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Viral Sequence Evolution in Acute Hepatitis C Virus Infection▽†

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CD8$^+$-T-cell responses play an important role in the containment and clearance of hepatitis C virus (HCV) infection, and an association between viral persistence and development of viral escape mutations has been postulated. While escape from CD8$^+$-T-cell responses has been identified as a major driving force for the evolution of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), a broader characterization of this relationship is needed in HCV infection. To determine the extent, kinetics, and driving forces of HCV sequence evolution, we sequenced the entire HCV genome longitudinally in four subjects monitored for up to 30 months after acute infection. For two subjects the transmission sources were also available. Of 53 total nonenvelope amino acid substitutions detected, a majority represented forward mutations away from the consensus sequence. In contrast to studies in HIV and SIV, however, only 11% of these were associated with detectable CD8$^+$ T-cell responses. Interestingly, 19% of nonenvelope mutations represented changes toward the consensus sequence, suggesting reversion in the absence of immune pressure upon transmission. Notably, the rate of evolution of forward and reverse mutations correlated with the conservation of each residue, which is indicative of structural constraints influencing the kinetics of viral evolution. Finally, the rate of sequence evolution was observed to decline over the course of infection, possibly reflective of diminishing selection pressure by dysfunctional CD8$^+$ T cells. Taken together, these data provide insight into the extent to which HCV is capable of evading early CD8$^+$ T-cell responses and support the hypothesis that dysfunction of CD8$^+$ T cells may be associated with failure to resolve HCV infections.

Cellular immune responses play a critical role in the outcome of hepatitis C virus (HCV) infection (11, 24, 47). Spontaneous clearance during the acute phase occurs only in 15 to 45% of subjects (42), and impairment of T-cell functions, general T-cell exhaustion over time (47, 52), dysfunctional dendritic cells (4), or suppression by regulatory T cells (9) have been discussed as possible contributors to the failure to clear chronic infection. In addition, the liver as the site of infection and antigen presentation has the potential to induce immunotolerance (33).

The inherent sequence diversity of HCV represents another contributor to viral persistence. Replication is characterized by a high mutation rate on the order of 1.5×10^{-3} to 2.0×10^{-3} nucleotide substitutions per site per genome per year (8). Mutations are believed to be influenced by both immune selection pressures (10, 15) and neutral sequence "drift" (44), although the relative contribution of these forces to sequence diversity remains to be defined. In human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections a few striking cases of viral escape mutations within immunodominant CD8 epitopes suggest that CD8$^+$ T-cell escape can play a critical role in both early and late loss of immune control against these viruses (3, 5, 23). Likewise, CD8$^+$ T-cell escape mutations have been postulated to decisively influence the outcome of acute HCV infection (13, 15).

Recent data on HIV and SIV now illustrate the extensive role that CD8$^+$ T-cell selective pressures have in shaping viral evolution, with greater than 50% of fixed amino acid substitutions arising after acute infection being associated with CD8$^+$ T-cell responses (1, 39). Other researchers have begun to document reversion of CD8$^+$ T-cell escape mutations upon transmission due to proposed viral fitness costs (19, 25, 31), thus purifying viral sequences in the population back toward a more fit viral consensus phenotype (25). Such reversions have also accounted for a substantial proportion of viral evolution within acutely HIV-infected individuals (32). Two recent studies now support a similar role for CD8$^+$ T-cell responses and reversions in driving HCV evolution (13, 41). However, no longitudinal data on full HCV genomes in acutely infected human study subjects are available, and the majority of studies have been carried out only on single detected epitopes (10, 12, 13, 15, 41, 46, 48, 49).

Knowledge of the immunological and virological factors governing the evolution of highly variable viruses will be important for identifying critical CD8$^+$ T-cell responses for targeting by vaccines. Thus, a broader characterization of the impact of positive and purifying selective forces driving

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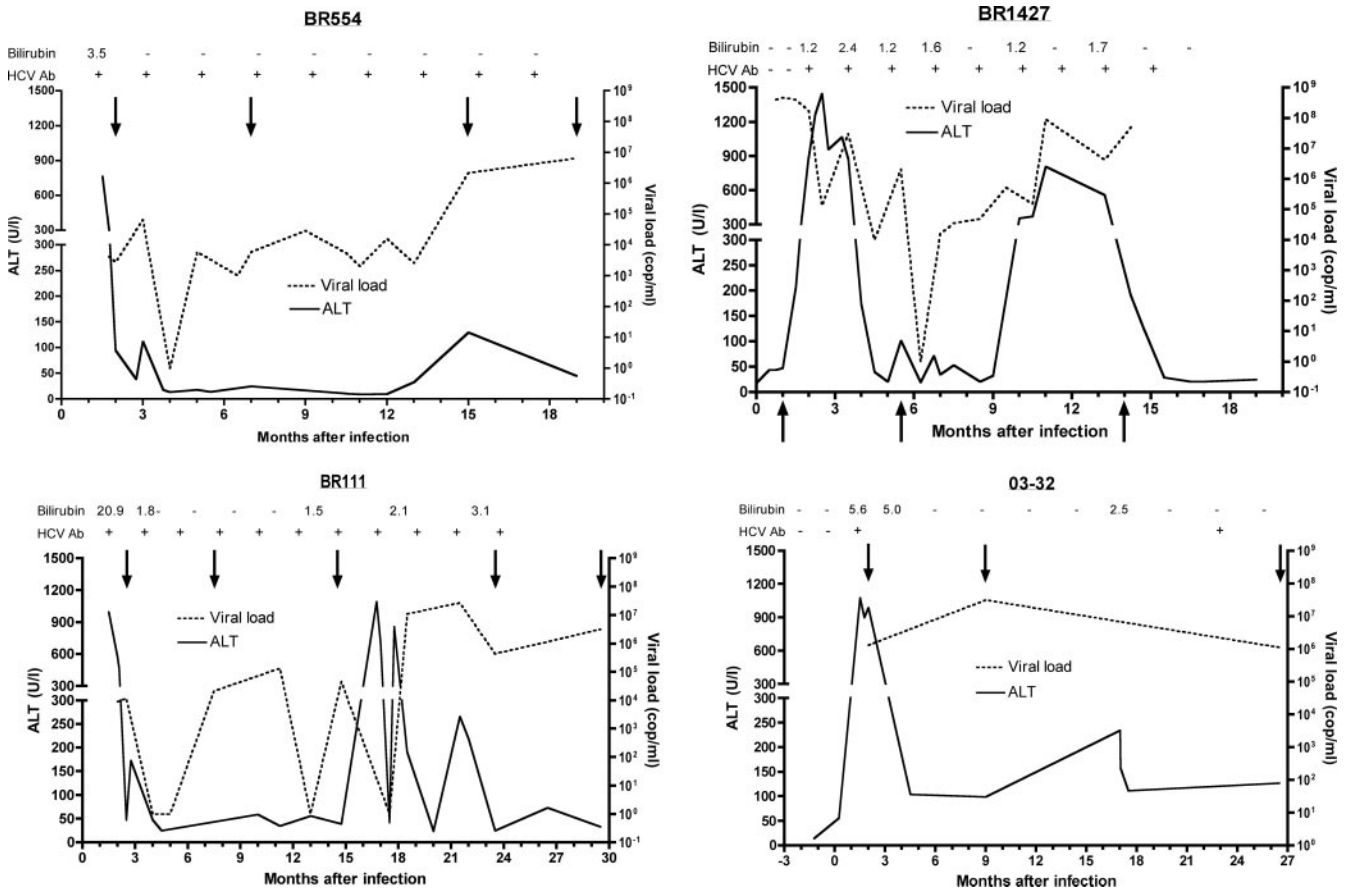

