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Acceleration of Hepatitis C Virus Envelope Evolution in Humans Is Consistent with Progressive Humoral Immune Selection during the Transition from Acute to Chronic Infection[▽]

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During the transition from acute to chronic infection in individuals persistently infected with hepatitis C virus (HCV), cellular responses initiate within the first 6 months of primary infection and collapse thereafter, whereas humoral responses activate later during the chronic phase. Whether and how this deviation of immune responses specifically influences HCV evolution are unknown. To determine the pattern of HCV evolution during this critical period, we conducted extensive sequence analysis on annual clonal hemigenomic sequences for up to 3 years in six well-characterized subjects, using statistical methods that accounted for repeated measures. Significantly different evolutionary rates were observed in envelope versus nonenvelope genes, with an increasing rate of nonsynonymous change (*dN*) in envelope genes and a stable *dN* in nonenvelope genes ($P = 0.006$). The ratio of the envelope to nonenvelope nonsynonymous rate increased from 2 in year 1 to 5 in years 2 and 3. Centripetal changes (reversions toward matching of the worldwide subtype 1a consensus sequence) were frequently observed during the 3-year transition from acute infection to chronicity, even in the presence of neutralizing antibody (NAb) pressure. Remarkably, sequences of hypervariable region 1 (HVR1) remained stable for up to 21 months in the absence of NAb pressure in one subject, followed by rapid changes that were temporally associated with the detection of NAb responses, which strongly suggests that HVR1 evolution is shaped by NAb pressure. These data provide the first systematic estimates of HCV evolutionary rates in multiple genes during early infection *in vivo* and provide additional evidence for deterministic, rather than random, evolution of HCV.

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) (2, 58). Following acute infection, which is usually asymptomatic, 60 to 80% of infected individuals develop persistent infection, which is still the leading cause of hepatocellular carcinoma and liver transplantation in the United States (33, 39, 53). Only pegylated alpha interferon and ribavirin are approved for treating this virus, and no successful vaccine has been developed (48).

The extraordinary evolution and diversity of HCVs are major challenges for vaccine design and drug development. HCV replicates to high levels using an error-prone polymerase (36), thereby generating in each host a spectrum of closely related but distinct viral variants called quasispecies (6, 34). Quasispecies distributions containing variants with a range of characteristics facilitate viral escape from selective pressure, balanced by fitness constraints that drive reversion to restore fitness, as has been demonstrated in controlled experiments involving simian immunodeficiency virus (SIV) under immune selective pressure and HCV under selective pressure from small-molecule inhibitors (17, 46). Similarly, HCV has been observed to escape selective pressure of the host immune response, demonstrating evidence of both escape and reversion and suggesting that intrinsic viral fitness also constrains HCV escape from immune selection *in vivo* (10, 12, 23, 25, 36, 42, 51, 57). However, available data are based on relatively short follow-up, single study subjects, or short amplicons (preventing accurate estimates of the relative rates of evolution of viral genes). As a result, little is known about HCV evolution in humans and about relative rates of change in structural and nonstructural proteins during the transition from acute to chronic infection.

The transition from acute to chronic HCV infection is poorly understood. During the acute phase, HCV RNA levels fluctuate, cellular responses to HCV reach a peak and then begin to wane and become dysfunctional, and neutralizing antibody (NAb) responses become detectable (9, 11, 35, 44, 54). Established chronic infection is associated with consistently weak cellular immune responses, the presence of antibodies that neutralize a wide variety of HCV isolates, and relatively stable HCV RNA levels between 500,000 IU/ml and 50 million IU/ml in 80% of individuals (3, 26, 50). The time between these two phases has rarely been studied even though it is likely that important viral adaptations occur during this transition from acute to chronic infection.

Studies of humoral immunity in HCV have advanced significantly due to development of model systems for studying neutralization, including retroviral pseudoparticles bearing HCV glycoproteins (HCVpp) and culture systems that support infection of a small number of HCV isolates (cell-cultured HCV, or HCVcc) (3, 21, 29, 56, 60). These systems generally give

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similar results though HCVcc-based assays tend to yield lower reciprocal neutralizing titers (47). Recent studies have suggested an important role for NAb in driving the evolution of HCV envelope proteins though their role in determining the outcome of acute HCV infection remains controversial (12, 22, 47). Thus, understanding the interaction between NAb response and HCV evolution is of great importance in defining the role of NAb on HCV control and in developing novel immune interventions for HCV-infected patients.

To better address these issues, we studied viral evolution across multiple genes subject to a variety of selective pressures from the onset of viremia in the acute phase to the early chronic phase over the first 3 years of infection in humans. We found discordant rates of evolution in envelope versus nonenvelope genes and evidence for immunologically driven evolution in the envelope genes. These results provide the first systematically obtained rates of evolution for the core, E1, E2, p7, NS2, and NS3 genes during the first 3 years of HCV infection *in vivo*.

MATERIALS AND METHODS

Study subjects. We studied specimens from subjects who had new-onset HCV viremia during follow-up in the Baltimore Before-and-After Acute Study of Hepatitis (BBAASH) cohort of injection drug users (IDU). Written informed consent was obtained from each subject. When acute infection was recognized, each subject was referred to an HCV treatment specialist not involved in this study. Even though evaluation and treatment were available to all subjects regardless of their ability to pay and even though counseling was provided regarding the benefits of early therapy, all declined. The BBAASH study protocol was approved by the institutional review board of the Johns Hopkins University School of Medicine.

To identify study subjects who were clinically well characterized and virologically evaluable with a single HCV infection, this intensive study used the following criteria: (i) strictly acute HCV infection with antibody seroconversion and with an interval between HCV RNA negativity and positivity of less than 3 months; (ii) at least 3 years of regular follow-up; (iii) sufficient HCV RNA in annual specimens for hemigenomic amplification (>1,000 IU/ml); (iv) no evidence of reinfection, mixed infection, or superinfection; (v) no treatment initiated; and (vi) negative for human immunodeficiency virus (HIV) and hepatitis B virus (HBV) antibodies.

