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TOPIC HIGHLIGHT

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Structure-function relationship in viral RNA genomes: The case of hepatitis C virus

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Abstract

The acquisition of a storage information system beyond the nucleotide sequence has been a crucial issue for the propagation and dispersion of RNA viruses. This system is composed by highly conserved, complex structural units in the genomic RNA, termed functional RNA domains. These elements interact with other regions of the viral genome and/or proteins to direct viral translation, replication and encapsidation. The genomic RNA of the hepatitis C virus (HCV) is a good model for investigating about conserved structural units. It contains functional domains, defined by highly conserved structural RNA motifs, mostly located in the 5'-untranslatable regions (5'UTRs) and 3'UTR, but also occupying long stretches of the coding sequence. Viral translation initiation is mediated by an internal ribosome entry site located at the 5' terminus of the viral genome and regulated by distal functional RNA domains placed at the 3' end. Subsequent RNA replication strongly depends on the 3'UTR folding and is also influenced by the 5' end of the HCV RNA. Further increase in the genome copy number unleashes the formation of homodimers by direct interaction of two genomic RNA molecules, which are finally packed and released to the extracellular medium. All these processes, as well as transitions between them, are controlled by structural RNA elements that establish a complex, direct and long-distance RNA-RNA interaction network. This review summarizes current knowledge about functional RNA domains within the HCV RNA genome and provides an overview of the control exerted by direct, long-range RNA-RNA contacts for the execution of the viral cycle.

Key words: Functional RNA domain; Cis-acting replicating element; Hepatitis C virus; Internal ribosome entry site; RNA-RNA interaction; Untranslatable region

Core tip: This review summarizes the main aspects of structurally conserved genomic RNA elements in the hepatitis C virus (HCV) genome and their role in the viral cycle. The genome of RNA viruses is a dynamic genetic entity endorsed with an information storage system defined by highly conserved, complex structural units, termed functional RNA domains. The genome of HCV contains several well-studied functional RNA domains that control essential viral processes, as well as transitions between them, by recruiting protein factors and also by establishing a complex, direct and long-range RNA-RNA interaction network.

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INTRODUCTION

The genomes of RNA viruses are not passive elements. The inherent high error rate of the viral polymerase during replication provides an important evolutive advantage by the generation of genotypically and phenotypically different virus pools on which natural selection operates[1]. By using this strategy, viruses have got RNA genomes with numerous signals overlapping protein coding sequences, thus achieving multiple levels of regulation throughout the infectious cycle. All this information is compactly packed in a minimal size for optimal propagation. Viral RNA genomes use a information storage system beyond the nucleotide sequence, defined by highly conserved regions that exhibit complex folding and play direct, functional roles in the viral cycle[2-4]. Two levels of structure or folding can be distinguished within an RNA molecule: (1) the secondary structure involves double and single stranded regions arrangements; and (2) the tertiary structure is determined by the relationships established between secondary structure elements. The combination of both conformational levels establishes the final shape of the RNA to generate the so-called functional RNA domains. These are dynamic elements since their structure can be selectively adopted from a wide variety of possible foldings to execute a specific function by recruiting protein factors, or modulating the conformation and function of distant regulatory elements[5]. These mechanisms achieve an active control of the gene expression. Therefore, RNA folding acts as a regulatory machine to diversify RNA genome functions with a minimal size.

Functional RNA domains are typically identified as one or more stem-loops with highly conserved sequence motifs located in the loops. These elements were initially described located in the 5'-untranslatable regions (5' UTRs) and the 3'UTRs of viral genomes, but now evidences are accumulating for their widespread distribution throughout the entire genomic RNA[5]. They can be organized, either as well-defined, phylogenetically conserved RNA structural motifs, or as sets of extensive folded regions throughout the whole viral genome [genome-scale ordered RNA structures (GORS)], following a clear structural pattern that may change even between closely related viruses[6,7].

The recent advent of novel bioinformatic tools and experimental techniques to probe and study RNA structure has provided high-resolution pictures of numerous viral RNA molecules. Among them, structural elements of the hepatitis C virus (HCV) genomic RNA are one of the best characterized from many different viruses. HCV infection affects to more than 3% of the world population, with high incidence of cirrhosis, hepatic steatosis and hepatocellular carcinoma. To date, no efficient vaccines have been developed against HCV and current treatments based on pegylated-interferon α and ribavirin are the standard of care (SOC) regimen with a limited efficacy of around 40% of the patients. Additionally, this therapy has important side effects. Recently, two direct-acting antiviral drugs targeting the viral protease NS3, telaprevir and boceprevir, have been approved by the United States Food and Drug Administration[8]. These compounds can be administered in conjunction with pegylated-interferon α and ribavirin for a short period of time to achieve an improved sustained virological response[8] with respect to the SOC. Unfortunately, prolonged treatments lead to the appearance of resistant variants. Other drugs targeting either the protease NS3 (simeprevir) or the viral polymerase NS5B (sofosbuvir) are currently being tested in Phase Ⅱ/Ⅲ clinical trials.

HCV belongs to the *Flaviviridae* family, which includes yellow fever virus, bovine diarrhea virus and dengue virus. The HCV genome shows such a variability that up to six different genotypes, with hundreds of subtypes and isolates, have been identified[9,10]. Viral genotype clearly affects the success of interferon therapy, although no clear correlation with virulence exists. Further, the HCV population infecting a patient is structured in terms of quasispecies. This term defines the closely related sequences of a heterogeneous viral population infecting a single individual[11]. Quasispecies structure has been associated with the failure of infected people to clear the virus and the subsequent development of a chronic infection[12]. Therefore, the identification of conserved therapeutic targets and the search for fully effective antiviral compounds is a major goal of HCV research. The functional importance of genomic structural elements for virus persistence and their high conservation rate suggests they might make good therapeutic targets. This review focuses in the main structural features of the HCV genomic RNA functional domains and their roles in the viral cycle.