FIG. 1. Clinical course of acute HCV infection. Four study subjects were monitored longitudinally from acute HCV infection. Alanine aminotransferase (ALT) levels, HCV viral loads, bilirubin levels of >1 mg/dl, and the presence of anti-HCV antibodies are shown. Arrows indicate time points from which viral sequences were generated.

HCV evolution in acute through chronic infection are needed. Here we have examined the relationship between genome-wide evolution of HCV and T-cell responses in four acutely infected subjects.

MATERIALS AND METHODS

Patients. In the present study we enrolled three patients from Rio de Janeiro (Brazil) and one from Boston (United States) who displayed acute HCV infection as defined by sudden onset of symptoms without a previous history of liver disease, a recent identifiable exposure, positive HCV RNA, and ALT levels 10-fold above the upper limit of normal. Seroconversion was documented in two patients (Fig. 1). There was no recent history of excessive alcohol intake or use of hepatotoxic drugs. Autoimmune hepatitis and infections with HAV, HBV, cytomegalovirus, Epstein-Barr virus, and herpes simplex virus types 1 and 2, as well as cases of leptospirosis and toxoplasmosis, were excluded by serological tests. The dates of infection for patient 03-32 (genotype [GT] 1a, monitored in short intervals because of known intravenous drug abuse) and BR554 (GT1a, sexual transmission), were estimated according to average values from the literature (14, 16, 38, 47) as 6 weeks before seroconversion (03-32) and 7 weeks before the onset of symptoms (BR554). For patients BR1427 (GT1b, needlestick injury) and BR111 (GT1a, infected during abdominal surgery) the time point of infection was known. All patients were untreated and unable to clear the virus during 14 to 29.5 months of follow-up. Putative sources of infection (BR601 and BR1430, donors) were identified for patients BR554 and BR1427 (recipients), respectively. Serum samples of these sources were available from the time point of transmission (BR1430/BR1427) or 2 weeks after the time point of the onset of symptoms in the recipient (BR601/BR554). A phylogenetic analysis of viral sequences from these samples verified BR601/BR554 and BR1430/BR1427 as transmission pairs (Fig. 2). No DNA samples for HLA typing could be obtained for either source patient. The present study was approved by the local Institutional Review Board, with all subjects giving written informed consent.

HLA typing and viral loads. HLA genotyping was performed by the Massachusetts General Hospital Tissue Typing Laboratory (Boston, MA) using standard molecular techniques. Viral loads were measured using the Cobas Amplicor HCV Monitor version 2.0 (Roche Molecular Systems, Branchburg, NJ).

Sequencing of autologous virus. Viral RNA was extracted from plasma samples using a vRNA extraction kit (QIAGEN, Hilden, Germany). A nested PCR with 12 external and 36 internal primer pairs spanning the entire HCV coding sequence amplified overlapping fragments of approximately 500 to 1.2 kb (see Tables S1 and S2 in the supplemental material). Using the QIAGEN One-Step RT-PCR kit, the RT-PCR cycling conditions were as follows: 50°C for 60 min and 95°C for 15 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 2 min at 72°C, with a final extension of 68°C for 20 min. Nested PCR conditions were 35 cycles of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C, with a final extension of 68°C for 20 min using Titanium *Taq* DNA-Polymerase (BD Clontech, Palo Alto, CA). PCR fragments were then purified (PCR purification kit; QIAGEN) and population sequenced bidirectionally on an ABI 3730 Prism automated sequencer. Sequence data are available from GenBank under accession numbers EF032883 to EF032900.

Sequence analysis. Sequencher (Gene Codes Corp., Ann Arbor, MI) and MacVector 4.1 (Oxford Molecular) were used to edit and align sequences. Mixed bases were called if the chromatogram showed two peaks at the same residue, with the lower peak reaching >25% of the dominant peak. By evaluation of several overlapping sequence fragments, only clear changes in the dominant base were identified as mutations. The majority of amino acid changes became fixed over time, although 16 mutations (16 of 137 [11.6%]) were only transiently observed and thus were excluded from further analysis.

