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p53-dependent transcriptional repression of p21^{waf1} by hepatitis C virus NS3

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Hepatitis C virus (HCV) NS3 protein is known to affect normal cellular functions, such as cell proliferation and cell death, and to be involved, either directly or indirectly, in HCV hepatocarcinogenesis. In this study, we demonstrated that NS3 protein could specifically repress the promoter activity of p21 in a dose-dependent manner. The effect was not cell type-specific and was synergistic when combined with HCV core protein. Repression of the p21 promoter by NS3 was almost completely lost when p53 binding sites present on the p21 promoter were removed. Furthermore, p53 binding sites were sufficient to confer a strong NS3 responsiveness to an heterologous promoter, suggesting that NS3 represses the transcription of p21 by modulating the activity of p53. Although the NS3 protein domain required for the majority of p21 repression was located on the protease domain, the proteinase activity itself does not seem to be necessary for repression. Both transcription and protein stability of p53 were unaffected by NS3, suggesting that NS3 might repress transcription of p21 by inhibiting the regulatory activity of p53 via protein–protein interaction(s). Finally, the growth rate of NS3-expressing cell lines was at least twice as fast as that of the parent NIH 3T3 cells, indicating that the repression of p21 is actually reflected by the stimulation of cell growth.

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 nonstructural protein NS3 is a multifunctional protein that is indispensable for virus replication. It has not only proteinase but also helicase activity, including ATPase activity (Suzich *et al.*, 1993), and the antibody titre against NS3 correlates with the severity of liver disease (Takegami & Hasumura, 1993). Also, NS3 has been reported to repress apoptosis induced by actinomycin D (Fujita *et al.*, 1996). Furthermore, Sakamuro *et al.* (1995) reported that NIH3T3 mouse fibroblasts become transformed after transfection with NS3 and are tumorigenic in nude mice. These properties suggest that NS3 affects normal cellular functions, such as cell proliferation and cell death, and is involved, either directly or indirectly, in HCV hepatocarcinogenesis.

Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (CDKs), which are subject to regulation by positive (cyclins) and negative (CDK-inhibitory proteins) effectors (Morgan, 1995). One such negative effector is the universal CDK inhibitor p21 (Xiong *et al.*, 1993). While p21 can be transcriptionally regulated by the p53 tumour repressor protein (El-Deiry *et al.*, 1993), and is thus believed to participate in the execution of p53 effects, its induction can also be activated via p53-independent pathways (Michiell *et al.*, 1994; Liu *et al.*, 1996; Zeng & El-Deiry, 1996). Through binding to cyclin-CDK complexes, p21 prevents CDK-dependent phosphorylation, and subsequent inactivation, of the retinoblastoma protein (RB), which negatively regulates cell cycle progression. p21 also interacts with proliferating cell nuclear antigen (PCNA) to inhibit PCNA-dependent DNA replication (Li *et al.*, 1994). Thus, p21 exerts antiproliferation activity through inhibition of cyclin-CDK complexes and/or PCNA functions (Luo *et al.*, 1996; Cayrol *et al.*, 1998).

Recently, Ishido & Hotta (1998) reported that the HCV

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NS3 could form a complex with wild-type p53, both in the absence and the presence of NS4a viral cofactor. Therefore, the initial aim of this study was to determine whether the complex formation between p53 and NS3 affects the *trans*-regulatory activity of p53 on the transcription of p21. Next, we tried to determine the region of the NS3 protein responsible for the transcriptional repression of the p21 promoter. For this purpose, we generated several mutants in which amino acid residues of NS3 were either deleted or changed into other residues and tested their activity on repression of p21 transcription. Finally, we investigated whether repression of p21 transcription by NS3 is connected to cell growth stimulation.