Amplification and cloning of the 5′ hemigenome. HCV RNA was extracted from serum or plasma (140 to 560 μl) with a QIAamp viral RNA mini-kit (Qiagen) following the manufacturer's instructions. The region from the 5′ untranslated region (UTR), through core, E1, E2, p7, and NS2 genes to the NS3/NS4A junction was reverse transcribed, amplified by nested PCR, and cloned as previously described (31, 57). Briefly, Superscript II reverse transcriptase (Invitrogen) was used to conduct the reverse transcription (RT) process with the RT primer 6080G1R-16 (5′-CCGGTTCATCCAYTGC-3′). Nested PCR was performed using Platinum *Taq* Polymerase High Fidelity (Invitrogen) and the same set of primers as described by Liu et al. (31), followed by gel purification, ligation, and transformation utilizing a TOPO XL PCR cloning kit (Invitrogen). Template resampling (30) is avoided by this method (57). Forty clones were randomly picked and cultured overnight, and 24 of these were randomly selected for further study.

Hemigenomic sequencing of representative clones. The 24 clones selected previously were amplified using a high-fidelity technique (TempliPhi; GE Healthcare Products, Inc.), following the manufacturer's instructions, and sequenced for identification of representative clones using the sequencing primer H77-1868a21 (5′-GAAGCAATAYACYGGRCCACA-3′), which covers a partial E1/E2 region of 603 bp, with 282 bp of E1 and 321 bp of E2, and contains hypervariable region 1 (HVR1). Phylogenetic trees were built based on these sequences to determine the representative clone(s) nearest the center of the tree (38). When more than one main cluster of clone sequences in a single sample was detected, one representative clone was selected from each main cluster. Representative clones were sequenced across the entire 5.2-kb hemigenomic region.

Sequence alignment and phylogenetic analysis. Sequence contigs were assembled and base called with CodonCode Aligner (version 2.0.6; CodonCode Corp., Dedham, MA) and analyzed using BioEdit (version 7.0.9.0 [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]), with alignment performed using ClustalX (version 2.0 [http://bips.u-strasbg.fr/fr/Documentation/ClustalX/]). These sequences were aligned and compared with a total of 388 well-defined human genotype 1a HCV complete genome reference sequences from GenBank (http://www.ncbi.nlm.nih.gov). Possible PCR-induced nucleotide mutations were excluded using CleanCollapse (version 1.6 [http://sray.med.som.jhmi.edu/SCRoftware/CleanCollapse]) by comparing sample sequences with each other and with all other reference sequences mentioned above. Divergence and rates of nonsynonymous (*dN*) and synonymous (*dS*) evolution were calculated using MEGA (version 4.1 [http://www.megasoftware.net]) with the maximum-composite-likelihood method (for calculation of divergence) and the Li-Wu-Luo method (for calculation of *dN* and *dS*). Phylogenetic trees were inferred using PhyML (version 3.0.1), using the general time-reversible (GTR) model and estimated gamma distribution, as suggested by ModelTest (41). Each tree was built using 103 full-length reference sequences for genotype 1 (39 of subtype 1a, 58 of subtype 1b, and 6 others) that were not epidemiologically linked as well as other genotypes. The transition/transversion ratio and proportion of invariable sites were also estimated using algorithms implemented in PhyML. Tree topology was searched using subtree pruning and regrafting (SPR) moves with five additional random starting trees and visualized using MEGA. VarPlot (version 1.7 [http://sray.med.som.jhmi.edu/SCRoftware/VarPlot]) was used to conduct sliding-window analysis and to analyze "toward" and "away" amino acid mutations between sequences. The following formula was used to characterize toward and away substitutions: $f = \log_2(\text{FreqFrom}) - \log_2(\text{FreqTo})$, where FreqFrom is the frequency of the original residue (the residue in the previous visit) in the reference alignment and FreqTo is the frequency of the substituted residue in the same reference alignment. Therefore, when f is positive, the substitution is a forward evolutionary change, directed "away" from the most common residues worldwide, whereas when f is negative, the substitution is a change from a less frequent residue to a more common residue according to the reference sequences and thus is considered a reversion or substitution directed "toward" higher-frequency residues. To assess HVR1 sequence changes and the corresponding clonal frequency in detail, 17 to 43 clones from each consecutive visit were sequenced for a partial E1E2 region (containing HVR1) as above. These partial E1E2 clonal sequences were then compared with those from initial viremia using VisSPA (version 1.66 [http://sray.med.som.jhmi.edu/SCRoftware/VisSPA]) to generate type 2 sequence logos (20), with the height of each amino acid determined by the $\log_2$ relative risk of observing it compared to the reference sequence(s).

Pseudoparticle production and neutralization assays. Autologous HCV E1E2-enveloped lentiviral pseudoparticles encoding the luciferase reporter gene were generated and used in neutralization assays as described previously (12). Briefly, 2-fold dilutions of heat-inactivated serum/plasma samples were incubated with pseudoparticles for 1 h at 37°C and added to Hep3B hepatoma cells in quadruplicate wells of a 96-well plate for 5 h, followed by measurement of luciferase activity at 72 h postinfection. Pseudoparticle infection resulting in luciferase activity was measured in terms of relative light units (RLUs) in the presence of test plasma (RLU_{test}) versus average infection in the presence of two or three separate HCV-negative specimens ($\text{RLU}_{\text{control}}$). Percent neutralization was calculated as follows: $100 \times [1 - (\text{RLU}_{\text{test}}/\text{RLU}_{\text{control}})]$. Results are reported as 50% inhibitory dilution (ID_{50}) values, i.e., the dilution of test plasma that resulted in a ≥50% decrease in pseudoparticle infectivity. As a control, all test plasma neutralized murine leukemia virus (MLV) pseudoparticles at ≤30%. To measure NAb responses in subject 29 (S29), autologous HCVpp were made based on E1E2 sequences from month 2 and month 25 samples, and neutralizing antibody (NAb) responses against these HCVpp were measured in consecutive autologous serum samples.

