Supplementary Information

Generation of plasmid constructs

The replicon-containing vector used to produce the constructs in this study, pFK5.1neo^{FAK} (from which the [FA]neo replicon is produced), contains both a FLAG-tag sequence within NS5A and a unique *Ase*I site at the NS4B5A boundary. Generation of this construct required removal of an existing *Ase*I site within the ampicillin coding region of the pFK5.1neo vector (26). Therefore the Kan^R gene from pCR-Blunt (Invitrogen) was amplified with primers Kan(fwd) and Kan(rev), digested with *Nde*I/*Spe*I, and cloned into *Ase*I/*Spe*I digested pFK5.1neo to generate pFK5.1neo^K. An *Ase*I site was introduced into the NS4B5A boundary of the 5.1 replicon sequence by a two-step PCR approach with the first round of amplification using primer pairs NS4B(Bst)fwd + AseOlap(rev), and AseOlap(fwd) + NS5A(MluI)rev. The second round PCR product, generated from the template made in the first round by combining NS4B(Bst)fwd and NS5A(MluI)rev primers, was digested with *Bst*API/*Mlu*I and cloned into *Bst*API/*Mlu*I cut pLRM-EGFP (McCormick *et al*. (2006) *J. Gen. Virol.* **87**, 635-640). The GFP cassette within the NS5A coding sequence of the resultant vector was replaced with a FLAG sequence by digestion with *Cla*I and ligation to a synthetic DNA fragment created by annealing primers FLAG1(fwd) and FLAG1(rev). Finally, digestion with *Nde*I and ligation enabled transfer of the NS coding region from this resultant vector into pFK5.1neo^K to generate pFK5.1neo^{FAK}. Replacement of the neomycin phosphotransferase ORF in pFK5.1neo^{FAK} with the *Photinus pyralis* luciferase ORF from pFK5.1luc was made possible by the presence of unique *Asc*I and *Pme*I sites flanking these genes, resulting in pFK5.1luc^{FAK} (from which the [FA]luc replicon was produced).

To introduce the coding changes into the NS4B5A boundary, the *Ase*I-*Mlu*I fragment from pFK5.1neo^{FAK} was replaced with an alternative PCR or plasmid-derived fragment. For the plasmids which encoded the [P3V]neo, [P4V]neo and [P43VV]neo replicons, these DNA fragments were generated using pFK5.1neo^{FAK} as a template with primer NS5A(MluI)rev in combination with primers 4B5AP3-V, 4B5AP4-V, 4B5AP3+4-V respectively. Identical DNA fragments were introduced into pFK5.1luc^{FAK} to generate equivalent luciferase-expressing constructs. For plasmids encoding replicons [P43VV/QGG], [P43VV/VGG], [P43VV/HLG], [P43VV/RVG], [P643GVV/TNI] and [P432VVS/QAE], the relevant *Ase*I-*Mlu*I fragments were directly obtained from cloned products derived from the P1′-3′ degenerate library polyclonal selection experiment. In the case of plasmids encoding [P3V/QGG], [P3V/VGG], [P43ST/QGG] and [P43ST/VGG], AseI-MluI fragments were instead obtained by PCR. Here, the PCR templates were the cloned products derived from the P1′-3′ degenerate library polyclonal selection experiment and the primers were NS5A(MluI)rev in combination with SV-QGG, SV-VGG, ST-QGG and ST-VGG respectively.

A similar cloning strategy to the above, involving use of primer pairs NS5A(MluI)rev and 4B5AP1′-3′, generated the plasmid library containing both a P43VV encoding region and random nucleotides at the P1′-P3′ encoding region of the NS4B5A boundary. An *Ase*I-*Mlu*I digested pool of PCR products was cloned into the *Ase*I-*Mlu*I cut vector encoding the [P43VV]neo replicon and plasmid DNA harvested directly from the pool (~4 x 10^4) of bacterial colonies that were obtained after ligation and transformation. The library pool of plasmids was validated with *Ase*I-*Mlu*I as well as sequencing to confirm that most plasmids contained the PCR insert and had nucleotide degeneracy at the P1′-P3′ coding positions.

