Comparison of Full-length Sequences of Interferon-sensitive and Resistant Hepatitis C Virus 1b

Sensitivity to Interferon is Conferred by Amino Acid Substitutions in the NS5A Region

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Abstract

We have previously demonstrated that sensitivity to interferon is different among hepatitis C virus (HCV) quasispecies simultaneously detected in same individuals and that interferon-resistant HCV quasispecies are selected during the treatment. To determine the genetic basis of their resistance to interferon, HCV genotype-1b was obtained from serum of three patients before and during interferon therapy, and their full-length nucleotide and deduced amino acid sequences were determined. Comparison of the pairs of interferon-resistant and interferon-sensitive HCV isolates in respective individuals demonstrated clusters of amino acid differences in the COOH-terminal half of the NS5A region (codon 2154–2383), which contained a common unique amino acid difference at codon 2218. Additional sequence data of the COOH-terminal half of the NS5A region obtained from six interferon-resistant and nine interferon-sensitive HCV confirmed the exclusive existence of missense mutations in a 40 amino acid stretch of the NS5A region around codon 2218 (from codon 2209 to 2248) in interferon-sensitive HCV. On the other hand, this region of interferon-resistant HCV was identical to that of prototype HCV genotype-1b (HCV-J, HCV-JTa, or HC-J4). We designated this region as the interferon sensitivity determining region. Thus, HCV genotype-1b with the prototype interferon sensitivity determining region appears to be interferon-resistant strains. The specific nature of these mutations might make it possible to predict prognostic effects of interferon treatment. (*J. Clin. Invest.* 1995. 96:224–230.) Key words: chronic active hepatitis • interferon sensitivity determining region • polymerase chain reaction • quasispecies • single strand conformation polymorphism

Introduction

IFN is so far the sole effective therapy for chronic hepatitis C virus (HCV)[1] infection. However, only half of the patients treated with high doses of IFN-α ($\sim$ 500 million units for 6 mo) show sustained responses to IFN with eradication of the virus (1, 2). Especially the response rate in patients with HCV genotype–1b infection, which causes 75% of chronic HCV infection in Japan, is only 40%, whereas that of HCV genotype–2 is $\sim$ 80% (1, 2). Therefore, mechanisms of IFN resistance in HCV-1b should be elucidated for achieving higher response rate of IFN therapy. Although determinants for IFN sensitivity in patients infected with HCV are not fully understood, the difference in IFN efficacy among different HCV genotypes suggests that certain differences in HCV genomes may determine the sensitivity to IFN even within the same genotypes.

HCV populations in vivo are composed of clones with various mutations throughout its genomes, so-called quasispecies (3). In a previous study, we demonstrated by the single strand conformation polymorphism (SSCP) analysis that the sensitivity to IFN is different among HCV quasispecies simultaneously detected in same individuals, and that IFN-resistant HCV quasispecies are selected during the treatment (4). Therefore, comparative nucleotide sequence analyses of the whole HCV genomes between IFN-resistant and IFN-sensitive HCV pairs from same individuals may reveal the specific genomic structure of IFN-resistant HCV.

In the present study, to elucidate the genetic basis of IFN resistance in HCV genotype–1b, we compared full-length nucleotide and deduced amino acid sequences of HCV genotype–1b before and during IFN therapy in three patients who did not respond to IFN. To obtain the predominant HCV sequences at each time point avoiding the influence of minor sequence heterogeneity derived from the low fidelity of *Thermus aquaticus* (Taq) polymerase or minor HCV quasispecies, we performed the direct sequencing of the PCR products from serum HCV-RNA. Since we found common clusters of amino acid differences in the COOH-terminal half of the NS5A in these pairs, we subsequently examined this region in IFN-resistant HCV from six nonresponders and IFN-sensitive HCV from nine complete responders, and identified the IFN sensitivity determining region (ISDR) in the COOH-terminal half of the NS5A region.

Methods

Patients. 18 patients with chronic hepatitis C who underwent IFN therapy were examined. 17 patients were treated with 240–774 million units of IFN-α for 17–26 wk intramuscularly, and 1 patient was treated with 252 million units of IFN-β for 6 wk intravenously. Clinical characteristics and dosage schedules are summarized in Table I. Nine patients did not respond to the IFN treatment (nonresponders; patients 1–9), and nine patients showed sustained biochemical responses to IFN with the elimination of HCV for at least 6 mo (complete responders; patients 10–18). All patients had biopsy-proven chronic active hepatitis with positive serum HCV antibodies (second generation assay) and serum

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1. *Abbreviations used in this paper:* HCV, hepatitis C virus; HVR, hypervariable region; ISDR, IFN sensitivity determining region.

