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The 5'UTR of the hepatitis C virus: structural, functional studies and clinical significance

Thesis Submitted for the Degree of
Doctor Philosophiae

Candidate:
Martina Gerotto

Supervisor:
Prof. Francisco E. Baralle

Academic Year 1995/1996

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ABSTRACT

The hepatitis C virus (HCV), the causal agent of 90% of the cases of parenteral hepatitis non-A, non-B, was cloned in 1988 from complementary DNA (cDNA) derived from the RNA of infectious schimpanzee plasma. HCV is a positive-stranded RNA virus which has been recently classified as a separeted genus of the *Flaviviridae*. The genome consists of one large open-reading frame of about 9400 nucleotides encoding for a polyprotein precursor of 3010 to 3033 amino acids. Several HCV isolates have now been cloned, and their sequence analysis revealed the existence of several HCV variants. Although HCV shows substantial nucleotide sequence variation throughout the viral genome, this sequence heterogeneity is not evenly distributed over the genome but differs between the regions, the 5' untranslated region being the most conserved region. Therefore, the 5'UTR has been the region of choice for the standardisation of a RT-PCR procedure to detect HCV RNA directly from the serum samples of the different populations of patients analysed in several studies by our group.

According to a recently proposed classification, there are six HCV genotypes which have been shown to have different geographical distribution. Distinction of HCV genotypes is important, since the activity and prognosis of HCV-related liver disease may be correlated to the infecting genotype, as well as the response to antiviral therapy.

At the time of our first study, reported here, data about the HCV epidemiology in Italy were not yet available . Thus, in the first part of this thesis, are reported the results of the analysis of the HCV genotypes infecting the Italian patients and their epidemiology in the different regions of Italy. The HCV genotype determination in large series of patients has been possible by the utilization of a rapid and sensitive dot blot hybridization assay with genotype-specific probes developed in our laboratory.

In line with the emerging relevance of the genotype determination in the HCV infection, we analyzed a series of well characterized groups of HCV infected patients in order to verify whether a relationship exists between the viral genotype and the clinical aspect of the disease. Interestingly, our results indicate that the HCV genotype can be considered as a predictive factor for the response to the antiviral treatment with alpha-interferon, since

factor for the response to the antiviral treatment with alpha-interferon, since patients infected with HCV genotype 2 or 3 respond better then those infected with genotype 1. Further studies in this direction strongly supported the idea that sequence variability between different HCV genotypes may influence the biological behaviour of the virus.
In this content, the second part of this thesis represents attempts to explain the characteristics of the virus infection *in vivo* and the relative pathogenicity of the different HCV strains in terms of the observed sequence variability in the 5' untranslated region (UTR) of the genomic RNA. The complex structural feature of the 5'UTR, in fact, provides the regulatory determinants for the internal initiation of translation of the viral mRNA. The possibility that this fundamental viral function can be influenced by the primary sequence, as well as by the secondary structure of the 5'UTR, is indicated by the results of our *in vitro* studies regarding the ability of different 5'UTRs to drive translation initiation of a reporter gene. The 5'UTrs of the three major HCV genotypes, two naturally occurring variants and artificially engineered mutants were utilised in this study.

Part of the results presented in this thesis have already been published in the following papers:

Genotypes of hepatitis C virus in Italian patients with chronic hepatitis C.
Tisminetzky S, Gerotto M, Pontisso P, Chemello L, Ruvoletto MG, Baralle F & Alberti A. *Int. Hepatol. Commun.* 2 (1994). 105-112

Analysis of the hepatitis C genome in patients with anti-LKM-1 autoantibodies.
Gerotto M, Pontisso P, Giostra F, Francesconi R, Muratori L, Ballardini G, Lenzi M, Tisminetzky S, Bianchi F B, Baralle F B and Alberti A. *Journal of Hepatology* 1994; 21: 273-276

Outcome of acute hepatitis C and role of alpha interferon therapy.
Alberti A, Chemello L, Belussi F, Pontisso P, Tisminetzky S, Gerotto M, Baralle F, and Simmonds P. *Viral Hepatitis and Liver Disease.* Proceedings of the Intenational Symposium on Viral Hepatitis and Liver Disease (1994). pp 604-606

Distribution of three major HCV genotypes in Italy.
A multicentric study of 495 patients with chronic hepatitis type C.
P.Pontisso, MG.Ruvoletto, M.Nicoletti, S.Tisminetzky, M.Gerotto, M.Levrero,M. Artini, M.Baldi, G.Ballardini, L.Barbara, P.Bonetti, S.Brillanti, C.Casarin, L.Chemello, A.Costanzo, MS.Demitri, C.Donada, MF.Felaco, M.Frezza, GB.Gaeta, F.Giostra, T.Iervese, O.Lo Iacono, M.Milella, L.Monno, F.Negro, F.Piccino, G.Russo, A.Vaccaro, F.Baralle and A.Alberti. *Journal of Viral Hepatitis.* 1995; 2,33-38

HCV-1a vs HCV-1b: the clinical point of view
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Comparison of genotyping and serotyping methods for the identification of hepatitis C virus types.
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Hepatitis C Genotypes in Patients with Dual Hepatitis B and C Virus Infection.
P. Pontisso, M. Gerotto, MG. Ruvoletto, G. Fattovich, S. Tisminetzky, F. Baralle and A. Alberti. *Juornal of Medical Virology* 48:157-160 (1996)

Discordant results of hepatitis C virus genotyping by two methodologies based on amplification of different genomic regions.
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CHAPTER 1

INTRODUCTION

1. 1. History

Viral hepatitis, as a disease entity, has been recognised for several millennia (Hollinger FB,1991). The first reference to jaundice as a symptom of the disease comes from the ancient Chinese literature. In the 17th, 18th, and 19th centuries reports of scattered outbreaks of jaundice affecting diverse populations were reordered more frequently. The disease was especially common among the military and was called "campaign jaundice".

The existence of a parenterally transmitted form of hepatitis was initially documented by Lurman in 1885. He reported the development of jaundice in a group of shipyard workers a few months after they had received smallpox vaccine prepared from human serum.

In 1912, Cockayne used for the first time the term "infectious hepatitis" to describe the epidemic form of the disease. Contemporary, McDonald suggested a viral aetiology of epidemic catarrhal jaundice.

In 1923, Blumer established the existence of one of the major forms of viral hepatitis now known as viral hepatitis type A.

Transmission of hepatitis by parenteral inoculation of human serum was firmly established in the late 1930s. At that time, Findlay and MacCallum described cases of jaundice occurring after inoculation of volunteers with a yellow fever vaccine prepared with the addition of human serum to stabilized the product. Fox and coworkers subsequently implicated human serum as the vehicle of transmission.

The differentiation of hepatitis A, then called infectious hepatitis, from hepatitis B, then called serum hepatitis, came principally from the studies of Murray and Krugman between the late 1950s and the 1970s. They demonstrated that the hepatitis A agent was transmitted primarily by the fecal-oral route while, the hepatitis B agent was transmitted

primarily by the parenteral route. The two agents were subsequently identified as HAV and HBV by transmission experiments in chimpanzees.

In the 1970s, with the development of sensitive and specific immunoassays for HAV and HBV, the non-B post transfusion hepatitis started to be elucidated.

The first evidence for the existence of the causative agent of one of these hepatitis, later designated as hepatitis D, came in 1978 by Rizzetto and colleagues. A previously unrecognised intracellular antigen was initially detected by immunofluorescence in liver biopsies from Italian patients with chronic HBV infection (Rizzetto,1985). The "delta" antigen was shown to be associated with the capsid protein of a defective virus, subsequently called the hepatitis D virus (HDV), requiring a helper function from HBV. The transmissible nature of HDV was established in 1980 by introduction of the virus to HBV-infected chimpanzees.

Although the epidemiological studies were suggesting the existence of a different form of fecal-oral transmitted hepatitis other that the hepatitis A, the peculiar nature of a second virus, the hepatitis E virus (HEV), has been confirmed only in 1983 after transmission experiments in non human primates. The first reported cases of this new form of hepatitis were probably during the massive epidemic that occurred in India in 1955-1956.

Initially described as a typical water-borne hepatitis A, a retrospective study confirmed the NANBH aetiology for the epidemic. Since the HEV could not be grown in cell culture, only a small amounts of virus could be recoverable from infected patients and experimentally infected animals, progress in the study of this virus came later with the utilisation of recombinant DNA technology (Reyes GR,1990).

The use of the diagnostic tests for hepatitis A and B in epidemiological studies on transfusion associated hepatitis, revealed that most of the post-transfusional hepatitis were not related either to HAV or toHBV.

NANBH was successfully transmitted from human sera to chimpanzees in 1978, but the identification of its main causative agent came only in 1989 when a new virus, called hepatitis C virus (HCV) was isolated and its genome was cloned and sequenced (Choo Q,1989).

At present, although sensitive and specific methods are available for the diagnosis of all the five known forms of hepatitis (from A

to E) 10 to 20 % of the acute and chronic hepatitis are still cryptogenic since it is not possible to attribute these hepatitis to any know specific virus or to metabolic and genetic conditions.

Finally, in 1995, the term "hepatitis X agent", previously coined to indicate the causative agent of cryptic hepatitis, was substituted by the hepatitis G virus (Simmons 1995, Linnen 1996). In fact, at that time, a new virus was isolated from the plasma of a patient with chronic hepatitis who was coinfected with the hepatitis C virus. From the sequence analysis of the HGV genome, it appeared that this virus, like the HCV, probably belongs to a new group of the family of Flaviviridae. Extensive epidemiological studies have still to be done to define if HGV is a true hepatitis virus and its clinical significance.

1. 2. The hepatitis C virus discovery

The term "non-A non-B hepatitis" (NANBH) was coined approximately 15 years ago to describe forms of viral hepatitis which were serologically non reactive to the then newly available tests for hepatitis virus A, B, and D. The first suggestion of the infectious nature of the NANB hepatitis came in 1979 when, for the first time, it was shown that the transmission of NANBH to chimpanzees occurred after i.v. administration of various human inocula (Prince 1974).

However, the lack of important experimental tools like specific viral antigens, antibodies, viral-like particles and tissue culture systems, represented the major impediment to characterise the causative agent. Only ten years later researchers of the Chiron Corporation (California) succeeded in the identification of NANBH specific nucleic acid using recombinant DNA cloning technologies (Choo 1989).

Large volumes of serum were collected from chimpanzees with relatively high infectious titer. Nucleic acids extracted from a crude viral pellet were used to generate a phage cDNA library that was screened with sera from patients diagnosed clinically with postransfusional NANBH. Screening 10^6 of the resulting recombinant phages led to the identification of a single positive cDNA clone. This clone was used as a probe in hybridisation experiments which demonstrated that its cDNA insert derived from an exogenous RNA molecule associated to NANBH infection and not from the host genome. In fact, the cDNA hybridised to RNA from infected

chimpanzee's liver and serum but not with DNA from the liver of the same infected animal and also not with the RNA extracted from the liver of uninfected chimpanzees.

Furthermore, the sequence of this clone revealed that this portion of RNA itself was encoding an immunoreactive polypeptide specific for NANBH infection. This particular sequence was cloned further to produce larger amounts of NANB-related protein. Later on, a modified form of this protein became the basis for the currently used enzyme-linked immunosorbent assay (ELISA) to detect circulating antibody against hepatitis C virus (Kuo 1989, Miyamura 1990). The complete sequence of the entire RNA genome of the first HCV isolate was than derived from overlapping clones.

Table 1: Virus taxonomy, genotypes, and serotypes of the human hepatotropic viruses

Item	HAV	HEV	HBV	HCV	HDV	HGV
Family	Picornaviridae	Uncertain *	Hepadnaviridae	Flaviviridae	Deltaviridae	?
Genus	Hepatovirus	Unnamed	Ortohepadnavirus	Hepacivirus	Deltavirus	?
Species	hepatitis A virus	hepatitis E virus	hepatitis B virus	hepatitis C virus	hepatitis D virus	hepatitis G virus
Genotypes	7	3	5	> 9	3	?
serotypes	1	1	1	?	?	?

*Taxonomic placement is difficult; HEV has characteristics of the *Caliciviridae* and *Togaviridae*

Table 2: Properties of the virions of the human hepatotropic viruses

Properties	HAV	HEV	HBV	HCV	HDV	HGV
Nucleic Acid Type	RNA	RNA	DNA	RNA	RNA	RNA
Strandedness	ss	ss	ds	ss	ss	ss
Linear or Circular	Linear	Linear	Circular	Linear	Linear	Linear
Sense	+	+	n.a.	+	−	+
Size of genome	7.5	7.8	3.2	9.4	1.7	7.5
Segmented	No	No	No	No	No	No
Site of Replication	Cytoplasm	Unknown	Nucleous	Cytoplasm ?	Nucleous	Unknown

1. 3. HCV structure and function

Most of the informations concerning the nature of the hepatitis C virus are derived from comparative analysis of the sequence of its genome. The organisation of the genome in itself and the derived virus structure are features that resemble those of *flaviviruses* and *pestiviruses*. For this reason, and because of limited sequence homology, HCV has been classified as a new genus (*hepaCvirus*) of the family in the *Flaviviridae*. The hepatitis C virion is small and enveloped, and it has a single-stranded, positive-sense RNA genome of about 9.5 kb. The genome contains a single, large open reading frame (ORF) which encodes for a viral polyprotein precursor of approximately 3010 amino acids. This ORF is flanked by 341 nucleotides that constitute the 5' end non translated region (UTR), and a 3' UTR of approximately 120 nt.

1. 3. 1. Genes and Proteins

Because no appropriate cell culture system has yet been developed to support the replication of hepatitis C virus, the identification and the functional analysis of individual HCV proteins has been possible only by the expression of HCV genes in a variety of *in vitro* and *in vivo* cDNA expression systems.

Similarly to the related *flavivirus* and *pestivirus*, the proteins of HCV are produced by cleavage of a polyprotein precursor by both host- and viral-encoded protease (see figure 1). The genetic order of the proteins on the HCV polyprotein precursor has been defined. The putative structural proteins, the nucleocapsid protein C (core) and the two enveloped glycosylated proteins E1, E2, are contained within the N-terminal quarter of the polyprotein in the order NH2-C-E1-E2. Currently, it appears that the these three proteins are processed co- and post-translationally by cleavage from the N-terminal region of the polyprotein at least in part by cellular signal peptidases.

1) *The "core" protein.* The nucleocapsid nature of the core protein, initially suggested by its highly basic constitution, has only been recently confirmed in immunoelectron microscopy studies of detergent-stripped virions. Recent studies revealed that two co-translational cleavages, catalysed in the endoplasmatic reticulum (ER) by lumen host

signal peptidases, are required for the release of the mature core protein from the viral polypeptide. In this way, with the contemporary release of an hydrophobic segment (H1 domain), the C-terminus of the core and the N-terminus of the E1 are generated.

It has been shown that the C-terminal hydrophobic region is responsible for the association of HCV core protein with the ER membrane (Landford 1993, Santolini 1994), and that the full-length core protein remains in the cytoplasm, whereas a truncated form of the core, devoid of the C-terminal hydrophobic domain, is translocated into the nucleus (Suzuki1995,Lo1995).

Recently, it has been suggested that, in addition to its nucleic acid binding activity, the core protein also partecipate in RNA packaging and virus assembly (Santolini 1994). Interestingly, Shin et al., have also shown that in HCV the phosphorylated form of the core protein can suppress *in vitro* replication and gene expression of the hepatitis B virus (HBV) (Shin 1993). Overall, these data indicate that the HCV core is a multifunctional protein.

2) *The glycoproteins E1 and E2.* The two putative envelope proteins gp31 (E1) and gp70 (E2) are transported to the lumen of the ER where these proteins appear to be anchored to the membrane. This is also supported by the presence of the two hydrophobic sequences H1 and H2 (respectively located upstream domain E1 and E2), which probably act as signal sequences to direct the integration of gp 31 and gp70 into the membrane of the ER.

Based on the genetic alignment with the *flaviviruses* and *pestiviruses*, it is currently assumed that all the proteins downstream the putative structural region encoding C, E1 and E2, represent non structural (NS) proteins.

3) *Proteolytic cleavage of the polyprotein.* The processing of the entire non structural portion of the polyprotein precursor appears to require the combined action of two viral encoded proteases. The first protease has been identified as the polypeptide product of the region spanning from the NS2 domain and the N-terminus of domain NS3. The NS2/NS3 is a autoproteinase responsible for cleavage at the NS2/NS3 site to generate the N-terminus of the NS3 protein (Grakoui 1993).

The second viral protease is encoded by the NS3 domain. As shown by different groups, this is a trypsin-like serine protease and it is

considered responsible of the cleavage at the NS3/NS4A, NS4A/4B, NS4B/NS5A, and NS5A/5B junctions. Residues His-1083, Asp-1107, and Ser-1155, numbered according to their location within the HCV polypeptide, are highly conserved among all the HCV strains sequenced so far. These residues were predicted to correspond spatially to the catalytic triad of the putative serine protease (Bartenshlager 1993, Tomei 1993, D'Souza 1995). Recently it has been shown, both by *in vitro* and *in vivo* analysis, that substitution of the Ser-1155 in the putative catalytic triad with an alanine inhibits the release of mature proteins NS3, NS4A, NS4B, NS5A, and NS5B. This observation implies that the NS3 protein is responsible for liberating itself from polypeptide precursor and catalysing cleavage reaction to release NS4 and NS5. Furthermore, the NS3 protein contains a nucleoside triphosphate-binding helicase domain that is presumably involved in unwinding of the RNA genome (Suzich 1993).

The NS4 polypeptide, located downstream the C-terminus of NS3, is processed in the two relatively hydrophobic proteins NS4A and NS4B. Their precise function is still to be defined. However, it has been shown that the C-terminus of NS4A interacts with NS2 and NS3 proteins for the processing of the presumed non structural portion of the polyprotein (Tomei 1995, Bertenschlager 1995). Furthermore, Shimotonho in a very recent study, suggested that NS4A might be directly involved in the phosphorylation of the NS5A protein. The analysis of several deletion mutants of the NS5A revealed the presence of an important region for NS4A-dependent phosphorylation in NS5A protein (Shimotonho et al. 1996).

3) *The RNA-dependent RNA polymerase*. The NS5 region, located in the C-terminal extremity of the polyprotein, contains the sequence encoding for the RNA-dependent RNA polymerase (RdRp). Similarly to *pestiviruses*, the NS5 is cleaved in two portions, NS5A and NS5B. On the basis of the presence of the characteristic sequence Gly-Asp-Asp (residues 2737 to 2739) and surrounding conserved motifs present in other polymerases, the NS5B was predicted to contain the RNA dependent RNA polymerase activity. This hypothesis seems to be confirmed from the results of a very recent study where it has been shown that NS5B alone is responsible for *in vitro* de novo synthesis of RNA (Behrens 1996).

The cloning of the hepatitis C virus was a molecular biology breakthrough where classical virology had failed. Indeed, the virus itself

has not yet been isolated and only few reports on its indirect visualisation by electron-microscopy (E.M.) are available. The rapid development of the antibody test to hepatitis C makes it the first such test to have been developed without the virus having been previously isolated or cultured.

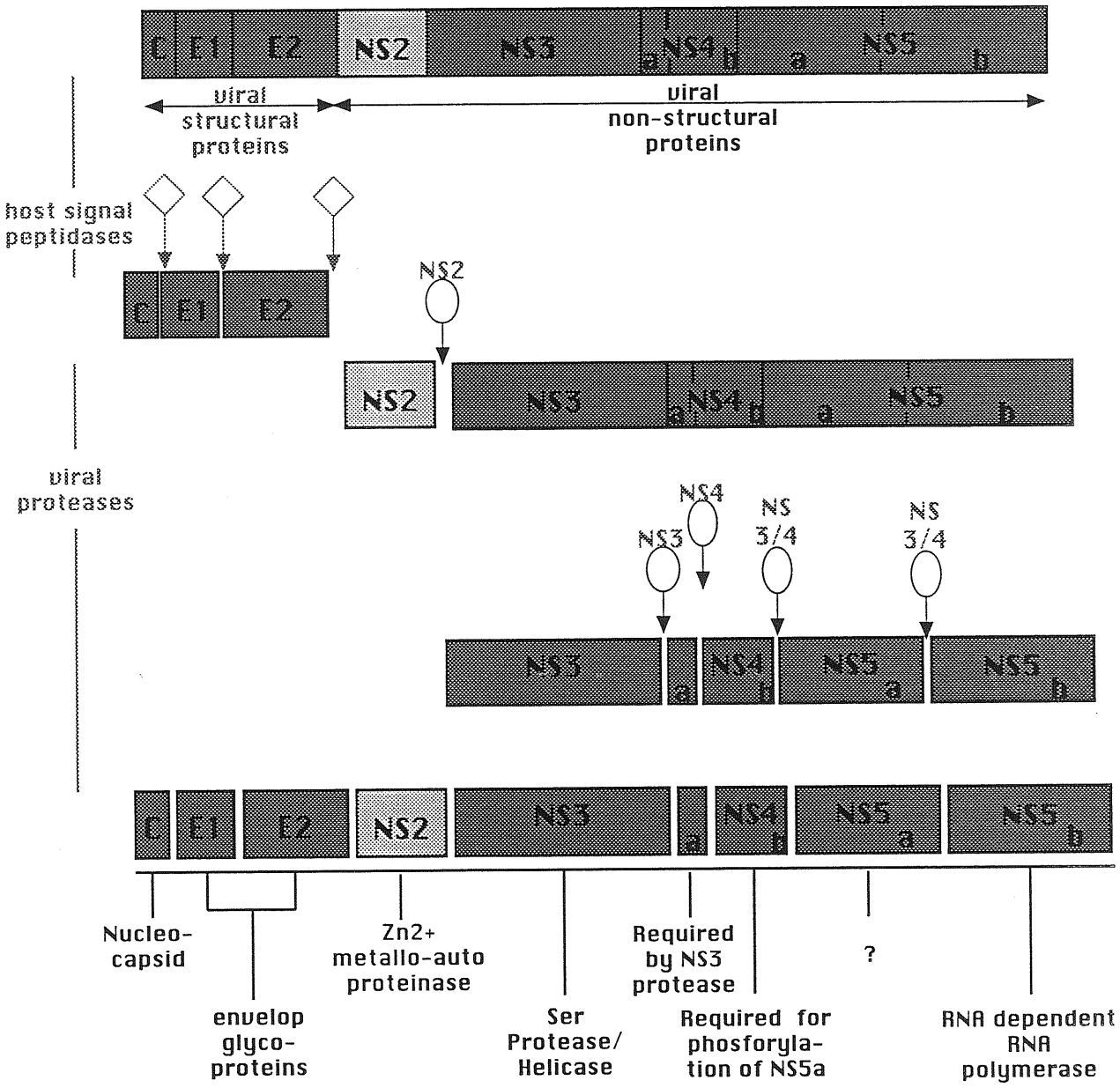


Figure 1: Schematic representation of the polyprotein encoded by the HCV genome and its putative processing scheme

1. 4. Structure of 3' terminus of the HCV genome

Due to also the little amount of virus normally present in cells of infected tissues and in blood, the isolation of the full-length HCV RNA genome directly from infected samples, has always been a limiting factor especially to obtain the entire sequence of the non translated region at the 3' end of the viral genome (3'UTR).

Since the replication of the HCV positive stranded RNA genome is initiated from the 3' terminus of the molecule, the determination of the exact sequence of its 3'UTR is essential to understand the molecular mechanism of the virus replication.

An important contribution to the identification of the complete sequence of the HCV 3'UTR came from a recent study by Tanaka and co-workers (Tanaka T, 1996). All the previous studies referred the 3'UTR to be a short sequence (from 27 to 66 nucleotides), followed by a presumed 3' terminal homopolymeric region that, in most of the isolates, was corresponding to a polyU sequence.

Thus, Tanaka reported for the first time the existence of a novel 98-nucleotide sequence, designed the "3'X tail", downstream to the polyU tract.

Furthermore, being the 3'X tail highly conserved even between the more phylogenetically distant related HCV strains, the authors, speculated that this sequence, as part of the 3' non translated region, plays an important role in the initiation of RNA replication. This is supported by the recent data from Pierangeli et al., demonstrating that the primary sequence of a stem-loop structure whithin the "Y" domain of the 3'UTR of poliovirus harbours an important signal for template recognition and viral replication (Pierangeli et al., 1995).

Preliminary structural analysis suggests that the 3'X terminus could form a complex stem-loop secondary structure. Although a detailed analysis of the biochemical\structural function of this element has not yet been done, this hypothesis would fit well with the possibility that this sequence is targeted by different viral and cellular cis-acting factors involved in the regulation of viral replication.

1. 5. The hepatitis C virus 5' untranslated region (UTR)

1. 5. 1. Structure and functions.

The 5' terminus of the HCV RNA genome is represented by a noncoding sequence of 341 nucleotides in length, generally indicated as the 5'untranslated region (UTR). The 5'UTR is the most conserved region of the HCV genome (sequence divergence in the 5'UTR of the two more distantly related HCV types is 6% compared with 33% over the whole genome)(Okamoto, 1991).

From the phylogenetic analysis of the 5' UTR sequence, HCV appears to be more closely related to *pestiviruses* than to *flaviviruses*.

It has been shown, for example, that HCV 5'untranslated region shares short segments of conserved primary nucleotide sequence with the corresponding region of the two animal *pestiviruses* responsible for bovine viral diarrhoea (BVDV) and hog colera (HChV) (Han et al., 1991).

Both, HCV and *pestiviruses* have fairly long 5'UTRs which contain several unused AUG codons preceding the authentic initiator codon, whereas flaviviruses have short 5'UTR without any AUG codon (Chambers,1990).