Methods

■ **Plasmid construction.** Plasmid pCI-neo-NS3 encodes the NS3 protein (aa 1007–1615) of the HCV-K isolate [genotype 1b (Cho *et al.*, 1993)] under control of the strong human cytomegalovirus immediate-early promoter. Plasmids pCI-neo-$NS3_{1027–1260}$ and -$NS3_{1027–1206}$ were constructed by deleting the region encoding the helicase/NTPase domain located at the 3′-terminal half of the gene, thus encoding truncated forms of the protein, a serine protease domain and a protease minimal domain of NS3 protein, respectively. For construction of pCI-neo-$NS3_{1027–1206}$ R1135G, in which Arg-1135 was replaced with a Gly residue, a point mutation was introduced into the coding sequence of $NS3_{1027–1206}$ by PCR-directed mutagenesis. A series of human p21 promoter CAT constructs and a p21-luciferase construct, WWW-luc, which contains a 2·4 kbp genomic fragment of the human p21 promoter, were described previously (El-Deiry *et al.*, 1993, 1995) and were kindly provided by Bert Vogelstein. pG13-luc was generated by inserting 13 copies of the p53 binding sequences into the pT81-luc reporter plasmid, which contains a basic promoter element (TATA box) (Kern *et al.*, 1992).

■ **Transfection and luciferase assay.** NIH3T3 and other cell lines used in this study 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 target and effector plasmid DNAs as previously described (Gorman, 1986). 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 was analysed by either CAT assay (Gorman, 1986) or luciferase assay (Otsuka *et al.*, 2000), depending on the reporter construct used, and values obtained were normalized to the β-galactosidase activity measured in the corresponding cell extracts. A quantitative estimate of chloramphenicol acetylation was obtained by excision of the substrate and products from thin-layer chromatography plates, and subsequent measurement by liquid scintillation counting in an LKB 216 scintillation counter. Each experiment was repeated at least twice, and although there were variations in the absolute reporter activity from one experiment to the next the profile of the response was always consistent.

■ **Generation of stable cell lines.** NIH3T3 cells (2×10^5 cells per 60 mm diameter plate) were transfected with 10 μg of plasmid DNA as described above. After 48 h, the transfected cultures were split in a ratio of 1:5, and were selected with 400 μg/ml of G418 sulfate (Gibco BRL) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum (FCS). After incubating for 2–3 weeks, colonies were selected and amplified to obtain cell lines. The expression level of transfected genes was checked by Western blotting analysis.

■ **Semi-quantitative RT-PCR and Western blotting analysis.** For RT-PCR, total cellular RNA was extracted from NIH 3T3 cell lines using Trizol (Gibco BRL). 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, 1 min – 56 °C, 1 min – 72 °C, 30 s; 72 °C, 5 min) using 5′ ATG GGA TCC CCG GAC GAT ATT GAA 3′ as sense primer and 5′ TCG CTT AGT GGA TCC TGG GGG CAG 3′ as antisense primer to compare the endogenous p53 RNA level. To determine the endogenous level of p21 RNA 5′ GGA TGG CTT CGA CAC CAT TC 3′ was used as sense primer and 5′ GAC AGC TTC TCT TCT GAC AGA 3′ as antisense primer. All PCR products were separated by 1·2% agarose gel electrophoresis, stained with 0·5 mg/ml ethidium bromide and photographed on a UV transilluminator. For Western blotting analysis, total cell lysates were prepared from NIH 3T3 cells and 10 μg of protein was separated by SDS-PAGE. The protein concentration of the extracts was measured using the BSA protein assay kit (Bio-Rad). The samples were loaded on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond PVDF; Amersham) by semi-dry electroblotting for 1 h at 15 V. The membranes were incubated in PBS with 0·1% (v/v) Tween 20 and 5% skimmed milk for 1 h at room temperature. p53, p21 and actin were detected using p53 monoclonal antibody (Santa Cruz Biotechnology), p21 rabbit polyclonal IgG (Santa Cruz) and actin mouse monoclonal IgG (Santa Cruz), respectively. The primary antibodies were detected by using the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham). Immunoblots were developed by addition of ECL-plus reagent (Amersham) according to the instructions given by the manufacturer and exposed to Hyperfilm ECL (Amersham).

