The VIIIth Report of the International Committee for the Taxonomy of Viruses (ICTV) currently classifies hepatitis C virus (HCV) and GB virus B as members of the *Hepacivirus* genus in the virus family, *Flaviviridae*.[1] Although the report recognizes the existence of 6 main genetic groups of HCV and designates them as "clades," it is beyond the remit of the ICTV to extend classification proposals below the level of species. Thus, separate arrangements are required for the standardization of genotype and subtype assignments of genetic variants of HCV.

A meeting was convened at the 11th International Symposium on HCV and Related Viruses, Heidelberg, Germany, October 2004. This was a successor to the first HCV classification meeting in Santa Fe, New Mexico, in 1997, with a similar membership of scientists from North America, Europe, and Japan working in the field of HCV sequence variation.[2] The purpose of the meeting was to analyze the current description, assignment, and nomenclature of HCV genetic variants and to review new developments in studies of HCV genetic variability and epidemiology. A new aim was to formally link genotype nomenclature proposals with the organization and sequences retrieval systems available on three HCV sequence databases that provide a resource to study genetic variability of HCV and its clinical, epidemiological, and therapeutic manifestations. The first database was created in Japan by Prof. Masashi Mizokami and co-workers (http://s2as02.genes.nig.ac.jp/), the second in the European Union by Prof. Gilbert Deleage et al.[3] (http://euhcvdb.ibcp.fr/), and the third in the United States by Dr. Carla Kuiken et al.[4] (http://hcv.lanl.gov/ or http://hcv-db.org). The accessibility of these databases and the provision for users to download and analyze annotated sequences make them ideal vehicles for reinforcing a standardized nomenclature system, and their support is an integral part of the outlined proposals. This support entails assisting users to avoid naming conflicts, providing advice and analysis support, ensuring that the nomenclature used in the 3 databases is standardized and follows the guidelines in this paper, and trying to increase awareness of these guidelines in the HCV research community and among journal reviewers and editors.

The meeting was convened with the following broad aims:

1. Standardize nomenclature for existing variants of HCV:
   - Develop consistent nomenclature for variants within each clade
   - Resolve conflicting subtype and genotype designations
   - Publish a complete list of currently classified recognized genotypes and subtypes, with acknowledgment of originating authors
2. Formulate agreed criteria for the designation of new HCV variants:
   - New genetic groups/clades/genotypes
   - Subtypes, recognizing that designation of subtypes may only be epidemiologically relevant in certain cases
   - Recombinant forms of HCV
3. Provide a classification scheme for HCV for research and database use:
   - Standardize nomenclature to provide a common interface for sequence retrieval from HCV databases
   - Provide a relevant classification for investigation of clinical and biological differences between HCV variants

Background

A standard system for HCV classification is of importance in studies of the epidemiology, evolution, and pathogenesis of HCV. Of particular clinical importance is the need to understand genotype-specific differences in response to interferon-α–based treatments. A classification system has to be robust, based on objective criteria, and able to accommodate new genetic variants and recombinant forms that are discovered in the future. To achieve this, the classification of HCV should be based, as with other biological systems, on its evolutionary history (as far as it is currently understood). The following section reviews current thoughts on the origins and epidemiology underlying the observed genetic diversity of HCV, and how these aspects may be incorporated into the proposed classification scheme.

HCV Sequence Variability. When the extent of the genetic heterogeneity of HCV was discovered in the early 1990s, a number of different methods were used for classifying variants.[5-12] These differed from each other in the methods used to delineate different genotypes (by pairwise distance measurements or by phylogeny), whether they incorporated the two levels of sequence variability in the nomenclature system, and finally, in the letters or numbers assigned to each recognized genetic group. Progress toward resolving these uncertainties in HCV classification was made by publication of a consensus paper in 1994,[13] proposing the classification of HCV by phylogenetic methods into 6 genotypes (updated phylogenetic tree shown in Fig. 1). These approximately equidistant genetic groups each contain a variable number of more closely related, genetically (and epidemiologically) distinct "subtypes." Genotypes differ from each other by

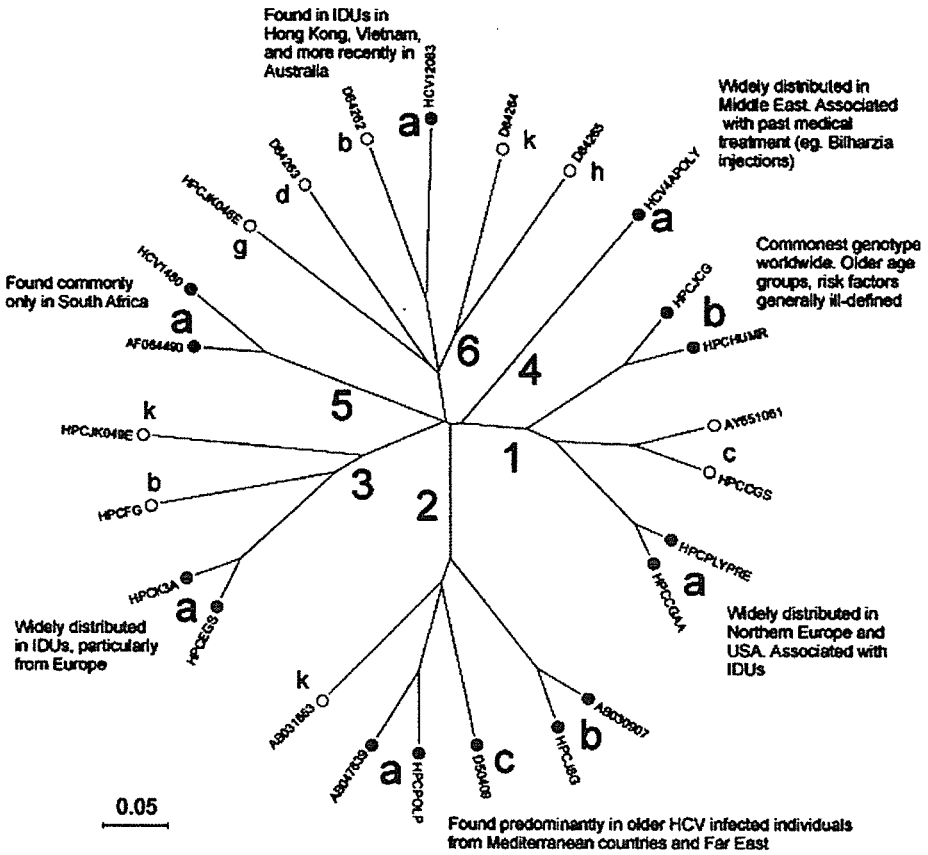

Fig. 1. Evolutionary tree of available complete open-reading frame sequences for each HCV genotype. Phylogenetic analysis was carried out on complete coding sequences of genotypes of HCV (maximum 2 where multiple sequences available; prioritized as in Table 1). The main identified risk groups for each genotype (IDUs, recipients of unscreened blood or blood products, other parenteral exposures) has been indicated where information is available (filled circles and accompanying text). These represent the main variants believed to have become prevalent in industrialized countries over the course of the 20th century. HCV genotypes 3k, 6d, 6g, 6h, and 6k are the re-assigned names of the previously described genotypes "10a," "7b," "11a," "9a," and "8b," respectively (Table 2). The tree was constructed by neighbor-joining as implemented in the MEGA package,[97] using Jukes-Cantor corrected distances.

31% to 33% at the nucleotide level, compared with 20% to 25% between subtypes. Despite the sequence diversity of HCV, all genotypes share an identical complement of co-linear genes of similar or identical size in the large open reading frame, and the genetic inter-relationships of HCV variants are remarkably consistent throughout the genome.[2] This has enabled many of the currently recognized variants of HCV to be provisionally classified, based on partial sequences from subgenomic regions such as core/E1 or NS5B.[14] The most conserved regions of the HCV genome are the 5′ untranslated region, and the terminal 99 bases of the 3′untranslated region. The inferred amino acid sequence of the core gene is also relatively invariant between genotypes. The most variable region of the HCV genome is the hypervariable region of E2.[15,16] Here, the large number of likely immune-selected amino acid changes[17-22] distorts the underlying phylogeny of HCV apparent from comparison of other genomic regions.

Each genetic group of HCV comprises varying numbers of more closely related variants, typically different from each other at 20% to 25% of nucleotides, compared with more than 30% between genotypes (Fig. 1). The most common variants found in Western countries have previously been classified with subtype labels, such as 1a and 1b in genotype 1; and 2a, 2b, and 2c in genotype 2. These variants have become very widely distributed over the past 50 to 70 years as a result of transmission through blood transfusion and various other invasive medical and surgical procedures, and by needle sharing between injection drug users (IDUs). They now represent the vast majority of infections in Western countries encountered clinically, and for which most information has been collected on disease progression and response to α-interferon–based treatment.

