+I 4 × TβRE was significantly induced (Fig. 2a). The elevated luciferase activity of the p21 promoter by brought about by TGF-β was specifically reduced by core protein in a dose-dependent manner. Furthermore, in the presence of Smad7, which is a known inhibitor of the TGF-β pathway and thus inhibits p21 induction by TGF-β (Pardali *et al*., 2000), the repression effect was almost completely abolished (Fig. 2b). These results indicate that HCV core protein inhibits a TGF-β pathway.

The Core-responsive region defined in this study also exactly overlaps the main butyrate responsive element be-

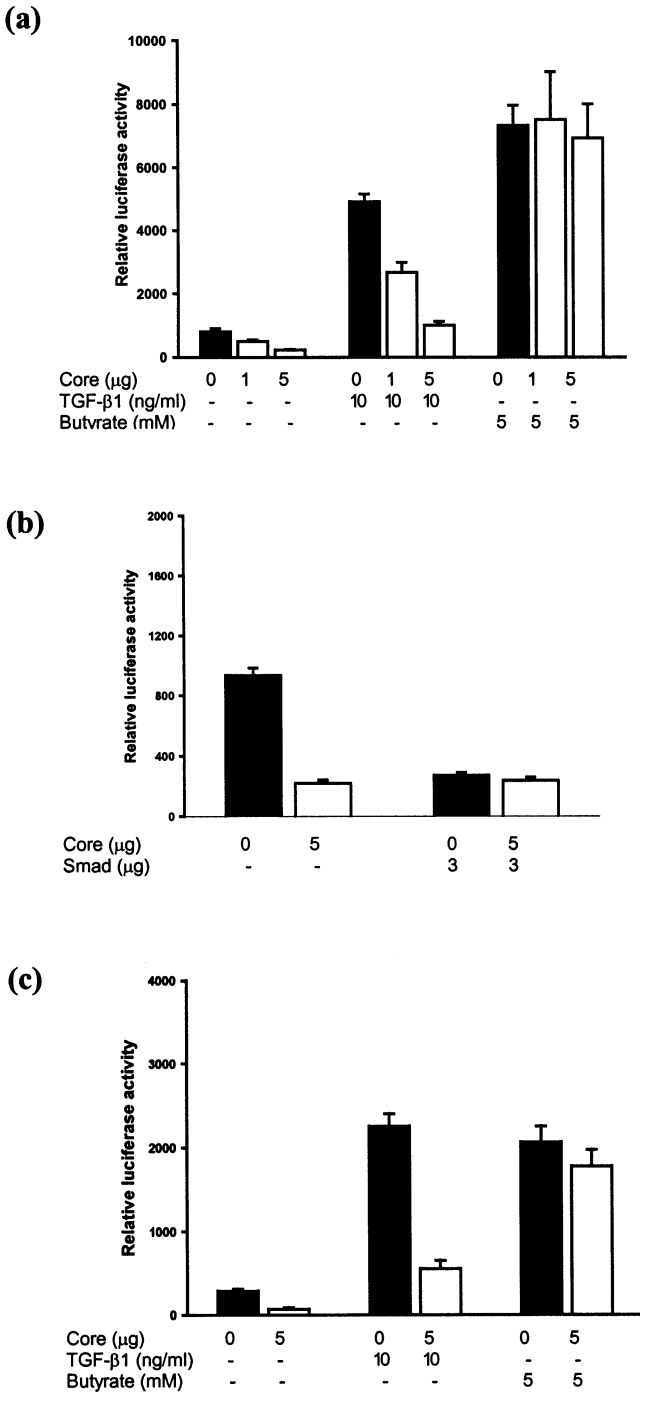

Fig. 2. Repression of p21 promoter by Core through a TGF-β pathway (a) NIH 3T3 cells were cotransfected with pGL2 T+I 4 × TβRE (Datto *et al*., 1995a) and increasing amounts of Core expression plasmid for 6 h and then treated with either TGF-β1 or butyrate at the indicated concentration for 48 h. (b) pGL2 T+I 4 × TβRE was cotransfected with Smad7-expressing plasmid (Hanyu *et al*., 2001) into NIH 3T3 cells in the presence or absence of Core. (c) p21P was cotransfected with an effector plasmid into NIH 3T3 cells as described in (a) and luciferase activity was assayed.

