# Tracking Hepatitis C Virus Quasispecies Major and Minor Variants in Symptomatic and Asymptomatic Liver Transplant Recipients

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To evaluate the possibility that distinct viral quasispecies play a role in the pathogenesis of progressive hepatitis C virus (HCV) infection, we performed a detailed evaluation of HCV quasispecies before and after liver transplantation in five patients infected with HCV genotype 1, three of whom developed severe recurrent hepatitis C and two of whom developed asymptomatic posttransplant infections with high-titered viremia. HCV quasispecies were characterized by using a combination of nucleotide sequencing plus heteroduplex tracking assay of the second envelope gene hypervariable region (HVR). An average of 30 HVR clones were analyzed per specimen; an average of five specimens were analyzed per patient over a 6- to 24-month study period. The complexity of HCV quasispecies in pretransplant serum varied, ranging from one to nine genetically distinct variants for the five patients. However, in all five cases, relatively homogenous quasispecies variants emerged after liver transplantation. In the three patients who developed recurrent hepatitis, quasispecies major variants present in pretransplant serum were efficiently propagated immediately after liver transplantation and were propagated throughout the course of acute and chronic hepatitis. In contrast, in the two asymptomatic cases, we observed rapid depletion of pretransplant quasispecies major variants from posttransplant serum, followed by emergence of new quasispecies variants by posttransplant day 30. Genetic analysis suggested that in these cases, the new quasispecies variants were derived from minor variants present at relatively low clonal frequency (less than 5% of HVR clones) within the pretransplant quasispecies populations. These data demonstrate that quasispecies tracking patterns are associated with the rapidity and severity of HCV-associated liver disease after liver transplantation. Further characterization of HCV quasispecies in animal model systems is warranted.

Hepatitis C virus (HCV) is an important cause of chronic liver disease leading to cirrhosis and end-stage liver disease in humans, and chronic hepatitis C is now recognized as a leading indication for orthotopic liver transplantation in the United States. Nearly 100% of HCV-infected patients who undergo liver transplantation develop recurrent viremia after the procedure (1, 5, 6, 8, 14, 24). However, only about 50% of such patients develop recurrent hepatitis in their liver allografts during the first few years posttransplantation, despite the high levels of viremia in most patients (8, 24).

HCV exists as a viral quasispecies in infected humans, characterized by extensive genetic mutation within the second envelope gene (E2) hypervariable region (HVR) (10, 21). The role of HCV quasispecies mutation in the pathogenesis of liver disease is uncertain. In the nonimmunosuppressed human host, HCV quasispecies mutations have been associated with viral persistence via antibody escape mechanisms (4, 12, 13, 19–22). Furthermore, one preliminary study has suggested that the degree of HCV genetic diversity within HVR is directly proportional to the extent of liver disease in the nonimmunosuppressed host (11), although a second study found no such correlation (16). Because of the lack of a suitable animal model or tissue culture system for HCV propagation, there have been no data to suggest that different HCV quasispecies

variants might differ in either cytopathic potential or the ability to cause persistent infection without overt liver disease.

Transplantation of an uninfected liver allograft into an infected person offers the opportunity to evaluate HCV quasispecies infection in a new tissue host. The present study describes tracking of HCV quasispecies major and minor variants before and after liver transplantation in five individuals, three of whom developed severe recurrence of chronic hepatitis, whereas two had only asymptomatic posttransplant infections. The five patients were selected because they were all infected with the same HCV genotype (genotype 1), and they all developed recurrence of high-titered HCV viremia within 30 days posttransplantation. In addition, at least five serial liver biopsies were available in each case to evaluate the histopathologic course of posttransplant infection. HCV quasispecies were characterized and tracked over time, using the techniques of nucleotide sequencing plus heteroduplex tracking assay (HTA) as originally described by Delwart et al. (3) for characterization of human immunodeficiency virus quasispecies and modified by our laboratory (23) for characterization of HCV quasispecies.

## MATERIALS AND METHODS

**Patients and clinical monitoring.** The five patients were selected from a larger cohort of HCV-infected patients who underwent orthotopic liver transplantation at the University of Washington Medical Center as described in detail elsewhere (8). In brief, all five patients had previous HCV infection as demonstrated by HCV antibody positivity and HCV viremia. They were monitored by a common protocol in which serum specimens were obtained immediately prior to liver

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TABLE 1. Clinical and virological features of HCV infection after orthotopic liver transplantation

| Patient no. | Pretransplant virologic features | | | Posttransplant virologic features | | | | | | Posttransplant clinical features | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | HCV RNA titer[b] | | | | | | Hepatitis on liver biopsy | | | | |
| | HCV genotype[a] | HCV RNA titer[b] | HCV genotype | Days | | | Months | | ALT level | Days | | Months | | |
| | | | | 7 | 14 | 30 | 6 | 12 | | 10 | 21 | 3 | 6 | 12 |
| 1[c] | 1b | 6.5 | 1b | 8.0 | 8.5 | 9.0 | 10.0 | NT | Elevated | No | No | Yes | Yes | NT |
| 2 | 1a | 8.0 | 1a | 8.0 | 9.0 | 8.0 | 10.0 | 8.5 | Elevated | No | No | Yes | Yes | Yes |
| 3 | 1a | 8.0 | 1a | 8.0 | 8.5 | 8.0 | 8.5 | 9.0 | Elevated | No | No | Yes | Yes | Yes |
| 4 | 1a | 5.5 | 1a | Neg | Neg | 8.5 | 9.0 | 9.5 | Normal | No | No | No | No | No |
| 5 | 1a | 7.5 | 1a | Trace | Trace | 8.0 | 8.0 | 10.0 | Normal | No | No | No | No | No |

[a] Simmonds nomenclature (18).

[b] Viral RNA levels are expressed as log transformed molecules per milliliter determined by quantitative competitive PCR (8). Trace, HCV RNA positive but less than 3 logs/ml, below the range of quantitation. Neg, HCV RNA negative by reverse transcription PCR. NT, not tested.

[c] Patient 1 expired at 7 months posttransplantation (see text for details).

transplantation and at regularly scheduled posttransplant intervals, including multiple days during the first posttransplant month, were separated from whole blood within 2 h of venipuncture, aliquoted, and immediately stored at −70°C until further virological testing.

