Hepatitis C virus (HCV), an enveloped positive-stranded RNA virus of the family Flaviviridae, is the major cause of post-transfusion hepatitis. HCV infection often leads to liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Saito et al., 1990). It is estimated that approximately 170 million people are infected with HCV worldwide (Zeuzem, 2000). The HCV genome is approximately 9600 nt in length (Robertson, 1998). The nucleotide sequences of genomes from HCV isolated from different parts of the world vary considerably and are quite heterogeneous. Six major genotypes of HCV have been described around the world, and show 30–50% variation in their nucleotide sequences. The sequence variability suggests that the HCV genome mutates frequently during replication and circulates in the serum as a population of quasispecies. The relative worldwide distribution of HCV genotypes varies considerably. For example, genotype 1a is common in the United States and Northern Europe. Genotypes 1b, 2a and 2b have a worldwide distribution. Genotype 3 is most frequent in the Indian subcontinent. Genotype 4 is the most common genotype in Africa and the Middle East. Genotype 5 is found in South Africa. Genotype 6 is found in Hong Kong and Southeast Asia (Simmonds et al., 1996). In the United States 75% of chronic hepatitis C cases belong to genotypes 1a and 1b, 13–15% to genotypes 2a and 2b, and 6–7% to genotype 3a (Mahoney et al., 1994; Lau et al., 1996; Alter et al., 1999). Alpha interferon (IFN-α), along with ribavirin, has been widely used as a standard treatment for chronic HCV infection all over the world. Interestingly, some clinical studies indicate that response to IFN therapy is related to the genotype of HCV. For example, patients infected with genotypes 2–6 can be efficiently treated with this regimen and the virus is cleared in more than 85% of cases. Patients with genotype 1 can have very good initial response followed by a very good second slope of viral clearance. This is not as frequent as with genotypes 2 or 3. However, this therapy is not very effective against HCV genotype 1, with only 50% response (Pawlotsky, 2000).

The genomic organization of all HCV strains is similar. All have a 5′ untranslated region (UTR), a large open reading frame of genomes from HCV isolated from different parts of the world vary considerably and are quite heterogeneous. Six major genotypes of HCV have been described around the world, and show 30–50% variation in their nucleotide sequences. The sequence variability suggests that the HCV genome mutates frequently during replication and circulates in the serum as a population of quasispecies. The relative worldwide distribution of HCV genotypes varies considerably. For example, genotype 1a is common in the United States and Northern Europe. Genotypes 1b, 2a and 2b have a worldwide distribution. Genotype 3 is most frequent in the Indian subcontinent. Genotype 4 is the most common genotype in Africa and the Middle East. Genotype 5 is found in South Africa. Genotype 6 is found in Hong Kong and Southeast Asia (Simmonds et al., 1996). In the United States 75% of chronic hepatitis C cases belong to genotypes 1a and 1b, 13–15% to genotypes 2a and 2b, and 6–7% to genotype 3a (Mahoney et al., 1994; Lau et al., 1996; Alter et al., 1999). Alpha interferon (IFN-α), along with ribavirin, has been widely used as a standard treatment for chronic HCV infection all over the world. Interestingly, some clinical studies indicate that response to IFN therapy is related to the genotype of HCV. For example, patients infected with genotypes 2–6 can be efficiently treated with this regimen and the virus is cleared in more than 85% of cases. Patients with genotype 1 can have very good initial response followed by a very good second slope of viral clearance. This is not as frequent as with genotypes 2 or 3. However, this therapy is not very effective against HCV genotype 1, with only 50% response (Pawlotsky, 2000).

