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Dynamic Analysis of Heterogeneous Hepatitis C Virus Populations by Direct Solid-Phase Sequencing

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In the present study, we used a semiautomated solid-phase direct sequencing method to analyze sequence diversity and variation of the hypervariable E2/NS1 region in the hepatitis C virus (HCV) genome in isolates from patients seropositive for HCV. A total of 24 isolates of various origins were sequenced. Six of the patients, not subject to any antiviral therapy, were monitored longitudinally, and rapid sequence variations were observed over a period of 14 months. The nucleotide change rate was found to be 0.1 to 0.2 nucleotide substitution per genome site per year. Furthermore, isolates from five of the patients were used for a comparative study of the direct solid-phase sequencing approach versus the frequently used approach of sequencing individual reverse transcriptase PCR clones. The advantage of direct solid-phase sequencing for studying dynamic changes in heterogeneous populations of HCV is discussed.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis (2, 9). HCV has a single positive-strand RNA genome of 9.4 kb encoding a large precursor polyprotein. It shares similarities in genome structure with flaviviruses and pestiviruses and is currently grouped into six major genotypes (12). A putative second envelope protein is encoded by the E2/NS1 region of the HCV genome, and a hypervariable region (HVR) is located within the E2/NS1 region. A high degree of sequence variation in the HVR within isolates and between isolates from different patients shows that this is a rapidly evolving region (4, 6, 7, 11). Antibody-neutralizing epitopes have been mapped to the HVR, and the data suggest that the HVR is under immune selection pressure (13, 16).

The high degree of heterogeneity of the HCV HVR encountered in many samples from patients probably requires a population-based approach for study and also makes it difficult to study the sequence variation of HVR with the frequently used method of sequencing a number of individual reverse transcriptase PCR (RT-PCR) clones. Here we describe the use of a semiautomated direct solid-phase sequencing approach for screening a large number of patients from the same geographical area in order to evaluate sequence diversity in virus populations and to monitor dynamic changes in HVR constitution during the natural course of infection in a number of patients. A comparison between this approach and the cloning approach is also made.

MATERIALS AND METHODS

Clinical samples and PCR. Blood serum samples were collected from 24 anti-HCV-positive patients and were stored at −20°C. The isolates of 21 of the patients had been genotyped as described previously (10). RNA extraction from 100 μl of serum was performed as described earlier, with a modified acid guanidinium thiocyanate-phenol-chloroform method (1, 17). The extracted RNA was frozen immediately and kept at −70°C until use. A 208-bp biotinylated PCR fragment was generated by seminested PCR. Reverse transcription and the outer PCR were performed in a single tube with 50 pmol (each) of the primers YU1 and YD1. The outer PCR product was diluted 1,000-fold. Inner PCR was carried out with 5 pmol of each primer (YU2 and YD1). Outer PCR was run with a program of 35 cycles of 95°C for 1 min, 45°C for 2 min, and 72°C for 3 min, and inner PCR was run with a program of 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. Five microliters of PCR product was analyzed on a 3% ethidium bromide-stained agarose gel. All primers used are described in Table 1.