HCV RNA GENOME ACTIVELY CONTROLS THE INFECTIVE CYCLE

The HCV genome is about 9600 nucleotides-long, single stranded positive RNA molecule[13-15] that encodes for a single open reading frame (ORF) flanked by two highly conserved UTRs (5'UTR and 3'UTR) (Figure 1A). The viral genome controls important processes of the infective cycle. During early infection, the genome acts as mRNA to generate the viral structural (core protein C, p7 and the envelope proteins E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). HCV translation is initiated by an internal ribosome entry site (IRES)-dependent mechanism[16,17] different to the cap-dependent method used for most cellular mRNAs. The IRES element is mostly located at the 5'UTR and spans a short stretch of the core coding sequence[18,19] (Figure 1B). Both the initiation translation step and the subsequent elongation phase are influenced by the presence of domains located at the 3' end of the HCV genome[20-25]. This process is dependent on the acquisition of a circular topology resembling the closed-loop structure adopted by cellular cap-mRNAs. Such architecture is achieved by both the recruitment of protein factors, able to simultaneously bind to the 5'UTRs and 3'UTRs of the genomic HCV RNA[20-23,26-28], and also by the establish-

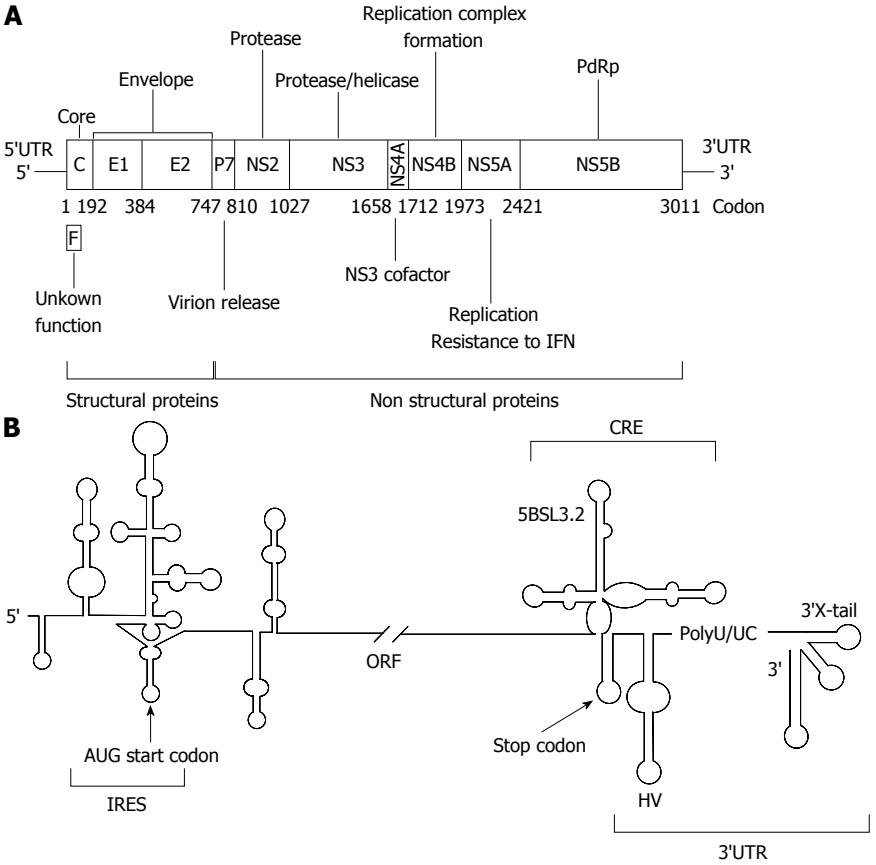

Figure 1 Genetic organization of the hepatitis C virus genomic RNA. A: A schematic view of the hepatitis C virus (HCV) genome, showing the 5' and 3' untranslatable regions (UTRs) and the genes encoding for the different viral proteins. Numbers allude to codon positions; B: A detailed diagram of the secondary structure proposed for the 5' and 3' ends is pictured. The region required for internal ribosome entry site (IRES) activity is indicated. The 3' end of the viral genomic RNA is organized into two well-defined structural elements: the cis-acting replicating element region containing the essential domain 5BSL3.2, and the 3'X-tail, separated by a hypervariable sequence (HV) and a polyU/UC tract. Start and stop translation codons, placed at positions 342 and 9371, respectively, are indicated by arrows. Numbers refer to the aminoacid positions according to the HCV Con1 isolate (GenBank accession number AJ238799). RdRp: RNA-dependent RNA polymerase; IFN: interferon; ORF: Open reading frame.

ment of direct, long-range RNA-RNA interactions[29-31]. Once viral proteins levels have reached a certain threshold, the genomic RNA serves as a template to initiate replication at the 3'UTR in a structure dependent manner. This process is also influenced by the 5' end of the HCV RNA[32,33]. The accumulation of viral genomes enhances the formation of homodimers by the interaction of two viral RNA molecules in the presence of the core chaperone protein[34-37]. Packaged genomic RNA is finally enveloped and released to the extracellular environment.