Results and Discussion

Repression of p21 promoter by HCV NS3 protein

To examine whether HCV NS3 protein represses transcription of p21, we investigated the effect of NS3 protein on the p21 promoter by using the p21A-CAT reporter, which contains the CAT gene under the control of the p21 promoter (El-Deiry *et al.*, 1995; see Fig. 2*a*). Initially, p21A-CAT and a full-length NS3-expressing construct were cotransfected into NIH 3T3 cells and CAT assay was assayed. The vector alone was also used as a negative control in the cotransfection assay. Results from this experiment suggested that HCV NS3 protein could significantly repress the promoter activity of p21 whereas it did not show a clear effect on transcription from CMV promoter (Fig. 1*a*). The repressive effect of HCV NS3 protein was dose-dependent (Fig. 1*b*) and was not cell type-specific (Fig. 1*c*).

As HCV core protein is also known to repress transcription of p21 (Ray *et al.*, 1998), we examined whether the combination of HCV core and NS3 results in a synergistic effect on the p21 promoter. As shown in Fig. 1(*d*), the CAT activity obtained from p21A CAT when both core and NS3 were expressed in combination was synergistically decreased, suggesting that the p21 promoter might be repressed by these two gene products via different pathways. The synergistic effect of core and NS3 on the transcription of p21 might play an important role in