Patients 1, 2, and 3 were selected because they developed biochemical and histologic evidence of chronic active hepatitis in liver allografts within the first 3 months after liver transplantation, which appeared unusually severe. In each case, either bridging fibrosis or bridging necrosis developed in the allograft within the first year after transplantation. Patients 4 and 5 were selected because they were infected with the same HCV genotype as the first three patients (HCV genotype 1) and had very similar patterns of high-titered HCV viremia after the first month posttransplantation (Table 1), but they had asymptomatic HCV infections for up to 2 years after transplantation. Asymptomatic HCV infection was defined as the absence of histologic evidence of liver injury on five consecutive protocol liver biopsies performed at 10 days, 21 days, 3 months, 6 months, and 12 months after liver transplantation and by normal values of alanine aminotransferase (ALT), aspartate aminotransferase, and other indices of hepatic function, which were monitored by routine posttransplant protocol as previously described (8).

**HCV RNA quantitation and genotype analysis.** Absolute levels of HCV RNA (molecules per milliliter of serum) were determined by quantitative competitive reverse transcription-PCR (RT-PCR) as previously described (7–9). Comparison of PCR products generated from internal control RNA (315 bp in size) and native HCV RNA (255 bp) allowed determination of native HCV RNA copy number. Two independent determinations were performed for each specimen. Quantitative competitive RT-PCR provided linear measurement of HCV RNA levels over a range of 3 to 10 log units (7, 9). HCV genotype was determined as described previously (8) and classified phylogenetically as proposed by Simmonds and colleagues (18).

**Cloning and nucleotide sequencing of the E2 HVR.** Total RNA was extracted from patient sera or liver biopsy specimens by the single-step guanidinium method (2), and HVR sequences were amplified by nested-set RT-PCR as described previously (23), using sense and antisense E2 HVR primers originally described by Weiner et al. (21). The size of the first-round PCR product was 244 bp, while the second PCR reaction yielded a 176-bp amplified product. Amplification products were purified (QIAQuick columns; Qiagen, Chatsworth, Calif.), and 6 to 8 ng was ligated into 50 ng of PCRII vector (TA cloning kit; Invitrogen, San Diego, Calif.) according to the manufacturer's protocol. Plasmid DNA was purified (QIAwell plasmid preparation system; Qiagen) and sequenced, using an Applied Biosystems (Foster City, Calif.) model 373A automated sequencer and M13 universal primers. When multiple parallel RNA extractions and RT-PCRs were performed on the same specimen, we observed highly similar or identical frequencies of major variants within quasispecies populations, indicating that minimal bias was occurring by our methods. Cloning of HCV quasispecies after 100-fold dilution of the HCV RNA also gave a consistent distribution of quasispecies variants, indicating the quasispecies pattern was not a function of HCV RNA concentration in these cases (data not shown). A quasispecies major variant was defined as any clone which was reproducibly detected at a frequency of greater than 10% of clones, while minor variants invariably comprised less than 5% of clones on repetitive sampling.

**Heteroduplex tracking assay (HTA).** HVR inserts were amplified by PCR from plasmid clones, and 20-ng aliquots of column-purified PCR product were end labeled with T4 polynucleotide kinase (Gibco BRL) plus 100 μCi of [32P] ATP (Amersham, Arlington Heights, Ill.) to generate probes. Probes (2 to 4 fmol; approximately $2.5 \times 10^4$ cpm/fmol) were then hybridized to a 5,000-fold molar excess of unlabeled HVR PCR products (10 to 20 pmol) derived either by direct amplification from patient serum or liver (heterogeneous HVR sequences) or from recombinant HVR-containing plasmids (clonal HVR sequences) prepared as described previously (23). Hybridization reaction mixtures consisted of unlabeled driver HVR sequences plus labeled HVR probe, 2 M NaCl, 100 mM

Tris (pH 8.0), and 20 mM EDTA. Reaction mixtures were denatured at 95°C for 5 min and annealed at 55°C for 2 h before the entire reaction volume was loaded on 1-mm-thick 6% polyacrylamide MDE gel (Baker) and electrophoresed for 18 to 20 h at 400 V. The gel was vacuum dried at 80°C on filter paper and exposed to X-ray film. Probe hybridized to itself (unlabeled) served as a marker for identification of homoduplexes. Hybrids with nucleotide mismatches showed aberrant migration and were identified as heteroduplexes.

## RESULTS

**Clinical and virological features of HCV infection after liver transplantation.** Table 1 summarizes the clinical and virological features of HCV infection before and after liver transplantation for the five patients. All five patients had active HCV infections with HCV genotype 1 both before and after transplantation; four patients were infected with HCV subgenotype 1a, while one patient was infected with HCV subgenotype 1b. Mixed genotype infections were excluded by nested PCR analysis of the HCV core gene with type-specific primers (17). Patients 1 to 3 each developed biochemical and histologic evidence of hepatitis in liver allografts by 3 months after liver transplantation. In all three patients liver biopsies performed at 10 days and 21 days posttransplantation showed no evidence of hepatitis. Biopsies performed at 2 to 3 months posttransplantation showed acute hepatitis in all three patients, and by 6 months posttransplantation, liver biopsies indicated severe hepatitis in all three. Patient 1 expired 7 months after liver transplantation as a result of an infectious pulmonary complication. Patients 2 and 3 had histologic findings of bridging fibrosis and bridging necrosis, respectively, in their liver allografts by 12 months posttransplantation. In patients four and five, liver biopsies performed at 10 days, 21 days, 3 months, 6 months, and 12 months after liver transplantation showed no evidence of hepatitis (Table 1), and serum ALT values remained normal.

The only difference in HCV viremia patterns between asymptomatic patients 4 and 5 and diseased patients 1 to 3 was observed during the first 2 weeks after liver transplantation. The diseased patients remained viremic on posttransplant days 7 and 14, with high viral RNA titers (greater than 7 log units/ml). In contrast, the asymptomatic patients had significant reductions in HCV viremia on posttransplant days 7 and 14, with HCV RNA undetectable by RT-PCR in patient 4 and detectable at only trace levels (less than 500 RNA molecules per ml) by RT-PCR in patient 5 (Table 1). However, by the end of the first posttransplant month, all five patients had developed high-titered HCV viremia (greater than 7 log units/ml) which persisted throughout the course of study in all cases (Table 1).

