Identification of a Key Determinant of Hepatitis C Virus Cell Culture Adaptation in Domain II of NS3 Helicase*

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Efficient replication of hepatitis C virus (HCV) replicons in cell culture is associated with specific sequences not generally observed in vitro. These cell culture adaptive mutations dramatically increase the frequency with which replication is established in vitro. However, replicons derived from HCV isolates that have been shown to replicate in chimpanzees do not replicate in cell culture even when these adaptive mutations are introduced. To better understand this apparent paradox, we performed a gain-of-function screen to identify sequences that could confer cell culture replication competence to replicons derived from chimpanzee infectious HCV isolates. We found that residue 470 in domain II of the NS3 helicase is a critical determinant in cell culture adaptation. Substitutions in residue 470 when combined with the NS5A-S232I adaptive mutation are both necessary and sufficient to confer cell culture replication to otherwise inactive replicons, including those derived from genotype 1b HCV-BK and genotype 1a HCV-H77 isolates. The specific substitution at residue 470 required for replication is context-dependent, with R470M and P470L being optimal for the activity of HCV-BK and HCV-H77 replicons, respectively. Together these data indicate that mutations in the NS3 helicase domain II act in concert with previously identified adaptive mutations and predict that introduction of compatible residues at these positions can confer cell culture replication activity to diverse HCV isolates.

The hepatitis C virus (HCV) is a major human pathogen for which both vaccines and broadly effective therapeutics are not available (1–3). HCV has a positive-sense RNA genome of ~9600 bases and expresses a polyprotein of ~3000 amino acids (4) that is processed by host and viral proteases to yield 10 mature proteins (5, 6). Subgenomic HCV RNAs from which the structural proteins have been excised but which express non-structural proteins and cis-encoded RNA elements essential for replication (replicons) (7–10) are capable of autonomous replication in cell culture (11–13).

An important feature of HCV is its high level of genetic variability, which is believed to be a consequence of the low fidelity of the viral polymerase. This variability is underscored by the identification of six major HCV genotypes (designated 1–6), more than 50 subtypes, and numerous quasi-species within each subtype (14). Several clones that are infectious in chimpanzees have been passaged in vitro (15–22). In contrast, replicons derived from only the HCV-con1 and HCV-N isolates have been shown to replicate robustly in cell culture thus far (11–13). Efficient replication in cell culture has been invariably associated with adaptive mutations that dramatically increase the frequency with which replication is established (12, 23–25). Adaptive mutations in the HCV-con1 replicon have been localized to various non-structural genes, although substitutions in NS5A, for example S232I, appear to be the most effective (23). Similarly a 4-amino acid insertion in NS5A that is not commonly observed in vivo is required for the replication in cell culture of an HCV-N replicon (12). Surprisingly, known infectious clones are not replication-competent even after the introduction of adaptive mutations (23). Interestingly, a recent study showed that cell culture adaptive mutations that confer cell culture replication competence to the HCV-con1 replicon abrogate or dramatically attenuate its infectivity in vivo (26).

Efforts to identify additional HCV sequences that replicate in cell culture remain a high priority, because availability of these sequences will not only contribute to a better understanding of HCV biology but will also facilitate the development of therapeutic agents that are broadly effective against all clinically relevant HCV genotypes. We described recently the development of a powerful HCV replication system, Bla-Rep, that employs HCV replicons expressing the β-lactamase reporter (bla) and subpopulations of Huh7 cells that are enhanced for HCV replication (27). Here we used this system and a systematic mutational approach to identify the restriction to replication of two known infectious HCV isolates. We report that the amino acid residue at position 470 in domain II of the NS3 helicase acts in concert with the NS5A-S232I adaptive mutation in determining the cell culture replication competence of HCV-BK and HCV-H77 isolates (genotypes 1b and 1a, respectively). The results presented here predict that substitutions in the NS3 helicase in combination with previously identified HCV-con1 adaptive mutations can confer replication competence to diverse HCV isolates.