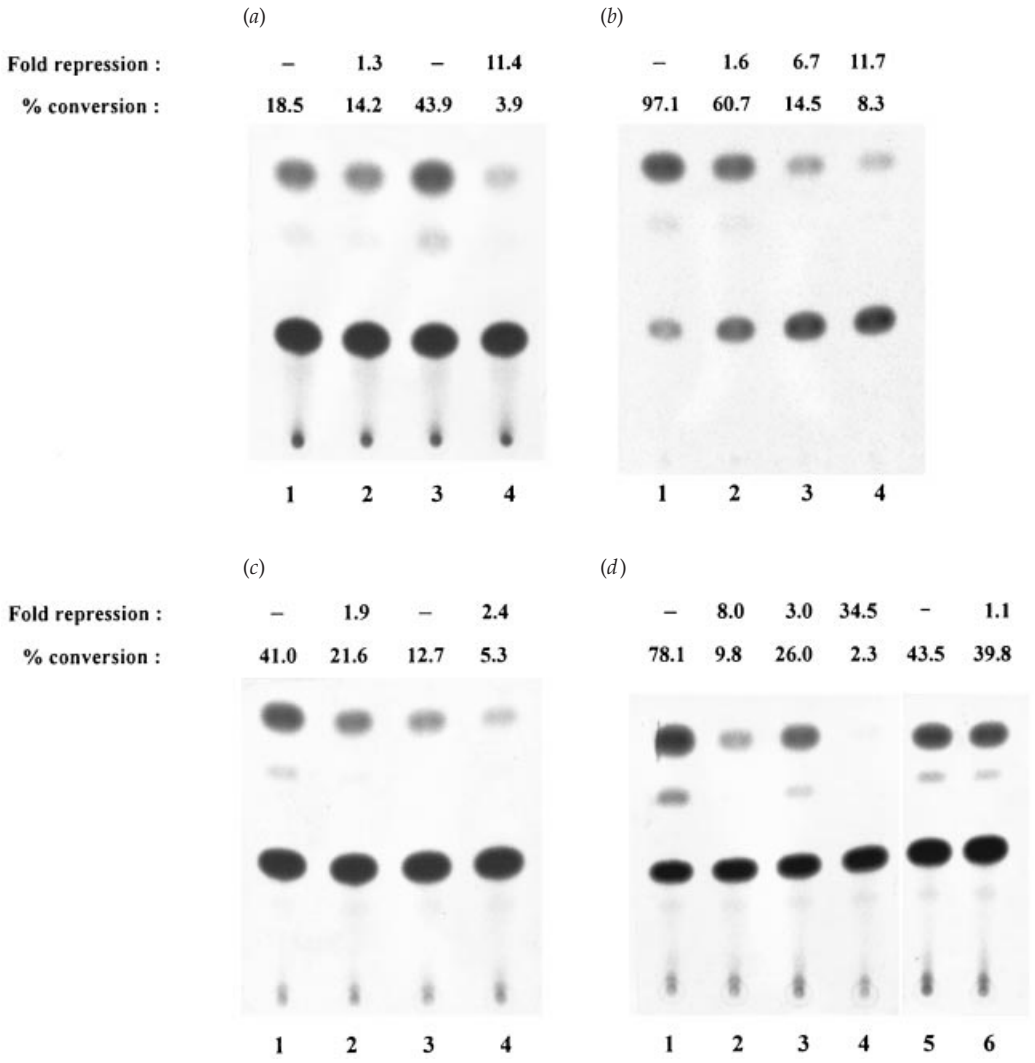

Fig. 1. Repression of p21 promoter by HCV NS3 protein. (*a*) Specific inhibition of p21 promoter by NS3 protein. Five μg of CMV-CAT (tracks 1 and 2) or p21A CAT construct (tracks 3 and 4) was cotransfected into NIH 3T3 cells with either an empty vector, pCI-neo (tracks 1 and 3) or the NS3-expressing plasmid pCIN-NS3$_{1007–1615}$ (tracks 2 and 4). CAT activity obtained from each transfectant was normalized to the *β*-galactosidase activity and indicated as -fold repression compared to that of the control. (*b*) Dose-dependent repression of p21 promoter activity by NS3 protein. NIH 3T3 cells were cotransfected with 3 μg of p21A CAT and 0 (track 1), 1 (track 2), 3 (track 3) or 5 μg (track 4) of NS3 expression plasmid and CAT activity was performed. (*c*) Cell-type specificity of p21 repression by NS3 protein. Three μg of p21A CAT was cotransfected with 5 μg each of an empty vector (tracks 1 and 3) or NS3-expressing construct (tracks 2 and 4) into either HepG2 (tracks 1 and 2) or HeLa cells (tracks 3 and 4). (*d*) Synergistic repression of p21 promoter by HCV core and NS3. Three μg of p21A CAT plasmid was cotransfected with 3 μg each of an empty vector (track 1), core- (track 2) and NS3- (track 3) expressing plasmids into NIH 3T3 cells. For tracks 4, 1·5 μg each of both core- and NS3-expressing plasmids were included. As a control, 3 μg of CMV-CAT plasmid was cotransfected with either an empty vector (track 5) or combination of core- and NS3- (track 6) expressing plasmids.

regulation of cell growth or other related events, because they are inevitably co-expressed during the natural course of virus infection.

p53-dependent repression of p21 by NS3 protein

Next, we tried to determine the regions of the p21 promoter responsible for the transcriptional repression by HCV NS3. The p53 binding sites present on the p21 promoter might be the most plausible candidate because binding of p53 onto one of these sites is very important for the induction of p21 transcription (Macleod *et al.*, 1995). To examine this possibility, a series of progressive 5′ promoter deletion mutants of the p21 promoter were tested (El-Deiry *et al.*, 1995; Fig. 2*a*). These constructs were transfected into NIH 3T3 cells in the presence or absence of the NS3-expressing construct and CAT activity was measured. CAT activity from p21A CAT, which contains a full-length p21 promoter with

