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Translational insensitivity to potent activation of PKR by HCV IRES RNA

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ABSTRACT

Translation of hepatitis C virus (HCV) is initiated at an internal ribosome entry site (IRES) located at the 5′end of its RNA genome. The HCV IRES is highly structured and greater than 50% of its nucleotides form based-paired helices. We report here that the HCV IRES is an activator of PKR, an interferon-induced enzyme that participates in host cell defense against viral infection. Binding of HCV IRES RNA to PKR leads to a greatly increased (20-fold) rate and level (4.5-fold) of PKR autophosphorylation compared to previously studied dsRNA activators. We have mapped the domains in the IRES required for PKR activation to domains III–IV and demonstrate that the N-terminal double-stranded RNA binding domains of PKR bind to the IRES in a similar manner to other RNA activators. Addition of HCV IRES RNA inhibits cap-dependent translation in lysates via phosphorylation of PKR and eIF2α. However, HCV IRES-mediated translation is not inhibited by the phosphorylation of PKR and eIF2α. The results presented here suggest that hydrolysis of GTP by eIF2 is not an essential step in IRES-mediated translation. Thus, HCV can use structured RNAs to its advantage in translation, while avoiding the deleterious effects of PKR activation.

1. Introduction

Viral RNAs control key aspects of both viral and host function. Beyond their function as genomic material, viral RNAs can act as *cis*-regulatory elements, as binding sites for proteins or other nucleic acids and their complexes. Hepatitis C virus (HCV) is a positive-sense RNA virus of the flaviviridae family and is the main causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Guidotti and Chisari, 2006). Translation of the HCV genome is initiated using an RNA element at its 5′end, known as an internal ribosome entry site (IRES) (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The host 40S ribosomal subunit binds directly to the IRES with high affinity, positioning the IRES start codon proximally to the ribosomal peptidyl-tRNA site (P-site) where the initiator tRNA codon–anticodon interaction forms (Otto and Puglisi, 2004; Pestova et al., 1998). The cap binding and scanning activities of a subset of initiation factors, including eIF4E and eIF4G, are not required for HCV IRES-mediated initiation (Otto and Puglisi, 2004; Pestova et al., 1998; Reynolds et al., 1996). IRES-40S complex formation leads to subsequent stepwise assembly of translationally competent complexes. Only a subset of host translation factors is required for HCV IRES-mediated initiation, including initiator $tRNA^{Met}$, the trimeric GTPase eIF2, as well as the IF2 orthologous eIF5B, another GTPase, and the large multiprotein assembly eIF3 (Ji et al., 2004; Otto and Puglisi, 2004; Pestova et al., 1998). Hydrolysis of GTP by eIF2 or eIF5B gates downstream events leading to assembly of the 80S initiation complex and translation of the HCV viral proteins (Locker et al., 2007; Pestova et al., 2001; Terenin et al., 2008).

The HCV IRES is highly structured and conserved among viral genotypes. The IRES contains a large fraction of based-paired secondary structure, with over 50% of the 372 nts involved in Watson–Crick or G–U pairing (Honda et al., 1999; Zhao and Wimmer, 2001). The secondary structure of the IRES consists of 4 structural domains rich in double-helical regions (domains I–IV) (Fig. 1A). Structural features of these domains have been determined using NMR spectroscopy and X-ray crystallography (Kieft et al., 2002; Kim et al., 2002; Lukavsky et al., 2000, 2003). The functional and structural roles of these domains in IRES mediated translation have been defined; domain I is thought to be dispensable for IRES function, whereas domains II–IV form the functional core. Domain III directs high-affinity contact with a protein-rich ribosomal surface near the tRNA exit site (E site) (Kieft et al., 2001; Kolupaeva et al., 2000; Lytle et al., 2001; Otto et al., 2002; Otto and Puglisi, 2004). The domain IIIabc junction, stem loop IIIe, and the RNA pseudoknot are essential for ribosomal interaction. Domain II is located near the ribosomal P-site codon, and likely modulates factor binding and function (Locker et al., 2007).