Direct solid-phase sequencing. The PCR products were sequenced directly with streptavidin-coated paramagnetic beads (Dynabeads M280-streptavidin; Dynal, Oslo, Norway). Ninety microliters of PCR product was incubated for 30 min with 300 μg of beads resuspended in 90 μl of washing-binding (W/B) buffer containing 1× TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), 2 M NaCl, 0.1% Tween 20, and 1 mM β-mercaptoethanol. The beads had been washed twice with W/B buffer prior to mixing and incubation. A neodymium-iron-boron permanent magnet was used to sediment the beads. After incubation, the beads were washed twice with 100 μl of W/B buffer and once with 80 μl of 1× TE. The DNA strands were melted apart by incubation with 10 μl of 0.1 N NaOH for 5 min, and after magnetic separation, the supernatant with single-stranded DNA was transferred to microtiter wells and neutralized by being mixed with 6 μl of 0.1667 N HCl. The beads with immobilized single-stranded DNA were washed once with 50 μl of 0.1 N NaOH, once with 100 μl of W/B buffer, and once with 80 μl of 1× TE buffer before being resuspended in 16 μl of sterile water and transferred to microtiter wells. The Sanger sequencing reactions were carried out with an automated Biomek-1000 robotic workstation (Beckman Instruments, Fullerton, Calif.) according to the protocol described by Hultman and coworkers (5). Both eluted and immobilized strands were sequenced. Briefly, a T7 sequencing protocol was followed with 0.9 × 4 U of T7 polymerase (Pharmacia Biotechnology, Uppsala, Sweden) and 2 pmol of 5′-end fluorescent-labelled sequencing primer for each sample (J1 and J2, respectively). The microtiter plate with single-stranded DNA (bound and eluted) was placed in a heating/cooling module (HCB-1000; Beckman Instruments). The robot was programmed to complete the annealing, sequencing, and formamide denaturation reactions with the same 96-well plate. The sequencing reaction mixtures were then loaded onto an automated laser fluorescent sequencing apparatus (Pharmacia Biotechnology), with a 6% polyacrylamide gel (ReadyMix gel; Pharmacia Biotechnology). Processed sequences were manually edited at the polymorphic sites caused by the heterogeneous virus populations and subjectively evaluated to determine the major consensus sequence and the minor variations at polymorphic sites as previously described by Leitner and coworkers for heterogeneous virus populations (8).

Cloning. Nonbiotinylated inner PCR product, generated with YD1 and a nonbiotinylated YU2 primer, was cloned with the Invitrogen TA cloning kit according to the manufacturer's instructions. Colonies were screened by PCR with 5 pmol (each) of one biotinylated and one nonbiotinylated primer (RIT 30 and RIT 27, respectively) (5), with a temperature profile of 5 min at 94°C, 30 cycles of 95°C for 15 s, and 72°C for 2 min and a final soak at 72°C for 10 min. Sequencing was performed with the solid-phase sequencing protocol described above, with the dye primers USP and RSP.

RESULTS

Direct sequencing of patient serum samples. Total RNA was extracted from blood serum samples collected from 24 patients, and RT-PCR was performed on viral RNA, followed by direct solid-phase sequencing of the PCR product as outlined in Fig. 1. The primers used are described in Table 1.

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TABLE 1. Characteristics of primers used in this study

Primer	Sequence[a]	Site of annealing (nucleotides)[b]
YU1	5′-CT(G/A) CTC CGG ATC CCA CAA GC-3′	1350–1369
YU2	5′-Biotin-(T/G)(C/T)T CCA TGG TGG GGA ACT GG-3′	1426–1445
YD1	5′-TCA TTG CAG TTC AGG GCC GT-3′	1614–1633
J1	5′-FITC-GGG GAA CTG GGC IAA GGT C-3′	1436–1454
J2	5′-FITC-TTC AGI GCI GTI CI(A/G) TTG ATG-3′	1604–1624
RIT27	5′-GCT TCC GGC TCG TAT GTT GTG TG-3′	
RIT30	5′-Biotin-AAA GGG GGA TGT GCT GCA AGG CG-3′	
USP	5′-FITC-CGT TGT AAA ACG ACG GCC AGT-3′	
RSP	5′-FITC-TTC ACA CAG GAA ACA GCT ATG ACC-3′	

[a] I, inosine; FITC, fluorescein isothiocyanate.
[b] Annealing sites according to the sequence for hcv-1 (GenBank accession number M62321).