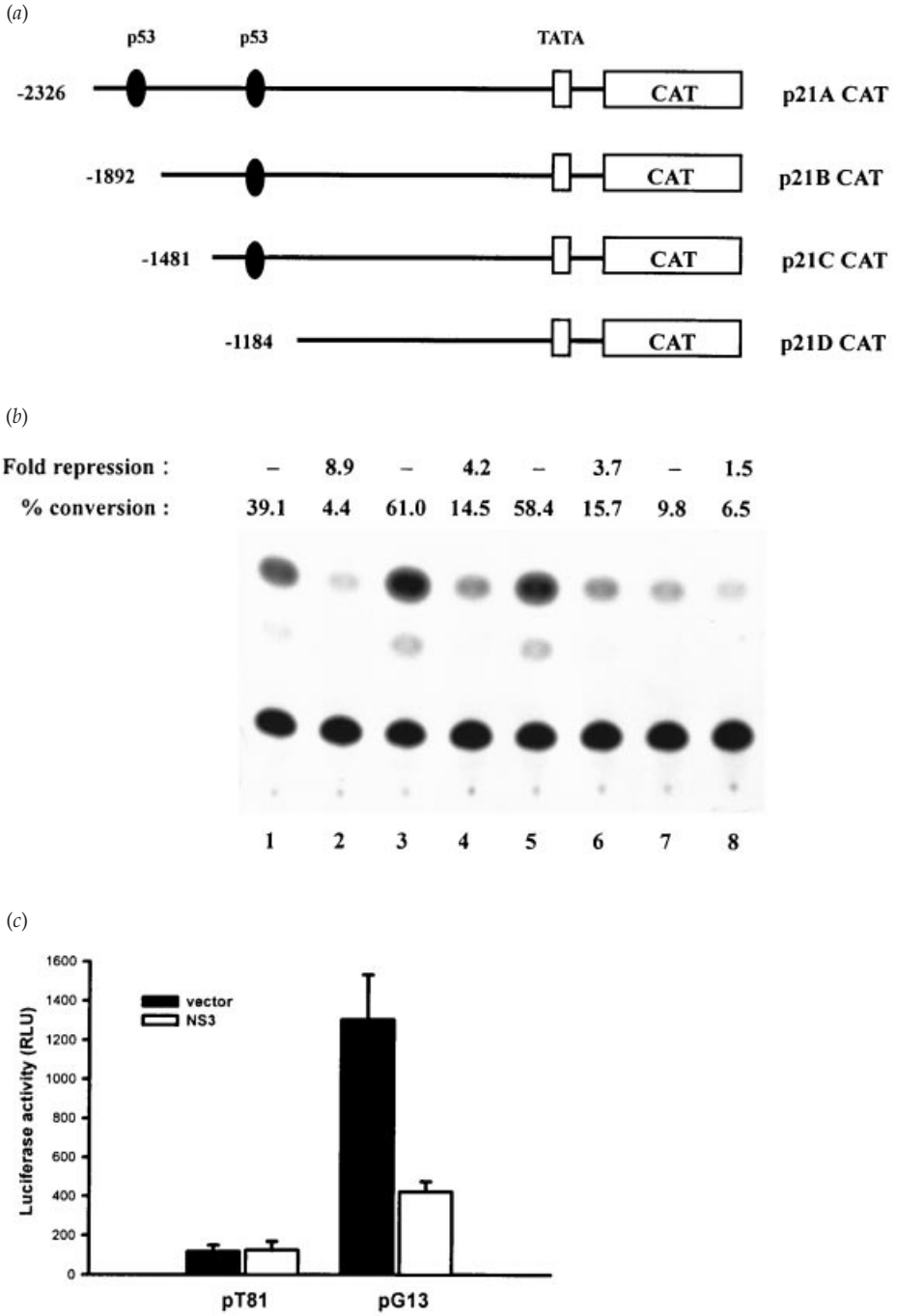

Fig. 2. p53-dependent repression of p21 by NS3 protein. (*a*) Schematic diagram of the various p21 promoter CAT constructs used in this study. The two p53 consensus binding sites located at the 5′-end of the full-length p21 promoter are indicated. (*b*) Full-length and truncated p21 promoter constructs were cotransfected into NIH 3T3 cells with either an empty vector (tracks 1, 3, 5 and 7) or NS3-expressing plasmid (tracks 2, 4, 6 and 8). p21 CAT constructs used were as follows: tracks 1 and 2, p21A CAT; tracks 3 and 4, p21B CAT; tracks 5 and 6, p21C CAT; tracks 7 and 8, p21D CAT. (*c*) Either pT81-luc reporter plasmid, which contains a basic promoter element (TATA box), or pG13-luc (Kern *et al*., 1992), which contains 13 copies of the p53 binding site in pT81-luc, were cotransfected into NIH 3T3 cells with either an empty vector or NS3-expressing plasmid. Luciferase activity obtained from each transfectant was normalized to the *β*-galactosidase activity.

two p53-binding sites, was repressed approximately 9-fold by NS3. Although removal of a p53 binding site reduced the effect of NS3, the repression from p21C CAT was still high, up to 3·7-fold. However, the repressive activity of NS3 was almost completely lost on p21D CAT, which does not contain any p53 binding site. Therefore, the regions of the p21

