Characterization of simple and complex hepatitis C virus quasispecies by heteroduplex gel shift analysis: correlation with nucleotide sequencing

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In infected humans, hepatitis C virus (HCV) exists as a quasispecies typically characterized by multiple nucleotide substitutions within the second envelope gene hypervariable region 1 (HVR1). In the current study, we used heteroduplex gel shift analysis (GSA) of HVR1 sequences amplified directly from patients' sera to define two patterns of HCV quasispecies: (i) simple quasispecies, which gave a mostly homogeneous gel shift profile with a single predominant band and (ii) complex quasispecies, which gave a gel shift profile with multiple bands. Recombinant HVR1 libraries were generated from two patients with complex HCV quasispecies (cases 1 and 2) and two patients with simple HCV quasispecies (cases 3 and 4), and 129 individual clones were analysed by either GSA, nucleotide sequencing or both techniques. In case 1 we identified a highly complex HCV quasispecies with 11 distinct HVR1 variants differing by 1-51 nucleotide changes. We found a general but not absolute correlation between GSA pattern and the number or position of nucleotide changes within HVR1. In case 2, the complex HCV quasispecies consisted of three distinct major variants; GSA of individual HVR1 clones allowed us to reconstruct the complex quasispecies pattern in vitro. In case 3, the simple quasispecies comprised 66% homogeneous clones and 33% unique minor variants differing by 1-3 nucleotides from the consensus sequence. In case 4, the simple quasispecies was 84% homogeneous, but six unique major shift variants were identified among 31 clones by GSA. In summary, HCV quasispecies can be characterized based on GSA profiles following direct PCR amplification of HVR1 sequences from patients' serum; the GSA profiles approximate the clonal population of HCV as determined by clonal analysis. GSA of HVR1 clones showed a strong correlation with nucleotide sequencing.

Introduction

Hepatitis C virus (HCV), the aetiological agent of chronic non-A, non-B hepatitis, infects an estimated three million individuals in the United States. Furthermore, greater than 90% of individuals with serological evidence of HCV infection have evidence of active viraemia by reverse transcription polymerase chain reaction (RT-PCR) (Gretch et al., 1992) and approximately 60% of infected individuals develop chronic liver disease (Alter et al., 1992). Patients with HCV infection are at increased risk for the development of liver cirrhosis and liver cancer (reviewed in Plagemann, 1991) and are also at increased risk for developing immune complex-mediated disorders (Johnson et al., 1993). Understanding the mechanisms of persistent HCV infection in humans is therefore an important clinical problem.

The genome of HCV is a positive-sense, single-stranded 9-4 kb RNA molecule. HCV has been demonstrated to exist as a quasispecies in infected individuals based on the finding of multiple mutations within the second envelope gene hypervariable region (HVR1) (Weiner et al., 1991; Martell et al., 1992; Kato et al., 1994). Virus quasispecies have been defined as a variable mixture of a master nucleotide sequence and a spectrum of mutant molecules isolated from a given host and are characteristic of certain classes of RNA viruses, such as lentiviruses and picornaviruses (Holland et al., 1992). The higher error frequency of RNA-dependent RNA polymerases is thought to be responsible for the generation of quasispecies and immune selection is thought to be a major factor driving quasispecies diversification (Weiner et al., 1992). It is tempting to speculate that quasispecies evolution contributes to HCV persistence and the high rate of resistance of HCV to interferon therapy (reviewed by Trepo et al., 1994).
Indeed, a recent report suggests that HCV quasispecies are differentially sensitive to interferon (Enomoto et al., 1994).

Quasispecies of HCV have previously been characterized by RT-PCR amplification of HVR1 and the cloning and sequencing of a large number of clones. However, these procedures are labour intensive, time consuming and expensive, which limits the number of viral isolates one can analyse. In the current report, we describe a more rapid method for analysing HCV quasispecies known as heteroduplex gel shift analysis (GSA), which has previously been applied to the analysis of human immunodeficiency virus (HIV) quasispecies (Delwart et al., 1993, 1994). Using GSA and nucleotide sequence analysis, we characterized simple and complex HCV quasispecies in four patients with active HCV infection. Compared to nucleotide sequencing, we found GSA to be a simple, rapid and inexpensive technique for the characterization of HCV quasispecies.