Since the original classification of HCV, further molecular epidemiology studies have revealed the existence of much greater diversity in certain regions of sub-Saharan Africa and in South and Southeast Asia (Fig. 2). Most new variants originate from specific geographical regions; for example, infections in Western Africa are predominantly by genotype 2,[23-27] whereas those in Central Africa, such as the Democratic Republic of Congo and Gabon, are by genotypes 1 and 4.[12,24,28-32] Taking this geographical mapping further, genotypes 3 and 6 show similar genetic diversity in South and Eastern Asia.[24,33-35]

These observations indicate the likely long-term presence in human populations in parts of Africa and Asia, distinct from HCV transmission patterns in Western and other non-tropical countries. The relatively recent ap-

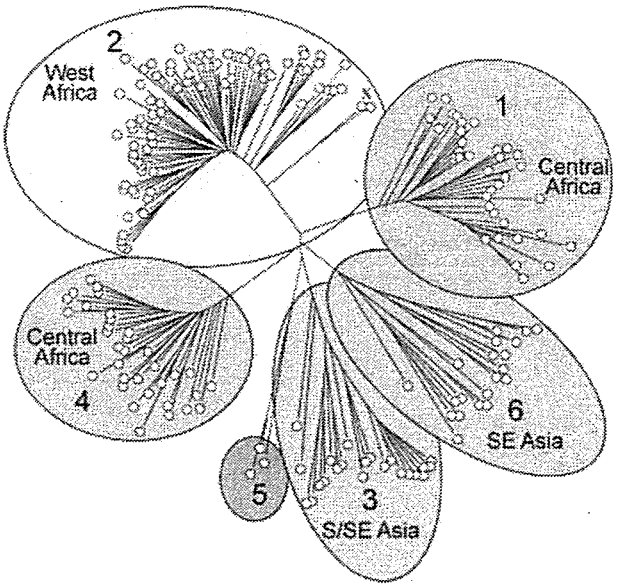

Fig. 2. Evolutionary tree of all available NS5B sequences of HCV. This phylogenetic analysis of the NS5B region of all publicly available nucleotide sequences in the region from 8276 to 8615 (numbered as in the H77 reference sequences, AF009606[63]) demonstrates that HCV variants still fall into 6 distinct genotypes but each contains numerous novel variants discovered in high-diversity areas in sub-Saharan Africa and Southeast Asia. The tree was constructed by neighbor-joining as implemented in the MEGA package,[97] using Jukes-Cantor corrected distances. More divergent members of genotype 2 are indicated with an "x."

pearance of new risk groups and routes of spread,[32,36] such as blood transfusion since the 1940s, the medical use of unsterilized needles for injections and vaccinations, and most specifically to industrialized countries, injecting drug use and the sharing of injection equipment,[32,37-39] has allowed the rapid spread and amplification of "founder" viruses. What we now call subtypes 1a, 1b, 2a, 2b, 3a, and 4a are likely to be the descendants of HCV variants that "seeded" these new, rapidly expanding transmission networks. As discussed later, HCV classification should both recognize the epidemiological associations of these "founder" viruses and incorporate their subtype names into the genotype nomenclature, while acknowledging that such labels are of little or no value in the description of HCV variants in high-diversity areas in sub-Saharan Africa and Southeast Asia.

Recombination. A recent discovery with implications for HCV classification is recombination between genotypes of HCV.[40,41] Homologous recombination in HCV could clearly be facilitated by the overlap in genotype distributions in many parts of the world. It also may be favored by the nature of HCV risk behavior, in which there may be frequent exposures around the time of primary infection (*e.g.*, repeated needle-sharing between several IDUs), and lack of protective immunity from reinfection during chronic HCV infection. Recently, a viable and rapidly spreading recombinant containing structural genes from genotype 2k and non-structural genes from genotype 1b was found in IDUs in St. Petersburg, Russia.[40,41] Inter-subtype (or intra-genotype) recombinants have also been described, such as a 1a/1b recombinant in Peru.[42] The true frequency of recombination may be underestimated because it would be difficult to detect if it occurred between variants of the same subtype. Similarly, it would be difficult to document inter-subtype recombinants where HCV is highly diverse, such as within genotype 2 in West Africa. Finally, although there is a comparative wealth of complete genome sequences of common HCV genotypes, such as 1b, most studies of HCV variability in high diversity areas are based on analysis of single sub-genomic regions, such as NS5B or core/E1, making detection of potential recombination events unlikely.

HCV Genotype Identification. Genotype identification is clinically important because genotypes 1 and 4 are more resistant than genotypes 2 and 3 to the current standard of care, pegylated interferon-α and ribavirin combination therapy.[43] Indeed, most treatment protocols require genotype information to tailor dose and duration of treatment. Genotyping assays are usually based on sequence analysis of an amplified segment of the genome, commonly the 5′untranslated region, because this region is targeted by most diagnostic assays for HCV RNA. Although this region is highly conserved, a well-characterized set of polymorphisms predict genotype and can be conveniently detected by probe hybridization,[44,45] changes in restriction sites[46,47] or by direct sequencing.[48]

For the purposes for which they are normally used (prediction of treatment response and dose scheduling),[43] currently used 5′UTR-based assays are acceptably accurate, with more than 95% concordance with genotypes identified by nucleotide sequencing in NS5B or other coding regions of the genome.[49-55] Several factors, however, preclude their use for definitive genotype identification, for identification of subtypes, and more generally, as an HCV classification tool:

- Although several genotype-specific nucleotide changes in the 5′UTR usually allow each of the 6 main genotypes to be differentiated from each other, there are exceptions. Some genotype 6 variants found in Southeast Asia have 5′UTR sequences identical to those of genotype 1a or 1b.[34,36,56] This illustrates a more general point that, even for genotype identification, the performance of genotyping assays is very much a property of the range of HCV variants tested. The currently used 5′UTR-based assays are unlikely to operate to the published level of accuracy (>95%; see above) in high-diversity areas.

- Even for well-characterized variants of HCV, such as those circulating in Western countries, sequence differences between subtypes may be variable or non-existent in the 5′UTR. For example, a sequence polymorphism at position 243 (numbered as in the H77 reference sequence), frequently used to differentiate subtypes 1a and 1b, is unreliable. In one of the original surveys, 6 (7.5%) of 80 subtype 1a sequences and 1 of 79 1b sequences would have been incorrectly identified on the basis of this polymorphism.[57] A related problem is that although some subtypes may be separately identifiable in the 5′UTR (such as 2a and 2b), others, such as 2c, may not, even though all 3 subtypes are approximately equally divergent from each other elsewhere in the genome (Fig. 1).
- Even relatively short coding regions of the HCV genome provide more definitive information on the genotype or subtype of an HCV variant than the 5′UTR. Although not necessarily required clinically, the nucleotide sequence of a sub-genomic region (including the conserved core gene) allows definitive identification of genotype and generally of the subtype, as well as being able to predict the existence of HCV variants not yet classified.
- For all genotyping assays, whether based on the 5′UTR or elsewhere in the genome, there is an intrinsic assumption that the genotype inferred from 1 region reflects that of the genome as a whole. Although few recombinant forms have been described, the spread of HCV variants such as the 2k/1b recombinant and the generation of further hybrid viruses in multiply exposed individuals would increasingly limit the accuracy of genotyping assays, and importantly for their clinical use, attenuate their predictive value for treatment response.

As should be evident from these points, HCV identification is an activity distinct from HCV classification. Classification provides the framework on which the specificity and accuracy of genotyping assays can be assessed, and for this purpose an agreed and consistent set of classification criteria, and a system of assigning genotype names is required. The following section discusses the issues in HCV classification in which consensus is required, and is followed by a series of classification and nomenclature proposals designed to maintain clarity in this field.

Current Issues to Resolve in HCV Classification

Several problems and uncertainties with current classification schemes for HCV have been identified and cause both inconsistencies with the nomenclature of HCV variants in published papers and difficulties for the organization and retrieval of HCV sequences from the 3 databases. These can be summarized as follows:

Diversity Within Genetic Groups. Although the primary division of HCV variants into 6 genetic groups is evident from phylogenetic analysis (Figs. 1 and 2), it has been increasingly recognized that there is considerably more genetic diversity within groups 2, 3, and 6 than found between the originally classified subtypes 1a and 1b, and 2a, 2b, and 2c.[34] In the past, it had been additionally proposed that more divergent variants within groups 3 and 6 qualify as separate major genotypes of HCV. At the HCV Classification meeting in Santa Fe, genetic group 6 was proposed to be re-designated as "clade 6," its variants retain their proposed genotype designations as genotypes 6, 7, 8, 9, and 11; similarly, "clade 3" should contain variants classified as genotypes 3 and 10.[2] In this scheme, the one-to-one correspondence between genetic group and genotype is lost.

The imposition of an additional tier of variability, however, leads to largely arbitrary classification decisions that compromised the simplicity of the original primary assignment of HCV genetic groups as genotypes. For example, both subtype 3b and the proposed new genotype 10a are both in genetic group 3 but are both highly divergent in sequence from subtype 3a, much more so than other subtypes of genotype 3 (Fig. 1). The decision to classify 10a as a genotype and 3b as a subtype was based on a difference in nucleotide sequence divergence in the coding region of only 3% (23% between 3a and 3b, 26% between 3a and 10a). This is much lower than the 31% to 34% divergence between variants in different genetic groups (such as between 1a and 2a). Divergence between the various proposed genotypes in group 6 is similarly consistently lower (mean, 27%; range, 21%-29%) than between the originally classified genotypes. Genetic group 2 may similarly contain more divergent sequences than the norm for subtypes (marked as "x" in Fig. 2). This might lead to the addition of further, equally arbitrary, genotype designations in a geographical region where otherwise genotype 2 variants are predominant in the population.