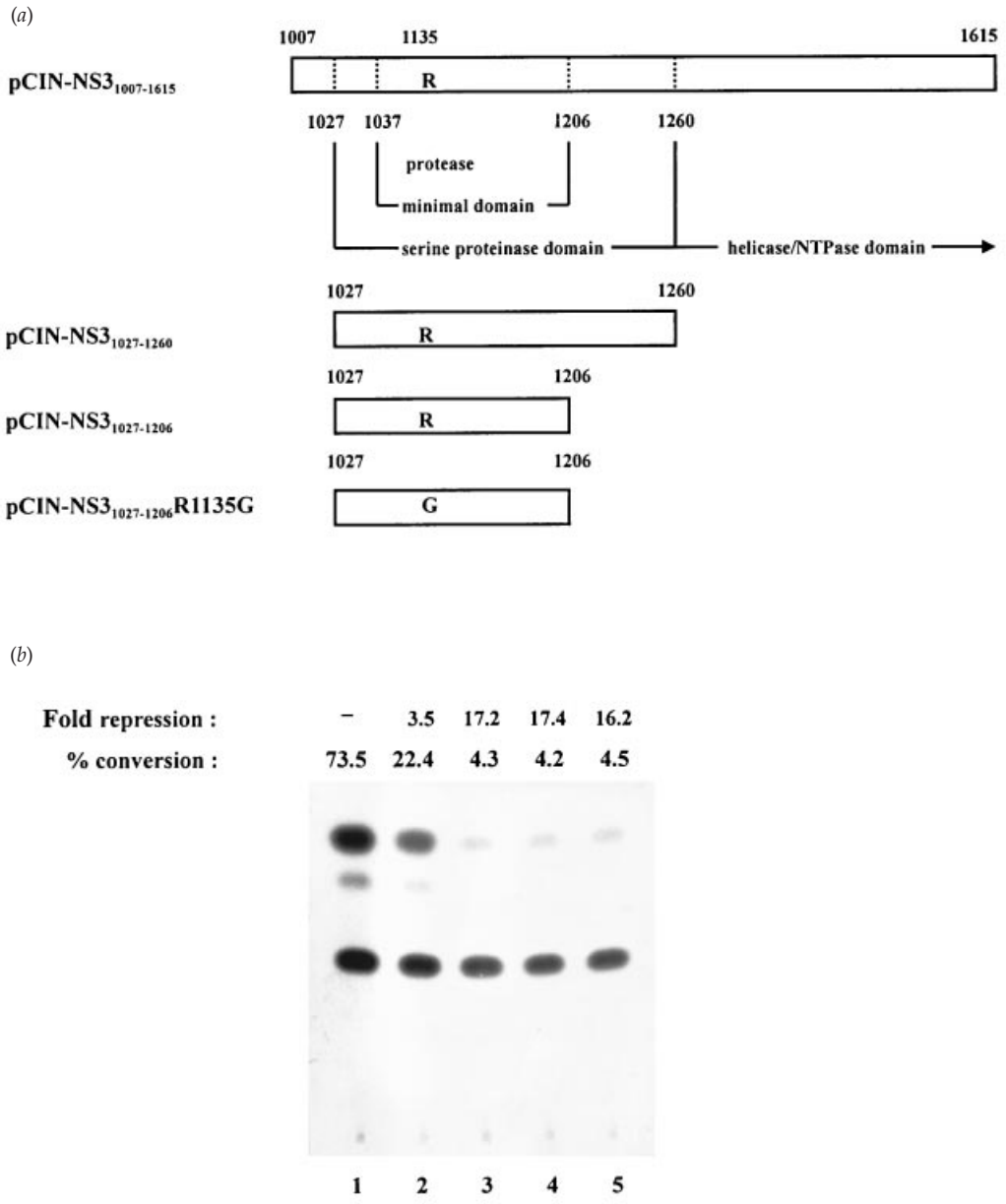

Fig. 3. NS3 domains responsible for the repression of p21. (*a*) Schematic diagram of the various NS3 constructs used in this study. The Arg residue at 1135 was site-specifically replaced with a Gly residue in pCIN-NS3$_{1027–1206}$ R1135G. (*b*) p21A CAT construct was cotransfected with either an empty vector (track 1) or NS3-expressing plasmid (track 2, pCIN- NS3$_{1007–1615}$; track 3, pCIN-NS3$_{1207–1260}$; track 4, pCIN-NS3$_{1027–1206}$; track 5, pCIN-NS3$_{1027–1206}$ R1135G) into NIH 3T3 cells and CAT activity was determined.

