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High levels of subgenomic HCV plasma RNA in immunosilent infections

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Abstract

A genetic analysis of hepatitis C virus (HCV) in rare blood donors who remained HCV seronegative despite long-term high-level viremia revealed the chronic presence of HCV genomes with large in frame deletions in their structural genes. Full-length HCV genomes were only detected as minority variants. In one immunodeficiency virus (HIV) co-infected donor the truncated HCV genome transiently decreased in frequency concomitant with delayed seroconversion and re-emerged following partial seroreversion. The long-term production of heavily truncated HCV genomes *in vivo* suggests that these viruses retained the necessary elements for RNA replication while the deleted structural functions necessary for their spread *in vivo* was provided in trans by wild type helper virus in co-infected cells. The absence of immunological pressure and a high viral load may therefore promote the emergence of truncated HCV subgenomic replicons *in vivo*.

Keywords

HCV; subgenomic; replicon; defective; serosilent

Introduction

Hepatitis C virus (HCV), a member of the Flaviviridae family, genus hepacivirus, is estimated to infect 170 million people worldwide (Alter et al., 1999;Wasley and Alter, 2000). It is the causative agent of an array of diseases from chronic hepatitis to cirrhosis and hepatocellular

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carcinoma (Alberti, Chemello, and Benvegnu, 1999;Alter et al., 1999;Paterson et al., 2000;Seeff, 2002;Zoulim et al., 2003).

Studies of cases of infection in transfusion recipients or in experimentally infected chimpanzees have provided insights into the kinetics of viremia and seroconversion in very early-phase HCV infection (Busch, 2001;Prince et al., 2004). Anti-HCV antibodies are usually detected three to twenty weeks after the initial exposure but, in rare cases, seroconversion can be delayed from six to nine months (Beld et al., 1999;Busch, 2001;Busch et al., 1995;Busch and Shafer, 2005;Prince et al., 2004). In contrast, over the last few years, five repeat blood donors have been identified who did not seroconvert while remaining viremic during one to five years of follow-up (Arrojo et al., 2003;Durand, Beauplet, and Marcellin, 2000;Durand et al., 2000b;Peoples et al., 2000;Stramer et al., 2004). These serosilent donors had normal liver enzymes, high levels of plasma HCV RNA ($>10^6$/ml) and appeared immunocompetent based on recall humoral responses against other pathogens (cytomegalovirus, Epstein Barr virus, herpes simplex virus, varicella zoster virus) and on T-cell responses in secretion and proliferation assays (Arrojo et al., 2003;Durand et al., 2000b). These chronically viremic serosilent HCV infections in healthy blood donors are therefore distinct from those of subjects who seroreverted to an antibody negative status decades after viral clearance (Morand et al., 2001;Takaki et al., 2000), injection drug users (IDU) with very low level of viremia for extended periods prior to seroconversion (Beld et al., 1999), immunosuppressed liver transplant recipients or AIDS patients with delayed or absent seroconversion (Maple et al., 1994), or serosilent cryptogenic HCV infection in the absence of plasma viremia where HCV RNA is only detected in the liver (Castillo et al., 2004;Schmidt et al., 1997). Several mechanisms may conceivably cause such serosilent infections in immunocompetent subjects in the face of high and chronic viremia including *in utero* infection or acquisition of HCV during blood transfusion as a newborn resulting in immunotolerance (Arrojo et al., 2003). A similar rare phenomenon of serosilent viremic infection has been reported for one HCV infected chimpanzee (out of 46) who only seroconverted after 5 years (Bassett, Brasky, and Lanford, 1998).

During the first three years following the implementation of nucleic acid testing for HCV RNA in US blood donations, 39 millions units were screened and over 16,000 HCV seropositive donors identified (Stramer et al., 2004). From 139 donations initially testing HCV RNA positive but HCV seronegative, 90 donors were enrolled and followed to seroconversion. Seroconversion occurred on average within 35 days. All but three of these 90 donors seroconverted within 250 days of follow-up (Stramer et al., 2004). These three donors consisted of one human immunodeficiency virus (HIV) co-infected first time blood donor who did not seroconvert to HCV for at least one year and two others, HIV negative, HCV viremic repeat blood donors who remained seronegative during two and a half to five years of follow up. In order to determine if unusual viral characteristics could account for or develop in such serosilent infections, we amplified by polymerase chain reaction (PCR) and sequenced the HCV genomes from the plasma of these three subjects. Also analyzed using PCR were HCV genomes in numerous liver and/or plasma samples from HCV seropositive subjects, cirrhotic patients and liver transplant recipients. We report here that in a subset of serosilent subjects we detected the long-term presence of highly truncated HCV genomes dominating the viral quasispecies with genetic characteristics reminiscent of defective interfering particles and autonomous intracellular replicons.

Results

Identification of serosilent blood donors

The longitudinal HCV viral loads and antibody test results of three blood donors who initially tested HCV RNA positive but did not undergo seroconversion within the next 250 days are

presented in Fig. 1. Subject TN9's viral load fluctuated between 2.9×10^6 and 2.8×10^7 copies/ml. For subject TN78, the HCV viral load varied between 3.3×10^5 to 1.4×10^7 copies/ml. No anti-HCV antibodies could be detected at any time points using either the enzyme immunoassay (EIA) 3.0 or the non-licensed research EIA (see materials and methods). For subject TN168, who was co-infected with HIV, seronconversion to HCV was detected after 13 months of follow-up concomitantly with anti-HCV therapy and several months following initiation of combination anti-retroviral therapy (see material and methods). The viral load remained high with a maximum of 4.5×10^7 copies/ml except for a one log drop following HCV seroconversion that only lasted for a few months before returning to the previous steady state level (Fig. 1).