Apart from the difficulty in placing this further dividing line between genotype and clade, the resulting classification in a subtype/genotype/clade hierarchy is geographically inconsistent. To many, the scheme has been confusing, because in some cases, a clade contains only 1 genotype and the terms are interchangeable (*e.g.*, genotype 1/clade 1); in others a clade may contain 5 or more genotypes (*e.g.*, clade 6, genotypes 6, 7, 8, 9, and 11). This confusion and lack of consensus has led to continuing nomenclature differences between publications

whenever variants from Southeast Asia and elsewhere are described.

Conflicting Subtype Designations. There are many examples of conflicting nomenclature within currently classified HCV variants. Most of these inconsistencies comprise 2 different subtypes being referred to by the same name, such subtypes "4a" found in Egypt[7] and Zaire.[12] Conversely, the same variant may be described with different subtype designation, such as VAT96, designated as "2k,"[58] and RU169 designated as "2j."[59] These occurrences will have to be resolved in an agreed catalogue of HCV variants, and for retrieval of sequences from the HCV databases.

Recombination. Currently no method exists for classifying recombinant forms of HCV. For database retrieval and for cataloguing the occurrence of recombinant viruses, a nomenclature system that recorded its genotype composition and provided unique identifiers for pattern of breakpoints would be of value. This system is in place for HIV-1 and might be used as a model for HCV.[60] Here, designation of inter-subtype recombinant viruses as (circulating) recombinant forms (RFs) requires detection and complete genome sequences of a recombinant virus from 3 or more independently infected individuals. Each new recombinant should have breakpoints in the same positions in each sequence. Each is then numbered sequentially in order of discovery, with subtype identification letters listed alphabetically to approximately indicate their composition. The HCV recombinant in St. Petersburg[40,41] would therefore be designated as RF 01_1b2k.

Table 1. Confirmed HCV Genotypes/Subtypes

Genotype*	Locus/Isolate(s)†	Accession number(s)	Reference(s)
Genotype 1			
1a	HPCPLYPRE, HPCCGAA	M62321, M67463	67, 68
1b	HPCJCG, HPCHUMR	D90208, M58335	69, 70
1c	HPCCGS, AY051292	D14853, AY051292	71
Genotype 2			
2a	HPCPOLP, JFH-1	D00944, AB047639	72, 73
2b	HPCJ8G, JPUT971017	D10988, AB030907	9, 74
2c	BEBE1	D50409	75
2k	VAT96	AB031663	58
Genotype 3			
3a	HPCEGS, HPCK3A	D17763, D28917	76, 77
3b	HPCFG	D49374	78
3k	HPCJK049E1	D63821	59
Genotype 4			
4a	HCV4APOLY	Y11604	79
Genotype 5			
5a	EUH1480, SA13‡	Y13184, AF064490	80, 81
Genotype 6			
6a	HCV12083, 6a33	Y12083, AY858526	82
6b	Th580	D84262	83
6d	VN235	D84263	83
6g	HPCJK046E2	D63822	59
6h	VN004	D84265	83
6k	VN405	D84264	83

NOTE. Tables 1, 2, and 3 were compiled by a working group of Donald Murphy, Erwin Sablon, and Phillipe Halfon.

*Consensus proposed genotype/subtype names. For instances in which multiple sequences of a HCV genotype are available, two sequences have been listed, prioritized by (1) publication date, or (2) submission date when unpublished.

†Locus (or isolate name, if locus is the same as the accession number).

‡Sequence obtained from acute phase plasma of a chimpanzee experimentally infected with (human-derived) isolate SA13.

Consensus Classification Proposals

Each of these issues in HCV classification was discussed, and the following consensus decisions were made. These are proposals for standardizing the nomenclature of currently described variants of HCV, and the future designation of new subtypes and genotypes as they are discovered.

Division of HCV Into Clades/Genotypes. The primary division of HCV variants remains the 6 genetic groups, irrespective of the hugely increased numbers of subtypes or variants since found within these groups. The consensus acknowledges that different levels of within-group diversity are found between genotypes, and different relationships within them. Nevertheless, varying degrees of diversity are becoming apparent in other genotypes (*e.g.*, among the genotype 2 variants from West Africa), and it is difficult and arbitrary to specify a degree of sequence divergence below which a subtype designation is made, and above which a new genotype is assigned. This difficulty is epitomized by the problems with the classifications of 3b and 10a within genotype 3 (see above).

The following points summarize the recommendations concerning the designation of HCV genotypes:

1. The primary division of HCV will henceforth be based on the 6 genetic groups apparent from Figs. 1, 2, and other published sequence analyses of HCV. Division of HCV variants into the 6 genetic groups of HCV is supported by each of the principal methods of phylogenetic analysis of the core/E1, NS5B, and complete genome sequences (Table 1). These comprise tree-building by: (i) neighbor-joining and unweighted pair group method with arithmetic mean from pairwise distances computed with a variety of substitution models, (ii) parsimony, and (iii) maximum likelihood. For distance-based methods, greater than 70% of trees (actually invariably greater than 90%) support the primary division of HCV variants into the 6 genetic groups, with no consistent support for any higher-level grouping. Consistency between phylogenetic methods is required for the assignment of new genotypes (see specific proposals below).

Table 2. Listing of HCV Variants With Proposed Changes in Genotype Nomenclature

Proposed Designation*	Published Designation	Status†	Isolate‡	Region Sequenced§	Reference(s)
Genotype 2					
2k	2j	C	RU169	NS5B (D86532), 3'UTR (D86532)	83
2j	2l	P	BA047	NS5B (D86530), 3'UTR (D86530)	83
2n	2e	P	NL50	C/E1 (L39309), NS5B (L44602)	84
2o	4f/2f	P	FR4	C/E1 (L38333), NS5B (L38373)	84
2p	2f	P	NL33	C/E1, (L39300), BS5B (L44601)	84
2q	2k	P	BA045	NS5B (D86529), 3'UTR (D86529)	83
Genotype 3					
3k	10a	C	HPCJK049E1	Complete genome (D63821)	59
Genotype 4					
4r	4a	P	Z4	C/E1 (U10236/L16652)	12, 64
			FrSSD120	C/E1 (AJ401097), NS5B (AJ291282)	93
4n	4 alfa	P	1359	C/E1 (AF271874)	65
4o	4 beta	P	2153	C/E1 (AF271882), NS5B (AF271815)	65
Genotype 6					
6c	7d	P	Th846	C/E1 (D37843), NS5B (D37857)	35
6d	7b	C	VN235	Complete genome (D84263)	83
6e	7a	P	VN540	C/E1 (D88474), NS5B (D87361)	34
6f	7e	P	BB7	NS5B (D28541)	96
6f	7c	P	Th271	C/E1 (D37844), NS5B (D37858)	35
6g	11a	C	HPCJK046E	Complete genome (D63822)	59
6h	9a	C	VN004	Complete genome (D84265)	83
6i	9b	P	Th555	C/E1 (D37849), NS5B (D37863)	35
6j	9c	P	Th553	C/E1 (D37848), NS5B (D37862)	35
6k	8b	C	VN405	Complete genome (D84264)	34
6l	8a	P	VN507	C/E1 (D88470), NS5B (D87357)	34

*Proposed new name based on revised criteria for genotype designations.
†Classification status; C: Confirmed; P: Provisional.
‡Example of isolates referred to in associated publications (last column).
§Regions sequenced (accession numbers in parentheses), prioritized for (1) complete genome; (2) Core/E1 and NS5B regions; (3) other regions where core/E1 and NS5B regions are not both available.

2. The genetic groups will be termed "genotypes." The previously proposed term "clade" to describe an HCV genotype might be regarded as an alternative, more descriptive term for genotype, and is currently used in the VIIIth ICTV Report.[1] However, for consistency with previous classifications of HCV and current clinical usage, we recommend the use of the term "genotype" for genetic group in HCV sequence databases and publications.
3. Variants of HCV currently designated with genotype numbers above 6 will be renamed according to the genotype group in which they fall, and with the next available subtype designation (Table 2). For example, genotype 10a will be re-classified as 3k, 7a as 6e, and so forth. The proposed changes to the nomenclature are presented in Table 2.
4. The identification of new genotypes will henceforth require demonstration of a consistent independent phylogenetic grouping away from any of the currently classified genotypes of HCV (see later discussion).