The NS4A 22S>C mutation was introduced by 2-step PCR mutagenesis, with 1st round amplification relying on primers FwdseqNS3rep + 4A22S>C(rvs), and NS5A(Mlu)rep + 4A22S>C(fwd). The PCR product from the 2nd round of amplification, generated from products of the first round in combination with primers FWDseqNS3rep + NS5A(Mlu)rep, was digested with *Mre*I/*Ssp*I and cloned into *Mre*I/*Ssp*I-digested [FA]luc or [P43VV/VGG]luc plasmids, creating [22S>C]luc and [22S>C/P43VV/VGG]luc, respectively.

For generating all replicon-containing pFB-derived baculovirus transfer vectors, the *Sfi*I-*Sfi*I fragment from pFBrep5.1neo (28-30) was exchanged with equivalent *Sfi*I-*Sfi*I fragments from the pFK5.1luc^{FAK}-based vectors.

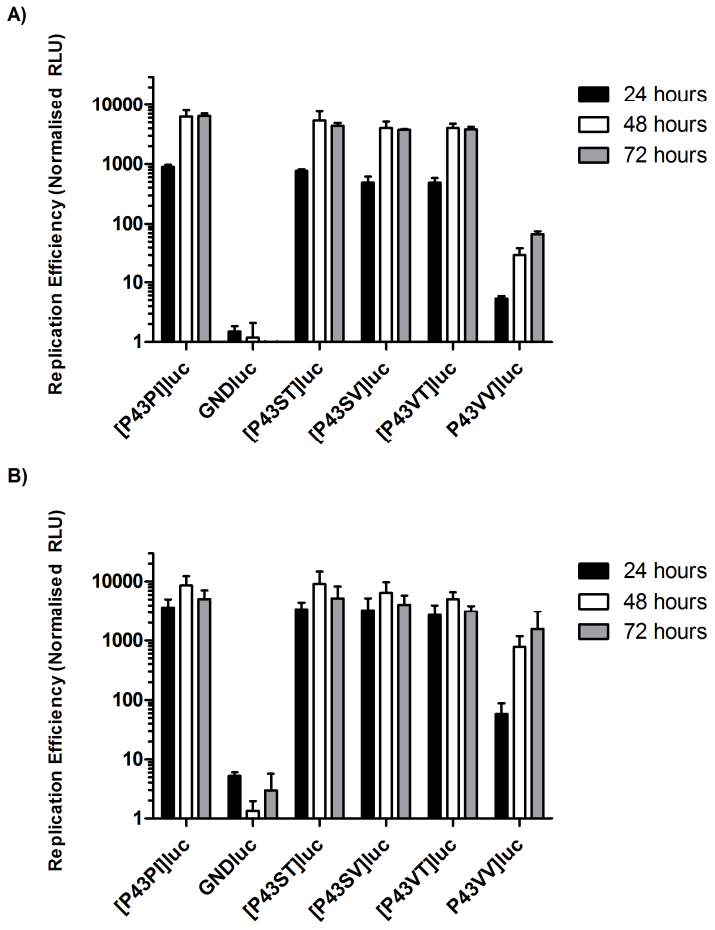
The bicistronic JFH-1 replicon construct used to assess the effect of NS4B5A boundary mutations on replication was a derivative of pSGR-JFH-1 (Kato *et al.* (2003) Gastroenterology 125:1808) that contained a tag sequence (GSSAIADYKDDDDKAIAGSS) between amino acid 429 and 430 of

NS5A incorporating a FLAG-tag (underlined) flanked by *AsiS*I restriction sites. This insertion had no effect on replicon replication (data not shown). The creation of this construct and introduction of mutations at the NS4B5A boundary was achieved using standard cloning procedures. This same construct, but with GFP introduced into NS5A via the *AsiS*I sites, was used as a template for PCR amplification of both the NS5AGFP coding region, the NS4B5AGFP coding region and NS3/4A coding region, these regions being flanked at the 5' end by a *Not*I restriction site and Kozak sequence (GCCACCATG), and the 3' end by a stop codon and *Not*I restriction site. In contrast, the JFH-1 NS4BGFP coding region which contained a 2xGSS hinge sequence between the COOH end of NS4B and NH2 end of GFP was generated by a two-step PCR reaction that resulted in a product with same Kozak sequence and a stop codon flanking the ORF, but where *Eco*RI restriction sites were placed at either end. All PCR products were cloned into the bacmid transfer vector pFBM (Adair *et al.* (2009) J Gen Virol. 90:833) via their respective *Not*I or *Eco*RI restriction sites to generate plasmids that could be used as bacmid transfer vectors, or directly as and mammalian expression vectors.