MATERIALS AND METHODS

Cell Culture—Huh7 human hepatoma cells were grown in Dulbecco’s modified minimal essential medium (Cellgro) supplemented with 2 mM GlutaMAX (Invitrogen), non-essential amino acids, 100 units/ml penicillin, 100 µg/ml of streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen). Media for culture of cell lines harboring replicon that expressed neomycin phosphotransferase were supplemented with G418 as indicated. Cells were grown at 37 °C and 5% CO2 and were passaged twice per week.

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‡‡ The abbreviations used are: HCV, hepatitis C virus; HDV, hepatitis D virus; bla, β-lactamase; MOPS, 4-morpholinepropanesulfonic acid; UTR, untranslated region.
Chimpanzee Infectious HCV-BK—HCV-BK comprises the genotype 1b BK sequence from the 5' -UTR through the KpnI restriction site in NS5B derived from a Japanese patient and a 3' -UTR (genotype 1a) derived from an Italian patient (28). Direct intrahepatic injection of in vitro transcribed HCV-BK RNA causes HCV infection in chimpanzees. Inoculation of a second chimpanzee with serum from an HCV BK-infected chimpanzee also results in infection, demonstrating that the BK sequences used in this study are infectious (29).

Replicon Constructs—HCV-con1 replicon constructs that express the bla reporter were as described previously (27). The HCV-BK subgenomic replicon was constructed by replacing the NS3 through the 3'-UTR sequence of the HCV-con1 replicon with the corresponding region from HCV-BK. The HDV ribozyme was added to the 3'-ends of both the HCV-con1 and HCV-BK replicons to facilitate the generation of native 3'-ends (30). In addition, a unique ClaI restriction site has been introduced at the NS5B-3'-UTR junction. Mutations were introduced as indicated using the QuickChange PCR mutagenesis kit (Stratagene). NS3 amino acid numbering is based on the mature sequence and does not include the initiator methionine present in the replicon constructs used.

Replication Assays—Replicon RNAs were transcribed and purified as reported previously (27) except that the templates were digested with XbaI, which cuts downstream of the HDV ribozyme. The integrity of the transcribed RNAs was checked by analytical agarose gel electrophoresis, and RNA was quantitated by absorbance.

Replication assays were performed as described previously (27). Bla-Rep assays were performed using MR2 cells, which are derived from Huh7 cells and are enhanced for HCV subgenomic replication (27). Cells were plated at 2.5 x 10^5 cells/well in six-well tissue culture plates and allowed to adhere overnight. Cells were lipotransfected with DMRIE-C reagent (Invitrogen) at the indicated concentrations of bla-replicon RNA using DMRIE-C. Medium for selection was supplemented with 250 μg/ml of G418. Colonies were counted 3 weeks after transfection. A "GAA" HCV-con1 replicon was used as the negative control for all replication assays. This replicon contains alanine residues in place of the aspartic acids at positions 318 and 319 in the GDD motif of NS5B that is essential for catalytic activity.

Purification, Reverse Transcription, and Sequencing of Viral and Replicon RNAs—HCV RNA was isolated from chimpanzee serum using the RNeasy RNA purification kit (Qiagen), and total RNA was purified from 10^6 replicon-harboring cells by TRIzol (Invitrogen) extraction followed by spin-column purification using the RNeasy RNA purification kit. cDNAs comprising the NS3 region were generated by reverse transcription using Superscript II reverse transcriptase (Invitrogen). cDNAs were subsequently amplified with Expand High Fidelity polymerase, subcloned into pETBlue1 (Novagen), and then sequenced with an ABI 373 Sequencer.

NS3 Helicase Expression and Purification—NS3 helicases were subcloned into the BamHI and HindIII restriction sites of pET-21B and expressed in BL21(DE3) cells. NS3 helicases were isolated from clarified bacterial lysates in three steps using immobilized metal-affinity chromatography, Q-Sepharose (Amersham Biosciences) chromatography, and poly(U)-Sepharose column chromatography. Purified helicases were stored at -20 °C in 20 mM Tris, pH 7.0, 10% glycerol. Protein concentrations were determined by absorbance at 280 nm using a calculated molar extinction coefficient of 47,480 M^-1 cm^-1.