promoter responsible for repression by NS3 were mapped to two segments ranged from −2326 to −1892 and from −1481 to −1184, respectively.

p53 binding sites present on the p21 promoter might play a critical role in the effect because successive removal of p53 binding site-containing segments resulted in a stepwise reduction in the NS3 responsiveness. Therefore, we could suggest that repression of the p21 promoter by NS3 is mediated by modification of p53 activity. To demonstrate that the p53 binding sites present on the p21 promoter are involved in the action of NS3, p53 binding sites were used in an attempt to confer NS3 responsiveness to an heterologous promoter. A luciferase construct (pT81-luc) containing a basic promoter element (TATA box) was not responsive to NS3 (Fig. 2*c*). However, as we expected, 13 copies of the p53 binding site in pG13 (Kern *et al.*, 1992) were sufficient to confer a strong NS3 responsiveness. Furthermore, when the p21A CAT construct was tested on Hep 3B cells, in which the p53 gene has been reported to be truncated and p53 protein absent, CAT activity from p21A CAT was not affected by co-

expression of NS3 (data not shown). Therefore, we concluded that NS3 represses the transcription of p21 by modulating the activity of p53.

NS3 domains responsible for repression of p21

To determine the region of NS3 protein responsible for the transcriptional repression of p21 promoter, several effector plasmids containing a deleted segment of the NS3 gene were generated (Fig. 3*a*). $NS3_{1027–1260}$ and $NS3_{1027–1206}$ encode a serine protease domain and a protease minimal domain of NS3 protein, respectively (Yamada *et al.*, 1998). They effectively repressed p21 promoter activity (Fig. 3*b*), indicating that the NS3 protein domain required for the majority of p21 repression is located on the protease domain and the helicase/NTPase domain located at the C-terminal half is dispensable for the effect. The repression activities of both $NS3_{1027–1260}$ and $NS3_{1027–1206}$ were actually much higher than that of full-length NS3 although no significant differences in their expression levels were observed (data not shown). It was a novel finding that $NS3_{1027–1206}$ has transcription repressor activity because no other functions except proteinase activity have been described so far for this region.

To investigate whether the proteinase activity of NS3 is essential for repression of p21, we constructed an expression vector, $NS3_{1027–1206}$ R1135G, in which Arg-1135 was replaced with a Gly residue. Arg-1135 is known to be situated near the oxyanion hole and seems to be essential for maintaining the conformation of the active centre of the NS3 proteinase; this activity is completely abolished when it is replaced with a Gly residue (Yamada *et al.*, 1998). When $NS3_{1027–1206}$ R1135G was tested, the p21 repression activity was still present (Fig. 3*b*), indicating that the proteinase activity of NS3 does not seem to be necessary for the repression of p21 promoter. Interestingly, a portion of NS3 (aa 1055–1200) which is similar to $NS3_{1027–1206}$ used in this study is known to form a complex with p53 (Ishido & Hotta, 1998). Therefore, the repressor activity of NS3 observed in this study might result from the inhibition of a p53 function which can activate p21 transcription via protein–protein interaction(s) between NS3 and p53. Because the minimal proteinase domain is more effective in repressing the p21 promoter than full-length NS3 (Fig. 2*b*), our suggestion might be true. We are currently investigating this mode of repression of p21 transcription by NS3.