Twenty of the patient serum samples were genotyped by PCR with genotype-specific primers based on the conserved core region. The results of the genotyping, together with the known transmission mode, are presented in Table 2. Phylogenetic analysis of the major sequence of the samples obtained from 21 patients of Swedish origin was performed. In parallel, three control patients of non-Swedish origin (Middle East) and four sequences retrieved from GenBank representing different genotypes were included. The phylogenetic analysis was performed on a 120-nucleotide sequence corresponding to the nucleic acid sequence of HVR and flanking regions with the PHYLIP software package. The result is presented in Fig. 2. The 21 isolates of Swedish origin were found to be rather divergent and do not group into any single easily identified cluster. Note that samples at two different time points (approximately 14 months apart) for five patients were included. For four of these patients (no. 2, 11, 13, and 20), the two samples group close together, but for the fifth patient (patient 8), the two samples group far apart, indicating a possible multiple infection with two major forms of the virus.

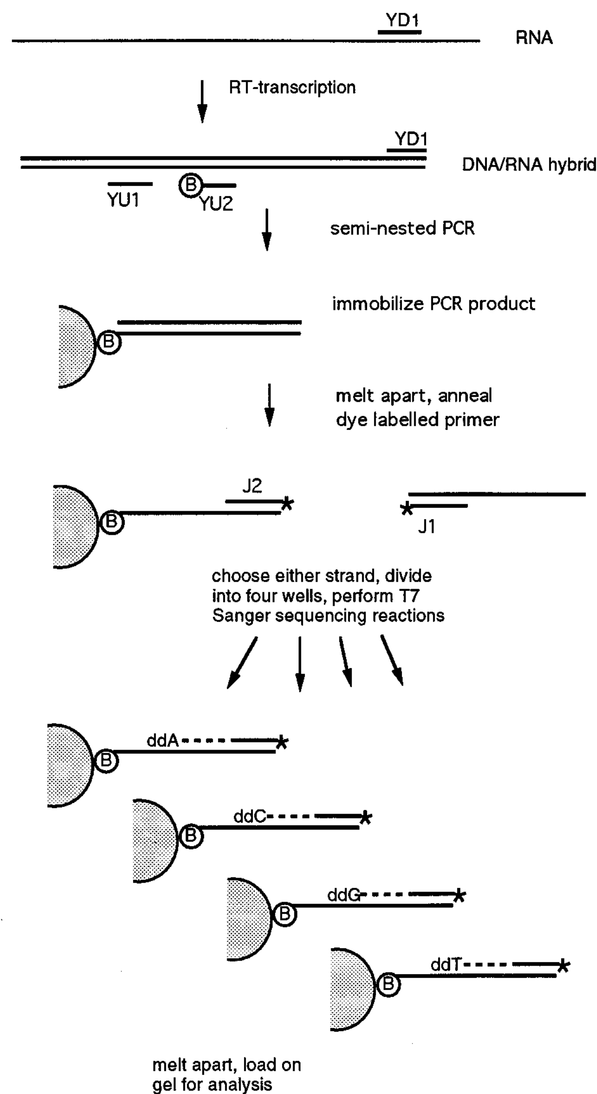

FIG. 1. Schematic drawing of the direct solid-phase sequencing concept used in this study. Sequencing of both strands was performed, although only the sequencing of the single strand immobilized onto the beads is illustrated.

TABLE 2. Characteristics of patients used in this study

Patient[a]	HCV transmission mode[b]	Genotype	GenBank accession no.
1	PTH	1a	U24599
2 (a, b)	PTH	1a	U24603, U24621
3	PTH	1a	U24614
4	PTH	2b	U24602
5	PTH	1a + 1b	U24606
6	IVDU	1a	U24616
7	PTH	1b	U24615
8 (a, b)	Sporadic	1a	U24600, U24258
9	PTH	1a	U24601
10	Sporadic	1a	U24613
11 (a, b, c)	Sporadic	1a	U24605, U24255, U24257
12	PTH	2b	U24604
13 (a, b)	IVDU	1a	U24612, U24617
14	PTH		U24598
15	PTH	1a	U24259
16	Sporadic	1a	U24618
17	PTH	1a	U24611
18	PTH	2b	U24608
19	PTH	1b	U24609
20 (a, b, c)	Sporadic	1a	U24597, U24256, U24622
21	PTH	1b	U24619
S1			U24620
S4			U24607
S5			U24610

[a] Patients S1, S4, and S5 are three control patients from the Middle East. All other patients are from Sweden. Letters (a, b, and c) in parentheses for patients 2, 8, 11, 13, and 20 represent different time points at which serum samples were taken (see text for details).
[b] PTH, posttransfusion hepatitis; IVDU, intravenous drug abuser; Sporadic, transmission mode unknown.

