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Genetic Screen for Monitoring Hepatitis C Virus NS3 Serine Protease Activity

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We have developed a genetic system to monitor the activity of the hepatitis C virus (HCV) NS3 serine protease. This genetic system is based on the bacteriophage lambda regulatory circuit where the viral repressor *c*I is specifically cleaved to initiate the switch from lysogeny to lytic infection. An HCV protease-specific target, NS5A-5B, was inserted into the lambda phage *c*I repressor. The target specificity of the HCV NS5A-5B repressor was evaluated by coexpression of this repressor with a β-galactosidase (βgal)-HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease construct. Upon infection of *Escherichia coli* cells containing the two plasmids encoding the cI.HCV5AB-cro and the βgal-HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease constructs, lambda phage replicated up to 8,000-fold more efficiently than in cells that did not express the HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease. This simple, rapid, and highly specific assay can be used to monitor the activity of the HCV NS3 serine protease, and it has the potential to be used for screening specific inhibitors.

The activity of specific proteases is essential in many fundamental cellular and viral processes. Viral polyprotein processing is indispensable in the replication and maturation of many viruses (6). Consequently, site-specific proteolysis has been an attractive target for the development of antiviral therapies based on potent and selective viral inhibitors. The generation of such therapies based on the inhibition of site-specific proteolysis has been clearly illustrated in the development of effective inhibitors of human immunodeficiency virus type 1 (HIV-1) (3, 8, 9).

The hepatitis C virus (HCV) is a positive-stranded RNA virus which is the causal agent for a chronic liver infection afflicting more than 170 million people worldwide. The infection is usually persistent, and after an asymptomatic period often lasting years, many patients develop chronic liver disease, including cirrhosis and hepatocellular carcinoma (1, 4). The HCV genome is approximately 9.6 kb long and encodes a polyprotein of around 3,000 amino acid residues. This polyprotein is processed into structural and nonstructural proteins by host signal peptidases and by two viral proteases, NS2/3 and NS3 (reference 23 and references therein). The role of the NS2/3 protease appears to be limited to the autoproteolytic cleavage of the NS2-NS3 junction in *cis* (22). The amino-terminal 180-amino-acid sequence of the NS3 protein encodes a serine protease which cleaves at the NS3/4A junction in *cis*, which is followed by cleavage at the NS4/4B, NS4B/5A, and NS5A/B sites in *trans* (10, 29). Of note, the NS3 serine protease requires an accessory viral protein, NS4A, for optimal cleavage activity. The contribution of NS4A to NS3 protease activity can be mimicked by a synthetic peptide encompassing amino acid residues 21 to 34 of NS4 (30). The three-dimensional structure of the NS3 protease domain (residues 1 to 181) complexed with a synthetic NS4A cofactor (residues 21 to 34) has shown that the NS4A peptide is an integral component of the NS3 protease structure (12).

It has previously been demonstrated that a bacteriophage lambda-based genetic screen can be used to monitor the activity and phenotype of the HIV-1 protease (2, 17, 26, 27). This genetic screen system is based on the bacteriophage lambda *c*I-cro regulatory circuit where viral repressor *c*I is specifically cleaved to initiate the switch from lysogeny to lytic infection (21). The inherent difficulties in the purification and characterization of the HCV NS3 protease by in vitro classical methodologies prompted us to explore this genetic system as a simple alternative approach for the characterization of HCV NS3 protease activity. Moreover, the different biological properties of the HCV NS3 protease and the HIV-1 protease (serine protease and aspartic protease, respectively) offered us the opportunity to explore whether this system can be used to characterize proteases with different mechanisms of action.

Using a unique restriction site (*Bss*H2) located in the linker domain of *c*I (26), the HCV NS5A-5B cleavage site was inserted into the λ *c*I repressor (cI.HCV5A5B) (Fig. 1). Oligonucleotides encoding the HCV proteolytic NS5A-5B cleavage site (EDVVCCSMSYTWTG, NS5AB sense [5′-CGCGCGCTAGT GAGGACGTCGTCTGCTGCTCAATGTCCTATACGTGGA CAGGCGCCG-3′], and NS5AB antisense [5′-CGCGCGGCGC CTGTCCACGTATAGGACATTGAGCAGCAGACGACGT CCTCACTAGCG-3′]) were inserted in frame in the bacteriophage λ *c*I repressor, pcI.Bss-cro, to generate pcI.HCV-cro. Similarly, a plasmid containing a mutant proteolytic NS5A-5B cleavage site (EDVVGGSMSYTWTG), pcI.HCVmt-cro, was also constructed. As shown in Fig. 2A, these two repressors efficiently repressed the infecting phage.