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frame (ORF) and a 3’UTR. The sequences present in the 5’UTR region form a highly ordered structure that can attach to host cell ribosomes and initiate translation by an internal ribosome entry site (IRES)-mediated mechanism. This type of cap-independent mechanism is known to be operative in certain other RNA viruses including picornaviruses (Collier et al., 1998; Saiz et al., 1999; Castet et al., 2002), and differs in several aspects from cap-dependent translation (Kozak, 2003). According to the reports of several different laboratories nt 40–370 of the HCV RNA genome are important for the IRES-mediated translation (Tang et al., 1999; Ali et al., 2000; Dasgupta et al., 2004). Therefore, sequence variability of the 5’UTR has important implications for the structural organization and function of the IRES element (Saiz et al., 1999). The RNA genome binds to the host cell ribosome and is translated to yield a large polyprotein of 3010 aa. This polyprotein is then cleaved intracellularly by the combined action of cellular and virally encoded proteases. Ten different mature proteins are generated. The structural proteins, core, E1 and E2 are required for virus assembly, export and binding to cellular receptors for infection. The non-structural proteins encode enzymes required for replication of the viral genome. Following the stop codon, the viral genome ends with sequences of variable length among different HCV genotypes, with a highly conserved 98 nt segment at the very terminus of the 3’UTR. These sequences are required for the initiation of ant igenomic-strand synthesis (Reed & Rice, 2000). We have reported previously that IFN-γ, IFN-β and IFN-α each target the highly conserved 5’UTR of the HCV genome, utilized by the virus to translate protein by an IRES-dependent mechanism (Dash et al., 2005).

We conducted this study to determine if quantitative differences in the IFN action of inhibiting IRES-mediated translation among different HCV genotypes could explain the viruses differential responses to treatment. We used standard polymerase chain reaction (PCR) and cloning methods to construct chimeric clones between the sequence encoding the green fluorescence protein (GFP) and HCV IRES sequences of different genotypes. High-level expression of GFP from different IRES clones was accomplished in Huh-7 cells using a two-step-transfection procedure. This method allows us to examine cells expressing GFP directly under a fluorescent microscope, without the requirement for immunological detection procedures. To examine the effect of IFN treatment on cap-dependent translation, Huh-7 cells were co-transfected with 1 μg IRES-GFP plasmid DNA along with 1 μg pDsRed2 red fluorescence protein (RFP) plasmid (BD Biosciences Clontech) using the FuGENE 6 (Roche Molecular Biology) transfection reagent. The effect of IFN on translation was determined at 24 h by examining the expression of GFP or RFP under a fluorescence microscope (Olympus), at 484 nm for the expression of GFP, 563 nm for the expression of RFP and 340 nm for DAPI. The percentage of GFP-positive Huh-7 cells was quantitatively measured using CELL QUEST computer software. As a control, we examined the specificity of IFN action by investigating IRES-mediated GFP translation in the presence of other cytokines [interleukin 1 (IL1), IL6, tumour necrosis factor alpha (TNF-α) and transforming growth factor beta 1 (TGF-β1)]. The stability of IRES-GFP mRNAs in the transfected-cell cultures was compared in the presence and absence of IFN treatment. Total RNA was isolated from IFN-treated cells and the levels of IRES-GFP mRNA were measured by a ribonuclease protection assay (RPA) using a probe specific for the GFP gene.

The IRES clones used in this study included nt 18–356 of HCV 5’UTR (Collier et al., 1998). Each clone contained the stem–loop II–IV domains, because these sequences are necessary for efficient IRES-mediated translation. These sequences were selected based on reports, which suggested that the first stem–loop structure (1–18) is not necessary for translation, and that the core coding sequences can modulate the IRES-activity of HCV (Tsukiyama-Kohara et al., 1992; Reynolds et al., 1996; Wang et al., 2000). The IRES nucleotide sequences from HCV of six different HCV genotypes (1–6) and two subtypes (1a, 1b, 2a and 2b) are shown in Fig. 1(a). The IRES sequences of HCV fold into stem–loop structures (II–IV) (Fig. 1b). As we previously demonstrated (Qi et al., 2003), any alteration in the primary or secondary structures in the stem–loop regions of the IRES severely affects translation efficiencies. Stem–loop II encompasses nt 43–120, stem–loop III encompasses nt 134–300 and stem–loop IV encompasses nt 300–354. Stem–loop structures IIA and IIB have 7 nt that display differential variations, 28 nt display variations in stem–loop structure II–IV and 5 nt display variations in stem–loop structure IV (Fig. 1b). The sequences of HCV 1a and HCV 1b are highly conserved in this region as compared to other genotypes. Thus, the majority of sequence differences in the IRES region among different HCV genotypes are in stem–loop III. We examined whether the nucleotide sequence differences in the stem–loop II–IV domains of the HCV IRES affect the expression of GFP in Huh-7 cells or not. We achieved