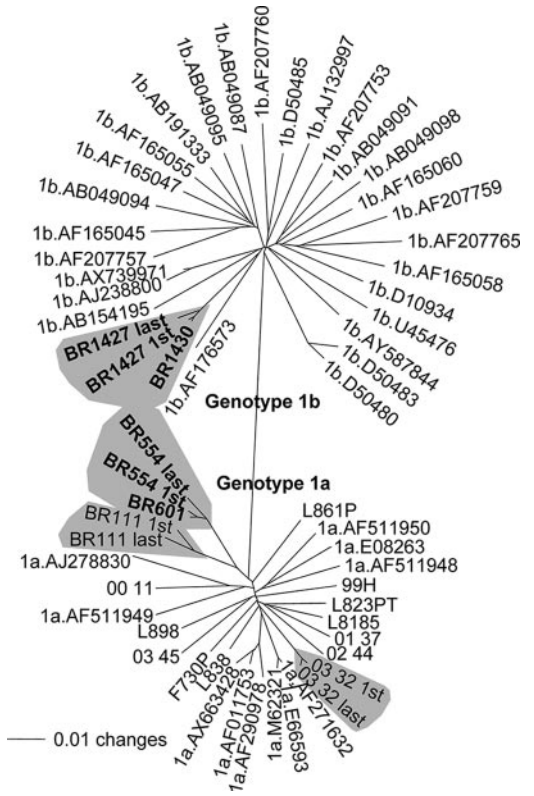

FIG. 2. Close phylogenetic relationship between viral sequences in transmission pairs. Using the neighbor-joining method, HCV sequences 2,960 amino acids in length were compared to 38 HCV genotype 1 sequences arbitrarily chosen from the Los Alamos HCV database and an additional 11 full-length GT1a sequences generated in-house (Timm et al., unpublished). Sequences of the earliest time points in the recipients (BR554 first, BR1427 first) are more closely related to sequences from the sources (BR601 and BR1430) than to consecutive sequences in the recipients (BR554 last, BR1427 last) or any other GT1 sequence from the database (bootstrap values 93 to 100), confirming transmission of the virus from BR601 to BR554 and from BR1430 to BR1427.

Phylogenetic analysis. Nucleotide sequences were translated into amino acid sequences and aligned to arbitrarily chosen full-length reference sequences from the Los Alamos National Laboratory (LANL) Database (http://hcv.lanl.gov, accession numbers provided in Fig. 2) and additional sequences generated in-house using CLUSTALX software. Phylogenetic trees were constructed by the neighbor-joining method with mean character distances on PAUP4.0b10 software, with calculation of bootstrap values based on 1,000 replications. Prior to the calculation of the ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site (dN/dS), mixed bases in the data set were converted into dominant base calls. To account for possible bottleneck effects upon transmission in the two transmission pairs, dominant base calls were chosen so that no change was counted between the donor and the recipient. SNAP (27), a web-based tool provided by the LANL Database, was then used for the calculation of dN/dS ratios.

Consensus sequences and conservation scores. Using 130 HCV1b (LANL) and 74 HCV1a (J. Timm et al., unpublished data) full HCV genome sequences HCV consensus sequences were generated separately for HCV genotypes 1a and 1b, and conservation scores for each amino acid residue in the coding region of HCV were calculated based on normalized Shannon Entropy (43), distinguishing 21 amino acids using a web-based algorithm (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl).

IFN-γ ELISPOT and ICS. HCV-specific $CD8^+$ T-cell responses were quantified by enzyme-linked immunospot (ELISPOT) assay on 200,000 peripheral blood mononuclear cells (PBMC) per well, stimulated with pools of 10 or 20 antigenic peptides at a final concentration of 10 μg/ml for each peptide as described previously (28). Responses were confirmed by single-peptide ELISPOT with 10 μg of peptide/ml, and assays were performed in duplicate for the quantitation of ex vivo responses. Responses were considered positive if the number of spots per well minus the background was at least 25 spot-forming cells (SFC)/10^6 PBMC with background levels of <15 SFC/10^6 PBMC. To verify $CD8^+$ T-cell responses, intracellular cytokine staining (ICS) for gamma interferon (IFN-γ) was performed by testing peptide-stimulated T-cell lines as described previously (28).

Bulk stimulation of PBMC. To establish $CD8^+$ T-cell lines, cryopreserved or fresh PBMC (4×10^6 to 10×10^6) were stimulated with 1 μg of synthetic HCV peptide/ml and 0.5 μg of the costimulatory antibodies anti-CD28 and anti-CD49d (Becton Dickinson)/ml in R10 media. Recombinant interleukin-2 (25 IU/ml; BD Biosciences) was added on day 2 and twice a week thereafter. Cells were restimulated with 2.5×10^7 irradiated PBMC after 2 weeks.

Statistical analysis. Fisher's exact test was used to compare mutation frequencies inside and outside epitopes, and a generalized estimating equation (54) with identity link function and sandwich-based standard error was used to assess mean differences in conservation scores between forward and reverse mutations. *P* values of <0.05 were considered significant.

RESULTS

Longitudinal sequencing of acute infected subjects and transmission pairs. To characterize viral evolution in our four acutely infected patients, we sequenced the entire viral genomes in sequential samples obtained during the acute infection and up to 29.5 months of follow-up (Fig. 1). By comparing viral sequences between patient samples from sequential time points, we identified 121 amino acid changes across the four chronically infected subjects, ranging from 12 to 46 mutations per individual (Fig. 3). Because mutations in envelope are substantially driven by humoral immune pressures (7), the envelope region was analyzed separately. Here, 68 (56%) mutations were observed within envelope and 53 (44%) outside envelope.

High rates of viral evolution early after transmission. The establishment of chronic infection versus clearance appears to be commonly determined within the first 6 months of infection (22). In order to assess potential differences in the kinetics of viral evolution during this acute phase versus the chronic phase of infection, viral amino acid changes between a baseline sequence and the sequence from a later time point were analyzed for three different periods: the transmission phase between the source sequence and the first time point in the recipient (patients BR554 and BR1427), an acute phase between the first time point in the recipient and a time point around six months of infection, and a longer (average of 15 months) chronic phase, including the remaining time of follow up for each patient. Due to limited sample availability the start and end points for the acute phase varied between 1 and 2.5 months and between 5.5 and 9 months after the time point of infection, respectively, in the different patients (Fig. 3).