Next we tested the target specificity of the HCV repressors by coexpressing these repressors with a β-galactosidase (βgal)-HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease construct (Fig. 1, 2A, and 3). The βgal-HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ construct contained NS4 residues 21 to 34 fused in frame via a short linker to the amino

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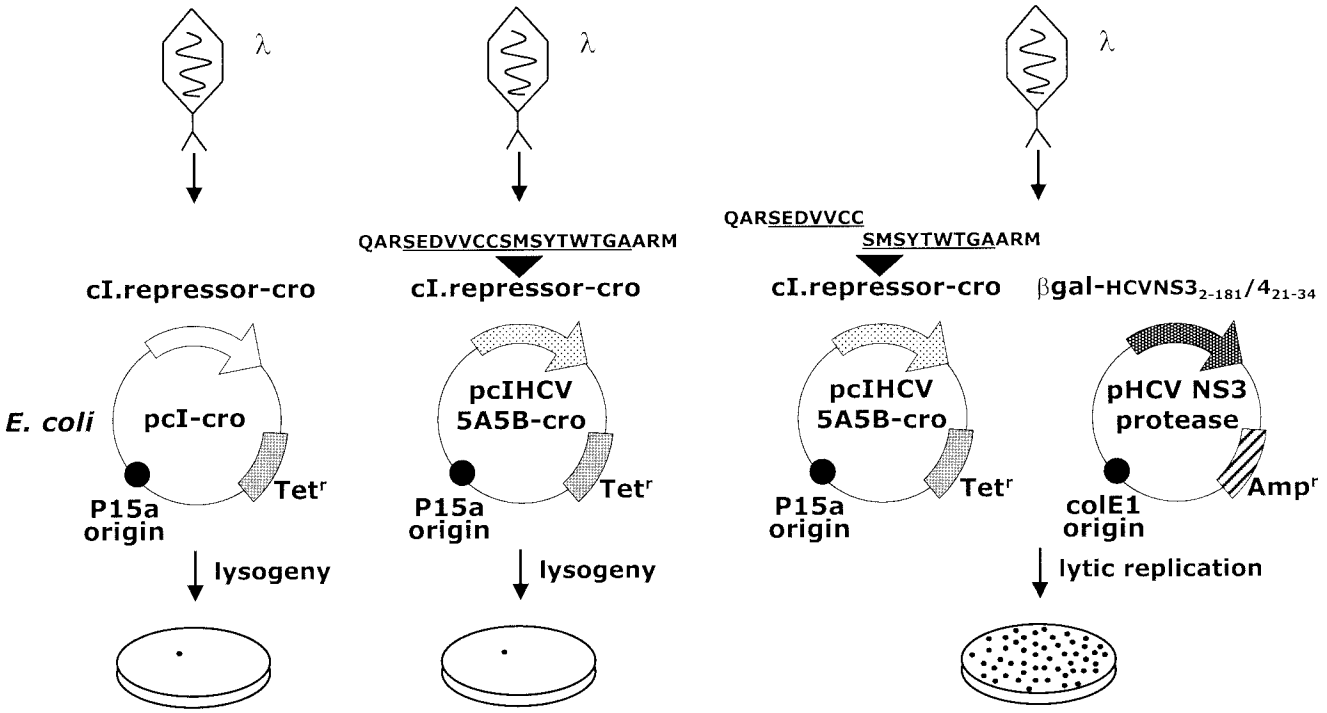

FIG. 1. Lambda-based genetic screen to monitor the activity of HCV NS3/4 protease. This genetic screen system is based on the bacteriophage lambda *c*I-cro regulatory circuit where the viral repressor *c*I is specifically cleaved to initiate the switch from lysogeny to lytic infection. When phages infect *E. coli* cells that express recombinant cI.HCV5A5B repressor and βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease, infection results in lytic replication. In contrast, phage replication is repressed in cells that do not express the specific βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease (lysogeny). The cI.HCV5A5B repressor contains the HCV NS5A-NS5B cleavage site sequence shown here.