Furthermore, the 5'end of the RNA genome of *flaviviruses* presents a m^7GpppG cap structure and it has been shown that in this case initiation of viral protein translation proceeds, as described for the majority of the eukaryotic mRNA (Kozak, 1992), by ribosomal binding to the 5' cap structure of the genomic RNA and scanning to the AUG codon (Chambers, 1990).

In contrast, in the case of HCV, like in *pestiviruses,* there are no evidences for the presence of a cap structure at the 5' end of the genome. All these characteristics, the lack of a cap structure, the length of the sequence, the presence of different unused AUG triplets, together with a proposed complex secondary structure of the HCV 5'UTR, are features incompatible with the cap-dependent scanning model for an efficient translation initiation.

For this reason, it has been suggested that HCV, like the *pestiviruses*, uses a different mechanism for the initiation of translation of its genome. This hypothesis has been later confirmed by a series of *in vitro* and *in vivo* translation experiments performed using synthetic dicistronic uncapped mRNAs. The results of these studies, combined with extensive

mutational analysis, provided experimental evidences that the hepatitis C virus 5' UTR is directly involved in promoting a cap-independent initiation of translation by internal ribosomal entry.

The cap independent model proposed for HCV RNA translation initiation is common to that previously well described for some naturally uncapped genomic RNAs of *picornaviruses*.

P*icornaviruses* RNAs are translated by an alternate mechanism involving internal entry of ribosome into the 5' untranslated region (R. Perez Bercoff, 1982a,b ; AM Degener 1983; J Pelletier 1988a). This mechanism involves binding of the ribosomes to an RNA sequence that folds into a complex secondary structure know as the internal ribosome entry site (IRES) (Jang, 1989). In this way, the translation initiation occurs independently from the presence or the absence of a cap structure at the 5'end of the messenger RNA (see figure 2).

1. 5. 2. The picorna model

Picornaviruses, especially polioviruses, are considered the best models for the analysis of the regulation of translation initiation of viral genomic mRNAs mediated by the 5'UTR through a cap-independent mechanism. In fact, most of our knowledge about the structure of specific elements within the 5'UTR and their role in the IRES function, derives mainly from the large number of studies on poliovirus genome translation and viral replication.

Although the 5' UTR of HCV is approximately half of the length of the RNA genome of same *picornaviruses* (such as poliovirus and encephalomyocarditis virus) and little sequence similarity, their 5'UTRs share same common features.

For example, a motif typically described in the 3' border of all the *picornavirus* IRES elements, which has been found repeated within the HCV 5'UTR, is a pyrimidine rich tract (Yn) separated from a downstream AUG triplet by a spacer (Xm) of 15-20 nucleotides (Jang, 1990). In *picornaviruses,* the AUG triplet of the Yn-Xm-AUG motif is the initiation codon in the so called type 2 IRES (encephalomyocarditis) but it is cryptic in the type 1 IRES (poliovirus) where the authentic AUG codon is located more than 150 nucleotides downstream to the next AUG (see figure 3). In *picornavirus*, deletion and mutational analysis demonstrated both the

importance of the Yn-Xm-AUG itself and of each single component of the motif (Pelletier J,1988b; Jang SK,1990; Meerovitch K,1991; Degener AM et al.,1995)).

Furthermore, in poliovirus, it has been shown that the primary sequence in the 5' UTR, which is strongly conserved among the different isolates (Poyry, 1992), has an important role, and that the introduction of same mutations within the 5'UTR effected both the neurovirulence of the virus and the cap-independent translation of the genome.

Deletion analysis of the 5'UTR of poliovirus permitted to identify the minimal sequence required for an efficient IRES function (Meerovitch, 1991). Once deduced, the 5' and the 3' boundaries of the IRES element, the ability of different mutations to effect the IRES function led to the conclusion that most part of this element is required either for direct interaction with trans-acting factors, or to maintain the conformation of structural features of the IRES necessary for such interactions.

1. 5. 3. The 5'UTR of HCV contains an internal ribosome entry site (IRES)

The first evidence of the presence of an internal ribosome entry site (IRES) within the 5'UTR of the hepatitis C virus comes from the *in vitro* translation studies of Tsukyiama-Kohara et al. (1992). Synthetic capped methylated dicistronic mRNAs, containing the CAT gene as first cistron and the first 1770 bases of the HCV genome as second cistron, were used in cell free protein synthesis systems. Both, CAT and HCV proteins, were efficiently translated in rabbit reticulocyte lysates, whereas, only the second cistron, containing the HCV protein, was translated in coxackievirus-infected Hela cells extract where translation of uncapped mRNAs is abolished as a consequence of the viral infection. These results suggested the presence, within the HCV sequence, of an internal ribosome landing pad, which allows the translation of the second cistron even when the ribosome entry at the 5' terminus of the dicistronic mRNA is blocked.

Similar experiments were performed *in vivo* by Wang and co-workers (1993). The 5'UTR of hepatitis C virus was inserted in a dicistronic vector between the CAT gene and the LUC gene. The T7 promoter before the CAT gene was used to produce synthetic dicistronic mRNAs. These RNAs were used to transfect both, HepG2 cells (a human hepatoma cell

line) and HeLa cells. Since the synthetic mRNA were uncapped, the CAT gene, within the first cistron, was so poorly translated that no CAT activity was detected in the cell extracts. On the contrary, independently from the translation of the CAT gene, the HCV 5'UTR located upstream to the second cistron was able to direct efficient translation of the LUC gene. Additionally, since the same results were obtained in both HepG2 cells and Hela cells it was suggested that HCV IRES does not have liver-cell specific preferences.

Similarly to what obtained *in vitro* by Tsukiyama-Kohara, Wang also demonstrated that, using these dicistronic uncapped mRNAs to transfect poliovirus infected HepG2 cells (where translation of uncapped messengers is impaired) only the second cistron was efficiently translated (Wang 1993). These data *in vivo*, give unequivocal evidences that the HCV 5'UTR contains an IRES element (Wang 1993).

Finally, the results obtained by Kattinen et al. (1993) further supported the conclusions of Tsukyiama-Koara and Wang. Kettinen provided important evidence for the cap-independence of HCV mRNA translation. He showed that, *in vitro*, the level of expression of the first cistron, from a dicistronic synthetic mRNA, could be increased by capping its 5' end, whereas the capping had no effect on the expression of the second cistron containing a portion of the HCV genome (including the 5' untranslated region) which allows translation to occur via the internal binding of ribosomes to the 5'UTR.

1. 5. 4. Structure of the HCV Internal Ribosome Entry Site

The exact nucleotide sequence and the secondary structure required for the HCV IRES have not yet been precisely defined.

The first attempts to identify the minimal sequence required for HCV IRES function, came from the group of Tsukyiama-Kohara (1992). They performed *in vitro* translation experiments using artificial mono- and dicistronic mRNAs containing different 5'UTR deletion mutants. They showed that deletions of the 5' non-coding sequence downstream to position 101 dramatically reduced the translation, suggesting that the putative IRES element was residing between position 101 and 332 of the HCV 5'UTR. Compared to the other known IRES sequences, that of HCV,

represented the shortest IRES so far discovered (picornavirus IRES is about 400 nucleotides in length).

A similar analysis was done by Wang et al. (1993). A series of deletions (both at the 5' and 3'ends) were produced within the HCV 5'UTR sequence cloned in front of the LUC gene in a dicistronic vector. *In vitro* synthesised mRNA was used to transfect cultured HepG2 cells. According to their results, the sequence downstream to the first 75 nucleotides from the 5'end, as well as the sequence immediately before the initiator codon, is essential for efficient translation since they proved that deletion of 9 nucleotides at the 3'end of the UTR can completely inhibit the translation.

Furthermore, the results obtained by Kettinen and coorkers (1993), suggested that the first few nucleotides of the coding sequence of the HCV capsid protein may also be involved in the HCV IRES function.

Recently, Rijnbrand et al. (1995), using site-directed mutagenesis, showed that almost the entire 5UTR of HCV is required for efficient translation initiation of uncapped mRNAs in poliovirus infected HepG2 cells (poliovirus translational host shut-off in the infected cells).

Finally, Honda et al. (1996), analysed the impact of a series of mutations within the HCV 5'UTR on the expression of the HCV capsid protein both *in vitro*, using rabbit reticulocyte lysates, and *in vivo* in Huh-T7 cells. According to their results, the 5' limit of IRES lies between nt 19 and nt 68 of the non-translated region. In this study, they further suggested that the 3' limit of the HCV IRES might extend downstream to the initiator AUG. They showed that, either deletion of some nucleotides before the AUG (from nt 333 to nt 337), or the insertion of 12 nucleotides at position 9 of the core sequence, inhibit HCV translation. The thermodynamic analysis, supported by the cleavage of synthetic RNA with single- and double-strand specific RNases, revealed the presence of a novel stem-loop structure (involving the nucleotide sequence between the UTR and the core gene) in which the initiator AUG is located within the loop. Interestingly, mutations within this sequence which are predicted to enhance the stability of the stem-loop structure, resulted in inhibiting HCV translation, whereas, decreasing the stability of the mutants by compensatory substitutions, was sufficient to restore efficient translation.

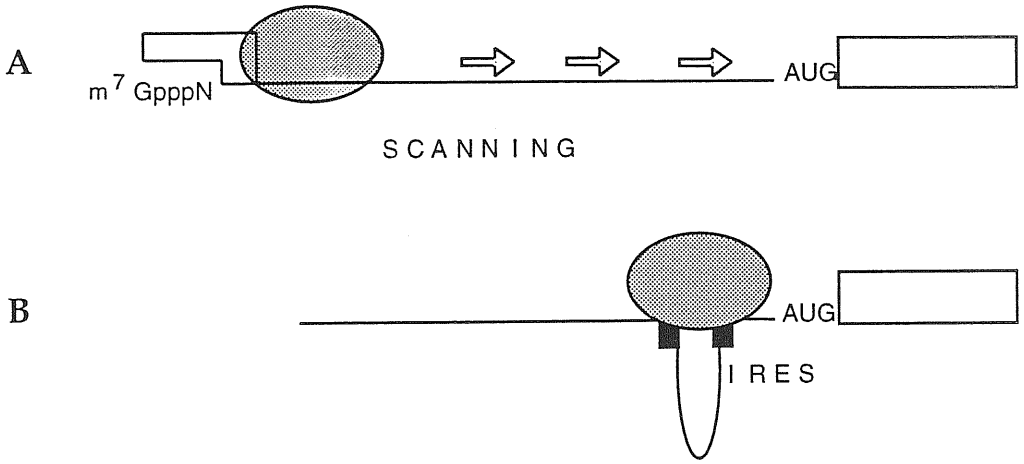


Figure 2: General models for cap-dependent and cap-independent initiation of translation of protein synthesis. A) migration (scanning) of the 40S ribosomal subunit and/or initiation factor(s) along mRNA, which has been proposed to occur during initiation of most capped mRNAs (Kozak 1978, 1980). B) Solid boxes are the IRES elements, determined either by the primary sequence or by higher-ordered structure within the 5'UTR of picornavirus and hepatitis C virus mRNAs.

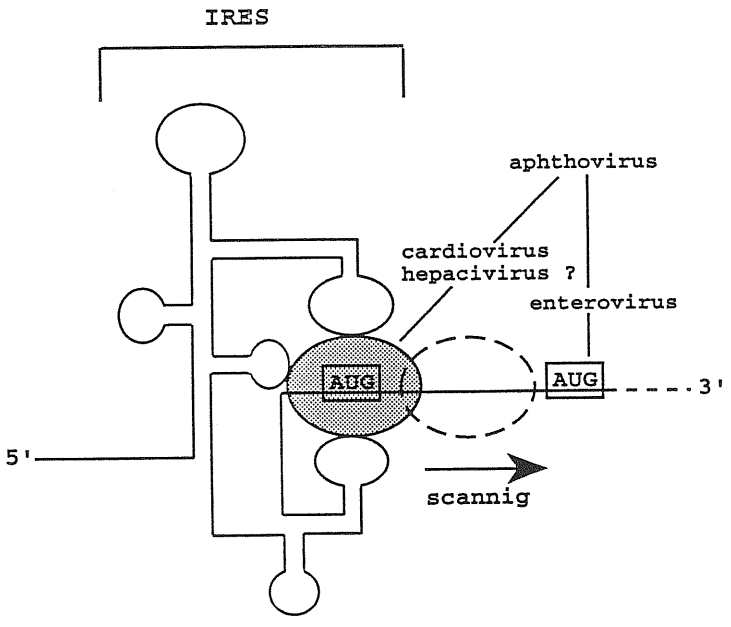


Figure 3: General model for internal initiation of translation on picornavirus RNAs (Jackson et al. 1990): the ribosome binding site is located at the 3' end of the IRES, and scanning from this binding site is necessary for initiation for aphthovirus and enterovirus; not on cardiovorus RNAs. The mechanism used by the hepatitis C virus is still to be defined. Shaded oval: a 40 S ribosomal subunit, with hypotetical factor(s) which recognises the IRES.

1. 5. 5. Secondary structure of HCV 5'UTR: the Brown's model

The structural model of the HCV 5'UTR proposed by Brown et al. (1992), was deduced initially by both, thermodynamic analysis of the primary sequence, and phylogenetic comparison of HCV 5'UTR derived structure to the secondary structure of the 5'UTR of two distantly related pestiviruses (HChV and BVDV) (see figure 4).

Additionally, nucleotide sequence alignment of different HCV strains, revealed the presence, within their 5'UTRs, of several covariant substitutions. The existence of covariant substitutions, since it is predictive of conserved base paired helical RNA structure, was used as an important parameter to further confirm the proposed structural model.

Finally, the definitive secondary structure of HCV 5'UTR was validated by cleavage of synthetic RNAs by double- and single-strand specific RNases.

Similarly to *pestiviruses*, four major structural domains have been identified in the HCV 5'UTR. The most relevant aspect derived from this analysis is the conservation of the structure of domain III of *pestiviruses* within the HCV structure. Domain III, located between position 125 to 323, is the largest secondary structure and it contains multiple stable stem-loops. Interestingly, the apical loop of domain III contains a short polypyrimidine tract which, together with a cryptic AUG located 17 nucleotides downstream, represents one of the two Yn-Xm-AUG motifs of the HCV 5'UTR. The other one, is constituted by a short pyrimidine rich tract immediately upstream the domain III. Although this pyrimidine sequence is not directly followed by an AUG at the primary sequence level, its probable base-pairing to the distal portion of the 5'UTR sequence, brings the sequence into closed proximity to the initiator AUG codon.

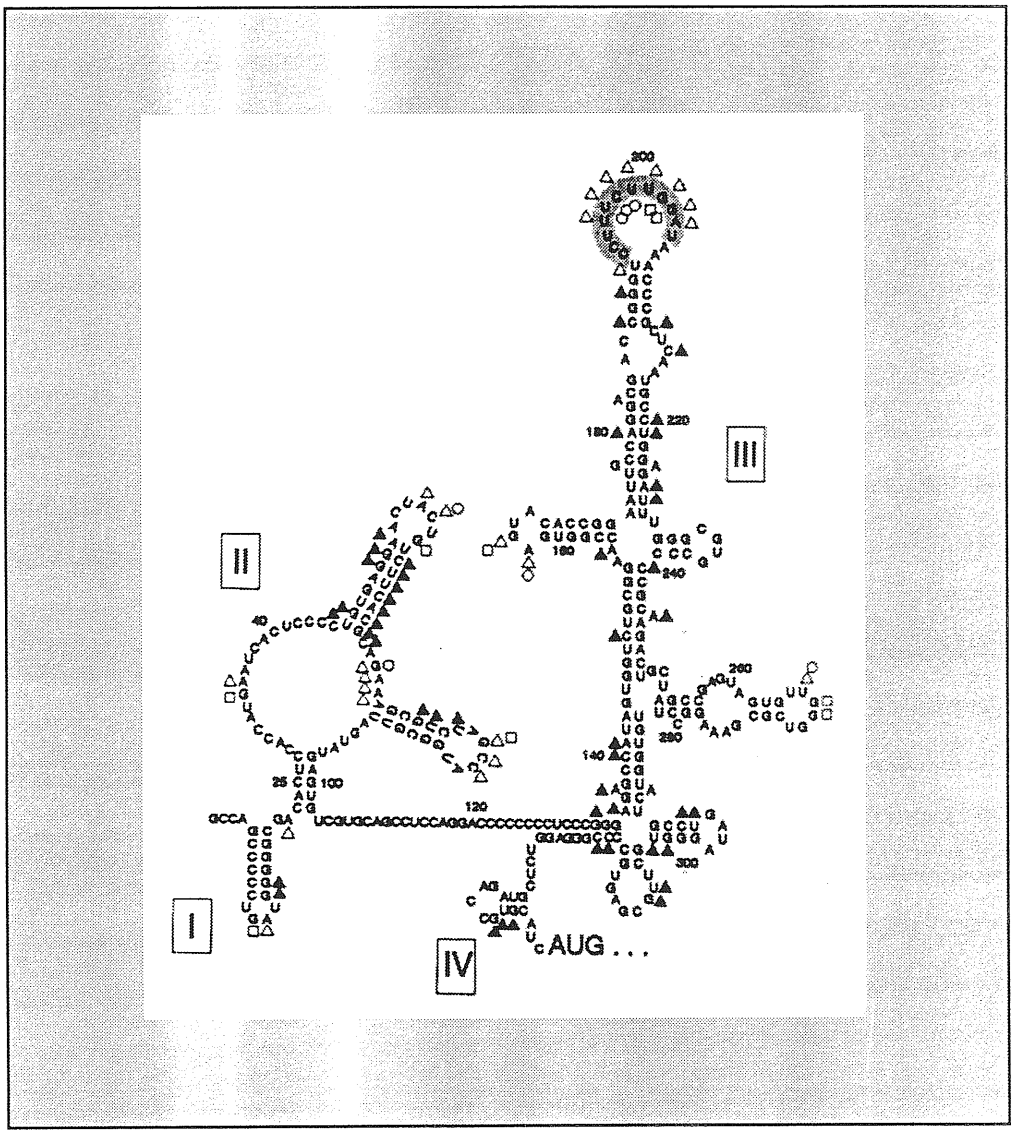


Figure 4: Secondary structure of the 5'UTR of HCV (strain AG94, genotype1a) proposed by Brown et al. (1992). Symbols adjacent to initial nucleotides indicate sites of nuclease cleaveges. The pyrimidine-rich tract within the apical loop of Domain III is indicated by a shaded backround

1. 6. Epidemiology

HCV infection occurs throughout the world with a relatively high prevalence in some countries like Japan, the southern part of USA, the Saudi Arabia, Spain and Italy, where 0.5-1.5% of the blood donors are HCV seropositive (van der Poel CL,1994).

HCV infection is mainly transmitted after direct percutaneous, parenteral exposure. Changes in disease transmission patterns for hepatitis C account for the changes in incidence.

Historically HCV infection was the main cause of transfusion-associated hepatitis. Typically, hemophiliacs receiving non inactivated blood clotting factors have been nearly all exposed to HCV. Fortunately, after the introduction of blood donor screening, the possibility to contract infection in this way has been drastically reduced.

The high prevalence of HCV seropositives between intravenous drug users (it has been estimated that 90% of them are infected), indicates that, today, this is one of the major route for the propagation of the infection (Alter MJ, 1993).

Other parenteral risk factors include dialysis, liver transplants, tatoons, exposure to multiple heterosexual partners and needle-stick accidents among healthcare workers.

Although a large number of studies have been performed, there are no unequivocal proofs of the transmission of infection in spouses of patients. In this case, in fact, an eventual transmission of infection might be due to other factors such as inapparent percutaneous exposure. On the other hand, it has been shown that 10% of the HCV infected mothers transmit the infection to their babies (Ohto H,1994).

MJ Alter, reported in a recent study that forty-five percent of American patients deny a known risk factor for hepatitis C, and low socioeconomic level is associated with a large portion of these patients. However, more than half of them report history of some type of high-risk behavior or contact but not in the six months preceding illness. Thus, undiscovered routes of transmission seem to be unluckly (Alter MJ, 1995).

Table 3: HCV seroprevalence in populations at high risk of infection.
(from Gaeta SB et al., 1993)

Drug abusers	80-90 %
Hemophiliacs	70-80 %
Thalassemics	70-80 %
Hemodialysis patients	15 %
Healthcare workers	7-5 %
Homosexualmales	8 %
Transplant recipients	3 %

1. 7. Pathogenesis

The acute clinical presentation of hepatitis C has mainly been documented in transfusion-associated cases in which, normally, only 20-30 % of the cases present the typical symptoms of the disease.

Because of the rarity of fulminant hepatitis C and the relatively mildness of its acute form, the importance of the HCV infection is to assign its tendency to become persistent and to induce chronic disease.

The infection persists in 80-90 % of the cases, and usually leads to chronic persistent (CPH) or chronic active hepatitis (CAH).

Data from different follow-up studies on HCV infected patients showed that 20-30 % of them developed cirrhosis. A further and very important possible sequela of HCV infection is primary hepatocellular carcinoma (HCC). Prospective studies showed a generally slow, gradual progression from chronic active hepatitis to cirrhosis and then to HCC in same patients after a mean interval of 10, 19, and 29 years post-transfection respectively (Tsukuma H, 1993).

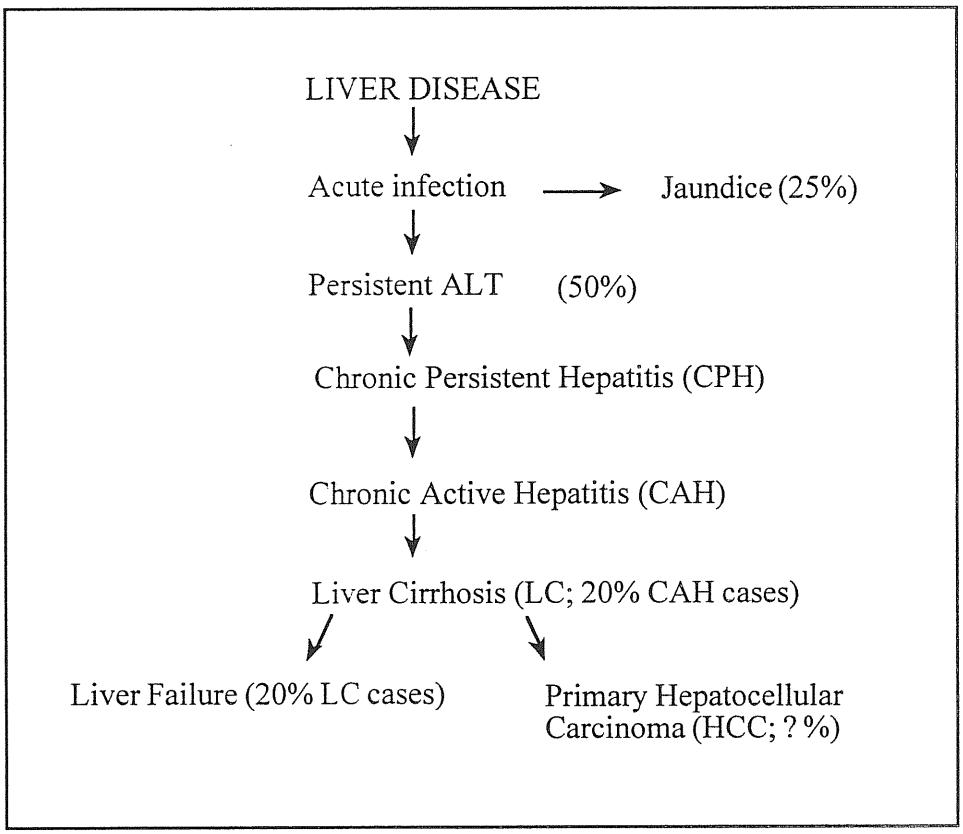
Disease progression is probably enhanced by other factors like hepatitis B virus co-infection and alcohol.

The mode of transmission may also influence the course of disease since it has been demonstrated that postransfusional hepatitis C infection assumed a more aggressive form than infection acquired by

intravenous drug use. This is probably related to the dose of the original virus inoculum.

Furthermore, since 1991, different studies demonstrated that the risk of progression towards a more severe disease is influenced by the particular HCV strain (Pozzato, 1991).

Table 4: Summary of potential clinical sequela of HCV infection



1. 8. The HCV genotypes

1. 8. 1. Distribution of HCV genotypes worldwide

By the analysis of the complete genomic sequence of seven HCV isolates (Kato 1990, Choo 1991, Okamoto 1991, Takamizawa 1991) Okamoto and coworkers (1992) initially found that all the HCV isolates available could be assigned to four genotypes: genotypes I (Choo 1991,

Inchauspe, 1991), II (Takamizawa,1991; Kato, 1990; Okamoto, 1990), III (Okamoto1991),IV(Okamoto1992).

Preliminary data from the analysis of the distribution of these HCV genotypes indicated that the distribution pattern was quite different among the countries studied.

It was shown that prevalence of type II (HCV-K1) was the highest in Japan (Okamoto 1991), China, Brazil and in some European countries (Takada et al. 1992). Type I (HCV-1) was found occasionally in Japanese and Chinese samples, but it was mainly found in the samples from Western countries suggesting that this type may be the Western type (Choo 1991, Ulrich 1990, Simmonds 1990, Ogata 1991). On the other hand, type III and type IV were scarcely found in the samples from Western countries but they were commonly found in Japanese and in Chinese samples suggesting that these may be Oriental types (Liu 1992; Takada 1992).

The sequence divergence in the entire HCV genome is reflected in various regions of the genome to a greater or lesser extent (see figure 5). As it is not easy to sequence the entire HCV genome, genotyping based on sequence analysis of partial gene regions, was used for identifying and classifying new genotypes.

More HCV groups were identified based on partial sequences derived from both structural and non-structural regions of the genome (Enamoto 1990, Bukh 1993, Tokida 1994, Bukh 1994, Stuyver 1994).