Outside envelope, in the two transmission pairs 7 of 26 (27%) mutations were observed upon transmission, 8 of 26 (31%) arose during the acute phase, and 11 of 26 (42%) arose during the chronic phase. When all four patients were combined, excluding the transmission event, 24 of 46 (52%) mutations appeared during the acute phase, and 22 of 46 (48%) mutations arose during the longer chronic phase window. To determine the kinetics of viral evolution after entry into a new host, we calculated the rate of amino acid substitutions between sequential time points. In the two transmission pairs BR601/BR554 and BR1430/BR1427 the highest rate of sequence changes outside envelope was observed upon transmis-

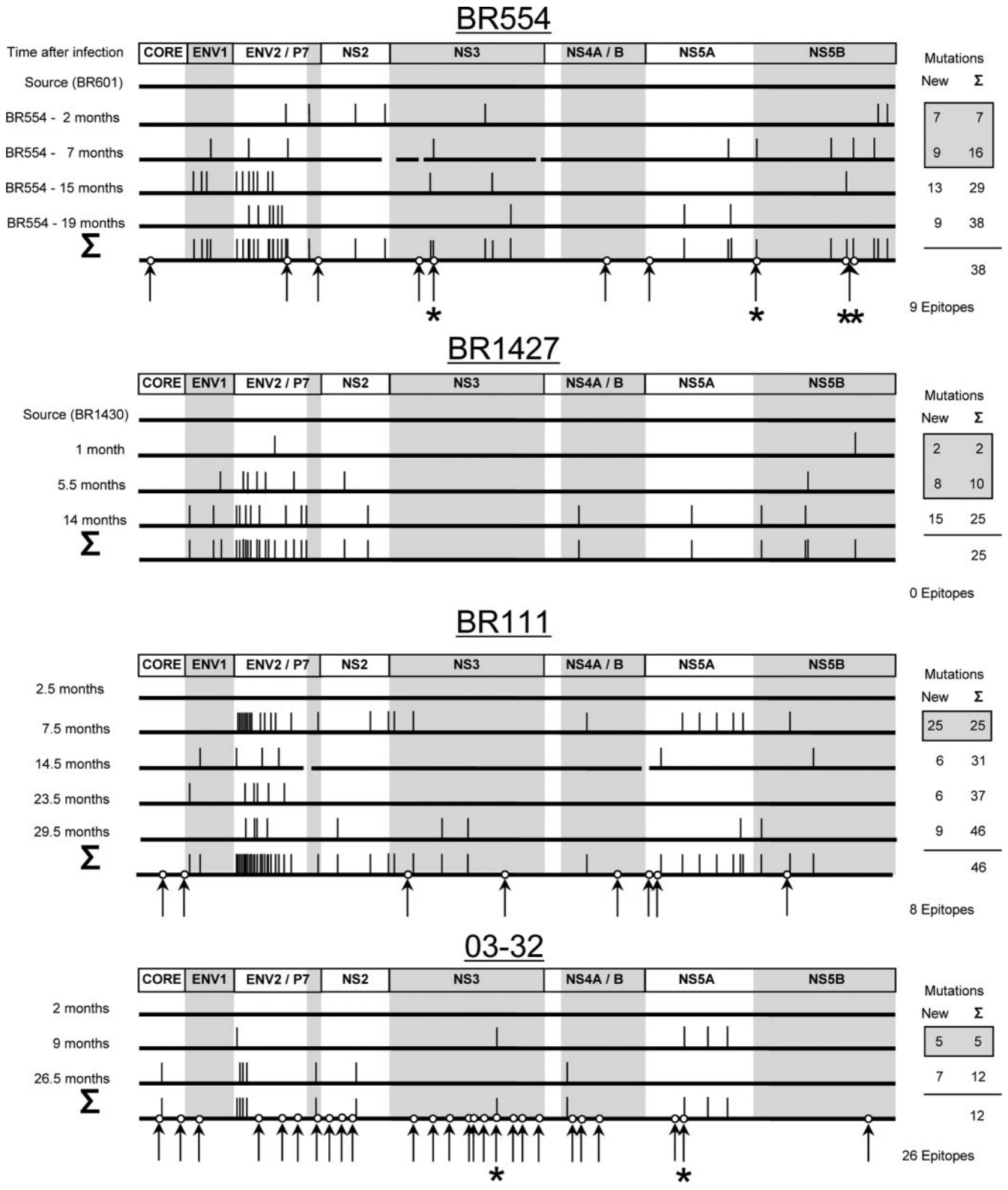

FIG. 3. Sites of sequence variation and detected $CD8^+$ T-cell responses. HCV viral genome sequences at multiple time points are illustrated as horizontal bars with time points shown on the left. Sequence changes are indicated as vertical dashes for each sequenced time point. The number of new mutations and the sum of mutations since the earliest available time point are listed on the right, with mutations considered to have happened during the early phase of the infection being framed in gray boxes. Detected $CD8^+$ T-cell responses are depicted as circles and arrows. Asterisks mark targeted epitopes in which sequence variations were observed over time.