Classification and Nomenclature of Previously Described Subtypes of HCV. The group believed the existing nomenclature of HCV genotypes and subtypes provided a valuable framework for ongoing studies of genetic variation. The following points summarize the group's decisions and recommendations for subtype designations:

1. Existing designations where they are consistent will be retained, irrespective of the criteria agreed for the designation of new subtypes (Tables 1 and 3).
2. Variants within genotypes 3 and 6 that have been re-designated as subtypes (see previous section) will be incorporated into the updated list.
3. HCV variants with conflicting names in the literature have been re-designated on consultation with the originating authors (Table 2).

Assignment of New Genotypes of HCV. Further variants of HCV likely will be discovered that merit their assignment as new genotypes, such as the candidate new genotype obtained from Central Africa.[61,62] To ensure their correct classification, it is essential to demonstrate that there is no significant grouping within any of the existing genotypes. This has to be demonstrated by rigorous phylogenetic analysis of a complete sequence of the coding region of the virus. This analysis will additionally

Table 3. Provisionally Assigned HCV Subtypes

		Accession Number(s)*		
	Isolate†	Core/E1	NS5B	Reference(s)
Genotype 1				
1d	HC1-N15, HC1-N16	L39299, L39302	L38377, L38372	84
1e	CAM1078, QC248	L38349(C), AY894555	L38361, AY894553	62, 84
1f	FR2	L38350	L38371	84
1g	2152, 1382	AF271822, AF271820	AF271798, AF271797	65
1h	98CM1521, QC94	AY256790(C), AY434131	AY257087, AY434132	32, 62
1i	FR16, QC77	n.a., AY434119	L48495, AY434120	62, 85
1j	QC2, QC89	AY434158, AY434128	AY434106, AY434129	62
1k	QC68, QC82	AY434112, AY434122	AY434113, AY434123	62
1l	98CM1383, 98CM1427	AY256789(C), AY256792(C)	AY257083, AY257091	32
Genotype 2				
2d	NE92, BN177	L39294, n.a.	L29634, AF037244	66, 86
2e	JK020, JK025	D49745, D49746	D49760, D49761	59
2f	JK081, JK139	D49754, D49757	D49769, D49777	59
2g	MED017	n.a.	X93323	26
2h	MED007	n.a.	X93327	26
2i	FR13, HN4	n.a., X76411/X76415	L48492, L48499	87, 88
2j	BA047, QC106	n.a., AY894528	D86530, AY894526	62, 83
2l	FR15	n.a.	L48494	85, 89
2m	QC76, QC104	AY434116, AY434143	AY434117, AY434144	62
2n	NL50	L39309	L44602	84
2o	FR4	L38333	L38373	84
2p	NL33	L39300	L44601	84
2q	BA045	n.a.	D86529	83
Genotype 3				
3c	NE048	D16612	D14198/D16613	33
3d	NE274	D16620	D14200/D16621	33
3e	NE145	D16618	D16619	33
3f	NE125, PK64	D16614, n.a.	D14203/D16615, L78842	33, 87
3g	IND1751, IND1452	X91423/X91307, X91306(C)	X91417, X91418	90
3h	QC29, SOM1	U33437(C), AF216792/AF216786	AF279120, AF216789	91, 92
3i	IND674, QC100	X91300(C), AY434137	X91422, AY434138	62, 90
Genotype 4				
4b	Z1	U10235/L16677	n.a.	12, 64
4c	Z6, GB358	U10238/L16678, L29606	n.a., L29607	12, 64, 66
4d	DK13, SD006	U10192/L16656, n.a.	n.a., D86537	12, 64, 83
4e	CAM600, GB809	L29589, L29629	L29590, L29626	66
4f	G22, FR12	L29595, L38332	L29593, L38370	66, 84
4g	GB549	L29620	L29621	66
4h	GB438, FrSSD35	L29610, n.a.	L29611, AJ291249	66, 93
4i	CAR4/1205	L36439	L36437	28
4j	CAR1/501	n.a.	L36438	28
4k	B14, FrSSD174	L39282, n.a.	L44597, AJ291294	84, 93
4l	SD002, 2116	n.a., AF271881	D86534, AF271816	65, 83
4m	SD035, 1797	n.a., AF271876	D86543, AF271813	65, 83
4n	1359, QC97	AF271874, AY434134	n.a., AY434135	62, 65
4o	2153, QC59	AF271882, AY434107	AF271815, AY434108	62, 65
4p	FrSSD158, QC139	AJ401099(E), AY434149	AJ291285, AY434150	62, 93
4q	QC86, QC107	AY434125, AY434146	AY434126, AY434147	62
4r	Z4, FrSSD120	U10236/L16652, AJ401097(E)	n.a., AJ291282	12, 64, 93
4t	98CM1458, QC85	AY256808(C), AY706996	AY257072, AY706997	32, 62
Genotype 6				
6c	Th846	D37843	D37857	35
6e	VN540, VN998	D88474, D31971	D87361, D30797	34
6f	Th271, EUTH36	D37844, U31261(C)	D37858, U31276	24, 35
6i	Th555, EUTH100	D37849, L50554(C)	D37863, L50535	35, 94
6j	Th553, EUTH1	D37848, L49473(C)	D37862, L49481	35, 56
6l	VN507, VN531	D88470, D88472	D87357, D87359	34
6m	EUBUR1, B4/92	L49480(C), D63943/D63944	L49484, D28543	56, 95
6n	D86/93, EUTH86	D63945, U31259(C)	D28545, U31275	24, 96
6o	VN4, QC33	L38341, AY894537	L38382, AY894535	62, 84
6p	VN12, QC123	L38340, AY894534	L38380, AY894532	62, 84
6q	QC57, QC176	AY754632, AY754617	AY754633, AY754618	62

*Accession numbers of sequences from the core/E1 and NS5B regions. Where two examples are listed, a comma divides the accession numbers from the two entries; "n.a.": not available; "/": denotes that the core/E1 or NS5B sequences are available from two different accession numbers; (C): only core sequence available; (E): only E1 sequence available.

†Listing of up to two examples of each provisionally assigned HCV subtype prioritized according to (1) availability of complete or near complete core/E1 and NS5B sequences, (2) publication date, (3) GenBank/EMBL/DDBJ submission date. Where possible, the isolate names referred to in associated publications (last column) are listed for ease of reference.

confirm the absence of recombination with sequences from other genotypes.

The following criteria were proposed for identification and designation of a variant of HCV as a new genotype:

1. *Provisional designation.* This requires one complete coding region sequence to be obtained, the demonstration of a separate grouping from other genotypes by phylogenetic analysis, and an absence of recombination. The sequence of a candidate new genotype should be independently analyzed by submission to one of the HCV databases. The sequence will be analyzed by a variety of phylogenetic methods described previously. This will allow the sequence to be assigned with the next available genotype number, and the subtype designation "a," for example, genotype 7a.
2. *Confirmed designation.* This requires coding sequences of 2 or more HCV variants from infections that are not directly linked epidemiologically. The sequences should further demonstrate a lack of grouping with current classified genotypes by the above methods. This further analysis, and any available sequences from subgenomic regions such as core/E1 and NS5B (see later discussion), will provide valuable reference information on the genetic heterogeneity within the newly designated genotype, the existence of subtypes, the geographical origins of the variants, and their likely designation in 5′UTR-based genotyping assays.

Assignment of New Subtypes of HCV. Different issues apply to the assignment of new subtypes. Some geographical regions contain so much diversity within genotypes that it is of little value to continue classifying them as subtypes. Elsewhere, however, subtype labels have particular epidemiological value and are widely used as genetic markers in studies of past and ongoing virus transmission of HCV in different risk groups.

To recognize this distinction, new subtype designations should only be provided where there is evidence for its spread in particular transmission networks, and where its identification would be of epidemiological value. The simplest method to achieve this distinction is to require evidence of infection with a new proposed subtype of HCV in several independently infected individuals.

The following criteria for assignment of new subtypes were proposed:

Provisional designation. Three or more examples of infection with a new proposed subtype are required for subtype designation. Sequences are required from both the core/E1 region (sequence data available from 90% or more nucleotides corresponding to positions 869 to 1292 in the H77 reference sequences, accession number AF009606)[12,63-65] and the NS5B region (data from 90% or more positions in the region 8276-8615 in H77).[7,8,66] The sequences of primers suitable for amplification of these regions from a wide range of HCV genotypes will be made available on the public databases.

Sequences will be analyzed by a variety of distance-based, parsimony, and maximum likelihood methods, and evidence sought for consistent phylogenetic grouping together and distinctness from other subtypes. Because currently classified subtypes of HCV differ in nucleotide sequence from each other by more than 15%, at least this level of divergence will be expected from other HCV variants within the genotype. However, as described in the Introduction, the existence of separately identifiable subtypes is primarily an epidemiological phenomenon associated with its recent spread. Because subtype designation are primarily epidemiological labels, it is clearly not appropriate or of value to develop formal criteria for their assignment. Indeed, the varying degrees of sequence divergence of variants within different genotypes would make the development of such criteria extremely difficult.

Candidate subtypes will be provisionally assigned with the next available subtype letter for the genotype on submission to one of the HCV databases. Sequences from the 5′UTR will be of value for assessment of their appearance in commonly used genotyping assays but are not required. Single or pairs of variants of HCV that would otherwise be designated as new subtypes by these criteria will not be assigned a subtype letter in the database.