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Host response to double-stranded RNAs is a hallmark of innate immunity. Double-stranded RNA-dependent protein kinase (PKR) is central to this response (Gale and Katze, 1998). PKR is a 551 amino acid protein, containing tandem double-stranded RNA binding domains (dsRBDs) at the N-terminus and a C-terminal serine-threonine kinase domain, connected by a flexible 80 amino acid linker (Clemens and Elia, 1997). Both dsRBDs are required for PKR binding to and activation by dsRNA (Bevilacqua and Cech, 1996; Kim et al., 2006; McKenna et al., 2007d). Upon RNA binding, self-association of PKR facilitates autophosphorylation of PKR at a key threonine in a canonical activation loop (Dey et al., 2005; McKenna et al., 2007d; Wu and Kaufman, 1997). The phosphorylated, catalytically active form of PKR regulates protein synthesis via efficient substrate phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) at Ser51, inhibiting the guanine nucleotide exchange activity of the eIF2 heterotrimeric complex and resulting in a reduction in translation efficiency (Gale and Katze, 1998).

Long stretches of double-stranded RNA are unusual in host RNAs. However, viral RNAs can be rich in secondary structure to accommodate packing into capsids. In addition, double-stranded RNAs can be formed during viral replication as intermediates. To probe how PKR responds to structured viral RNAs, we have investigated the activation of PKR by the HCV IRES. Here we show that the HCV IRES is an extremely potent activator of PKR kinase activity. We have mapped the domains in the IRES required for activation to domains III–IV, which mediate interaction of the IRES with the ribosome, and demonstrated that the dsRBDs of PKR bind to the IRES in a similar manner as to other RNA activators. We show that addition of HCV IRES RNA to translation extracts leads to potent inhibition of canonical cap-mediated translation. However, HCV IRES-initiated translation is not affected by PKR-mediated eIF2 phosphorylation. Thus, HCV is able to use structured RNAs to its advantage in translation, while avoiding the deleterious effects of PKR activation.

2. Materials and methods

2.1. Plasmid architecture

Plasmids for transcription of HCV IRES, pT7HCVIRES (J1), (JFH-1), (H77c), and (S52) carry the cDNA of nucleotides 1–374 of HCV J1 (genotype 1b), JFH-1(2a), H77c(1a), and S52(3a) under a T7 promoter, respectively. A PCR product containing, in order, a HindIII site, T7 promoter, HCV IRES sequence, BsmBI site, and EcoRI site was cloned into HindIII and EcoRI sites of pUC118. Plasmids for the transcription of HCV IRES domains, pT7II, pT7III–IV, pT7IIIb, pT7IIIacd, and pT7IIIef-IV carry cDNA of nucleotides 45–117, 119–354, 178–221, (137–178 and 221–287), and (119–139 and 285–354) of HCV J1were designed as above, with the exception that the BsmBI and EcoRI sites are replaced with a BsaI site for domain II and BbsI site for domains III–IV, IIIb, IIIacd, and IIIef–IV.

DNA fragments for luciferase reporter RNAs are under the control of a T7 promoter; pT7HCVLuc carries nucleotides 1-374 (5′UTR and 33nt of core protein-coding region of HCV J1) and nucleotides 9372-9549 (3′UTR) of HCV J1 at the 5′ and 3′end of the firefly luciferase gene, respectively. pT7EMCVLuc contains EMCV IRES (nucleotides 271–831) at the 5′end of Firefly luciferase gene (Shimoike et al., 1999). pT7HCVLuc, pT7EMCVLuc, and pRL-null that carry the Renilla luciferase gene under control of a T7 promoter (Promega) are linearized with BamHI, XhoI, and XbaI, respectively.