Supplementary Table 1. Sequences of primers used.

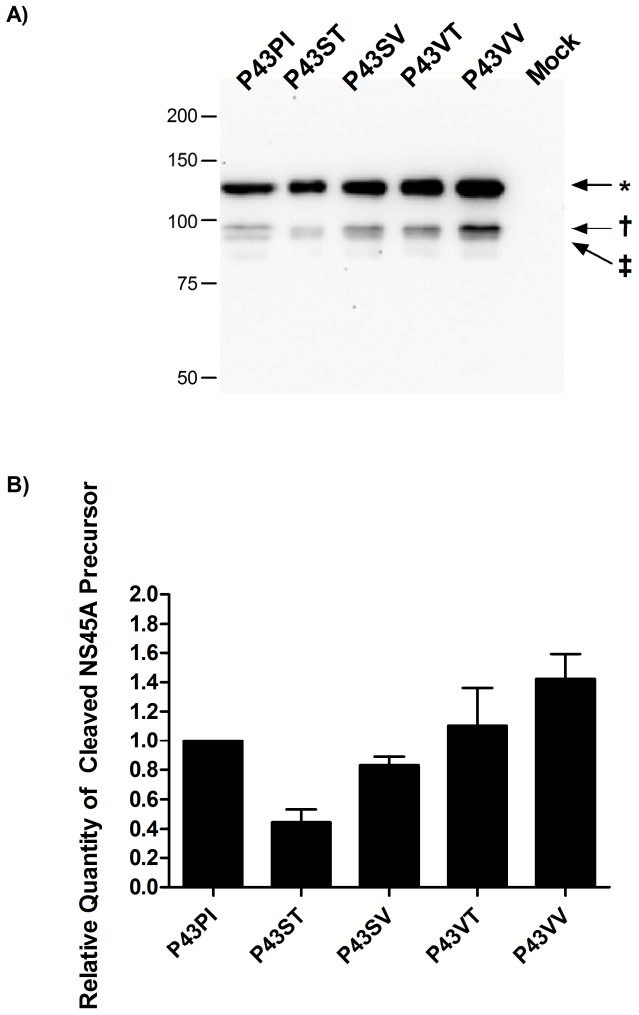
Primer name	Sequence
NS16/2	5′-CACTTGACCTACCTCAG-3′
Kan(fwd)	5′-GTGTGTACTAGTCACGTAGAAAGCCAGTCCGCAG-3′
Kan(rev)	5′-GTGTGTCATATGTCAGAAGAACTCGTCAAGAAGGC-3′
NS4B(Bst)fwd	5′-CTGATAGCGTTCGCTTCGCGG-3′
AseOlap(rev)	5′-CAGTCCTCATTAATCCACTGGTGAAGCCTCTTCAG-3′
AseOlap(fwd)	5′-CCAGTGGATTAATGAGGACTGCTCCACGCCATG-3′
NS5A(MluI)rev	5′-CCCTAGAATAATTTGGCGCC-3′
FLAG1(fwd)	5′-CGATTGATTACAAGGATGACGATGACAAAT-3′
FLAG1(rev)	5′-CGATTTGTCATCGTCATCCTTGTAATCAAT-3′
4B5AP3-V	5′-GGGGGGATTAATGAGGACTGCTCCGTGCCATGCTCCGGCTCGTGG-3′
4B5AP4-V	5′-GGGGGGATTAATGAGGACTGCGTCACGCCATGCTCCGGCTCGTGG-3′
4B5AP3+4-V	5′-GGGGGGATTAATGAGGACTGCGTCGTGCCATGCTCCGGCTCGTGG-3′
SV-QGG	5′-ACCAGTGGATTAATGAGGACTGCTCCGTGCCATGCCAGGGAGGTTGG-3′
SV-VGG	5′-ACCAGTGGATTAATGAGGACTGCTCCGTGCCATGCGTGGGGGGATGG-3′
ST-QGG	5′-ACCAGTGGATTAATGAGGACTGCTCCACGCCATGCCAGGGAGGTTGG-3′
ST-VGG	5′-ACCAGTGGATTAATGAGGACTGCTCCACGCCATGCGTGGGGGGATGG-3′
4B5AP1′-3′	5′-CCAGTGGATTAATGAGGACTGCGTCGTGCCATGCNNNNNNNNNTGGCTAA GAGATGTTTGGGATTG-3′
4B/5A(prec_fwd)	5′-GAATTCTAATACGACTCACTATAGGAAGCCACCATGGAAGCCTTCTGGGC GAAGCATATG-3′
4B/5A(prec_rev)	5′-TTTTTTTTTTTTTTTTCACCCCACGATCCTCATGGAACC-3′
NS5A(Mlu)rep	5′-TCAGCAGCCACCCGCCAC-3′
4B5Ajct(fwd)	5′-CGTGTGCGCAGCGATACTG-3′
4B5Ajct(rvs)	5′-ACCTGACACGGGCACTTTAC-3′
FwdseqNS3rep	5′-GTGCACATGCTTTACATGTG-3′
RevseqNS4Arep	5′-GTTTGCAGCACCCCGATTGC-3′
5.1(3940-3959)	5′-CTATGGGAACCACTATGCGG-3′
Con1(5082-5063)	5′-CTCCTGCCTGCTTAGTCTGG-3′
Int_prot_seq	5′-CGGCACCTTAGTGCTCTTGC-3′
Int_4A_seq	5′-ACCTAAACACACCAGGGTTG-3′
4A22S>C(fwd)	5′-GACAACAGGCTGCGTGGTCATTGTGGGCAG-3′
4A22S>C(rvs)	5′-ATGACCACGCAGCCTGTTGTCAGGCAATAC-3′

