Mechanisms of Internal Ribosome Entry in Translation Initiation

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During eukaryotic translation initiation, the 40S ribosomal subunit must be recruited to a messenger RNA (mRNA) and positioned at the correct initiation codon. In most mRNAs, this is achieved via a series of intermolecular events involving a group of protein factors that assemble on the capped 5′ end of the mRNA. This assembly recruits the 40S ribosomal subunit and enables it to scan to the translational start site (Merrick and Hershey 1996). The mRNA is thought to play a passive role in this process and typically lacks significant secondary structure in the 5′-untranslated region that might interfere with scanning (Fig. 1). In contrast, an alternative mechanism of translation initiation involves active roles of the 5′- and in some cases the 3′-untranslated regions of an mRNA (Jackson 1996; Sachs et al. 1997). In these cases, the untranslated regions are often highly conserved, may extend for several hundred nucleotides, and appear to contain extensive secondary and tertiary structures. Here we describe structural features of the 5′-untranslated region of hepatitis C virus (HCV) that enable its function as an internal ribosome entry site (IRES). We also discuss evidence for IRES-mediated translation in certain cellular genes, and the possible roles of conserved 3′-untranslated regions of HCV mRNA in translational control.

IRES: CONTROL AT THE 5′ END

IRESs occur in the 5′-untranslated regions of numerous viral genomic RNAs as well as some eukaryotic cellular RNAs. In contrast to the 7-methyl-guanosine cap-dependent mechanism used by most eukaryotic messenger RNAs, these RNA structures recruit and activate the translation machinery independent of the 5′ end of the mRNA (Fig. 1) (Sachs et al. 1997). IRES RNAs have varied sequences, proposed secondary structures, and cofactor requirements, but all IRES RNAs induce translation initiation in the absence of the 5′ cap structure and in the absence of a 5′ terminus (they are cap- and end-independent).

IRESs were first identified in the picornaviruses, and these provided the initial paradigm for understanding internal translation initiation (Jackson and Kaminski 1995). Within this family of viruses, several different IRES classes were identified based first on secondary structure and later on cofactor requirements. Site-directed mutagenesis of these IRESs, which are between 300 and 500 nucleotides in length, led to the proposal that IRESs form complex three-dimensional tertiary structures that interact with the translation machinery using contacts located throughout the IRES RNA sequence.

HEPATITIS C VIRUS IRES STRUCTURE AND FUNCTION

Recently, intense interest has focused on the IRES of HCV, a viral pathogen of worldwide health concern. This IRES was first identified by Wang et al. (1993), and since that time, extensive mutation and deletion analysis coupled with translation assays both in vivo and in cell-free extracts have identified the sequences and secondary structures necessary for translation initiation (for review, see Rijnbrand and Lemon 2000). The minimal HCV IRES is ~330 nucleotides in length and is highly conserved both in terms of primary sequence and secondary structure. An important step forward in understanding the mechanism of HCV IRES action (and viral IRESs in general) came when Pestova et al. (1998) demonstrated that

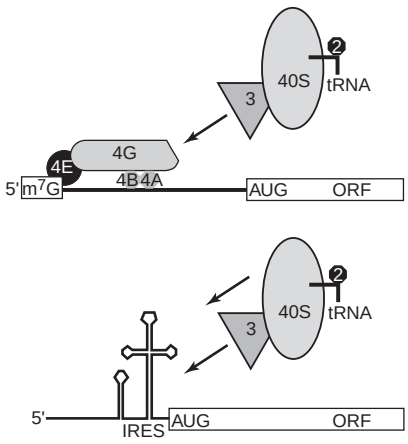

Figure 1. Schematic representation of cap-dependent (*top*) and HCV IRES-driven internal initiation of translation (*bottom*). Cap-dependent initiation requires a set of protein factors that interact with the 5′ cap structure which are not required for 40S subunit recruitment by the HCV IRES RNA.