terminus of the NS3 protease domain (residues 2 to 181) (Fig. 3). After viral RNA was isolated from an individual infected with HCV genotype 1b (patient 1), 10 μl of resuspended RNA was reverse transcribed at 42°C by using the avian myeloblastosis virus reverse transcriptase (Promega) and the oligonucleotide HCVproR1 (antisense) (5′-GGATGAGTTGTCTGTG AAGAC-3′; residues 3966 to 3984 of the BK strain). An aliquot of the reverse transcriptase product was amplified by PCR with AmpliTaq Gold DNA polymerase (Applied Biosystems) using the buffers and conditions specified by the manufacturer. The oligonucleotides used for the amplification were HCVproL1 (sense) (5′-GCAAGGGTGGCGACTCCTTGC-3′; residues 3401 to 3421 of the BK strain) and HCVproR1. Nested PCR was then performed with a 5′ oligonucleotide (HCVproL2) encoding an *Eco*RI site, residues 21 to 34 of NS4, and a dipeptide linker, Gly-Gly, along with residues 2 to 8 of NS3 (residues 3433 to 3446 of the BK strain) (5′-GGGTTGA ATTCTATGGCTCCTATTGGATCTGTTGTTATTGTTGG AAGAATTATTTTGTCTGGAAGAGGAGGACCTATCAC GGCCTACTCCCAA-3′). The 3′ oligonucleotide (HCV proR2) was complementary to residues 175 to 181 of NS3 (residues 3942 to 3962 of the BK strain) and encoded an in-frame stop codon flanked by an *Xho*I site (5′-GGGAGGG GCTCGAGTCAAGACCGCATAGTAGTTTCCAT-3′). The 621-bp fragment was digested with *Eco*RI and *Xho*I and inserted into pBSK− (Stratagene) to generate a βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease fusion protein (pHCVNS3$_{2-181}$/4$_{21-34}$ protease).

Escherichia coli JM109 cells containing plasmid pcI.HCV-cro or pcI.HCVmt-cro were then transformed with plasmid pHCVNS3$_{2-181}$/4$_{21-34}$ protease (Fig. 1). The resulting cells were grown overnight at 30°C in the presence of 0.2% maltose, harvested by centrifugation, and resuspended to an optical density at 600 nm of 2.0 per ml in 10 mM $MgSO_4$. To induce the expression of HCV NS3$_{2-181}$/4$_{21-34}$ protease, cells (200 μl) were incubated in 1 ml of Luria-Bertani (LB) medium containing 12.5 μg of tetracycline, 20 μg of ampicillin, 0.2% maltose, 10 mM $MgSO_4$, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. Thereafter, cell cultures were infected with 10^7 PFU of λ phage. After 3 h at 37°C, the titer of the resulting phage was determined by coplating the cultures with 200 μl of *E. coli* XL-1 Blue cells (adjusted to an optical density at 600 nm of 2.0 per ml in 10 mM $MgSO_4$) on LB plates using 3 ml of top agar containing 12.5 μg of tetracycline per ml, 0.2% maltose, and 0.1 mM IPTG. After incubation at 37°C for 6 h, the resulting phage plaques were counted in order to score growth. In the experiments in which *E. coli* cells express the HCV NS3$_{2-181}$/4$_{21-34}$ protease (Fig. 2A), λ phage replicated up to 8,000-fold more efficiently than in cells that did not express the HCV NS3$_{2-181}$/4$_{21-34}$ construct.

The target specificity of the HCV repressor was further analyzed by coexpression of a control mutant cI.HCV5Abmt-cro target site (EDVVGGSMSYTWTG) with the βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease construct. As shown in Fig. 2A, the mutant cI.HCV5Abmt-cro target site prevented phage replication, arguing that the cI.HCV5AB-cro degradation was spe-