Truncated HCV genomes in serosilent infections

The HCV polyprotein coding region was amplified in two overlapping PCR fragments representing the 5' and 3' halves of the HCV genome (4.7 kb and 4.5 kb respectively), and sequenced as described in Materials and Methods and illustrated in Fig. 3. We analyzed the plasma viral genome in a total of 6, 2 and 9 samples from subject TN9, TN78 and TN168 respectively. The corresponding time points are indicated with shaded squares in Fig.1. Following agarose gel electrophoresis, the nPCR products corresponding to the 3' half of the HCV genome gave the expected single band of 4.5 kb in all samples analyzed (data not shown). For TN9 and TN168, 5' half genome amplification products were generated that were significantly shorter than expected. To ensure the reproducibility of the viral quasispecies population sampling in our PCR amplifications, we performed the nPCR in duplicates using independent inputs of viral cDNA (Fig. 1). For all 6 plasma samples from subject TN9, a major band of about 2.8 kb was observed in addition to a faint band of the expected full length size at 4.7 kb (Fig. 2A). After corrections for PCR efficiency and fluorescent dye staining, the percentage of plasma virus consisting of the HCV deletion mutant fluctuated between 53 and 78 % of the quasispecies (see materials and methods). For subject TN168, a similar phenomenon was observed with a 3.6 kb band dominating for the first four time points (Fig. 2B: lanes B, i, ii, iii) alongside the expected full length band of 4.7 kb. Another faint band of intermediate size was also detected, which was revealed to be a heteroduplex between the full length and the truncated amplicons (Fig. 2B). The following three time points of TN168 (Fig. 2B: lanes iv, v and vi), collected starting two months after the initiation of HCV treatment, showed the presence of only the full-length 4.7 kb PCR product. Delayed HCV seroconversion, seen as rising EIA titers, started three months after the initiation of anti-HCV therapy and nine months after starting anti-HIV therapy and was associated with the emergence of the full-length genomes (Fig. 1B and Fig. 2B). Concomitantly with a later decrease in the anti-HCV EIA signals, the full-length genomes then underwent a strong decline in frequency relative to the shorter defective genomes (Fig. 1B and Fig. 2B). The fluctuating presence of truncated genomes in TN168 was therefore temporarily associated with fluctuating levels of anti-HCV antibodies. The percentage of plasma virus consisting of the deletion mutant fluctuated between 42% and 66% of the quasispecies during time points B through iii, fell below detectable amount during iv through vi before rising again to 65% to 70% during vii and E (Fig. 2B and materials and methods). In the third blood donor TN78, both the earliest and latest samples showed exclusively full-length genome amplification products (Fig. 2C).

Truncated genomes retain open reading frame

To obtain sequence information on the truncated HCV genomes, we subcloned the 5' PCR amplicons from the baseline and exit time points of subject TN9 and TN168. The amplicon from time point iv of TN168 when only the full length fragment was detectable was also directly sequenced. Plasmids containing the full-length and the deleted 5' genomes were selected by sizing the PCR products derived from plasmid subclones and five full lengths and five deletions subclones were sequenced from each time points. All subclones of the shorter amplicons from

both time points from either subject contained the same deletion. Arrows in figure 2 highlight the time points and amplicons sequenced.

The truncated PCR products in subject TN9 resulted from a 2010 nucleotide (nt) deletion (HCV genome position 979-2988) covering most of E1, E2, p7 and the 5' end of NS2 (amino acids 213 to 882) (Fig. 3) and was uninterrupted by any stop codon. The short 5' amplicons from TN168 were the result of a 1080 nt in frame deletion (positions 1550-2629) including most of E2 and p7 (amino acids 403-763) and was also uninterrupted by any stop codon (Fig. 3). The full-length subclones from both subjects at all time points showed open reading frames.

The PCR products representing the 3' half genomes of these five time points were also directly sequenced. Sequences from both genome halves were assembled to obtain the sequence of the HCV polyprotein coding region. No frame shifting or stop codons were seen in the 3' PCR products.

Mutation rate in full length genomes from serosilent blood donors

In order to examine the potential impact on viral evolutionary rates of the absence or late appearance of anti HCV antibodies we examined longitudinal sequence changes in the full-length genome sequences from all three blood donors. Consensus sequences of the 5' amplicons were used for TN9 and TN168 whose PCR products were subcloned prior to sequencing (see materials and methods). We determined the number of synonymous (S) and non-synonymous (NS) mutations per year as well as the dN/dS ratio (Nei and Gojobori, 1986;Ota and Nei, 1994)(Table 2). As controls for subjects who exhibited normal seroconversion and had full genome sequence data available, we used the full genome longitudinal sequence data published by Nagayama et al (Nagayama et al., 1999). Sequences from subject TN78 showed a yearly rate of synonymous mutations within the range of the control sequences reflecting ongoing viral replication but the lowest rate of NS mutations and consequently the lowest dN/dS ratio. In case of TN9, the values we obtained were within the range of the regular seroconverters. For TN168, who underwent late seroconversion, the rate of S and NS mutations as well as the dN/dS derived by comparing the initial to the intermediate time points (TN168 t1-2) were higher than any of the controls, while comparison between the initial and the last time points (TN168 t1-3) were within the range of seroconverting controls. The full-length t2 genome clustered phylogenetically with those in the flanking time points (t1 and t3) and was therefore not the result of re-infection with another genotype 1a strain, but was nonetheless highly divergent from these variants (data not shown). Rather than being a descendant of the initially sampled t1 genome the highly divergent t2 genome therefore represents the outgrowth of a pre-existing HCV variant and the comparison between the initial and the last time point (Table 2: TN168 t1-3) better reflects ongoing mutation rates than that between t1 and t2 (Table 2: TN168 t1-2).

When similar analyses were performed using the E1E2 region alone, which encodes the envelope proteins that are the main antibody targets, TN78 again showed the lowest dN/dS ratio (no NS mutations were detected in E1E2) while the dN/dS for TN9 and TN168 fell within the range of the seroconverting controls.

HCV specific T-cell responses in HCV serosilent blood donor TN9

Because of the absence of HCV-specific antibodies, we also investigated whether any T-cell responses could be measured. Blood samples from TN9 obtained one year after the last sample shown in figure 2A was still seronegative and was tested for T-cell responses. ELIspot assays were performed to quantitate the number of lymphocytes in the blood that produced interferon (IFN)-gamma in response to (i) a panel of overlapping HCV genotype 2b peptides that covered the complete NS2 and NS3 sequence and (ii) recombinant HCV proteins of genotype 1

sequence. A pool of 32 cytomegalovirus (CMV), Epstein Barr Virus (EBV) and influenza A virus peptides which are known T cell epitopes was tested for comparison. Whereas no significant T-cell response was detectable against any of the HCV peptides or proteins when testing freshly isolated lymphocytes, a response against the CMV, EBV and influenza A virus peptide control pools was readily detectable (>260 IFN-gamma spots per million PBMC). Such findings were confirmed with a second assay with frozen and thawed lymphocytes (data not shown).