the HCV IRES binds directly to the 40S ribosomal subunit and eukaryotic initiation factor-3 (eIF3) in the absence of any additional protein factors (Fig. 1). Quantitative biochemical and biophysical analysis of these interactions then identified the presence of a tertiary fold within the HCV IRES RNA and the mechanism by which this fold recruits the translational machinery (Kieft et al. 1999, 2001; Kolupaeva et al. 2000). Binding of the 40S subunit to the HCV IRES RNA involves intermolecular contacts in all three independently folded tertiary domains of the IRES, yet only two of these domains contribute substantially to binding affinity. Within the two domains that provide affinity for the 40S subunit, multiple intermolecular contacts are made. In contrast, binding to eIF3 involves only a single IRES tertiary domain and is weaker than binding to the 40S subunit, and mutations that disrupt the eIF3 IRES interaction but not the 40S–IRES interactions are less detrimental to IRES translation initiation activity. Thus, recruitment of the 43S particle (which contains the 40S subunit, eIF3, eIF2, initiator met-tRNA, and GTP) is driven primarily through multidomain contacts between the 40S subunit and the IRES RNA, suggesting that the strategy of HCV IRES 40S subunit recruitment is fundamentally different from both prokaryotic and canonical eukaryotic mechanisms (Kieft et al. 2001).

The novel nature of HCV IRES translation initiation suggests that behind this mechanism lies an equally unique RNA structure. Detailed, high-resolution studies of the HCV IRES have been hindered by the fact that the IRES RNA is large, multidomained, somewhat flexible, and forms an extended scaffold structure even when folded. These challenges have thus far dictated a "divide and conquer" type of approach to high-resolution structural determination. Recently, nuclear magnetic resonance (NMR) structures of several isolated stem-loops have been solved (Klinck et al. 2000; Lukavsky et al. 2000), and we have recently solved the structure of a folded IRES RNA junction by X-ray crystallography (J.S. Kieft and J.A. Doudna, unpubl.). These structures yield insight into the local folding of individual parts of the IRES, and could be useful in future drug development strategies. However, in order to understand the determinants of IRES affinity and specificity for the translation machinery, structures of IRES RNA bound to its biological targets are needed.

We recently reported the determination of two structures of HCV IRES RNA bound to isolated 40S ribosomal subunit from rabbit, solved by cryo-electron microscopy (cryo-EM) in combination with the single particle approach (Spahn et al. 2001). The two structures were of the full-length IRES RNA and a mutant IRES RNA lacking domain II (Fig. 2). Domain II contacts the 40S subunit and is important for full IRES activity, but contributes little to binding affinity (Kieft et al. 2001). The 20 Å resolution cryo-EM structures reveal that the IRES RNA binds to the head and platform of the 40S subunit in a single extended conformation, making intermolecular contacts that match biochemical predictions. Furthermore, the HCV IRES RNA induces a conformational change in the 40S subunit that involves fusion of the ribosome density in the head and shoulder regions. This conformational change was not observed in the complex with the domain II deletion mutant, and thus it is the presence of this IRES RNA structural domain (domain II) that induces the conformational change. Domain II contacts the head near or in the E site for tRNA binding and effectively transforms the mRNA decoding channel into a tunnel. In addition, the contact induces fusing of ribosome head to shoulder density at the site where mRNA enters the decoding cleft. Previous biochemical data suggested that domain II affects the way in which the 40S subunit and the IRES RNA interact (Pestova et al. 1998; Kolupaeva et al. 2000); the cryo-EM images provide a plausible structural explanation.

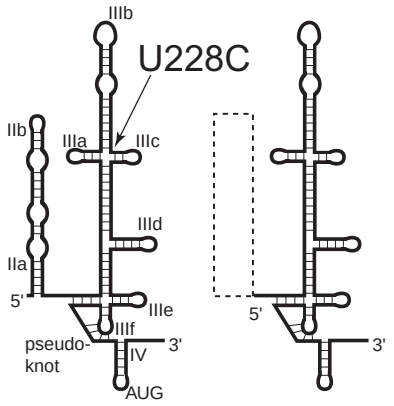

Figure 2. Schematic secondary structures of the HCV IRES constructs used in the study. Mutant U228C has a single point mutation in junction IIIabc (*left*, indicated with *arrow*). The domain II deletion mutant is shown on the right, with a dashed box indicating the location of the deleted domain.