Confirmed designation. One or more complete genome sequences will be required for confirmed designation. This will allow the degree of sequence divergence from other subtypes over the whole genome to be assessed as well as confirming an absence of recombination.

Assignment of Recombinant Forms of HCV. It is important that the classification scheme for HCV genotypes should be able to incorporate HCV recombinants. However, with the current description of only 2 or 3 confirmed or possible recombinants in the literature, it was deemed to be of less immediate importance to classify these formally, and to develop rules for nomenclature. Until review at a subsequent classification meeting, sequences with evidence of recombination will be annotated as such in the databases, with options to include or exclude them from downloads or analyses of sequences.

Interface With HCV Sequence Databases. The HCV sequence databases are in a unique position to support the effort to make the HCV nomenclature more uniform. By assigning geno/subtypes to the sequences that people retrieve and download, they can influence the

commonly used nomenclature of existing sequences, whereas they can have a coordinating role in assigning new geno/subtypes and keeping track of these, especially before journal publication. The databases are also committed to assist in the naming of new geno/subtypes, through helping researchers name proposed new geno/subtypes, by checking existing names for consistency and correcting any inconsistencies that are found, by making it easy for the field to keep track of which geno/subtype names have already been assigned, and by providing tools for genotype or subtype identification and detecting recombinants.

The HCV database websites will provide access to the criteria for assignment of new genotypes and subtypes of HCV developed in this consensus paper, and make HCV researchers, reviewers, and journal editors aware of these guidelines. They will provide the listing of current assigned subtypes and genotypes (based on Tables 1 and 3), but will be automatically updated as sequence data are submitted, showing which designations exist in the databases, but not those that have been given out and not yet published. The distinction between "provisional" and "confirmed" designations will also be implemented in the databases through the provision of a separate field for this category. The genotype name re-assignments in Table 2 will similarly be made available, and the 3 databases will keep in continuous contact to ensure that the nomenclature of currently existing sequences is uniform and free of conflicts.

Summary

This report describes a series of proposals for the classification of HCV variants into genotypes and subtypes. It addresses both current problems with the nomenclature of existing variants, and incorporates our improved understanding of the genetic diversity and epidemiology of HCV into the revised criteria for the designation of new genotypes and subtypes. The consensus meeting provided the opportunity to compile for the first time a full listing of currently described variants of HCV (Tables 1 and 3), and the opportunity to perform the minimum number of genotype and subtype name re-assignments to create consistency in nomenclature (Table 2).

Finally, these proposals serve as a framework for access to the 3 databases, which will follow the revised nomenclature presented here for sequence retrieval, and to use the revised criteria for classification in their coordinating role in the assignment of new genotypes and subtypes; this will be of major value in preventing future inconsistencies in nomenclature.

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Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3

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Background. Interferon regulatory factor (IRF)-3 plays an important role in initiating cellular interferon-stimulated gene-mediated antiviral responses. In the present study, we evaluated the effects of IRF-3 expression and activation on intracellular hepatitis C virus (HCV) replication using an HCV replicon system. ***Methods.*** An HCV replicon was constructed that expressed a neomycin-selectable chimeric firefly luciferase reporter protein. A small interfering (si) RNA oligonucleotide directed against IRF-3 mRNA was designed and synthesized. A eukaryote expression plasmid vector was constructed that expressed IRF-3 mRNA under control of the cytomegalovirus early promoter/enhancer. To evaluate transcriptional activity of the interferon-stimulated genes, a reporter vector was used that expressed firefly luciferase under control of the interferon-stimulated response element (ISRE). ***Results.*** The baseline expression of IRF-3 did not significantly differ between cells with and without expression of the replicon. Transfection of an IRF-3 expression plasmid into the cells raised the ISRE-luciferase activities. The increase of ISRE activity was significantly more potent in the replicon-expressing cells than in cells without replicon expression. Concomitantly, the overexpression of IRF-3 suppressed HCV replication levels. In contrast, siRNA knockdown of IRF-3 suppressed ISRE activity by 38% ± 2%. Interestingly, the suppression of IRF-3 resulted in a significant increase of HCV replication, by up to twofold, depending on the IRF-3 suppression levels. ***Conclusions.*** IRF-3 negatively regulated intracellular HCV replication, and was partially activated in cells that expressed the HCV replicon. Thus, IRF-3 is a key molecule controlling HCV replication through modulation of host interferon gene responses.

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Introduction

Hepatitis C virus (HCV) is a worldwide health-care problem causing a spectrum of liver disease ranging from an asymptomatic carrier state to liver cirrhosis and hepatocellular carcinoma.[1] Currently available anti-HCV treatments are based on high-dose administration of a major antiviral cytokine, interferon (IFN)-α. However, even with the most efficient regimen of pegylated interferon in combination with ribavirin, almost half of all cases are refractory to the treatment and fail to eradicate the virus.[2] Without the IFN therapy, HCV is associated with persistent infection and replication in the liver in spite of intact host immune systems; these features lead us to speculate that HCV escapes from or attenuates host antiviral responses.

Type I IFN plays a central role in eliminating viruses not only in therapeutic applications but also as a na-tural cellular antiviral defense mechanism.[3,4] IFNs mediate antiviral responses by inducing expression of interferon-stimulated genes (ISGs), including those encoding 2,5-oligoadenylate synthetase, double-stranded RNA-dependent protein kinase R, and MxA proteins, resulting in the degradation of cellular RNA, general repression of protein synthesis, and apoptotic cell death.[5] Also, a DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of ISGs, including those encoding cytokines and chemokines, was the principal reaction during the course of the viral infection and its clearance and that a considerable proportion of the genes were inducible by IFNs.[6]

The expressional control of ISGs is directed by receptor-mediated stimuli of type I IFNs.[7] Binding of

the IFNs onto their receptors activates receptor-associated janus kinases, which phosphorylate signal transducer and activator of transcription (STAT) 1 and STAT2. The phosphorylated STATs, 1 and 2, recruit IFN regulatory factor (IRF)-9 to form a complex with IFN-stimulated gene factor-3, which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of ISGs, and activates expression of ISGs.[3,4,8]

Other than by type I IFNs, expression of ISGs is controlled by binding ISRE with other molecules, including IRF-1, IRF-3, and IRF-7. Among them, IRF-3 is a transducer of virus-mediated signaling and plays a critical role in the induction of cellular antiviral responses.[8–11] IRF-3, which is ubiquitously expressed in the cytoplasm, is subjected to phosphorylation by virus infection, double-stranded RNA, and bacterial lipopolysaccharides. The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus, and predominantly activates expression of the IFN-β gene and certain ISGs.[4,12,13]

The IRF-3-mediated IFN pathway might be a target of viruses to counteract antiviral responses and to promote virus replication in the infected cells. Ebola virus, bovine diarrhea virus, and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins.[14–16] In the case of HCV, some reports suggest interaction of virus proteins with the cellular IFN system. The viral NS5A protein has been reported to interfere with cellular IFN signaling.[17] It has recently been reported that HCV NS3/4A fusion protein blocks virus-induced activation of IRF-3.[18] Taken together, these findings indicate that IRF-3 is not only a key molecule of cellular innate immune responses but also might be a target of antiviral strategies. However, the mechanisms of IRF-3 activation by HCV infection in hepatocytes have not been explored yet, nor have the effects of the activated IRF-3 on HCV been satisfactorily studied.

An HCV subgenomic replicon is an in vitro model that simulates cellular autonomous replication of HCV genomic RNA. The development of the replicon system has partly overcome the problem of a lack of HCV replication models.[19] Replication of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs,[20–22] suggesting intact IFN receptor-mediated cellular responses. However, in the absence of the exogenous interferon, persistent and high-level expression of the replicon has caused us to speculate that intracellular virus-induced antiviral responses become attenuated or malfunction as a result of the expression of viral proteins. We have previously reported that the baseline activity of ISG expression is substantially decreased in cells expressing replicon and that this decrease is partly attributable to the transcriptional suppression of IRF-1.[23] In the present study, we extended our observations by investigating the effects of IRF-3 expression and activation on HCV replication.

Materials and methods

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 2mM L-glutamine and 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 300μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneodelS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib.[22] The replicon pRep-N was reconstructed by replacing the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase genes (pRep-Feo, Fig. 1).[24,25] RNA was synthesized from pRep-N and pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-N and Huh7/Rep-Feo, respectively). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A, and NS5A protein expression levels and with the replicon RNA expression levels.