Statistical analysis. To account for the multiple observations per person, the longitudinal data on HCV nonsynonymous evolutionary rates were analyzed by linear regression models utilizing generalized estimating equations (GEEs) and an exchangeable correlation structure (59). For multivariate GEE analysis, covariates were added in a stepwise manner, and covariates that were significant ($P < 0.05$) in the stepwise models were retained in the final model.

Nucleotide sequence accession numbers. Nucleotide sequences described in this report have been submitted to the GenBank and have been assigned accession numbers HM000514 through HM001192.

RESULTS

Characteristics of subjects. To investigate HCV evolution during the transition from acute to chronic infection, we identified subjects from a prospective cohort designed to detect acute HCV infection according to the following criteria: (i)

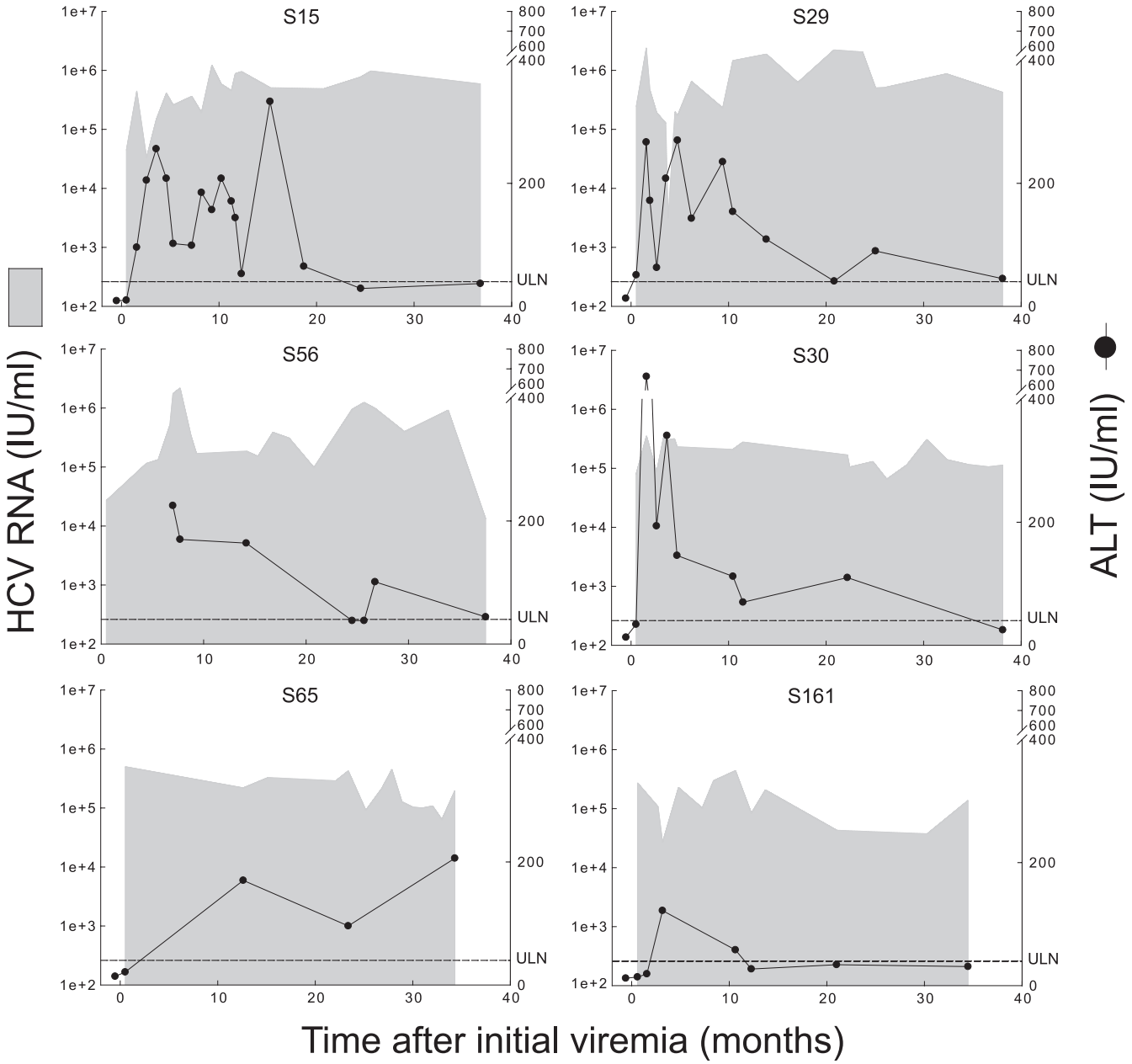

FIG. 1. HCV RNA and ALT levels of six subjects during the 3-year transition from acute to chronic infection. The time of initial viremia (time zero) was calculated as the midpoint between HCV RNA-negative and -positive visits. ALT levels prior to initial viremia were available for all but subject 56 (S56). For subject 65, no serum samples were available for more frequent ALT testing between initial viremia and the year 1 visit.

anti-HCV antibody seroconversion to exclude initially chronic infection, (ii) an interval between HCV RNA negativity and positivity of no more than 3 months to ensure that the first viremic specimen was truly from the acute phase, (iii) annual sampling to ensure that evolutionary rates would not be skewed by irregular sampling intervals, and (iv) 3 years of follow-up to assess early chronic infection. Six subjects satisfied all of the inclusion criteria. As is typical for our cohort (11), all were self-identified as Caucasian, and all were infected with genotype 1a HCV, with a median age of 25 at seroconversion (ranging from 19 to 30). Four annual samples, including a specimen from the initial viremia, were studied. The time of initial viremia was defined as the midpoint between HCV RNA-negative and -positive specimens. The dynamics of HCV RNA and alanine aminotransferase (ALT) levels of these subjects are shown in Fig. 1.