RESULTS Subgenomic Replicons Comprising HCV-BK Non-structural Proteins and 3'-UTR Are Not Cell Culture Replication-competent—We focused our efforts on HCV-BK and HCV-H77, two isolates that are infectious in chimpanzees but that have failed to replicate in cell culture. Because they are infectious, all the activities and cis elements required for replication that are encoded by both sequences are known to be functional. We generated replicons that comprise the NS3-NS5B non-structural genes and the 3'-UTR from HCV-BK to test for cell culture replication. These constructs were engineered with an HDV ribozyme, which autocatalytically cleaves itself from the 3' -end of the replicon to yield replicon RNA transcripts with native 3'-ends. HCV-con1 replicons that are transcribed with the HDV ribozyme show the same replication activity as replicons that are transcribed from a Scal digested template without the ribozyme (data not shown). Replicons were generated either with the bla reporter for transient replication assays or with the neomycin phosphotransferase gene for selection experiments. In addition, the S232I mutation in NS5A that confers cell culture adaptation to the HCV-con1 replicon (23) was engineered into the HCV-BK replicon. As shown in Fig. 1, the wild-type replicon derived from the BK chimpanzee infectious genome did not replicate in the transient replication assay (Fig. 1). The activity of the HCV-BK replicon containing the S232I mutation is not significantly different from that of the replication-deficient HCV-con1 replicon, indicating that this adaptive mutation does not confer replication competence to HCV-BK (Fig. 1). Similar analyses were performed by colony formation assay using corresponding neo' replicons. The wild-type HCV-BK replicon did not yield any colonies. Introduction of the S232I adaptive mutation conferred modest replication competence to HCV-BK (~100 colonies/μg) compared with corresponding HCV-con1 replicons (~5000 colonies/μg). These results indicate that the introduction of the NS5A-S232I adaptive
Point mutations S196T and R470M in NS3 helicase confer replication competence to BK replicon.

Each of the 11 amino acids that differ in the NS3 helicases of HCV-BK and HCV-con1 were mutated by PCR mutagenesis. MR2 cells were transfected with HCV-BK replicon RNAs containing the indicated NS3 helicase mutations and NS5A-S232I and then assayed by Bla-Rep 3 days later. HCV-con1 subgenomic replicons containing the NS5A-S232I mutation with or without the inactivating GAA mutation in NS5B were tested in parallel. The results are averages of three or more independent experiments. Em460/Em530, the ratio of fluorescence emission at 460 and 530 nm.

Restriction to Cell Culture Replication of HCV-BK Replicon Maps to NS3—To identify the block to replication in the BK replicons, we generated chimeras in which the various non-structural proteins of HCV-BK and HCV-con1 were swapped. These swaps were initially made using HCV-BK replicons that had the S232I mutation, because this mutation modestly improved BK replicon replication in the colony formation assay described above. Substitution of NS4A, NS4B, NS5A, or NS5B either alone or in combination had modest or no effects on the replication of either replicon, suggesting that these regions did not account for the differences in replication competence (data not shown). However, as shown in Fig. 1, replacement of the NS3 coding region in the BK replicon with the con1 NS3 resulted in a replicon that replicated with essentially the same activity as the HCV-con1 replicon with the NS5A-S232I mutation. Conversely, introduction of the HCV-BK NS3 into the HCV-con1 replicon essentially abolished replication activity (Fig. 1). In contrast, none of the other con1 regions yielded any improvement in replication of the HCV-BK replicon (data not shown).