Methods

Clinical specimens. Serum samples were obtained from four patients with active HCV infections who were participating in ongoing studies at the University of Washington Medical Center. The diagnosis of active HCV infection was determined by positive serological testing for HCV antibodies (EIA2, Abbott Laboratories and RIBA II, Ortho Diagnostics) by positive testing for HCV RNA by RT-PCR using primers specific to the S' non-coding region and by abnormal biochemical markers as described previously (Gretch et al., 1992, 1993). Serum was separated from whole blood within 2 h of venipuncture and stored at −70 °C to allow optimal recovery of viral RNA.

RNA extraction and PCR. Total RNA was extracted from patients' sera by the single-step guanidinium thiocyanate method (Ausubel et al., 1991) with slight modification: 100 μl serum was added to 500 μl denaturing solution (4 m-guanidinium thiocyanate, 25 mm-sodium citrate, pH 7.0, 0.1 m-mercaptoethanol and 0.5% N-laurylsarcosine) and vortexed briefly. Next, 50 μl of 2 m-sodium acetate pH 4.0, 500 μl water-saturated phenol and 100 μl 49:1 chloroform:isoamyl alcohol were added, respectively, and mixed well by vortexing. This extract was then incubated on ice for 15 min before centrifugation at 12,000 r.p.m. for 15 min at 4 °C. The aqueous supernatant was precipitated with 1 volume of 100% isopropanol at −20 °C for 30 min before centrifuging at 14,000 r.p.m. for 20 min at 4 °C. The pellet was dissolved in 250 μl denaturing solution and precipitated with 250 μl 100% isopropanol, incubated at 20 °C for 30 min and centrifuged at 14,000 r.p.m. for 20 min at 4 °C. The pellet was then washed with 500 μl 75% ethanol, centrifuged, air dried and resuspended in 10 μl diethylpyrocarbonate-treated distilled water. After incubation at 70 °C for 5 min, the entire 10 μl RNA was reverse transcribed in a 25 μl reaction containing 100 pmol each of sense and antisense primers (Weiner et al., 1992), 6 mM-MgCl2, 1 mmol each deoxynucleoside triphosphate (dNTP), 1 mM-DTT, 75 mM-KCl, 5 mM-Tris-HCl pH 8.3, 20 U RNase inhibitor (Pharmacia LKB) and 130 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL). The mixture was incubated at 37 °C for 1 h, then at 95 °C for 5 min.

A first round PCR reaction was performed as follows: 10 μl of the cDNA was added to a 40 μl PCR mixture containing 1·13 mM-MgCl2, 23·5 mM-Tris-HCl pH 8.3, 35·5 mM-KCl and 1·5 U AmpliTaq polymerase (Perkin Elmer). The reaction was amplified for 35 cycles (94 °C for 10 s, 50 °C for 30 s and 72 °C for 30 s) in a Perkin Elmer 9600 thermocycler. The size of the first round PCR product was 244 nucleotides.

A hot start, nested PCR was then performed using identical cycling conditions and internal primers, as previously described (Weiner et al., 1991), which yielded a 176 bp amplified product. For nested PCR, the lower reaction mixture contained 3 mM-MgCl2, 200 μmol each dNTP, 10 mM-Tris-HCl pH 8.3, 15 mM-KCl and 100 pmol each primer and was separated by a wax layer (AmpliWax; Perkin Elmer) from the upper reaction mixture containing 40 mM-Tris-HCl pH 8.3, 60 mM-KCl, 1·5 U AmpliTaq polymerase (Perkin Elmer) and 0·1% of the first round product. The amplified product size was confirmed by gel electrophoresis using 2% agarose and DNA molecular weight standards (Gibco BRL).