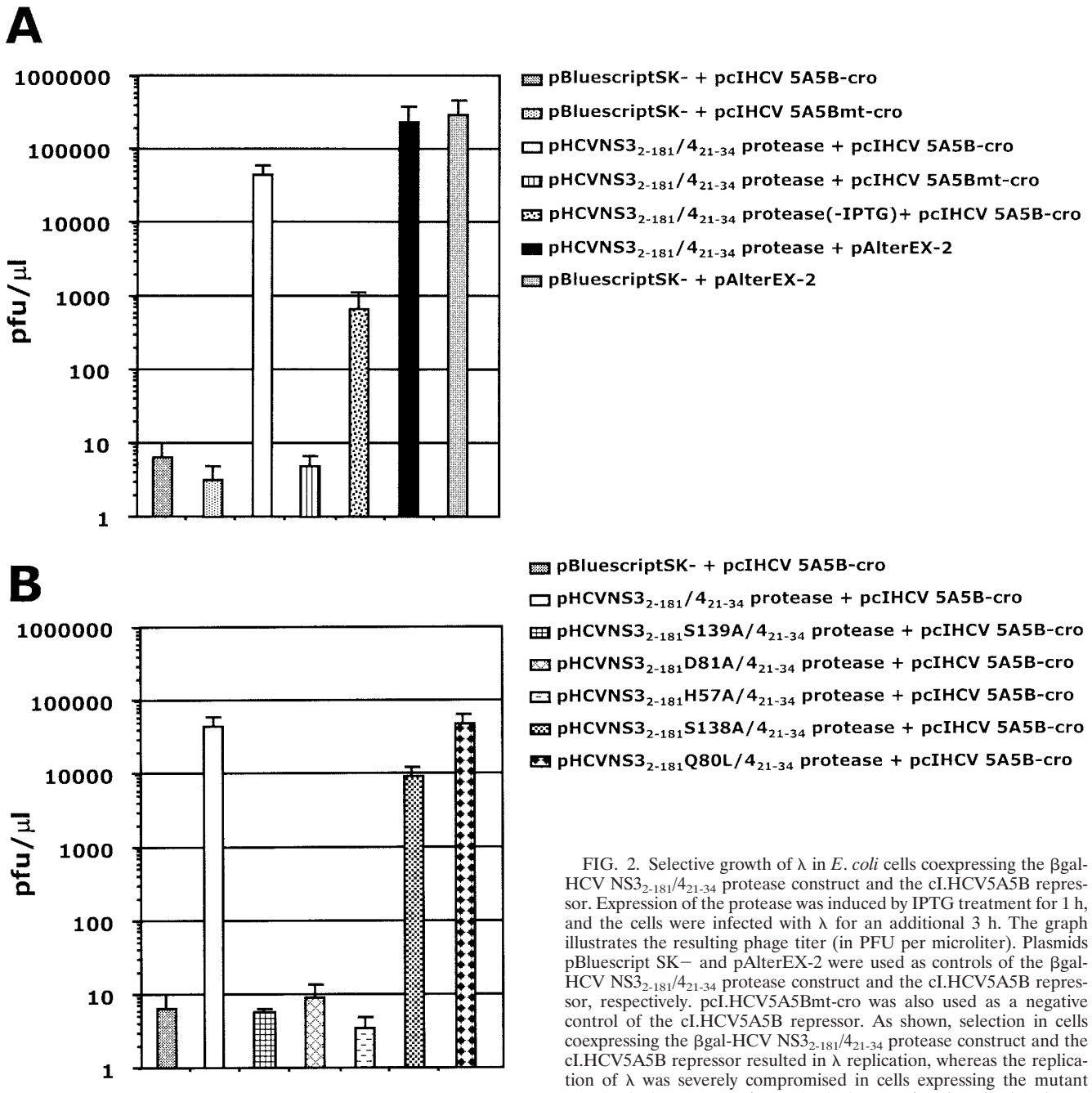

FIG. 2. Selective growth of λ in *E. coli* cells coexpressing the βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease construct and the cI.HCV5A5B repressor. Expression of the protease was induced by IPTG treatment for 1 h, and the cells were infected with λ for an additional 3 h. The graph illustrates the resulting phage titer (in PFU per microliter). Plasmids pBluescript SK− and pAlterEX-2 were used as controls of the βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease construct and the cI.HCV5A5B repressor, respectively. pcI.HCV5A5Bmt-cro was also used as a negative control of the cI.HCV5A5B repressor. As shown, selection in cells coexpressing the βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease construct and the cI.HCV5A5B repressor resulted in λ replication, whereas the replication of λ was severely compromised in cells expressing the mutant cI.HCV5A5B repressor (pcI.HCV5A5Bmt-cro) or in cells that do not express active proteases. Absence of protease induction with IPTG also compromised λ replication. Values are the means ± standard deviations (error bars) of at least four experiments.

cifically mediated by the HCV NS3$_{2-181}$/4$_{21-34}$ protease. Finally, when the expression of βgal-HCV NS3$_{2-181}$/4$_{21-34}$ protease was not induced with IPTG, phage replication was significantly reduced (Fig. 2A).