Bukh et al. (1993), analysed 576 bp of the E1 gene of HCV from isolates collected wordwide, and found that they could be divided into six major genetic groups. In parallel, Simmonds et al., (1993) compared a sequence of 222 bp within the NS5b region of HCV isolates, and proposed the classification into six major types and a series of subtypes. In this classification, the types correspond to the major genetic groups identified by Bukh et al. (1993), and Tokida (1994), and the subtypes corresponded to genotypes.

During the same time that these works were in progress, we also chose a similar experimental methodology to analyse the different 5'UTR sequences in Italian patients. The following comparison of the sequences that we collected in this study were seen to belong to three major groups, which were later found to be identical to Simmonds' genotypes 1, 2, and 3 (Tisminetzky et al., 1994).

The nomenclature of HCV variants has been the subject of considerable controversy until Simmonds proposed a "consensus" one based on the phylogenetic analysis of isolates. Its main advantage consists in the fact that it can take new isolates by numbers (e.g. 1, 2 , 3, etc.), which further divide into subtypes designed by letters (a, b, c, etc.), and is gaining wide application (Simmonds et al. 1993, Simmonds 1995).

Sequence divergence of the HCV genomes occurs in three different strata. The largest variation can be observed between any two HCV genomes of different major genetic groups (31-35%), the middle variation between any two genomes of the same group, but with different genotypes (20-23%), and the smallest variation between any two genomes of the same genotype (1-10%). Assuming that sequence divergence higher than 20% in the entire genome represents different genotypes, the middle variation would be adopted as genotypic differences.

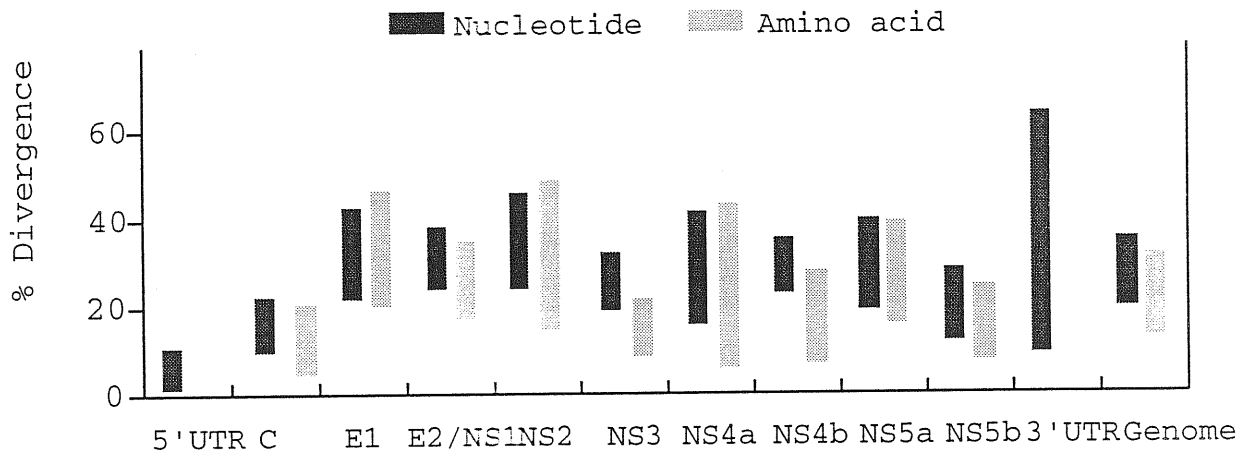


Figure 5: Sequence divergence of HCV. The percentage divergence in nucleotide and amino acid sequence in various regions of the genome, as well as the entire genome, of six representative HCV isolates of different genotypes are shown. The HCV genome under comparison are: HC-J1 (genotype 1a); HC-J4 (1b); HC-J6 (2a); HC-J8 (2b); NZL1 (3a); and HCV-Tr (3b) (Miyakawa Y. et al., 1995)

1. 8. 2. Clinical application of HCV genotypes

1) Diagnosis of HCV infection

The existence of different HCV genotypes had an important bearing on the development of serological diagnosis of HCV infection. Early ELISAs for detecting anti-HCV antibodies used the recombinant

C100-3 protein, deduced from the NS3/NS4 region of the prototype HCV-1 of genotype I (Choo 1991). ELISA-1 was not very sensitive, missing viremia in some blood units that could transmit HCV infection to recipients (Esteban 1990, Donhane 1992) or in sera in which HCV RNA was later detected by PCR (Wiener 1990). In fact the C100-3 sequence, derived from the NS3 and NS4 regions, was poorly conserved among the isolates of type I, II, III and IV. For this reason, new generation of ELISA and RIBA tests, were developed to obtain more sensitive tests in detecting antibodies raised by the infection of different genotypes. Two new proteins C33 and C22-3 were included along with the previous used C100-3 protein in the new ELISA and RIBA tests (van der Poel 1991, McHutchison 1992). The amino acid sequence of these two proteins (derived from the NS3 and from the core gene respectively) are more conserved among different genotypes. The advantage from the identification of common sequences in immunodominant epitopes of different HCV genotypes was demonstrated by the superiority of these second generation tests.

2) Clinical aspect of the disease with different HCV genotypes.

It has been known that there is a relationship between the HCV genotype and the severity of liver disease. In this sense, preliminary data, obtained by different groups, from patients infected by different HCV genotypes, showed that, in non-A, non-B related disease the ratio between the HCV types I- II (HCV-1, HCV-K1), compared to HCV types III-IV (HCV-K2a, HCV-K2b) was increasing with the severity of liver disease. In fact, types I and II were more frequently associated with chronic active hepatitis, cirrhosis and also to the progression of the disease in hepatocellular carcinoma (HCC). At the same time, HCV types III and IV infection was prevalent in younger patients, having a low frequency of history of blood transfusion than in those having HCV types I and II (Pozzato 1991, Takada 1992).

Although the number of patients analysed was still too small, these results suggested the possibility that the HCV infecting genotype may influence the clinical feature of the disease.

3) HCV genotypes and response to interferon treatment

Initially, in controlled clinical trials of individuals with hepatitis C, it was shown that the recombinant alpha interferon induces

response in about 50% of the recipients at the completion of therapy, and in 25% after at least six months thereafter as judged by normalisation of aminotransferase levels (Davis 1989, Di Bisceglie 1989). Therefore, host and viral factors that were likely to produce a sustained response to interferon therapy were analysed in a number of studies.

Evidences started to accumulate indicating that the response to interferon is dependent on HCV genotypes (Pozzato 1991, Yoshioka 1992). Patients infected with genotype II were reported to be less responsive to interferon than those infected with HCV of other genotypes (Takada 1992, McOmish 1992). The failure of patients infected with genotype II to respond to interferon has been reported predominantly in Japan, where genotype I is virtually nonexistent. Therefore, the results obtained were still insufficient to distinguish any difference between infection with genotype I and II of the same major genetic group. Additional clinical studies are needed to define whether or not the determination of HCV genotypes is a predictive factor for the response to the therapy.

1. 9. Objectives of the present study

In view of the emerging significance of the HCV genotypes and their possible implication in the clinical aspect of the disease, we decided to characterize, by sequencing of the 5'UTR of the viral genome, different HCV strains prevalent in the Italian population and to determine the pattern of distribution of these genotypes in different regions of Italy.

This information was further exploited to verify whether there is any relation between the infecting genotypes and the clinical aspects of the disease by analysing clinically well defined groups of patients.

Since the results suggested that the different HCV genotypes have distinct biological properties, we wished to analyse the significance of sequence variation within the 5'UTR as far as the efficiency of translation initiation is concerned. The 5'UTRs of the three major HCV genotypes, two naturally occurring variants and artificially engineered mutants were utilised in this study.

CHAPTER 2

MATERIALS and METHODS

1. Cells and bacteria

HeLa cells were routinely maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and streptomycin (100 μg/ml).
Cultures of *Escherichia coli* DH5α were transformed following treatment with calcium chloride and selected for ampicillin resistance following standard procedures.

2. Serum samples

Most of the serum samples included in this work were referred from patients with chronic hepatitis C collected from different areas of Italy. We have also used serum samples from a selected group of patients with autoimmune hepatitis type 2, and from symptomless HCV 'carriers'. Specimens of serum samples were immediately frozen in liquid nitrogen and stored at -80°C.

3. Amplification of HCV RNA by reverse transcription and polymerase chain reaction (RT-PCR)

To detect HCV RNA, 3 μl of serum was mixed with 17 μl of buffer containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of each dNTPs (Pharmacia, Uppsala, Sweden), 50 pmol of antisense outer primer (primer AS1), heated at 95°C for 2 min., and cooled rapidly on ice. After addition of 0.1U/μl of RNasin (Boehringher Mannheim, Mannheim, Germany), and 10 units of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, BRL, Milan, Italy), the mixture was incubated at 37°C for 60 min.

For amplification by PCR, a 100 µl mixture was prepared containing 1X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2.5 units of Taq polymerase (Boehringer Mannheim, Germany), and 50 pmol of both antisense and sense outer primer (primer S1). PCR was performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CO, USA). The first cycle entailed denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min, and extension 72°C for 1 min. Then, 29 further cycles were run at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 45 seconds. For the second round of PCR, 3 µl of the first PCR reaction mixture was amplified using 50 pmol of internal sense (primer S2), and internal antisense primers (primer AS2). Amplified products were analysed by electrophoresis on 1.5% agarose gel.

To reduce the risk of contamination in PCR analysis, solutions and aliquots were prepared in a laboratory not used to perform HCV analysis. RNA manipulation and transfer of samples were performed under sterile conditions. Unused and sterile reagents were usedfor new reactions. cDNA synthesis, PCR amplification, and gel electrophoresis of PCR products were performed in separated areas. All solutions were pipetted with disposable pipettes and pipette tips containing aerosol barrier. Gloves were always changed after contacts with possible contaminating samples. Water and HCV a negative serum sample were included in each test to monitor contaminations.

4. Oligonucleotide primers

Synthetic DNA olgonucleotides were purchased by Primm s.r.l. (Milan, Italy).

S1:	5'-GCCATGGCGTTAGTATGAGT-3'
S2:	5'-GTGCAGCCTCCAGGACCC-3'
AS1:	5'-GTGCACGGTCTACGAGACCT-3'
AS2:	5'-GGCACTCGCAAGCACCCTAT-3'
HAS1:	5'-TTAAGCTTGGTGCACGGTCTACGAGACCT-3'
K297S:	5'-TTGGTACCTGTGAGGAACTACTGTCT-3'
LOOP-S:	5'-CGGGTCCGGGGGGGGATCAA-3'
LOOP-AS:	5'-TTGATCCCCCCCCGGACCCG-3'
STASC-S:	5'-GGGTGACGCCCACCTTTCT-3'

STASC-AS: 5'-AGAAAGGTGGGCGTCACCC-3'
STDIS-S: 5'-TGGAACATGGGCCTAATAC-3'
STDIS-AS: 5'-GTATTGAGGCCCATGTTCCA-3'

5. Cloning and sequencing of HCV-specific PCR products

PCR products were separated on agarose gels, fragments were excised from the gel and purified using microspun columns (Pharmacia). After phosphorylation (20 min, 37°C, 5 units of T4 polynucleotide kinase), they were treated with the Klenow fragment (20 min, 37°C, 2 units polymerase), and cloned into SmaI site of the pUC18 vector. Three independent clones were isolated and nucleotide sequence of both plus and minus strand were determined by dideoxynucleotide chain termination method (T7DNA Polymerase Sequencing System). Electrophoresis was performed on 6% polyacrylamide/7 M-urea sequencing gels.

6. Direct sequencing of PCR products

The PCR products were purified by microspun columns (Pharmacia). One-quarter of the purified products was heat-denaturated for primer annealing and then used in sequencing reactions with T7 DNA polymerase (Sequenase; United States Biochemicals) performed according to the manufacturer's instructions.

7. Dot blot hybridisation assay with type specific-probes

For HCV genotyping by dot blot assay, 10 µl of the PCR amplified products from each serum sample were heat-denaturated at 95°C for 5 min., and spotted on nitrocellulose filters in triplicate, after addition of 40 µl of 20XSSC (3M NaCl. 0.3M Na-citrate). Filters were then incubated at 42°C for 1 hour in a pre-hybridisation solution containing 6XSSC, 0.25% milk powder. Using the same solution, filters were than incubated for at least three hours at 42°C with the ^{32}P, 5'-end labelled genotype-specific oligonucleotide probes described below.

For each probe, 50 ng of oligonucleotide was added to 50 µl of mix containing 1X kinase buffer, 10U T4 polynucleotide kinase and 20 µCi $\gamma^{32}P$-dATP, and incubated at 37°C for 30 min. Radiolabeled

oligonucleotides were purified from unincorporated nucleotides by ethanol precipitation. Type 2a and type 2b probes were often used in the same hybridisation solution and HCV sequences hybridising with the two mixed probes were generally classified as genotype 2.

Filters were washed twice with two changes of 6XSSC/0.1% SDS at room temperature (RT) for 10 min., twice in 3XSSC/0.1% SDS at RT for 10 min., and twice in 1XSSC/0.1% SDS at 55°C for 15 minutes. Autoradiography was performed using Kodak X-OMAT AR films, with overnight exposure at -80°C.

HCV genotype-specific oligonucleotide probes.
HCV-G1: 5'-CGCTCAATGCCTGGAGAT-3'
HCV-G2a: 5'-CACTCTATGCCCGCCAT-3'
HCV-G2b: 5'-CACTCTATGTCCGGTCAT-3'
HCV-3: 5'-CGCTCAATACCCAGAAAT-3'

8. HCV subtype 1a and 1b detection

At the 5'UTR sequence level, HCV type 1a and 1b can be distinguished from each other by a sequence polymorphism at position 244 (numbering as Cha, 1992) with an adenosine (A) residue in type 1a viruses and a guanosine (G) in subtype 1b. The presence of a G at position 244 produce a restriction site in the RT-PCR amplified products which is recognised by BstUI enzyme. Digestion of the 211 bp long 5'UTR fragment, obtained by nested PCR as previously described, with BstUI enzyme generates three fragments of 137 bp, 30 bp, and 44 bp in the case of HCV subtype 1b, and two fragments of 167 bp and 44 bp for subtype 1a.

9. HCV RNA quantitation

Viral load was assessed by a recently developed PCR system which includes a quantitation standard (QS) that is co-amplified with the target HCV-RNA (Amplicor HCV Monitor, Roche Molecular System, Inc., Branchburg, NJ) (McGuiness 1996). The assay was performed according to the manufacturer's instructions.

10. Computer assisted sequence analysis

Multiple sequence alignments were performed with the program GENALIGN (Miller 1990). RNA secondary structure in the 5'UTR of HCV wild type and variants were predicted using the program FOLD (Devereux et al. 1984).

11. RNA transcription

Plasmid DNA (1mg), purified either by CsCl-density gradient or by Qiagen anion-exchange column, was transcribed with T7 RNA polymerase (Promega) in a 30 µl reaction mixture, generally in accordance with the manufacturer's instructions. Nucleic acids were extracted with phenol/chloroform and precipitated with ethanol, and a portion was analysed on agarose gel electrophoresis followed by staining with ethidium bromide. The concentration of RNA was estimated by spectrophotometry.

12. In vitro transcription and translation

Translation reactions were carried out in rabbit reticulocyte lysates RRL (Promega). RRL were programmed with either 50 ng/µl of synthetic RNA or 50 ng/µl of plasmid DNA in presence of T7 RNA polymerase (Promega). Translation products were labelled with ^{35}S-methionine (ICN Trans^{35}S label) and analysed by 13.5% SDS-PAGE gel electrophoresis. Gels were treated with Amplify (Amersham) for 15 min at RT, dried and X-ray films were exposed at -80^{o}C overnight.

13. DNA transfection

Human cervical carcinoma cells (HeLa) were maintained in Dulbecco's modified Egle's medium with 10% fetal calf serum (DMEM). Nearly confluent cell monolayers (80%) in 35mm-diameter dishes were washed, fed with 1.5 ml of DMEM without serum, and infected for 30 min at 37^{o}C with recombinant vTF7-3 vaccinia virus (which express T7 RNA polymerase) at a multiplicity of infection of 20 p.f.u. Cells were washed, transfected with 1mg of CsCl-gradient purified plasmid DNA in 10 ml of Lipofectin (Gibco\BRL), and further incubated at 37^{o}C.

14. CAT Assay

The cells were harvested 24 hours after transfection, washed in ice cold PBS, and resuspended in 250 mM Tris (pH 7.8). Whole cell extracts were prepared by four repeated freeze-thaw cycles followed by centrifugation to remove cell debris. The amount of cell extract to be assayed for CAT activity was a function of protein concentration (expressed as O.D.) over transfection efficiency. Protein concentration of cell lysates was determined using Bradford assay (Bradford, 1976). Samples were incubated for 1 hour at 37°C with 5 µl ^{14}C-1-deoxychloramphenicol (50 mCi/mmol, Amersham), 70 µl 1M Tris-HCl pH 7.8, 20 µl 4 mM Acetyl-CoA in a final volume of 150 µl. The reaction was stopped by extracting chloramphenicol with 1 ml of ethyl acetate and, following lyophilization, samples were resuspended, spotted on TLC silica gel plates and developed by ascending chromatography in a 95% chloroform-5% methanol mixture. Autoradiography resulted in qualitative informations, whereas quantitation was achieved by cutting out the corresponding areas of the TLC plates and counting the radioactivity in the scintillation counter using a liquid scintillation cocktail.

15. Plasmids construction

The molecular cloning of the cDNA representing the 5'UTR of different HCV strains was performed after RT-PCR amplification (as described before) of a fragment encompassing the sequence between nt 35 and nt 341 (numbering as Cha, 1992). Primer K297S and HAS1 were used for PCR amplification; the resulting PCR products are flanked by a KpnI and a HindIII site respectively at the 5' and 3' ends. The KpnI and HindIII digested PCR fragments were inserted, after agarose gel-purification, into KpnI-HindIII site of plasmid pULB3475 (kindly provided by d'Adda di Fagagna, I.C.G.E.B., Trieste), under regulation of the T7 promoter and immediately upstream the CAT coding sequence. Only two nucleotides of vector sequence remained between the HCV 5'UTR and the CAT coding sequence.

All the constructs, and the HCV genotype or strain from which the 5'UTR/insert was derived are listed below:

Genotype	name of plasmid
HCV-1b	pULB5'UTR1
HCV-2a	pULB5'UTR2
HCV-3	pULB5'UTR3
HCV-1b variant	pULB5'UTR1-205
HCV-2a variant	pULB5'UTR2-181/184/215/220

Digestion of plasmid DNA, dephosphorylation by alkaline phosphatase treatment, purification of DNA fragments by agarose gel electrophoresis, ligation of proper DNA fragments, and propagation of the constructs in competent bacterial cells were performed by standard procedures (Sambrook et al., 1989). DNA-modifying enzymes were used according to the manufacturer's instructions.

16. Oligonucleotide-directed and PCR mutagenesis

HCV 5'UTR mutated sequences were generated in PCR driven by pairs of complementary oligonucleotides containing the desired mutation. Two cDNA fragments were generated in separate reactions by a first round of PCR amplifications (primers used for each construct are indicated below) using plasmid pULB5'UTR3 as template and, following purification, these were allowed to hybridise to the overlapping sequences, followed by extension and amplification by PCR. The mutated linear DNA fragments were gel-purified, digested with enzymes KpnI and HindIII (which cut only the 5' and 3' extremities of the fragment) and introduced into KpnI/HindIII digested, phosphatase-treated and gel purified plasmid pULB5'UTR3475. The orientation and correct insertion of the mutated fragments was ascertained by DNA sequencing.

Primers used for site directed mutagenesis and relative derived construct:

K297S/LOOP-AS;LOOP-S/HAS1	pULB5'UTR3/195-200
K297S/STDIS-AS;STDIS-S/HAS1	pULB5'UTR3/208
K297S/STASC-AS;STASC-S/HAS1	pULB5'UTR3/189-208*

* The double-mutation within the 5'UTR sequence contained in plasmid pULB5'UTR3/189-208 has been obtained using plasmid pULB5UTR3/208 as template.

CHAPTER 3

RESULTS-PartI

3. 1. 1. Identification of distinct HCV genotypes in Italian patients with chronic hepatitis type C.

Althogh the existence and geographical distribution of multiple HCV genotypes had been reported by several groups, when we started this study, no such data were available about the epidemiology of HCV in Italy.

To identify the HCV genotypes prevalent in our country we determined the sequence of the 5'UTR of the virus from a series of consecutive patients with chronic hepatitis C.

The patients were all of Italian origin, living in the North-East part of Italy. These patients were recruited following admission to the Clinica Medica II of the University of Padova. The age range was between 22 and 69 years and the male\female ratio was 1.8. They were all anti-HCV (Ortho Diagnostics, Raritan, NJ, USA) positive confirmed by second generation RIBA (Chiron Corporation; Emeryville CA, USA). They were all HCV RNA positive in the serum as detected by RT-PCR using primers specific for the 5'UTR (see chapter 2).

A fragment of 211 nt representing the HCV 5'UTR from nucleotides 95 to 306 (numbered as Cha; 1992) was amplified from serum samples of 21 Italian patients with chronic hepatitis C.

Serum RNA was used as template for cDNA synthesis, the product of which, in turn, served as template for nested PCR. Two sets of primers were designed, primer AS1 and S1 for outer reaction, primer AS2 and S2 for the nested reaction (primer sequences are described in chapter 2). These primers were chosen to match the published HCV RNA sequences corresponding to genotype 1, 2, and 3 (Kato 1991, Choo 1991, Okamoto 1991). After reverse transcription followed by PCR amplification

(RT-PCR), a fragment of 211 bp resulting from the second amplification was purified by electrophoresis on a 1.5 % agarose gel.

The PCR products were isolated, blunt-ended and inserted into pUC18 cloning vector by standard procedure. Once the PCR products had been cloned in pUC18 vector, there was still the possibility to find different sequences from the same isolate, due to either Taq polymerase errors or to virus heterogeneity itself. Thus, to verify the accuracy of the sequences, at least four clones for each RT-PCR product were sequenced. Furthermore, for each serum sample, RNA extraction and RT-PCR was done at least twice. Samples were meticulously handled to reduce contamination. Water and normal serum were always used as controls to monitor false positive results.

For each one of the 21 RNAs, three independent cDNA clones were isolated, and their sequence, determined by the dideoxy chain termination method with T7 DNA polymerase (Sequenase, United States Biochemical, Denver, CO). The sequence was compared with the consensus sequence of HCV-1 (Choo 1991). Genaligne program was used for sequence alignments and comparison (IntelliGenetics). The sequences alignment is shown in figure 6.

By sequence homology we identified three main groups of sequence: 8 out of 21 HCV isolates were included in group 1, 11 in group 2 and 2 in group 3. Two cases of group 1 had complete sequence homology to HCV-1 (Choo 1991), five to HCV-J (Kato 1990) and a single case had a sequence identical to South African genotypes SA3 and SA4 (Cha 1992). Patients in group 2 had significant sequence homology with HCV-K2a and HCV-K2b (Enamoto 1990). The isolates of group 3 were identical to the published British E1 sequence (Chan 1992).

3. 1. 2. Development of a dot blot hybridisation assay with type-specific probes for HCV genotypes determination.

We developed a dot-blot hybridisation assay to rapidly screen, with type-specific probes, the genotype of infecting HCV in patients with chronic hepatitis C.

Genotype-specific oligonucleotide probes were synthesised on the basis of the three nucleotide patterns of the 5'UTR resulting from the sequencing of the HCV isolates infecting the first 21 patients. The position

of the characteristic probes was chosen in a region of the 5'UTR with relatively little similarity between genotypes, but good conservation within each of the genotype (see figure 6). Sequences of the three oligonucleotides corresponding to the three genotype-specific probes are described in chapter 2

For the dot blot procedure, from each sample an aliquot of the PCR amplified product (nested PCR was performed as described before using AS1, S1 and AS2, S2 primers) was heat denatured and spotted on nitrocellulose filters in triplicate. Each filter was incubated with one of the three different ^{32}P labeled, 5'-end specific probes for three hours. Hybridisation of PCR products to type specific probes was revealed by autoradiography.

Figure 7, shows the results of dot blot assay for the 21 HCV isolates (previously characterised by cloning and sequencing) in which the three probes hybridised to each HCV cDNA with 100% sensitivity and 100% specificity.

The dot blot assay was then used to screen the infecting HCV genotypes of 58 additional patients with chronic hepatitis. In this new series, 25 sera of group 1, 25 of group 2, and 1 sera of group 3 were found. PCR fragments amplified from the sera of seven patients failed to react with type-specific probes and remained unclassified.

Furthermore, the genotypic attribution was always confirmed by direct sequencing when the HCV 5'UTRs from ten patients, randomly selected among these 58 cases, were analysed.

The prevalence of HCV genotypes within the entire population of 79 patients screened up to now are summarised in table 5. The results indicate that 42% of the HCV isolated were ascribed to group 1 sequences , 45% to group 2 and 4% to group 3, while 9% of them remained unclassified.

The main demographic, epidemiological and clinical features of the 79 patients analysed in this study are also summarised in table 5. Patients were grouped according to the infecting HCV genotype. Patients of different groups did not present significant differences about sex distribution, possible cause of infection, and duration of liver disease. On the other hand, patients infected with type 3 virus were significantly younger than those infected by type 1 and type 2. The mean levels of alanine amino transferase were similar in patients of all the three groups.

One third of patients, infected either by HCV type 1 or type 2, had chronic persistent hepatitis (CPH), one third had chronic active hepatitis (CAH), and one third had cirrhosis. Patients of group 3, instead had milder liver histological features since all of them were found to have chronic persistent hepatitis (CPH).

3. 1. 3. Distribution of HCV genotypes in Italy.