ANALYSIS OF 48S AND 80S PREINITIATION COMPLEX FORMATION WITH THE HCV IRES

Structural and biochemical studies of the HCV IRES provide a foundation for understanding the stepwise mechanism of internal initiation (Fig. 3). In this model, the IRES serves as a binding site for the components of the 43S particle through contacts with eIF3 and the 40S subunit. Once the 43S complex is bound to the IRES (Fig. 3, [4]), the mature 48S complex is formed, with the tRNA anticodon base-paired to the mRNA initiator codon. The 48S complex can then recruit the 60S subunit in a GTP hydrolysis-dependent step, releasing eIF2 and forming the 80S complex. In many ways, this mechanism is very similar to the canonical cap-dependent mechanism, differing only at the step of 43S particle recruitment (Fig. 3, going from [1] to [4]). The realization that HCV IRES RNA binding to the 40S subunit alters the conformation of the ribosomal subunit suggests a role for the IRES RNA as an active manipulator of the translation machin-

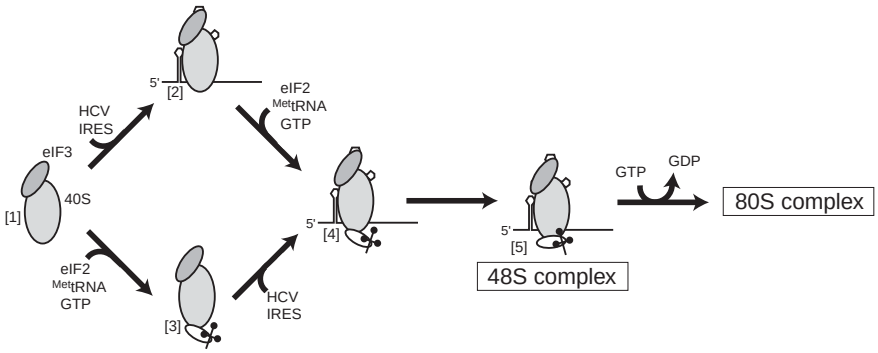

Figure 3. Simplified general mechanism of translation initiation by the HCV IRES. Initiation involves the binding of the components of the 43S particle directly to the IRES RNA ([1] through [4]), formation of the codon–anticodon interaction to form the mature 48S complex ([5]), and subsequent hydrolysis of eIF2-bound GDP, which allows 60S subunit joining. (Adapted from Pestova et al. 1998.)

ery beyond its ability to bind the 40S subunit and eIF3. This observation also raises several new questions regarding the detailed mechanism of HCV IRES-driven internal initiation of translation, including:

- What is the purpose of the 40S subunit conformational change induced by the IRES RNA?
- What are the contributions of other factors on the mechanism of HCV initiation?
- What other dynamic events does the IRES RNA orchestrate within the preinitiation complexes?

To begin a preliminary investigation of these questions, we have taken advantage of the ability of the HCV IRES to assemble preinitiation complexes in rabbit reticulocyte lysate (RRL), and of the ability of small molecule inhibitors to capture these complexes. Specifically, the addition of a nonhydrolyzable GTP analog prevents eIF2-driven GTP hydrolysis and subsequent dissociation of eIF2 from the ribosome, leading to a buildup of 48S complexes. The complexes can then be fractionated via ultracentrifugation through a sucrose gradient, enabling the quantitation of relative amounts formed under different conditions. Incubation of radiolabeled wild-type HCV IRES RNA in RRL in the absence of any small-molecule inhibitors leads to the sucrose gradient profile shown in Figure 4a. After 15 minutes, both 48S and 80S complexes are present, and there is virtually no free IRES RNA remaining. Thus, assembly of preinitiation complexes by the HCV IRES is quite efficient. The efficiency of assembly drops, however, when mutations are introduced into the IRES RNA (Fig. 4b). Two different mutants are shown, one with a single U to C point mutation at nucleotide 228 and the other with domain II completely deleted (Fig. 2). Of these two mutations, mutant U228C is the most inhibited, with a relatively large amount of free RNA remaining after 15 minutes in RRL. This mutant has been shown to initiate translation at only 5% of wild-type level and has reduced affinity for both the 40S subunit and eIF3 (Kieft et al. 1999; Kieft et al. 2001). In contrast, the domain II deletion mutant appears to incorporate into 48S complexes more readily than U228C, but still below wild-type level. Interestingly, this mutant has