Cloning. PCR product was purified using QIAquick PCR product purification columns (QIAGEN) according to the manufacturer's protocol. Purified product was quantitated by ethidium bromide staining (Ausubel et al., 1991) using DNA standards as controls and 6·8 ng was directly ligated into 30 ng PCR II vector (TA cloning kit; Invitrogen). Transformation of recombinant plasmid DNA into Escherichia coli INV F' competent cells was performed according to manufacturer's protocol (Invitrogen) and transformants were grown on ampicillin plates.

Sequencing. Nucleotide sequence of the N-terminal region of the E2 gene, defined as the hypervariable region 1 (HVR1) (Weiner et al., 1991) was determined by sequencing a total of 162 HVR1-insert clones from three patients. Plasmid DNA was prepared for sequencing using QIAwell plasmid prep system according to the manufacturer's protocol (QIAGEN) and sequenced by fluorescence-based Taq 'dye-deoxygen' terminator cycle sequencing system (ABI) using M13 universal primers. Sequencing reactions were analysed on an Applied Biosystems model 373A automated sequencer (ABI).

Heteroduplex gel shift analysis. Heteroduplex gel shift analysis (GSA) was performed as follows. A representative clone containing an E2 HVR1 insert of interest was amplified by PCR, purified and 32P-end-labelled to generate a probe. The probe was then hybridized to unlabelled E2 HVR1 PCR product either derived by direct PCR from serum (heterogeneous) or from HVR1-containing plasmid DNA (homogeneous). Hybrids were analysed by agarose gel electrophoresis and autoradiography. Probe hybridized to itself (unlabelled) served as a marker for the identification of homoduplexes. Hybrids with nucleotide mismatches showed aberrant migration and were identified as heteroduplexes.

Homogeneous PCR product was generated either from 25 μl of bacterial lysate, or from 100 ng of plasmid DNA from which the nucleotide sequence was previously determined. For bacterial lysates, colonies were placed into 100 μl distilled water, mixed with 10 μl 1 M-NaOH, incubated for 1 h at 37 °C and 10 μl 1 M-HCl was added and mixed before performing PCR with nested primers. Radiolabelled probes were generated from 20 ng of clonally derived PCR product, which was column purified and end labelled using T4 polynucleotide kinase (Gibco BRL) and 100 μCi of [32P]ATP (Amersham).

Hybridization reactions were performed by combining 2·4 μl unlabeled homogeneous or heterogeneous PCR product (80 pmol) of DNA with 0·5 μl (2×10⁴ c.p.m. ; 2·4 fmol) DNA labelled probe, 2·1 M-NaCl, 100 mM-Tris-HCl pH 8·0 and 20 mm-EDTA. This ratio of probe to target DNA (1 : 3000) was optimal for tracking HVR1 heteroduplexes (data not shown). Reactions were denatured at 95 °C for 5 min and annealed at 60 °C for 2 h before the entire reaction volume was loaded on 1 mm thick, 6% polyacrylamide MDE gel (Baker) and electrophoresed for 18–20 h at 400 V. The gel was vacuum dried at 80 °C on filter paper and exposed to X-ray film.
Results

Correlation between nucleotide sequencing and GSA of HCV HVR1

Nucleotide sequence analysis of the HCV HVR1 has indicated that HCV may persist in serum as rapidly mutating viral quasispecies, which produce a large number of genetic variants over time (Weiner et al., 1991; Martell et al., 1992; Kato et al., 1994). Recently, Delwart and coworkers (1993, 1994) have described GSA to be a more rapid method for characterization of viral quasispecies during chronic HIV infection. Fig. 1 illustrates the strengths and limitations of the GSA to probe 1.1, variable shifts in the hybrid banding patterns of duplexes formed between both strands of probe and unlabelled DNA from clone 1.1 and was 32p-end-labeled as described in Methods. When probe 1.1 was hybridized with probe 1.1. Clones 1.12 and 1.13 differed by 51 and 52 nucleotide changes from probe 1.1, respectively (Fig. 1a) and displayed very large shift patterns when hybridized with probe 1.1 (Fig. 1b; rightmost lanes). The background homoduplex band was slightly stronger for clones 1.12 and 1.13 where base differences are 50 and 51 nucleotides, respectively. This may be due to a decrease in the efficiency of hybridization of probe 1.1 to clones 1.12 and 1.13.