The dot blot hybridisation assay developed in our laboratory was used for a large scale analysis of the HCV genotypes in a wide population of patients with chronic hepatitis type C.

For this study serum samples were collected from 23 medical centres distributed all over Italy. The patients were not pre-selected for any particular criteria such as severity of the disease, level of progress of the illness, age, sex or risk groups. Each group represents consecutive patients (all anti-HCV positive in serum by second generation ELISA) with chronic hepatitis C reported at the respective centre. A total of 888 serum samples were found HCV RNA positive by RT-PCR using primers specific for the conserved 5'untranslated region (the protocol used is described in chapter 2). The PCR products were used in the dot blot assay for HCV genotype determination. Table 6 summarises the characteristics and the clinical features of the patients.

Patients	888
Age	45±14 (mean±SD) 18-94 years (range)
Sex	
Males	571
Females	351
Diagnosis	
Asymptomatic carriers	10%
CPH	18%
CAH	60%
Cirrhosis	11%
HCC	1%

Table 6 CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; HCC, hepatocellular carcinoma

The results of the genotype analysis are schematicaly represented in figure 8.

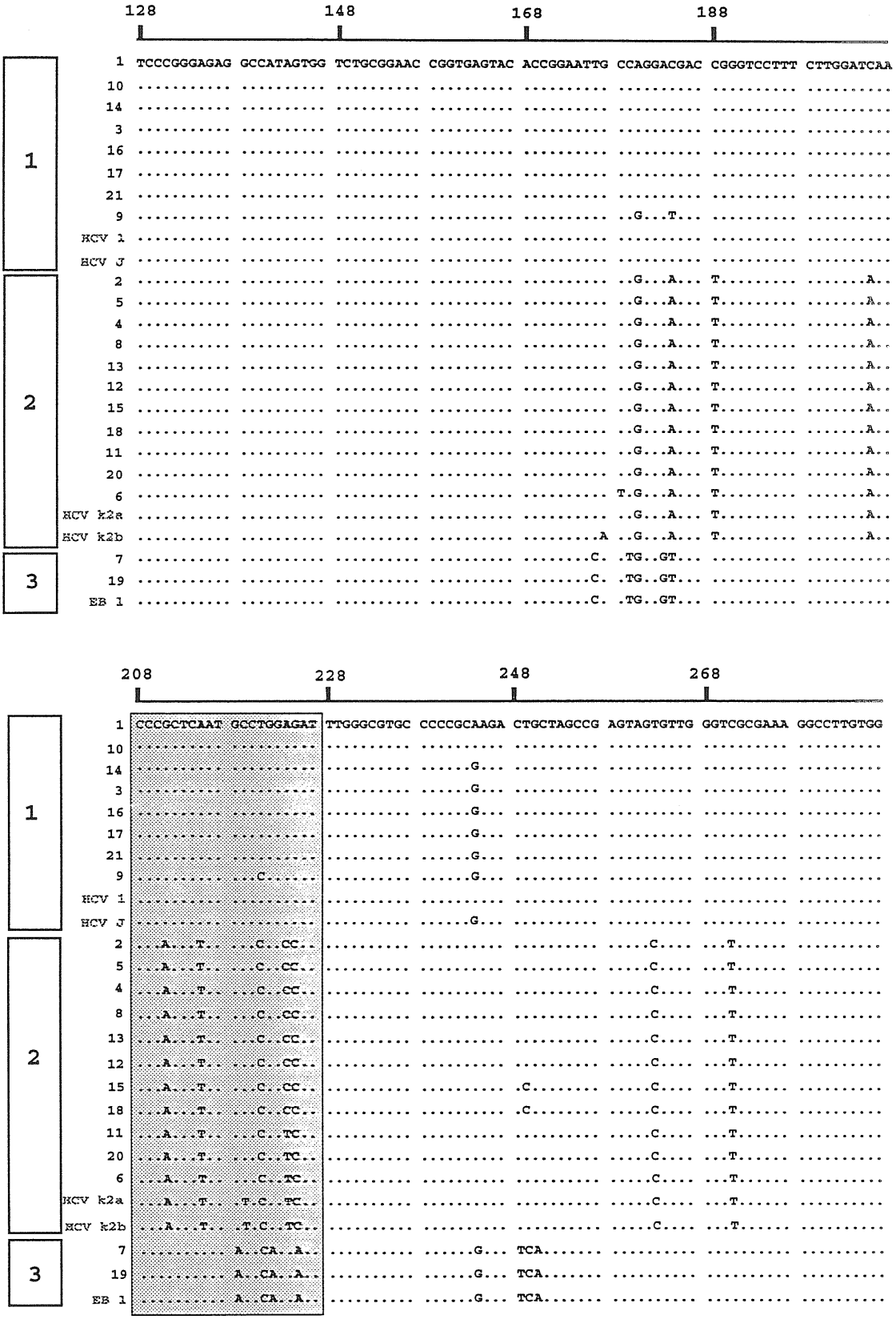


Figure 6: Comparison of necleotide sequence in the 5'UTR from 21 Italian patients with previously published HCV sequences. Dots indicate identity with sequence 1 (top line), nucleotide substitutions are indicated. Nucleotides are numbered according to Cha et al. (1992). Coloured area corresponds to the region from which type-specific oligonucleotide-probes were derived for the dot blot hybridisation assay.

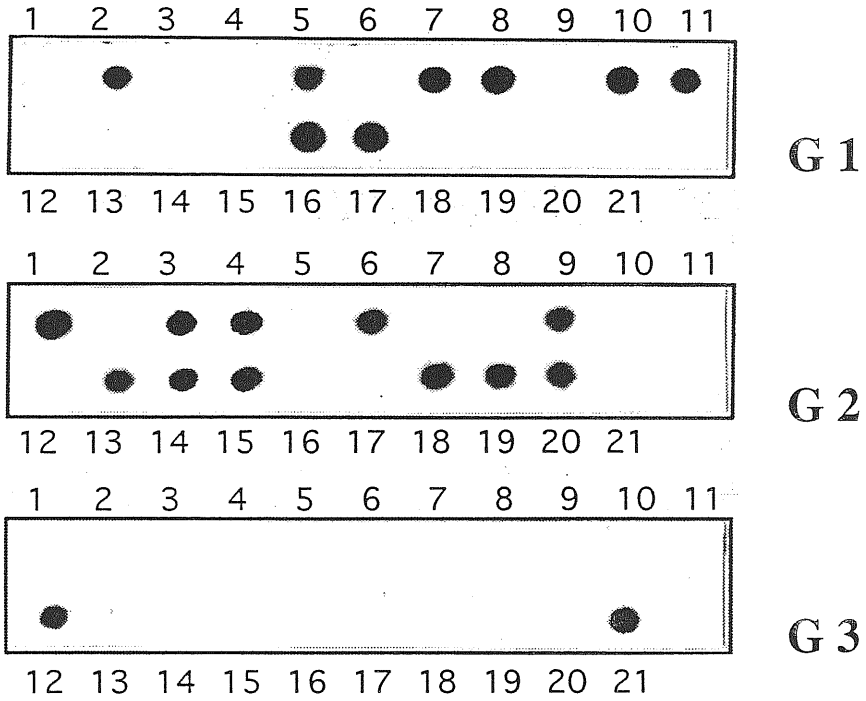


Figure 7: Dot Blot Assay. Hybridisation of the HCV cDNAs from 21 patients with chronic hepatitis using genotype-specific probes

	HCV genotype			
	genotype 1	genotype 2	genotype 3	undefined
No cases	33 (42%)	36 (45%)	3 (4%)	7 (9%)
M/F	16/17	13/23	2/1	5/2
Age (mean yrs±SD)	46.4±11.6	48.68±19.7	26.6±5.7	43.2±12.2
Duration of liver disease (yrs)	7.2±4.2	7.9±6.5	6±3	7.5±2.1
Type of exposure				
Blood transfusion	29%	38%	33%	43%
Other high risk	33%	22%	33%	28%
Unknown	38%	40%	34%	29%
Mean ALT	191.6±114.7	220.4±169.6	170.3±93.3	189.4±86.3
Histology				
CPH	33%	32%	100%	33%
CAH	46%	36%		50%
Cirrhosis	21%	32%		17%

Table 5:
Epidemiologic and clinical features in 79 patients with chronic hepatitis C classified according to the infecting HCV genotype

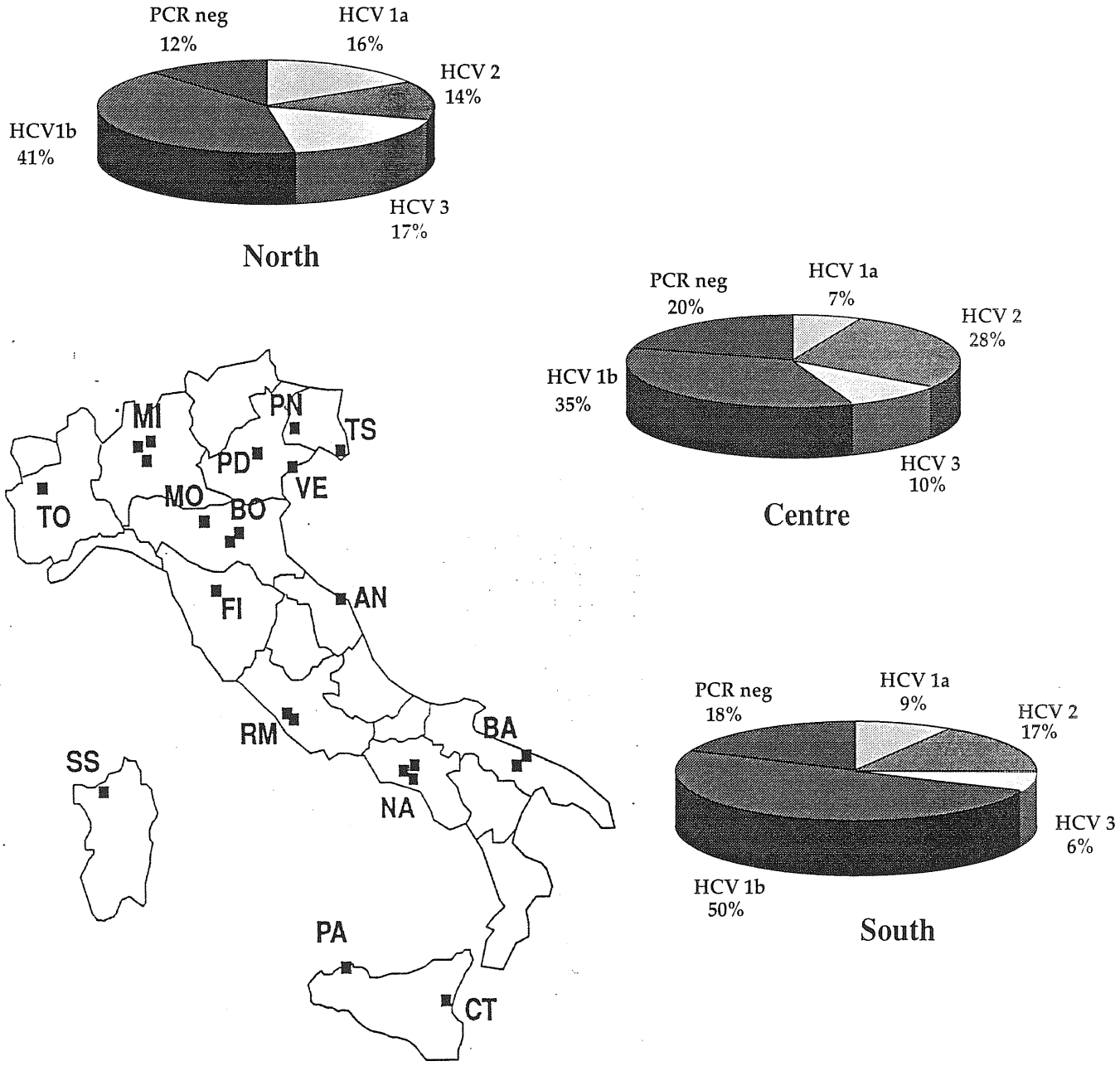


Figure 8: HCV genotypes distribution in Italy. The 23 medical centres partecipating to the study are indicated on the map. A total of 888 patients, with chronic hepatitis C, were found HCV RNA positive in the serum by nested PCR and were used for genotype detemination by dot blot assay. Patients anti-HCV positive but HCV RNA negative (the percentages are shown) could not be genotyped. Patients were grouped by birth region, and the prevalence of the main HCV genotypes (1a, 1b, 2a, and 3a) are expressed in percentage.

3. 1. 4. Dot Blot Assay: an efficient method for HCV genotyping

3. 1. 4. 1. Introduction

The sequence divergence of the entire HCV genome is reflected in various region of the genome to a greater or lesser extent, thus, not only the sequencing of the complete genome, but also part of it, is the most reliable method to determine infecting genotype. As even partial sequencing of the genome is not practical in clinical and epidemiological studies, HCV genotypes are usually determined by:

A) Restriction fragment length polymorphism (RFLP). The amplified cDNA corresponding to the 5'UTR of the viral genome is digested by restriction enzymes that recognise sequence polymorphisms between HCV genotypes. Resulting fragments, of different sizes, are then separated on high resolution agarose gels by electrophoresis (Davidson 1995).

B) Hybridisation of amplified 5'UTR or NS5B sequences to oligonucleotides probes that are specific for particular genotypes: the dot-blot and line probe methods (Tisminetzky 1993, Stuyver 1994).

C) Selective amplification by PCR of portions of the NS4 or core sequences by genotype-specific primers (Okamoto 1992).

D) Serotyping. Different from the others, this method is based on the study of the antibody response to different HCV types infection, and not on the molecular characterisation of the viral genome. Sequence variations in the NS4 region, which are specific for major genetic groups, allowed Simmonds at al. (1993) to develop an ELISA test with synthesised oligopeptides that have serotype-specific sequences.

3. 1. 4. 2. Comparison between the dot blot hybridisation method with the serotyping method for the identification of HCV genotypes

With this study we wanted to verify if the molecular method, i.e. the dot blot hybridisation with specific probes, and the determination of the serotypes, have similar value to identify the infecting genotype in HCV positive sera samples.

Sera from 127 patients with chronic hepatitis C were analysed. All the patients had abnormal transaminase levels for at least 6 months and liver histology showed features of chronic persistent hepatitis (CPH) in 30

patients, chronic active hepatitis (CAH) in 59 and cirrhosis in 38 patients. Serum samples were tested in parallel using the two techniques briefly described below:

A) Dot blot was performed, as described in the previous paragraph, by hybridisation of genotype-specific probes to the 5'UTR amplified sequences.

B) For the serotyping, branched oligopeptides based on the epitopes sequences mapping in the NS4 region, were used to develop an enzyme-linked immunosorbent assay (ELISA) that serologically distinguishes infection by HCV of genotype 1, 2 and 3 (Simmonds 1993).

The results are summarised in table 7. The two techniques used are almost equivalent. Only in two cases with genotype 2 and in two cases with genotype 3, the serotyping method gave discordant results indicating genotype 1 sequences. The observed discrepancies might be due, either to the detection by serotyping of past antibodies in patients recently infected with other genotypes, or to the variations of the type 2 and 3 epitopes with respect to the peptides used for the ELISA.

Table 7
Comparison of serotyping vs genotyping in 127 patients with chronic hepatitis type C

	Serotype				
	1	2	3	Unclassified	Total
Genotype					
HCV 1	44	0	0	5	49
HCV 2	2	33	0	5	40
HCV 3	2	0	14	0	16
Unclassified	2	1	0	0	3
PCR negative	12	3	4	0	19
Total	62	37	18	10	127

For this reason, we verified the sensitivity of the dot-blot method for the detection of mixed infections. One HCV genotype 1 positive serum and one HCV genotype 2 positive serum, with the same RNA level, (RNA level was established by end point dilution of the initial serum) were

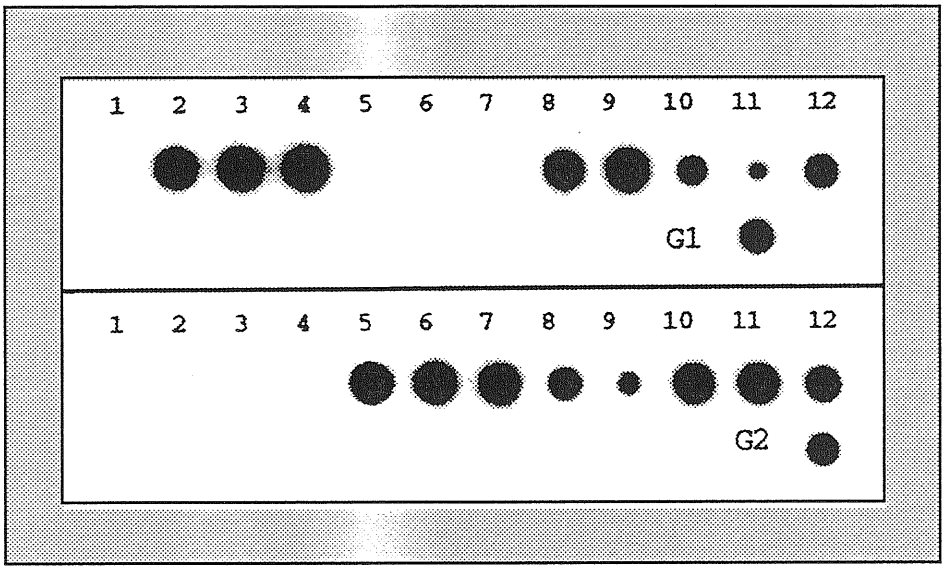


Figure 9: Sensitivity of Dot Blot Assay in detection of mixed viral infection. Position 2 shows undiluted HCV-type 1 positive serum and position 5 undiluted HCV-type 2 positive serum. Two serial 10-times dilutions of each HCV-positive serum were performed using one HCV-negative serum (position 3 and 4 for HCV-1 positive serum, 6 and 7 for HCV-2 positive serum, 1 for negative control). Alternatively, two 10-fold dilutions of HCV-2 positive serum in the corresponding HCV-1 positive serum (8 and 9) and of HCV-1 positive serum in the HCV-2 positive serum (10 and11) were carried out before PCR amplification and hybridisation with type-specific probes. In position 12, the same amounts of the two sera were mixed. A) Samples hybridising with the HCV-1 specific probe. B) samples hybridising with the HCV-2 specific probes. G1, HCV-1 positive control; G2, HCV-2 positive control.

mixed together in serial ten time dilutions before PCR amplification and hybridisation with genotype-specific probes.

This approach allowed the definition of the sensitivity of the system for the detection of multiple infection by different genotypes, which could be identified in the same serum sample by dot-blot assay, even when one of the two sequences was 100 times less frequent than the other (figure 9).

3. 1. 4. 3. Comparison between two HCV genotyping methods based on the PCR amplification of different genomic regions

The aim of this study was to compare the results of HCV genotyping by means of PCR amplification of the core region with type specific primers (the method originally described by Okamoto et al. 1992), with the results obtained by dot-blot hybridisation of genotype specific probes to amplified 5'UTR sequences.

The genotype of HCV infecting 144 Italian patients (106 from drug addicts patients and 38 from patients having chronic hepatitis C but no history of drug abuse) were analysed in parallel by the two methods.

The amplification of the HCV 5'UTR and the dot-blot assay were performed as described before.

To amplify part of the HCV core gene, directly from 200 µl of each serum, the same primers and the same RT-PCR conditions indicated by Okamoto et al. (1992) were used.

The results indicated that PCR amplification of the HCV core region was much less efficient than amplification of the 5' untranslated region, allowing genotyping of only 69/144 of the infecting HCV. On the other hand, dot-blot assay allowed the correct identification of the HCV genotype in all the 144 samples. Concordant results from the two methods were found in 61% of the cases. A more precise description of the results is shown in table 8.

The genotype determination by PCR amplification with core-specific primers resulted in an overestimation of the mixed infections as it came out from the comparison with the results of the dot-blot assay with genotype-specific probes and by direct sequencing of some of these PCR products.

Furthermore, the high rate of indeterminate samples obtained in this study using the method developed by Okamoto might be the result of mismatch between the primers used for the RT-PCR (their sequences were deduced from sequence alignment of mainly Japanese isolates) and the sequence of the core gene in most of our samples.

This last finding suggests that geographic origin of the samples, which results in sequence divergences and/or different prevalence of each genotypes, is probably the cause of discrepancies between the results obtained from these two methods.

Table 8: Comparison between HCV genotyping by PCR amplification of HCV 5'UTR and amplification of the Core region from sera of 144 patients with chronic hepatitis C

	5'UTR assay					
Core region assay	Type 1a (n=34)	Type1b (n=37)	Type2a (n=36)	Type2b (n=5)	Type3a (n=29)	Type4a (n=3)
Core negative (n=55)	7	10	17	4	12	3
Not classified (n= 16)	2	4	5	1	4	-
Type I (n=31)	18	6	4	-	3	-
Type II (n=25)	2	15	7	-	1	-
Type III (n=1)	-	-	-	-	1	-
Type IV (n=3)	-	1	1	-	1	-
Type V (n=8)	-	-	2	-	6	-
Mix (n=7)	5	1	-	-	1	-

3. 1. 5. Classification of HCV genotypes, an update.

Till date, at least 34 subtypes within nine genetic groups (genotypes) have been reported. As shown in figure 10, HCV genotypes have distinct geographical distributions. Some genotypes (types 1a,1b, 2a, and 2b) are broadly worldwide distributed, whereas others, such as type 5a and 6a, are only found in specific geographical regions.

From a practical point of view, only five of the 34 genotypes identified up to now, prevail in the most areas of the world: the five genotypes 1a, 1b, 2a, 2b, and 3a. Furthermore, only few of them have been analysed and found to be associated with severe hepatic disease and poor response to interferon. For these reasons, we considered, in our studies, the three major HCV groups 1, 2 and 3 which are also more represented in Europe and particularly, in Italy.

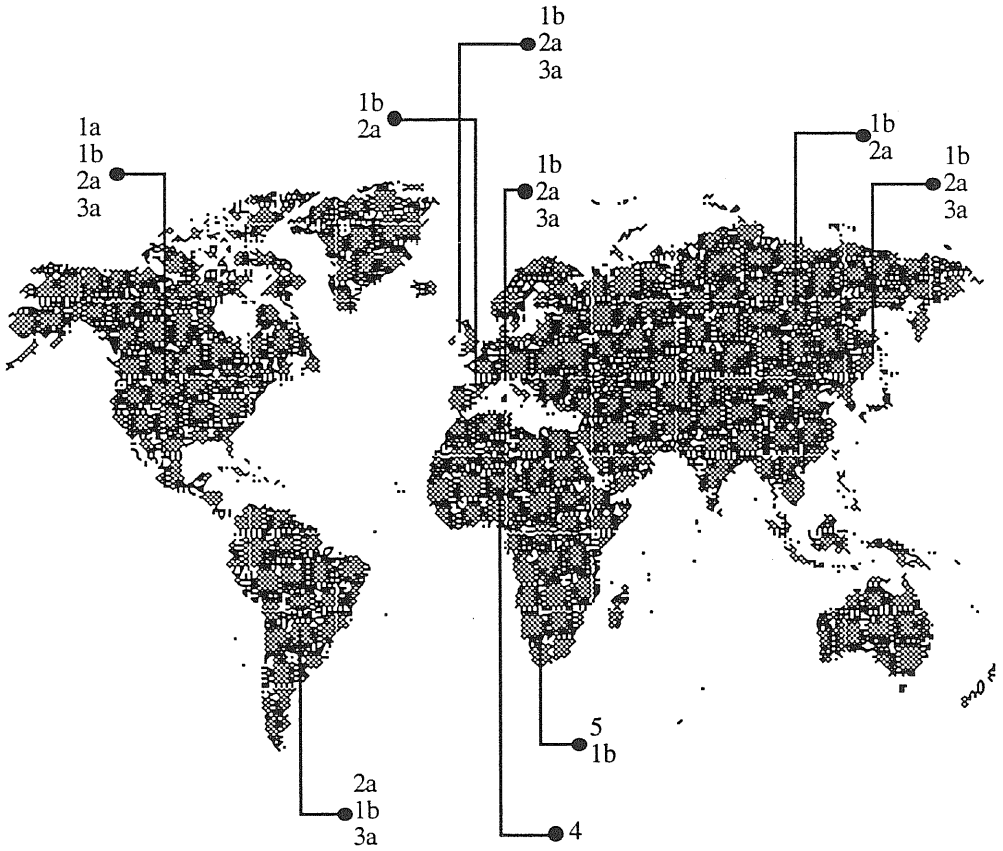


Figure 10: The geographical distribution of predominant HCV genotypes in different areas of the world, indicated in order of decreasing prevalence.

3. 2. HCV genotypes and response to interferon therapy

3. 2. 1. Introduction

The first study on the treatment of the chronic non-A,non-B hepatitis with recombinant human interferon (IFN) was done by Hoofnagle et al. in 1986. This pilot study demonstrated that: 1) interferon was active at lower doses than those required for the treatment of the hepatitis B and D; 2) immediately after the beginning of the treatment, interferon was able to induce a substantial reduction of the alanine transaminase (ALT) levels indicating an anti viral activity of IFN; 3) IFN was not able to eradicate the virus since most of the cases showed disease relapse after the treatment was interrupted. All these observation were later confirmed by a series of controlled studies (Davis 1989, Marcellin 1991).

With the discovery of the hepatitis C virus, which is responsible for the majority of the non-A, non-B hepatitis, and the availability of sensitive techniques for its detection, today it is known that the biochemical response to interferon (in terms of normalisation of ALT levels) is associated to a drastic reduction of the viremia (Brillanti 1991, Shindo 1991). Thus, the primary response to therapy is due to the anti viral effect of IFN. However, the high percentage of relapses confirms that IFN is unable to eradicate completely the virus which, indeed, persists at such low levels that it can not even be detected with the available techniques (Shindo 1992).