**Characterization of HCV quasispecies variants.** Figure 1

**A**

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        363 ---------+---------+---------+---------+---------+------- 420
Pre-Tx      SMVGNWAKVLVVMLLFAGVDGQTHVTGGTHGRGAHGITSLFTPGASQKIQLVNTNGSW   MV-1
(n=17)
```

**B 6 Months Post-Tx Clones**



**C**



FIG. 1. Analysis of a simple HCV quasispecies before and after liver transplantation in patient 1 (diseased). The E2 HVR was amplified from sequential clinical specimens by RT-PCR and analyzed by either nucleotide sequencing (A) or HTA (B) as described in Materials and Methods. (A) Deduced amino acid sequences of 73 HVR clones from pretransplant serum (Pre-Tx) or from posttransplant sera (post-Tx) obtained at 5 days (5D), 1 month (1M), and 6 months (6M) posttransplantation or from a liver biopsy specimen obtained at 6 months posttransplantation. The consensus amino acid sequence of MV-1 is in boldface 1 (see text for details). (B) HTA analysis of 10 representative HVR clones isolated from patient 1 serum at 6 months posttransplantation, using radiolabeled MV-1 sequences as the probe. PCR amplification products were prepared from HVR clones and hybridized to a $^{32}$P-labeled probe derived from a consensus clone (MV-1), and the hybrids were analyzed by gel electrophoresis and autoradiography as described in Materials and Methods. Lane 7 contains the homoduplex shift control. (C) Bar graph comparing percentage of HVR clones identical to the consensus clone (MV-1) by either sequence analysis (black bars) or HTA (gray bars) at various time points before and after liver transplantation.

illustrates characterization of HCV quasispecies variants at multiple time points before and after liver transplantation in patient 1. A total of 73 independent HVR clones from six different time points were analyzed by nucleotide sequencing (Fig. 1A). In pretransplant sera, 9 of 17 (53%) of the HVR clones had identical amino acid sequences, while the remaining 8 clones each had a single unique amino acid change relative to the consensus pretransplant sequence. Thus, patient 1 had a single quasispecies major variant in pretransplant serum, designated MV-1, plus numerous minor variants which were all

posttransplantation, 16 of 18 (89%) and 9 of 10 (90%), respectively, of the HVR clones had the consensus MV-1 amino acid sequence. At 6 months posttransplantation, 9 of 15 (60%) of the serum-derived clones and 10 of 13 (77%) of the liver-derived clones had the consensus MV-1 sequence. Therefore, after liver transplantation, the consensus quasispecies major variant (MV-1) remained unchanged, and none of the minor variants identified in pretransplant serum showed evidence of emergence or propagation after liver transplantation. Figure 1B illustrates the gel shift profile of 10 representative HVR clones isolated from patient 1 serum at 6 months posttransplantation: although very similar gel shift patterns were ob-

served for all 10 clones relative to the MV-1 probe, slight shifts in mobility were detected for the clones in lanes 4, 6, 9, and 11, suggesting minor differences from the MV-1 probe (Fig. 1B, lane 7).

The bar graph in Fig. 1C summarizes the strong correlation observed between predicted amino acid sequence and HTA for assessing the relatedness of HCV quasispecies variants over time. Over the course of study of patient 1, a total of 167 individual HVR clones were analyzed by the HTA technique: while the majority of HVR clones were identical in mobility to the probe homoduplex control, a subset of quasispecies variants were distinguishable from MV-1 by HTA. The percentage of quasispecies variants which were slightly different from MV-1 fluctuated over time. For example, gel shift patterns appearing identical to that for MV-1 were observed for approximately 90% of HVR clones obtained from four independent RT-PCRs at either 5 days or 1 months posttransplantation. Amino acid sequence patterns were also 90% homologous at these time points (Fig. 1A). In contrast, identical-appearing HTA and amino acid patterns accounted for 54 to 58% of pretransplant HVR clones and 68 to 80% of HVR clones isolated from liver and serum at 6 months posttransplantation (Fig. 1C).