Cured Huh7 cells

To establish cured Huh7 cells (cHuh7), from which replicon RNA was eliminated, Huh7/Rep-Feo was treated

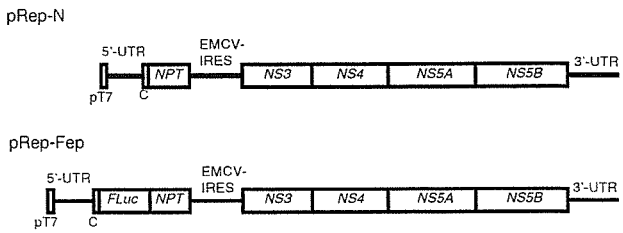

Fig. 1. Structures of the hepatitis C virus (HCV) replicon plasmids. *UTR*, untranslated region; *pT7*, T7 promoter; *C*, truncated HCV core region (nucleotides 342–377); *EMCV*, encephalomyocarditis virus; *Fluc*, firefly luciferase gene; *NPT*, neomycin phosphotransferase gene; *NS3*, *NS4*, *NS5A*, and *NS5B*, genes that encode HCV nonstructural proteins

with 100 U/ml of IFN-α for 14 days. The absence of replicon RNA was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and by the loss of resistance to G418.[23]

Small interfering RNAs

Three small interfering RNAs (siRNAs) directed against IRF-3 were synthesized: siRNA1 (5′-gug gga gac agg acg cug cTT-3′), siRNA2 (5′-gcc aga cac cuc ucc gga cTT-3′), and siRNA3 (5′-ggu ugu gcc cac gug ccu cTT-3′). A control siRNA was used as previously described (5′-ucg ggg cac ugc uag auc cTT-3′).[24]

Plasmids

The expression plasmid vector pcDNA3.1-IRF-3 expresses the human IRF-3 open reading frame, which was cloned from human hepatocyte mRNA by RT-PCR using primers IRF-3/5′ (5′-CAC CAT GGG AAC CCC AAA GCC ACG GAT CCT-3′) and IRF-3/3′ (5′-GCT CTC CCC AGG GCC CTG GAA ATC CAT G-3′). The PCR product was inserted into the pcDNA3.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) as instructed, and the nucleotide sequence was confirmed. The plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. The plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. pRL-CMV (Promega), which expressed *Renilla* luciferase protein, was used for correction of transfection efficiency.

Transient transfection

DNA and siRNA transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To perform reporter assays to determine the effect of IRF-3 on ISRE in the cells, a total of 5×10^4 Huh7, cHuh7, and Huh7/Rep-N cells were subcultured onto 24-well plates the day before transfection. A total of 100 ng of pISRE-TA-Luc and various amounts of pcDNA3.1-IRF-3 with empty vector and 0.1 ng of pRL-CMV, to a total mass of DNA of 400 ng, were transfected by using 2 μl of Lipofectamine 2000.

Western blotting

Cytoplasmic and nuclear fractions of cell lysates were prepared as described elsewhere.[26] The purity of the cytoplasmic and nuclear fractions was monitored by immunoblotting using an antibody directed against a nuclear protein, USF-2 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Twenty micrograms of protein was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) or anti-His antibodies (Invitrogen), and detected by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

Immunocytochemical staining

Cells seeded onto tissue culture chamber slides were washed with phosphate-buffered saline (PBS) and fixed with 99% cold acetone for 10 min. After rinsing with PBS, cells were incubated with an anti-IRF-3 antibody at a dilution of 1/500 or an anti-His antibody at a dilution of 1/200 in PBS/3% goat serum. After 3 h, cells were washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. Cells were then washed and mounted with VectaShield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescence microscopy was carried out with an Olympus BX50.

Luciferase reporter assays

Luciferase activity was measured by luminometer (Lumat LB9501; Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were done in triplicate, and the results were expressed as means ± SD.

MTS assays

To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t* test; *P* values less than 0.05 were considered statistically significant.

Results

Expression level of IRF-3 in cells with and without HCV replication

First, we evaluated the expression levels of endogenous IRF-3 in Huh7 cells with or without expression of the HCV replicon. Western blotting analysis showed no sig-

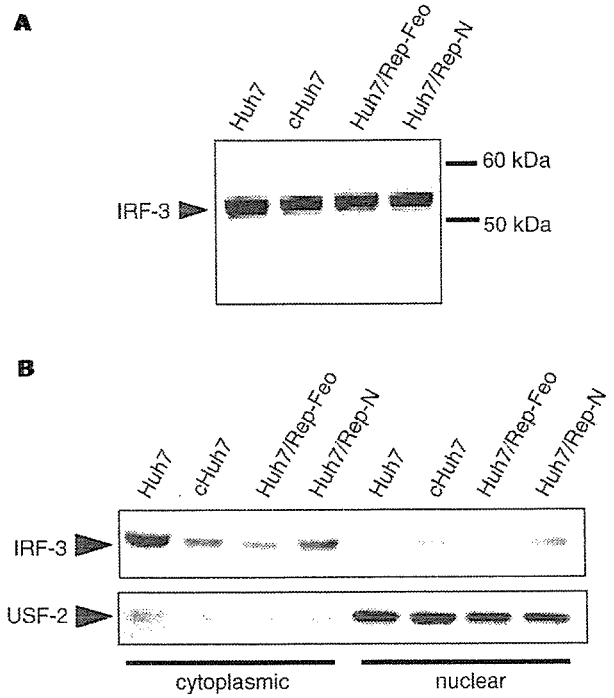

Fig. 2A,B. Expression of endogenous interferon regulatory factor-3 (*IRF-3*) in cells with and without expression of the HCV replicon. **A** Western blotting. Whole cell lysates from Huh7 and Huh7/Rep-Feo were prepared. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting, the expression of IRF-3 protein was detected by a monoclonal anti-IRF-3 antibody. **B** To detect IRF-3 translocated to the nucleus, we prepared the cytoplasmic and the nuclear fractions of cell lysates from naïve Huh7, Huh7/rep-Feo, Huh7/Rep-N, and cured Huh7 (*cHuh7*) cell lines, and detected IRF-3 expression by Western blotting. The purity of cellular fractionation was tested by immunoblotting for USF-2. The differences in the IRF-3 expression levels were due to different yields from the preparation of cytoplasmic and nuclear fractions. However, the ratio of nuclear to the respective cytoplasmic IRF-3 remained equal among the cell lines tested

nificant difference in expression levels of IRF3 between Huh7 and Huh7/Rep-Feo (Fig. 2A). Similarly, levels of IRF-3 mRNA were not significantly different between Huh7 and Huh7/Rep-Feo.

IRF-3, once activated by site-specific phosphorylation, translocates into the nucleus. To examine the nuclear translocation of the activated IRF-3, we prepared nuclear and cytoplasmic fractions of cell lysates from naïve Huh7, cured Huh7, and two Huh7 that expressed the replicon, Huh7/Rep-N and Huh7/Rep-Feo. Western blotting of the cell fractions showed that most IRF-3 protein was localized in the cytoplasm in each cell line, and that there was no obvious increase in nuclear IRF-3 in any of the cell lines, nor were there differences in the nuclear IRF-3 levels between cells with and without the HCV replicon (Fig. 2B). Similarly, the immunocytochemistry analysis showed that IRF-3 was mainly localized in the cytoplasm, and there were no differences in the patterns of IRF-3 staining (data not shown).

Overexpression of IRF-3 and effects on ISRE activity and HCV replication

Because IRF-3 is a strong inducer of ISGs on activation, a slight change in the IRF-3 activation level could affect ISRE enhancer activity. Thus, we examined ISRE reporter activities of cells with and without the replicon, and evaluated the effects of IRF-3 by overexpression. Transfection of an IRF-3 expression plasmid, pcDNA-IRF-3, resulted in expression of 6xHis-tagged IRF-3, which was confirmed by Western blotting using an anti-IRF-3 antibody (Fig. 3A) and an anti-His antibody (Fig. 3B). Immunohistochemistry showed cytoplasmic expression of the transfected IRF-3 (Fig. 3C). Cotransfection of pcDNA-IRF-3 with an ISRE-luciferase reporter plasmid, pISRE-TA-luc, into Huh7, cHuh7, and Huh7/Rep-N cell lines resulted in a significant increase of ISRE activity in cells in which IRF-3 was overexpressed (Fig. 3D). Interestingly, the cell line expressing the replicon, Huh7/Rep-N, showed a significantly higher ISRE induction ratio by IRF-3 overexpression than naïve Huh7 or cured Huh7 (28.7-fold vs. 8.9- or 11.7-fold, $P < 0.01$), suggesting partial activation of IRF-3 in the replicon-expressing cells. Concomitantly with the ISRE activation, transfection of pcDNA-IRF-3 into Huh7/Rep-Feo resulted in a significant decrease of internal luciferase activities to $19.6 \pm 1.8\%$ of control, indicating suppression of cellular HCV replication by IRF-3 overexpression (Fig. 3E). MTS assays of the IRF-3 transfected cell lines showed no obvious effects on cell growth or viability, indicating that these effects of IRF-3 overexpression were not due to nonspecific effects or to cytotoxic cell death (Fig. 3F).