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Table I. Clinical Characteristics of the Patients

Patient No.	Sex	Age (yr)	IFN	Total dose (million units)	Dosage schedule*
Nonresponders					
1	female	59	natural α	267	6 M daily for 1 wk, 3 M thrice weekly for 25 wk
2	female	55	natural α	240	3 M daily for 2 wk, 3 M thrice weekly for 8 wk, 6 M thrice weekly for 7 wk
3	male	56	α 2a	468	6 M thrice weekly for 26 wk
4	male	45	α 2a	722	9 M thrice weekly for 26 wk
5	female	25	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
6	male	53	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
7	female	48	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
8	male	27	α 2a	774	9 M daily for 2 wk, 9 M thrice weekly for 24 wk
9	male	59	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
Complete responders					
10	female	63	natural α	258	3 M daily for 2 wk, 3 M thrice weekly for 24 wk
11	male	67	α 2a	774	9 M daily for 2 wk, 9 M thrice weekly for 24 wk
12	male	57	α 2a	774	9 M daily for 2 wk, 9 M thrice weekly for 24 wk
13	male	52	α 2a	258	3 M daily for 2 wk, 3 M thrice weekly for 24 wk
14	female	44	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
15	male	48	α 2a	342	9 M daily for 2 wk, 3 M thrice weekly for 24 wk
16	male	60	β	252	6 M daily for 6 wk
17	male	42	natural α	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk
18	male	38	α 2a	516	6 M daily for 2 wk, 6 M thrice weekly for 24 wk

* M, million units. wk: weeks.

HCV-RNA of genotype-1b (5, 6). They were negative for serum HBs antigen, HBc antibody, and antinuclear antibodies, and had no other causes of hepatitis including excessive alcohol intake or hepatotoxic drugs. Sequential serum samples were obtained from each patient and stored at −70°C until use.

A pairwise comparison of the full-length HCV genomes before and during IFN treatment was performed by direct sequencing in three nonresponders (patients 1–3). In these patients, single strand conformation polymorphism analyses of the E2-hypervariable region (HVR) (4) revealed that they had two groups of quasispecies before IFN treatment. During IFN treatment, the quasispecies that was major before the therapy disappeared, and the other minor quasispecies remained and became dominant, indicating that the major HCV quasispecies before the therapy was IFN-sensitive and that HCV quasispecies during the therapy was IFN-resistant. Therefore, comparison of HCV quasispecies before IFN treatment with those during IFN treatment would make it possible to identify the genetic mechanism of IFN resistance. Detailed single strand conformation polymorphism analyses of the fluctuation of HCV quasispecies during IFN treatment in patients 1 and 2 were described in our previous report (4). A part of the NS5A region was sequenced in the other 15 patients (patients 4–18). To obtain sequences of IFN-resistant HCV, amino acid sequences were determined at the end of the IFN treatment in six nonresponders (patients 4–9) who were treated with large doses of IFN (516–774 million units). To obtain sequences of IFN-sensitive HCV, amino acid sequences just before the treatment were determined in nine complete responders (patients 10–18). NS5A sequences before the IFN treatment were also determined in patients 4–9.

RNA extraction. Serum RNA was extracted by the acid-guanidinium-phenol-chloroform method (7). Briefly, 150 μl of plasma was mixed with 400 μl of the guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.9], 0.5% sarkosyl, and 1% 2-mercaptoethanol) and extracted with water saturated phenol. The aqueous phase was extracted once with chloroform. RNA was ethanol-precipitated with 20 μg of glycogen (Boehringer Mannheim, Mannheim, Germany) as a carrier. Obtained RNA was dissolved in 6 μl of distilled water and stored at −80°C until use.

cDNA synthesis. 5 μl of the reverse transcription mixture were adjusted to contain 1 μl of the RNA solution, 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, MD), 10 units of RNase inhibitor (Promega Corp., Madison, WI), 50 pg of random hexamers (Takara, Kyoto, Japan) or 10 pmol of a poly-A10 primer (AAAAAAAAAA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. The mixture was incubated at 37°C for 45 min.