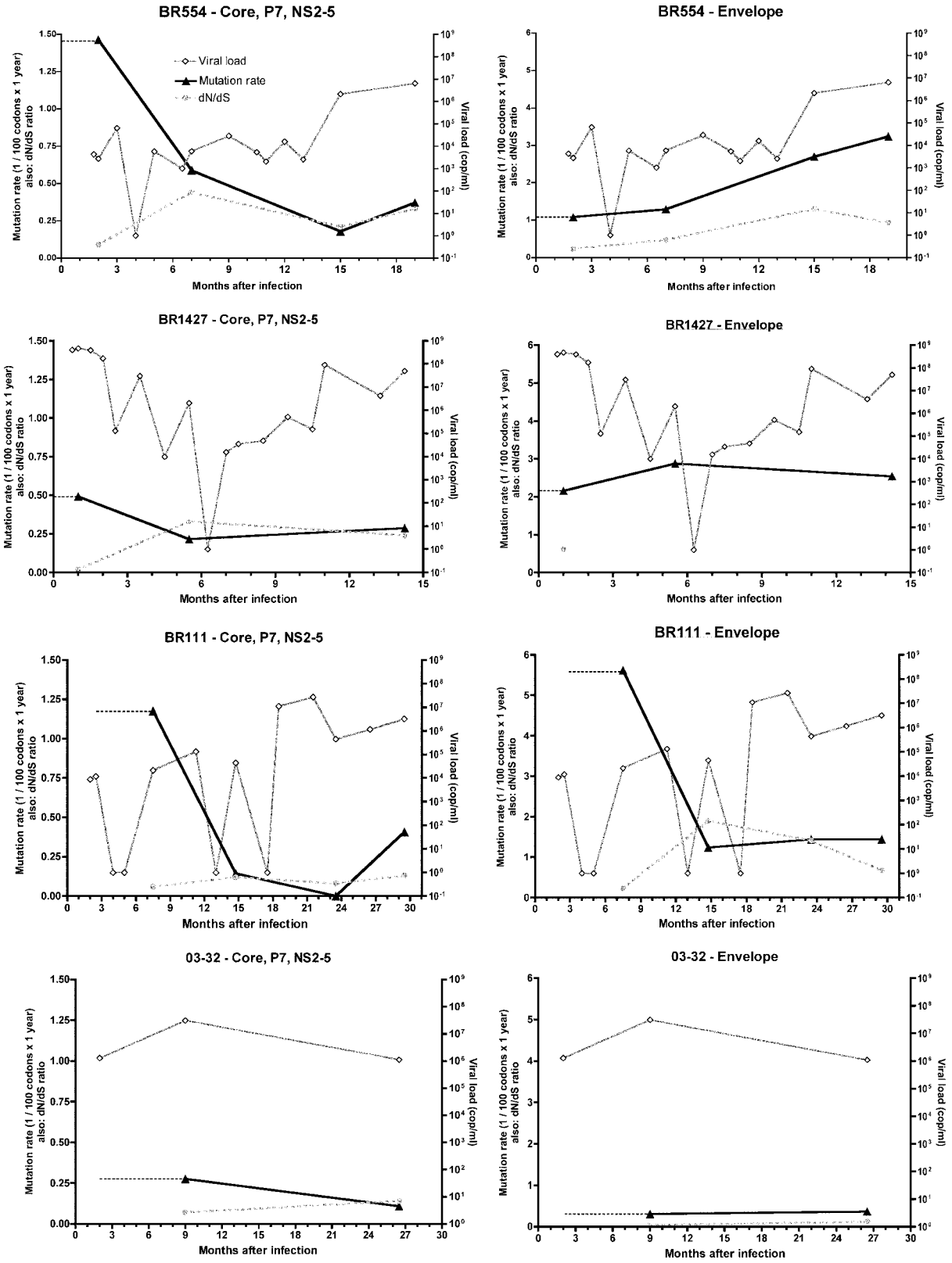

FIG. 4. Rates of nonsynonymous changes decline over the course of infection. Rates of nonsynonymous changes in the dominant viral sequence (per year, normalized per 100 codons) are indicated separately for envelope and for the rest of the HCV genome. Independent of the viral loads, the rate of sequence changes is highest during the acute phase of the infection and decreases thereafter. dN/dS ratios are low upon transmission and fluctuate around individually higher levels thereafter.

sion (Fig. 4). After transmission, the mutation rate was highest during the acute phase of infection and declined two- to four-fold thereafter in patients BR554, BR111, and 03-32. Within patient BR1427, however, the rate of evolution was stable at a low level after transmission.

The dN/dS ratio is commonly used to differentiate between random genetic drift and evolution driven by selective pressures. In order to evaluate the hypothesis that high initial mutation rates might represent early adaptation to selection pressures in the new host, we calculated dN/dS ratios between all sequenced time points and analyzed these in conjunction with the viral loads over time. dN/dS ratios were low for the transmission event in both transmission pairs but remained relatively constant later in all patients (Fig. 4), suggesting higher rates of random genetic drift during the early phase after transmission. Notably, however, in patient BR554 this observation did not coincide with high replication rates, which would have allowed for more random errors by the HCV RNA-dependent RNA polymerase. During the later course of the infection intraindividual changes in the mutation rates were similarly independent of the viral loads, although these varied dramatically between patients, as well as within patients over the course of infection (Fig. 4). In summary, HCV sequence evolution showed considerable variation between patients but seemed to appear in large part early after transmission. Although dN/dS ratios suggested random errors during viral replication as a possible cause for the high mutation rates upon transmission, a lack of correlation with the rate of viral replication did not support this hypothesis.

Role of HCV specific CD8$^+$ T-cell responses in driving viral evolution. In HIV and SIV 53% to 60% of amino acid mutations outside envelope appear to be driven by CD8$^+$ T-cell responses (1, 39). Evidence for a strong link between CD8$^+$ T-cell pressures and viral amino acid mutations in HCV was similarly demonstrated by a 7- to 13-fold higher frequency of these mutations within CD8$^+$ T-cell epitopes (13, 15). In order to provide a comprehensive analysis of the relationship between host cellular immune pressures and HCV sequence evolution, we performed a genome-wide analysis of CD8$^+$ T-cell responses. These were tested ex vivo by ELISPOT assay in PBMC derived from the first sequenced time point using a set of overlapping 15- to 18-mer peptides spanning the entire HCV-H77 proteome (genotype 1a), as well as 100 described optimal HCV epitopes (28, 30, 51). All detected responses were confirmed on peptide-stimulated T-cell lines by ICS. In addition, responses were also tested after stimulation of PBMC in tissue culture with a set of peptide-epitopes matching the patients' HLA alleles (15 each for patients BR111 and BR554, 5 for patient BR1427; Table 1). Here, due to limited resources, PBMC from different time points were used (Table 2). Using this approach a total of 41 CD8$^+$-T-cell responses (31 ex vivo, 10 after stimulation) were detected. For the two transmission pairs we also synthesized 17 autologous 21-mer peptides in the regions undergoing viral evolution where our peptide set differed from the autologous viral sequence. Although T-cell lines were stimulated with these autologous peptides, only two additional CD8$^+$ T-cell responses were detected, accounting for a total of 43 epitopes in the four patients (Fig. 3 and Table 1).