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**Fig. 1.** (a) Sequence variation of the 5’UTR in different HCV genotypes and subtypes. Alignment of the HCV 5’UTR sequences used in this study. The sequences are 18–356 nt. The consensus sequence shows the residues that are conserved in all eight sequences used in this study and the predicted stem–loop structures are indicated. (b) Predicted secondary structure of the HCV 5’UTR. The sequence shown is that of genotype 1a 5’UTR, HCV-H (Inchauspe et al., 1991), and the structure is based on those proposed by Brown et al. (1992), Honda et al. (1996) and Komishita et al. (1997). Stem–loop structures are labelled for reference. The chimeric clones were made by fusing the GFP-encoding sequence, including a poly(A) tail, after the CCU sequence of the 5’UTR by overlapping PCR.
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high-level expression of GFP from different IRES clones in Huh-7 cells using a replication-defective adenovirus that expresses T7 RNA polymerase. These high levels of GFP expression from different IRES constructs were comparable, indicating that all the IRES sequences are equally efficient in mediating the translation of GFP, even though they have

**Fig. 2.** IFN-α2b-inhibited translation of GFP from the IRES sequence of eight different HCV chimeric clones. Huh-7 cells were seeded in 12-well plates. On the following day, cells were incubated with 1ml D-MEM, 2% fetal bovine serum, 2 μl replication-defective adenovirus expressing T7 RNA polymerase for 2 h, and then co-transfected with 1 μg HCV-GFP chimeras and 1 μg pDsRed2 plasmid using the FuGENE 6 transfection reagent. Immediately after DNA transfection, cells were treated with different concentrations of IFN diluted in serum-free media. IFN-α2b-inhibited the expression of GFP fusion proteins at between 1–1000 IU IFN ml⁻¹. IFN-α2b had no effect on the expression of the RFP clone (pDsRed2-N1). Photographs were taken at ×20 magnification at 24 h after IFN treatment.
minor nucleotide sequence variations in the stem–loop II–IV regions.

The direct effect of IFN treatment on cap-dependent and cap-independent translation was assessed by co-transfecting two plasmid constructs, one expressing GFP by an IRES-dependent mechanism and the other expressing RFP by a non-IRES-dependent mechanism. Expression of RFP and GFP was examined in the presence and absence of IFN-

no-IRES-dependent mechanism. Expression of RFP and GFP was examined in the presence and absence of IFN-

a2b treatment using eight different IRES clones from HCV. The results of these experiments indicate that IFN inhibits expression of GFP in a dose-dependent manner (Fig. 2). All the IRES clones were found to be sensitive to IFN-a2b. We also determined that IFN treatment did not alter the expression of RFP, suggesting that there was no effect on cap-dependent translation. We then quantified differences in the level of GFP-positive cells after IFN treatment by flow cytometric analysis (see Supplementary Fig. S1 available in JGV Online). These results showed that IFN treatment inhibited GFP expression from all the IRES sequences with equal efficiency. IFN treatment did not alter the percentage of cells expressing RFP in Huh-7 cells. This suggests that IFN-a inhibits GFP expression in all IRES clones in a dose-dependent manner. Viral infection induces a robust production and secretion of IFN and many inflammatory cytokines that are important in generation of antiviral response. Some of these cytokines protect cells from virus infection, as well as mediating both the adaptive and the innate immune response. The IFN-induced JAK-STAT signal transduction pathway in a cell is also activated by some of these cytokines. We determined that IL1, IL6, TNF-a and TGF-b1 did not inhibit expression of GFP at a concentration of 2 μg ml^{-1}. In fact, none of the cytokines inhibited expression of RFP or GFP in Huh-7 cells (see Supplementary Table S1 available in JGV Online). We then examined whether IFN treatment could have degraded IRES messages, which could have abolished GFP translation. The results of these experiments indicated that there were no significant differences in the levels of IRES-GFP mRNA among HCVs of different genotypes. The antiviral effect of IFN on RNA stability and translation of GFP are presented in Fig. 3, which indicates that IFN-a preferentially blocks protein translation without altering the stability of IRES-GFP mRNA.