PCR. 1 μl of the cDNA solution was made up to 50 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of each PCR primer (outer primer), 200 μM each d-nucleoside triphosphate (dNTP), 0.01% gelatin and 0.5 units of Taq polymerase (AmpliTaq; Cetus Corp., Emeryville, CA) (8). PCR consisted of 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min.

To obtain whole HCV genomes, PCR primers of 25-mer were designed based on the sequence of HCV-J (9) and were used to amplify 19 fragments of HCV cDNA spanning ~ 700 bp overlapping each other (nucleotides 1–400, 303–923, 796–1450, 1261–1692, 1578–2193, 2029–2673, 2536–3237, 3129–3748, 3639–4289, 4159–4743, 4624–5286, 5146–5769, 5653–6270, 6168–6792, 6703–7320, 7226–7800, 7730–8329, 8198–8874, and 8760–9401; nucleotide positions are numbered according to HCV-J) from the random hexamer-primed cDNA. The cDNA for the extreme 3′-end of HCV genomes was also amplified from the poly-A primed cDNA by the 3′-RACE method (10). PCR primers for the extreme 5′-end cDNA amplification were designed based on the nucleotide 1–12 of HC-J4/83 (11), which located upstream of the 5′-end of the HCV-J sequence. For determination of the COOH-terminal half of the NS5A region from patients 4–18, HCV-cDNA of nucleotide 6703–7800 was amplified as described above. PCR primers and sequencing primers were synthesized with a DNA synthesizer model 391 (Applied Biosystems Japan, Tokyo, Japan).

Direct sequencing of PCR products. 1 μl of the first PCR products of each fragment was transferred to the second PCR reaction with nested 5′- and 3′-primers. M13-forward (5′-TGTAAAACGACGGCCAGT-3′) and M13-reverse (5′-CAGGAAACAGCTATGACC-3′) sequencing primer sequences were attached to the 3′ terminus of 5′- and 3′-

Table II. Region-dependent Nucleotide (5′- and 3′-Noncoding Region) or Predicted Amino Acid (Coding Region) Differences between IFN-sensitive and IFN-resistant HCV‡ from Three Patients*

Region	Patient 1	Patient 2	Patient 3
5′-noncoding region (330 nt§)	0	0	0
Core (191 aa‖)	2	0	2
E1 (192 aa)	1	0	2
E2 region (426 aa)	13	10	6
NS2 region (217 aa)	7	1	5
NS3 (631 aa)	1	2	4
NS4A (54 aa)	0	0	0
NS4B (261 aa)	0	0	1
NS5A (447 aa)	16	5	8
NS5B (591 aa)	1	3	3
3′-noncoding region (41 nt)	0	3	3
Total amino acid differences	41	21	31

* Samples for sequencing were obtained just before the start of IFN treatment. ‡ Samples for sequencing were obtained at 4, 17, and 26 wk after the start of IFN therapy from patient 1, patient 2, and patient 3, respectively. § nt, nucleotides; ‖ aa, amino acids.

nested primers, respectively, to facilitate the direct sequencing by an automated DNA sequencer model 373A (Applied Biosystems Japan). The composition of the PCR mixture was the same as the first PCR mixture except for the primers. After residual dNTPs and primers were removed using a spin filtration column (Suprec-02; Takara) 7 μl of the PCR products were sequenced by the dye termination method according to the manufacturer's instruction. Sequencing primers were M13-forward primers for the sense strand and M13-reverse primers for the antisense strand.

Results

Comparison of full-length sequences between IFN-resistant and IFN-sensitive HCV in same patients. Distribution of nucleotide differences in noncoding regions and amino acid differences in coding regions in three nonresponders (patients 1–3) are summarized in Table II. HCV genomes from patients 1–3 consisted of 9,401 nucleotides and contained an open reading frame of 3,010 amino acids. Organizations of the genomes were similar to those of HCV-J, and processing patterns of HCV polyproteins are described according to the previous reports on HCV genotype-1b (12–14). In patient 1, 36 out of 41 amino acid differences were found in three regions of the HCV genome, the E2, the NS2, and the NS5A regions. In other regions, there were only five amino acid differences. In patient 2, amino acid differences in the structural region were exclusively found in the E2 region, and 5 out of 11 amino acid differences in the nonstructural region were detected in the NS5A region. In patient 3, although amino acid differences were more scattered, amino acid differences were most frequently found in the NS5A region among nonstructural regions (8 of 21). The 5′-noncoding region was identical between IFN-sensitive and IFN-resistant HCV in each patient. In patients 2 and 3, there were three nucleotide differences in the 3′-noncoding region while no differences were found in patient 1.