In summary, GSA of this set of 13 HVR1 clones isolated from a highly complex HCV quasispecies showed excellent correlation with nucleotide sequencing: we observed a unique gel shift pattern for each clone containing a different number of base mismatches relative to the probe. Furthermore, we observed unique gel shift patterns for clones with the same number of mismatches in different positions. There was a general (but not absolute) correlation between the degree of gel shift and the number of DNA base differences relative to the probe. When each heteroduplex gel was run a constant distance, the individual gel shifts seen in different experiments correlated on a consistent basis with a given range of nucleotide changes (data not shown).

Investigation of a complex HCV quasispecies following direct amplification from serum

One potential application of GSA would be the evaluation of HCV quasispecies following direct PCR amplification of HVR1 from patient’s serum. This application is illustrated in Fig. 2 for patient 2, whose serum contained a complex HCV quasispecies. Nucleotide sequence analysis of 31 HVR1 clones isolated from the serum of patient 2 revealed three distinct major sequence variants within HVR1 in approximately equal proportion. Representative HVR1 sequences of these three major variants are shown in Fig. 2(a; designated clone 2.1, clone 2.2 and clone 2.3). Clones 2.2 and 2.3 differed from the reference clone 2.1 by 10 and 11 nucleotide changes within HVR1, respectively. Furthermore, clones 2.2 and 2.3 differed from each other by eight nucleotide changes within HVR1.
Fig. 1. Comparison of gel shift analysis with nucleotide sequencing in the characterization of a complex HCV quasispecies. RT-PCR was performed on serum from patient 1 using primers specific for the second envelope gene hypervariable region 1 (HVR1) and amplification products were cloned into vectors as described in Methods. (a) The nucleotide sequence of 13 HVR1 clones identified within this patient's HCV quasispecies, in order of increasing nucleotide divergence relative to the reference clone (clone 1.1). (b) Heteroduplex gel shift analysis (GSA) of the 13 clones when clone 1.1 was used as a probe (probe 1.1). The position of the clone 1.1 homoduplex band is noted by the arrow. Experimental conditions of GSA are given in Methods.
Hepatitis C virus quasispecies

(a)

Clone

1087

2.1 TCCAGTGTTGGGAACTGGC~AAAGGTCTTGGTAGTGCTGT TGCTAT TCGCCAGCGT CGACGCGCACACCCACGT CACCGGGGGAAGTAG

2.2 ................................................................. T ..................... GC

2.3 ....................................................................................... GT

(b)

Band 5

Band 4

Band 3

Band 2

Band 1

Fig. 2. In vitro reconstruction of a complex HCV quasispecies pattern using gel shift analysis. HVR1 sequences were isolated from the serum of patient 2 by PCR and cloned as described for Fig. 1. Nucleotide sequence analysis of 30 clones revealed three major sequence variants; consensus nucleotide sequences of the three major sequence variants (designated clones 2.1, 2.2 and 2.3) are listed in (a). (b) Reconstruction of the complex quasispecies pattern observed in the serum of this patient using gel shift analysis in vitro. Lane 1, HVR1 was directly amplified from patient 1 serum and hybridized to probe 2.1 (derived from clone 2.1 by end-labelling). The position of the homoduplex band (band 1) as well as heteroduplex shift bands (bands 2-5) are indicated to the left of lane 1. Lane 2, probe 2.1 hybridized to unlabelled DNA from clone 2.1 (homoduplex control). Lanes 3-6: homoduplex and heteroduplex shift patterns obtained when probe 2.1 was hybridized to equimolar mixtures of clones 2.1 and 2.2 (lane 3), clones 2.1 and 2.3 (lane 4), clones 2.2 and 2.3 (lane 5) and clones 2.1, 2.2 and 2.3 (lane 6).