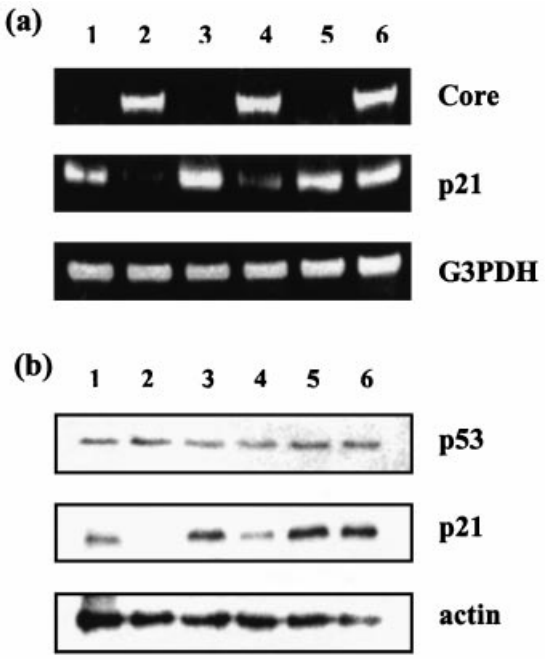

Fig. 3. Effect of Core on expression of endogenous p21. NIH 3T3 cells were transfected with either an empty vector (lanes 1, 3 and 5) or Core-expression vector (lanes 2, 4 and 6) and then treated with either 10 ng/ml of TGF-β1 (lanes 3 and 4) or 5 mM butyrate (lanes 5 and 6) as described in Fig. 2. (a) RT–PCR analysis of p21, Core and G3PDH transcripts was performed as described previously (Ahn *et al*., 2001). (b) The level (protein) of p53, p21 and actin was measured by Western blotting.

tween −87 and −72, which mediates the effect of butyrate on the p21 promoter (Nakano *et al*., 1997). Butyrate arrests cell growth by activating p21 promoter through specific Sp1 sites in a p53-independent fashion. In contrast to the effect of core protein on the TGF-β pathway, Core does not repress the effect of butyrate (to induce p21 transcription) at all (Fig. 2a, c), suggesting that core protein does not act through a butyrate pathway. This result is consistent with the mutational analysis of p21 promoter because the Sp1 sites on the p21 promoter were not related to the effect of core protein.

Next, we tried to confirm that inhibition of the TGF-β pathway by core protein, as demonstrated with the TβRE-luciferase construct, is responsible for the repression of p21 promoter by core protein. As expected, in the presence of either TGF-β or butyrate, the core protein inhibited only the effect of TGF-β on the p21 promoter (Fig. 2c). We therefore concluded that Core represses p21 transcription through inhibition of a TGF-β pathway.

Repression of endogenous p21 gene expression by HCV Core

To elucidate whether expression of the endogenous p21 gene is actually repressed by HCV core protein at the transcriptional level, we measured the level of endogenous p21 RNA. According to a semi-quantitative RT–PCR analysis, p21 was repressed by expression of the core protein (Fig. 3a, lanes

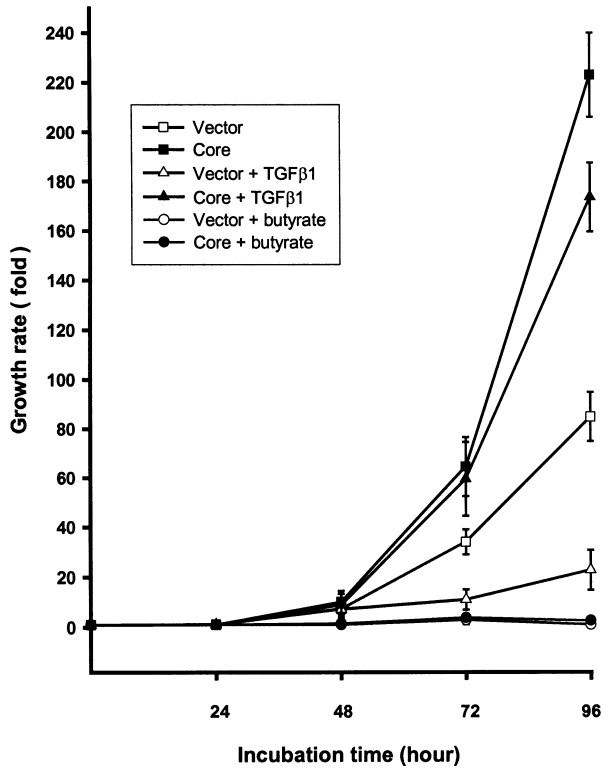

Fig. 4. Stimulation of cell growth rate by HCV core protein. The growth rate of either NIH 3T3 cells or Core-expressing stable cells in the presence of either 10 ng/ml of TGF-β1 or 5 mM butyrate for the indicated period was measured.