The distribution of differences in amino acid residues between IFN-sensitive and IFN-resistant HCV in three patients are schematically illustrated in Fig. 1. Vertical lines in each rectangle for HCV polyproteins of three patients indicate positions of predicted amino acid differences between isolates before and during the IFN therapy. In patient 1, amino acid differences between IFN-sensitive and IFN-resistant HCV showed a striking accumulation in the COOH-terminal half of the NS5A region (codon 2154–2383). Amino acid differences in the NS5A region in patients 2 and 3 were also clustered in the COOH-terminal half of the NS5A region. The comparison of three patients revealed that amino acid differences in individual patients were commonly found in the COOH-terminal half of the NS5A region and the E2-HVR. No other regions had common amino acid differences among three patients.

Amino acid differences between IFN-resistant and IFN-sensitive HCV in patients 1–3 are summarized in Table III. In the center of the COOH-terminal half of the NS5A region where amino acid differences were clustered in three patients, there was an unique amino acid residue (codon 2218) which was commonly different between IFN-resistant and IFN-sensitive HCV in three patients. This residue was histidine in three previously published HCV genotype-1b isolates including HCV-J. IFN-resistant HCV in three patients also had histidine in this position, while IFN-sensitive HCV had amino acid substitutions other than histidine. No other amino acid residues were commonly different between IFN-resistant and IFN-sensitive HCV in the whole HCV genomes.

Differences in nucleotide sequences of the 5′- and 3′-noncoding regions are shown in Table IV. No common nucleotide differences were present among three patients in the noncoding regions.

Comparison of COOH-terminal half of the NS5A in IFN-treated patients. Subsequently, amino acid sequences of the COOH-terminal half of the NS5A region were determined in additional 15 IFN-treated patients (patients 4–18) infected with HCV genotype-1b, six nonresponders (patients 4–9) and nine complete responders (patients 10–18). Amino acid sequences of IFN-resistant HCV were obtained at the end of the IFN treatment in nonresponders and those of IFN-sensitive HCV were obtained just before the treatment in complete responders. Fig. 2 demonstrates multiple amino acid sequence alignments of this region in 12 IFN-sensitive HCV and 9 IFN-resistant HCV from patients 1–18, along with three published HCV genotype-1b isolates (HCV-J, HC-J4/84 [11], and HCV-JTa [15]) as well as prototype HCV genotype-2a (HC-J6) (16) and genotype-2b (HC-J8) (17). The direct sequencing from patients 4–18 showed no mixtures of different sequences.

The most part of this region showed a variety of differences as shown in Fig. 2, whereas predicted amino acid sequences between codon 2209 and codon 2248 showed a distinct correlation with IFN sensitivity (IFN sensitivity determining region, ISDR). All IFN-sensitive HCV had amino acid substitutions in this region compared to HCV-J, whereas amino acid sequences of this region in prototype HCV genotype-1b (HCV-J, HCV-JTa, and HC-J4) and IFN-resistant HCV were completely conserved. On the other hand, the other portions in the COOH-terminal half of the NS5A region were polymorphic among HCV isolates, and showed no distinct association with IFN sensitivity. Codon 2218 encoding the sole amino acid residue commonly different in HCV pairs from patients 1–3 existed in the middle of the ISDR. This amino acid residue was most frequently substituted in this region (9 of 12 IFN-sensitive HCV), and 9 of 12 IFN-sensitive HCV had additional substitutions around codon 2218. In patient 18, there was a unique