Evolution of HCV envelope glycoprotein-encoding genes is dominated by divergence during the establishment of chronicity. To characterize HCV evolution during the transition from acute to chronic infection at the population level, we sequenced the partial E1/E2 region containing HVR1 from 504 hemigenomic clones, with a median of 21 clones for each visit

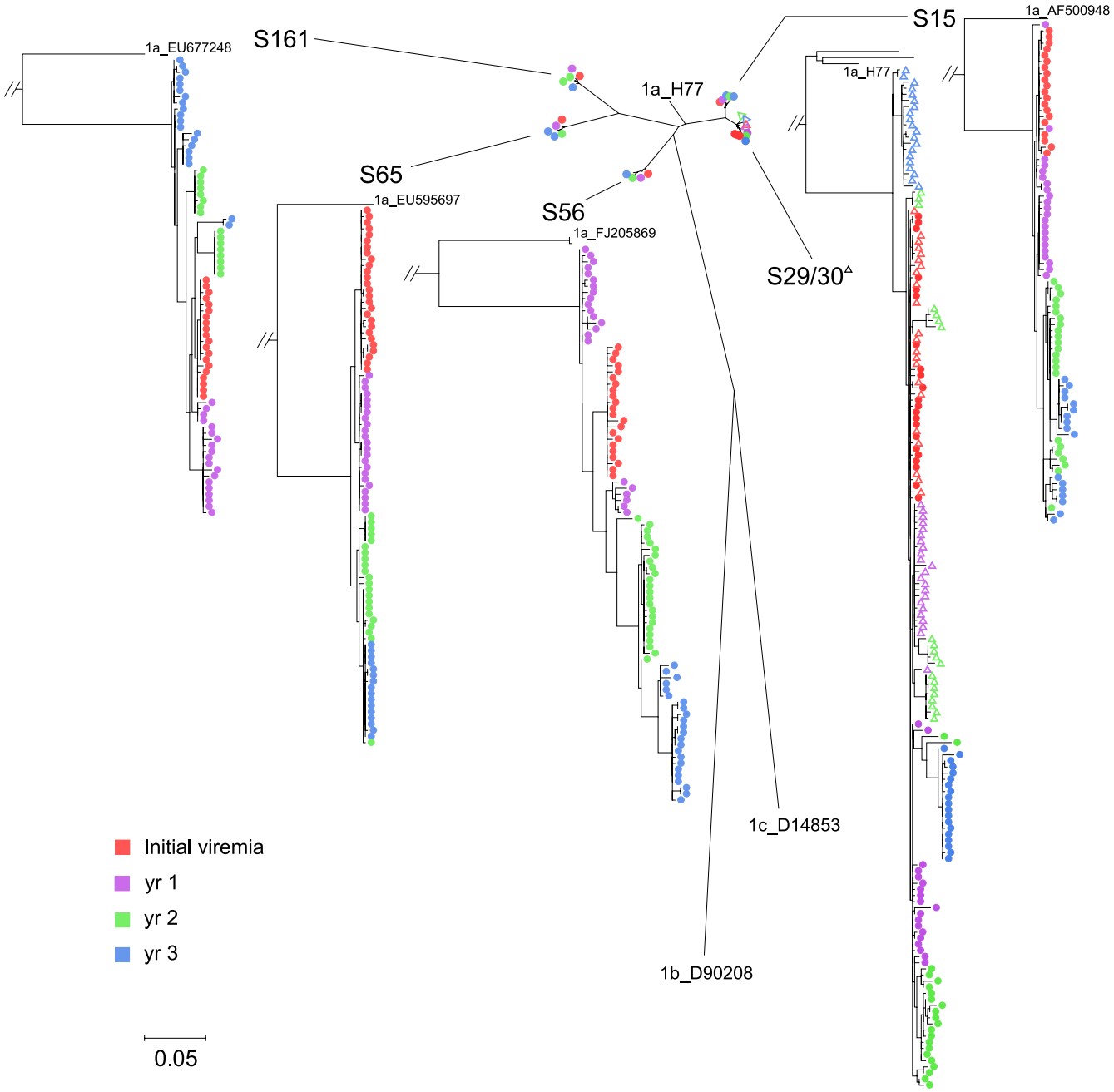

FIG. 2. Phylogenetic trees of hemigenomic and partial E1E2 sequences. The center unrooted maximum-likelihood tree demonstrates the phylogenetic relationship among the hemigenomic sequences from different subjects. All sequences are represented by circles except for those of subject 30, whose sequences are represented by triangles to differentiate them from epidemiologically linked subject 29. Subtype 1a, 1b, and 1c sequences obtained from GenBank are included as references, and accession numbers are given, except for H77, for which the accession number is AF009606. For each study subject, a maximum-likelihood tree was constructed based on partial E1E2 sequences from approximately 20 clones for each visit, with 103 reference sequences and genotype 7 used as an outgroup. For clarity, only the clade containing the subject-specific sequences and nearest reference sequence neighbors is shown.

(ranging from 17 to 43). The overall structure of maximum-likelihood trees built using these partial E1/E2 sequences was consistent with parallel analysis of the hemigenomic clones (Fig. 2). Sequences from sibling subjects 29 and 30 clustered together in one clade, consistent with their self-report of needle sharing and contemporaneous infection.

Evolutionary trends were temporally ordered (shifting to new clades from one visit to the next) but not in a pattern that strictly adhered to changes toward or away from the worldwide consensus. Here, "toward" means centripetal or convergent changes back toward the center of the worldwide tree, and "away" means centrifugal or divergent mutation, as discussed previously (1, 8, 25, 28, 51). Subjects 29 and 30, which followed similar evolutionary courses initially, diverged during the third year (Fig. 2).