**Propagation of quasispecies major variants after liver transplantation in symptomatic patients.** Persistence of quasispecies major variants after liver transplantation was also seen in patients 2 and 3, both of whom developed severe hepatitis in their liver allografts within the first 3 months after liver transplantation. In patient 2, nucleotide sequencing of 46 HVR1 clones isolated from pre- and posttransplant sera also indicated a single quasispecies major variant in this case, designated MV-2 (data not shown). Figure 2A illustrates a quasispecies tracking experiment using MV-2 as a probe, after hybridization with heterogeneous HVR sequences amplified from pretransplant serum (lane 2) and posttransplant serum obtained at 6 days (lane 3), 1 month (lane 4), 2 months (lane 5), 6 months (lane 6), and 12 months (lane 7) after surgery. Mobility of the heterogeneous HVR sequences appeared highly similar or identical to that of the MV-2 probe control at all time points except 6 months posttransplantation, when a small but reproducible gel shift pattern was evident. Clinically, patient 2 had a sharp peak in serum ALT values (357 IU/liter) at the 6-month time point, with findings of extensive inflammation and hepatocellular necrosis on liver biopsy. At this time, 13 of 30 (43%) of the HVR1 clones showed detectable shifts by HTA (e.g., Fig. 2C, lanes 3, 5, 7, 8, 9, 13, and 14), compared with only 1 of 30 (3%) HVR clones obtained at 2 months posttransplantation (Fig. 2B). The minor variants observed at 6 months posttransplantation were not observed at 12 months posttransplantation by HTA using heterogeneous HVR sequences (Fig. 2A, lane 7) or by either HTA or sequence analysis of 39 different HVR clones generated from two independent RT-PCRs (Fig. 2D).

These data confirm that patient 2 was also infected with a relatively homogeneous HCV quasispecies both before and after liver transplantation and that severe hepatitis occurred during the time period of quasispecies homogeneity. We observed a transient increase in quasispecies minor variants during the peak of hepatitis, as reflected by high ALT values and liver biopsy findings; however, these minor variants were not propagated.

Analysis of pretransplant serum in patient 3 revealed a complex HCV quasispecies pattern consisting of three major sequence variants, designated MV-3a, MV-3b, and MV-3c, in roughly equal proportions (Fig. 3A, lanes 1 to 3). The doublet shift pattern observed in lanes 2 and 3 of Fig. 3A resulted from
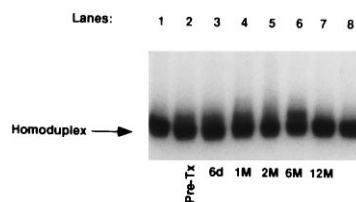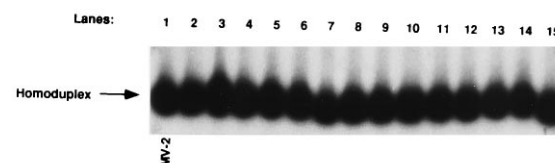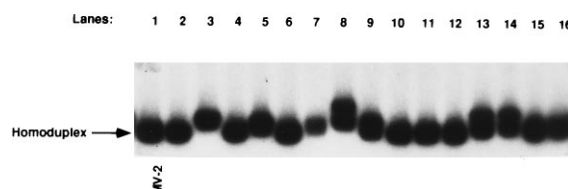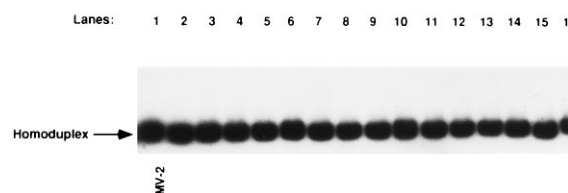


FIG. 2. Analysis of a simple HCV quasispecies over time in patient 2 (diseased). HVR sequences were amplified from sequential serum specimens obtained before and after liver transplantation, and HTA was performed as described in the legend to Fig. 1. (A) HCV quasispecies tracking experiment. HVR sequences were amplified from pretransplant serum (Pre-Tx; lane 2) and posttransplant sera at 6 days (lane 3), 1 month (lane 4), 2 months (lane 5), 6 months (lane 6), and 1 year (lane 7) after liver transplantation. Heterogeneous HVR amplification products were hybridized to a $^{32}$P-labeled probe derived from the pretransplant consensus clone (MV-2), and hybrids were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lanes 1 and 8 contain the homoduplex shift control. (B to D) Clonal frequency analysis of 45 individual HVR clones isolated from patient 2 serum at 2 (B), 6 (C), and 12 (D) months posttransplantation (Post-Tx), after hybridization with MV-2 probe. Lane 1 contains the probe homoduplex control.

hybridization of both strands of the radiolabeled DNA probe to target DNA strands and was highly reproducible and characteristic of a particular quasispecies variant. Figure 3D compares the consensus amino acid sequences of the three major variants in pretransplant serum: MV-3a differed from MV-3b by five amino acids and 10 nucleotides; MV-3a differed from MV-3c by seven amino acids and 11 nucleotides; and MV-3b differed from MV-3c by six amino acids and 8 nucleotides. The HTA patterns were consistent with the differences in nucleotide sequences between the three quasispecies major variants, regardless of which major variant was used as the probe (data not shown).
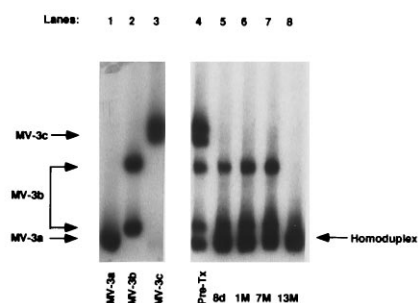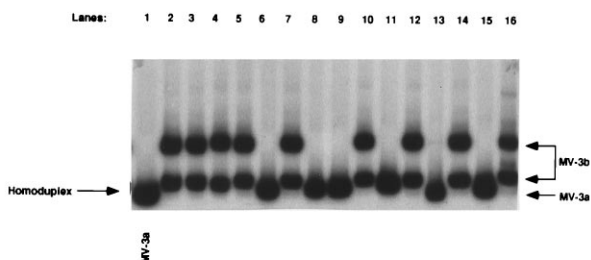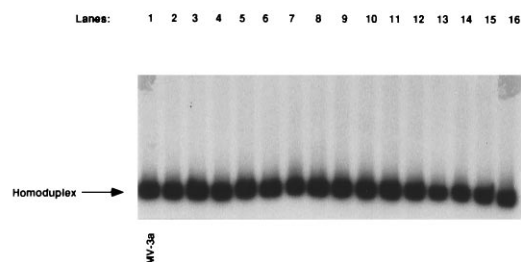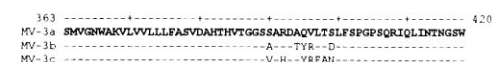
FIG. 3. Analysis of a complex HCV quasispecies before and after liver transplantation in patient 3 (diseased). (A) Heteroduplex tracking assay of the HCV quasispecies. Lanes 1 to 3 show individual HTA profiles of the three quasispecies major variants (MV-3a, MV-3b, and MV-3c, respectively) after amplification and cloning from pretransplant serum. Radiolabeled MV-3a was used as a probe. Lanes 4 through 8 illustrate a HCV quasispecies tracking experiment. HVR sequences were amplified from pretransplant serum (Pre-Tx; lane 4) and posttransplant sera at 8 days (lane 5), 1 month (lane 6), 7 months (lane 7), and 13 months (lane 8) after liver transplantation. Heterogeneous HVR amplification products were hybridized to a $^{32}$P-labeled probe derived from MV-3a, and hybrids were analyzed by polyacrylamide gel electrophoresis and autoradiography. (B) Analysis of 15 HVR clones isolated from patient 3 serum at 7 months posttransplantation (Post-Tx), using radiolabeled MV-3a as the probe. Lane 1 contains the homoduplex shift control. Nine of 15 clones gave gel shift patterns identical that of to MV-3b, while 6 of 15 clones gave patterns indistinguishable from that of the MV-3a homoduplex. (C) Analysis of 15 HVR clones isolated from patient 3 serum at 13 months posttransplantation. All 15 clones gave gel shift patterns that were indistinguishable from that of the MV-3a probe control. (D) Predicted amino acid sequences of the quasispecies major variants MV-3a, MV-3b, and MV-3c.