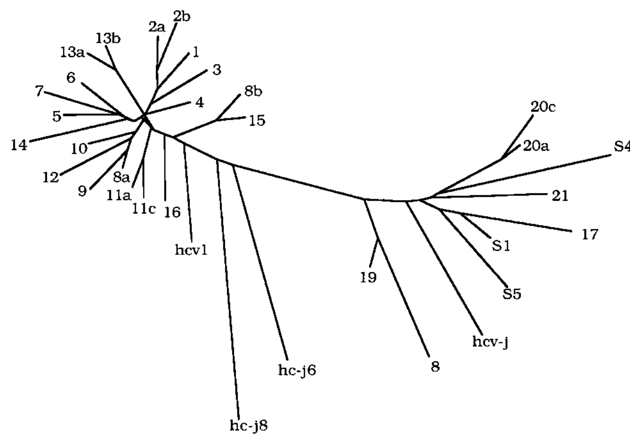

FIG. 2. Phylogenetic tree analysis based on the HVR of HCV in samples from different patients. The tree was constructed with the NEIGHBOR program in the PHYLIP package (version 3.5), kindly provided by J. Felsenstein, which clusters a matrix of nucleotide distances previously estimated with the program DNADIST, which with the D option was set to use the stochastic model of maximum likelihood. For five patients (no. 2, 8, 11, 13, and 20), samples from two different time points approximately 14 months apart have been included. Four sequences retrieved from GenBank, representing genotypes 1a, 1b, 2a, and 2b, have been included (accession numbers are in parentheses): hcv-1 (M62321), hcv-j (D90208), hc-j6 (D00944), and hc-j8 (D01221).

Comparison of direct versus cloned sequencing. To evaluate the potential advantage of direct solid-phase sequencing compared with the cloning approach, samples from patients (no. 1, 4, 7, 10, and 17) with virus populations having various degrees of polymorphism in the HVR were amplified by PCR and the fragments were cloned into a plasmid. Approximately 10 to 15 clones of each sample were sequenced, and the sequences were compared with those obtained by direct sequencing of the same sample. Good agreement between the consensus sequences obtained with the two methods was observed, although the major sequence obtained by direct sequencing was in some cases found to make up less than 50% of the clones analyzed (Fig. 3).

Substitution rate in vivo. A longitudinal study of six patients was performed to estimate the nucleotide substitution rate of viruses in vivo. The rate was found to be between 0.095 and 0.21 nucleotide substitution per genome site per year. The corresponding amino acid substitution rate was found to be approximately 9 of 27 amino acids in 14 months. The result is shown in Fig. 4. An example of the raw data for part of the HVR from one patient at three different time points is shown in Fig. 5. An example of a new sequence variant can be observed as the amino acid residue alanine is gradually changed to a glycine. This shows the usefulness of the technique for studying dynamic changes in a heterogeneous virus population over time.