In addition, proliferation assays were performed using the recombinant HCV proteins of genotype 1 (Core, NS3, helicase, NS4, NS5A, NS5B) and freshly isolated peripheral blood mononuclear cells (PBMC). Consistently with the ELIspot results, there was no significant HCV-specific proliferative response (data not shown). This lack of detection of HCV-specific CD8 or CD4 response to HCV epitopes indicated an absence, or anergy, of the circulating HCV-specific T-cells, while the T-cell response against other viruses was preserved.

Full-length HCV genomes in acute or chronic infections and in cirrhotic and post-transplant viremic livers

In order to control for the possibility of artifactual generation of deletion products during PCR, the plasma from 12 recently HCV infected seropositive IDUs (see materials and methods) were similarly amplified. Both HCV 5' and 3' half amplicons of these three genotype 3a and nine genotype 1a HCV genomes had the expected size on agarose gel (4.7 kb and 4.5 kb respectively) (Fig. 2C for 5' half genomes and data not shown for 3' half genomes) indicating that the shorter amplicons generated from TN9 and TN168 were not a PCR artifact and were not present in these seropositive subjects. To test whether longer-term infection might be associated with the generation of large genomic deletions, we also tested plasma samples from ten seropositive, first time, healthy blood donors (see materials and methods). Again all ten plasma samples (eight 1a and two 3a genotypes) showed only full-length 5' genome PCR amplicons. We next tested liver and matched plasma samples from HCV genotype 1a infected cirrhotic patients (see materials and methods). In all nine cases only the full-length 5' HCV genome PCR products were detected in both liver and plasma quasispecies. Last, we tested six plasma samples and two liver biopsies from four liver transplant patients (three 1a and one 1b genotypes) under immunosuppressive therapy with a wide range of post-transplant viral loads (5×10^4–8×10^7). Only full-length 5' HCV genomes were detected (data not shown).

Full-length genomes in other HCV serosilent HIV co-infected patients

As part of the REACH study (Hall et al., 2004), two additional subjects were identified as co-infected with HIV and HCV (SM09012 and BH01129) while remaining EIA 3.0 seronegative for HCV for at least 4.1 and 5 years respectively (see materials and methods). A final case of HCV/HIV co-infection, TL0220, was identified in the UFO cohort (Hahn et al., 2002). This case remained HCV viremic and serosilent for the last 4.5 years of observation (see materials and methods). These three additional HIV-HCV co-infected subjects were also tested for the presence of truncated HCV genomes. Half genome PCR were performed on two to three time points from each subjects revealing only the presence of full-length genomes (data not shown).

Discussion

Serosilent HCV infections are extremely rare (Stramer et al., 2004). For serosilent donor TN78 the lack of antibody response may have been due to *in utero* infection since her only risk factor was an HCV positive mother. Absence or delayed appearance of anti-viral antibody following fetal exposure or infection at delivery has been reported for bovine viral diarrhea virus (BVDV) (Charleston et al., 2001;Peterhans, Jungi, and Schweizer, 2003) and hepatitis B virus (HBV) (Yuen and Lai, 2000). Why donor TN9 remained seronegative for over five years and showed

no sign of cellular immune response remains unknown. He possibly became infected through IDU but was otherwise healthy and had normal immune responses to other infectious agents. The initially HCV seronegative status of TN168 may have resulted from HIV induced immunodeficiency. In the two long-term HCV serosilent cases previously reported in the literature, the recipients of contaminated blood donations both seroconverted indicating that serosilence was limited to the blood donors (i.e. host-specific) and not directly related to the nature of their viruses (Arrojo et al., 2003;Durand, Beauplet, and Marcellin, 2000;Durand et al., 2000a).

The serosilent blood donor in whom only full-length genomes were found (TN78) showed a lower ratio of dN/dS mutations in both the full ORF and E1E2 regions than found in a control group of seroconverting subjects (Nagayama et al., 1999) possibly reflecting reduced selection for CTL and antibody escape mutations as expected in a immunosilent state (Bowen and Walker, 2005a;Bowen and Walker, 2005b;Grakoui et al., 2003;Rehermann, 2000;Rehermann and Nascimbeni, 2005;Shimizu et al., 1994;Timm et al., 2004). A reduced rate of amino acid changes in the hypervariable region (HVR) of the E2/NS1 region has also been reported in hypogammaglobulinemic subjects consistent with reduced antibody mediated selection for epitope changes in this region (Booth et al., 1998). Therefore, although the TN78 virus accumulated a typical number of synonymous mutations over the years, indicating ongoing viral replication (Neumann et al., 1998), the low dN/dS ratio and the absence of any amino acid changes in E1E2 indicated that little or no selection pressure was being applied to this viral population.

A phenomenon of defective viral genomes akin to that described here for TN9 and TN168 has been extensively documented (Barrett and Dimmock, 1986;Huang and Baltimore, 1970;Roux, Simon, and Holland, 1991). Defective interfering animal RNA viral particles can readily evolve after passages at a high multiplicity of infection (MOI) in tissue culture. Complementation in trans by a wild-type helper virus render these defective genomes dependent on and competitive with the parental virus genome and can result in both attenuation or aggravation of viral cytopathic effects. Trans-complementation can occur as long as all the cis required elements needed for RNA replication and packaging into virions are retained on the defective genomes and that a helper virus co-infects the same producer cell.