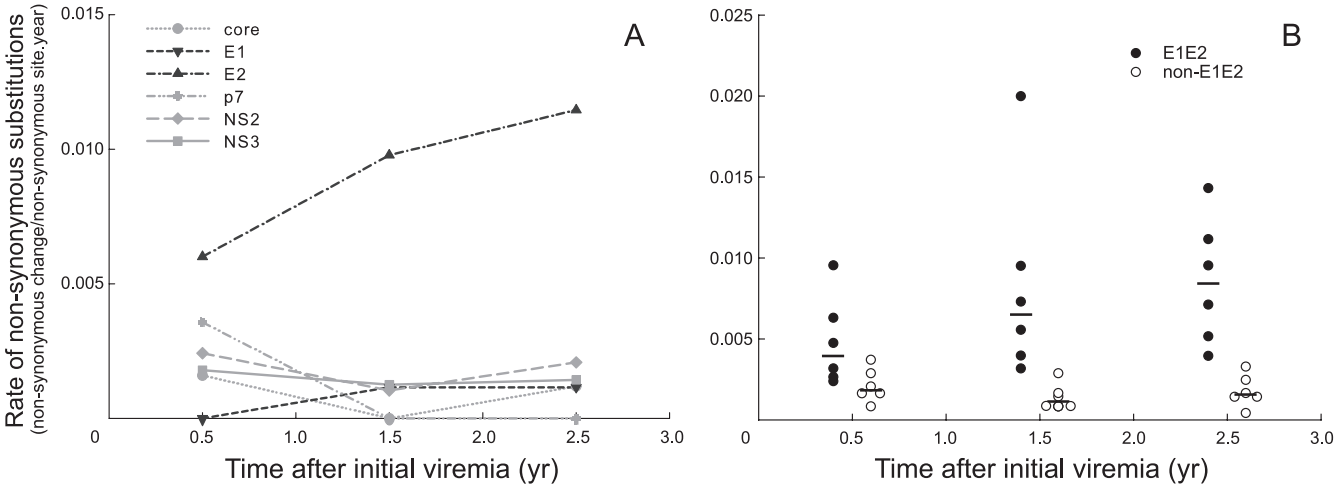

FIG. 3. Rate of nonsynonymous evolution during 3 years of follow-up after primary HCV infection. Plotted are the rates of nonsynonymous change in each gene of the HCV hemigenome, represented by a median value from six individual subjects (A), and in envelope (E1 and E2) versus nonenvelope (core, p7, NS2, and NS3) genes for each individual, with medians represented by horizontal lines (B). Rates are plotted along the horizontal axis at the midpoint between the two annual visits being compared. The difference between E1E2 and non-E1E2 rates was significant ($P = 0.006$) based on a GEE model that corrects for multiple measurements per individual.

Increasing rate of nonsynonymous evolution in envelope genes versus a decreasing rate in nonenvelope genes. Because acute HCV infection that progresses to chronicity is generally associated with early initiation of cellular immune responses that later weaken (9, 10, 27, 44), in contrast to neutralizing antibody responses that have delayed onset but are maintained in chronicity (12, 32, 35), we anticipated a deviation between the evolutionary trends of envelope and nonenvelope genes. To test this hypothesis, we compared the divergence, rate of nonsynonymous evolution (*dN*), and rate of synonymous changes (*dS*) of each gene of the 5.2-kb hemigenome individually as well as by clustering the envelope (E1E2) and nonenvelope (core, p7, NS2, and NS3) genes. A total of 28 clonal hemigenomic sequences, i.e., one or two such sequences from each annual visit of these six subjects, were analyzed, allowing direct comparison of the rate of evolution between different genes. For a summary of the number and distribution of both nucleotide and amino acid mutations, see Tables 1 and 2 posted at http://sray.med.som.jhmi.edu/Liu_supp/JVI-2265-09_supp_Tables.pdf). Overall, the 5.2-kb amplicon has a mutation rate of 5.6×10^{-3}/site/year; the rate is 7.7×10^{-3}/site/year for structural genes and 4.5×10^{-3}/site/year for nonstructural genes.

Divergence of each gene of the hemigenome accumulated over time (data not shown), indicating continuous evolution from the initial quasispecies. However, the rates of nonsynonymous substitution (resulting in amino acid substitution and considered to result from selective pressures at the protein level) showed different trends among genes (Fig. 3). Although they differed in the absolute rate of change, the envelope glycoprotein-encoding E1 and E2 genes shared similarly increasing *dN* rates, whereas nonenvelope genes (core, p7, NS2, and NS3) showed stable or decreasing *dN* rates. The decreasing trends were observed mainly during the first 2 years during which the transition from acute to chronic infection occurs (Fig. 3A) (9, 10). The data for the combination of envelope (E1 and E2) genes and for the cluster of nonenvelope (core, p7, NS2, and NS3) genes as prospectively intended revealed distinct trends of nonsynonymous evolution. In the E1E2 region, the median annual rate of change per nonsynonymous site was 0.0040, 0.0064, and 0.0083 during years 1, 2, and 3, respectively. However, in the non-E1E2 region the median annual rate of change per nonsynonymous site was 0.0018, 0.0011, and 0.0015 for years 1, 2, and 3, respectively. To assess differences in evolutionary rates, a multivariate model was used that included terms for the main effects of time and genomic region as well as an interaction between the two and accounted for multiple observations per person (59). It was found that the rate of nonsynonymous evolution in the E1E2 region was significantly higher than that in the non-E1E2 region ($P = 0.0064$), and this rate in E1E2 increased over time ($P = 0.0016$), in contrast to that in the non-E1E2 region, which did not vary with time ($P = 0.61$) (Fig. 3B).

Analysis of hemigenomic sequences demonstrates that HCV evolution comprises both centripetal and centrifugal substitutions. Like HIV and SIV, amino acid substitutions of HCV can be defined as either centrifugal (forward) or centripetal (reverse) evolution (17, 18, 25, 42). In characterizing the differences in evolution between envelope and nonenvelope genes, we classified changes in the 5′ hemigenome as either centrifugal or centripetal substitutions, relative to a reference alignment of 388 full-length genotype 1a sequences from GenBank (Fig. 4).