IFN-a has been the standard therapy for chronic HCV infection. It has been observed that patients infected with certain genotypes of the virus respond less often than others do (Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004). The mechanism for the differential antiviral action of IFN against HCV is not fully understood. We reported that IFN-a inhibits replication of the HCV replicon as well as the full-length HCV clone by blocking at the level of IRES-mediated translation; other laboratories have confirmed this specific effect of IFN on IRES-mediated translation, but the effect has been related only to the HCV1a and 1b genotypes (Dash et al., 2005; Prabhu et al., 2004; Kato et al., 2002; Shimazaki et al., 2002). In our study, we demonstrated that IFN-a2b treatment directly inhibited translation of GFP mediated by the IRES from all genotypes in a concentration-dependent manner. We also showed that IFN-a2b did not inhibit cap-dependent expression of RFP (from pDsRed2) in the transfected Huh-7 cells. Inhibition of GFP expression from the IRES clones by IFN is not due to a direct effect on the expression of adenovirus T7 polymerase. We determined that efficient expression of GFP was achieved from the IRES sequence of HCVs of six different genotypes even though there is some variation in their nucleotide sequences in the stem–loop II–IV region of the IRES structure. As a control, we examined the effect of IRES-mediated translation by TNF-a and IL1 and IL6. Some of these cytokine levels are increased during viral infection (Daniels et al., 1990; Tilg et al., 1992; Dumoulin et al., 1999). IL1 is one of the most prominent cytokines and its level increased during
inflammation. It has been documented as having a role in viral clearance and the host immune response. Signalling pathways activated by IL1 involve the activation of IRAK-α and IRAK-2 kinases (Cao et al., 1996). This signalling step involves the mitogen-activated protein kinase pathways and NF-κB activation. In this study, we examined whether or not activation of this pathway blocks the IRES-mediated translation of GFP in Huh-7 cells. We found that IRES-mediated translation of GFP is not sensitive to IL1 or IL6 (see Supplementary Table S1 in JGV Online). It has been shown that liver-infiltrating T lymphocytes and hepatocytes produce TNF-α during chronic HCV infection. TNF-α levels are elevated in the serum of patients with chronic HCV infection. TNF-α has been shown to have an antiviral effect against certain viruses including encephalomyocarditis virus, hepatitis B virus, vesicular stomatitis virus, adenovirus and herpes simplex virus. TNF-α binds to its receptor on the cell surface and activates several major transcription factors including NF-κB and AP-1 (Liu et al., 2001). Our studies suggest that TNF-α treatment has no effect on HCV IRES-dependent translation (see Supplementary Table S1 in JGV Online). These experiments lead us to the conclusion that the IRES-mediated GFP translation was not inhibited by the signal transduction pathways activated by these pro-inflammatory cytokines in a human-liver-derived cell line.

IFN-α induces the transcriptional activation of the 2’5′ (A) synthetase, which polymerizes ATP into 2’5′-linked oligoadenylates of variable lengths. This enzyme can activate a latent ribonuclease, Rnase L, present in all animal cells. The activated RNase L can then cleave single-stranded RNAs. We examined the stability of IRES-GFP messages after IFN treatment using an RPA, and found that the levels of IRES-GFP mRNA did not significantly differ in cells with 1–1000 IU IFN ml–1 or without IFN treatment (see Supplementary Fig. S2 in JGV Online). This excludes the possibility that inhibition of GFP expression from the IRES clones is due to extensive degradation of IRES-GFP mRNA. However, our study does not exclude the involvement of RNase L pathways induced by IFN and their role in IFN therapy. We also cannot rule out the possibility that some of the IRES mRNA could have undergone partial hydrolysis by RNase L pathways. A study performed by David Barton’s group (Han & Barton, 2002) indicated that certain HCV genotypes are more sensitive to RNase degradation than others. In their analysis, HCV genotypes 2a, 2b, 3a and 3b were more sensitive to RNase-mediated degradation than HCV 1a and 1b. The RNA of genotypes 2a, 2b, 3a, 3b was degraded to fragments of 200–1000 bases in length. We did not observe this preferential degradation in our investigation using IRES clones. Taken together, results from this study suggest that IFN treatment inhibits translation of all IRES mRNA without RNA degradation. Studies are underway to investigate alternative pathways that block translation without degradation of IRES mRNA in IFN-treated cells. We used Huh-7 cells as a model cell line to understand the mechanisms of IFN action against HCV. This cell line efficiently supports HCV replication, and numerous investigators have used this cell line to study the antiviral effects of IFN. It is important that future studies should be performed to confirm these observations using other human hepatic cell lines, including primary human hepatocytes.

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