In examining the location and kinetics of mutations outside envelope, 6 of 53 (11%) mutations arose within regions targeted by detectable CD8$^+$ T-cell responses. From a different perspective, 5 of 38 (13%) detected epitopes exhibited sequence variation over time. This represents a 3.5-fold-higher likelihood for mutations to appear within detected epitopes than within other regions of the genome ($P = 0.01$). For mutations falling within detected CD8$^+$ T-cell epitopes the impact of these mutations on epitope recognition was studied on PBMC derived from the time points immediately before the mutation appeared. After culturing T-cell lines in the presence of wild-type peptide their reaction against the original and variant peptides was tested in ELISPOT titration assays. A difference of $\geq$10-fold in the peptide concentration necessary to induce 50% of the maximum IFN-γ response was considered significant. Here, four out of five variant peptides elicited significantly weaker responses compared to the wild type, validating the impact of these mutations on CD8$^+$ T-cell recognition (data not shown).

Since it has been hypothesized that escape mutations might be decisive to the outcome of the acute HCV infection (13, 15), we assessed whether escape mutations might appear at higher rates within the acute phase. As illustrated in Fig. 3, five of six mutations within targeted CD8 epitopes arose during the acute phase, with mutations being a mean 5.3-fold more likely to appear within detected epitopes ($P = 0.004$). In contrast, during the chronic phase only one of six CD8-associated mutations were observed. Notably, none of the seven sequence changes upon transmission were located within detected CD8$^+$ T-cell epitopes. Among all mutations, the rate of mutations associated with CD8$^+$ T-cell responses also tended to be higher during the acute phase (5 of 24 mutations [20.8%] compared to the chronic phase (1 of 22 mutations [4.5%]) (not significant). Thus, CD8$^+$ T-cell responses represented an associated driving force for viral mutations predominantly during the acute phase of HCV infection but did not demonstrably influence sequence changes upon transmission. Notably, however, the majority of targeted CD8$^+$ T-cell epitopes remained invariant over time.

Reversions similarly contribute to HCV sequence evolution. In addition to immune selection pressures influencing viral evolution, recent studies have begun to document the ability of mutations to revert to consensus residues upon transmission to a new host (2, 19, 31, 48). Although due to a lack of samples we could not directly assess immune driven mutations or mutations in adaptation to other selective forces in the viral donors, we used the direction of sequence mutations away from or toward an HCV genotype specific consensus sequence as an estimate measure to identify putative reversions from previous selection pressures in our data set. Here, 10 of 53 (19%) mutations outside envelope in our patients evolved toward consensus consistent with reversion. Notably, all six of the CD8$^+$ T-cell associated sequence mutations described above were forward mutations. In examining the kinetics of reversions over time, 8 of 10 (80%) reversions arose upon transmission or during the acute phase of the infection versus only 2 of 10 (20%) in the chronic phase. Although mutation rates are higher in the acute phase in general, a declining trend (not significant) for reversions was also seen among all mutations over time, where 2 of 7 (29%) mutations upon transmission, 6 of 24 (25%) mutations during the acute phase,

TABLE 1. Detected epitopes[a]

Patient	Epitope sequence	Variant sequence	H77 position	HLA restriction[b]
BR111	NEGCGWAGW		88–96	B44
	LLALLSCLTV		178–187	A2
	CINGVCWTV		1073–1081	A2
	KLVALGVNAV		1406–1415	A2
	ILAGYGAGV		1851–1859	A2
	VLSDFKTWL		1987–1995	A2
	RQVGDFHYV		2094–2102	A2
	ALYDVVSKL		2594–2602	A2
BR554	YLLPRRGPRL		35–44	A2
	YRLWHYPCTI		613–622	Cw7
	LYGMWPLLL		790–798	Cw7
	CINGVCWTV		1073–1081	A2
	HAVGIFRAA	HVVGIFRAA	1175–1183	A2
	LFFNILGGWV		1807–1816	A2
	VLSDFKTWL		1987–1995	A2
	SLLRHHNLVYSTTSRSA	SLLR**N**HNLVYSTTSRSA*	2449–2465	
	THFFSVLIARDQ	TH**L**FSVL**M**ARDQ*	2847–2858	
03-32	NEGCGWAGW		88–96	(B44)
	LLALLSCLTV		178–187	(A2)
	NASRCWVAM		234–242	B35
	*RLADFA*QGW		461–469	(B53)
	NTRPPLGNWF		541–550	(B57)
	YRLWHYPCTI		613–622	Cw7
	FYGMWPLLL		790–798	Cw7
	LSPYYKRYISW		831–841	(A25)
	HPTLVFDITKL		881–889	
	AVFGPLWIL		894–902	
	CINGVCWTV		1073–1081	(A2)
	VTRHADVIPV		1133–1142	(A2)
	HAVGIFRAA		1175–1183	(A2/A68)
	ATLGFGAYMSKA		1260–1271	A3
	GVDPNVRTGVRT		1273–1284	
	YDIIICDECHSTDATSIL		1310–1327	(B38)
	HPNIEEVAL	H**S**NIEEVAL*	1359–1367	B35
	ATDALMTGF		1436–1444	A1
	CVTQTVDFSLDPTFTIETTTL		1457–1477	
	TLTHPITK		1636–1643	B8
	LPYIEQGMML		1715–1724	B35
	VITPVVQTNW		1745–1754	
	LTTSQTLLF		1801–1809	(B57)
	HRFAPPCKPLLR		2131–2142	(B27)
	EPEPDVAVL	EPEPDVA**W***	2163–2171	B35
	LGVPPLRAWR		2912–2921	(B57)

[a] Epitope sequences and their positions according to the H77 reference sequence are indicated. Two peptide epitopes specifically synthesized according to the wild-type viral sequence are printed in italics in column 2. Variant peptides that resulted in reduced antigen recognition by ELISPOT titration assay are marked with an asterisk. The specific difference is indicated in boldface. No epitopes were detected for patient BR1427.