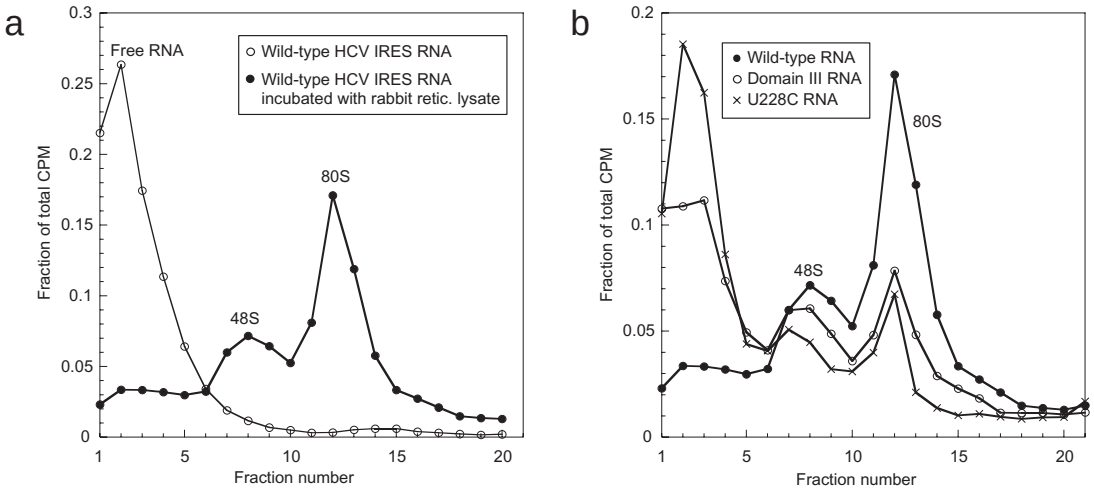

Figure 4. Sucrose gradient profiles of wild-type and mutant IRES RNAs incubated in rabbit reticulocyte lysate for 15 minutes in the absence of small-molecule inhibitors. The locations of free RNA, the 48S complex, and 80S particle are shown.

reduced initiation activity, but has nearly wild-type binding affinity for the 40S subunit and eIF3. This suggests that its reduced ability to form 48S complexes is not due to reduced affinity for the translation machinery. Rather, the fact that domain II induces a conformational change in the 40S subunit through contacts near the tRNA/eIF2-binding site may indicate the presence of additional assembly steps dependent on this IRES structure. It is worth noting that not only do both mutants show a reduced ability to form 48S complexes, but both mutants also display a reduced ratio of 80S:48S when compared to wild-type IRES RNA. Thus, it appears that the assembly pathway may be affected at multiple steps, both before 48S formation and between 48S and 80S formation. Once again, it seems possible that the IRES must do more than just bind the translation machinery; it must somehow interact in very specific ways that signal the assembly of the higher-order complexes.

The above-described analysis reveals the relative populations of free IRES, 48S, and 80S at a single time point, in a fully dynamic system. To focus on the specific step of initial 48S formation, we used a nonhydrolyzable GTP analog to capture 48S complexes and analyzed these IRES RNA-RRL reactions at more than one time point. Figure 5a contains the gradient profiles of wild-type and domain II deletion mutants treated in this manner. Again, after 15 minutes, wild-type RNA is nearly completely incorporated into the 48S complex, while the mutant still displays a large amount of free RNA. Interestingly, after 90 minutes, the mutant RNA has "caught up," now appearing nearly identical to wild-type IRES RNA. Thus, there is a difference in the *rate* of 48S buildup between these two mutants, despite the fact that their *affinities* for the ribosome are virtually the same. Furthermore, this may explain the high amount of variability in reported translation initiation values for mutants involving domain II, as measurements taken at different time points could reveal radically different values. This behavior is in stark contrast to the U228C mutant, shown in Figure 5b. Even after 90 minutes of incubation, this mutant is unable to build up 48S complexes to wild-type levels. Thus, although the two mutants both fail to assemble 48S com-