In Fig. 2(b), lane 1, HVR1 was amplified by RT–PCR directly from patient 2 serum and hybridized to radioactive probe 2.1 (which had been generated from clone 2.1 by end labelling). A complex gel shift pattern with five major bands (Fig. 2b, lane 1; bands 1–5) was observed, confirming the genetic complexity of HVR1 isolated from this patient’s serum. We next examined the GSA profiles generated when all possible combinations of the three major variants (clones 2.1, 2.2 and 2.3) were hybridized with probe 2.1. A homoduplex shift pattern was observed following hybridization of probe 2.1 to clone 2.1 (Fig. 2b, lane 2). When probe 2.1 was hybridized to an equimolar mixture of DNA from clone 2.1 and clone 2.2 (Fig. 2b, lane 3), the homoduplex pattern from clone 2.1 was apparent, plus a unique heteroduplex shift pattern attributed to clone 2.2 (a doublet). When probe 2.1 was hybridized to an equimolar mixture of DNA from clone 2.1 plus clone 2.3 (Fig. 2b, lane 4), the homoduplex pattern of clone 2.1 was again apparent, plus a unique heteroduplex shift pattern attributed to clone 2.3 (a doublet with a greater gel shift). When probe 2.1 was hybridized to an equimolar mixture of DNA from clone 2.2 plus clone 2.3 (Fig. 2b, lane 5), the two unique doublet shift patterns were readily apparent (clone 2.2 shift pattern plus clone 2.3 shift pattern). Finally, probe 2.1 was hybridized to an equimolar mixture of DNA from clone 2.1 plus clone 2.2 plus clone 2.3 (Fig. 2b, lane 6). The heteroduplex and homoduplex shift patterns observed in this complex mixture experiment closely reflected those patterns observed in lanes 2–5.

Importantly, the GSA pattern observed following direct PCR amplification of HVR1 from serum was faithfully reproduced by in vitro mixing of all three clones.
Fig. 3. Analysis of a simple HCV quasispecies present in the serum of patient 3. Direct amplification of hypervariable region 1 (HVR1) from serum followed by gel shift analysis (GSA) showed a single band (lane 1). Lanes 2–21 demonstrate GSA of 20 independent HVR1 clones isolated from the same serum specimen. Clone 3.1 was end-labelled and used as probe (probe 3.1). This simple quasispecies contained 66% homogeneous HVR1 sequences and 33% minor sequence variants (1–3 nucleotide changes) based on nucleotide sequence analysis of 35 independent clones obtained from the serum of patient 3 over a 1 month period (see text for details).

Fig. 4. Detection of low-frequency major shift variants within a simple quasispecies by heteroduplex gel shift analysis (GSA). The leftmost lane demonstrates a single strong band plus a series of low-intensity shift patterns observed when hypervariable region 1 (HVR1) was amplified from patient 4 serum and directly hybridized with probe 4.1 (derived from end-labelled clone 4.1). The remaining lanes represent GSA profiles obtained using probe 4.1 plus DNA from 31 independent HVR1 clones generated from this patient's quasispecies (clones 4.1 to 4.31). Note the distinct heteroduplex gel shift patterns observed with clones 4.2, 4.7, 4.16, 4.21, 4.23, and 4.26.
homogeneous pattern (Fig. 3, leftmost lane), suggesting
were obtained when clones 2.2 and 2.3 were used
homoduplex (related to clone 2.1), whereas bands 2 and
5 represent variants related to clone 2.3. Identical results
were obtained when clones 2.2 and 2.3 were used
separately as probes in this experiment (data not shown).
These experiments further illustrate the utility of GSA
for analysing complex HCV quasispecies following direct
amplification of HVR1 from patient serum and for
resolving complex quasispecies in vitro.