DISCUSSION

The phylogenetic analysis of the HVR shows that the different virus populations isolated from different Swedish patients are diverse in terms of the nucleic acid sequence. They group into two broad clusters: one smaller group including the hcv-j strain (genotype 1b) together with the strains from the three Middle East patients and one larger group including the prototype hcv-1 strain (genotype 1a). The two other sequences retrieved from GenBank, hc-j6 and hc-j8, corresponding to genotypes 2a and 2b, do not group with any of the samples analyzed. The grouping based on the HVR does not correlate with genotype (based on the more conserved core region). The discrepancy is not very surprising, since the genotyping based on the conserved core region reflects the evolutionary origin of the virus, while the grouping based on the nucleotide sequence of the HVR probably can be said to reflect the immunological history of the virus, i.e., the selection pressure it has encountered in different hosts along its transmission path. Since the HVR most likely is under selective pressure, it is not fully suitable for strict phylogenetic analysis, as illustrated by this example. However, it can be a tool to analyze HVR subtype switching (as indicated by results for patient 8) in longitudinal analysis, not evident by core region genotyping. Note that the phylogenetic analysis for this study was performed with a population-based approach, since it is based on direct DNA sequencing.

Note that 4 of the 27 amino acids in the HVR (amino acids 2, 6, 23, and 26, as denoted in Fig. 4) are constant in all samples analyzed as well as in the four GenBank sequences retrieved and other published sequences (3, 4, 10, 11, 13, 16). However, the nucleotides in the third position in some of the codons for these four amino acids differ between some isolates from different patients in this study and in some cases between samples from the same patient taken at different time points. This strongly suggests that there exists a selective pressure to conserve these four amino acid residues. Patient 11 (Fig. 4) shows an interesting feature: a four-amino-acid insertion just upstream of the HVR found in samples from all three time points, indicating the rather flexible three-dimensional structure of the region. It has previously been pointed out that the amino terminus of the HCV E2 domain appears to be a flexible

Direct sequence: Major	F	A	G	V	D	A	E	T	H	T	T	G	G	S	A	A	R	A	T	F	G	I	A	N	F	F	T	P	G	A	K	Q	N	I	Q	L	I	N	T
minor															V			T	A	S	A	L	T	S	L		A	S			R		D	V					
minor																					V						S							L					
Sequence of cloned PCR products: Clone 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	A	-	-	-	R	-	D	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	T	S	L	-	S	-	-	-	-	-	D	-	-	-	-	-	-
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5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	A	-	-	L	-	-	L	-	S	-	-	-	-	-	D	-	-	-	-	-	-
6	-	-	-	A	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	L	T	-	L	-	A	-	-	-	-	-	D	V	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	S	V	L	-	-	L	-	D	S	-	-	-	-	K	-	-	-	-	-	-
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11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	L	T	-	L	-	A	-	-	-	-	-	D	V	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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FIG. 3. Comparison of direct sequencing versus sequencing of cloned PCR products. At the top is the deduced amino acid sequence of the major strain, determined by direct solid-phase sequencing, with the minor variants indicated just below. Below this are the sequences of the 14 clones analyzed, which originated from the same sample. Note that only four of the cloned sequences are identical to the major consensus sequence obtained by direct sequencing and that among the 14 clones, 10 different species are found.