Connection between p21 repression and stimulation of cell growth

Because the tumour repressor p21 protein is a 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 NS3 protein may result in stimulation of cell growth. To test this possibility, we prepared several NIH 3T3 cell lines which stably express NS3 and measured their growth

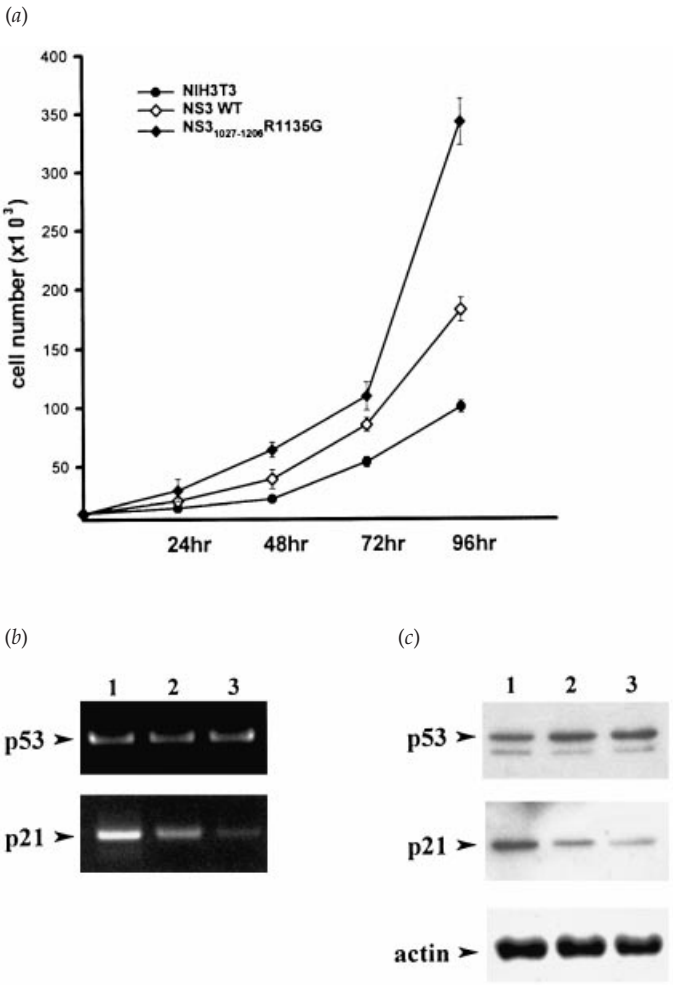

Fig. 4. Cell growth stimulation by HCV NS3 protein. (*a*) Growth rate of NS3-expressing cell lines. The growth rate of each cell line expressing a full-length or truncated form of NS3 was compared to that of the parent NIH 3T3 cells. Growth rate was determined by plating $1{\cdot}0 \times 10^4$ cells in duplicate wells of six-well trays in DMEM containing 10% FCS and counting the cells with a haemocytometer after 24, 48, 72 and 96 h. (*b*) Effect of NS3 on the level of p53 and p21 RNA. The level of p53 and p21 RNA in either full-length NS3- (track 2) or $NS3_{1027–1206}$ R1135G- (track 3) expressing cell lines were compared with those in the parent NIH 3T3 cells (track 1). (*c*) Effect of NS3 on the level of p53 and p21 protein. The level of p53 and p21 protein in either full-length NS3- (track 2) or $NS3_{1027–1206}$ R1135G- (track 3) expressing cell lines were compared with those in the parent NIH 3T3 cells (track 1). The level of endogenous actin in each cell line was used as a control.

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 NS3-expressing cell lines was at least twice as fast as that of the parent NIH 3T3 cells (Fig. 4*a*). Of the NS3-expressing cell lines, those expressing $NS3_{1027–1206}$ R1135G showed the highest growth rate. Considering that $NS3_{1027–1206}$ R1135G repressed the p21 promoter most strongly in transient transfection experiments

(Fig. 3*b*), it seems likely that the repression of p21 is accurately reflected by the stimulation of cell growth. Furthermore, we investigated whether NS3 affects expression of the endogenous p53 gene. According to a semi-quantitative RT-PCR analysis of the cell lines (Fig. 4*b*), the cellular p53 RNA level was not affected by expression of NS3. A similar result was obtained when the level of p53 protein was measured in the same cells (Fig. 4*b*). These results, in addition to a previous report (Ishido & Hotta, 1998), strongly suggest that NS3 represses transcription of p21 by inhibiting the regulatory activity of p53 via protein–protein interaction(s) but not by affecting the protein level of p53 through either transcriptional repression or protein degradation.

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