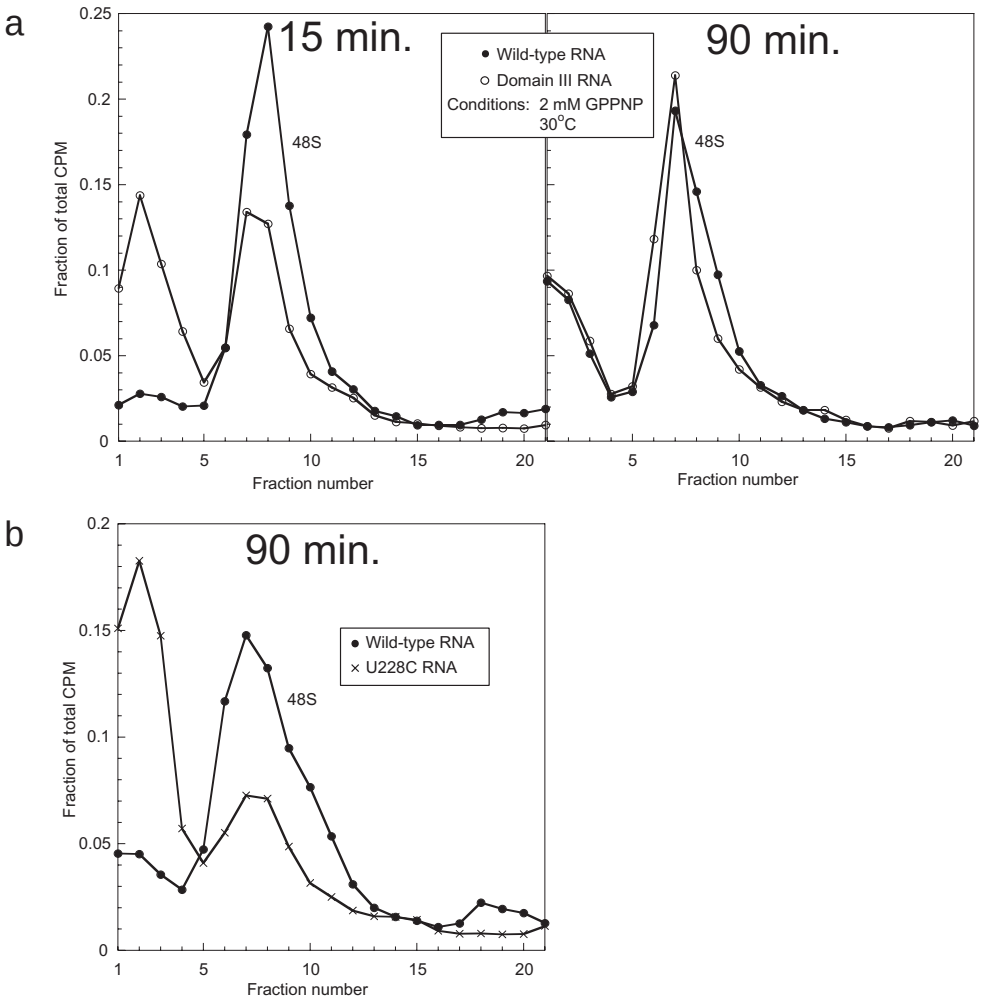

Figure 5. Sucrose gradient profiles of wild-type and mutant IRES RNAs incubated in rabbit reticulocyte lysate in the presence of a nonhydrolyzable GTP analog (GPPNP) for various times.

plexes as well as the wild type, they are failing for different reasons.

ADDITIONAL LAYERS OF COMPLEXITY

From the above-described results it appears that IRES-mediated preinitiation complex formation is an ordered, multistep process. It is possible that factors that do not bind directly to the IRES may transiently contact the IRES RNA or influence IRES-driven assembly. Good candidates for these types of interactions are eIF2 and eIF5. eIF2 should lie near the mRNA-binding groove, close to domain II of the IRES. Indeed, one subunit of eIF2 has been implicated as being important from HCV IRES function in a functional genomics assay (Kruger et al. 2000). eIF5, on the other hand, has been described as a "proofreader" that allows 48S complexes to form 80S particles based on correct formation of the codon–anticodon interaction. Either one of these factors could be receiving or sending signals to the HCV IRES RNA, a possibility that remains to be explored.