As a consequence, it is possible that the effect of IFN in those patients which respond to the therapy, is due to the suppression and not to the complete eradication of the virus.

The observations derived from those first studies are important and they have to be taken into consideration for the development of more efficient therapeutic approaches.

Recently, clinical and biological researches on the hepatitis C virus started to elucidate some important and useful topics for the therapy.

For example, the possibility that HCV genotype and/or viral titre (Hagiwara 1993, Mita 1994) may influence the likelihood of achieving remission has been suggested. In particular, it has been shown that the infecting HCV genotype might influence both, the natural history of the disease, and the response to IFN treatment.

Therefore, the study of the differences in the response to IFN therapy in relation to different genotypes is to be considered a pivot in the clinical research.

3. 2. 2. Identification of the HCV genotype in a population of Italian patients. Analysis of the response to IFN related to the genotype.

In our experience, the first evidence of a relationship between the HCV genotypes and the response of the patients to the interferon therapy came from a previous study in which we analysed 79 Italian patients with chronic hepatitis type C. We observed that during the therapy with IFN the transaminase levels declined to nearly normal levels in 26% of patients infected with HCV genotype 1, in 80% of patients infected with genotype 2, and in 100% of patients with genotype 3. Due to the relative small number of cases analysed at that time, we extended this study to a larger group of Italian patients.

In this study, we analysed 495 consecutive patients with chronic hepatitis C treated with interferon-alpha obtained from nine different Universities or General Hospitals homogeneously distributed in Italy (Bologna, Padova, Pordenone, Torino, Trieste, Roma, Napoli, Bari and Palermo).

All patients had been diagnosed as having chronic hepatitis C with or without cirrhosis and had completed a course of IFN-alpha therapy with schedules which were ranged from 3MU given three times weekly for 6 or 12 months, to 6 MU given three times weekly for 6,12 or 24 months. All patients were followed for at least 12 months after cessation of treatment.

HCV RNA was determined directly from each serum sample by nested PCR after reverse transcription, using primers from the 5' untranslated region as previously described.

To identify the infecting HCV genotypes, we used the dot blot hybridisation assay with type-specific probes standardised in our laboratory. Having previously observed that in Italy most of the patients with chronic hepatitis C are infected with genotype 1, 2 and 3, we used probes specific for these three major types to screen the 495 serum samples analysed in this study.

Although all 495 patients were anti-HCV positive in serum by second-generation ELISA, 17% of them were HCV RNA negative in serum by nested PCR and could not be genotyped.

The amplified cDNA from each positive sample was tested by dot blot hybridisation with the three HCV genotype-specific probes. HCV 1 was found in the 57% of the cases, HCV 2 in 31%, and HCV 3 in 8%. Two sera were found to contain a mixed infection being equally reactive with HCV 1 and HCV 2 specific-probes.

Direct sequence performed in 93 of these samples confirmed the results obtained with the dot-blot method. Furthermore, twelve samples (3%) which had been not recognised by any of the three probes, after sequence analysis were found to contain sequences of the HCV 4 genotype (infections with this type are typically described in Egypt).

The only relevant data obtained by the relation between genotypes and possible cause of infection (table 10) indicates that HCV 3 is highly represented in patients with history of drug abuse being found in 53% of those cases.

Results from the analysis of the response to interferon therapy in relation to the genotype are described in table 9.

Table 9: Response to therapy in relation to total IFN-alpha dose

	All patients(%)			<600 MU (%) (271 cases)			>600MU (%) (140 cases)		
	NR*	TR	SR	NR*	TR	SR	NR*	TR	SR
HCV 1	53	32	15	59	41	10	32	36	32
HCV 2	20	33	47	21	31	48	19	30	51
HCV 3	0	32	68	0	43	57	0	0	100

*NR, non-responders; TR, transient responders; SR, sustained responders.

	HCV1	HCV2	HCV3	PCR negative (%)
Blood transfusion	23	32	5	27
Surgery	24	28	21	25
Hystory of drug addiction	9	2	52	30
Unknown	44	38	22	18

Table 10: Risk factors distribution in relation to HCV genotype in chronic hepatitis type C

Compared to patients infected with HCV 2 and HCV 3, the number of non-responders was higher in patients infected with HCV 1 in which also the rate of sustained responses (patients with ALT normalisation for 12 months after therapy) was significantly lower.

As the patients were treated with different schedules of IFN, we could analyse the sensitivity of the different HCV genotypes to IFN-alpha. To do that, patients were divided into two groups considering the total amount of IFN received. The higher dose of IFN substantially increased the number of responses in patients with genotype 1 (from the 15% of responders in cases treated with less than 600MU, to 32 % in those given more than 600MU), and led to sustained response in all patients with genotype 3. No substantial modifications were seen in patients with genotype 2 even when high doses of IFN were used.

3. 2. 3. The clinical significance of subtype 1a and subtype 1b

In the previous study, we showed that genotype 1 is generally less responsive to IFN treatment. In particular, while other studies were suggesting that subtype 1b is also associated to a more severe stage of disease, little was known about the behaviour of subtype 1a.

Since subtypes 1a and 1b are broadly distributed world-wide (subtype 1b is found in the majority of the Japanese patients, while, subtype 1a is mostly represented in the American population), it was important to investigate if significant differences exist between the two

HCV types and, above all, if it is important to distinguish them for clinical assessment.

In an attempt to define the clinical profile of patients infected with HCV 1a and HCV 1b, we analysed 159 patients with chronic hepatitis C previously recognised (using the dot blot assay) to be infected by HCV genotype 1.

Sequences of subtype 1a can not be distinguished from 1b with the genotype-specific probes used in the previously described dot-blot assay, since their 5'UTR nucleotide sequences differ only in a single position (type 1a has a A at position 244 while type 1b has a G).

On the other hand, comparison of the 1a and 1b 5'UTR sequences revealed that , in the case of subtype 1b, the G at position 244 creates a restriction site for the BstUI enzyme. Accordingly, the subtypes of genotype 1, 1b produces three DNA fragments of 137 bp, 30 bp, and 44 bp and that of 1a only two fragments of 167 bp and 44 bp respectively (as shown in figure 11).

Due to the small sizes, different fragments were separated by electrophoresis on 10% polyacrylamide gel and visualised by UV light after ethidium bromide staining.

We identified 144 patients (91%) as being infected by HCV 1b and the rest (9%) as being infected by HCV 1a. These results were confirmed by direct sequence of 38 of these samples.

Unfortunately, a great difference in number of subjects in the two groups did not allow significant statistical analysis. However, there were not substantial differences when age, histological findings, and risk factors were compared. Interestingly, primary interferon response was different between the two groups, confirming the low response in patients infected with HCV 1b. However, sustained response in patients with HCV 1a was still lower than that described in patients infected with genotypes 2 and 3 (Chemello 1994, Dusheiko 1994).

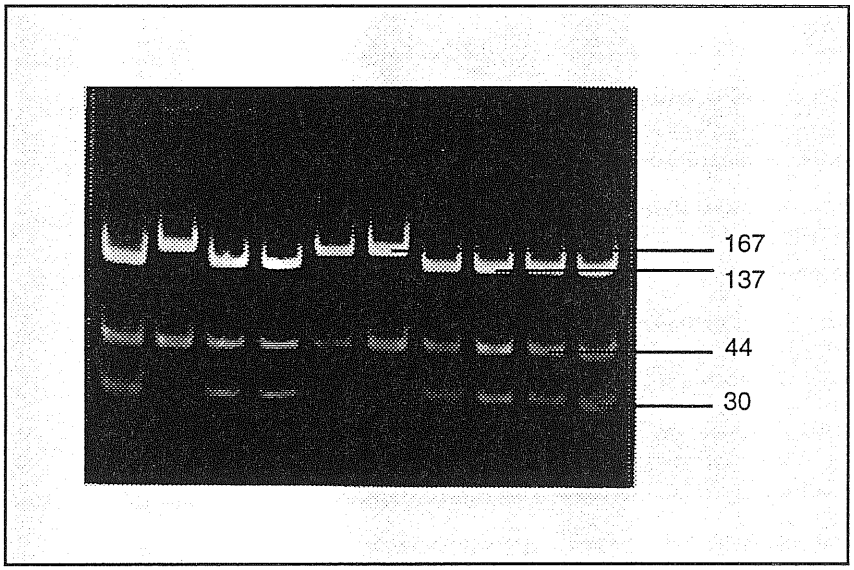


Figure 11: Electrophoresis on 10% polyacrilamide gel of PCR products, representing HCV 5'UTR (from position 105 to 316), after digestion with BstUI enzyme for determination of HCV subtypes 1a and 1b. HCV1a: two fragments of 167 pb, and 44 pb; HCV-1b: three fragments of 137 pb, 44 pb, and 30 pb.

3. 3. HCV infection and autoimmune chronic liver disease

3. 3. 1. Introduction

There is a complicate relationship between HCV infection and autoimmune chronic liver disease.

In Italy, anti-HCV antibodies were generally found in patients with autoimmune chronic hepatitis (Magrin 1991, Lenzi 1991) regardless of the type of autoimmunity. Additionally, the HCV antibodies reactivity found in Italian patients with autoimmune features was often associated with HCV RNA detection by RT-PCR (67%).

Similarly, HCV RNA was found in Japanese patients positive for HCV antibodies and with autoimmune chronic hepatitis even if in a lower percentage (19%) (Nishiguchi 1992).

In contrast, only 1 of the 17 (6%) U.S. patients analysed with autoimmune chronic hepatitis and positive for serological markers of HCV infection, were found HCV RNA positive (Fried 1993).

However, the association between HCV and type 2 autoimmune chronic liver disease suggests that humoral autoimmune reaction might be triggered by the virus infection. In type 2 autoimmunity, autoantibodies to liver and kidney microsomes (anti-LKM) are found, while type 1 autoimmune hepatitis is characterised by anti-nuclear antibodies. Anti-LKM reactivities, with different fine antigenic specificities, are indicated as anti-LKM1 (recognises cytochrome P-450IID6), anti-LKM2 (associated to ticrynafen-induced liver disease), and anti-LKM3 (found in patients with delta virus infection).

Patients with anti-LKM1 autoantibodies frequently have antibodies to HCV and HCV RNA detectable (Lenzi 1991, Garson 1991).

3. 3. 2. Analysis of the HCV genotype in Italian patients with anti-LKM1autoantibodies

In this study we investigated whether type 2 autoimmune chronic hepatitis might be associated with one particular HCV genotype or subtype.

Twenty-two patients were analysed (9 males and 13 females) with a median age of 50 years. They were all selected on the basis of

1) elevated ALT levels for at least 12 months and histological evidence of chronic hepatitis; 2) anti-HCV seropositivity confirmed by second generation ELISA (Orto Diagnostics, Raritan NJ, USA) and RIBA 2 (Chiron Corporation, Emmeryville, CA, USA), 3) serum positivity for anti-LKM1 by indirect immunofluorescence (Lenzi 1990). For the majority of these patients the source of infection was varied, with two patients having a history of intravenous drug abuse, and two patients being recipient of blood transfusion.

Seventy-nine patients with chronic hepatitis C, negative for autoimmune markers, were included in the study as control group.

HCV RNA was detected in the sera of patients by RT-PCR using primers specific for the 5'UTR as described before. For the genotype determination, PCR amplification products were hybridised with genotype-specific probes by dot blot assay. To confirm the results obtained by dot-blot assay, in ten cases, the nucleotide sequence of the PCR product was determined by direct sequencing.

All the 22 anti-HCV and anti-LKM1 sera samples were positive for HCV RNA. Results from hybridisation with probes specific for HCV genotype 1, HCV genotype 2, and HCV genotype 3 are shown in figure 12. 17 anti-LKM1 positive patients were found infected with HCV genotype 1 (77%), 4 patients with HCV genotype 2 (18%), and 1 patient with HCV genotype 3 (5%).

Prevalence of genotype 1 and 2 were significantly different from those obtained from the analysis of the 79 anti-LKM1 negative patients of the control group. HCV of genotype 1 was much more frequently represented in anti-LKM1 positive patients than in patients with only HCV infection.

Patients infected with all the three genotypes did not represent substantial differences in sex, transaminase levels, and titres of anti-LKM1 antibodies.

Furthermore, as shown by the dot blot hybridisation assay in figure 12, three of the four samples reacting with genotype 2 probe, gave only a weak hybridisation signal. This phenomena, previously observed in about 2.5% of all the samples analysed by the dot-blot method, is generally associated to sequence variation within the area of the 5'UTR recognised by the probes.

Four anti-LKM1 samples reacting with probe 2, five samples hybridising with probe 1, and one reacting with probe 3, were analysed by direct sequencing.

After alignment with the prototype sequences of genotypes 1, 2, and 3, all the three sera which gave a weak signal with HCV 2 probe were found to contain nucleotide substitutions in the area recognised by the specific probe. On the other hand, sequences obtained from seven additional samples of genotype 1, one of the four samples of genotype 2, and one of genotype 3, showed complete identity to their respective reference strain.

An interesting finding was that two of the four variants of genotype 2 had the same nucleotide substitutions (G to A in position 180, C to T in position 221), and that, up to date, not one identical 5'UTR was found in patients with chronic hepatitis C infected by HCV of genotype 2.

According to the model of the 5'UTR proposed by Brown et al.(1992), the region recognised by the genotype-specific probes, where the mutations of the genotype 2 sequence were observed, plays an important role in the determination of the secondary structure of the 5'UTR.

The importance of the secondary structure of the 5'UTR, which has been reported to show discrete variations among different genotypes, was stressed for the first time by Tsukyiama-Kohara (1992), who suggested its possible role in controlling the translation initiation of the HCV RNA genome.

By computer assisted sequence analysis, using the RNA FOLD program, we obtained the secondary structure of the 5'UTR derived from the three HCV isolates of genotype 2 containing nucleotide substitutions, and the results were then compared to the structure obtained from the reference genotype strain.

In all cases the stem-loop structure, derived from the portion of the 5'UTR sequence analysed, is maintained. In particular, the single substitution found in one of the three HCV genotype 2 variants had no effects on the base pairing within the proposed stem-loop; while, the two compensatory substitutions found in the other two genotype 2 variants, resulted in an increase in the stability of the same structure.

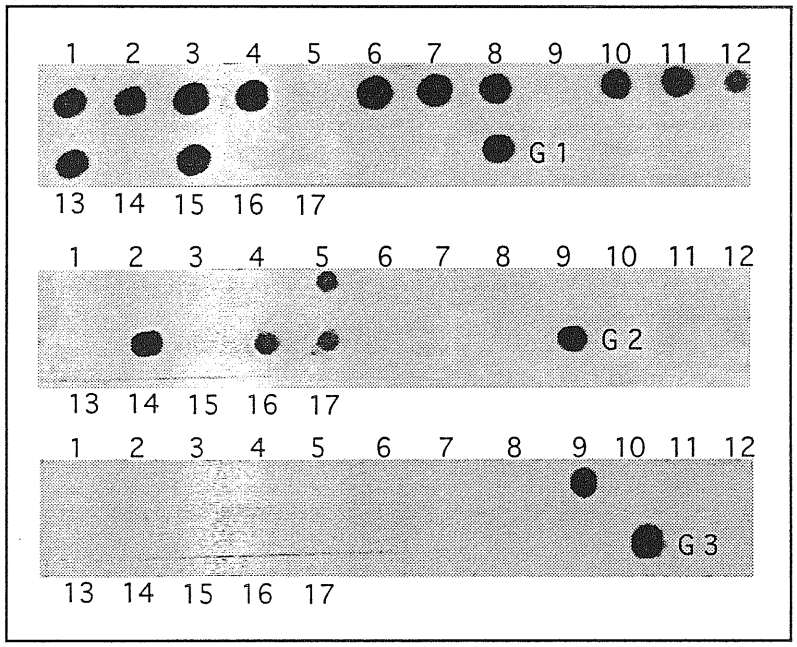


Figure 12: HCV genotyping by Dot Blot Assay. Genotype-specific probes are hybridised with cDNA/PCR fragments from anti-HCV, anti-LKM-1 positive patients with autoimmune chronic hepatitis. The weak signals from samples 12, 5, 16, and 17, are due to sequence variations within the area recognised by the probes.

3. 4. Persistent hepatitis C viremia without liver disease: possible implication for HCV genotype and level of viremia.

Initially, the introduction of tests for screening for hepatitis C virus in donated blood led to the recognition of asymptomatic blood donors positive for anti-HCV antibodies. Later on, the development of the amplification of the HCV nucleic acid sequences by polymerase-chain reaction (PCR) permitted to identify, within this group, HCV "asymptomatic carriers" having antibodies against HCV and HCV RNA despite having no symptoms of liver disease. Moreover, asymptomatic carriers include patients that responded to interferon therapy in which HCV viremia has been found to persist even in absence of liver disease symptoms.

Little information was available so far about symptomless carriers and about the eventual correlation between virological parameters of HCV infection and the clinical course.

The aim of this study was to investigate whether the prevalence of HCV viremia and genotypes might influence the course of infection in HCV-RNA positive patients with no evidences of hepatitis as assessed by liver function tests.

Two distinct groups of asymptomatic carriers were analysed in this study: a first group of long-term responders to IFN therapy which remained HCV-RNA positive, and a second group of HCV carriers with persistetly normal transaminse values, despite anti-HCV and HCV RNA positivity.

3. 4. 1. Analysis of HCV RNA in a group of sustained responder patients before and after interferon therapy.

A serie of 114 patients (all treated in randomised clinical trials conducted in the Clinica Medica II of the University of Padova), which had shown biochemical response to interferon, in terms of persistent normal ALT levels maintained up to 12 months after cessation of therapy, were tested for HCV-RNA positivity by RT-PCR. The 27 patients (corresponding to the 24%), who were persistently HCV-RNA positive despite ALT normalisation after therapy, were selected for this study.

3. 4. 1. 1. HCV genotyping

Viral HCV-RNA was detected with RT-PCR using two sets of primers for the amplification of the 5'UTR sequence as described in chapter 2. Infecting HCV genotypes were identified by dot-blot hybridisation using three genotype-specific probes (corresponding to genotype 1, 2 and 3).

The dot-blot assay showed that only 1 out of the 27 patients was infected by HCV genotype 1, while all the others were infected by HCV genotype 2.

Furthermore, compared to what was previously observed from the same genotype analysis in the whole group of 114 biochemical sustained responders before treatment, these 27 patients conserved the same viral genotype after the therapy. Nevertheless, the distribution of genotype 2 within these 27 patients was significantly higher then that observed in the original group.

3. 4. 1. 2. Quantification of serum HCV RNA

The level of viremia was determined in the 27 HCV RNA positive patients before and six months after the treatment using a recently developed PCR system: the branched DNA (bDNA) signal amplification (Amplicor HCV Monitor, Roche Molecular System, Inc., Branchburg, NJ). This method includes the co-amplification of a quantitiation standard (QS) and the target HCV-RNA. Briefly, HCV RNA extracted from the serum was amplified by RT-PCR in the same tube as the quantitiation standard using rTth polymerase. Amplified products were heat denatured and loaded onto the detection plate. Serial 5 fold dilutions were carried out, followed by parallel hybridisation with the probes specific for the 5'UTR and for the quantitation standard previously coated on the microwell plate. Computer assisted analysis of the results obtained from the enzymatic reaction and the O.D. measurement at 450 nm, allowed to identify from 10^3 to 10^5 copies of HCV-RNA/ml in a linear range. Paired sera from the same patient, obtained before and from 6 up to 36 months after treatment, were analysed in the same run of assay.

Despite a wide range of HCV-RNA levels in each patients, the viral RNA load was either unchanged or elevated after treatment in majority of the patients (table 11).

Table 11: HCV RNA levels observed in 27 sustained biochemical responder patients with ongoing HCV replication

	Before treatment * (Copy number /ml)	After treatment ** (Copy number /ml)
Mean ± SD	38,637 ± 96,459	97,880 ± 213,195
Median	7,642	30,716
Range	2,000 - 404,472	2,000 - 894,413

* VS ** p=0.0045 (Wilcoxon matched pairs test)

3. 4. 1. 3. Sequence analysis of the HCV 5'UTR

The 5'UTR was analysed by direct sequencing of the PCR products obtained from, both, pre- and post-treatment serum samples of 14 of the 27 HCV-RNA patients.

Direct sequence of the amplified products was performed using the Sequenase PCR Product Sequencing Kit (Amersham, UK) according to manufactures instructions. Sense primer S2, and antisense primer AS2 (the inner primers for the "nested" PCR) were used to determine the sequence in both the directions.

In some cases, the simultaneous finding of two nucleotide within the same sequence, revealed the presence of a mixed infection. This phenomena was repeatedly found in different patients, either before or after treatment and was generally associated with higher viremic values. Furthermore, in some of these cases, sequence from a third serum sample (drown one or two years after the cessation of the therapy) showed, again, the selection of one of the two sequences (an example is shown in figure 13).

In the case of one patient, we could analyse the 5'UTR sequence of the infecting virus corresponding to a late biochemical reactivation documented about four years after therapy. As shown in figure 14, in correspondence to the increased ALT value in the serum, associated

to an higher viral titre, this patient was having a mixed infection. Few months later, the patient (who in spite of the normalization of the ALT level, was still HCV RNA positive) and showed again a single sequence which was identical to the one found before the therapy.

Compared to the reference sequence (all but one where classified as genotype 2a), single nucleotide variations were found in several patients, and they were often conserved in the sequences derived from both, the pre- and post-treatment serum samples.

When localised within the secondary structure of the 5'UTR, these nucleotide changes did not affect the proposed structure of the stem-loop of domain III (Brown et al. 1992) where all of them were located.

Furthermore, the 5'UTR of the single isolate classified as genotype 1 found in this study showed a peculiar mutation compared to the HCV-1 prototype sequence. The same mutation, an adenosine inserted in position 205 (within the upper portion of the same stem-loop structure), was conserved in the viral sequence found in this patient before and after the IFN treatment.

3. 4. 2. Analysis of HCV RNA in a group of healthy-carriers

The five patients included in this group were discovered anti-HCV positive (by second-generation enzyme immunoassay and recombinant immunoblot assays) after an occasional laboratory check-up.

In all the five cases HCV RNA was detected, in the same serum sample used for serology, by nested RT-PCR using primers localised in the 5' UTR sequence. Although they had a high titre of anti-HCV antibodies and were positive for HCV-RNA detection, these patients had persintently normal alanine (ALT) and aspartate (AST) aminotransferase activities. Furthermore, physical examination, and a battery of liver-function tests revealed no sign of liver disease.

Clinical records revealed that during the previous years (up to seven years in one case) abnormal ALT values were not found. The source of infection was unknown in three; two had possible occupational exposure to HCV.

3. 4. 2. 1. HCV genotyping and sequence analysis of the 5'UTR

Determination of the HCV genotypes, by dot-blot hybridisation assay, showed that two patients were infected by genotype 1b and three patients were infected by genotype 2.

Direct sequencing was performed using the same PCR amplification products. In all the five cases the results obtained by dot-blot analysis were confirmed, and in four of them the 5'UTR sequence was identical to the prototype sequence.

In one case the HCV isolate, classified as genotype 2, showed four nucleotide changes compared to the reference genotype 2a sequence (G to A in position 181, A to G at nt 184, T to C at nt 215, and C to T at nt 220), and not one identical sequence has been described so far. These mutations, which were localised within the same stem-loop structure previously considered, as compensatory substitutions, resulted to preserve this structure intact.

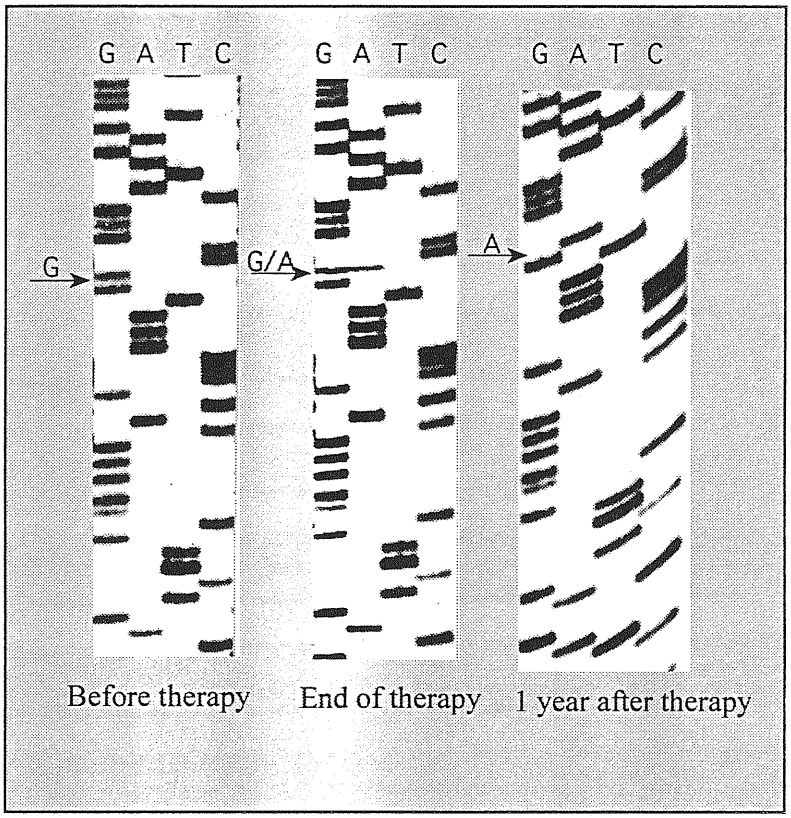


Figure 13: Direct sequence of the HCV 5'UTR from three sera samples obtained before and after IFN treatment in one patient with biochemical sustained response to the therapy.