Tracking of this relatively complex HCV quasispecies before and after liver transplantation by using the HTA technique is illustrated in Fig. 3A, lanes 4 to 8. Lane 4 illustrates HTA of heterogeneous HVR sequences amplified directly from pretransplant serum: the three gel shift patterns representing MV-

3a, MV-3b, and MV-3c are clearly evident. Lanes 5 to 8 illustrate tracking of the three quasispecies major variants after liver transplantation. MV-3a was detectable in all posttransplant serum specimens and was the only quasispecies major variant detectable at 13 months posttransplantation (lane 8). MV-3b was detected at 8 days, 1 month, and 7 months after liver transplantation (lanes 5 to 7) but was no longer detected at 13 months (lane 8). MV-3c was not detectable in any of the posttransplant serum specimens by the HTA tracking technique (lanes 5 to 8).

The clonal frequency of the three major variants was detected both before and after liver transplantation, as illustrated in Fig. 3B and C. On the basis of HTA of 59 HVR clones isolated from pretransplant serum, MV-3c was the quasispecies major variant of highest clonal frequency before transplantation (50% of HVR clones), followed by MV-3a (28% of clones) and MV-3b (22% of clones). At 8 days posttransplantation, MV-3a represented approximately 66% of HVR clones, MV-3b comprised approximately 30% of clones, and MV-3c represented less than 3% of clones. At 1 and 7 months posttransplantation, MV-3b became the most prevalent quasispecies major variant in serum, comprising 52 and 67%, respectively, of clones (Fig. 3B). At 13 months posttransplantation, MV-3b was not detected in any of the 30 HVR clones analyzed, and MV-3a was the only quasispecies major variant identified (Fig. 3C).

**Emergence of quasispecies minor variants after liver transplantation in asymptomatic patients.** Characterization of HCV quasispecies before and after liver transplantation in the two asymptomatic patients (patients 4 and 5) is summarized in Fig. 4. HTA analysis of 30 HVR clones isolated from pretransplant sera in patient 4 indicated this patient had a relatively simple quasispecies pattern, with a single major variant designated MV-4$_{pre}$ representing greater than 95% of clones (Fig. 4A, left panel). In contrast, analysis of 40 HVR clones from pretransplant serum in patient 5 revealed a more complex quasispecies pattern (Fig. 4A, right panel): two distinct quasispecies major variants, designated MV-5a (45% of clones; represented by lanes 4, 10, 11, and 12) and MV-5b (18% of clones; lanes 2 and 6) were identified. For this experiment, an MV-5b-derived HVR clone was randomly picked as a probe (lane 1). Numerous minor variants were also detected in the pretransplant quasispecies of patient 5, each at a frequency of less than 3% of pretransplant clones (e.g., lanes 3, 5, 8, and 9).

Figure 4B illustrates quasispecies tracking by the HTA technique after liver transplantation in patients 4 and 5. In patient 4, MV-4$_{pre}$ was used as a probe, and in patient 5, MV-5b was used as a probe. In both experiments, the posttransplant HTA patterns were clearly different from the pretransplant HTA patterns, indicating emergence of new quasispecies which appeared homogeneous over time. Figure 4C illustrates HTA of multiple HVR clones isolated from posttransplant sera of patients 4 and 5, using the same probes as used for Fig. 4A and B. Analysis of more than 90 HVR clones isolated from four different posttransplant time points yielded indistinguishable HTA patterns in 99% of clones in both cases (Fig. 4C), confirming that the posttransplant HCV quasispecies were remarkably homogeneous at the clonal level with respect to HVR sequences. These patterns were not due to sampling or selection bias, because (i) the same patterns were observed at multiple time points over a 1- to 2-year period and (ii) the same patterns were obtained with diluted and undiluted specimens (data not shown). Importantly, the pretransplant quasispecies major variants identified in patients 4 and 5 were not found in any of the posttransplant sera tested in either case.

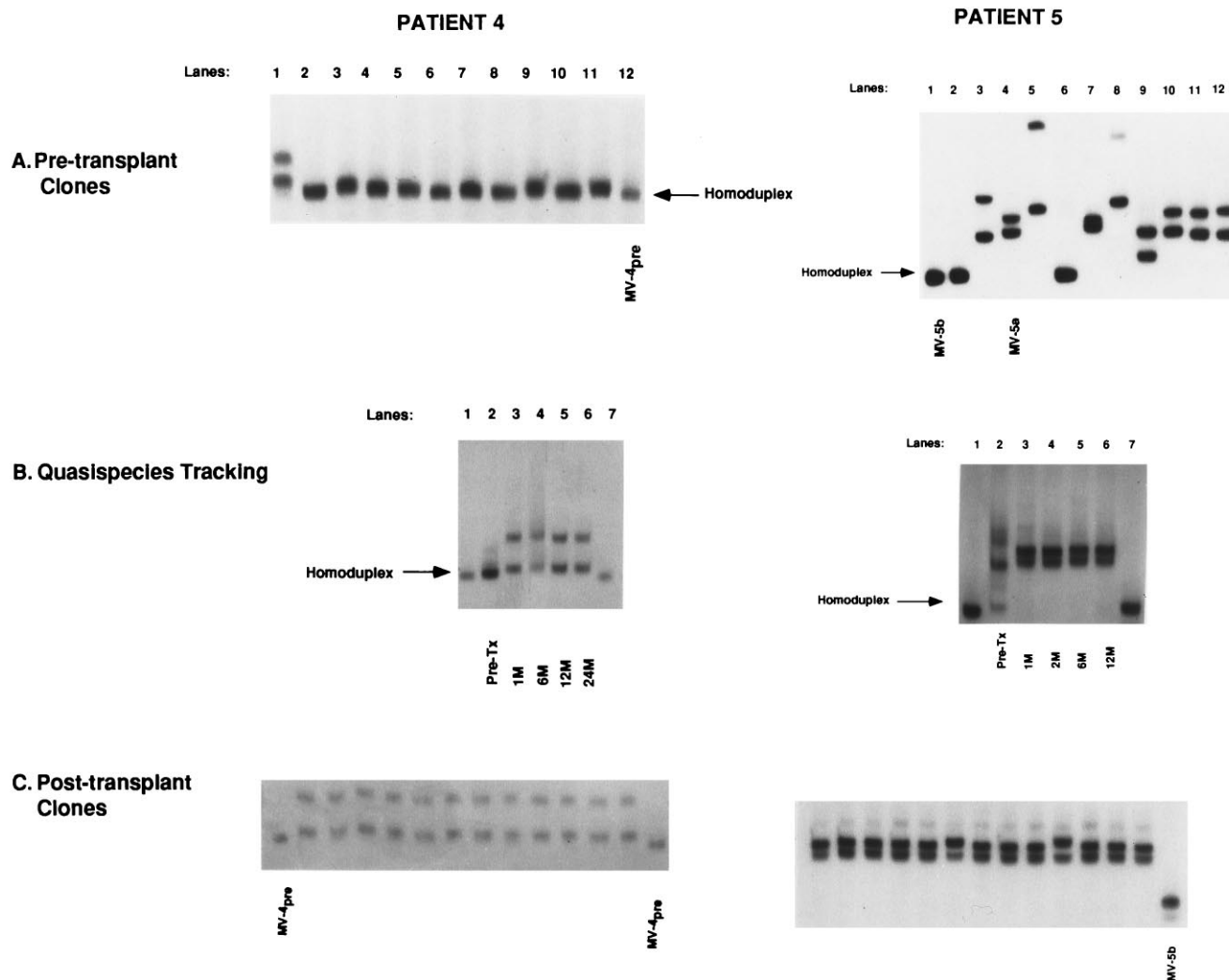We next performed a series of experiments to determine the