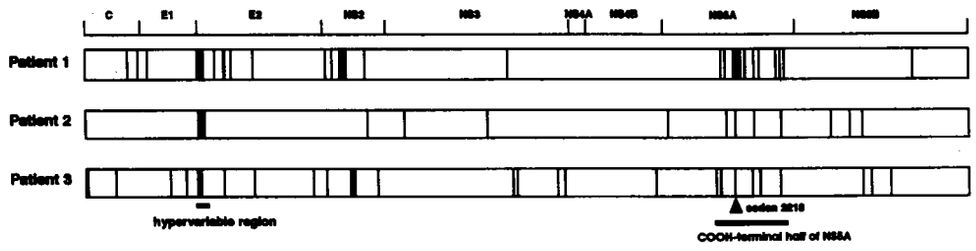

Figure 1. Scheme of distribution of amino acid differences between IFN-sensitive and IFN-resistant HCV in patients 1–3. Vertical lines in each rectangle for HCV polyproteins indicate positions of amino acid differences between HCV pairs of each patient. Solid lines below rectangles represent the E2 region–hypervariable region and the COOH-terminal half of the NS5A region where amino acid differences were found commonly in three patients. An arrow head indicates codon 2218 which is the sole amino acid residue commonly different in three pairs. Sequence data of these HCV isolates are deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483 for HCV-K1-S1 (IFN-sensitive HCV from patient 1), D50480 for HCV-K1-R1 (IFN-resistant HCV from patient 1), D50485 for HCV-K1-S2 (IFN-sensitive HCV from patient 2), D50481 for HCV-K1-R2 (IFN-sensitive HCV from patient 2), D50484 for HCV-K1-S3 (IFN-resistant HCV from patient 3), and D50482 for HCV-K1-R3 (IFN-resistant HCV from patient 3).

insertion of 8 amino acids between codon 2221 and codon 2222. In HCV isolates from patients 15–17, amino acid residue 2218 was histidine that was same as HCV-J, while these HCV had multiple substitutions other than residue 2218. Pretreatment amino acid sequences of the ISDR in patients 4–9 were also identical to HCV-J except residue 2218 of patient 9, which was arginine and changed to histidine at the end of the treatment. The amino acid sequences of the ISDR in HCV-2a (HC-J6) and HCV-2b (HC-J8) also contained multiple amino acid substitutions as well as a deletion of four amino acids. In addition, the region from amino acid 2219 in the ISDR to the COOH-terminal end of the NS5A showed very low homology between HCV-1b and HCV-2 (2a and 2b) with insertions of 4 and 20 amino acids.

Discussion

In the present study, we identified clusters of amino acid differences in the COOH-terminal half of the NS5A region between IFN-sensitive and IFN-resistant HCV genotype–1b obtained from three patients by comparative analyses of full-length nucleotide sequences of HCV genomes. All HCV pairs commonly contained an unique amino acid difference at codon 2218. Additional sequence data of the COOH-terminal half of the NS5A region obtained from six nonresponders and nine complete responders confirmed the exclusive existence of amino acid substitutions in a part of the NS5A region between codon 2209 and codon 2248 in IFN-sensitive HCV, whereas IFN-resistant HCV remained after the IFN therapy in nonresponders had an identical sequence to that of prototype HCV genotype–1b. Thus, HCV genotype–1b with the prototype sequence in this region is IFN-resistant while HCV with mutations in this region is IFN-sensitive. We designated this region ISDR.

Comparing the full-length nucleotide and deduced amino acid sequences of HCV genomes before and during IFN treatment, we found that amino acid substitutions were almost confined in the E2 region, the NS2 region, and the NS5A regions in patient 1. In this patient, responses of two HCV quasispecies to the IFN treatment were clearly different; one quasispecies (IFN-sensitive HCV) became undetectable shortly after the start of IFN and the other did not change throughout the IFN treatment (4). Such a striking contrast of IFN effects seems to originate from differences in the HCV genomes themselves, because other background factors that might influence IFN effects including host immune surveillance systems and liver histology are absolutely identical for these quasispecies. Therefore, genetic basis of the difference in IFN effects could be elucidated by comparing these HCV genomes. Such data could only be obtained in in vitro experiments with recombinant viruses under controlled conditions, which is so far impossible to be done. Although amino acid substitutions were not so confined in patient 2 and 3 as patient 1, only the NH_2-terminal of the E2 region and the COOH-terminal half of the NS5A region (codon 2154–2383) commonly contained amino acid differences in three patients. Therefore, it is suggested that IFN resistance may be conferred by these amino acid sequences. Especially, IFN-sensitive HCV all had an amino acid substitution at codon 2218 compared to the prototype HCV genotype–1b (HCV-J), while IFN-resistant HCV had no mutations in this position. It was the only amino acid residue that was commonly different between IFN-sensitive and IFN-resistant HCV in patients 1–3 throughout the whole genome.