IRESs IN CELLULAR mRNAs

In addition to viruses, certain cellular mRNAs appear to utilize an IRES mechanism to facilitate protein expression under conditions where cap-dependent translation may be compromised. Such genes include those encoding human immunoglobin heavy-chain binding protein (BiP) (Macejak and Sarnow 1991), human fibroblast growth factor (FGF-2) (Vagner et al. 1995), insulin-like growth factor (IGF)II (Teerink et al.1995), eIF4G (Gan and Rhoads 1996), platelet-derived growth factor (PDGF)2 (Bernstein et al. 1997), the proto-oncogene c-*myc* (Nanbru et al. 1997), and the vascular endothelial growth factor (VEGF) (Stein et al. 1998). These mRNAs characteristically contain 5′UTRs longer than 200 nucleotides, often with numerous AUGs upstream from the initiation codon. The presence of an IRES in these 5′UTRs was established by the use of dicistronic constructs containing the putative IRES sequence inserted between two protein-encoding cistrons, or by the ability of the 5′UTR in question to initiate synthesis of a reporter protein during picornavirus infection (when cap-dependent translation is compromised) (van der Velden and Thomas 1999).

Although cellular IRESs have not been studied as extensively as the viral IRESs, initial differences between them can be noted. When compared to picornavirus IRESs, which can stimulate expression of the second cistron by 100-fold, most cellular IRESs only modestly increase second cistron expression (2- to 20-fold) (although the c-*myc* IRES stimulates expression ~70-fold) (Stoneley et al. 1998). In addition, some cellular IRESs demonstrate an ability to modulate activity in response to the cell cycle (Cornelis et al. 2000; Pyronnet et al. 2000). Furthermore, no secondary structural homology appears to exist between viral and cellular IRESs, although the structural organization of cellular IRES elements is currently poorly defined. There may be functional differences between the viral and cellular IRESs, as well. For example, deletion of the 3′ end of viral IRESs abolishes IRES activity, whereas activity is only gradually lost as segments are deleted from the c-*myc* IRES.

An intriguing question is why a certain subset of mRNAs has evolved to be translated by both the "conventional" cap-dependent mechanism and the IRES-mediated cap-independent mechanism. Interestingly, the genes implicated in using an IRES encode factors involved in cell growth, transcription, and translation initiation. Expression of such proteins must be tightly regulated during growth, differentiation, and apoptosis. IRES-mediated translation of these cellular mRNAs may represent a vital regulatory mechanism for the survival and proliferation of cells under acute but transient stress conditions. It remains to be discovered whether cellular IRESs represent a unique strategy for recruiting ribosomes and whether all cellular IRESs use the same mechanism to interact with the translational machinery. In addition, it will be important to identify protein components mediating IRES-dependent translation of cellular mRNAs, and to understand how these IRES complexes assemble and manipulate the translation apparatus.

REGULATORY ELEMENTS IN VIRAL 3′-UNTRANSLATED REGIONS

Like 5′UTRs, the 3′UTR of RNA virus genomes is often conserved and has roles in the translation, replication, and packaging of viral RNA (Chambers et al. 1990; Gale et al. 2000). In HCV, the 3′UTR consists of a variable region (30–40 nucleotides) and a homopolymeric poly(U)/polypyrimidine tract (20–200 nucleotides) followed by the 3X region (98 nucleotides), which is highly conserved across all serotypes (Chambers et al. 1990). Chemical and biochemical studies together with modeling (MFOLD) suggest that the 3X region consists of three stem-loops (SL1, SL2, and SL3, Fig. 6), although chemical probing data indicate that the SL3 and SL2 region is structurally heterogeneous and may have alternate conformations. In chimpanzees, both the poly(U) and the 3X region are necessary for viral infection and replication, whereas the variable region appears to be dispensible (Yanagi et al. 1999; Kolykhalov et al. 2000).

A common aspect of mechanistically distinct cellular and viral translation mechanisms is synergistic interac-

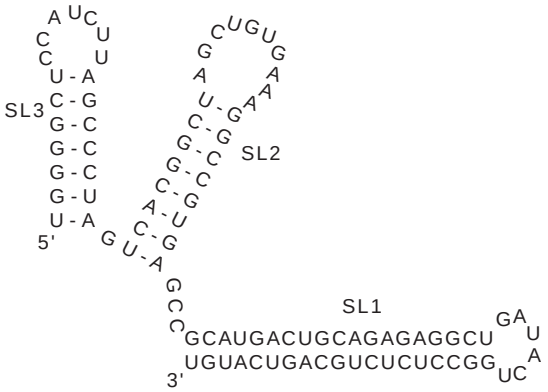

Figure 6. Secondary structure of the HCV 3X region.