Analysis of simple HCV quasispecies by GSA

GSA was also useful for evaluating HCV quasispecies
with minor genetic changes (simple quasispecies) as
illustrated in Figs 3 and 4. GSA of HVR1 amplified from
the serum of patient 3 demonstrated a relatively
homogeneous pattern (Fig. 3, leftmost lane), suggesting
the presence of a single major variant. This HCV
quasispecies was then cloned, and multiple random
clones were analysed by either GSA (Fig. 3) or nucleotide
sequence analysis (data not shown). In the GSA
experiment, 20 HVR1 clones were analysed in addition
to the reference clone (clone 3.1). All 20 HVR1 clones
appeared very similar to the probe (homoduplex pattern),
although 3 of 20 clones (clones 3.4, 3.18 and 3.19)
showed very slight but detectable shifts relative to
reference clone 3.1 (Fig. 3). Sequence analysis of 35 new
HVR1 clones obtained from three independent RT–PCR
cloning experiments performed at three time points
with a 1 month period confirmed that 66% (23/35) of
clones were identical to clone 3.1, whereas 33% of
clones differed by 1–3 nucleotides relative to clone 3.1
(data not shown). Thus, nucleotide sequencing and GSA
of individual clones confirmed the presence of a mostly
homogeneous genetic population of HCV in this patient
(simple quasispecies), although nucleotide sequencing
was clearly more sensitive for detecting genetic variants
with minimal sequence changes (minor variants) relative
to the consensus variant. The clinical significance of this
simple quasispecies is discussed elsewhere (Gretch
et al., 1995).

In a fourth case (patient 4), direct PCR amplification
of HVR1 from serum followed by GSA (leftmost lane;
Fig. 4) revealed a predominant single band plus low
intensity shift patterns (designated shift A and shift B in
Fig. 4), suggesting a predominantly simple quasispecies
plus a low frequency of genetic variants with appreciable
genetic heterogeneity. Next, 31 HVR1 clones were
generated from the serum specimen and analysed by
GSA (Fig. 4): 81% (25/31) of the clones gave homo-
duplex gel shift patterns relative to the reference clone,
whereas six clones gave unique heteroduplex shift
patterns (clones 4.2, 4.6, 4.16, 4.21, 4.23, and 4.26).
Upon careful examination of the six heteroduplex shift
patterns, clones 4.21 and 4.25 displayed doublet shift
patterns very similar to that observed by GSA of HVR1
directly amplified from serum (leftmost lane; Fig. 4).
None of the six variants appeared to be identical and all
appeared to have shift patterns consistent with 4–20
nucleotide differences relative to reference clone 4.1.
This experiment confirmed that the HCV quasispecies
from patient 4 had a single predominant variant
representing approximately 80% of clones (major vari-
ant). However, in contrast to patient 3, the patient 4
quasispecies also contained a population of low-fre-
quency major variants, each of which appeared at a
frequency of less than 5% of clones in this experiment
and displayed an apparently unique gel shift pattern.

It is important to note that in case 4 (Fig. 4) some of
the major variants detected by GSA of individual clones
did not appear in the original GSA of heterogeneous
PCR product amplified directly from serum. Therefore,
although direct PCR from serum followed by GSA was
useful for screening the overall complexity of HCV
quasispecies, subcloning of the HVR1 and GSA analysis
of individual clones was a more sensitive method for
detecting low-frequency major variants within the quasi-
species.

Discussion

HCV appears to behave as a rapidly evolving virus
quasispecies in the infected human host; however, the
clinical significance of this complex virological phenom-
emon in terms of viral persistence, transmission and
pathogenesis is largely unknown. The current study
demonstrates the utility of heteroduplex gel shift analysis
(GSA) for the rapid and extensive characterization of
HCV quasispecies isolated from the serum of four
representative patients, two with apparently homo-
genous (simple) quasispecies and two with more
heterogeneous (complex) quasispecies. In the current
study, nucleotide sequence analysis was performed on
over 100 discrete HVR1 clones to confirm the genetic
complexities observed by GSA and to determine the
relationship between altered gel shift and the degree of
genetic change relative to reference clones.