2.2. RNA and protein preparation

For the preparation of HCV IRES (J1, JFH-1, H77c, and S52), II, III–IV, IIIIb, IIIacd, IIIef–I, TAR (Kim et al., 2006), and VA_I RNA (McKenna et al., 2006), pT7HCV IRES, II, III–IV, IIIb, IIIacd, IIIef-IV, pTAR, and pVA_I were linearized with BsmBI (HCV IRES), BsaI (II, VA_I), BbsI (III–IV, IIIb, IIIacd, IIIef-IV), or BstZI (TAR), respectively. All viral RNAs were prepared via *in vitro* transcription as described

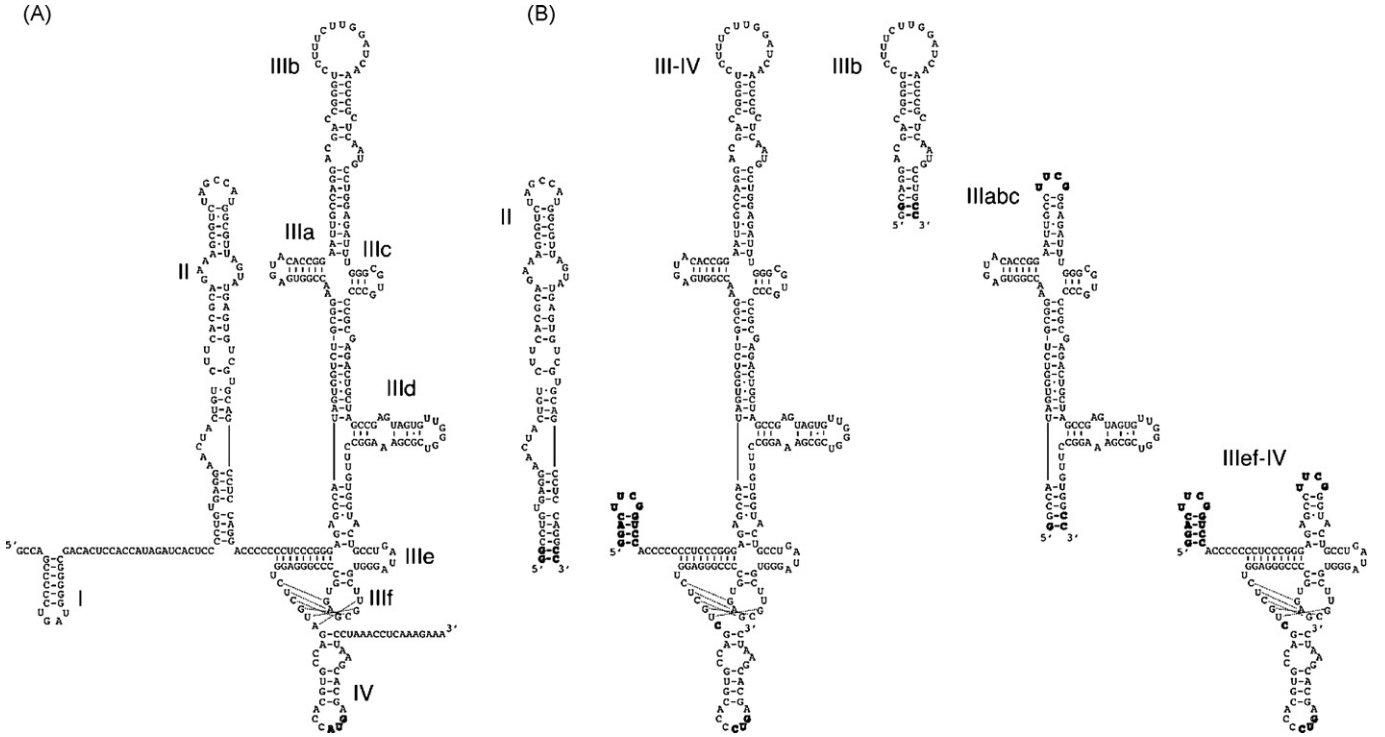

Fig. 1. Sequences and secondary structures of HCV IRES RNA. (A) Predicted secondary structure of HCV 5′UTR and immediately downstream the open reading frame (denoted as "HCV IRES", 1–374 nts) (Honda et al., 1999; Zhao and Wimmer, 2001). Individual stem-loop structures are indicated by roman numerals, and the translation initiation codon (AUG) is highlighted in bold font. (B) Truncations of HCV IRES RNA employed, including domain II (II), domains III and IV (III–IV), domain IIIb (IIIb), domains IIIa, IIIc, and IIId (IIIacd), and domains IIIe, IIIf, and IV (IIIef–IV). Nucleotide modifications to stabilize the secondary structure of each construct are indicated in bold.