Supplementary Figure 1



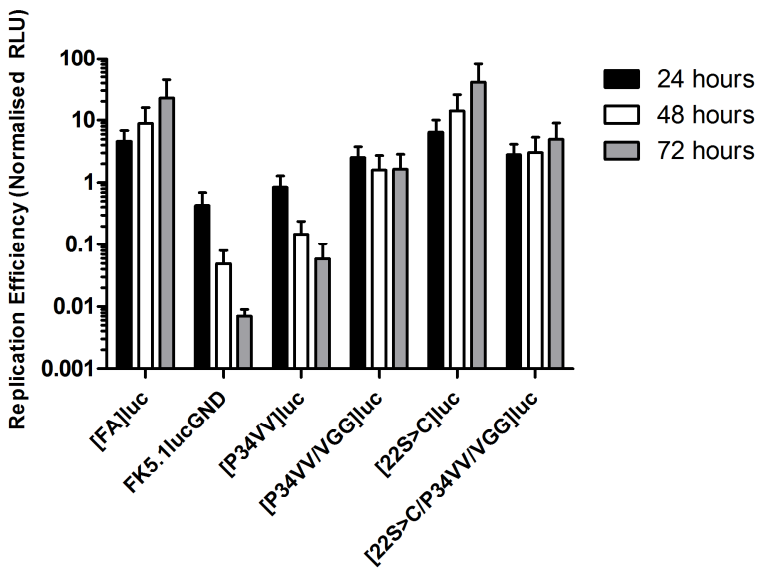
Supplementary Fig. 1. The effects of valine residues at the P3 and P4 positions within the NS4B5A boundary on HCV JFH-1 replication. (A) Huh7 cells were electroporated with a JFH-1 based bi-cistronic replicon transcript expressing luciferase and containing either the wildtype P43PI sequence at the NS4B5A boundary or mutant boundary sequences P43ST, P43SV, P43VT and P43VV. A luciferase-expressing polymerase knockout control transcript (GNDluc) was also included. Cells were prepared at 4, 24, 48 and 72 hours post electroporation and luciferase activity determined. Data shown represents mean luciferase activity (n=2, +/- S.D.) at 24, 48 and 72 hours normalised to the 4 hours time point. (B) The same experiment in (A) repeated using Huh7.5 cells (n=2, +/- S.D.).