The maintenance of a proper balance between these processes involves fine regulation mechanisms, which involve the interplay of functional RNA domains located throughout the entire ORF[38,39]. The 5' core coding sequence helps in the preservation of structures important for IRES activity and replication (Figure 1B)[19,40-43]. Within the 3' end of the NS5B coding sequence, the stem-loop 5BSL3.2 is embedded in a cruciform structure that has been identified as a *cis*-essential element for viral RNA synthesis [cis-acting replicating element (CRE)] (Figure 1B)[44,45] and as a regulatory partner of the IRES function[25].

An interesting feature of all these functional RNA domains is that they do not operate only by recruiting protein factors. Instead, they establish a complex and dynamic network of contacts, which fits viral necessities to promote the consecution of different steps of the viral cycle, as well as the switch between them. Furthermore, this interacting web provides important benefits, such as minimizing protein requisites.

Next sections will outline the current knowledge about different HCV functional RNA domains and their involvement in the complex interaction network that governs the initiation of essential viral events and the transitions between them.

THE HCV IRES REGION

The initiation of the HCV protein synthesis is driven by the high affinity interaction IRES-40S[46-48]. This primary contact promotes conformational changes that directly clamp the viral RNA to the ribosomal subunit and thus position the appropriate start codon in the P site[49]. The further binding of eIF3 aids the incorporation of the ternary complex eIF2-GTP-tRNA$_i^{Met}$ to yield the 48S particle[48,50]. The formation of the active translation complex 80S is assessed by the GTP hydrolysis for the concurrent release of eIF2 and eIF3[51] and the final joining of the

60S subunit. It is noteworthy that this mechanism is primarily accomplished by functional RNA domains, thus minimizing protein factor requirements and simplifying the pathway for the assembly of the fully active ribosome.

The secondary structure of the HCV IRES region was originally proposed by Brown *et al*[52] and latter refined to include several new motifs and interactions. Under physiological magnesium conditions, the HCV IRES folds into two major domains with well defined functions (Ⅱ and Ⅲ; Figure 2)[53], plus a short stem-loop containing the start codon (domain Ⅳ)[54]. Rather than forming a tightly packed element, domains Ⅱ and Ⅲ are extended and aligned at both sides of a complex double pseudoknot structure (PK1 and PK2; Figure 2)[49,55]. The 3D architecture and several single RNA structural elements are highly conserved among other closely related viruses from the *Flaviviridae* family[46,56,57].

Domain Ⅱ is an autonomously folded module composed of two short helical segments, the basal subdomain Ⅱa and the apical subdomain Ⅱb, separated by a highly conserved internal E-loop[56,58] and capped by an apical loop (Figure 2). Domain Ⅱ adopts an overall distorted L-shape conformation[59] because of the twist forced by the internal E-loop. This folding is conserved in HCV and related viruses[51].

While domain Ⅱ is not essential for 40S recruitment[47,60,61], it has been shown that its deletion decreases viral protein synthesis yield up to five-fold by blocking the formation of the translationally active 80S complex[46,48,60,62,63]. Analysis by cryo-EM have demonstrated that the bend in domain Ⅱ is a requisite for changing the conformation of the 40S ribosomal subunit[49,64], in a reminiscent manner to that shown by eIF1 in the canonical cap-dependent translation initiation mechanism[65]. The apical loop placed in subdomain Ⅱb would also contribute to this structural rearrangement in the ribosome[66]. Remarkably, all these conformational reorganization events do not only account on ribosomal proteins but also on the 18S rRNA. This could be the result of the coordinated action mediated by long-distant contacts established between domains Ⅱ and Ⅳ[67,68]. Ribosome folding rearrangements further induce eIF2-GTP hydrolysis, triggering the release of protein factors and the recruitment of the 60S subunit to constitute the 80S complex[48,51,63,64].

The large, highly branched domain Ⅲ consists of six hairpins (designated subdomains Ⅲa to Ⅲf) organized around three- and four-way junctions (Figure 2)[52], which can be identified as recruiting centers for the translational machinery. The apical Ⅲabc junction is the platform for the binding of eIF3[50,69]. The main goal of this interaction seems to be the relief of the competition between eIF3 and the IRES for a common site in the 40S ribosomal subunit, as well as avoiding the formation of canonical 43S translational complexes[70]. This assesses that HCV mRNA translation is specially favored over that of host mRNAs.

The middle section of domain Ⅲ is defined by a three-way junction that contains the essential G-rich subdomain Ⅲd (Figure 2). This element is the core 40S binding center[60,71,72]. Its structure is that of a dynamic stem-loop with an internal E loop motif and an apical loop with typical U-turn geometry[73,74]. This architecture exposes the bases placed in the apical loop and favors their interaction with viral and host ligands, both nucleic acids and proteins. Further, the subdomain Ⅲd seems to be a determinant partner in the acquisition of the functional folding of the surrounding domains[53].

The basal fragment of domain Ⅲ (subdomains Ⅲe and Ⅲf) includes the highly conserved, complex double-pseudoknot motif (PK1 and PK2; Figure 2)[55], which defines a four-way junction to constrain the position of the AUG codon at the P-site of the 40S ribosomal subunit. Remarkably, the spatial distance between the pseudoknot and the AUG firmly resembles to that observed between the canonical Shine-Dalgarno motif and the initiation codon in prokaryotic mRNAs[75]. As noted above, the structural element PK1-PK2 also guides domains Ⅱ and Ⅲabc in an extended conformation to get the easy access of protein factors.