tion between the 5′ and 3′ ends of mRNA, which affects translational efficiency. In several cases a physical link between the 5′ and 3′ ends via protein and/or RNA contacts leads to circularization of the mRNA, and this may be a general phenomenon (Wells et al. 1998). Although there are regions of sequence complementarity in the 5′ and 3′ UTRs of HCV, no RNA:RNA interactions have been detected (Blight and Rice 1997). Similarly, studies with a replicon of the related *pestivirus* bovine diarrhea virus (BVDV) showed that there were no RNA:RNA interactions occurring between the 5′ and 3′ UTRs (Yu et al. 1999). This raises the possibility that circularization of the HCV genome, if it does occur, may involve a linkage involving protein:RNA interactions.

Proteins that have been shown to bind elements of the HCV 3′UTR include La (Spangberg et al. 2001), hnRNP C (Gontarek et al. 1999), HuR (Spangberg et al. 2000), glyceraldehyde 3-phosphate dehydrogenase (Petrik et al. 1999), viral NS3 (Banerjee and Dasgupta 2001), ribosomal protein L22 (Wood et al. 2001), and PTB (pyrimidine tract binding protein) (Ito and Lai 1997; Tsuchihara et al. 1997; Chung and Kaplan 1999), but the biological relevance of these interactions remains unclear. Both La and GAPDH recognize the polypyrimidine tract, and La may have a role in maintaining stability of the RNA (Spangberg et al. 2001). PTB and the ribosomal protein L22 are the only proteins identified so far that appear to bind specifically to the 3X region. Interestingly, L22 has also been shown to bind to EBER RNA of the Epstein-Barr virus, an RNA that has no sequence homology with the HCV 3X region (Toczyski et al. 1994). A specific interaction between PTB and the 3X RNA has been demonstrated by a number of investigators (Ito and Lai 1997; Tsuchihara et al. 1997; Chung and Kaplan 1999). PTB has also been shown to play a role in the translation of several RNAs (Hunt and Jackson 1999; Gosert et al. 2000) and to interact with the HCV IRES (Anwar et al. 2000). The region of the 3X RNA that interacts with PTB appears to be limited to stem-loops SL2 and SL3, and there is a requirement for the integrity of the structure and sequence of the stem-loops (Ito and Lai 1997; Chung and Kaplan 1999). However, the sequence immediately prior to stem-loop SL3 is partially homologous to the consensus sequence for cellular polypyrimidine tract targets of PTB recognition, and there are conflicting results as to its requirement for efficient PTB binding of 3X (Tsuchihara et al. 1997). Since L22 and PTB are relatively promiscuous and bind other RNA targets that are dissimilar to 3X, it seems unlikely that these proteins are responsible for the high of degree of conservation observed in the 3X region. Furthermore, no quantitative data are available yet regarding the affinity and specificity of these interactions.

Sequences that interact with a specific protein and enhance translation have been identified in the 3′UTR of a number of cellular mRNAs. In in vitro translation assays, the 3X region enhances the efficiency of both cap-dependent and IRES-mediated translation 3- to 4-fold (Ito et al. 1998; Michel et al. 2001). However, recent work which examined these effects in the context of the complete 3′UTR found that the presence of the 3X region depressed translation (Murakami et al. 2001). Perhaps the use of HCV replicons will enable these conflicting results to be resolved and should facilitate investigations into the role of the HCV 3′UTR in the viral life cycle (Blight et al. 2000).

CONCLUSIONS

Regulation of translation via 5′- and 3′-untranslated regions is ubiquitous, yet the molecular mechanisms of these processes are not well understood. Structural and biochemical analyses of viral IRES elements and 3′ UTRs will reveal details of such pathways that will both illuminate basic virus biology and provide the basis for rational design of antiviral therapeutics. These investigations may also shed light on fundamental mechanisms of translational control present in eukaryotic cells.

ACKNOWLEDGMENTS

The authors thank the National Science Foundation and the National Institutes of Health for training grant support to A.G. and a Program Project grant to J.A.D. J.A.D. is a fellow of the David and Lucile Packard Foundation.

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