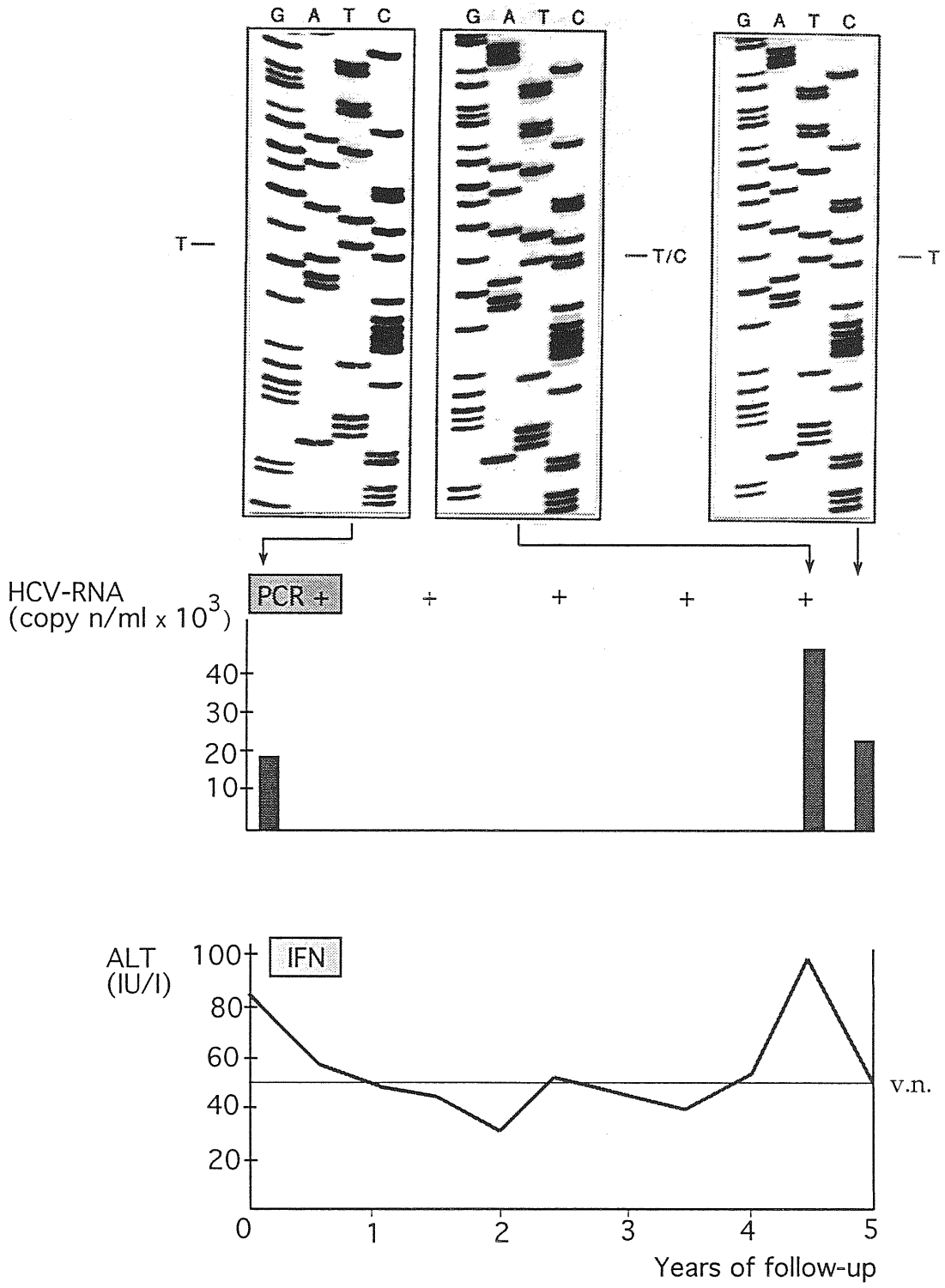


Figure 14: 5'UTR sequences observed in sequential serum samples of a sustained responder with late reactivation in relation to clinical profile.

RESULTS-Part II

3. 5. Functional implication of different 5' untranslated regions of hepatitis C virus RNA: influence on reporter gene expression.

The HCV genotypes have been associated with differences in the course of disease and in the response to interferon treatment. However, from a virological point of view, it is impossible to predict whether the degree of sequence variability between genotypes would have any influence on the biological behaviour of the virus.

The complex structural feature of the 5'UTR provides the regulatory determinant for several essential viral functions. Recognition signals for viral or cellular factors that mediate viral replication are thought to reside in this portion of the RNA genome.

Furthermore, a considerable number of studies provided important evidences about the presence, within the 5'UTR, of an internal ribosomal entry site (IRES) element (Tsukiyama-Kohara 1992, Kettinen et al. 1993, Wang et al. 1993, Reynolds et al. 1994).

Therefore, the sequence variability in the 5'UTR may be one of the determinants of the characteristics of the viral infection *in vivo* and of the relative pathogenicity between genotypes.

The complex 5' untranslated region is far more conserved within the virus groups (6% sequence divergence between type 1 and type 2 sequences compared with 33% over the whole genome). Comparison of the 5'UTR sequence from different HCV reveals that the variation is limited to particular sites (Chan 1992, Bukh 1992).

Despite the little nucleotide variation observed among the 5'UTRs of different HCV genotypes, the highly ordered structure of the RNA 5' terminus is conserved in all cases. Significantly, most of the nucleotide changes found in the different HCV strains are thought to be involved in the maintenance of the base-pairing (covariant substitutions) (Simmonds 1993b, Tsukiyama-Kohara 1992) of the region between 140 and

290, the proposed structure of which forms an extended stem-loop with several side branches (Brown 1992).

The proposed interactions of both viral and cellular proteins with the 5'UTR of viral RNA clearly constrains possible sequence variability in this region.

As a consequence, the little sequence divergence existing between the 5'UTRs of different genotypes and subtypes, may have implications for theories about the structure of the HCV 5'UTR and its function as internal ribosome entry site.

Our interest in this respect, was to analyse the translational efficiency of HCV genotypes 1, 2, and 3, since nucleotide variations within the 5'UTR sequences of these genotypes may provide some insight into functionally important structures of internal initiation of translation.

We also analysed the possible effects of naturally occurring variations we found in the 5'UTR sequence of some isolates, located at those sites where the 5'UTR secondary structure is remarkably conserved.

Finally, we used site-directed mutagenesis to map structural elements within the 5'UTR which have an important role in the IRES-mediated internal initiation of translation.

3. 5. 1. Translation directed by 5'UTRs of genotype 1, 2, and 3.

From the comparison of the 5'UTR sequences of different HCV genotypes and subtypes can be referred that most of the type-specific polymorphisms are located within the stem structure immediately downstream of the apical loop of domain III, which represents the largest secondary structure of the 5'UTR.

Several lines of evidences suggest a functional role of domain III and, in particular of some conserved elements within the apical stem-loop structure, in the IRES mediated initiation of viral protein translation.

In order to analyse the efficiency of different 5'UTRs to initiate protein translation, the 5'UTR sequences of HCV genotype 1b, 2a, and 3a were linked to the coding sequence of chloramphenicol acetyl transferase (CAT) mRNA and the level of expression of CAT protein was assessed both *in vitro* and *in vivo*.

A series of constructs was generated in which 5'UTR sequences of different HCV strains were joined in frame to the CAT gene of

plasmid pUL3475 (kindly provided by d'Adda di Fagagna, ICGEB, Trieste) immediately downstream to the T7 bacteriophage RNA polymerase promoter. A fragment representing the 5'UTR sequence from position 35 to position 341 was amplified by RT-PCR directly from positive serum samples of patients infected with HCV genotype 1b, 2a, and 3a only. Primers used for both the reverse transcription and PCR amplification are describes in chapter 2. The PCR product, which contains a KpnI and a Hind III site at the 5' and 3' terminus respectively, was ligated in the KpnI-Hind III site of plasmid pULB3475 polylinker in order to eliminate plasmid sequences between the HCV 5'UTR and the CAT gene.

The expression of the CAT gene in the derived plasmids pULB5'UTR1, pULB5'UTR2, and pULB5'UTR3 is dictated by the HCV 5'UTR of genotype 1b, 2a, and 3a respectively.

3. 5. 1. 1. *In vitro* experiments

In order to determine the translational profile of these synthetic RNAs *in vitro* we adopted two distinct strategies:

A) Plasmid DNAs, prepared both by CsCl density gradient centrifugation and by Qiagen anion-exchange column, were used to perform *in vitro* transcription and translation of various CAT expressing mRNAs in rabbit reticulocyte lysate (RRL) with T7 TNT kit (Promega) according to the manufacturer's instructions.

B) Each RNA was synthesised by transcription of the DNA sequence of various plasmids with T7 RNA polymerase. The RNAs produced were designed to have a polyA tail (SV40 polyA) at the 3' end to increase its stability in the RRL reaction mix. This approach allows an efficient production of a large amount of RNA with uniformly defined 5' and 3' ends. Equimolar quantities of various RNAs were used in translation experiments performed in a cell-free expression system using rabbit reticulocyte lysate (Promega).

All the RNAs generate CAT protein of the expected size, and the results obtained with both the strategies (A and B) were comparable. The results of these *in vitro* experiments, can be summarised as follows: the amount of CAT protein obtained with RNAs from plasmid pULB5'UTR2 and plasmid pULB5'UTR3 were virtually indistinguishable from another, while a slight decrease was observed in the level of CAT protein expressed

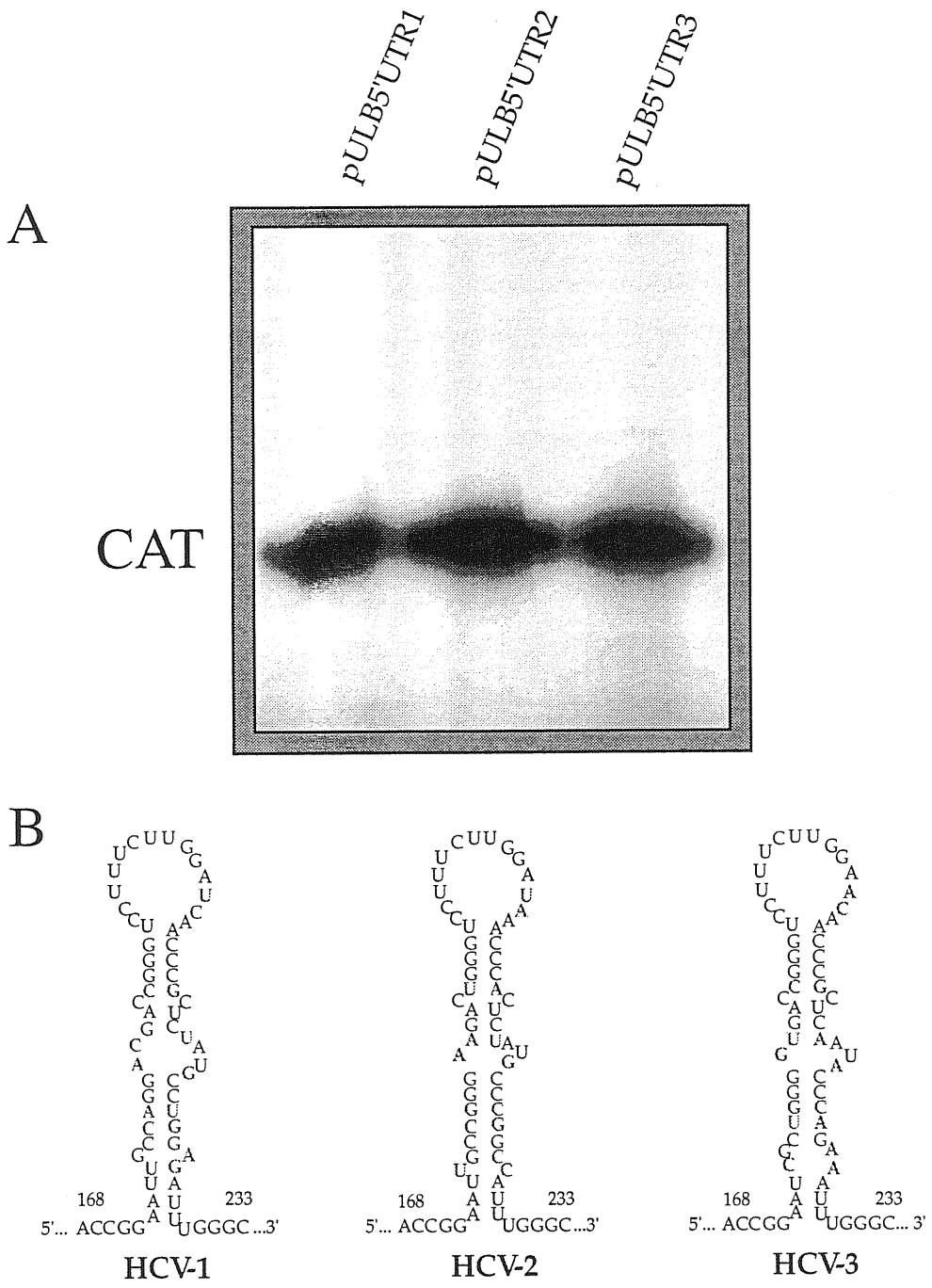


Figure 15: In vitro translation of rabbit reticulocyte lysetes programmed with transcripts encoding CAT under translational control of the HCV 5'UTR. A) Fluorography of SDS-PAGE gel showing products of in vitro translation reactions programmed with transcripts derived from plasmids pULB5'UTR1, pULB5'UTR2, and pULB5'UTR3, which contain the 5'UTR sequences of HCV genotype 1, genotype 2, and genotype 3 respectively. B) Schematic representation of the predicted secondary structure of the RNA sequences from nucleotide 168 to 233 of the 5'UTR of HCV genotype 1, 2, and 3. Genotype-specific nucleotide variations are indicated.

by RNA derived by plasmid pULB5'UTR1 (figure 15 shows the results of the *in vitro* translation experiment using synthetic RNAs).

3. 5. 1. 2. *In vivo* experiments

In order to determine the translational profile of these monocistronic constructs *in vivo*, we transfected plasmid DNA into HeLa cells using Lipofectin. Cells were previously infected with vTF7-3, a vaccinia virus that express the T7 DNA dependent RNA polymerase (Fuerst 1986). After the virus was left to adsorb for 45 minutes at room temperature, equimolar amounts of plasmids pULB5'UTR1, pULB5'UTR2, and pULB5'UTR3 were transfected into cells. As a positive control, we transfected cells with the same molar amount of the original vector pULB3475. 24 hours post-transfection the cells were lysed and CAT activity was normalysed against protein concentration in the extract.

The CAT expression level of each transfected plasmid containing the HCV 5'UTR sequences was quantified by scintillation counting and optical scanning. The values of CAT expression with plasmids containing the 5'UTR sequences from genotype 2 and 3 (pULB5'UTR2, pULB5'UTR3) were calculated relative to the activity of plasmid pULB5'UTR1, containing the 5'UTR sequence of genotype 1, and expressed in percentage. The calculated values are representative of three independent experiments

The results (shown in figure 16) are as follows: pULB5'UTR1, 100%; pULB5'UTR2, 132%; pULB5'UTR3, 44%. These results were consistent with those obtained by *in vitro*, exept for the case of plasmid containing the 5'UTR of genotype 3. Althogh plasmid pULB5'UTR3 was as efficient as plasmid pULB5'UTR2 *in vitro*, *in vivo* this plasmid resulted in a three-fold reduction of CAT expression compared with plasmid pULB5'UTR2.

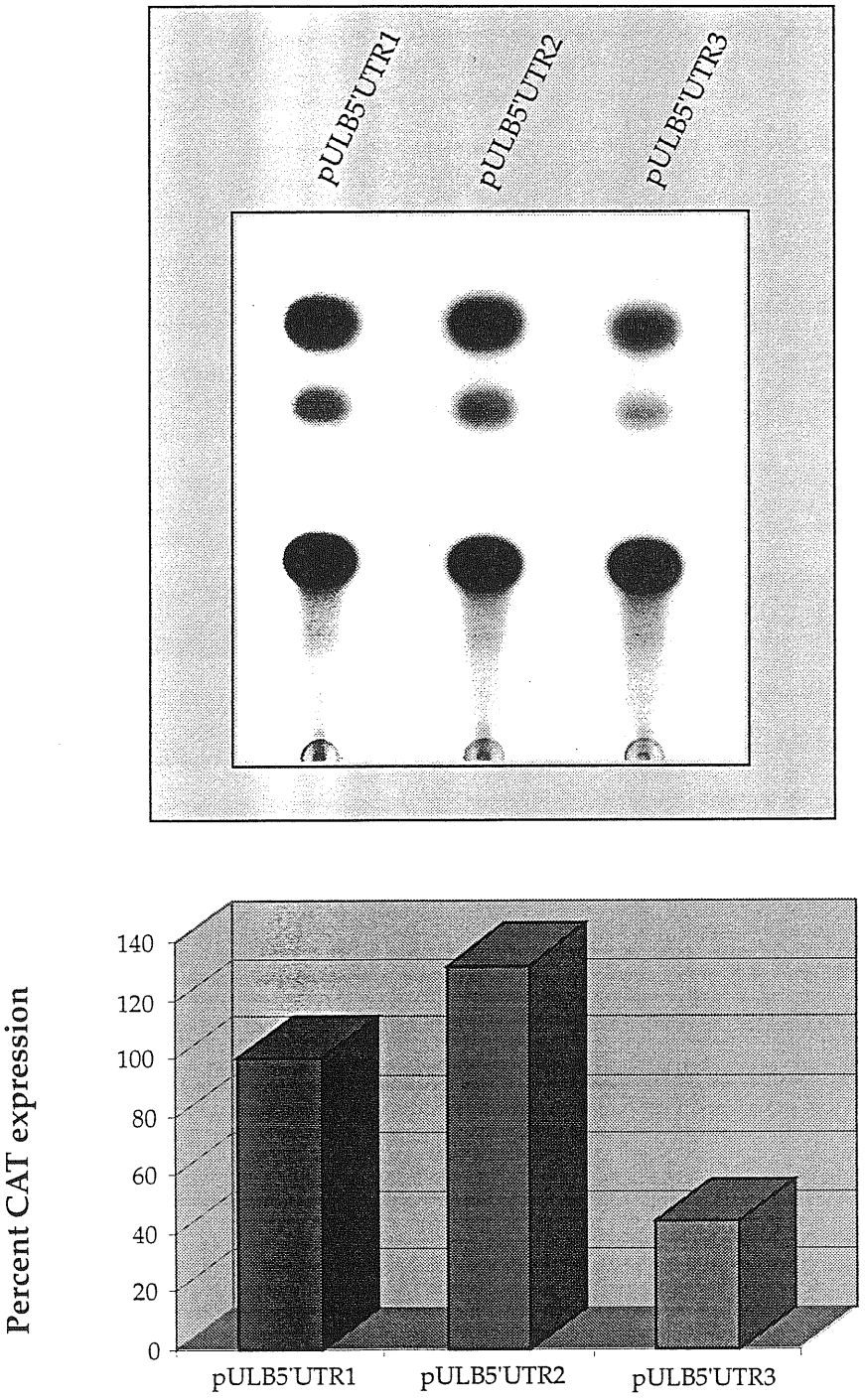


Figure 16: A) CAT expression in HeLa cells after transfection with the indicated constructs (pULB5'UTR1, pULB5'UTR2, and pULB5'UTR3) in which CAT gene expression is under control of the 5'UTR of HCV genotype 1, 2, and 3 respectively.
B) CAT activities, determined as described in Materials and Methods, are shown as values relative to that with the 5'UTR of HCV genotype 1 (100%).

3. 5. 2. Translation directed by 5'UTRs of two naturally occurring variants of HCV type 1 and type 2.

The two serum samples used in this study were selected from a previously analysed population of asymptomatic HCV infected individuals (see paragraphs 3.4.1.3. and 3.4.2.1.). HCV RNA was detected in sera samples by RT-PCR amplification using sets of primers specific for the 5'UTR. Dot blot analysis with type-specific probes, allowed to classify one of the HCV isolate as genotype 1, and the other as genotype 2. The PCR fragments representing the 5'UTR of both the isolates were cloned, sequenced, and compared to their reference HCV sequences (HCV-J for genotype 1, Kato 1990; HC-J6 and HC-J8 for genotype 2, Takamizawa 1991, Okamoto 1991). Sequence analysis revealed an adenine inserted in position 205 in the 5'UTR sequence of type 1, infecting the first patient whereas, the 5'UTR sequence of type 2, found in the second patient, had 4 different mutations (G to A at nt 181, A to G at nt 184, T to C at nt 215, and C to T at nt 220).

The sequences were confirmed by the analysis of new clones derived from at least two new RT-PCR amplifications from each serum sample.

Interestingly, the nucleotide variations found in both the isolates, were located within the highly variable region of the 5'UTR which was predicted to form the large stem-loop structure containing important cis-acting elements for the IRES function.

In particular, computer assisted analysis of the 5'UTR sequence variant of type 2, revealed that the 4 mutations found were not affecting either the shape or the stability of the stem being compensatory substitutions (position 181 contains an A residue which can be predicted to bind to T at nt 220, and at position 184 an A substituted for G maintains base pairing with C substituted by a T at position 215).

On the other hand, the nucleotide insertion described in the type 1 variant was localised to the non-base-paired terminal loop and would therefore have no effect on base pairing within the proposed stem structure (see figure 17B).

However, since no one of these mutations have been described so far, we decided to use these two 5'UTR sequences to analyse, both *in vitro* and *in vivo*, the possible influence of these two unusual

sequence variations on the efficiency of the 5'UTR to drive cap-independent translation initiation.

The 5'UTR sequence from nt 35 to nt 341 of the HCV variants of type 1 and type 2 were obtained, directly from the serum, by reverse transcription of viral RNA and PCR amplification using the same primers and reaction conditions previously described. The amplified 5'UTR sequences were inserted between the T7 promoter and the CAT gene of vector pULB3475 using the same cloning strategy adopted in the previous study. The two derived constructs were named as follows: pULB5'UTR1-205 for plasmid containing the sequence variant of type 1; pULB5'UTR2-181\184\215\220, plasmid containing the sequence variant of type 2.

3. 5. 2. 1. *In vitro* experiments

Plasmid pULB5'UTR1-205 and plasmid pULB5'UTR2-181\185\215\220 were tested for their efficiency to express CAT protein in rabbit reticulocyte lysates (RRL) in presence of T7 RNA polymerase using the transcription-translation Kit (T7 TNT Kit, Promega). In each experiment, the efficiency of CAT expression of these constructs was compared to that of plasmid pULB5'UTR1 and plasmid pULB5'UTR2 containing the corresponding wild type 5'UTR sequences.

Furthermore, we performed similar *in vitro* translation experiments in which equimolar amounts of synthetic RNAs, (previously obtained with T7 DNA dependent RNA polymerase) were translated in rabbit reticulocyte lysates (Promega).

The results obtained with these two methods were comparable and they are represented in figure 17A. Both RNAs from plasmid pULB5'UTR1-205 and plasmid pULB5'UTR2-181\185\215\220 showed a lower efficiency of CAT expression when compared with RNA from plasmids pULB5'UTR1 and pULB5'UTR2 respectively.