Domain Ⅳ exposes the AUG start codon, at nucleotide 342, in an apical loop enclosing a helical motif (Figure 2). This structure is not conserved in other HCV-like IRESs[76]. In fact, the stem must be unwound to allow for the recognition of the AUG codon, which could entail some disadvantages. This is in good agreement with data demonstrating that the stability of stem-loop Ⅳ is inversely correlated to IRES translational efficiency[54].

Therefore, the HCV IRES is defined by a set of RNA domains that replace the functions played by many host factors to provide a simplified way for the initiation of the viral proteins synthesis. Moreover, these functional domains are able to manipulate the translational machinery to assess the preferential reading of the HCV mRNA.

ESSENTIAL 3'UTR

The HCV 3'UTR is of primary importance for the initiation of the minus RNA strand synthesis during the viral replication step[33,77,78] and also may act as enhancer of the IRES function[20-24]. It is about 240 nts long sequence placed at the 3' end of the viral genome[79], with evolutionarily conserved secondary structure elements that define three functionally and conformationally independent modules (Figure 3). From 5' to 3': (1) A poorly conserved sequence of around 40 nts, termed hypervariable region at the 5' end of the 3'UTR. It folds as a single stem-loop, which is not completely required for viral replication[33,78]; (2) A polyU/UC tract, whose length and composition is a critical determinant of efficient HCV replication in cell culture[80]. It has been proved that a minimum of 26 U nts homopolymer is enough for efficient amplification of the viral RNA[33,78]. Further, it can act outside of its usual molecular context, thus suggesting that this is not only a linker region[80]. The polyU/UC stretch also interacts with host factors related to cellular protein synthesis, such as polypyrimidine tract-binding protein[81,82], the La autoantigen[83], heterogeneous nuclear ribonucleoprotein C (Gon-

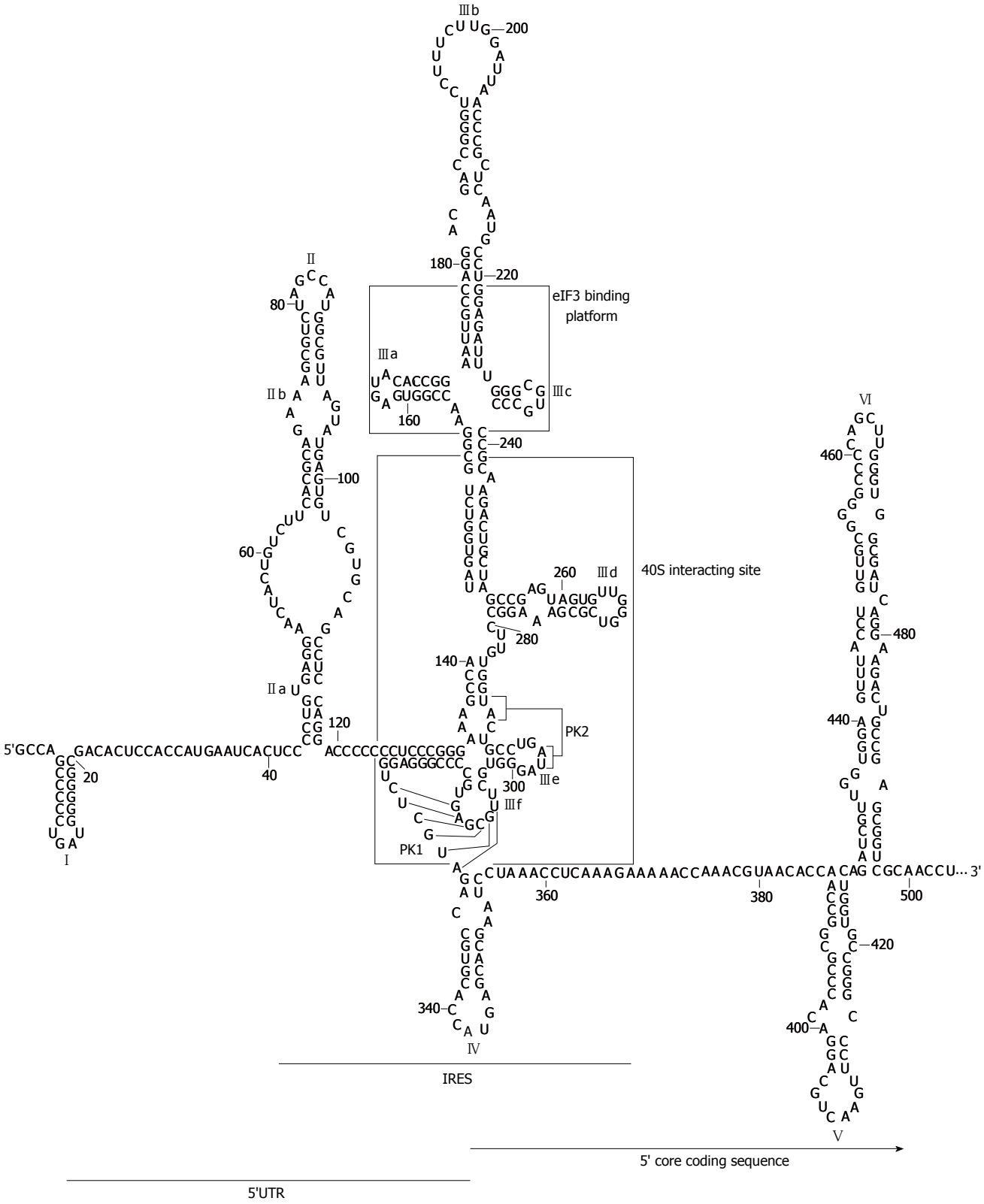

Figure 2 Secondary structure proposed for the hepatitis C virus internal ribosome entry site. The 5' untranslatable region (UTR) plus domains Ⅴ and Ⅵ located at the core coding sequence are included. Minimum region for internal ribosome entry site (IRES) activity is depicted. Domains involved in the interaction with eIF3 factor and ribosomal subunit 40S are marked in boxes. Pseudoknot elements are indicated as PK1 and PK2. The translation start codon is shown in bold. Nucleotide numbering corresponds to hepatitis C virus con1 isolate.