[b] Known HLA restrictions for each epitope are given. Described HLA restrictions not matching our patients' HLA types are given in parentheses.

and 2 of 22 (9%) mutations in the chronic phase of infection represented reversions. In summary, reversions as the result of purifying selective pressure seemed to importantly contribute to viral evolution and illustrated a trend toward a stronger influence during transmission and the early phase of infection.

The kinetics of viral evolution are influenced by the inherent conservation of residues. Escape mutations in CD8 epitopes are considered influential to the outcome of acute HCV infection but have been observed to also arise late during the chronic phase of the infection in chimpanzees (15). This variability may be influenced not only by the strength of the cellular immune response but also by the impact of escape mutations on viral replication capacity. Functional and structural constraints may prevent certain sequence changes and therefore result in the preservation of optimal residues (1, 34). To examine whether structural constraints were impacting the kinetics of viral evolution, we calculated conservation scores for all amino acid residues across HCV as a measure of residue stability (43). Conservation scores were then compared between early (including the transmission event) and late arising mutations and also between forward and reverse mutations. As depicted in Fig. 5, early arising forward mutations preferentially arose at more variable residues compared to late arising mutations ($P = 0.01$). In contrast, early reversions showed a trend toward arising faster at highly conserved residues (not significant). Therefore, functional or structural constraints appeared to influence the kinetics of forward mutations and

TABLE 2. ELISPOT screening time points[a]

Patient	ELISPOT screenings (mo)	HLA type		
		HLA-A	HLA-B	HLA-Cw
BR111	2.5/5.5/7.5/19#/46.5#	02/23	44/44	04/05
BR554	2#/3.5#/7/11#/19	02/31	39/51	07/15
BR1427	2/2.5/4#/14.5#	26/29	15/15	02/03
03-32	2/9/26.5	01/03	08/35	04/07

[a] Time points of ELISPOT screenings for $CD8^+$-T-cell responses (at months posttransmission) are given, and visits that included ELISPOT screening of peptide-stimulated T-cell lines are marked (#). The patients' HLA types are also indicated.

possibly also reversions over the course of infection, limiting the rate of forward mutations while enhancing the rate of reversions.

Different patterns of viral evolution observed in envelope. High rates of viral evolution in the envelope glycoproteins, especially hypervariable region 1 (HVR1), have been described as a function of humoral immune pressures (7, 50). We investigated here whether $CD8^+$ T-cell responses targeting envelope (28) might also contribute to its sequence diversity. Of the 68 envelope mutations, 45 (66%) mutations were situated in HVRs. Eight $CD8^+$-T-cell responses, all outside the HVR, were detected, but none of these CD8 epitopes overlapped with one of the remaining 23 mutations. These data suggest that cellular immune pressures may have little effect on driving sequence diversity in the more conserved regions of HCV envelope, whereas we cannot make a statement for the HVR since our consensus peptide set may easily have missed $CD8^+$ T-cell responses in this region. The rates of sequence evolution did not show a common trend over time in the four patients, and no relationship between mutation rates and viral replication levels could be observed (Fig. 4). Surprisingly, however, the influence of reversions on envelope was quite profound, with 27 of 68 (40%) mutations evolving toward consensus. In summary, these results indicate a remarkable impact of reversions on intrahost viral evolution in envelope, whereas we could not detect a significant influence of $CD8^+$ T-cell driven forward mutations, revealing striking differences in the evolution of envelope compared to the rest of the viral genome.

DISCUSSION

In the present study we describe the extent and kinetics of viral sequence evolution after acute HCV infection and the impact of $CD8^+$ T-cell responses and purifying pressures. Outside the envelope, 30% of all mutations were driven by $CD8^+$ T-cell responses or purifying pressures, both influencing viral evolution predominantly during the acute phase of the infection (20.8 and 25% of all mutations, respectively), compared to the chronic phase (4.5 and 9%). The kinetics of both forward and reverse mutations correlated with the degree of conservation of the mutating residue on a population level. Outside the envelope, the rate of sequence evolution declined over time in three of the four patients. In contrast, viral evolution in the envelope displayed different kinetics in each individual, and, moreover, 40% of the mutations represented reversions, whereas no influence of $CD8^+$ T-cell escape mutations was detected. Therefore, overall different forces were contributing to the evolution of envelope versus the rest of the HCV genome.

Similar to HIV and SIV, viral escape from $CD8^+$ T-cell responses is also a frequent phenomenon in HCV infection (13, 15, 41, 48, 49). Previously, Cox et al. examined viral evolution in hemiviral genomes over the first 6 months of infection in five to eight acutely HCV-infected subjects (13) and observed that 25% of sequence mutations were associated with detectable $CD8^+$ T-cell responses. These values are similar to our findings of 20.8%. In contrast, however, the percentage of epitopes displaying sequence evolution over time between the Cox et al. study and ours (58% versus 13%, respectively) and the likelihood for mutations to appear within $CD8^+$ T-cell epitopes (13-fold versus 5.3-fold, respectively) were both markedly higher in the Cox et al. study. Considering the substantial variation between patients and the small sample size in both studies, these differences may simply be explained by natural variation between individuals. Alternatively, they may be due