Further sequencing studies with 15 IFN-treated patients revealed that there was an apparent relationship between IFN sensitivity and the amino acid sequence around codon 2218 (amino acid 2209–2248, ISDR) in the NS5A region. Amino acid substitutions in the ISDR were exclusively found in the pretreatment HCV from nine IFN responders, whereas six nonresponders had HCV with prototype ISDR (identical to HCV-J) at the end of the large dose of IFN treatment. Thus, we conclude that HCV genotype–1b with the prototype ISDR is an IFN-resistant strain and HCV genotype–1b with mutant ISDR is IFN-sensitive. An amino acid residue at codon 2218 seemed to have a critical effect on IFN sensitivity. All IFN-resistant HCV from patients 1–9 had histidine at this site while six of nine IFN-sensitive HCV had an amino acid other than histidine. Although three IFN-sensitive HCV (patients 15–17) had histidine in this position, they contained other substitutions in the ISDR. IFN-sensitive HCV from patient 18 had an unique insertion of eight amino acids just next to codon 2218. These results suggest that codon 2218 is most important, while the secondary structure of the entire ISDR would influence the sensitivity to IFN.

Although amino acid substitutions in the ISDR was most closely associated with IFN effect, there were several amino acid residues other than the ISDR in the COOH-terminal half of NS5A which seemed to be partially correlated to the IFN responsiveness (Fig. 2). For example, specific amino acid residues at amino acid 2171 (arginine), 2187 (valine), and 2413 (glycine) were mainly observed in the IFN-sensitive HCV, while they were also observed in IFN-resistant HCV. These observations suggest that the region around the ISDR may also

Table III. Amino Acid Differences between IFN-sensitive (sen) and IFN-resistant (res) HCV Genotype-1b in Patient 1–3

Regions (codon*)	Codon	HCV-J	Patient 1		Patient 2		Patient 3	
			sen	res	sen	res	sen	res
Core (1–191)	10	K					Q	K
	106	S					N	S
	147	A	T	A				
	183	S	P	S				
E1 (192–383)	217	A	Q	E				
	292	T					A	T
	345	V					M	V
E2 region (384–809)	384	H	A	N	R	N		
	386	H			T	Y	R	H
	388	T			T	S		
(HVR: 384–410)	390	G	G	A				
	392	V			Q	K		
	393	A			G	S		
	394	S	H	R				
	396	T	T	V				
	398	S			R	G	G	S
	399	L	V	L			L	F
	400	V	A	T	A	V		
	402	W	L	I				
	404	S			T	A		
	405	Q	S	P	F	P		
	410	K			R	K		
	445	H	H	R				
	475	D	T	A			A	V
	479	S	N	D				
	500	S	S	Q				
	577	N	D	N				
	579	L					L	F
	781	R					R	K
NS2 region (810–1026)	821	A	V	A				
	824	V	V	I				
	827	V					I	M
	841	R	R	K				
	871	D	G	D				
	879	A	V	A				
	892	L	M	V				
	907	T					I	T
	908	R					R	K
	921	A					H	C
	957	R	Q	R				
	962	A			A	T		
	1001	P					P	A

Regions (codon*)	Codon	HCV-J	Patient 1		Patient 2		Patient 3	
			sen	res	sen	res	sen	res
NS3 (1027–1657)	1093	P			S	P		
	1370	T			I	T		
	1444	F	Y	F				
	1462	V					D	V
	1475	T					R	T
	1612	I					T	I
	1636	T					I	T
NS4A (1658–1711)			(ND‡)		(ND)		(ND)	
NS4B (1712–1972)	1950	S					S	N
NS5A (1973–2419)	1989	S			T	S		
	2155	L					P	A
	2169	A	A	T			T	A
	2171	L					I	L
	2185	T	T	A	A	T		
	2211	L	S	L				
	2215	C	Y	C				
	2216	T	I	T				
	2218	H	Q	H	R	H	C	H
	2219	H	Y	H				
	2224	A	P	A				
	2251	V	V	I				
	2252	I	T	I				
	2279	R					K	R
	2283	P	S	P	P	R		
	2300	S	P	S			P	S
	2303	D					A	D
	2356	G	D	G				
	2372	A	T	A			P	T
	2374	D			N	S		
	2383	E	E	G				
NS5B (2420–3010)	2546	L			L	Q		
	2608	P			P	A		
	2650	N			S	N		
	2654	T					V	T
	2681	V					I	V
	2750	E					G	E
	2820	R	R	K				

Amino acids are denoted by single letter codes. Codon 2218 is underlined. * codons are numbered according to HCV-J. ‡ ND, no differences.

influence the IFN sensitivity in a cooperative manner to some extent. In addition, there are other regions that may potentially contribute to the IFN response, such as the NS2 region in patient 1 and 3. Further studies on the role of these regions in IFN sensitivity are needed.