3. 5. 2. 2. *In vivo* experiments

To determine whether the 5'UTR sequences contained in plasmids pULB5'UTR1-205 and pULB5'UTR2-181\185\215\220 influence the efficiency of translation initiation *in vivo*, we transfected these plasmids into HeLa cells. Cells were first infected with recombinant vaccinia virus to

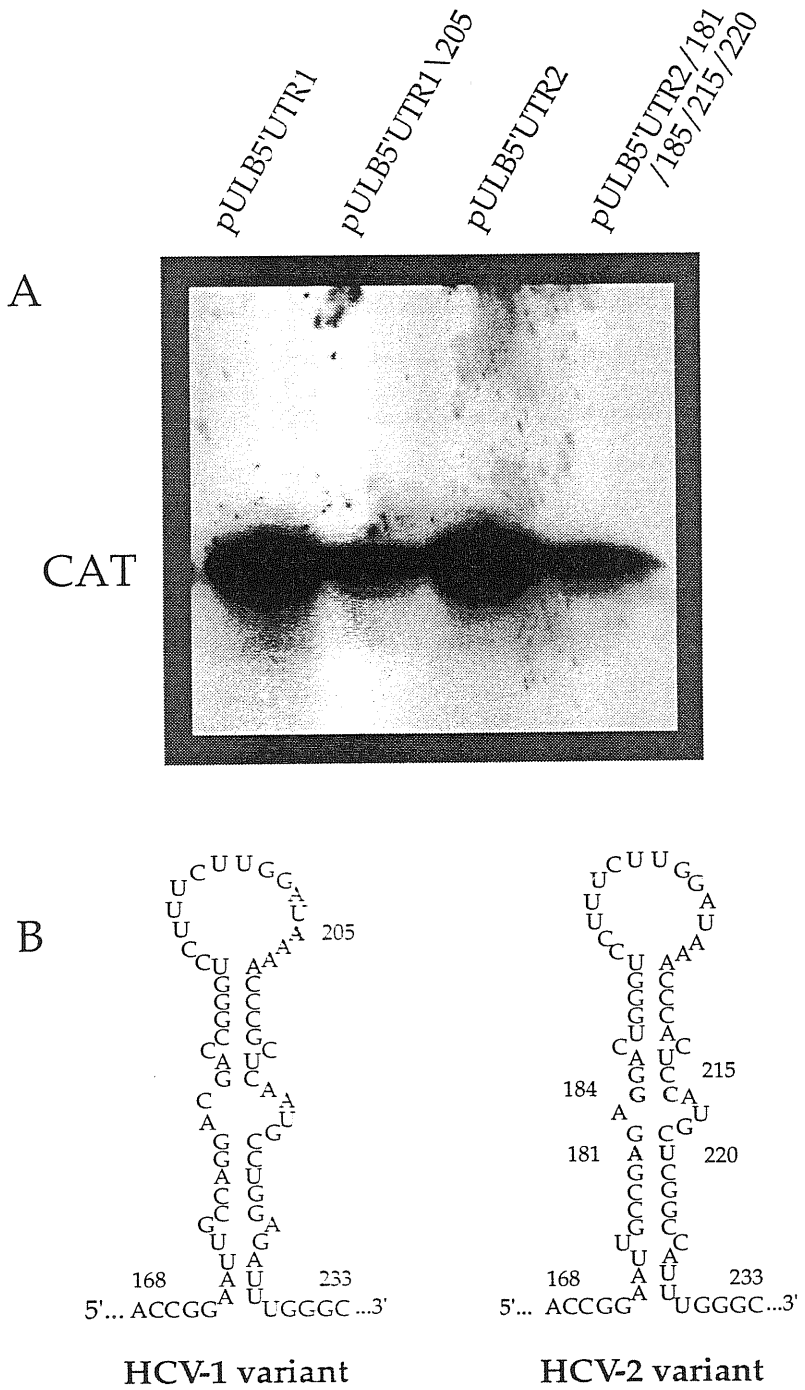
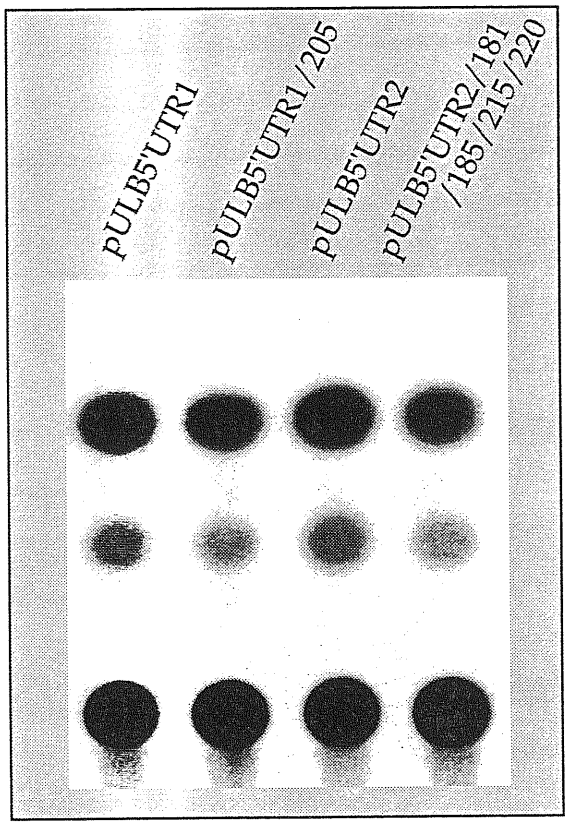


Figure 17: In vitro translation in rabbit reticulocyte lysates programmed with transcripts encoding CAT under translational control of the HCV 5'UTR. A) Fluorography of SDS-PAGE gel showing products of in vitro translation reactions programmed with transcripts derived from plasmids pULB5'UTR1, pULB5'UTR1/205, pULB5'UTR2, pULB5'UTR2/181/185/215/220, which contain the 5'UTR of genotype 1, of a naturally occurring variant of genotype 1, the 5'UTR of genotype 2, and of a naturally occurring variant of genotype 2, respectively. B) Schematic representation of the predicted secondary structure of the RNA sequences from nucleotide 168 to nucleotide 233 of the 5'UTRs of the HCV 1, and HCV 2 variants (position of nucleotide variations are indicated).



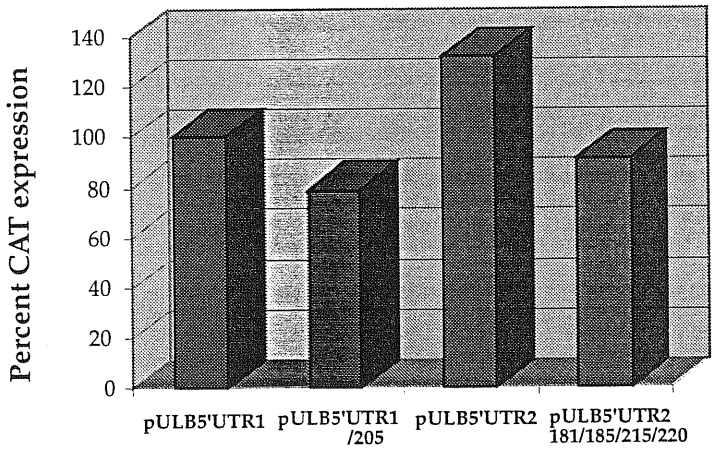


Figure 18: A) CAT expression in HeLa cells after transfection with the indicated constructs (pULB5'UTR1, pULB5'UTR1/205, pULB5'UTR2, pULB5'UTR2/181/185,215/220) in which CAT gene expression is under control of the 5'UTRs of genotype 1, genotype 2 (in plasmids pULB5'UTR1, and pULB5'UTR2 respectively), and of two 5'UTRs variants (pULB5'UTR1/205, and pULB5'UTR2/181/185/215/220). B) CAT activities are shown as value relative to that with the wt 5'UTR sequence of HCV genotype1

produce T7 RNA polymerase, transfected with Lipofectin and tested for CAT enzymatic activity as previously described.

Figure 18 shows the results of CAT assay. The CAT activity values, was calculated relative to that obtained with plasmid pULB5'UTR1, and is representative of three independent experiments. These are as follows: pULB5'UTR1, 100%; pULB5'UTR1-205, 79%; pULB5'UTR2, 132%; pULB5'UTR2-181\185\215\220, 91%. The results obtained from the *in vivo* experiments were coincident to those obtained *in vitro* although in the later case we could not exactly quantify the amount of CAT protein obtained.

3. 5. 3. Translation directed by HCV 5'UTR mutants

In order to identify sequences and structural elements within the 5'UTR that may contribute to the control of translation initiation , a series of point mutations were introduced within this sequence. We focused our analysis on specific sequence components of the stem-loop structure of domain III which are shared from the different HCV genotypes.

3. 5. 3. 1. Mutational analysis of the pyrimidine tract Py-II. *In Vitro* experiments.

Of particular interest is the nucleotide sequence between nt 191 and 199 which constitutes the short, single stranded pyrimidine-rich tract (Py II) located within the loop at the top of domain III structure. Py II is part of a Yn-Xm-AUG motif (Y, pyrimidine; X, any base), here represented by the 'UCCUUUCUU' sequence which precedes a cryptic AUG triplet.

Similar elements have been described in the *picornaviruses* RNAs, were the functional significance of the Yn-Xm-AUG motif in translation initiation has been amply demonstrated by extensive mutational analysis (Jang, 1990; Pilipenko, 1992; Silveira Carneiro, 1995; Degener, 1995).

The basic plasmid for mutagenesis experiment was pULB5'UTR3 (see paragraph 3.5.1). PCR with mutagenic oligonucleotides was used to introduce base-substitution into the Py-II motif of the genotype 3 5'UTR; the derived plasmid, pULB5'UTR3/195-200, contains 6 nucleotide changes between nt 195 and nt 200 (UUUCUU to GGGGGG) (see figure 19B).

In vitro translation experiments were performed in rabbit reticulocyte lysates (RRL) using both, synthetic RNAs obtained with T7 RNA polymerase, or plasmid DNAs with TNT T7 polymerase Kit (Promega), as previously described. As shown in figure 19A, the nucleotide substitutions within the Py-II motif of plasmid pULB5'UTR3/195-200 caused a dramatic decrease in amount of CAT protein synthesised compared to that obtained with plasmid pULB5'UTR3.

3. 5. 3. 2. *In vivo* experiments

To determine whether the mutation within the Py-II sequence effects the translation initiation *in vivo*, plasmid pULB5'UTR3/195-220 was used to transfect Hela cells using the same protocol as in the previous experiments. The CAT enzymatic activity was measured in cell extracts 24 post-transfection and expressed as a percentage with respect to the plasmid pULB5'UTR3 containing the original 5'UTR sequence. Results can be summarised as follows: pULB5'UTR3, 100%; pULB5'UTR3/195-200, 13% (see figure 20).

Thus, the results obtained *in vivo* showed that alteration of the polypyrimidine sequence between nt 195 and nt 200 significantly impaired the ability of the HCV 5'UTR in directing translation initiation.

3. 5. 3. 3. Mutational analysis of the stem-loop structure of domain III. *In vitro* experiments.

The stem structure, immediately downstream to the previously considered loop of domain III, formed by base-pairing between the sequence from nt 173 to 191 and sequence 208 to 230, is present in all HCV genotypes. However, most of the predicted base pairings involve genotype-specific nucleotide changes which, in most of the cases, are compensatory substitutions that allow the preservation of the structure.

To determine whether the primary sequence and/or the stability of the structure derived from this sequence are important for IRES function, we substituted, by site directed mutagenesis, the sequence ACCCG from nt 207 to 211 with sequence TGGGC. This mutation, which prevents the original base-pairing, is expected to significantly effect the stem structure in proximity of the loop. This point mutation, introduced within the 5'UTR sequence of genotype 3, led to the development of a new construct derived from plasmid pULB5'UTR3, named pULB5'UTR3/207.

Results from *in vitro* transcription and translation with the TNT T7 Kit (Promega), as well as from the translation of synthetic RNAs in RRL, showed that this structural alteration drastically impaired the synthesis of CAT protein.

In light of this result, we further introduced a second mutation at position 188 to 192, were the sequence CGGGT was substituted by

sequence GCCCA. In this way we obtained a compensatory mutant, pULB5'UTR3/188/207, in which the proposed base-pairing between the sequences of the two arms of the stem, are restored. As shown in figure 19A, *in vitro,* the IRES mediated translational efficiency of plasmid pULB5'UTR3, is partially recovered by the restoration of the secondary structure of the 5'UTR sequence contained in plasmid pULB5'UTR3/188/207.

3. 5. 3. 4. *In vivo* experiments

The effects of the mutations in both plasmids pULB5'UTR3/207 and pULB5'UTR3/188/207 were tested *in vivo*. HeLa cells were transfected with equimolar amounts of plasmids pULB5'UTR3/207, and pULB5'UTR3/188/207, as previously described. The efficiency of translation of the mutants RNAs were evaluated by comparison to the CAT enzymatic activity measured in cells transfected with plasmid pULB5'UTR3 (figure 20 shows the results of CAT assay). The relative values, expressed in percentage, were: pULB5'UTR3, 100%; pULB5'UTR3/207, 9%; pULB5'UTR3/188/207,68%.

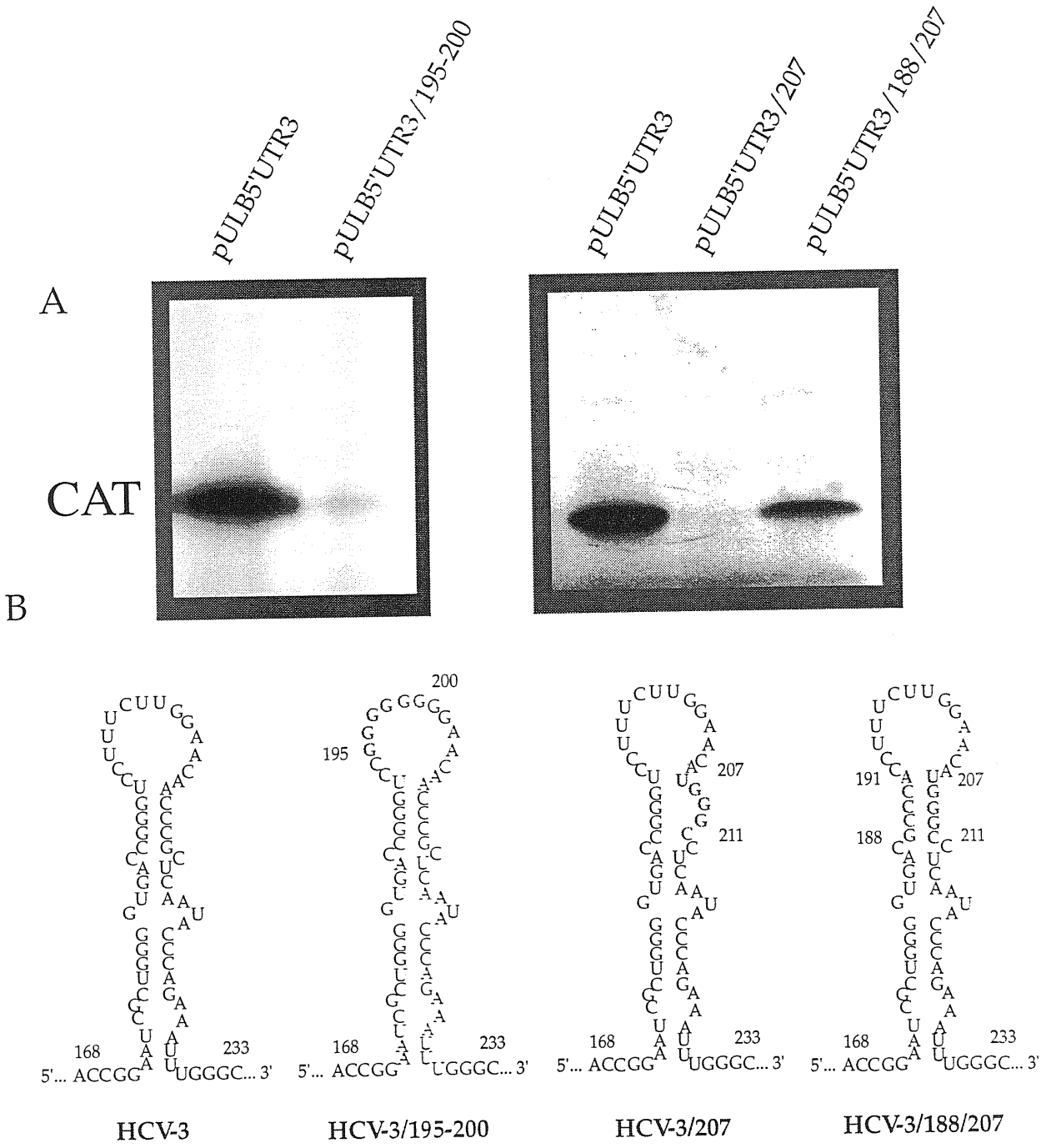


Figure 19:In vitro translation in rabbit reticulocyte lysates programmed with transcripts encoding CAT under control of the HCV 5'UTR. A) Fluorography of SDS-PAGE gelshowing products of in vitro translation reactions programmed with transcripts derived from plasmids pULB5'UTR3, pULB5'UTR3/195-200, pULB5'UTR3/207, pULB5'UTR3/188/207. Here are shown the effects of a six bases substitution within thepolypyrimidine tract of domain III (pULB5'UTR3/195-200), the influenceof a mutation leading to the disruption of the base-pairing in the upper portion of stem-loop III pULB5'UTR3/207), and the effect of the introduction of a second compensatory mutation which restore the original secondary structure pULB5'UTR3/188/207. B) Schematic representation of the predicted secondary structure of the RNA sequences from nucleotide168 to nucleotide 233 of the 5'UTRs of the HCV of genotype 3, and of the derived mutants.

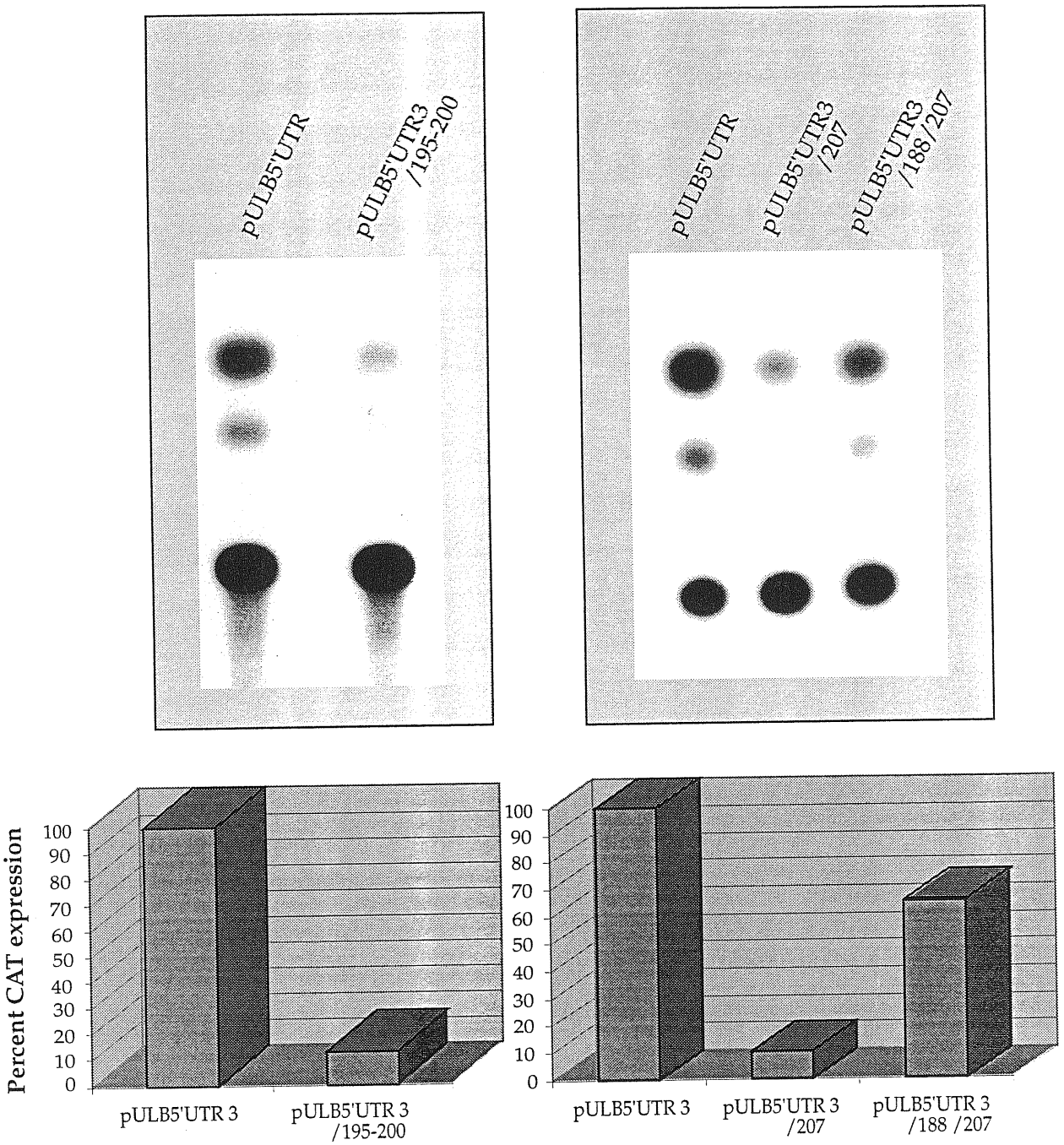


Figura 20: A) CAT expression in HeLa cells after transfection with the indicated constructs (pULB5'UTR3, pULB5'UTR3/195-200, pULB5'UTR3/207, pULB5'UTR3/188/207) in which CAT gene expression is under control of the 5'UTR3 in case of plasmid pULB5'UTR3, and of the same 5'UTR mutated in the polypyrimidine tract (position 195-200) in plasmid pULB5'UTR3/195-200. In plasmid pULB5'UTR3/207, 5 bases have been sobstituted (207 to 211) within the 5'UTR type 3, in order to disrupt the secondary structure of the upper portion of stem-loop III. In plasmid pULB5'UTR3/188/207 the secondary structure of stem-loop III is restored by a second compensatory mutation (bases from 188 to 193 have been substituted). B) CAT activities are shown as values relative to that with the 5'UTR of HCV genotype 3.

CHAPTER 4

DISCUSSION-Part I

4. 1. The HCV genotypes

After the discovery by Choo et al. (Choo, 1989), identifying HCV as the major cause of parenterally transmitted non-A, non-B hepatitis, a number of other HCV isolates from different areas of the world were obtained and their genomes sequenced.

In 1991, when Okamoto et al. (Okamoto, 1991) reported the entire sequence of the genomic RNA of a new Japanese isolate (named HCV-J6), the complete genome sequences of at least three HCV isolates had been already determined. These include sequences from the American prototype HCV-1 (Choo, 1991) and sequences from two Japanese strains HCV-J (Kato, 1990) and HCV-BK (Takamizawa, 1991).

Contemporarely, using always pooled plasma samples from non-A, non-B hepatitis patients and potential HCV carriers, some partial HCV sequences from individual Japanese samples were reported (Takeuchi, 1990a). In particular, partial nucleotide sequences of the 5'UTR and of non structural regions (NS3, NS4 and NS5) were reported by several Japanese groups (Takeuchi, 1990 b; Enamoto, 1990; Nakao, 1991).

Viruses have been traditionally classified into serotypes on the basis of their antigenic characterisation.

In the case of the hepatitis C virus, the lack of a straightforward method for *in vitro* culture, prevents its classification using traditional virological methods. For example, there is no neutralisation assay to show whether distinct serotypes of HCV exist among the known variants. However, with the development of molecular biology, it has been possible to make a genotypic classification of the hepatitis C virus. Classification of HCV has indeed relied almost entirely on nucleotide sequence comparison of complete genomes or subgenomic fragments between variants.

Preliminary sequence alignment analysis showed that most of the Japanese HCV isolates varied markedly from the American prototype HCV-1, suggesting already at that time, the existence of multiple strains or different genotypes of the virus.

The analysis performed by Enamoto et al. (1990) also indicated that the Japanese isolates of HCV could be classified into at least two types, initially designed as HCV-K1 and HCV-K2. Sequence comparison of part of the core gene in HCV-K1 revealed 96 % homology to prototype HCV-1. HCV-K2 was found to be distantly related to both HCV-K1 and HCV-1 since it showed only 67 % homology at nucleotide level.

HCV-K2 was divided in two subtypes, named HCV-K2a and HCV-K2b, which showed 80 % nucleotide homology (Enamoto, 1990).

However, despite the numerous HCV sequences reported from Japan and the United States, information about HCV nucleotide sequences in other parts of the world were scarce.

At that time, in literature, the terminology used to distinguish different genetic variants of HCV was not consistent, and classification based on an average percent homology was ambiguous.

4. 2. The HCV genotypes 1, 2, and 3 are the most frequently represented in the Italian population

In early 1992, only little information was available about the epidemiological distribution of the hepatitis C virus in patients from European countries, and in particular from Italy. Initially, the aim of our research was to define the types of HCV circulating in our country. We started by the analysis of the HCV RNA from a restricted number of patients (21) with chronic hepatitis C, all coming from the North of Italy.

Being the most conserved region of the viral RNA genome, we chose the 5'UTR as target sequence for RT-PCR amplification, cloning and sequencing procedures. On the bases of their sequence homology, the HCV isolates from these patients where grouped into three major clusters of sequences (HCV type 1, type 2, and type 3) (see figure 6).

The region between nt 209 and 226, containing type-specific polimorphisms, was chosen to derive complementary oligonucleotide probes. In this way we developed a rapid and sensitive dot-blot assay with

type specific probes which allowed us to screen a larger number of HCV infected patients.

With this method we determined the distribution of the HCV genotypes in a total of 79 patients. In this series, we found a similar prevalence of patients infected with HCV type 1 and HCV type 2, while HCV type 3 had a much lower frequency (table 5). The isolates of nine out of 79 patients analysed were not genotyped by our method. This is not surprising as single nucleotide variations within the sequence complementary to our probes will destabilize hybridisation and give negative results. Subsequent sequencing of the dot blot negative samples, later identified as genotype 4, confirmed sequence variation within the area recognised by the probes.

The identification of three major HCV types in the Italian patients and the possibility to screen large numbers of infected individuals, induced us to assess whether the definition of the HCV type in patients with chronic hepatitis C, in addition to its importance for epidemiological studies, had also some clinical implications.

4. 3. Comparison between different methods for HCV genotypes determination

The introduction of a series of different and indirect methods for the determination of the HCV genotype, allowed the researchers to perform important epidemiological studies on large numbers of patients from different areas of the world. However, as it appeared from our studies and from those of other groups, not always the results obtained with different genotyping methods were coincident and sometimes were not even comparable.

In the first study, we compared the Dot blot assay standardised in our laboratory with the ELISA based HCV-serotype determination assay developed by Simmonds et al. (1994) The serotyping method can differentiate HCV types on the bases of the variable amino acid composition of the NS4 protein.

The results indicated that these two techniques used for genomic characterisation of HCV are almost equivalent (see table 7). The serotyping method is more easy to perform and it is less expensive. However, some discrepancies observed were probably ascribed to the

detection, by serotyping method, of past antibodies in patients recently infected with a different HCV genotype. In few cases HCV genotype 2 and HCV genotype 3 were classified, by serotyping, as genotype 1. This might be due to antigenic variation amongst genotype 2 and genotype 3 isolates which, produced novel determinants not represented amongst the peptides used in the ELISA, and led to spurious reactivity to genotype 1 peptides.

Furthermore, some cases of cross reactivity observed by serotyping were not due to a multiple infection, as demonstrated by direct sequencing of the 5'UTR/PCR samples, but were probably ascribed to structure alteration of coating peptides and thus, to incorrect antibody recognition.

In the second study we compared the results of HCV genotyping by PCR amplification of the core region (method originally developed by Okamoto et coworkes) with the results obtained by dot-blot assay (table 8). PCR amplification of the core did not allow the identification of about 50% of the initial samples. Furthermore, concordant results between the two methods were found in only 61% of the classified samples. This indicated that the genotyping by PCR amplification of the core region, when applied to samples from Italian patients, gave a large proportion of unclassified results.