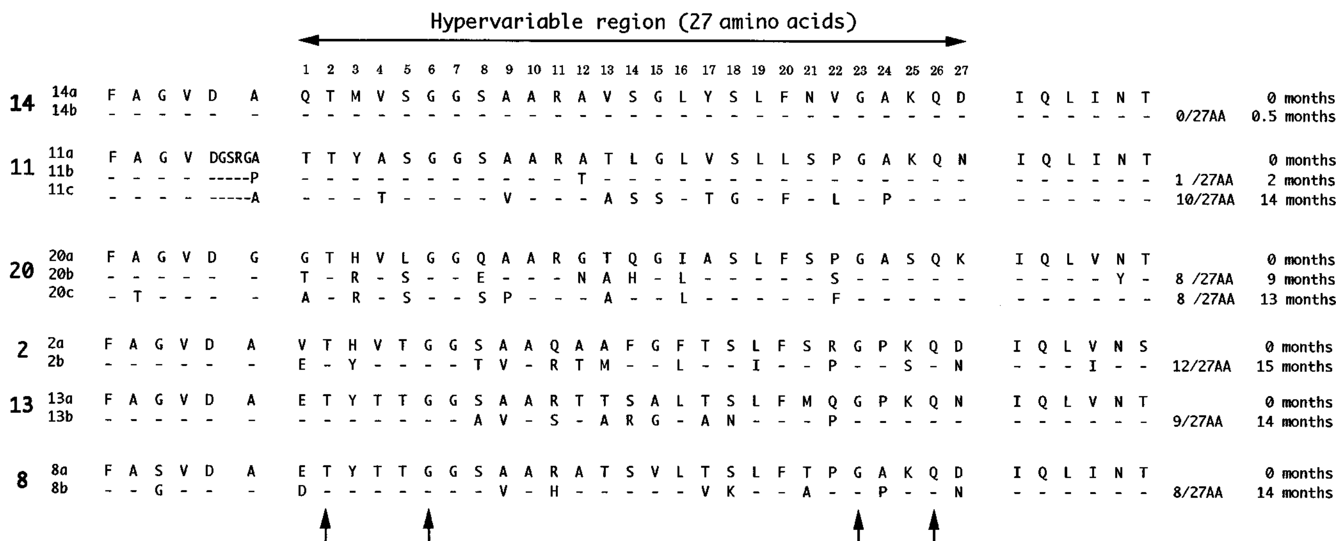

FIG. 4. Change in major sequence over time for six patients. None of the patients was subject to antiviral treatment. Arrows indicate amino acid positions that were constant in all patients. The region shown here corresponds to nucleotides 1473 to 1589 according to the nucleotide designations for hcv-1 (GenBank accession number M62321). AA, amino acids.

end with no apparent structural constraints (16). Another observation is that no defective virus with stop codons, as described by Higashi and coworkers (4), has been observed in the major or minor sequences obtained in this study. An explanation for this can be that defective quasispecies present in small proportions (less than 10% of the total population) can be found only by RT-PCR cloning and not by direct DNA sequencing.

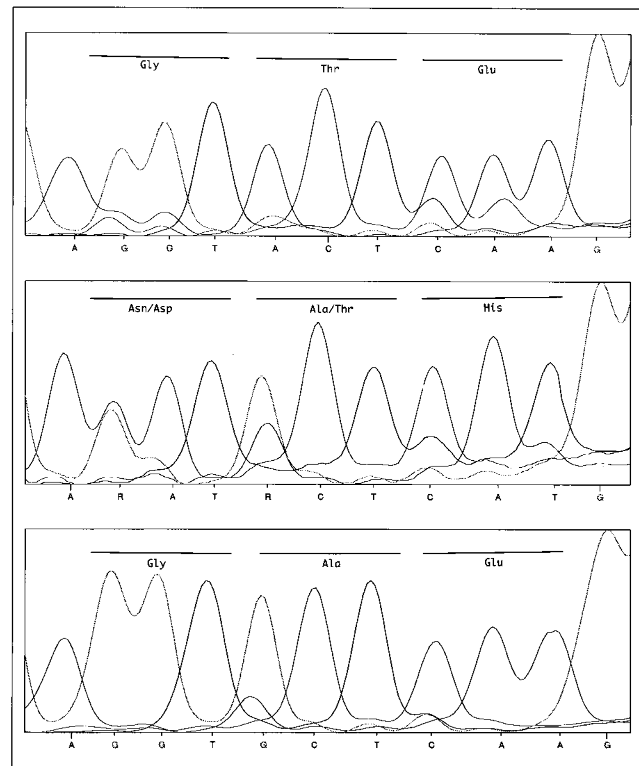

FIG. 5. Raw data from direct sequencing of serum from patient 20 at three different time points: 0 months (top), 9 months (middle), and 13 months (bottom). At the left and the right amino acid sites, a substitution is found at the intermittent time point, but it has reverted at 13 months. At the middle site, a gradual substitution of alanine for threonine can be observed. The sequence shown corresponds to nucleotides 1523 to 1533 according to the nucleotide designations for hcv-1 (GenBank accession number M62321).