Both centrifugal and centripetal substitutions emerged frequently during the first 3 years of infection in all subjects, with 118 centripetal and 130 centrifugal substitutions, demonstrating that both forms of substitution contribute to HCV evolution during the first 3 years of infection (Fig. 4). The rates of both centrifugal and centripetal substitutions differed between envelope and nonenvelope genes, with much higher and increasing rates in envelope genes and stable trends in nonenvelope genes (Table 1). The HVR1 region contained half of all centrifugal mutations (42/85, or 49.4%) and over half of all centripetal mutations (47/78, or 60.3%) in the envelope genes

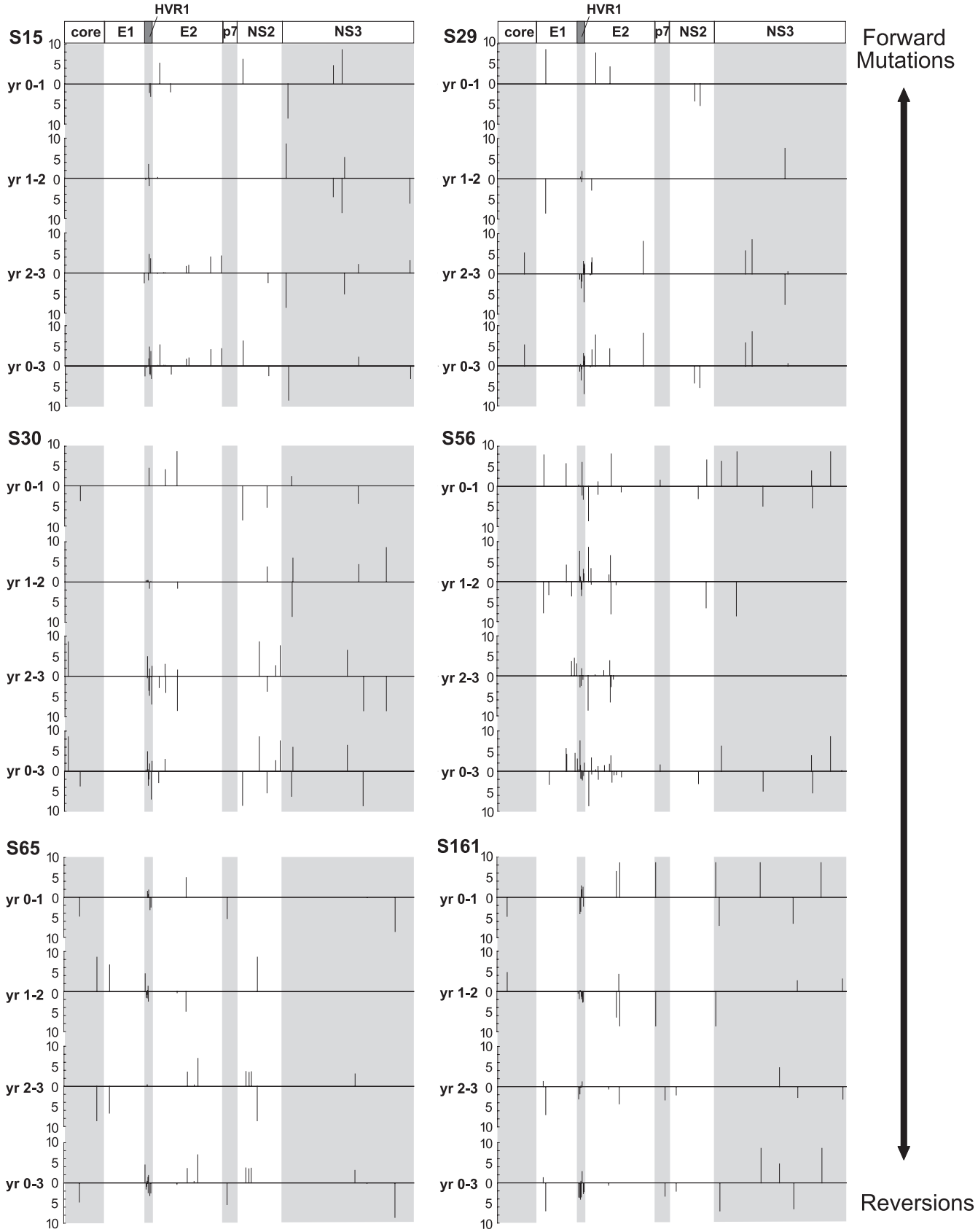

FIG. 4. Forward (centrifugal) and reverse (centripetal) amino acid substitutions during the transition from acute to chronic HCV infection. HCV viral hemigenomic sequence comparisons are depicted as horizontal bars, with the time interval shown on the left. Amino acid substitutions are indicated as vertical lines such that the height and direction are proportional to the change in amino acid frequency among 388 subtype 1a reference sequences, with relative change away from consensus shown above and change toward consensus shown below the horizontal bar, on a base 2 logarithmic vertical scale.

TABLE 1. Summary of median centripetal and centrifugal amino acid substitution rates in different regions during the 3 years of transition from acute to chronic infection

Time period (yr)	Median amino acid substitution rate by site and type (10^{-3} substitutions/site/year)							
	Non-E1E2		E1E2		HVR1		E1E2-non-HVR1[a]	
	Centrifugal	Centripetal	Centrifugal	Centripetal	Centrifugal	Centripetal	Centrifugal	Centripetal
0–1	2.3	3.2	7.2	4.5	55.6	74.1	3.8	0.9
1–2	1.8	1.4	6.3	9.0	92.6	92.6	1.9	3.8
2–3	2.7	2.3	10.8	10.8	92.6	129.6	6.6	3.8

[a] The region of E1E2 excluding the HVR1 region.

(data not shown). The non-HVR1 region of the envelope genes followed an irregular pattern of evolution, probably due to double selection of both cellular and humoral immunity, balanced by functional constraints and resulting in complex evolutionary patterns (9, 10, 14, 23, 25, 55). Notably, the rate of change in E1E2 excluding HVR1 was not significantly different from the rate of change in nonenvelope regions.

HVR1 sequence changes in accordance with neutralizing antibody responses reveal immune-driven evolution. Sliding-window analysis of nonsynonymous changes according to sampling interval with median values of the six subjects are shown in Fig. 5. While the rates of synonymous change were comparable across the 5′ hemigenome (data not shown), as previously noted in chronic infection (42), the rates of nonsynonymous change varied among different regions, with HVR1 having the highest median rate of nonsynonymous change during each of the three annual intervals; notably, this rate increased over time in contrast to the rate for nonenvelope genes (Fig. 5).