tarek, 1999 #1925) and glyceraldehyde-3-phosphate dehydrogenase[84], among others[85,86]. It seems likely that the recruitment of these factors could contribute to regulate viral translation mediated by the IRES region[20,21]; and (3) The 3'X-tail is a highly conserved, 98-nts long sequence, located at the 3' termini of the HCV genome. It theoretically folds into two alternate and mutually exclusive conformations[35] (Figure 3). Both predicted structures pre-

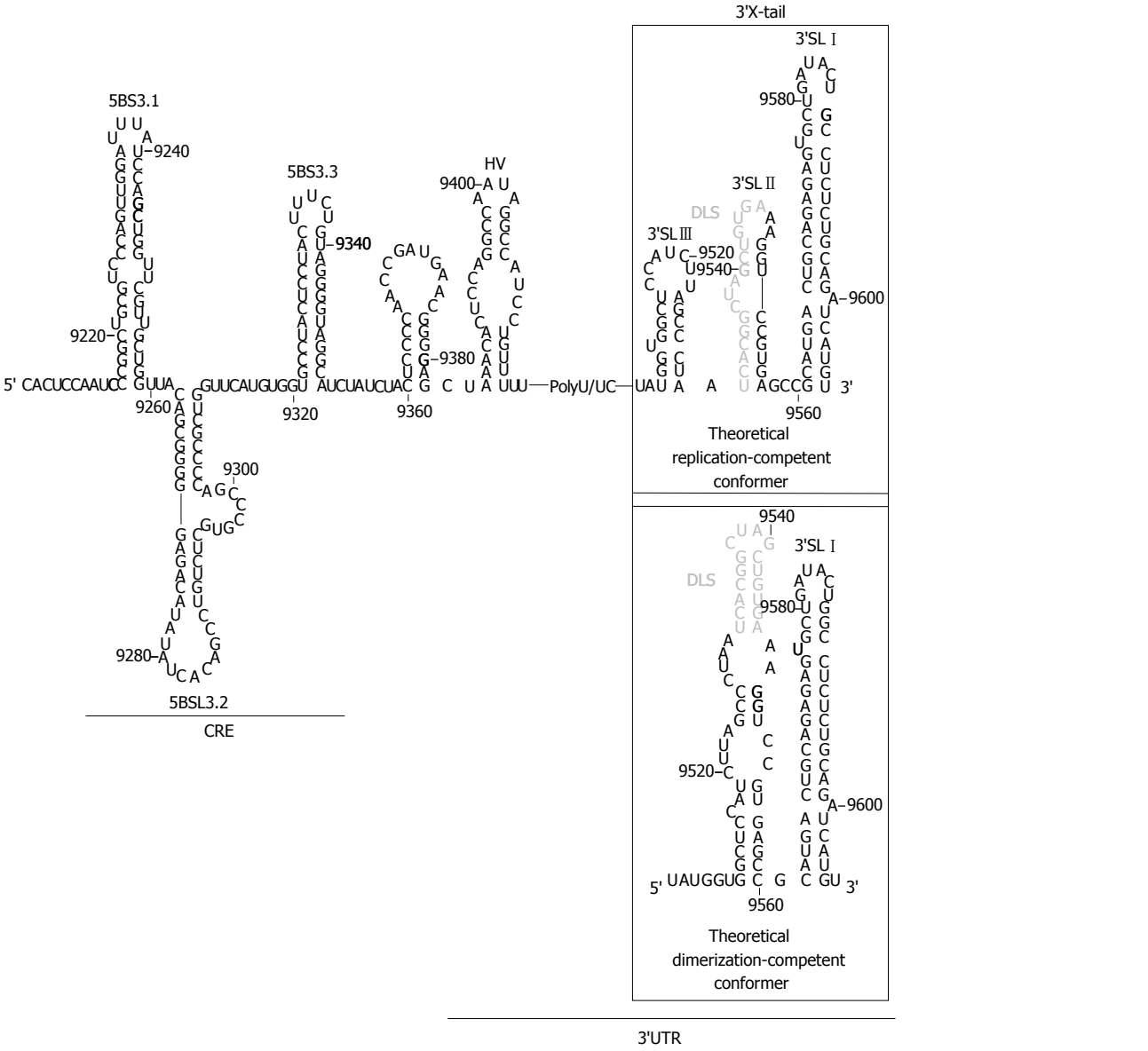

Figure 3 Theoretical folding of the 3' end of the hepatitis C virus genomic RNA. The figure shows the entire 3' end containing the cis-acting replicating element (CRE) region plus the 3' untranslatable region (UTR). The 3'X-tail folds into two different conformers with distinct functional roles. Dimer linkage sequence (DLS) is shown in grey. Translation stop codon in position 9371 is indicated in bold. Nucleotide numbering is as noted in Figure 2.

serve the essential 3'SL I placed at the very 3' end, which has important implications for the initiation and specificity of the viral RNA replication[87-89]. The 55 nts segment placed upstream of 3'SLI folds either as two stem-loops, named 3'SL II and 3'SL III, or as a single stem-loop exposing a 16 nts long palindromic sequence [dimer linkage sequence (DLS)] (Figure 3). Both conformers assume different functionalities during the HCV cycle and are therefore related to transitions between different steps of the viral infection.