Mutations in NS3 Helicase Confer Cell Culture Replication Competence to HCV-BK—The results obtained from the chimeric experiments demonstrated that the block to HCV-BK replication in cell culture maps to NS3. An alignment of the HCV-BK and HCV-con1 sequences revealed that there are 12 amino acid differences in NS3, with 1 mutation mapping to the protease domain and the remaining 11 mapping to the helicase domain. To identify which of the amino acid differences accounted for the dramatic differences in replication efficiency, each of the residues in the NS3 coding region of the BK replicon with the con1 NS3 that differed from that in HCV-con1 was individually mutated to the residue found in HCV-con1. The resulting replicons were then tested for replication activity by Bla-Rep. As shown in Fig. 2, two mutations restored replication competence to the BK replicon. The introduction of an R470M mutation into the HCV-BK NS3 helicase resulted in a replicon that had significantly higher replication efficiency than the HCV-con1 S232I replicon. The NS3-S196T mutation also enhanced HCV-BK replicon activity but with lower efficiency than the R470M mutation. At all other positions tested, introduction of the corresponding HCV-con1 residue had only modest effects. When the NS3-S196T and R470M mutations were combined, a modest but reproducible additivity in transduction efficiency was observed (data not shown). To determine the nature of the higher level of reporter gene expression observed in the HCV-BK replicons containing the R470M mutation, we measured the fraction of the cell population harboring replicon. We then calculated reporter activity normalized to the number of replicon-harboring cells (27). The increase in reporter gene expression was found to be because of both increases in the frequency with which replication is established (the fraction of cells that expressed reporter) and to a modest (<2-fold) increase in the replicon copy number per cell (data not shown).

Although the NS3-R470M mutation dramatically enhances the replication activity of HCV-BK replicons, different residues are observed at this position in other isolates. To determine whether arginine at this position is incompatible with replication or rather that the methionine at this position is unique in conferring cell culture replication competence, HCV-BK replicons containing R470P and R470G mutations were tested. Proline, glycine, and leucine are frequently seen in genotype 1 HCV isolates at this position (Table I). As shown in Fig. 3, replicons containing either proline or glycine in place of Arg-470 replicated albeit with modestly lower efficiency those with methionine at this position.

To assess the effect of BK residues at positions 196 and 470 on HCV-con1 replication fitness, corresponding HCV-con1 replicons were engineered to have the T196S or M470R mutation or both and were tested in Bla-Rep. As shown in Fig. 4, T196S and M470R caused a modest and dramatic reduction in replication activity, respectively, whereas the combination of both mutations essentially abrogated replication activity. These results demonstrate that residues 196 and 470 both contribute to the replication of HCV-con1 replicons.

Mutations in NS3 Are Not Sufficient to Confer Cell Culture Replication Competence—As mentioned above, the S232I adaptive mutation in NS5A had minimal effects on the replication competence of the BK replicon. However, robust replication of BK replicons was observed when this mutation was combined with R470M and to a lesser extent with S196T-NS3 helicase mutations. The corresponding replicons without the S232I mutation in NS5A were tested in transient replication assays to assess whether S196T or R470M helicase mutations could by themselves confer replication competence to HCV-BK replicons. Replicons containing either of the NS3 helicase mutations but not the S232I-NS5A adaptive mutation did not replicate (Fig. 5A), demonstrating that cell culture replication of both con1 and BK replicons is dependent on both the NS3 and NS5A adaptive mutations. Additional analyses using a colony formation assay confirmed the results shown in Fig. 5A but also indicated that residue 470 makes a significantly greater contribution to replication activity than residue 196 (Fig. 5B).

NS3 Helicase Domain Mutations at Positions 196 and 470 Confer Replication Competence to HCV-H77 (Genotype 1a) Replicons—Replicons derived from the chimpanzee infectious genotype 1a isolate HCV-H77 also failed to replicate efficiently in cell culture with the introduction of the single adaptive mutation S232I in NS5A (3, 12). The results obtained with HCV-BK replicons prompted us to address whether mutations at posi-
tions 196 and 470 would confer cell culture replication competence to HCV-H77 replicons as well. The wild-type HCV-H77 NS3 helicase has a serine at position 196 and a proline at position 470, a combination that is compatible with efficient replication in HCV-BK replicons as described above. We generated a series of HCV-H77 replicons containing either the wild-type serine or a threonine at position 196 and either the wild-type proline, methionine, or leucine at position 470 in NS3 with and without the S232I adaptive mutation in NS5A. These replicons were assayed by Bla-Rep, and the data are summarized in Fig. 6. The H77 replicon containing Ser-196 and Pro-470 failed to replicate as did replicons containing the point mutations S196T and P470M in combination with S232I. However, HCV-H77 replicons containing both P470L and NS5A-S232I became replication-competent, thus demonstrating the importance of this region of NS3 helicase in the cell culture adaptation of HCV-H77.