FIG. 4. Analysis of HCV quasispecies before and after liver transplantation in the asymptomatic liver transplant recipients 4 and 5. (A) Heteroduplex tracking assay of pretransplant quasispecies. The left panel illustrates HTA profiles for 11 HVR clones isolated from patient 4 pretransplant serum, after hybridization with patient 4 pretransplant probe MV-4$_{pre}$. Lane 12 contains the homoduplex shift control. The right panel illustrates HTA profiles for 11 HVR clones isolated from patient 5 pretransplant serum, after hybridization with the patient 5 pretransplant probe MV-5b. Lane 1 contains the homoduplex shift control. (B) HCV quasispecies tracking before and after liver transplantation in patients 4 and 5. HVR sequences were amplified from pretransplant serum (Pre-Tx) and posttransplant sera at 1 month (1M), 2 months (2M), 6 months (6M), 12 months (12M), and 24 months (24M) after liver transplantation. Heterogeneous HVR amplification products were hybridized to $^{32}$P-labeled probes derived from pretransplant HVR clone MV-4$_{pre}$ (patient 4) or pretransplant HVR clone MV-5b (patient 5). Homoduplex gel shift controls are present in lanes 1 and 7 for both experiments. (C) HTA profiles of representative HVR clones isolated from patient 4 serum at 1 month posttransplantation, after hybridization with pretransplant probe MV-4$_{pre}$, and from patient 5 serum at 12 months posttransplantation, after hybridization with pretransplant probe MV-5b. Homoduplex shift controls are indicated.

relationships between the pre- and posttransplant quasispecies in patients 4 and 5. First, a representative posttransplant HVR probe was prepared for both patients; the probes were designated MV-4$_{post}$ for patient 4 (Fig. 5A) and MV-5$_{post}$ for patient 5 (Fig. 5B). The posttransplant probes were hybridized with large panels of either pretransplant or posttransplant HVR clones and analyzed by the HTA technique. In patient 4, all posttransplant clones tested gave HTA shift patterns which appeared identical to that of the MV-4$_{post}$ homoduplex control (Fig. 5A, lanes 8 to 10, for example) but gave different HTA shift patterns for all 87 pretransplant HVR clones tested (Fig. 5A, lanes 2 to 4, for example). Although we found no pretransplant clones identical in mobility to MV-4$_{post}$, we did identify three pretransplant minor variants (clones 25, 61, and 87) which gave HTA shift patterns which were intermediate between those of MV-4$_{pre}$ and MV-4$_{post}$ (Fig. 5A, lanes 5 to 7).

The right panel of Fig. 5A compares the consensus amino acid sequences of the pretransplant quasispecies major variant (lanes 2 to 4), the pretransplant minor variants (lanes 5 to 7), and the posttransplant quasispecies major variant. Relative to the pretransplant major variant (MV-4$_{pre}$), three amino acid changes were observed in 100% of posttransplant HVR clones: an Arg to Gly at position 384, a Leu to Phe at position 403, and an Ile to Val at position 414. Furthermore, the predicted amino acid sequences of two pretransplant minor variants (clones 61 and 87) both contained the Arg-to-Gly mutation at position 384 but no change at position 403 or 414 relative to MV-4$_{pre}$. Clones 61 and 87 differed from each other by three nucleotides, which accounted for the differences in HTA shift patterns. The nucleotide sequences suggest that MV-4$_{post}$ may have been derived from or related to pretransplant minor variant clone 87 (data not shown).

FIG. 5. Relationship between pre- and posttransplant quasispecies in patients 4 and 5 (asymptomatic patients). (A) HTA profiles and amino acid sequences of HCV quasispecies variants isolated from patient 4 serum before and after liver transplantation. A consensus posttransplant HVR clone (MV-4$_{pre}$) was radiolabeled with $^{32}$P and hybridized to HVR sequences isolated from three pretransplant (Pre-Tx) major variant clones (lanes 2 to 4), three pretransplant minor variant clones (lanes 5 to 7), and three posttransplant (Post-Tx) major variant clones (lanes 8 to 10). Lanes 1 and 11 contain homoduplex gel shift controls; deduced amino acid sequences of the HVR clones are summarized on the right. (B) HTA profiles of 20 HVR clones isolated from patient 5 pretransplant serum, using a consensus posttransplant HVR clone as the probe (MV-5$_{post}$). The rightmost lane contains the homoduplex shift control. Pretransplant clone *21 gave a homoduplex shift pattern. Amino acid sequences of HCV quasispecies variants in patient 5, including the consensus pretransplant quasispecies major variants MV-5a and MV-5b, the consensus posttransplant quasispecies major variant MV-5$_{post}$ (clones 1, 2, and 3), and pretransplant clone *21, are shown on the right. The positions and identities of amino acids are shown relative to the MV-5a sequence.