Effect of IRF-3 siRNA oligonucleotides on ISRE-luc and HCV Feo-replicon cells

To investigate the effects of suppression of IRF-3 synthesis on HCV replication, three synthetic siRNAs, siRNA1, siRNA2, and siRNA3, were used. Western blotting showed that transfection of each siRNA into Huh7 cells resulted in a decrease of the IRF-3 protein level, by 39.5%, 57.8%, and 37.4%, respectively. To study the effects of IRF-3 suppression on HCV replication, siRNAs were transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 4 days of transfection. The siRNAs upregulated HCV replication to various extents (Fig. 4A). Thus, we used siRNA3, which was the most efficient, for the following assays. Cotransfection of ISRE-TA-Luc with siRNA3 or a

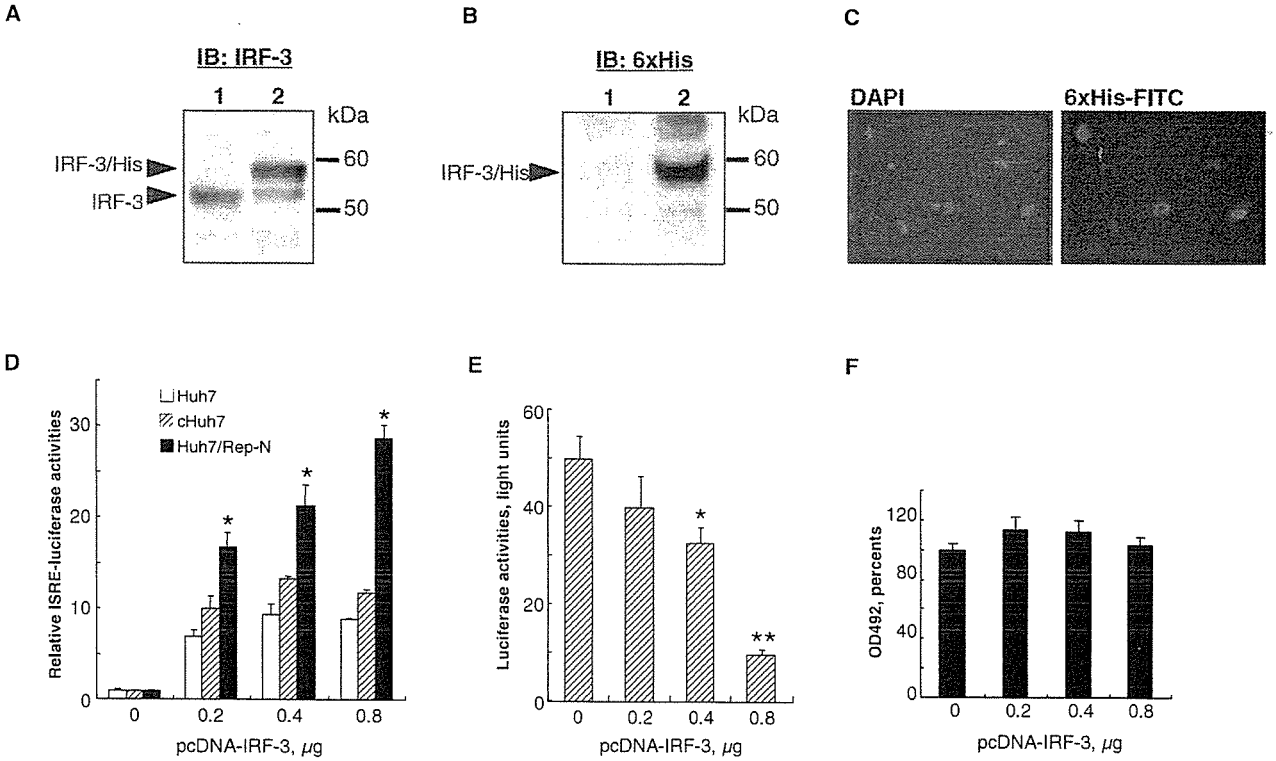

Fig. 3A–F. Effects of IRF-3 overexpression on the regulation of interferon-stimulated response element (*ISRE*) activity and HCV replication. An IRF-3-expression plasmid, pcDNA-IRF-3, was transfected into Huh7 cells (*lane 2*), and Western blotting analyses were performed using monoclonal anti-IRF-3 antibody (**A**) and anti-His antibody (**B**). Untransfected Huh7 is shown as a control (*lane 1*). **C** Fluorescence microscopy. The pcDNA-IRF-3 was transfected into Huh7 cells, and the cells were stained with 4′,6-diamidino-2-phenylindole (*DAPI*) and with anti-His antibody followed by fluorescein isothiocyanate (*FITC*)-labeled secondary antibody. The figure shows DAPI staining for nuclei (*left panel*) and transgenic IRF-3 expression (*right panel*). Magnification, ×40. **D** Effects of transgenic IRF-3 expression on ISRE reporter activity. The pcDNA-IRF-3 and ISRE-TA-luc reporter plasmids were cotransfected into Huh7 (*white bars*), cured Huh7 (*gray bars*), and Huh7/Rep-N cells (*black bars*), and luciferase activities were measured 24 h after the transfection. *Error bars* denote means + SD (*$P < 0.01$ relative to Huh 7 cells and cured Huh7 cells; Student-Newman-Keuls test). **E** Effects of transgenic IRF-3 expression on the levels of HCV replication. The indicated amounts of pcDNA-IRF-3 were mixed with empty pcDNA plasmid to adjust the total amount of DNA, mixed with Lipofectamine 2000, and transfected into Huh7/Rep-Feo cells seeded onto 24-well culture plates, and the luciferase activities were measured 24 h after transfection. *Error bars* denote means + SD (*$P = 0.006$; **$P = 0.0001$ relative to transfection with the empty vector). **F** MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays. The indicated amounts of pcDNA-IRF-3 were transfected into Huh7/Rep-Feo cells with the same conditions described above, and MTS assays were performed. *Error bars* indicate means + SD

control siRNA into Huh7 and Huh7/Rep-N resulted in significant suppression of ISRE-luciferase activities, by 50% in both Huh7 cells and Huh7/Rep-N (Fig. 4B). To study the relation between the suppression level of IRF-3 by siRNA3 and HCV replication, siRNA3 was transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 2 days of transfection. siRNA3 upregulated HCV replication in a dose-dependent manner (Fig. 4C).

Discussion

Persistent virus replication in host cells is the function of the interplay between the cellular antiviral system and the counteraction of the virus to evade host antiviral responses.[17] In our present study, even though IRF-3 expression levels were mostly similar between cells with and without HCV replication (Fig. 2A), overexpression (Fig. 3A–C) and knockdown (Fig. 4A) of IRF-3 were associated with up- and downregulation of ISG expression, as indicated by ISRE reporter activities (Figs. 3D and 4B), and were inversely correlated with HCV subgenomic replication levels (Figs. 3E and 4C). These

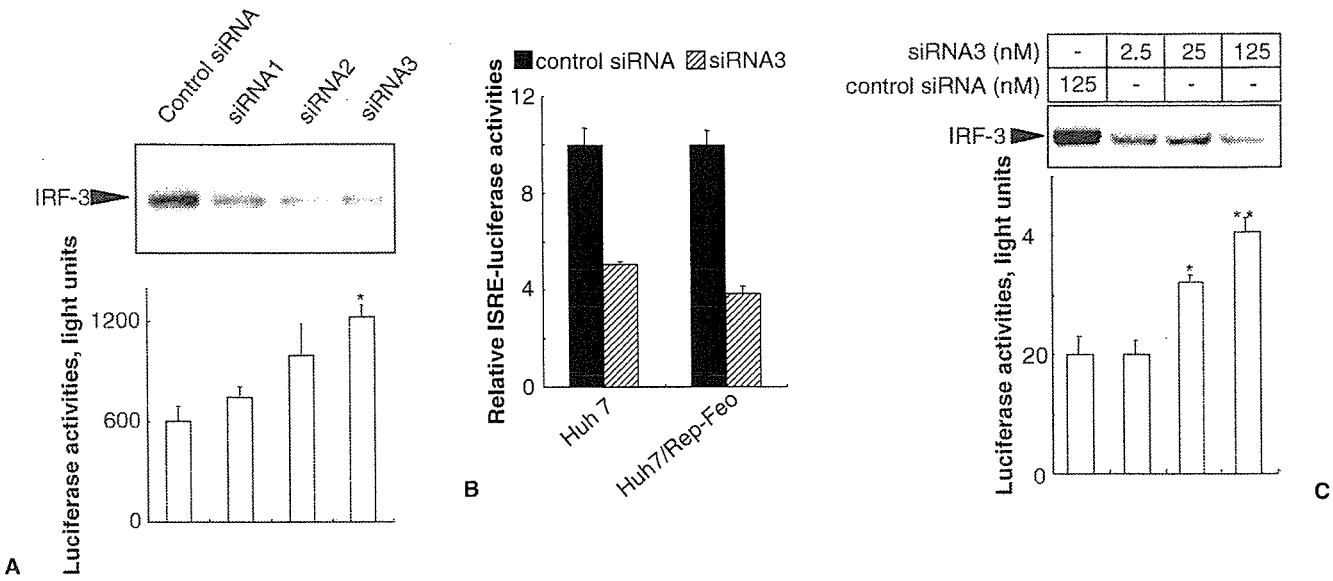

Fig. 4A–C. Suppression of IRF-3 expression by siRNA and effects on ISRE activity and HCV replication. **A** Suppression of endogenous IRF-3 expressions by IRF-3-directed siRNAs. Three siRNAs, siRNA1, siRNA2, and siRNA3, were transfected into Huh7 cells, and IRF-3 were detected by Western blotting using monoclonal IRF-3 antibody. Transfection of the three siRNAs substantially inhibited expression of IRF-3 protein. Three siRNAs and a control siRNA were transfected into Huh7/Rep-Feo cells that was plated onto 24-well plates. Graph: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.0007$ relative to transfection with a control siRNA). Suppression of IRF-3 by siRNA increased HCV replication. **B** Effects of an IRF-3-directed siRNA, siRNA3, on ISRE-reporter activity. The siRNA and a control siRNA were individually transfected with pISRE-TA-luc reporter plasmid into Huh7 and Huh7/Rep-Feo cells, and luciferase activities were measured 24 hours after transfection. *Error bars* denote means + SD (*$P < 0.001$ relative to transfection with a control siRNA). **C** The correlation between the suppression level of IRF-3 by siRNA and HCV replication. The indicated amounts of siRNA3 or a control siRNA were transfected into Huh7/Rep-Feo cells, which were plated onto 24-well plates. The Western blotting shows dose-dependent suppression of IRF-3. *Graph*: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.003$; **$P = 0.0007$ relative to transfection with a control siRNA)