Although the S196T had essentially no effect in isolation, the introduction of this substitution into the HCV-H77 replicon containing P470L further enhanced replication activity. Similar data were obtained in colony formation assays using H77 replicons that express neor. No colonies were observed with either wild-type replicons or replicons that contained either single NS3 or NS5A mutations in isolation. Replicons containing the combination of the NS3-P470L and NS5A-S232I mutations yielded 140 colonies/μg of replicon RNA. Addition of the S196T mutations to this replicon further improved colony formation efficiency to 460 colonies/μg of replicon RNA. For comparison in the same sets of experiments, HCV-con1 replicons containing the NS5A-S232I mutation yielded 3000 colonies/μg of RNA. These data indicate that the NS3 helicase residues at positions 196 and 470 influence the replication potential of genotype 1a replicons.

### TABLE I

| Residue | Frequency |
|---------|-----------|
| Arg     | 44        |
| Gly     | 28        |
| Leu     | 27        |
| Pro     | 20        |
| Ser     | 16        |
| Ala     | 10        |
| Met     | 8         |
| His     | 7         |
| Ile     | 2         |
| Thr     | 2         |
| Gln     | 1         |

*Frequency is the number of times the residue is observed at NS3 position 470 in 165 independent full-length HCV sequences in GenBank.

**FIG. 3.** Proline and glycine at position 470 in NS3 helicase are compatible with efficient replication. HCV-BK subgenomic replicons were engineered to contain substitutions at NS3 helicase position 470 with residues frequently seen in other isolates. Replication was determined by Bla-Rep at day 3 after transfection. Results are averages of three or more independent experiments. $Em_{460}/Em_{530}$, ratio of fluorescence emission at 460 and 530 nm.

**FIG. 4.** Residues present at positions 196 and 470 in HCV-BK NS3 attenuate replication of the HCV-con1 replicon. T196S, M470R, or both mutations were introduced into the NS3 of the HCV-con1 replicon. MR2 cells were transfected with replicon RNAs and assayed by Bla-Rep 3 days later. Results are averages of three or more experiments. $Em_{460}/Em_{530}$, ratio of fluorescence emission at 460 and 530 nm.

**FIG. 5.** NS3 helicase mutations are not sufficient to confer cell culture replication competence. HCV-BK replicons containing the indicated substitutions in NS3 with and without the NS5A-S232I adaptive mutation were assayed by Bla-Rep using replicons containing the bla reporter (A) and by colony selection using replicons expressing neo' (B). A, MR2 cells were transfected with the indicated replicon and assayed 3 days later. The results are averages of three or more independent experiments. B, for colony selection experiments, $2 \times 10^5$ Huh7 cells were transfected with the indicated RNA. After 24 h, cells were placed under selection with 250 μg/ml G418 for 3 weeks, at which time the colonies were counted. $Em_{460}/Em_{530}$, ratio of fluorescence emission at 460 and 530 nm.
NS3 Helicase Mutations Do Not Affect Helicase Unwinding Activity—To explore the effects of the cell culture adaptive mutations on helicase unwinding activity, HCV-BK NS3 helicase domains with and without the S196T and R470M mutations were expressed and purified to homogeneity from Escherichia coli. Helicases were then compared in unwinding assays using double-stranded DNA substrates. As shown in Table II, the rates of unwinding were comparable for each protein. This fact, with the fact that both NS3 mutations map to the protein surface, suggests that these mutations mediate interactions with other viral- or host-encoded proteins involved in viral replication.

NS3 Residue 470 Substitutions Required for Replication in Cell Culture Are Not Observed in the Serum of an Infected Chimpanzee—It was of interest to investigate whether revertants containing substitutions in NS3 470 and or NS3 196 could be found in the sera of HCV-BK infected chimpanzees. Sequence analyses of 10 NS3 sequences rescued from the serum of an HCV BK-infected chimpanzee (5780 genome equivalents/ml) (29) did not reveal substitutions in these positions relative to the parental clone, consistent with the suggestion that substitutions at these positions are specifically required for cell culture adaptation.