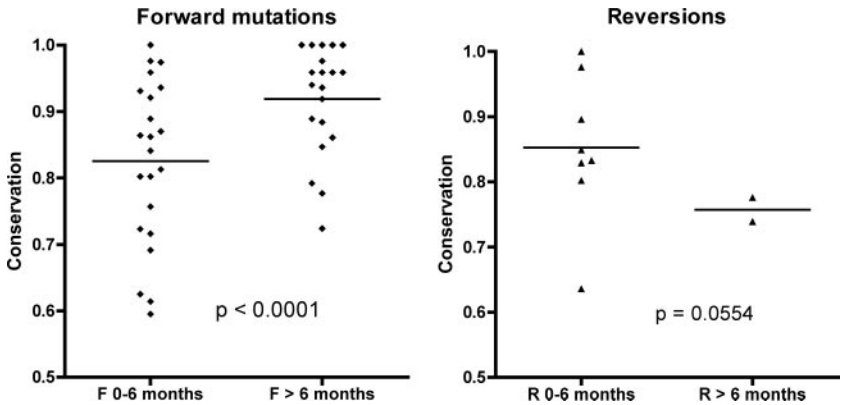

FIG. 5. The rate of forward and reverse mutations correlates with the conservation of amino acid residues on a population level. Conservation scores were plotted for the residues in which forward and reverse mutations were detected, comparing mutations arising between the first six months of infection and the remaining time of follow up. Early arising forward mutations were located within less conserved residues compared to late arising mutations, while reverse mutations tended to arise faster (nonsignificant) within amino acid residues exhibiting a higher degree of conservation.

to differences in the patient characteristics between cohorts (e.g., primarily i.v. drug use in the Cox et al. study). Although a clear correlation between the magnitude of the cellular immune response and emergence of escape variants has not been described, it is also possible that use of the in vitro peptide stimulation protocol in our study may have preferentially identified weak immune responses that may be less likely to induce viral escape. More notable, perhaps, is that in HIV and SIV $CD8^+$ T-cell selective forces are associated with upward of 60% of non-Env mutations (1, 39) compared to the significantly lower percentages we (4.5 to 20.8%) and Cox et al. (25%) have observed in HCV. This difference may be influenced by a difficulty to detect $CD8^+$ T-cell responses in HCV (29), possibly due to sequestration of specific cell populations in the liver (53). However, by stimulation with autologous peptides our assays should have been highly sensitive. Moreover, in an additional analysis of mutations within HLA-matched described epitopes and of mutations adjacent to detected epitopes that have been shown to possibly impair T-cell recognition by altering antigen processing, neither increased the number of CD8-associated viral mutations in our study to the percentages described for HIV (1; data not shown). Recent studies have now begun to identify HLA class I-associated viral sequence polymorphisms at the population level (6, 21, 37). These analyses do not depend on the detection of cellular immune responses and are not limited to the detection of IFN-γ as effector molecule. Therefore, such sequence-based approaches may additionally help to reveal the role of $CD8^+$ T-cell escape mutations in driving the evolution of HCV.

The hypothesis has been put forward that $CD8^+$ T-cell escape may decisively influence the outcome of HCV infection (13, 15). In our study five of six mutations associated with $CD8^+$ T-cell responses did appear during the acute phase of infection, possibly influencing the outcome of acute HCV infection. In contrast, however, patients 03-32 and BR111 progressed to chronicity without substantial escape in targeted epitopes. Although this observation does not preclude escape as a vitally important mechanism to virus persistence, these data suggest that it may only be one of various different avenues leading to chronicity.

Mutations associated with $CD8^+$ T-cell responses were detected preferentially during the early phase of infection in our study, and mutation rates outside envelope overall declined over the course of infection. Therefore, as a consequence of T-cell dysfunction in HCV infection (12, 47), escape mutations may appear only in epitopes against which the respective $CD8^+$ T-cell response remains functionally preserved (49). Declining mutation rates over time have similarly been observed in chimpanzees acutely infected with a single defined strain of HCV (18). Notably, no synonymous mutations were detected between this infecting strain and sequences obtained after 26 weeks (18). This is in contrast to the mostly synonymous, random evolution suggested by the initial low dN/dS ratios observed upon transmission in our study. However, random drift only exists in nonfunctional pseudogenes. In expressed genes the rate of nonsynonymous changes also reflects functional constraints acting in a dynamic interplay between conservative and evolutionary selective pressures (26), as observed in the correlation between conservation scores and mutation kinetics in our study (Fig. 5). Moreover, in humans HCV is transmitted as a mixture of quasispecies. Viral sequence changes upon transmission likely represent selective transmission or outgrowth of particular clones of the quasispecies ("bottleneck phenomenon") rather than de novo mutations (18, 34, 40), with unselective acceptance of synonymous sequence polymorphisms but conservation of the predominant, functional amino acid sequence. We believe these changes are reflected in low dN/dS ratios upon transmission in our patients.

Data in SIV (17, 19, 20) and HIV (36) have shown that viral sequence mutations may have a significant effect upon viral replication capacity and revert rapidly upon transmission to a new host (19, 20). A recent study in HCV similarly suggests the potential for immune-selected mutations to impact viral fitness (45). However, only very few studies have described reversion of escape mutations in HCV infection (13, 41, 48). In the present study, we identified 37 viral mutations that evolved toward consensus, possibly indicating reversion to a more fit viral phenotype in the new host. In correlating the rates of reversions with conservation scores, rapidly reverting residues tended to arise at more conserved positions; this is possibly indicative of a more deleterious impact of these mutations upon viral replication. If true, rapidly reverting residues may help to highlight immune-driven mutations in HCV that may be exacting a high cost to viral fitness and therefore represent attractive targets for vaccines.

In summary, in characterizing the extent, kinetics, and driving forces of viral evolution in four patients acutely infected with HCV, we observed that escape mutations driven by $CD8^+$ T-cell responses, together with reversions, accounted for 30% of all viral mutations outside envelope. As a possible correlate of developing T-cell dysfunction as reported in other studies, high initial and later declining rates of viral evolution were observed. Notably, correlations between the kinetics of forward and reverse mutations with the conservation of residues on a population level suggest that viral protein structure and function are influencing viral evolution. Finally, chronic outcome was not strongly associated with CD8 escape in our patients. Taken together, these data provide insight into the extent to which HCV is capable of evading early $CD8^+$ T-cell responses and support the hypothesis that both CD8 escape and isolated dysfunction of $CD8^+$ T cells may be associated with failure to resolve HCV infections.

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