In patient 5, the posttransplant probe (MV-5$_{post}$) also gave homologous HTA patterns with all posttransplant HVR clones tested (data not shown) but gave clearly different HTA shift patterns for 28 of 30 pretransplant clones (Fig. 5B). However, MV-5$_{post}$ did give homologous-appearing HTA shift patterns for two pretransplant HVR clones (e.g., clone 21 in Fig. 5B). The right panel of Fig. 5B compares the consensus amino acid sequences of the pretransplant quasispecies major variants in this patient (MV-5a and MV-5b), the pretransplant minor variant (clone 21), and the posttransplant quasispecies major variants (MV-5$_{post}$ clones 1, 2, and 3). The amino acid sequence of pretransplant minor variant 21 was identical to that of the posttransplant quasispecies major variant, implying that the posttransplant HCV quasispecies was derived from a pretransplant minor variant in patient 5.

In summary, these experiments demonstrate that in asymptomatic patients 4 and 5, the pretransplant HCV quasispecies major variants were genetically different from the posttransplant quasispecies major variants. Analysis of the HVR sequences in the asymptomatic patients suggested that the posttransplant quasispecies were closely related to or derived from pretransplant minor variants. The HCV quasispecies patterns in these five patients are summarized by bar graphs in Fig. 6.

## DISCUSSION

This study used the techniques of HTA and nucleotide sequencing to investigate patterns of HCV quasispecies before and after liver transplantation in five patients who were infected with HCV genotype 1. Three of the patients developed severe recurrence of liver disease during the first few months after liver transplantation, while the other two patients developed high-titered yet asymptomatic posttransplant HCV infections. In the three diseased patients (patients 1 to 3), quasispecies major variants present in pretransplant serum were efficiently propagated after liver transplantation (Fig. 6). In the two asymptomatic patients (patients 4 and 5), consensus sequences of the quasispecies major variants present in pretransplant serum were not detectable at any time after liver transplantation, and by 1 month posttransplantation, new quasispecies major variants of the same genotype had emerged in both cases; these quasispecies appeared to be more closely related to pretransplant quasispecies minor variants, as judged from HTA patterns and sequence analysis.

This study shows that quasispecies major variants were efficiently propagated immediately after liver transplantation in the three diseased patients but not in the asymptomatic patients. We selected our patients so that other virological characteristics associated with disease progression (genotype and level of viremia) were similar. These data may suggest that in some instances, certain HCV quasispecies have a higher pathogenic potential than others, although further testing of this concept in animal models is needed. It is conceivable that the quasispecies described in the current study differ in regions of the HCV genome other than the HVR, a possibility which is currently under investigation.

It was of interest to characterize HCV quasispecies propagation before and during the acute and chronic phases of hepatitis in our diseased patients. The pretransplant HCV quasispecies were either relatively homogeneous, with a single major variant (patients 1, 2, and 4) or complex, with 2 or more major variants (patients 3 and 5), and the complexity of HCV quasispecies in pretransplant serum did not correlate with the posttransplant disease course. Similarly to Martell et al. (15), we observed remarkably homogeneous propagation of HCV quasispecies major variants in patients 1 and 2, indicating that

quasispecies genetic diversity posttransplant is not directly proportional to disease activity per se. In patient 1, the pretransplant HCV quasispecies was 54% homogeneous at the amino acid sequence level immediately prior to liver transplantation and became approximately 90% homogeneous during the first week posttransplantation, which might be explained by a burst of new viral replication in the transplanted liver allograft, as would be expected from a highly hepatotropic quasispecies variant. Over time, the quasispecies accumulated minor mutations, although the major variant remained unchanged. In patient 2, we observed a transient increase in HVR heterogeneity due to an accumulation of quasispecies minor variants at 6 months posttransplantation, but at 12 months posttransplantation, we observed reversion to a homogeneous pattern resembling the pretransplant quasispecies major variant (Fig. 6B).