**DISCUSSION**

An unbiased mutagenesis approach was used to identify the block to cell culture replication of subgenomic replicons derived from two HCV isolates that have been shown to replicate in chimpanzees. We report that combination of the previously identified con1 NS5A adaptive mutation S232I with substitutions in NS3 helicase residue 470 is necessary to confer cell culture replication competence to the HCV-BK and 1a-H77 HCV replicons. Residue 470 maps to the surface of the NS3 helicase and is not in the nucleotide, metal binding, or nucleic acid binding sites, suggesting that this position might be part of an interaction interface for binding to other viral proteins or host-encoded factors (32–34). Furthermore the demonstration that mutations at this position do not affect the in vitro unwinding activities of purified NS3 helicase is consistent with a role in mediating interactions with other factors.

Residue Ser-196 is located in domain I of the NS3 helicase and appears to play a secondary role compared with residue 470. Mutations in domain I that map near to this position in the helicase have previously been shown to improve transduction efficiency of the HCV-con1 subgenomic replicon. For example, mutations E177G and T255I increase the transduction efficiency of HCV-con1 replicons severalfold either alone or in combination with the NS5A adaptive mutation S225P (25). Likewise the mutations R258G and K583E were shown to increase the efficiency of colony formation in G418 selection experiments with HCV-con1 replicons, either with or without the NS5B adaptive mutation R465G (24). In these previous examples, the improvement in replication efficiency was relatively modest, resulting in up to 10-fold increases in colony formation efficiency. Similarly, in G418 selection experiments using HCV-BK neomycin replicons, colony formation efficiency improved nearly 8-fold for replicons containing both the NS3-S196T and NS5A-S232I adaptive mutations compared with those with only NS5A-S232I (Fig. 5B). These results suggest that the NS3-S196T mutation has modest effects, comparable with those engendered by the previously reported domain I mutations and that it may act by a similar mechanism.

In contrast, residue 470 is localized to domain II and its critical involvement in cell culture replication has not been recognized previously. Methionine, proline, and glycine substitutions at this position are all able to confer replication competence to HCV-BK replicons. Interestingly, the 1b HCV-N isolate, which exhibits a high transduction efficiency, also has a glycine at this position and a 4-amino acid insertion in NS5A that appears to be functionally equivalent to the S232I adaptive mutation. Surprisingly, we find that neither methionine nor proline substitutions at this position are compatible for replication in the context of HCV-H77 replicons but instead a leucine substitution conferred replication competence to this isolate. These results suggest that the residue required at this position for optimal replication is context-dependent and may vary among different genotypes. More detailed analyses are likely to identify additional NS3 helicase domain II residues that are part of an interface and which may also influence cell culture replication activity.

The biological activity attributed to NS3 helicase residues 196 and 470 is absolutely dependent on the presence of the NS5A adaptive mutation S232I. In turn the ability of the NS5A S232I mutation to confer replication competence is also dependent on its cooperation compatibility with NS3 helicase. The observation that the optimal residue at position 470 appears to be isolate-specific suggests that this residue might influence viral replication via a direct interaction with another viral protein. An alternative interpretation to these observations is that there are potentially two independent restrictions to cell culture replication, one of which can be addressed by the introduction of a mutation in residue 470 of NS3 helicase. The other restriction requires an adaptive mutation in either NS4B, NS5A, or NS5B. Additional work will be required to better understand the mechanism by which substitutions in these residues can confer replication activity in cell culture. Regardless of the mechanism by which these mutations mediate cell culture adaptation, the findings described here provide a rational and simple approach to generate and characterize isolates of other genotypes that are replication-competent in cell culture. The availability of clinically relevant isolates that replicate in cell culture will facilitate the development of broadly active HCV therapeutic agents.
Cell Culture Adaptive Mutation in NS3 Helicase Domain II

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