The specificity of this *trans*-cleavage reaction was further demonstrated by the lack of phage replication in cells expressing mutated forms of the HCV NS3$_{2-181}$/4$_{21-34}$ protease that included substitutions within the catalytic triad, that is, H57A, D81A, and S139A (Fig. 2B). NS3 protease single mutants were constructed by site-directed mutagenesis using the overlap extension protocol (24). Any of the former substitutions within the catalytic triad completely abolished the proteolytic activity detected with the genetic system presented here. Interestingly, the substitution S138A, which is not part of the catalytic triad, reduced phage growth up to five times, showing the lower catalytic efficiency of this mutant protease (Fig. 2B). In contrast, the Q80L substitution, a very frequent polymorphism found between HCV NS3 proteases of genotype 1, did not affect the catalytic efficiency of the enzyme (Fig. 2B). These findings demonstrate that quantitation of the protease activity

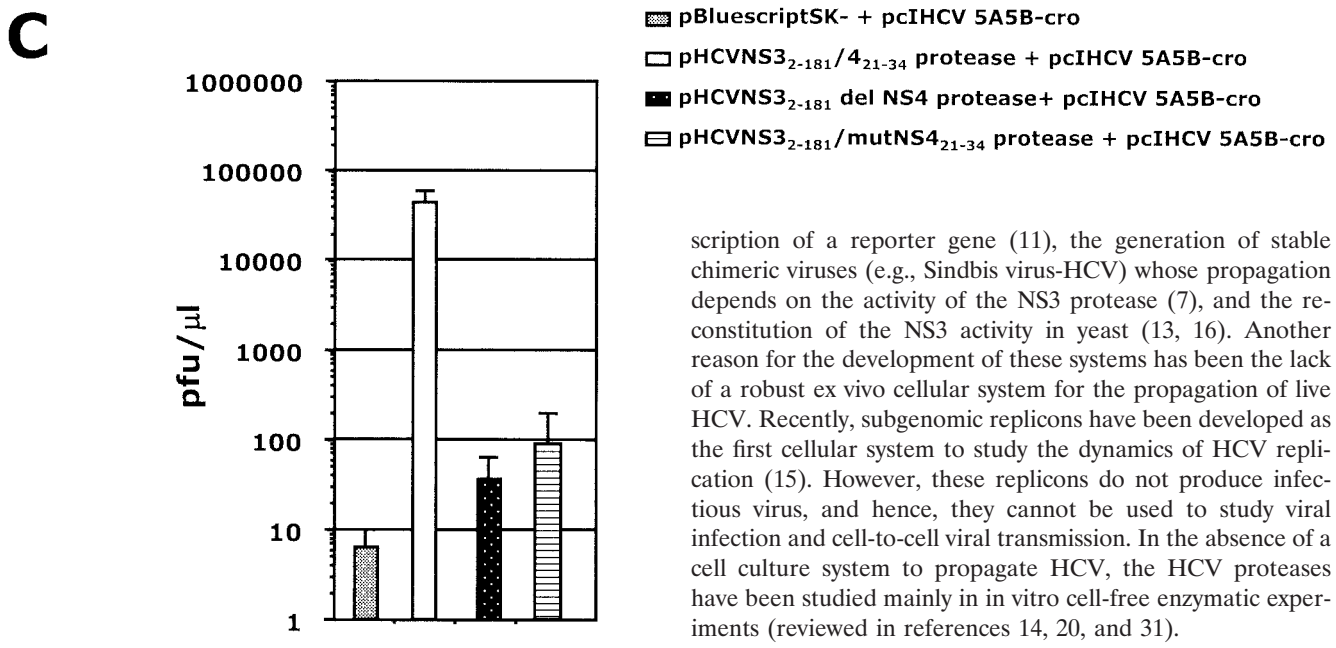

FIG. 2—*Continued*.

with this lambda-based genetic system makes it possible to monitor HCV NS3 proteases with different catalytic efficiencies.