1 and 2). The endogenous p21 protein level was also decreased by HCV Core (Fig. 3b, lanes 1 and 2). In addition, the core protein could clearly repress the expression of endogenous p21 gene elevated by TGF-β (Fig. 3, lanes 3 and 4) but not by butyrate (lanes 5 and 6), which is consistent with the results of reporter assay. The expression level of p53 was not significantly affected by core protein under the conditions used in this study.

Connection between p21 repression and stimulation of cell growth

Because the tumour repressor p21 protein is an universal inhibitor of cyclin-CDK complexes and DNA replication that induces cell cycle arrest at the G_1–S checkpoint, the repression of p21 by HCV core protein may result in growth stimulation of the cells. To test this possibility, we prepared several NIH 3T3 cell lines which stably expressed Core and measured their growth rates. Five different cell lines with each plasmid were selected and tested to show that differences in their growth rates are not just due to the chance selection of cell clones that grow at different rates. As expected, the growth rate of Core-expressing cell lines was approximately twice as fast as that of the parent NIH 3T3 cells (Fig. 4). The stimulation of cell growth by Core was maintained in the presence of TGF-β, but was abolished by butyrate. Considering the effect of core protein on the expression of p21 in the presence of either TGF-β or butyrate in transient transfection experiments (Fig. 2c and Fig. 3), it seems likely that the repression of p21 by the core protein through a TGF-β pathway is properly reflected by the stimulation of cell growth. TGF-β is known to inhibit cell proliferation by repressing expression of the proto-oncogene c-*myc* and by inhibiting the activity of cyclin CDKs which leads to arrest of the cell cycle at an early G_1 phase (Alexandrow & Moses, 1995; Reynisdottir *et al.*, 1995). In particular, the transcriptional induction of the gene for the universal CDK inhibitor p21 is considered an important mechanism to execute the anti-proliferative action of TGF-β (Hu *et al.*, 1998). Therefore, it is possible to postulate that HCV core protein stimulates cell growth rate by blocking the stimulatory effect of TGF-β on p21 transcription, as demonstrated in this study.

Recently, however, Yoshida *et al.* (2001) reported that HCV core protein inhibited p21 expression post-transcriptionally, but not at the transcription level. Furthermore, entirely opposite results – that core protein upregulates expression of p21 by enhancing p53 function and prolongs the G_1 to S transition – have been reported (Otsuka *et al.*, 2000; Lu *et al.*, 1999). We do not understand how cells respond oppositely to the same protein for expression of this important cell cycle regulator. One possibility is the differences in the core proteins used. Because HCV can induce either hepatitis or HCC, and it takes a long period to induce HCC after the initial infection of hepatic cells, it could be speculated that the potential of Core to regulate p21 expression changes during the course of viral infection. Therefore, it might be interesting to test whether the p21-regulatory activity of Core derived from patients with either hepatitis or HCC differs.

This study may provide a clue to elucidate the transcription mechanism of p21. The p21 gene is regulated by a rapidly growing list of physiological and pathological factors. In particular, the region between −83 and −74 in the p21 promoter has been shown to be required for induction of p21 by several factors such as TGF-β (Datto *et al.*, 1995a), Ca^{2+} (Prowse *et al.*, 1997), butyrate (Nakano *et al.*, 1997) and lovastatin (Lee *et al.*, 1998). Among these, TGF-β and butyrate inhibit cell proliferation and induce G_1 cell cycle arrest in various cell types (Alexandrow & Moses, 1995; Datto *et al.*, 1995b; Nakano *et al.*, 1997; Reynisdottir *et al.*, 1995). Sp1 and Sp3 bind to this region in TGF-β- and butyrate-treated cells (Li *et al.*, 1998; Matsukawa *et al.*, 1997; Moustakas & Kardassis, 1998), suggesting that stabilization of Sp1 binding mediates induction of the p21 promoter by TGF-β and butyrate. However, it is still questionable because the pattern of Sp1 binding in TGF-β- and butyrate-treated cells was not much different from that in untreated cells (Pardali *et al.*, 2000; Nakano *et al.*, 1997). Furthermore, the two pathways showed different responses to HCV core protein for expression of p21 gene, as demonstrated in this study. This study may provide a

good model system to study the TGF-β pathway because it specifically responds to Core protein. Another important point to be elucidated is how Core protein inhibits the TGF-β pathway. Core protein may inhibit the function of intracellular effectors of TGF-β such as Smad3 and Smad4, either directly or indirectly by protein–protein interactions, or may augment the activity of inhibitory signalling molecules such as Smad6 and Smad7. To provide an answer to this question, more detailed studies on the mechanism by which Core regulates the TGF-β pathway should be carried out.

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