The molecular basis of the 3'UTR functioning are not well understood. Several reports have described the binding of both viral and host factors to the different structural elements of the 3'UTR[81,90-97], but these findings do not provide completely satisfactory explanations for many of the experimental observations. The involvement of the 3'UTR in a long range RNA-RNA interaction network with other genomic elements would likely fill the gaps in the complex functioning of this region[30,31,33,98,99].

FUNCTIONAL RNA DOMAINS WITHIN THE CODING SEQUENCE

Cis-acting elements within the core coding sequence

Advances in novel bioinformatic tools have allowed for the extensive search of evolutionarily conserved RNA domains, resulting in the identification of domains distinct from those present in the UTRs. Comparative analyses of numerous HCV isolates sequences revealed an unusual high degree of conservation in the 5' end of the core protein coding sequence[100]. Interestingly, this conservation could not be explained only by the preservation of the amino acid sequence since synonymous substitutions were suppressed. This finding entails a functional

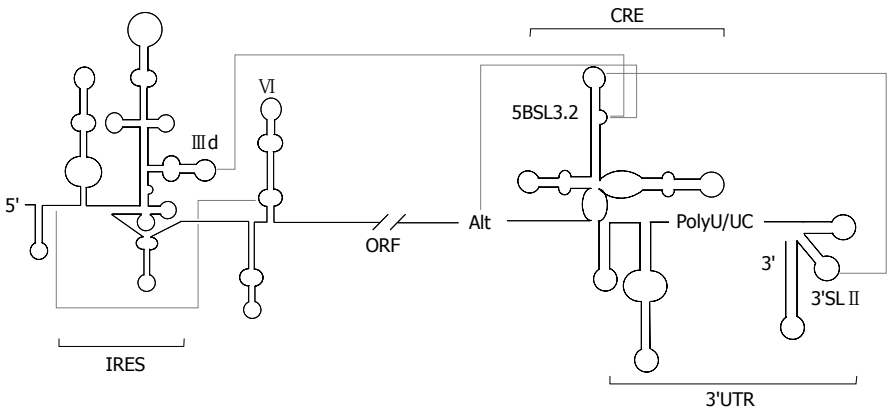

Figure 4 Long-range RNA-RNA interactions in the hepatitis C virus genome. Detailed diagram of the interacting network in the genomic hepatitis C virus RNA. The minimum region for internal ribosome entry site (IRES) activity is marked. The 3' end of the viral genome, containing both the cis-acting replicating element (CRE) and the 3' untranslatable region (UTR), is included. Functional RNA domains involved in the establishment of long-distant contacts are indicated. Figure adapted from[5].

constrain that was related to the presence of an alternative ORF coding for the so-called protein F[101,102] (Figure 1A); and to the existence of structural RNA domains with functional roles in the HCV cycle[38] (Figure 2). While the production and biological role of protein F is still a controversial issue[103], it has been demonstrated that the 5' core coding sequence folds as two stem-loops (domains V and VI; Figure 2) important for IRES activity and viral replication[19,40,42,43].

The mechanism of action of domains V and VI is unclear. It has been proposed their participation in a long-range RNA-RNA interaction involving nucleotides 24-38 of the linker region between domains I and II in the 5'UTR, and 428-442 placed in domain VI (Figure 4)[40,41]. This contact would render a locked conformation of the IRES, which could be released by the interaction of the liver-specific microRNA miR-122 with nucleotides 22-28 of the HCV RNA[104]. This hypothesis provides a mechanism for the involvement of domain VI in viral translation, as well as supporting the essential role of miR-122 in HCV infection[105,106]. Alternatively, Roberts *et al*[107] found that viral translation regulation mediated by miR-122 is strictly dependent on Argonaute proteins and does not involve the structural transition previously proposed. It should be noted that investigations were performed with different experimental tools and model systems. Hence, it is not possible to discard any of the proposals; neither they are mutually exclusive in a cellular context.

Cis-acting replicating element in the viral RNA polymerase coding sequence

In addition to the core coding sequence, the 3' end of the HCV ORF also harbors evolutionarily conserved structural RNA elements. Up to six different stem-loop motifs have been identified by using a combination of sequence alignment and thermodynamic folding softwares, as well as classical comparative analysis[38,39,108,109]. One of these structural elements, the so-called domain 5BSL3.2 or SL9266, is embedded into a cruciform structure delimited by two adjacent stem-loops, 5BSL3.1 and 5BSL3.3 (CRE, Figure 3). While the essentiality of 5BSL3.2 for virus replication has been largely demonstrated[44,45,98,109], the role of the two additional domains 5BSL3.1 and 3.3 is still unclear[80].