By analysing individual HVR1 clones by both GSA
and nucleotide sequencing, we show that the distance of
the heteroduplex gel shift correlates in general with the
extent of nucleotide divergence between any two clones;
however, the distance of gel shift was not absolutely
proportional to the number of genetic changes,
suggesting there is a strong positional effect (i.e.,
dependence on where the mutation occurs). Differences
of 3 or more nucleotides relative to the reference sequence
(probe control) were reliably detected as shift variants by GSA. Furthermore, minor variants with one or two nucleotide changes were occasionally detected as minimal shift variants by GSA. The sensitivity of GSA for detecting minor genetic changes appeared much better if the probe was reasonably different from the target clones (i.e., 4 to 15 nucleotide changes). For example, in the case of patient 1 (Fig. 1), clones which had an identical number of genetic changes relative to the probe were resolved as different from one another by GSA when the genetic changes occurred in different positions. Furthermore, all clones with unique HVR1 sequence composition were resolved as different by GSA in this experiment. In contrast, as illustrated for patient 3 (Fig. 3) clones that were very similar to the probe were often difficult to resolve from the homoduplex pattern, even when they contained only two or three nucleotide changes.

The overall limit of resolution of GSA was approximately 1.7% divergence between any two HVR1 sequences, which is similar to that reported for the HIV-1 envelope gene hypervariable region (Delwart et al., 1993, 1994). Since detection of one or two nucleotide changes relative to the probe was less reliable by GSA than by nucleotide sequence analysis, it appears that GSA will be less useful than sequencing for detecting minor sequence variants. However, the utility of GSA as a technique for screening multiple HVR1 clones to find low-frequency major variants is especially evident from the analysis of patient 4 (Fig. 4). In this case, 31 clones were screened from an apparently homogeneous quasispecies in a single experiment, six unique HVR1 variants were identified, each of which was present at a frequency of less than 5% in the quasispecies. Thus, GSA promises to be a useful tool for screening for low-frequency genetic variants within a quasispecies population. In a similar fashion, GSA will allow faster evaluation of larger numbers of clones than nucleotide sequencing and should thus provide more accurate information about clonal frequency for future studies of the role of HCV quasispecies in pathogenesis. An alternative method for analysing HCV quasispecies variants is single-strand conformation polymorphism analysis (SSCP) as recently described by Enomoto et al. (1994). This technique has the advantage of assessing overall genetic heterogeneity using direct PCR products. However, determination of individual quasispecies variants requires excision and sequencing of DNA bands.

At present, the biological significance of mutation within HVR1 of HCV is only speculative. It has been suggested that mutations in this region permit HCV to escape from both the humoral and cell-mediated immune responses directed against the virus. In this respect, it has been shown that antibodies are produced against HVR1 in an isolate-specific manner and that the production of different anti-HVR1 antibodies varies during different episodes of disease (Weiner et al., 1992, Sekiya et al., 1993; Kato et al., 1994). GSA may be of particular value for screening patient serum for low-frequency immune escape variants as an adjunct to both humoral and cellular immunological studies.

Analysis of other regions of the HCV genome by GSA should also be possible. For example, nucleotide sequencing followed by phylogenetic analysis has demonstrated the existence of at least six major HCV genotypes and multiple subtypes (Simmonds et al., 1993). The overall complexity of the HCV genome appears adequate in several regions for genotype analysis by GSA. Furthermore, GSA may be particularly useful for epidemiological studies, for example tracking related HCV variants within a population of infected individuals or tracking the transmission of HCV from one infected individual to another (Weiner et al., 1993).

Finally, GSA technology may prove extremely useful for the tracking of HCV quasispecies over time, in the context of active disease (pathogenesis research) and during antiviral therapy. HCV infection is a common indication for liver transplantation and persistent infection of the transplant recipient occurs in the majority of cases. Tracking HCV quasispecies variants in such patients may improve our understanding of the mechanisms of persistent infection and the pathogenesis of liver disease in this population (D. Gretch et al., unpublished results). Similarly, tracking HCV quasispecies during interferon therapy may improve our understanding of the problems of resistance and relapse during and after interferon therapy. In this respect, HCV quasispecies which are differentially sensitive to interferon therapy have been reported (Enomoto et al., 1994). It is also possible that GSA of one or several HCV regions may eventually provide prognostic information regarding response or resistance to various antiviral therapies.

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References


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