The NH_2-terminal of the E2 region is known as the hypervariable region and suggested to encode neutralizing antibody epitopes (18–20). Recent works described that heterogeneity of the HVR is associated with poor IFN responses and that some HVR quasispecies are selected during IFN treatment (21, 22). However, this region is so variable that no specific sequence correlates with IFN effects (23). In addition, we observed that HCV quasispecies with identical amino acid sequences in the HVR differently responded to IFN, indicating that regions other than the HVR should confer the resistance to IFN (unpublished observation). Therefore, the selection of HVR quasispecies during IFN treatment might be secondary to the selection of IFN-resistant HCV with the prototype ISDR. Patients with heterogeneous HVR quasispecies may have more diverse HCV genomes and may have more chance to harbor

Table IV. Nucleotide Sequence Differences in the 5′- and 3′-Noncoding Regions between IFN-Sensitive (sen) and IFN-resistant HCV (res) in Patients 1–3

Regions	nt*	HCV-J	Patient 1		Patient 2		Patient 3	
			sen	res	sen	res	sen	res
5′-noncoding region			(ND‡)		(ND)		(ND)	
3′-noncoding region	9364	C			C	T		
	9368	G					G	A
	9372	T			T	C	T	C
	9375	C			A	C		
	9388	A					T	A

* nt, nucleotides are numbered according to HCV-J. ‡ ND, no differences.

IFN-resistant HCV. Otherwise, HCV with heterogeneous HVR has high mutation rates in vivo and more likely to become IFN resistant with changes in the ISDR during IFN therapy.

The correlation of NS5A structures with IFN effects demonstrated in this study might make it possible to develop assays to predict IFN resistance in each patient. Reliable identification of IFN-sensitive or IFN-resistant HCV by genotyping of the ISDR would be very useful for improving the current ambiguity of IFN sensitivity prediction in the same genotype by serum HCV titer or liver histology (2). However, some patients treated with IFN show transient responses followed by a deterioration (breakthrough) suggesting the emergence or selection of IFN-resistant HCV as seen in patient 1–3 and 9 in the present study, thereby the detection of IFN-sensitive HCV before treatment may not necessarily predict the favorable outcome of IFN therapy. Further studies on the correlation between pretreatment ISDR and IFN effectiveness, and on the emergence of IFN-resistant HCV during IFN therapy should be carried out to define the clinical significance of ISDR.