Supplementary Figure 2



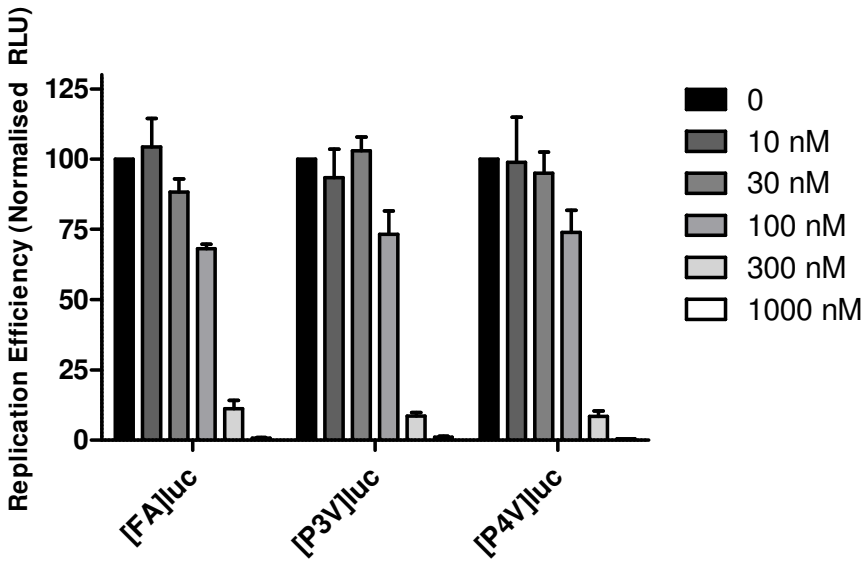
Supplementary Fig. 2. Analysis of JFH-1 NS4B5A cleavage resulting from P43 boundary mutations. (A) Plasmid constructs were generated for expression of a JFH-1 NS4B5AGFP precursor with either a wildtype P43PI boundary, or mutant boundary sequences P43ST, P43SV, P43VT and P43VV. Plasmids were transfected into HepG2 cells using polyethylenimine according to a standard protocol (Zanta *et al.* (1997) Bioconjug. Chem. **8**:839). Sixteen hours later the same cells were transduced for 2 hours with $5x10^7$ PFU/ml of a baculovirus construct expressing JFH-1 NS3/4A and allowed to recover for a period of 5 to 8 hours prior to lysis. Lysates were probed by western blot using goat anti-GFP antisera (Serotec). Figure (A) shows a representative blot from three separate experiments with the position of uncleaved precursor (*), as well as NS3/4A dependent (†) and NS3/4A independent (‡) cleaved precursors marked. (B) Graph detailing the extent of cleavage of the various JFH-1 derived NS4B5AGFP precursors in the experiments described in A (n=3, +/- SEM)

Supplementary Figure 3



Supplementary Fig. 3. Consequences of the NS4A 22S>C mutation on replicon replication. Huh7.5 cells were electroporated with [FA]luc and [P43VV/VGG] as well as equivalent vectors containing the NS4A 22S>C mutation. A polymerase defective control experimental group was also included. Cells were harvested at 4, 24, 48 and 72 hours post electroporation and luciferase activity determined. Data shown represents mean luciferase activity (n=2, +/- S.D.) at 24, 48 and 72 hours normalised to the 4 hours time point.

Supplementary Figure 4



Supplementary Fig. 4. The effect of cyclosporin A on replication of replicon constructs with differing NS4B5A boundaries. Huh7.5 cells were electroporated with [FA]luc, [P3V]luc and [P4V]luc before being treated with a serial dilution of cyclosporin A at 4 hours post transfection. Cells were harvested at 72 hours post electroporatation and luciferase activity determined. Data shown represents mean luciferase activity (n=2, +/- S.D.) normalised to untreated control values.