The 5BSL3.2 stem-loop consists of two G-C rich helices connected by an eight-base bulge, and capped by a 12-base apical loop (Figure 3)[45,98]. Disruptions in either the sequence or its folding lead to replication-incompetent HCV genomes[45,98]. Moreover, subtle changes in the apical loop prevent RNA replication, indicating that sequence specificity is required for interaction with protein factors, such as the NS5B protein (viral RNA dependent RNA polymerase)[110] and, more likely, distal RNA functional elements[29,98,99,109]. Relocation of 5BSL3.2 was only possible to the 3' variable region preceding the poly(U/UC) tract, involving a functional link with the 3'UTR[98]. Domain 5BSL3.2 has been also shown to act as an inhibitory element of the viral IRES function[25], even in the presence of a translational enhancer such as the HCV 3'UTR. This action is strictly dependent on the sequence and the structural integrity of the bulge, pointing again to the existence of interactions with distant functional RNA domains of the viral genome.

LONG-RANGE RNA-RNA INTERACTION NETWORK IN THE HCV GENOME

As it has been mentioned, the preservation of a proper equilibrium among different viral process and the adequate transitions between them must be assessed for reaching adaptive fitness and virus persistence. To accomplish this, the available functional genomic RNA domains establish an intricate and dynamic interacting web that is mediated, not only by the well-known protein-related 5'UTR-3'UTR bridges[21-23,27,28,111], but more importantly by the formation of direct RNA-RNA contacts that minimize protein requisites.

The domain 5BSL3.2 is a good example of an all-RNA-based mechanism. This element participates in

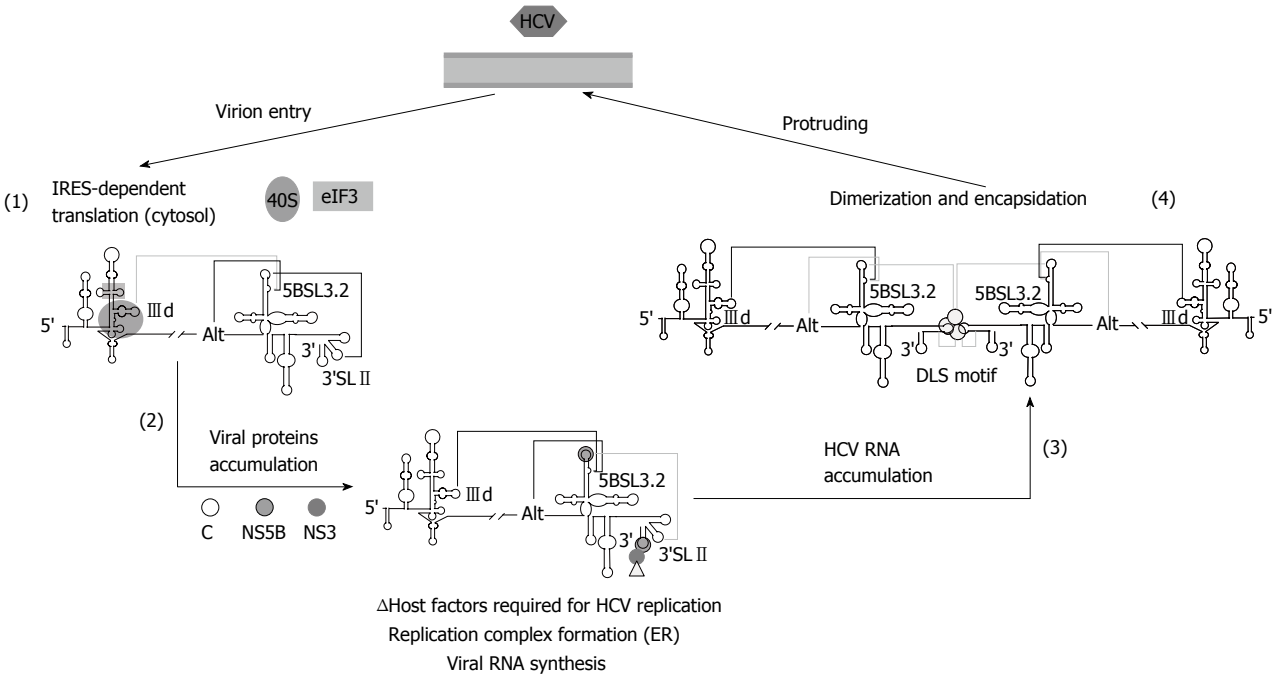

Figure 5 Proposed model for the participation of long-distant RNA-RNA interactions in the consecution of the hepatitis C virus infective cycle. Virions penetrate inside the cell and the genomic RNA is released in the cytoplasm to initiate viral translation (1). The 40S ribosomal subunit binds to the IIId subdomain of the internal ribosome entry site (IRES) region and avoids its interaction with domain 5BSL3.2, thus favoring the contacts Alt-5BSL3.2-3'SL II. Once protein levels have surpassed a certain concentration (2), replication complexes are fixed on the surface of the endoplasmic reticulum (ER) to initiate the genomic RNA amplification. The recruitment of protein factors at the 3'X-tail would hide the 3'SL II domain. This would displace the interactions balance toward the IIId-5BSL3.2 contact, thus impeding efficient translation. Importantly, a considerable pool of molecules should alternatively display a preferred folding defined by the interaction Alt-5BSL3.2, which is indispensable for replication. The accumulation of newly naked viral genomes (3) could induce a replication-defective state by the acquisition of a favored conformation involving the interaction IIId-5BSL3.2, which would yield the dimer linkage sequence (DLS) exposed in an apical loop. The presence of the viral core chaperone protein would participate in the formation of dimeric genomes (4), which would be finally encapsidated, enveloped and released to the extracellular medium. Improbable contacts are indicated by grey lines at each step. Figure adapted from[31].