We further noticed that the higher rate of unclassified samples were found when the infecting genotype was other than genotype 1. For this reason we explained these results with the much higher prevalence of HCV genotype 2 and HCV of genotype 3 within the Italian population, compared to the Japanese population where, indeed, the most prevalent is genotype 1.

The poor concordance between these two methods of genotyping is thus to be ascribed to the sequence diversity in HCV isolates of different geographic origin. Our results indicate that caution is needed to choose the proper method for HCV genotype determination, and it has to be considered that those methods distinguishing a few genotypes at most would suffice for clinical managment.

4. 4. The HCV genotypes 1, 2, and 3 have different geografical distribution in Italy

The three groups of HCV sequences we found in the previous epidemiological studies, were comparable to the three main cluster of viral sequences identified by Simmonds in a group of Scottish blood donors (Simmonds1993).

Simmonds and coworkers also suggested that, unlike the 5'UTR, where there are only three distinct groups, each of the coding regions shows prominent differentiation of group 1 and group 2 into two further separate clusters of sequences. As consequence, the three major groups were designed as genotype 1, genotype 2, and genotype 3, whereas the more closely related subgroups within type 1 and type 2 were designed as subtypes 1a, 1b, 2a, and 2b. At the same time in Japan, Okamoto and Mori determined the entire nucleotide sequences of the prototypes of genotypes I, II, III, IV, and V corresponding to Simmonds' types 1a, 1b, 2a, 2b and 3 (Okamoto 1991, 1992 a,b,c, Mori 1992). Finally, as it has been pointed out by Bukh et al., five common genotypes (I/1a, II/1b, III/ 2a, IV/ 2b, and V/3a) were found across the world in varying degrees (Bukh 1993). In addition, particular genotypes were indigenous to specific areas; for example, genotype 4 was commonly found in the North and centre of Africa, and genotype 5 in South Africa.

In view of the growing number of new informations about HCV genotype distribution and classification, we performed a second and more extensive analysis of the HCV sequences from about 890 Italian patients coming from 23 clinical centres homogeneously distributed over the entire country. For each HCV RNA positive patient the infecting genotype was determined by dot blot hybridisation assay with type specific probes.

The genotype prevalence was determine in each region and the results clearly showed that HCV type 1b is the most represented in our territory with an higher prevalence (50%) in the South of Italy. Type 1a was slightly more frequent in the North (16%), whereas type 2a and type 2b, all together, were 14% in the North, 28% in the centre, and 17% in the South. Genotype 3 was rare in the South , whereas in the North it was the second most frequently represented (17%) after genotype 1b (see figure 8). These

results were consistent with pervious reports according to which, genotype 1b is the most represented worldwide.

The differences observed in the distribution of some genotypes, like type 1b and type 3, may be due to different time and routes of infection. For example the higher number of genotype 3 isolates found in patients from the North of Italy may be related to the large proportion of patients with history of intravenous drug abuse found in this population. Drug addicts, in fact, differ from the general population in the relative prevalence of HCV genotypes being mainly infected with genotype 3 (McOmish 1993, Silini 1995). On the other hand, genotype 1b, the transmission of which in the past occurred frequently in patients receiving blood and blood-derived factors, may be especially diffused in the South of Italy where the number of patients with post-transfusional hepatitis is higher.

4. 5. The HCV genotype: a predictive factor for response to the interferontherapy

Interferon therapy has been shown to be effective in the treatment of patients with chronic hepatitis C (Davis 1989, Di Bisceglie 1989). In several years after the licensing of interferon-alpha for chronic hepatitis C, it has been shown that a higher proportion of patients have improvements in serum aminotransferases, HCV RNA levels and hepatic histology during therapy, but only a small percentage have a long-term response. As a consequence, if one could predict which patient would ultimately have a long term-response to IFN therapy, one might treat only those whose likelihood of a response warranted the expense and the discomforts of several months of therapy.

Recent studies indicated the HCV infecting genotype is a possible predictive factor for the response to IFN treatment (Yoshioka 1992, Chemello 1994). However, at the time of this study, only little information was available about the genotype distribution in the different countries as well as about their clinical significance.

We used the dot blot hybridisation assay to determine the HCV genotype of 495 Italian patients from different clinical centres who where included in IFN-alpha trials. The genotypes distribution was the same as that shown in our previous epidemiological studies, HCV 1b and

HCV 2a being the main subtypes. The discrete variations found in the prevalence of each genotype were comparable with our previous observations.

Our analysis revealed that patients infected with HCV genotype 1 have a significantly higher rate of non-response compared to those infected with genotype 2 or genotype 3.

These patients were treated with different schedules of IFN so that we could analyse the sensitivity of different HCV genotypes to IFN-alpha by dividing patients into two groups in relation to the total amount of IFN given.

Patients infected with HCV genotype 2 and genotype 3 showed higher rates of sustained response compared to those infected with genotype 1, independent of the dose of IFN received.

Overall, the rate of substantial responders was 15% in patients infected with genotype 1, 47% in patients infected with genotype 2, and 68% in those with HCV genotype 3 (see table 9).

Furthermore, we noticed that, in the case of patients infected with HCV genotype 1, the rate of sustained response was also related to the dose of IFN-alpha. It was low (15%) in cases treated with a total dose of 600MU but increased to 32% in patients which received more than 600MU.

Our data has confirmed by a recent report from Kohara et al., who demonstrated that sensitivity to IFN-alpha can be predicted by the infecting HCV genotype. Interestingly, in this study it was also shown that the higher sensitivity of patients infected with HCV genotype 2 is related to a higher rate of viral RNA reduction during IFN treatment (Kohara 1995).

All together, these results suggest that the higher rate of response to IFN-alpha, together with the decrease of HCV RNA during the therapy, and the higher probability of total elimination of HCV RNA from the serum by IFN treatment, can be explained by a major sensitivity of HCV genotype 2 to IFN-alpha compared to HCV genotype 1. On the other hand, although the high response rate among the patients with HCV genotype 3 is noteworthy, the number of cases examined is still too low to make conclusions. In particular, data about the HCV RNA levels of genotype 3 during the IFN therapy are awaited.

In addition, the determination of HCV genotype before IFN treatment may be useful to predict the response to the therapy and also to

decide a proper schedule, in particular if a patient is infected with HCV genotype 1b.

4. 6. Relationship between HCV infection and autoimmune chronic liver disease

Type 2 autoimmune chronic liver disease has been associated to the infection with the hepatitis C virus.

In Italy, patients with autoimmune hepatitis type 2, anti-LKM-1, and anti-HCV positive, were often found positive also for HCV RNA (Lenzil991).

In this study, we have determined by dot blot hybridisation assay the HCV genotype of 22 Italian patients with autoimmune hepatitis type 2.

Our results indicate that all the three major HCV genotypes involved in the etiology of the hepatitis C in Italy, genotype 1, 2, and 3, are present in patients with autoimmune hepatitis type 2. Although this indicates that the autoimmune reaction is not associated to a specific type of HCV, it was noticed that an unexpected higher prevalence of genotype 1 was found (17/22). This may suggest that HCV genotype 1, which is generally found in patients with a more severe form of chronic liver disease and who have a lower possibility to respond to the interferon therapy, may induce anti-LKM-1 antibodies more easily than other HCV types.

An interesting finding was that 3 of the 4 HCV genotype 2 isolates showed peculiar nucleotide variations compared to their reference sequence and of other closely related HCV strains. These nucleotide substitutions, identical in two cases, do not change the features of the predicted secondary structure, but resulted in a change of the negative free energy value calculated for the sequence forming the upper portion of stem-loop III, where they are located (DG values changed from -11.7 of the wt to -13.8 and -15.2 kJ/mol). This sequence variability at the 5'UTR level suggests a possible modification of the overall structure of important elements involved in the control of translation initiation and which may thereforeeffect the viral translational efficiency.

On the other hand, these differences in the 5'UTR sequence may reflect further nucleotide variations in other regions of the viral RNA genome and thus, represent new variants of HCV genotype 2.

4. 7. HCV genotypes and levels of viremia in HCV heathy carriers

In chronic HCV infection, the question whether the persistence of virus is always associated to hepatic damage is still a controversial issue.

In this study we have analysed two different groups of patients: group 1 includes patients having a sustained biochemical response to IFN therapy although they show persistent HCV RNA positivity; group 2 is represented by 5 healthy carriers which were occasionally discovered positive for both HCV antibodies and HCV RNA.

In the experiment that attempted to determine the relationship between persistent HCV RNA positivity and absence of liver disease, all the treated patients showed unvariable or increased levels of circulating HCV RNA before and after the interferon therapy.

Thus, we excluded the possibility that the absence of disease in these patients is due to a trivial viral load.

Analysis of the HCV genotype revealed a much higher prevalence of genotype 2 with respect to the other genotypes, especially in group 2 patients (26/27). This data is concordant with a previous report about the HCV genotypes distribution in viremic patients with normal ALT levels (Silini 1994). In another study, Ichimura et al., showed that genotype 1b was found more often in patients with chronic liver diseases than in HCV carriers who were identified among apparently healthy blood donors, whereas the opposite relation was seen for genotype 2a (Ichimura 1994).

On the other hand, these results were expected since genotype 1, which is less responsive to antiviral therapy (Chemello 1994, Tsubota 1994), is more frequently found in patients with elevated alanine aminotransferase (ALT) levels and who have an evidence of chronic liver disease (Kobayashi 1996).

Thus, we considered the possibility that the 'healthy carrier' condition of these patients might be due to the presence of 'non-virulent' or less aggressive HCV strains. In particular we analysed the conserved 5' untranslated region which has been demonstrated to have an active role in the control of viral genome translation and replication.

In some cases, sequence analysis of the 5'UTRs from group 1 patients revealed the presence of single nucleotide variations with respect to the wild-type sequence. Single nucleotide substitutions were found

alternatively in the pre- and/or post-treatment serum samples. Direct sequencing of some of these samples revealed simultaneous presence of two sequences differing by a single nucleotide. Mixed viral populations detected at the 5'UTR level, found in this study, were probably reflecting the quasispecies nature of HCV infection indicated by several groups (Martell 1992, Okada 1992). In this sense, the high sensitivity of the direct sequencing method might explain our findings: in fact, heterogeneous sequences were detected in concomitance to higher levels of viremia, were the predominant strains can be detected by direct sequencing approach.

In one case (the only HCV isolate of genotype 1) an adenosine was found inserted at position 205 which may leads to a structural change in the upper portion of the stem-loop of domain III. The same nucleotide variation was found in both the pre-and post-treatment serum samples of the patient.

HCV isolates from the second group of patients (two of genotype 1 and three of genotype 2) had the same 5'UTR sequence as their reference strain with the exception of one genotype 2 isolate which showed four compensatory substitutions located within the two arms of stem-loop III.

From the computer assisted secondary structure analysis of this sequence it was concluded that, although there is not variability in the shape of the stem, this variant showed a substantial change in the negative free energy value (predicted DG values changed from -11.7 to -13 kJ/mol).

Although the exact mechanism used by HCV for translation initiation remains unknown, recent experiments showed that different sequence motifs within the IRES element of its 5'UTR, can influence the efficiency of viral initiation of translation (Tsukyiama-Kohara 1994, Chan 1994).

To further verify this hypothesis, the 5'UTRs of the two naturally occurring HCV variants described here, were later chosen to perform *in vitro* and *in vivo* experiments to asses their translational efficiency.

In conclusion, we cannot ascribe the absence of liver disease to a particular HCV genotype or strain, even though our finding suggests that genotype 2 might have some peculiar characteristics.

DISCUSSION-Part II

4. 8. Intruduction

The HCV RNA genome contains a 5' untranslated region (UTR) of approximately 341 nucleotides which is by far the most conserved region of the genome among different HCV strains and isolates (Bukh 1992). The reasons why this region has to be so conserved between HCV types, to the extent that the sequence subtypes cannot be differentiated from each other, are currently unclear. However, as the 5'UTR adopts a secondary structure with presumed regulatory roles in the initiation of translation of the viral genome, the requirement for internal base pairing may considerably restrict the degree of possible variability.

Translation of synthetic bicistronic mRNAs revealed that a function of the HCV 5'UTR is to promote initiation of translation by internal ribosome entry. It has been shown that the 5'UTR sequence of HCV RNA contains a genetic element functionally related to that of *picornaviruses*: the internal ribosome entry site or IRES, which drives translation initiation of the viral mRNA in a cap-independent way.

Initially, Tsukiyama-Kohara et al. (1992), fixed the 5' boundary of the HCV IRES about 200 nucleotides upstream the initiator AUG codon. Subsequent experiments from other groups extended the 5' boundary to a region between nt 30 and 70 of the RNA, implying a larger IRES of about 300 nucleotides. Recently, evidences have started to accumulate about the requirement of the first 10 codons of the polyprotein for efficient internal initiation (Raynolds 1995).

Parallel studies of HCV 5'UTR with the 5'UTRs of some *picornaviruses* revealed that, despite the little or no sequence similarity, their 5'UTRs were marked by the presence of pyrimidine-rich tract/spacer/AUG motifs (Xm-Yn-AUG), that represent the only universally conserved elements in all the IRESs.

HCV IRES has been suggested to have also one more feature in common with the IRES elements of cardiovirus/aphthovirus group of *picornaviruses*. The proposed internal ribosome entry site of HCV is also at, or very close, to the authentic initiator codon and translation is probably initiated without scanning (Reynolds 1995).

Within the different groups of HCV strains isolated, there is a strong conservation of IRES secondary structure which reflects the high degree of homology at the primary sequence level. The secondary structure maps predicted for the 5'UTRs of different HCV genotypes, show similar overall structure, with only small differences in the details of particular stem-loop domains.

In this sense, analysis of sequence variations found in isolates of the major groups revealed extensive structure-conserving substitutions within stems (with 'hot spots' of sequence divergence localised mainly in domain III), and a high degree of sequence conservation in the loops (Brown 1992).

A typical feature of the HCV 5'UTR sequence is the presence of several small open reading frames (ORFs), the number of which depends on the particular strain. The functional status of the ORFs in the 5'UTR of HCV is still unknown. However, since all the HCV sequences show at least one of these small ORFs, it has been suggested that they are maintained because of their role in control of translation (Han JH 1991).

4. 9. Different efficiency in translation initiation mediated by the HCV 5'UTR of genotype 1, 2, and 3.

On the basis of the presence of little but perhaps significant differences in the primary sequence as well as in the secondary structure of the 5'UTR, we compared IRES functions of HCV RNA directed by cDNA clones of genotype 1, 2, and 3.

In vitro transcription and translation experiments, performed either by the addition to rabbit reticulocyte lysates of plasmid DNA in presence of T7 RNA polymerase, or by addition of synthetic RNAs, indicated that translation initiation directed by the 5'UTR of genotype 1 is slightly less efficient compared to genotype 2, whereas sequences from genotype 2 and 3 are equally efficient (3.5.1.1. figure 15). The results obtained by *in vivo* experiments with genotype 1 and genotype 2 mirror

those obtained *in vitro* (3.5.1.2. figure 16). In contrast, translational efficiency from genotype 3 sequence resulted in a three fold reduction compared to that from genotype 2.

Our data from *in vivo* experiments with 5'UTRs of type 1 and 2, confirm the results obtained *in vitro* by Tsukyiama-Kohara et al., who in a previous study compared the IRES functions of the 5'UTR of genotypes 1 and 2 (Tsukyiama-Kohara 1992). Furthermore, the same group demonstrated that any replacement of 5'UTR RNA segments of genotype 1 by the corresponding segment of HCV genotype 2, resulted in a much higher translation initiation (Namoto 1994).

The greatest disparity in our data between the *in vitro* and *in vivo* assays was obtained from the genotype 3 5'UTR . When the genotype 3 specific IRES was assayed by *in vitro* transcription and translation experiments, no detectable difference was observed compared to genotype 2 IRES efficiency, whereas, *in vivo*, type 3 sequence was about 35% as efficient as type 2.

A possible explanation cames from a previous work of Siddiqui et al. They demonstrated that a mutation of the non initiator AUG triplet at nt 215, within the 5'UTR of genotype 1, causes a decrease in translation efficiency both *in vitro* and *in vivo*, with a greater inhibitor effect *in vivo*, indicating that the 5'UTR IRES element is more sensitive to this mutation in the cellular environment.

Interestingly, the same AUG triplet, which represents, together with the previous polypyrimidine tract, one of the two Yn-Xm-AUG motifs of the HCV IRES, is missed in the 5'UTR sequence specific of genotype 3 (where the G at nt 217 becomes an A) while, it is present in both genotype 1 and genotype 2.

In this sense, since our results *in vivo* showed an even greater reduction of translation efficiency from genotype 3 compared to genotypes 1 and 2, we suggest that the disruption of this Yn-Xm-AUG motif may have a different effect on IRES function when inserted in the sequence context specific to genotype 3.

Our results, in parallel to those from the site directed mutagenesis performed by Wang et al. (1994), suggest that the AUG at nt 215 is required for maximum levels of HCV translation but is not used for initiation of viral protein synthesis and thus is not essential for translation initiation.

4. 10. Efficiency of translation initiation mediated by the 5'UTR sequences of two naturrally occurring HCV variants.

In this study we have included the 5'UTR sequences of two naturally occurring HCV variants, one of genotype 1 and one of genotype 2, which were previously isolated from a group of symptomless patients.

Both, genotype 1 and genotype 2 like 5'UTRs, showed peculiar point mutations within the stem-loop III sequence (3.5.2.1. figure 17B).

For type 1 variant, an adenosine was found inserted in position 205, in correspondence to the single stranded stretch of nucleotides forming loop III. When tested, both *in vitro* and *in vivo*, the translational efficiency of this 5'UTR was always slightly lower compared with that of genotype 1 5'UTR, with an estimated reduction of 20% for the *in vivo* protein synthesis (3.5.2.2. figure 18)

In the case of the 5'UTR of genotype 2 variant four covariant substitutions were found at position 180/219 and 183/214, localised in the upper portion of stem-loop III. From the *in vitro* and *in vivo* experiments the IRES containing this 5'UTR is 40% less efficient in driving translation initiation compared to the wt 5'UTR of genotype 2.

Taken together, our results, strongly relate the pattern of variability described in the 5'UTR of these HCV isolates and the IRES mediated translational efficiency.

In this sense it is tempting to hypothesise that the nucleotide insertion in position 205 of type 1 sequence, which leads to a structural change in the loop III, is effecting the role of the same stem-loop. Undirected evidences for this model come from different previous observations which indicated that loop III is a putative binding site for regulatory factors like PTB protein (Naushad 1995).

Furthermore, this insertion is located within the 'spacer' sequence between the polypyrimidine tract PyII (at nt 191-207), and the AUG codon at nt 215, which together form one of the two Xm-Yn-AUG motifs found in the HCV IRES. The role of the 'spacer' sequence in Xm-Yn-AUG motif was suggested in a study by Jang and colleagues, regarding a similar Xm-Yn-AUG motif present in poliovirus IRES (Jang 1990). They stressed the importance of the distance between the polypyrimidine tract at nt 558 and the AUG codon at nt 586 in poliovirus 5'UTR. Pilipenko et al.

showed that if the distance of 22 nucleotides is changed, translational efficiency decreases (Pilipenko 1992).

This suggests that factors may exist which are capable not only of recognising specific sequences and secondary structures but which also require the proper spacing of these entities.

Also, these results, together with the results we obtained from the mutational analysis of the polypyrimidine tract PyII, suggest that this Xm-Yn-AUG motif in HCV may have an important function as in poliovirus and EMCV IRESs. The recognition of the polypyrimidine tract properly spaced from the downstream AUG may provide a nucleation site for the final assembly of a translation initiation complex.

In the case of the type 2 5'UTR variant, the four compensatory substitutions observed were not apparently affecting the overall structure of stem-loop III. However, the analysis of the secondary structure which could be formed within the 56 bp extending from 172 to 228, revealed that the four nucleotide substitutions generated a stem-loop structure of DG= -14, whereas the wild type sequence showed a DG= -10.4. Thus, the covariant substitutions described at positions 180/219 and 183/214 increase the stability of the stem-loop structure.

In any case, consistent with the dual structural and primary sequence requirements for efficient initiation of transation we can hypothesize that IRES efficiency can be due either to the structural disturbance of base-paired stem or to the abolishment of factors binding because of an altered nucleotide sequences.

4. 11. Identification of *cis*-elements in the HCV 5'UTR, involved in IRES mediated initiation of protein translation.

In *picornavirus* considerable attention has been attributed to the role of the oligopyrimidine tracts and to the sequences between these tracts and the following AUG triplets.

Because of its similarity to *picornaviruses*, we analysed the single stranded stretch of pyrimidine (named Py-II) located within the upper portion of the stem-loop III of the HCV 5'UTR. In the different HCV strains the total number of consecutive pyrimidine residues in loop III is well conserved. On the contrary, the sequences immediately before and after this polypyrimidine tract are unusually variable, being, amongst different

HCV strains, the most variable regions of the whole IRES. Typically, a non initiator AUG is located 13 nucleotides downstream to the Py-II motif.

We examined the influence of these stretch of six pyrimidine residues within the Py-II motif of the HCV IRES on the efficiency of internal translation initiation. The substitution mutant constructed to examine this question was designed to preserve the proposed structure of loop III (see figure19B).

We demonstrated by site-directed mutagenesis that the primary sequence representing the pyrimidine stretch between nt 195 and nt 200 is critical for HCV translation both *in vitro* and *in vivo* (figures 19A, and 20). We showed, in fact, that the six nucleotide substitutions introduced abrogated almost completely the IRES activity.

Our results demonstrated that this conserved pyrimidine stretch in HCV is functionally equivalent to that of poliovirus type 2, of FMDV, and of EMCV. Deletions of the polypyrimidine stretch in EMCV and point mutations in the pyrimidine rich tract of poliovirus type 2 and EMCV dramatically decreased the translational efficiency in vitro and in vivo (Meerovitch 1991, Jang 1990, Kuhn 1990). More recently, Silveira Carneiro et al., showed that also the 5' distal polypyrimidine rich-tract of HAV RNA, reminiscent of the poly(C) tract of *cardio-* and *aphthoviruses*, is involved in process of translation, and that it may constitute an important cis-element recognised and bound by cellular factor(s) at initiation of translation (Silveira Carneiro 1995).

Furthermore, as suggested by Degener et al. (1995), the pyrimidine-rich tract and a highly ordered secondary structures of the 5'UTR of *picornavirus* RNAs, are *cis*-acting elements reminescent of those of some coordinated cellular mRNAs, the translation initiation of which is probably modulated by the interaction between some cellular *trans*-acting factor and the pyrimidine-rich tract of their 5'-untranslated region (Klausner and Hardford, 1989; Levy et al., 1991; Cardinali et al., 1993). This suggests that the mechanism of internal initiation of translation of *picornavirus* RNAs, as well as HCV RNAs, may be adjuvated by factor(s) present in the host cell.

On the other hand, it has been recently reported by Wang that substitution of three pyrimidines by purines in the pyrimidine-rich tract of loop III, resulted only in a negligible decrease in translational efficiency (Wang 1994).

In light of our results, a more extensive disruption of the pyrimidine stretch is required for the production of transcripts with the minimal level of activity (13% of wt) observed.

This is of importance since the polypyrimidine tract Py-II could be, by analogy to that of *picornavirus*, one of the major binding sites for the polypyrimidine-tract-binding protein (PTB). Among the various cellular trans-acting factors, PTB (also known as p57), is probably involved in spliceosome assembly (Patton 1991), and has been shown to be functionally required for *picornavirus* IRES, where it binds at one or more sites (Hellen 1994, Luz 1991).

Moreover, Naushad et al. recently provided evidence that the role of PTB is functional indispensable in the HCV-IRES-mediated translation (Naushad 1995). They found that PTB interacts with three non contiguous regions of the HCV 5'UTR, even if they were not able to determine the exact sequences, of the Py-II motif, with which PTB makes contact.

From all these data, it is tempting to hypothesise that the failure of Wang et al. to detect a substantial inhibitory effect on translation efficiency after mutation of the polypyrimidine tract is related to the small number of nucleotide changes. Only the substitution of a larger number of consecutive pyrimidines leads to a strong inhibition of translation initiation. In this case, although there is not enought evidence, the mutation could be sufficiently extended to prevent the binding of the PTB protein which is certainly a critical element in the regulation of viral protein translation.

The crucial role of domain III for initiation of translation has been demonstrated by Wang et al. since they showed that a deletion of 127 nucleotides, that eliminate the upper portion of domain III (including the polypyrimidine tract Py-II), resulted in a dramatic reduction of translation efficiency (Wang 1994).

After we demonstrated the importance of the primary sequence within the loop III for HCV IRES function, we analysed whether the integrity of the stem structure supporting this sequence is also important for translation efficiency. We mutated part of the stem to destabilize the helix structure (figure 19B). When five proximal base-pairing nucleotides of the stem were altered, internal initiation was almost completely abolished both *in vitro* and *in vivo* (see figures 19A and 20).

Failure of this mutant HCV 5'UTR to initiate translation efficiently, strongly suggests the importance of the relevant secondary structure.

A second mutant, in which a compensatory mutation was generated to restore the predicted secondary structure, provided conclusive evidence in support to our previous findings. In fact, the translational efficiency of this second mutant RNA was recovered, with an estimated efficiency of 65% with respect to the wt sequence.

The mutational analysis presented here shows that the Py-II motif is associated to a stable stem structure, which has to be maintained for efficient initiation of translation. From these results we concluded that both, the maintenance of a stable stem structure and its primary sequence in itself, are required for efficient translation initiation. In fact, if only the secondary structure was important we would have expected to obtain a complete recovery of the translational efficiency from the compensatory mutant mRNA. The sensitivity of the IRES to subtle mutations within this portion of the 5'UTR is consistent with the proposed fundamental role of stem-loop III for internal initiation of translation.

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