Next, we investigated the influence of the central region of NS4A (residues 21 to 34) in this genetic system. We constructed a deletion form of the HCV NS3$_{2-181}$/4$_{21-34}$ protease construct in which the NS4 residues were absent; we also constructed a mutated form in which the I residues at positions 25 and 29 of NS4A were replaced by A residues (Fig. 3). NS4 mutants were generated by PCR by directly amplifying the pHCVNS3$_{2-181}$/4$_{21-34}$ construct with the appropriate mutated HCVproL2 oligonucleotide. Although the NS3 protease has proteolytic activity of its own, complex formation with the viral NS4A polypeptide is essential for efficient processing of the NS3-NS4A and NS4B-NS5A sites and improved cleavage at the NS4A-NS4B and NS5A-NS5B junctions (reviewed in reference 31). Moreover, NS4 amino acids I25 and I29 seemed to be critical for NS4 activation of NS3 protease (25). The former two constructs HCV NS3$_{2-181}$delNS4 and HCV NS3$_{2-181}$/mutNS4$_{21-34}$, tested here using the lambda-based genetic system showed very little proteolytic activity (Fig. 2C). Although the HCV NS3$_{2-181}$/mutNS4$_{21-34}$ construct showed 10 times more activity than the negative control, its activity represented only 0.2% of the wild-type HCV NS3$_{2-181}$/4$_{21-34}$ construct activity (Fig. 2C). These results indicate that the NS4A peptide is essential in our system for the NS3 cleavage of the NS5A-NS5B junction. In short, we have developed a simple, rapid, and highly specific assay that can be used to monitor the activity of the HCV NS3 serine protease.

Because the NS3 protease has been an attractive target in developing effective therapeutic agents against HCV, several assay systems have been developed to identify specific inhibitors. These assay systems have included a system in mammalian cells in which NS3 protease cleavage is required for transcription of a reporter gene (11), the generation of stable chimeric viruses (e.g., Sindbis virus-HCV) whose propagation depends on the activity of the NS3 protease (7), and the reconstitution of the NS3 activity in yeast (13, 16). Another reason for the development of these systems has been the lack of a robust ex vivo cellular system for the propagation of live HCV. Recently, subgenomic replicons have been developed as the first cellular system to study the dynamics of HCV replication (15). However, these replicons do not produce infectious virus, and hence, they cannot be used to study viral infection and cell-to-cell viral transmission. In the absence of a cell culture system to propagate HCV, the HCV proteases have been studied mainly in in vitro cell-free enzymatic experiments (reviewed in references 14, 20, and 31).

The simplicity of our system can be seen as a complement to the classical biochemical approach for monitoring NS3 proteolytic activity. An interesting result of the present study has been the finding that our system allows the characterization of enzymes with different proteolytic activities. The activity of the protease carrying the S138A substitution is reduced fivefold compared to wild-type protease (Fig. 2B). Since this mutant shows a proteolytic activity 1,000 times higher than the inactive mutants carrying substitutions within the catalytic triad, the genetic system used here is sensitive enough to allow the quantification of a wide range of different proteolytic activities. The reproducibility showed by this assay (Fig. 2) and previous work that we have performed with λ-based HIV-1 expression vectors (2, 17) strongly suggest that the different proteolytic activities observed here with the S138A variant (Fig. 2A) were not due to different expression levels of HCV protease variants but resulted from the lower catalytic efficiency of the S138A mutant. Coupling mutant sequence libraries with this positive genetic selection system will allow the study of a huge number of functional mutants (2, 18, 19, 27). Finally, as we have previously demonstrated for the HIV-1 protease (2, 17), this system may be used to search for new protease inhibitors and to predict the protease inhibitor resistance profile in the course of viral infection and antiviral therapy.

Site-specific proteolysis plays a critical role in regulating a number of cellular and viral processes. The system described here in which proteases with different mechanisms of action can be monitored should be useful to characterize cellular, viral, or other infectious agent proteases with different activities and specificities.

Nucleotide sequence accession number. The NS3 nucleotide sequence from the HCV-infected individual (patient 1) reported in this paper has been deposited in the GenBank database under accession number AF510039.