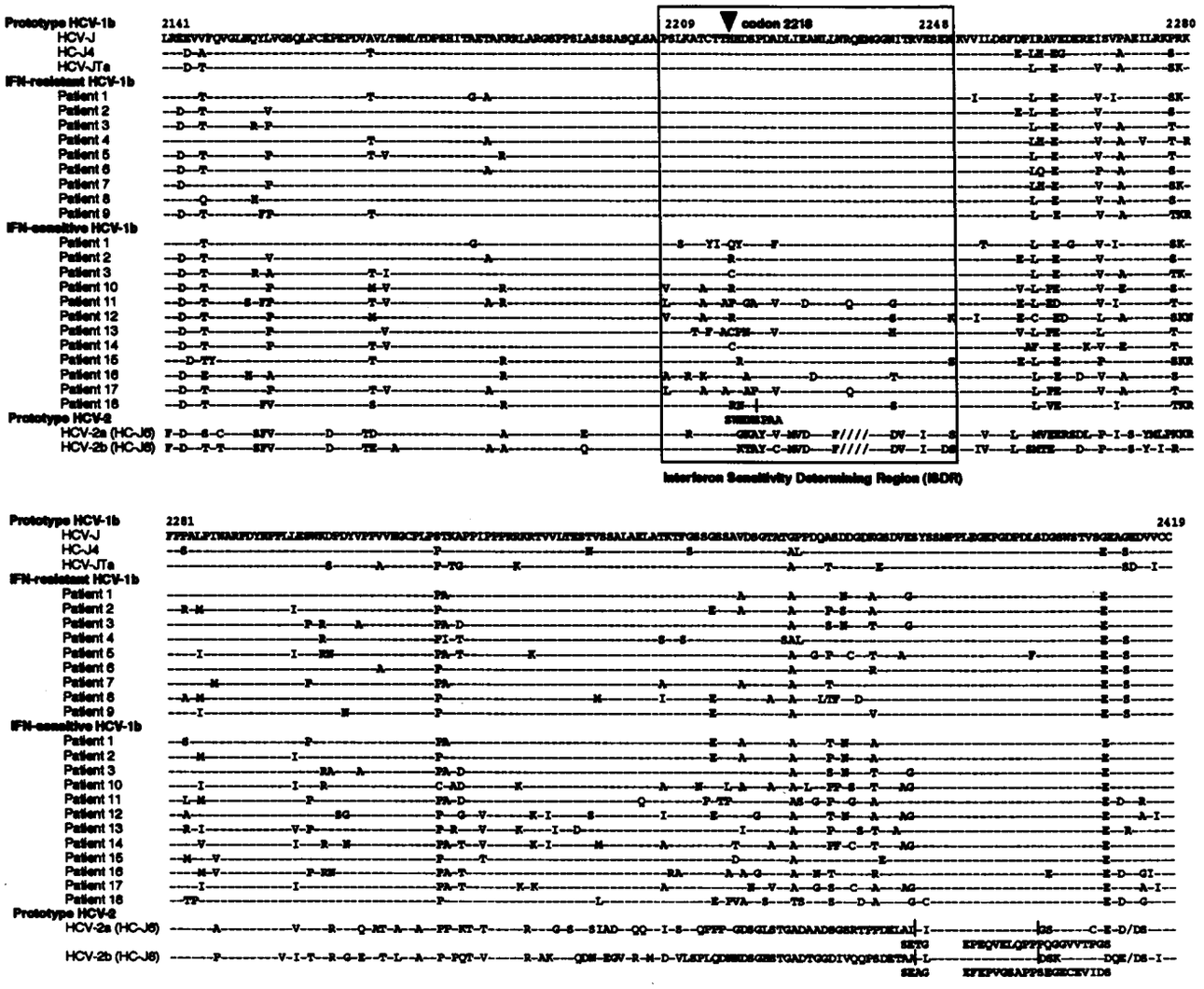

Figure 2. Amino acid sequence alignments of the COOH-terminal half of the NS5A region in patients 1–18 together with published HCV genotype-1b, -2a (*HC-J6*), and -2b (*HC-J8*) sequences. Dashes indicate amino acids identical to those of HCV-J. IFN sensitivity determining region (*ISDR*) is boxed. An arrow head indicates codon 2218. Slashes in HCV-2a and -2b sequences indicate the deletion of amino acids. Vertical bars in patient 16, HCV-2a, and HCV 2b indicate the insertion of amino acids shown below each bar. Amino acids are denoted by single letter codes, and amino acid sequences are numbered according to HCV-J.

The COOH-terminal half of the NS5A including the ISDR (amino acid 2219–2419) has been known to show marked diversity among different genotypes (1a, 1b, 2a, 2b, and 3a) (24). Especially, HCV genotype–2 had a deletion of four amino acids and multiple amino acid substitutions in the ISDR, as well as numerous amino acid substitutions including insertions of 4 and 20 amino acids at the COOH-terminal end of the NS5A (Fig. 2). The association between NS5A structure and IFN sensitivity in HCV-1b observed in the present study suggests that different IFN sensitivity among different genotypes might also be derived from the differences in the NS5A. The same approach described in this study would elucidate the existence and structure of IFN-resistant HCV in other genotypes.

Precise functions of each nonstructural protein as well as the NS5A region are not fully elucidated yet. The requirement of the NS5A region for the efficient processing of NS4 proteins by NS3 protease is suggested (14), but it has not been confirmed. Elucidation of the function of the NS5A region and the effect of its heterogeneity on the function is needed for better understanding of IFN resistance and for the development of effective treatments. Interestingly, published HCV genotype–1b isolates (HCV-J, HC-J4, and HCV-JTa) have prototype ISDR. Since each isolate was directly cloned from a single patient without PCR amplification, these patients are supposed to have relatively high serum levels of HCV-RNA. Recent investigations have shown that high titers of HCV are associated with the resistance to IFN among the same genotypes (25, 26), implying that these prototype HCV might be IFN resistant with prototype ISDR. These findings suggest that the NS5A region might influence the rate of HCV replication. The relationship between ISDR structures and HCV replication remains to be further investigated.

In conclusion, we have identified the IFN sensitivity determining region in the NS5A region by comparing full-length sequences of IFN-sensitive and IFN-resistant HCV genotype–1b in the same patients. HCV genotype–1b with prototype-ISDR shows resistance to IFN, whereas missense mutations in this region are associated with increased sensitivity to IFN. The specific nature of these mutations might make it possible to predict prognostic effects of IFN treatment.

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