Defective genomes of numerous flaviviridae family viruses have been reported including West Nile virus (WNV) (Brinton, 1983;Debnath et al., 1991), dengue (Aaskov et al., 2006;Wang et al., 2002), classical swine fever virus (CSFV)(Meyers and Thiel, 1995) and others. Murray Valley encephalitis virus (MVEV) with large 2.4-2.5 kb in frame deletions from core or prM to NS1 rapidly dominated in persistently infected, Vero cells (Lancaster et al., 1998). For the pestivirus bovine viral diarrhea virus (BVDV), large deletions in the 5' of the RNA genomes of *in vivo* isolates resulted in a defective virus dependent on wild-type helper virus (Kupfermann et al., 1996;Tautz et al., 1994). Deletions of the BVDV structural genes up to NS3 left all the cis elements necessary for intra-cellular replication (Behrens et al., 1998). BVDV trans-complementation was not possible for NS3, NS4A, NS4B and NS5B mutations but could take place for structural genes mutations (Grassmann et al., 2001). For Kunjin virus defective genomes, cis required sequences needed for genome replication and packaging were mapped to NS3 and NS5 and protein expression of parts of NS3 was required in cis for genome replication (Khromykh, Sedlak, and Westaway, 2000;Liu et al., 2002;Pijlman, Kondratieva, and Khromykh, 2006). Subgenomic replicons of Kunjin virus were also generated with Core through E deletions (Khromykh and Westaway, 1997), providing models for the specifically engineered HCV subgenomic replicons (Lohmann et al., 1999). Subgenomic HCV replicons with mutations in non-structural genes could also not be complemented in trans (Evans, Rice, and Goff, 2004) possibly explaining why the non-structural reading frame of the truncated

HCV genomes described here remained open (i.e. are required in cis for truncated genome RNA replication).

The presence of in frame E1-NS2 truncated HCV genomes resembling those reported here has been recently reported in liver biopsies of 4/24 hepatitis patients and 2/2 hepatocarcinoma (HCC) patients (Yagi et al., 2005). Interestingly the ratio of truncated to full-length genomes appeared to be 100 fold greater in liver than serum. In these patients the 3' breakpoints of their deletions were located close to that found here in NS2 for TN9 (−21 to +5 amino acids) (Yagi et al., 2005). Truncated HCV genomes were also detected in the serum of 4/12 recent liver transplant recipients with recurrent high-level viremia (Iwai et al., 2006). Deletions extended from the 3' end of the core gene to p7 or NS2 and in two cases fell within 4 amino acids of the TN9 NS2 deletion (Iwai et al., 2006). There is therefore a preference for the 3' end of the truncation in vivo to occur within the same region of NS2. NS2 and the NS2/NS3 junction are also favored sites of natural inter-genotype recombinants (Kageyama et al., 2006;Moreau et al., 2006;Noppornpanth et al., 2006)(Legrand-Abravanel et al., 2007)(Kalinina et al., 2002) possibly reflecting numerous interactions amongst the proteins encoded either upstream or downstream of this recombination hot spot . That the natural subgenomes reported retain the complete sets of proteins downstream of NS2 may therefore also reflect the multiple interactions required among these proteins, possibly ocurring co-translationally, and limited capacity for transcomplementation of non-structural functions (Appel, Herian, and Bartenschlager, 2005). The long-term stable detection of HCV subgenomes in plasma reported here may therefore reflect the occasional generation of intra-cellular replicons whose deleted structural functions, including envelope glycoproteins, can be provided by trans-complementation in cells co-infected with wild-type helper HCV. Resulting subgenome containing particles released into the bloodstream from co-infected cells would be competent for cell entry and further RNA replication into new target cells thereby amplifying truncated genomes and sustaining the high and chronic plasma levels seen here.

In the HIV co-infected blood donor (TN168), defective HCV genomes were temporarily displaced by full-length ones following the initiation of anti-HCV combination therapy and immediately prior to detectable seroconversion. It is unknown whether the immunomodulatory effects associated with anti-HCV treatment, increasing CD4 due to antiretroviral therapy, the late seroconversion to HCV and/or the temporary drop in plasma viral loads changed the conditions previously favoring defective genomes. The detection of similarly truncated genomes in an otherwise healthy HCV seronegative subject (TN9) does indicates that a generalized state of immunosuppression is not required but rather that a lack of specific anti-HCV immune responses together with a high viral load may favor the outgrowth of defective genomes. The rapid re-emergence of truncated genomes in TN168 was associated with a measurable drop in antibody titers and a slight rebounding of HCV viremia. Overall these observations are consistent with the absence of immune selection pressure against HCV favoring the outgrowth of defective HCV genomes possibly by producing conditions analogous to the high MOI known to promote the emergence of defective interfering particles in tissue culture. Viremia was robust in these two donors (10^6 to 10^8HCV RNA/ ml) indicating that high level viremia may be involved possibly by increasing the probability that a cell will be co-infected with both helper and defective genomes. Immune responses may also prevent the outgrowth of defective variants by continually selecting for novel immune response escape variants resulting in serial population bottlenecks as immune escape variants are repeatedly selected. Serial immune response-induced population bottlenecks may therefore select against defective genomes in a manner analogous to a low MOI virus passage in tissue culture preventing the emergence of defective interfering viral particles.

Materials and Methods

Serological assays

The serological test used throughout was the HCV 3.0 enzyme-immunoassay (EIA) (Ortho Clinical Diagnostics, Raritan, New Jersey) which includes recombinant proteins representing linear epitopes from the Core, NS3, NS4 and NS5 regions (Chien et al., 1999;Lin et al., 2005) and is used to screen approximately 80% of the blood supply in the United States (US) for antibodies to HCV. Three unlicensed EIAs containing antigenic proteins not present in HCV 3.0 EIA 1) MEFA 7.1-NS3/4a, 2) F and Core, and 3) E1/E2 proteins were also used for a subset of samples (Tobler et al., 2007). MEFA 7.1 contains the linear epitopes used in licensed EIAs, including the latest EIA-3.0, in combination with genotype 1-3 specific epitopes. NS3/4a is a conformational protein retaining protease and helicase enzymatic activities. E1/E2 proteins are derived from Chinese hamster ovary cells (Chien et al., 1999;Lin et al., 2005).

Subjects

Subjects TN9, TN78 and TN168 have previously been reported as sero-silent infections using HCV EIA 3.0 with normal plasma alanine aminotransferase (ALT) (Peoples et al., 2000;Stramer et al., 2004). Samples from subject TN9 and TN78 were also tested by three unlicensed EIA and were also seronegative (Tobler et al., 2007).