Although HVR1 is well known for its high variability, the mechanism of its rapid change is poorly understood. Available evidence suggests that the envelope genes, especially the HVR1 region of E2, are both less constrained than and under different selective pressures from nonenvelope genes (14, 25, 55). This was further illustrated by the different trends of evolution observed in the current study during the transition from acute to chronic infection. Given evidence that HVR1 sequence evolution may be driven by humoral immunity, i.e., neutralizing antibody responses, we compared NAb profiles with HVR1 sequence evolution in a subject from whom appropriate samples had been obtained and infectious autologous HCV E1E2-expressing lentiviral pseudoparticles (HCVpp) had been developed (12) (Fig. 6). During the first 21 months after primary infection, there was no detectable neutralizing antibody response. Surprisingly, there were no amino acid substitutions during that time in HVR1 despite high-level viremia (RNA levels of 0.5 to 1.0 $\times$ 10^6 IU/ml) (Fig. 1). At month 25, when NAb responses were first detected against autologous month 2 HCVpp, amino acid substitutions were also first observed in the HVR1 region. With increasing magnitudes of NAb responses against both month 2 and month 25 HCVpp detected at subsequent visits, HVR1 sequences continued to diverge from both the month 2 and month 25 sequences. Sequence changes after month 25 showed both divergence and diversification, with dominant sequences sharing T395A, R398G, and T400A substitutions but different substitutions at other sites. Interestingly, the R398G and T400A substitutions as well as the T391S and M408K substitutions that had emerged by month 38 could be considered centripetal changes, based on comparison with 388 reference sequences from subtype 1a (depicted at the top of Fig. 6). These data also illustrate transient changes, such as L402F (month 25) and multiple changes at month 38.

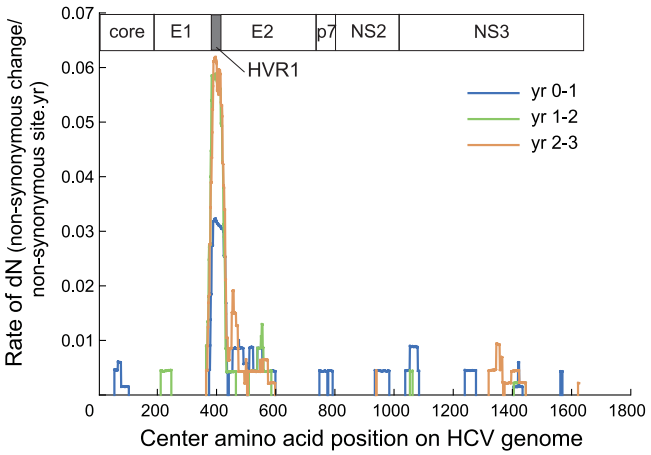

FIG. 5. Nonsynonymous change during 3 years of follow-up, showing median values of the six subjects calculated in a sliding window 50 codons wide, with one-codon increments, and comparing sequential annual visit sequences using the Nei-Gojobori model. The 5′ hemigenomic map is depicted at the top for orientation.

DISCUSSION

In this study, we conducted sequential analysis of HCV hemigenomic sequences for six well-characterized human subjects who declined treatment as their infections progressed from initial viremia to early chronicity. Hemigenomic sequences and annual sampling allow, for the first time, direct comparison of evolutionary rates for different genes and different subjects during the transition from acute to chronic infection. Markedly nonlinear evolutionary rates in E2 were investigated mechanistically using autologous HCVpp to measure NAb titers, revealing that evolutionary stasis of HVR1 was associated with a lack of neutralization, whereas the onset of rapid evolution was explained by rising NAb titers.

In HCV-infected individuals who progress to chronicity, neutralizing antibody responses do not seem to initiate until the chronic phase is established (12, 40), whereas cellular responses are readily detected during the acute phase but lose function during prolonged infection for reasons that are