results are consistent with a previous report that IRF-3 mediates expression of the antiviral gene via ISRE.[12] Furthermore, the replicon-expressing cells showed a significantly higher rate of ISRE activation than naïve or cured cells when IRF-3 was overexpressed (Fig. 3D). These results suggest different activation levels of the IRF-3-mediated pathway by replication of the HCV subgenome. Because IRF-3 is a strong inducer of interferon-β production,[4,12,13] it is possible that the effects of IRF-3 on HCV replication were predominantly mediated by interferon production, which led to activation of ISRE-dependent transcription. Collectively, our results suggest that replication of the HCV subgenome was closely correlated with expression and activation levels of IRF-3 and that IRF-3 was a key cellular factor controlling ISRE-regulated ISG expression and cellular antiviral responses.

Foy et al.[18] reported that the HCV NS3/4A fusion protein substantially blocks phosphorylation and nuclear translocation of IRF-3 by experimental virus infection. We have also confirmed that double-stranded RNA-induced activation of the IRF-3 pathway was abolished in cells expressing the HCV replicon (unpublished data). Our present results reinforce the reports that IRF-3 is a key molecule of the cellular innate immune responses against HCV and that it may constitute a target of antiviral strategies.

Although our findings suggest activation of the cellular IRF-3-IFN pathway along with HCV replication, there are still unsolved questions: Which molecule is the sensor of HCV? Which viral component is the target of the detection? How is the triggered signal transduced to the IRF-3 activation leading to IFN responses? Studies of the virus-induced IFN signaling pathway are making progress in the discovery and elucidation of these issues. Several molecules have been recently identified that are involved in innate immune responses against various pathogens, including viruses: toll-like receptor (TLR) families, which recognize viral components of double- or single-stranded RNAs and lipoproteins;[27] two kinases, IKKe and TBK1, which catalyze phosphorylation of IRF-3;[28,29] and TRIF,[30] which mediates Myd88-independent TLR signaling. TLR3 has been reported to recognize double-stranded RNA and to activate IRF3-

mediated IFN signal transduction, suggesting that TLR3 could be a candidate receptor for innate immune responses against viruses.[8,11] However, our preliminary studies have shown that treatments with polyinosinic polycytidylic acid [poly(I-C)] and lipopolysaccharides, which are ligands of TLR3 and TLR4, respectively, have no effect on cellular ISRE activities or on HCV subgenomic replication (data not shown), a part of which is consistent with the findings of previous studies.[31] More recently, a DExD-box helicase, RIG-I, has been identified as a cytoplasmic receptor molecule that recognizes double-stranded RNA.[32] Speculatively, unknown molecules may recognize HCV genomic replication in cells and activate the IRF-3-mediated antiviral pathway.

Because our present study was based on the HCV subgenomic replicon system, which expresses only viral nonstructural proteins and not structural proteins, our results may have limited implication for the association between HCV infection and the innate immune system. Moreover, Huh7 cells, which are the host of the HCV replicon, are of human hepatoma origin.[33] Most hepatomas arise from chronic viral hepatitis and liver cirrhosis.[1] Although little information is available on what Huh7 cells were derived from, it is possible that these cells have been primed by past HCV infection, which could modify the cellular innate immunity continuously. To address these possibilities, further investigation using other cell lines that stably support HCV replication may be warranted.

Although in vitro HCV replication is highly sensitive to exogenous IFN, in clinical settings, a majority of HCV-infected patients are resistant to IFN treatments.[2] Our results suggest that an IRF-3-mediated innate immune system response might be activated by HCV infection in hepatocytes. This initial reaction in the host cells against the virus may determine the activities of the cellular and humoral immune responses that follow, and the clinical course of the infection thereafter. At present, few reports correlate clinical features with the function of IRF-3 in the HCV-infected liver. Castelruiz et al.[33] have reported that patients with chronic hepatitis C show a significant increase in IFN-β mRNA in liver tissue. Thus, one of our next objectives is to elucidate how the innate immune system participates in the whole clinical process of HCV infection, and whether individual differences in the innate immune response influence clinical features.

In conclusion, our results demonstrate that IRF-3 negatively regulates HCV replication in vitro, possibly through IRF3-mediated ISG expression pathways. Therefore, IRF-3 might be a key molecule not only as a mediator of the host antiviral responses against HCV but also as a potential therapeutic target to control HCV replication.

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Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication

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SUMMARY. Type-I interferons (IFNs) and the interferon-stimulated genes (ISGs) play a major role in antivirus responses against hepatitis C virus (HCV) infection. In this study, we studied expression profiles of ISGs in cells supporting subgenomic HCV replication (Huh7/Rep), and screened their activities to suppress HCV replication. Real-time PCR analyses showed that the expression levels of 23 ISGs were significantly lower in Huh7/Rep than naive Huh7 cells due to transcriptional suppression of the interferon-stimulated response element (ISRE). Furthermore, the expression level of ISGs was also decreased in the cured Huh7 cells in which replicon had been eliminated (cHuh7), indicating adaptation of the cells to support HCV replication by downregulating ISGs. On the other hand, expression of HCV replicon was significantly suppressed by overexpression of several ISGs including PKR, MxA, IRF-9, GBP-1, IFI-6-16, IFI-27, 25OAS and IRF-1. Knock down of GBP-1, IFI-6-16 and IFI-27 by short hairpin RNA resulted in increase of HCV replication. Thus, we conclude that downregulation of ISG expression is required in the host cells supporting HCV replication and that several ISGs directly suppress HCV replication. The search for ISGs that regulate HCV replication may help to elucidate the cellular antiviral defence mechanisms against HCV infection.

Keywords: guanylate binding protein-1, hepatitis C virus, interferon-induced protein 6–16, interferon-inducible protein-27, interferon-stimulated gene, replicon.

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Abbreviations: HCV, Hepatitis C virus; IFN, interferon-alpha; ISG, interferon-stimulated gene; 25OAS, 2′, 5′-oligoadenylate synthetase; MxA, myxovirus resistance 1; PKR, double-stranded RNA-dependent protein kinase R; IFI-56K, interferon-induced protein 56; IRF, interferon regulatory factor; GBP-1, guanylate binding protein-1; IFI-6-16, interferon-induced protein 6–16; IFI-27, interferon-inducible protein 27; ISGF-3, interferon-stimulated gene factor-3; TAP1, transporter ATP-binding cassette, major histocompatibility complex 1; IFP35, interferon inducible protein 35kD; PLSCR1, phospholipid scramblase 1; LMP7-E1, major histocompatibility complex encoded proteasome subunit LMP7-E1; eIF2-alpha, eukaryotic initiation factor-2 alpha; eIF3, eukaryotic initiation factor-3; STAT, signal transducer and activator of transcription; NS5A, nonstructural protein 5A; Fluc, firefly luciferase; ISRE, interferon-stimulated response element; GAS, interferon-gamma activation site; AP1, activator protein 1; NF-kappa B, nuclear factor-kappa B; Rluc, renilla luciferase; shRNA, short hairpin RNA; IRES, internal ribosome entry site.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality [1,2]. HCV is characterized by persistent infection in the liver that leads to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses not only as therapeutic applications [3] but also as natural cellular antiviral defence mechanisms [4,5]. DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of the interferon-stimulated genes (ISGs), including cytokines and chemokines, was the principal reaction during the course of the viral infection and its clearance and that a considerable proportion of the genes were inducible by IFNs [6].

Interferons are naturally produced in response to virus infection, and to cellular exposure to IFN itself [7]. The expressional control of the ISGs is directed by receptor-mediated stimuli of type-I IFNs [8]. Binding of the IFNs onto their receptors activate receptor associated janus kinases, which phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STATs 1 and 2 recruit IFN regulatory factor-9 (IRF-9) to form a