These three blood donors were followed for 1782, 906 and 706 days respectively. TN9 had a history of IDU, and may have been exposed to blood from his partner who was known to be HCV positive. Lookback identified a recipient of his blood products who was viremic at day 22 with the same genotype (2b) but the inability to test the recipient at a later date precluded a determination of whether this recipient seroconverted (Peoples et al., 2000). Subject TN168 was initially identified as co-infected with and seropositive for HIV. He initiated antiretroviral combination therapy (ART) at day 114 consisting of d4T, 3TC and efavirenz following the initial virus detection and anti-HCV treatment with Peg-interferon alpha-2b and ribavirin at day 301. On day 362, he was still seronegative for HCV but became borderline positive (EIA 3.0 S/Co of 1.07) at day 398. S/Co then rose and stayed above 4 until day 615 before falling to 2-3. At day 463, he became indeterminate by recombinant immunoblot assay (RIBA^{TM} HCV 3.0 Strip Immunoblot Assay, Chiron, Emeryville, CA) due to reactivity to the c33c band only. TN78 remained seronegative throughout the period of study. Her only reported risk factor was her HCV seropositive mother.

Three other HIV co-infected subjects were also analyzed (SM09012, BH01129, and TL0220). SM09012, BH01129 were identified from a community study of homeless person in San Francisco (Hall et al., 2004) while TL0220 was from a study of young San Francisco IDU (Hahn et al., 2002). Two plasma samples from both SM09012 and BH01129, collected 4.1 and 5 years apart respectively, were analyzed. Both patients were seropositive for HIV but tested consistently negative for HCV-specific antibodies using EIA 3.0, and maintained a HCV viral load in the 10^6-10^7copies/ml range with normal ALT levels. SM09012 was sporadically compliant under changing antiretroviral therapies with an HIV viral load range of 10^5 to 7×10^5 and a CD4 count that declined from 336 to 96 cells/ml. BH01129 was under continuous but changing antiretroviral therapies with an HIV viral load range of 10^3 to 10^5 and a CD4 count that declined from 608 to 38 cells/ml. TL0220 was confirmed positive for HCV RNA by nucleic acid test (NAT) screening (Procleix^{TM} Assay, Gen Probe, San Diego CA and Chiron, Emeryville, CA) but the HCV viral load was not determined. Plasma samples obtained during the initial visit and then at 2.2 and 4.5 years later were analyzed. Informed consent was obtained from all subjects.

The recently infected seropositive IDUs have been previously described and had been infected a minimum of 155 to 519 days before sampling (Herring et al., 2004). The seropositive blood donors were healthy blood donors whose first blood donation was HCV seropositive and viremic and were therefore infected for an unknown period of time. Nine liver biopsies and associated plasma samples were taken from nine cirrhotic patients on the day of transplantation of new livers. Six plasma and two liver biopsies were also taken from four post-liver transplantation patients under immunosuppressive regimen with HCV viral loads ranging from 5×10^4 to 2.8×10^7 HCV RNA copies/ml and low to moderately elevated ALT. The study was reviewed and approved by the UCSF IRB.

Full genome plasma HCV amplification and sequencing

Plasma aliquots (280 μl) were centrifuged for 30 sec at 14,000 rpm to clarify the samples. Viral RNA was extracted from the supernatant using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) and collected in a final volume of 50 μl of elution buffer containing 40 U of Protector RNAse inhibitor (Roche Diagnostic Corporation, Indianapolis, IN). First strand cDNA synthesis was initiated using 14 μl of RNA extracted the same day and 0.5 μg of genotype specific primers, either HCVwg8 for the 5' half genome or HCVwg6 for the 3' half genome, and 200-400 U of murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a final volume of 25 μl, according to the manufacturer's instructions. For amplification of each half genome, a nested PCR was performed using 5 μl of each cDNA and TaKaRa Ex Taq DNA polymerase (TaKaRa Shuzo Co., LTD, Shiga, Japan) following the manufacturer's recommendations. For the 5' half genome, the primers used were HCVwg1 and HCVwg8 for the first round and HCVwg2 and HCVwg9 for the second round. For the 3' half genome, the primers used were HCVwg3 and HCVwg6 for the first and HCVwg4 and HCVwg7 for the second round respectively. Primers for genotypes 1a and 3a have been described elsewhere (Bernardin et al., 2006). Primers HCVwg1 and HCVwg2 were used for all three genotypes represented in this study (1a, 2b and 3a). Primers HCVwg8 and HCVwg9, specific for the 3a genotype, were also compatible with genotype 2b (Bernardin et al., 2006). All other amplification primers for genotype 2b were genotype-specific (Table 1). Primers were designed using the consensus sequences from the Los Alamos National Library HCV database (http://hcv.lanl.gov/content/hcv-db/GET_ALIGNMENTS/alignments.html). The PCR cycles were run as follows: 2 min at 94°C, followed by 10 cycles of 15 sec at 94°C, 30 sec at 57°C, 4 min at 68°C, and 20 cycles of 15 sec at 94°C, 30 sec at 57°C, 4 min plus 10 extra seconds added at each new cycle at 68°C. The final PCR products were run in a 0.8% agarose gel. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and eluted in a final volume of 50 μl. PCR products (100 ng) were then mixed with 5 pmol of a sense or antisense primer for sequencing reactions. Sequencing primers were genotype specific except for SEG2 and SEG13, which were used for 1a and 3a genomes (Bernardin et al. 2006 and Table 1).

When direct sequencing was not possible due to co-amplification of truncated with full-length genomes, the PCR products were purified and subcloned into pGEM-T Easy vector System I (Promega). Whites E. coli colonies were randomly picked and mixed with a second round PCR mix for amplification of the insert. The resulting PCR products were purified and prepared for sequencing as mentioned above.

Sequencing reactions were performed with ABI Big Dye dye terminators (Applied Biosystems, Foster City, CA) and run on an ABI 3700 automated capillary sequencer (Applied Biosystems). Sequences were edited manually using EditView and were assembled into a single contig and then aligned using the Seqman and Megalign programs, respectively (DNAstar, Inc., Madison, WI). GenBank sequence accession numbers are DQ430811-DQ430820.