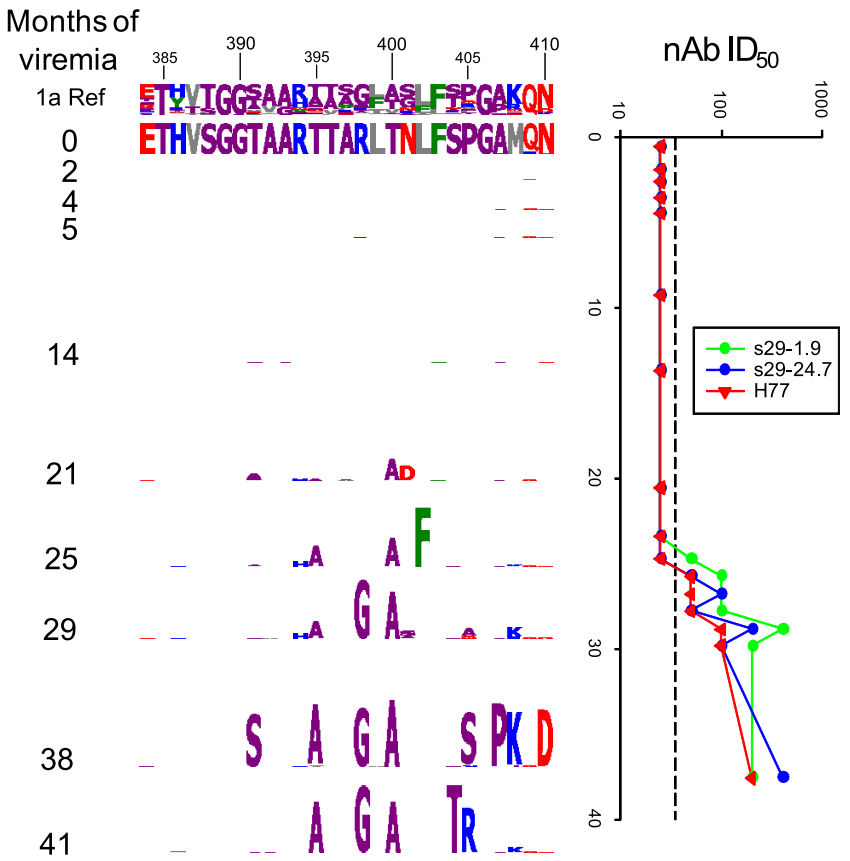

FIG. 6. HVR1 evolution correlated with neutralizing antibody responses in subject 29. Autologous neutralization during 3 years of follow-up from acute to chronic HCV infection is depicted on the right, with the corresponding amino acid sequence evolution on the left. Type 1 sequence logos were used to demonstrate the variability among 388 reference sequences (1a Ref) as well as the initial viral quasispecies from subject 29 (month 0). Amino acid positions are depicted above according to H77 sequence (GenBank accession number AF009606). For months 2 through 41, type 2 logos (20) were used to compare amino acid sequences to month 0 sequence, with the height of each amino acid determined by the $\log_2$ unlikelihood of an amino acid at a given position relative to the initial sequence. To determine NAb ID_{50} titers, HCVpp expressing E1E2 from month 2 and month 25 visits were incubated with serial 2-fold dilutions of autologous plasma. When 50% neutralization was not detected at the starting plasma dilution of 1:50 (dashed line), the result was recorded as one-half this value, a titer of 1:25. Neutralization results represent a single experiment in which both autologous HCVpp samples were assayed concurrently with the full array of plasma samples.

poorly understood (9, 10, 24, 27, 44). Across the HCV genome, CD8 T-cell epitopes have been mapped, and all genes seem to be potential targets of cellular immune pressure (9, 10, 25, 49). Although T-cell epitopes have been mapped within the envelope gene, CD8 T-cell responses appear to have minor relative impact on driving sequence evolution in this region (25). In contrast, the envelope gene is a well-accepted target for humoral immune selection, which appears to be the main selective force driving envelope gene evolution (5, 12, 40, 55). Therefore, the differential timing and targeted genomic locations of humoral and cellular pressure are the most likely mechanisms of uneven evolutionary rates in envelope and nonenvelope genes during the transition from acute to chronic infection.

The current study demonstrates for the first time in a systematic way in humans that HCV envelope and nonenvelope genes have significantly different trends in nonsynonymous evolution ($P = 0.006$), with increasing *dN* values in envelope genes and stable or decreasing *dN* values in nonenvelope genes during the transition from acute to chronic HCV infection. These findings are consistent with our current understanding of the host immune response to HCV, coupled with immune-driven evolution. Previously, Kuntzen et al. reported similar findings showing that there are more T-cell-driven mutations outside the envelope gene during the acute phase of HCV infection than during the chronic phase (25). That they did not observe a trend for envelope evolution may have been due to irregular sampling intervals and a smaller number of subjects. The current findings are also consistent with prior studies of hypogammaglobulinemic humans and with our study of chimpanzees that have poor humoral responses to E2, which revealed evolutionary stasis in HVR1 (4, 5, 43).

In HIV (16), SIV (17), and HCV infection (23, 45), viral immune escape may incur a fitness cost, reducing replication capacity (17, 19) or infectivity (23). Transmission to a new host or reduction in immune pressure in the same host may be associated with reversion to a sequence associated with higher intrinsic fitness, which is generally represented by the population consensus (17, 18, 25, 42, 51); such changes are called centripetal in the current study. Though there are exceptions in which the immune escape variant has become

dominant in a population (37), immune selection is highly individual, as reflected by the evolutionary divergence of sisters S29 and S30 in the current study and in a pair of concurrently HIV-infected monozygotic twins in a prior study (13). In the current report, with well-characterized subjects from whom hemigenomic sequences were obtained annually, we have demonstrated that both centrifugal and centripetal substitutions emerge frequently and contribute to viral evolution during the transition from acute to chronic infection in all six subjects, regardless of the phase of infection or the region of the HCV genome (Fig. 4). This finding is consistent with evidence that escape and reversion both occur during acute infection (25).

It is still controversial whether humoral response plays an important role in controlling HCV infection (7, 12, 15, 40, 52, 55). Earlier studies, primarily of chimpanzees, detected delayed and weak humoral responses, and incomplete protection in animal studies as well as evidence of cell-to-cell spread of HCV in tissue culture has been reported (7, 15, 52). In contrast, a recent human study suggested that spontaneous resolvers tend to have early induction of NAb responses, whereas chronically evolving subjects have delayed initiation of NAb responses (40), arguing for a positive role (or at least correlation) for NAbs in HCV control. With a more sensitive autologous HCVpp method, continual neutralization escape during chronic infection was demonstrated in a single individual (55), supporting an impact of NAbs on viral sequence evolution *in vivo*. However, due to limited availability of samples, virological data were missing for a gap of 14 years, and viral evolution could not be investigated during this crucial period in this study (55). Using the same method, we have recently demonstrated in an acutely infected cohort that virus-specific NAbs drive sequence evolution and correlate with the outcome of infection (12). Here, we further demonstrated the detailed interaction between NAb response and HCV quasispecies evolution during the transition from acute to chronic infection. With autologous HCVpp and sequencing of population HCV clones for each visit, we showed that HVR1 remained stable for 21 months without NAb pressure but kept changing away from the initial sequence after the initiation of a NAb response. These data strongly support the hypothesis that HVR1 evolution is largely driven by the NAb response.

This study is limited by the number of people studied, the lack of information regarding the source (i.e., transmitter) sequence, and inclusion of only half of the HCV genome. Though we are confident that more can be learned by addressing these limitations in the future, we do not believe these limitations biased our results; in fact, our prospective sampling, stringent inclusion criteria, and detailed analysis of hemigenomic clone sequences at standardized intervals make this the largest and most representative study of viral evolution in humans during acute HCV infection to date. It is unfortunate that detailed immunological results are not available for most subjects because inclusion criteria for this study were focused on annual sampling rather than on the availability of a large volume of blood draws (9).

In summary, we determined the first three annual rates of evolution of the core, E1, E2, p7, NS2, and NS3 genes during acute infection in humans. Envelope and nonenvelope genes had distinctly different evolutionary trends, consistent with temporal patterns of immune selection. The accelerating rate of envelope evolution was striking, which is consistent with mounting pressure from neutralizing antibodies, with the nonsynonymous rate ratio of envelope to nonenvelope genes increasing from 2 in year 1 to 5 in years 2 and 3. These findings extend our current knowledge and quantitative understanding of host-HCV interaction during the establishment of chronicity.

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