viral translation and replication by its integration in a complex network of interactions with distant regions of the HCV genomic RNA (Figure 4). The apical loop of 5BSL3.2 is complementary to the apical loop of the 3'SL II within the 3'X-tail[98,99,112]. The resulting kissing loop contact contributes to the structural organization of the 3'X-tail and is essential for HCV replication[98]. The 8-nts bulge may establish two different interactions: (1) one with the apical loop of the subdomain IIId of the IRES region[29,112], which is related to the aforementioned translational inhibitory effect[25]; (2) the second with the Alt sequence, centred around position 9110, upstream of the CRE element[99,109,112]. This interaction is again critical for the synthesis of the viral genomic RNA[109]. Analyses by different biochemical techniques have proved that the complex interplay IRES-5BSL3.2-3'X-tail influences the global architecture of the affected regions and the surrounding functional RNA elements[30,31,99]. Thus, the 3' end of the HCV RNA genome, which contains both the CRE and the 3'UTR elements, fine-tunes the three dimensional structure of the IRES region[30], which could be associated to the regulation of viral translation[25]. Conversely, the interaction IIId-5BSL3.2 induce structural rearrangements in the 3'X-tail that finally lead to the conformational transition of the essential domains 3'SL II and 3'SL III, which switch to a single stem-loop folding that exposes the DLS motif in an apical loop[31] (Figure 3). Importantly, it has been recently reported that all these interactions are equally probable[112]. Therefore, choosing between different contacts might depend on the presence of additional host and/or viral proteins.

Based on these findings, it has been recently proposed a working model[31], which integrates current knowledge concerning to RNA-RNA interactions in the HCV genome, and their implications for the consecution of the viral cycle (Figure 5). In the first stage of the infection, the HCV IRES would be occupied by the translational machinery, thus avoiding any contact with the 5BSL3.2 domain. This would favor the establishment of the interactions 5BSL3.2-3'SL II, which occludes the DLS motif, and 5BSL3.2-Alt. After protein synthesis, the CRE and the 3'X-tail would recruit the viral polymerase (NS5B) and other replication complex factors (both RNA and proteins)[92,109,111,113-117]. In this context, both the 5BSL3.2-IIId and 5BSL3.2-Alt interactions could be equally feasible. Swapping between them could contribute to the creation of a translational repressed state[25] and an enhanced replicative process[109]. The subsequent amplification of viral RNA molecules would displace the structural equilibrium between the 5BSL3.2-IIId and 5BSL3.2-Alt interactions toward the long-range IRES-CRE contact. This would increase the proportion of RNA genomes exposing the DLS motif in the apical loop of the dimerizable conformation, leading to the formation of dimeric

genomic particles in the presence of the core chaperone protein[34].

Therefore, domain 5BSL3.2 would occupy the central position in a complex and dynamic interacting web that would help to bring the ends of the HCV genome into close proximity to support the formation of a biologically favoured close-loop topology. Swapping between different RNA structural partners through the viral cycle would thus control the course of the infectious process.

EXTENSIVE STRUCTURED REGIONS IN VIRAL RNA GENOMES

The search for novel conserved RNA structural units in viral genomes has been prompted in recent years by the appearance of bioinformatic tools that allow the study of the secondary structure of whole RNA genomes. Initial investigations based on the study of folding free energies in many positive stranded animal and plant viral RNA genomes identified extensive secondary structure regions that followed well-defined patterns[6,39]-the so-called GORS. They were initially related to different mechanisms for controlling viral replication, yet their prevalence appeared to be quite variable among different genera. For example, extensive base-pairing within the coding sequence was thermodynamically predicted for the hepacivirus genome, while in the closely related *Pestivirus* and *Flavivirus* genera this pattern was clearly absent. Since replication strategies are usually conserved among the members of a same family, it is unlikely that GORS work as fundamental base for the execution of the viral cycle. Remarkably, GORS are strongly associated to viral persistence[7], thus raising the question whether they can be involved in the suppression of innate intracellular defence mechanisms. Thermodynamic predictions and phylogenetic studies based on base-pairing rules have recently been combined with oligonucleotide probe accessibility and atomic force microscopy studies to investigate the link between theoretical predictions and the 3D conformation of viral genomes with and without GORS in solution[7]. The results showed that the HCV genome is a tightly compact molecule, in contrast to RNA genomes that lack GORS, such as that of poliovirus which folds pleomorphically, commonly involving long single stranded stretches. These studies have contributed to understand how RNA conformation could be related with a virus defence system. Thus, it seems likely that extensive folding areas could interfere with the antiviral cellular pathways triggered by double-stranded RNA, such as the interferon production during the initial infection, in an analogous manner to the expression of structured RNA transcripts by large DNA viruses[118]. Though much remains to be investigated about this phenomenon, it has an undeniable relevance for virus-host interactions.

CONCLUSION

During last years, the great advances in the fields of RNA structure determination by high-throughput techniques and bioinformatic tools have enabled the first pictures for the structural organization of the eukaryotic transcriptome, the so-called structure. In molecular virology, these advances have gained special relevance for their implication in the identification of functional RNA domains. These structurally conserved RNA elements interact with protein factors and other RNA domains to direct and regulate essential viral functions as well as switching between different steps of the viral cycle. Interfering with the functioning of these structural domains offers a potential means of treating viral infections, such as that caused by the HCV. Further implementations of the current methodologies will undoubtedly improve the identification and validation of functional RNA domains in the near future, thus extending our knowledge of RNA-mediated regulation not only in viral systems, but also in many cellular processes.

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