Extraction and amplification of liver HCV

Biopsies were obtained from surgically removed liver tissues from chronically infected patients presenting end-stage HCV-associated cirrhosis or from post-liver transplant HCV patients. About 30 mg of tissue were grinded using a disposable tissue grinder (The Kendall Company, Mansfield, MA). After addition of 600 μl of lysis buffer (RNeasy mini kit, Qiagen), the suspension was further disrupted and homogenized by centrifugation through a QIAshredder column (Qiagen). The tissue lysate was then centrifuged and the supernatant subjected to RNA extraction according to the manufacturer's instructions (RNeasy mini kit, Qiagen). The isolated RNA was collected in 50 μl of elution buffer containing 40 U of RNAse inhibitor (Roche Diagnostic Corp.). First strand cDNA synthesis was initiated using 2 μg of RNA and 0.5 μg of HCVwg8. Conditions for the reverse transcription as well as for the 5' half genome nPCR were identical to those for plasma RNA.

Relative frequency of truncated and full-length genomes

Because shorter PCR amplification products are generated more efficiently than longer products, we tested whether the apparent ratio of the truncated to full length amplicons from TN9 and TN168, as determined by agarose gel electrophoresis and ethidium bromide staining fluorescence measurements, resulted in an over-estimation of the frequency of truncated genomes. Reconstitution PCR experiments using the second round PCR primers were performed using mixtures of full-length and truncated product plasmid subclones. Amplification products were then run on agarose gel and the relative fluorescence intensity of each band stained with ethidium bromide was measured (Syngene, Cambridge, UK). Three bands were visible, the third one being of an intermediate size, identical to what was observed after amplification of the original TN168 material (Fig. 2b). Because only the two subcloned variants were used to initiate the PCR, we interpret the intermediate band as reflecting the presence of DNA heteroduplexes between the full length and truncated amplification products. When the two independently generated TN168 clonal products were mixed, denatured and re-annealed the same 3-band pattern was seen by agarose gel electrophoresis (data not shown). Only two bands were seen for TN9 whose larger deletion may prevent efficient annealing of different size amplicons. After co-amplification, we estimated that the shorter fragments were amplified two (TN168, 1080 nucleotide deletion) to four (TN9, 2010 nucleotide deletion) times more efficiently than full-length fragments. In order to correct for the reduced ethidium bromide staining of the shorter PCR products we also adjusted for that effect based on the respective length of the PCR products.

Analysis of virus-specific T cell responses

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as previously described (Takaki et al., 2000) and immediately used for analysis. Enzyme-linked immunospot (ELIspot) assays were performed to quantitate the number of PBMC producing IFN-gamma in response to (i) a panel of overlapping 18 mer peptides (NMI Peptides, Reutlingen, Germany) that covered the complete NS2 and NS3 sequence of HCV genotype 2b (11 amino acid overlap, 7 amino acid offset between peptides) and (ii) recombinant HCV core, NS3, helicase, NS4, NS5A and NS5B proteins of genotype 1 sequence (Mikrogen, Germany) and (iii) a panel of 32 cytomegalovirus (CMV), Epstein Barr virus (EBV) and influenza A virus peptides which are known T cell epitopes presented by 13 HLA-A and -B molecules (CEF control peptide pool from DAIDS, NIAID, NIH AIDS Research and Reference Reagent Program). IFN-γ ELIspot assays were performed as described (Rahman et al., 2004) using duplicate cultures of 300,000 PBMC stimulated with (1) pools of HCV peptides or the CEF peptide pool containing each peptide at 1 μg/ml, (2) individual HCV proteins at 10 μg/ml, and the respective (3) DMSO and (4) protein buffer controls. The number of spot-forming cells was determined with a KS ELISpot Reader (Zeiss, Thornwood, NY). Antigen-specific spot-

forming cells (SFC in the presence of antigen minus SFC in buffer or DMSO controls) were counted.

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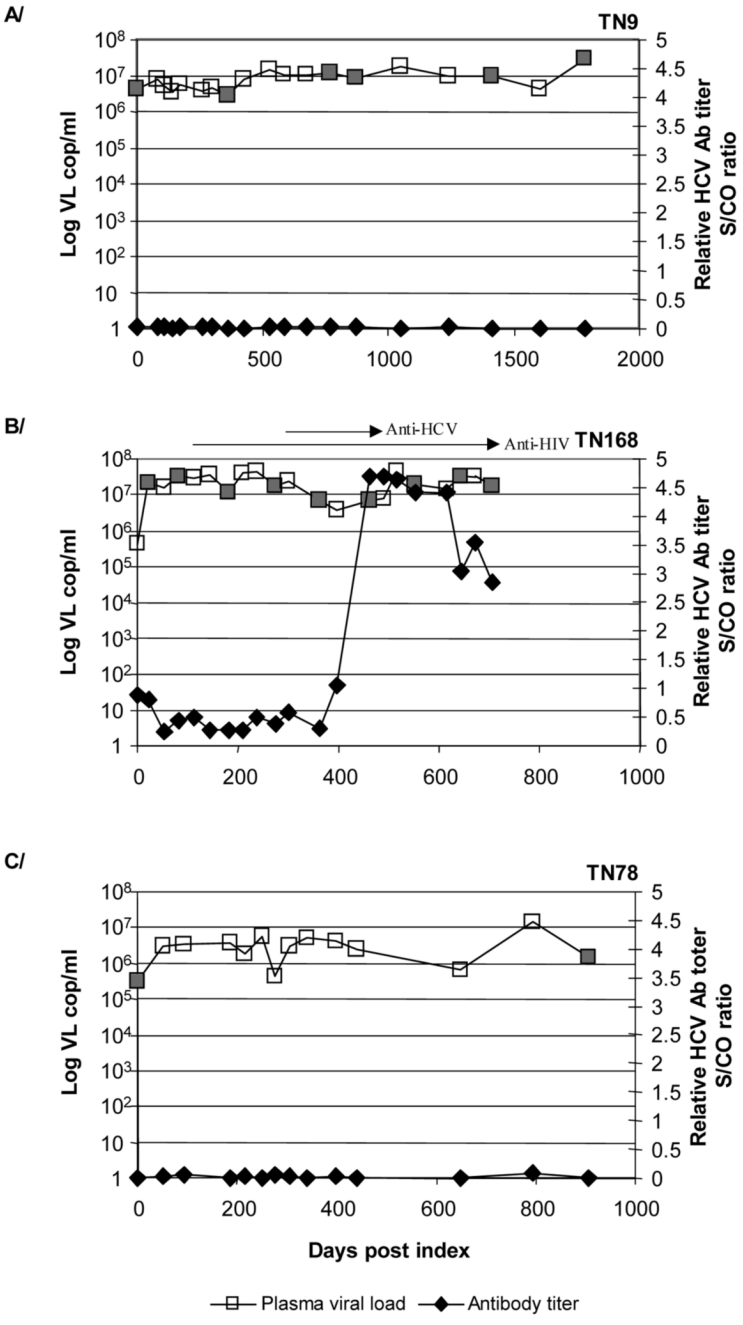

Fig 1. Virological and serological follow-up for subjects TN9, TN168 and TN78

Plasma HCV viral loads (VL) are expressed as HCV RNA/ml (left y axis). Antibody titers, determined by EIA 3.0, are expressed as signal over cut off ratios (S/CO, right y axis). Time points selected for amplification by RT-nPCR are indicated by shaded squares within the VL data points, the first and last being the baseline and exit time points respectively. Durations of anti-HIV and anti-HCV treatments are indicated for TN168.