One of the major advantages of direct sequencing compared with sequencing of cloned RT-PCR products is illustrated in Fig. 3. Direct sequencing allows for rapid determination of the major sequence of a heterogeneous virus population (i.e., the consensus sequence reflecting the relative populations of the different strains present in a virus population), giving a rough estimate of the polymorphism of the region analyzed. By comparison, using cloned RT-PCR products requires a large number of clones to be sequenced to get a representation of the virus population, and it still would not be possible to rule out cloning bias due to secondary structures of the PCR fragment and misincorporation caused by *Taq* polymerase. Figure 3 also illustrates another important feature in terms of comparing the direct sequencing and cloning approaches. Of the 14 clones analyzed, only 4 show a sequence identical to the major sequence, and of the other 10 clones, only 2 are identical. This illustrates the fact that, with direct sequencing, the major nucleotide seen at one polymorphic site can be the additive result of several minor strains present in the population, identical at this position but differing at others. The major sequence seen is thus the consensus sequence of the heterogeneous virus population and is not necessarily the sequence of the most abundant strain. For studying dynamic changes in heterogeneous virus populations, a population-based approach is probably more appropriate. The versatility of solid-phase direct sequencing for these types of studies, which has been documented for human immunodeficiency virus (8, 14, 15), is demonstrated here for the HVR of HCV.

Mutation rates in the HVR of between 0.013 and 0.91 nucleotide substitution per genome site per year have been reported, with the higher mutation rates associated with flare-ups of the infection and with progression of the disease towards the chronic stage (7, 11). Our results are well within this broad interval. Note that in two patient serum samples, some nucleotide substitutions were found to revert at a later time point, exemplified in Fig. 5. This suggests that because of relatively infrequent sampling, the mutation rate probably is

somewhat higher than determined. More frequent sampling and shorter time intervals would be necessary to establish the actual mutation rate or change in virus population constitution in a patient over time. It must be pointed out, however, that the corresponding amino acid substitutions found at the middle time point, but that later had reverted, are accompanied by permanent substitutions of flanking amino acids (patients 11 and 20 in Fig. 4 and nucleotide sequence of patient 20 in Fig. 5). This change thus represents a new quasispecies. It is also important to point out that the changes do not represent the actual evolution of a single virus clone, since the predominant sequence at a later time point is not necessarily the direct progeny of the predominant sequence at the first time point. It can very well be one of the minor populations expanded from a pool of genomes present and undetected at the first time point. It has previously been shown that a minor variant constituting 10% of a heterogeneous virus population corresponds to the reliable limit of detection, when direct sequencing is used (8). None of the six patients studied here received antiviral therapy before or during this period, and the observed variation therefore represents the natural history of the HCV infection in these patients.

The most prominent nucleotide sequence divergence over time was found in patient 8, who had 5 silent and 15 nonsilent nucleotide substitutions resulting in substitutions of 8 of the 27 amino acids over a period of 14 months (Fig. 4). The results from patient 8 suggest that a clonal alteration has occurred, in which a heterogeneous preexisting minor variant has a growth advantage compared with the earlier predominant HCV variant. This hypothesis is supported by the results from the phylogenetic analysis (Fig. 2) when samples from patients 2, 8, 11, 13, and 20 from a second time point are included. Samples a and b (or c) from patients 2, 11, 13, and 20, representing the first and second time points for these different patients, respectively, group closely together, suggesting that the virus in the b samples (or c samples) has evolved from the virus in the corresponding a samples. In contrast, samples 8a and 8b, representing the first and second time points for patient 8, are found far apart and do not show any close relationship, which could partly explain the appearance of the five silent nucleotide substitutions in these samples. For patients 2, 11, 13, and 20, the nucleotide substitutions observed were preferentially in the first or second codon position, resulting in amino acid substitutions. This is in agreement with findings from previous reports (3, 7, 11). In conclusion, the present study shows that dynamic analysis of heterogeneous HCV populations can be performed by direct solid-phase sequencing.

ACKNOWLEDGMENT

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