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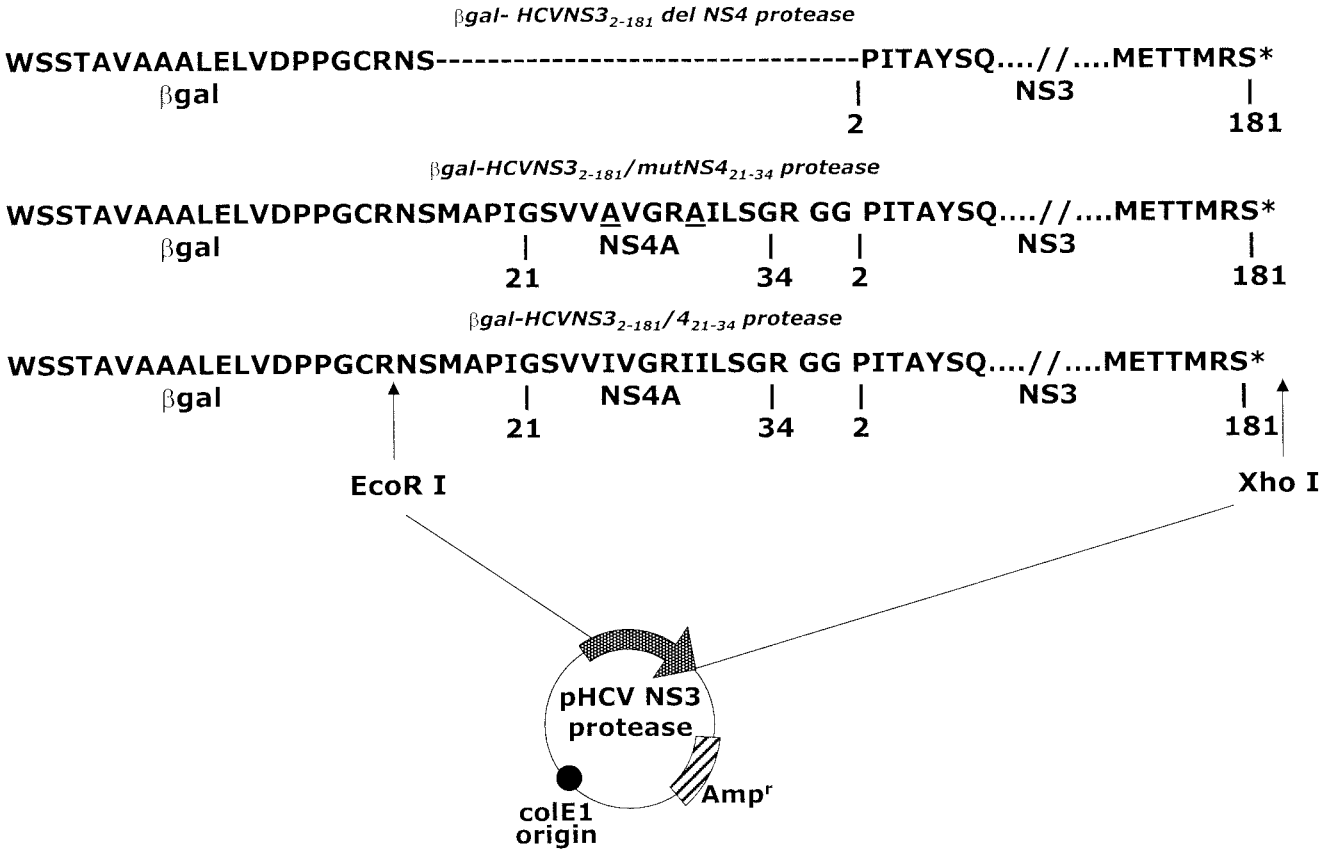

FIG. 3. The βgal-HCV $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease construct contains NS4 residues 21 to 34 fused in frame via a short linker to the amino terminus of the NS3 protease domain (residues 2 to 181). It has been previously demonstrated that the kinetic parameters of a single-chain protein containing the NS4A cofactor and the HCV NS3 protease were identical to those of the bona fide $NS3_{1\text{-}631}/NS4A_{1\text{-}54}$ protein complex generated in eucaryotic cells (5, 28). The HCV $NS3_{2\text{-}181}$/mut$NS4_{21\text{-}34}$ and HCV $NS3_{2\text{-}181}$delNS4 protease constructs are also shown.

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