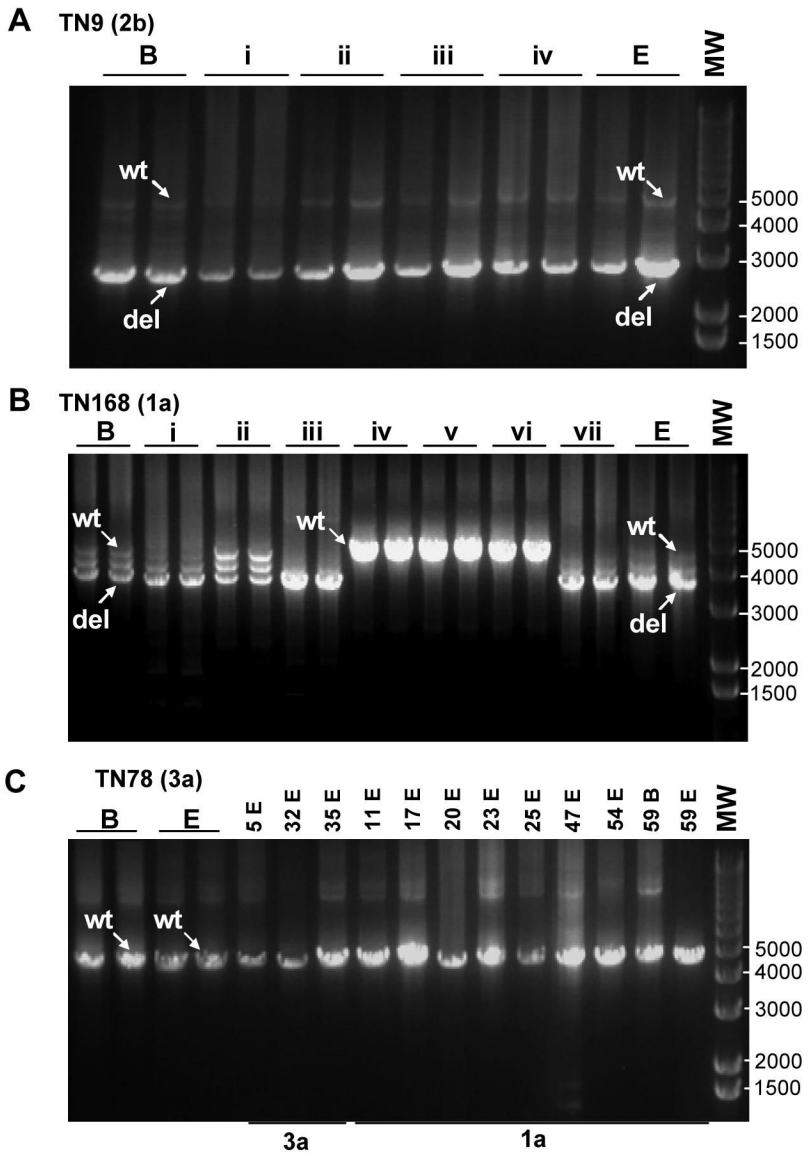

Fig 2. Detection of truncated HCV genomes

The amplified HCV PCR products corresponding to the 5′ half of the genome were separated by agarose gel electrophoresis. Full-length and deletion containing amplicons are labeled by white arrows (A and B). C: Amplification of the 5′ half genomes of subject TN78 and of regular seroconvertors. Time points are as follows: B, baseline; i-vii, intermediate; E, exit. MW: molecular weight marker in nucleotides. The 5′ amplicons selected for sequencing are highlighted by arrows.