The two patients who developed asymptomatic posttransplant infections (patients 4 and 5) had dramatic reductions in HCV viremia during the initial 2 weeks after surgery, followed by emergence of new quasispecies major variants by 1 month after liver transplantation. In both cases, the emergent quasispecies major variants were most likely derived from quasispecies minor variants present in pretransplant serum. It is of considerable interest that in these two patients, the emergent quasispecies variants did not cause apparent liver disease, even though they were replicating at high titer, and both represented HCV genotype 1, which is considered relatively pathogenic. Perhaps the HCV quasispecies variants were attenuated as a result of mutation, being efficient at replication but inefficient with regard to the induction of hepatitis. A second possibility is that the emergent quasispecies variants have a reduced hepatotropism. Additional quantitative in situ experiments are under way to determine the relative contributions of hepatic and extrahepatic replication in patients with and without disease. Another possibility is that the major HVR sequences identified in pretransplant serum represented defective (noninfectious) virus derived from the explanted liver. It will be important to establish the relative infectivity of these isolates in tissue culture and small animal models once developed.

It is likely that the differences in viral quasispecies phenotypes and disease patterns are strongly influenced by host factors in these patients. For example, patients 1 to 3, HCV viremia remained high during the initial 2 weeks immediately after liver transplantation, raising two possibilities: (i) highly efficient extrahepatic replication was occurring at the time of liver transplantation in these cases and (ii) the quasispecies major variants had a high affinity for hepatocyte receptor recognition on the transplanted allograft, or the allograft may have been highly susceptible to these major variants for unknown reasons. Regardless, high-titered viremia during the first weeks posttransplantation has been identified as a marker for early recurrence of liver disease in these and other HCV-infected patients whom we have studied (8). In asymptomatic patients 4 and 5, HCV viremia was greatly reduced during the first 2 weeks after liver transplantation, which may suggest that either extrahepatic replication was not highly active at the time of liver transplantation or the HCV RNA in the pretransplant serum was not indicative of highly infectious virus, or perhaps the liver allograft was not susceptible to the pretransplant quasispecies in these cases. Finally, quasispecies selection by immunological mechanisms at the time of liver transplantation is also a factor. All liver transplant recipients receive intense induction immunosuppressive therapy at the time of transplantation. Whether selective differences in humoral or cellular immune responses are associated with the different patterns of
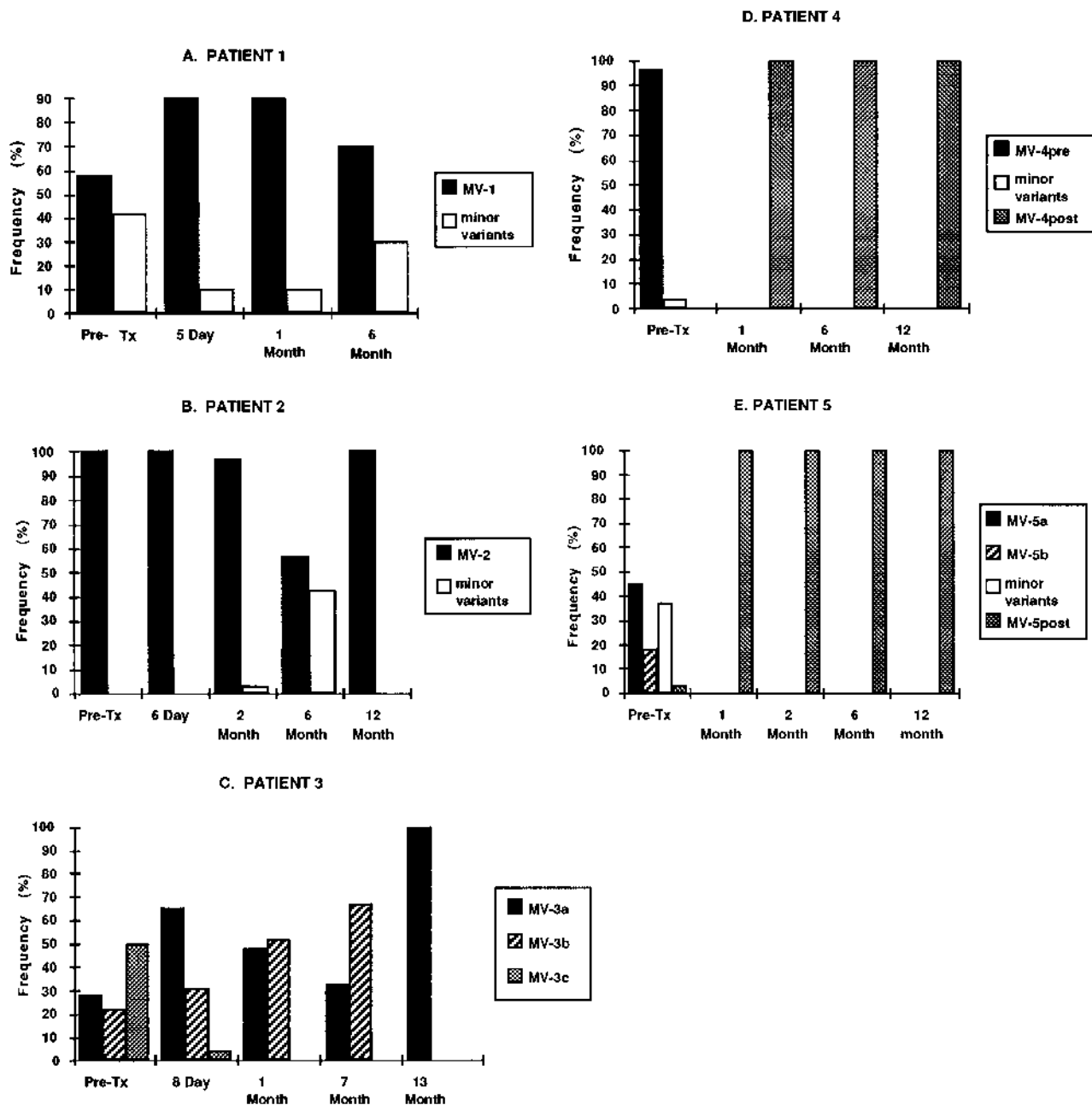
FIG. 6. Bar graphs comparing percentage of HVR clones related to the quasispecies major variants in patients 1 through 5. The frequency of quasispecies variants was determined either by clonal frequency analysis using the HTA technique, as described for Fig. 1 and 2, or by amino acid sequence analysis, using serum obtained prior to transplantation (Pre-Tx) or at sequential times after liver transplantation. Patients 1 and 2 were similar in that the vast majority of HVR clones isolated either before and after liver transplantation appeared identical to a consensus quasispecies major variant present in pretransplant serum; this major variant was designated MV-1 in patient 1 and MV-2 in patient 2. In patient 3, the pretransplant quasispecies consisted of three distinct major variants: MV-3a, MV-3b, and MV-3c. MV-3a and MV-3b were both propagated after liver transplantation, whereas MV-3c was not. In asymptomatic patients 4 and 5, the quasispecies major variants present in pretransplant serum, MV-4$_{pre}$ and MV-5$_{pre}$, were not propagated after liver transplantation. Instead, new quasispecies major variants designated MV-4$_{post}$ and MV-5$_{post}$ emerged after liver transplantation. The predicted amino acid sequence of MV-5$_{post}$ was identical to that of a minor variant identified in pretransplant serum. See text for details.

quasispecies variation that we encountered remains to be defined.

This study demonstrates the utility of the HTA technique for characterizing and tracking HCV quasispecies major and minor variants, since hundreds of HVR sequences can be screened for mutation within a relatively short period of time. This technique may be particularly useful for identifying im-

mune escape variants during vaccine studies, for characterizing persistent HCV quasispecies during interferon therapy in humans, or for tracking HCV quasispecies variants during animal challenge experiments. The technique is also potentially valuable for rapidly assessing other regions of the HCV genome. When combined with careful clinical studies, such an approach may ultimately lead to identification of new and important

genetic markers of HCV persistence, virulence, and resistance to therapy.

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