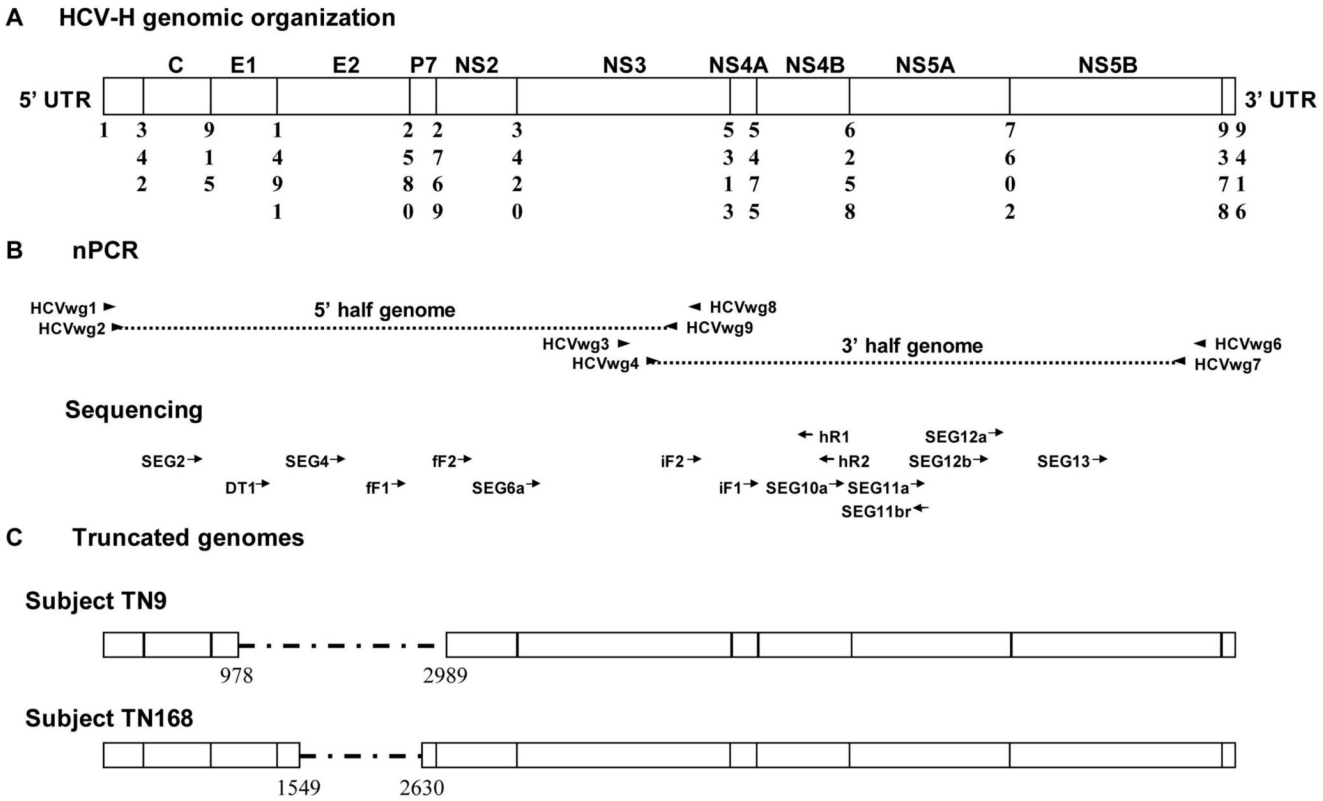

Fig 3. Genome organization of the deletion mutants from subjects TN9 and TN168

A: genomic organization of the HCV reference strain. B: Full HCV genome amplification and sequencing strategy diagram showing the location of the nPCR primers and sequencing primers. C: positions of the truncations observed in subjects TN9 and TN168.

Table 1

Primers used for amplification and sequencing of the HCV 2b genomes

			Consensus 2b pos.
nPCR Primers (2b)[a]			
HCVwg1	+	GGCGACACTCCACCATGAATCACT	minus 20-4[c]
HCVwg2[b]	+	RAYCACTCCCCTGTGAGGAAC	minus 3-18[c]
HCVwg3[b]	+	GAGACCGCAGGTGTCAGGCTAGTGG	4325-4339
HCVwg4[b]	+	TTCAAAGAAGAAGTGCGATGAGC	4501-4523
HCVwg9[b]	−	GTGAATCCAGTCATGAGGGCGTC	4628-4650
HCVwg8	−	AGGTGGGGTCCAGGCTGAAGT	4704-4724
HCVwg7[b]	−	AGAGTATGTGTGCAGTGAAAAGGC	9041-9064
HCVwg6	−	GGCGCTCCAAGTTTTCTGAGAG	9093-9114
Sequencing (2b)			
SEG2	+	TCGCCGACCTCATGGGGTAC	693-712
DT1	+	GGCCACTGGGGTGTGGTGTTTGG	1355-1377
SEG4	+	GTGCCCCACAGACTGTTTTAGG	2056-2077
fF1	+	GGTGGTCCTCCTTTTCTTGTTGTTGG	2470-2495
fF2	+	GCCGAGGCCCAGATTCAGCAATGGG	2873-2897
SEG6a	+	AGCGCAGAGGGGGACCTCGTGGGATGGC	3623-3650
iF1	+	CCTTTGATGAGATGGAAGAATG	5427-5448
hR1	−	TGGGAGGCCAACCATCCCCCCATG	5749-5772
SEG10a	+	GTCCAGTGGATGAACAGACTG	6053-6073
SEG11a	+	CCTTGCGACCCTGAGCCGGAC	6797-6817
SEG12a	+	CTCCATGCCTCCCCTTGAGG	7474-7493
SEG13	+	CCAATTGACACAACTATCATGGC	8027-8049

[a] HCVwg1 and HCVwg2 were used for all three genotypes 1a, 2b and 3a.
HCVwg8 and HCVwg9 were used for genotypes 2b and 3a.

[b] Primers used for nPCR and sequencing. + Sens primers; − antisens primers.

[c] Consensus sequence does not include a complete 5′ extremity. Primer sequence derived from available GenBank HCV 2b 5′ UTR sequences.

Table 2

Mutation rate in serosilent subjects

		Full length genomes				E1E2		
	Subject ID	**dt (days)[a]**	**dN/dS[b]**	**#S/year[c]**	**#NS/year[d]**	**dN/dS**	**#S/year**	**#NS/year**
Subjects from Nagayama et al. 1999	MD1	911	0.08	14	3.6	0.17	4.4	3.2
	MD2	484	0.06	26.4	4.5	0.08	6	1.5
	MD3	673	0.07	35.3	3.8	1.22	0.5	2.2
	MD4	491	0.08	52.8	11.9	0.23	13.4	8.9
	MD5	400	0.06	94	20.1	0.08	30	8.2
	MD6	675	0.12	15.7	5.9	0.42	1.6	2.2
	MD7	886	0.04	48.6	4.9	0.06	8.2	2.1
	MD8	1114	0.07	22.6	4.9	0.38	2.6	3.6
	MD9	1012	0.08	44.4	10.1	0.09	6.9	2.2
	MD10	386	0.25	12.3	7.6	0.63	1.9	3.8
Serosilent subjects	**TN78**	**906**	**0.01**	**25**	**1.6**	**NA [e]**	**3.6**	**0**
	TN9	**1782**	**0.16**	**16.4**	**7.8**	**0.15**	**3.7**	**2.9**
	TN168 t1-2	**339**	**0.33**	**103.4**	**34.5**	**0.13**	**25.8**	**10.8**
	TN168 t1-3	**683**	**0.28**	**74.3**	**30**	**0.12**	**11.2**	**4.3**

[a] dt: time interval in days.

[b] dN/dS: ratio of the number of non-synonymous mutations per non-synonymous site over the number of synonymous mutations per synonymous site.

[c] #S/year: number of synonymous mutations per year.

[d] #NS/year: number of non-synonymous mutations per year.

[e] NA: not available as no NS mutation detected.