TOPIC HIGHLIGHT

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Establishment of chronic hepatitis C virus infection: Translational evasion of oxidative defence

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Abstract

Hepatitis C virus (HCV) causes a clinically important disease affecting 3% of the world population. HCV is a single-stranded, positive-sense RNA virus belonging to the genus *Hepacivirus* within the *Flaviviridae* family. The virus establishes a chronic infection in the face of an active host oxidative defence, thus adaptation to oxidative stress is key to virus survival. Being a small RNA virus with a limited genomic capacity, we speculate that HCV deploys a different strategy to evade host oxidative defence. Instead of counteracting oxidative stress, it utilizes oxidative stress to facilitate its own survival. Translation is the first step in the replication of a plus strand RNA virus so it would make sense if the virus can exploit the host oxidative defence in facilitating this very first step. This is particularly true when HCV utilizes an internal ribosome entry site element in translation, which is distinctive from that of cap-dependent translation of the vast majority of cellular genes, thus allowing selective translation of genes under conditions when global protein synthesis is compromised. Indeed, we were the first to show that HCV translation was stimulated by an important pro-oxidant-hydrogen peroxide in hepatocytes, suggesting that HCV is able to adapt to and utilize the host antiviral response to facilitate its own translation thus allowing the virus to thrive under oxidative stress condition to establish chronicity. Understanding how HCV translation is regulated under oxidative stress condition will advance our knowledge on how HCV establishes chronicity. As chronicity is the initiator step in disease progression this will eventually lead to a better understanding of pathogenicity, which is particularly relevant to the development of anti-virals and improved treatments of HCV patients using anti-oxidants.

Core tip: Oxidative stress inhibits canonical translation, however, emerging evidence suggests that oxidative stress can actually stimulate alternative translation from select internal ribosome entry site (IRES) elements including that involved in redox regulation and in persistent virus infection *e.g.*, human immunodeficiency virus and hepatitis C virus (HCV). We postulate a novel role of oxidative stress-activated IRES-mediated translation in redox homeostasis and virus persistence. In the case of HCV, we explore the idea that HCV exploits oxidative stress to activate its own translation as a novel means of evading the host oxidative defence to establish chronicity.

INTRODUCTION

Hepatitis C virus (HCV) causes a clinically important disease affecting 3% of the world population[1]. About 75% of the infection will develop into chronic hepatitis, which can then progress into fibrosis, cirrhosis and hepatocellular carcinoma. A vaccine is not available. Current interferon (IFN) treatments are expensive, with numerous side effects and are particularly ineffective against the predominant genotype 1 in America and European countries[2]. The newly approved protease inhibitors likely promote the emergence of drug resistant mutants, owing to the high mutation rate of the HCV genome[2]. Thus there is a pressing need for alternative HCV therapies. HCV establishes a chronic infection in the face of an active immune response and the host oxidative defence. A number of mechanisms have been proposed to account for evasion of the antibody and cellular immunity and the natural killer, IFN and Toll-like receptor innate immunity[3-5]. However, little is known about how the virus can survive in a highly oxidative environment given that oxidative stress is such a prominent clinical feature associated with hepatitis C infection[6-12]. Adaptation to oxidative stress is key to virus survival. We postulate that adaptation can be at the level of translation as HCV uses an internal ribosome entry site (IRES) element for translation, distinctive from that of cellular translation[13]. Indeed, we were the first to show that translation from the HCV IRES was stimulated by an important pro-oxidant-hydrogen peroxide (H_2O_2) in hepatocytes, suggesting that HCV is able to adapt to and utilize host anti-viral response to facilitate its own translation thus allowing the virus to thrive under oxidative stress condition to establish chronicity[14]. Anti-oxidants are now in clinical trials in the treatment of HCV patients[15-17]. Understanding the mechanisms of how HCV evades host oxidative defence at the translational level may help shape the formulation of improved and new anti-oxidant treatments for HCV.

HCV

HCV is a *Hepacivirus* belonging to the family *Flaviviridae*[18]. As a single-stranded, positive-sense RNA virus, translation is the first step in the life cycle of HCV upon infection of a susceptible cell. Its 5' untranslated region (UTR) contains an IRES element used to translate the 9.6 kb RNA genome into a single polypeptide which is then cleaved by the host and viral proteases into structural proteins core, envelopes E1 and E2, and non-structural (NS) proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 1)[19]. The RNA polymerase, NS5B, then catalyzes replication of the viral genome. The genome of HCV undergoes a high mutation rate giving rise to genetic variants, thus HCV is divided into genotypes and sub-types and is populated as "quasispecies"[20,21]. A "quasispecies" is a cloud of diverse, genetically linked mutants that function cooperatively and behave as a unit for natural selection[22]. Thus, a population of mutants with

similar fitness values will out-compete those with a broad range of fitness values even though the latter includes mutants of high fitness values. This constitutes the basis of "the survival of the flattest" in "quasispecies" theory in contrast to Darwinian "the survival of the fittest"[23]. However, there is much debate on whether HCV or any RNA virus ever exists as a "quasispecies" in evolutionary term as the mutation rate of HCV is never high enough to lead to "quasispecies" dynamics[23]. Nevertheless, this "quasispecies"/intra-host variants phenomenon has great impact on virus persistence, pathogenesis, anti-viral treatment and vaccine design. However, different regions of the genome exhibit different degrees of sequence variability, with the envelope E2 region being the most variable harbouring hypervariable regions and the 5' UTR the most conserved[21,24,25]. Thus, targeting 5' UTR may be a solution to solve the problem of sequence variability in anti-viral therapies[26,27].

TRANSLATION

The vast majority of proteins is synthesized by a process known as cap-dependent translation, so named because it requires a 7-methyl guanosine (m^7G) cap-structure at the 5' end of the mRNA[28]. Translation is initiated when the cap is bound by the cap-binding complex eukaryotic initiation factor (eIF) 4F, which consists of eIF4E, eIF4A and eIF4G (Figure 2A). eIF4E is the cap-binding protein. eIF4A is a helicase, its unwinding activity is promoted by another initiation factor, eIF4B. eIF4G is the scaffold protein, which functions to recruit the 40S ribosomal subunit-eIF3-eIF2 pre-initiation complex to the 5' end of the mRNA via protein-protein interaction between eIF4G and eIF3. The ribosomal complex, primed by eIF1/1A, then scans a short distance (50-100 nucleotides) to (usually) the nearest AUG triplet within a favorable (Kozak) sequence context to initiate translation[29].

IRES mediates an alternative form of translation distinctive from that of cap-dependent translation of the vast majority of cellular genes, thus allowing selective translation of genes under conditions when global protein synthesis is compromised *e.g.*, virus infection, stress[30-32]. IRES translation is an important strategy employed by a subset of virus, mainly that of RNA viruses belonging to the *Picornaviridae* family, to continue viral protein synthesis during host translational shut off. IRESs found in cellular mRNAs mainly serve the function of regulating cellular processes such as apoptosis, differentiation, angiogenesis, thus their activity is usually tightly regulated and many are only responsive to stress. Studies on viral IRESs suggest that the IRES element forms a direct landing pad for the ribosome, therefore, the secondary and tertiary structures of the IRES are important for its activity (Figure 2B)[33-36]. As a result, the viral IRES element normally spans a considerably longer 5' UTR that folds into a higher order structure and is interspersed with multiple AUG triplets[37]. However, short sequence motif rather than secondary structure is important in
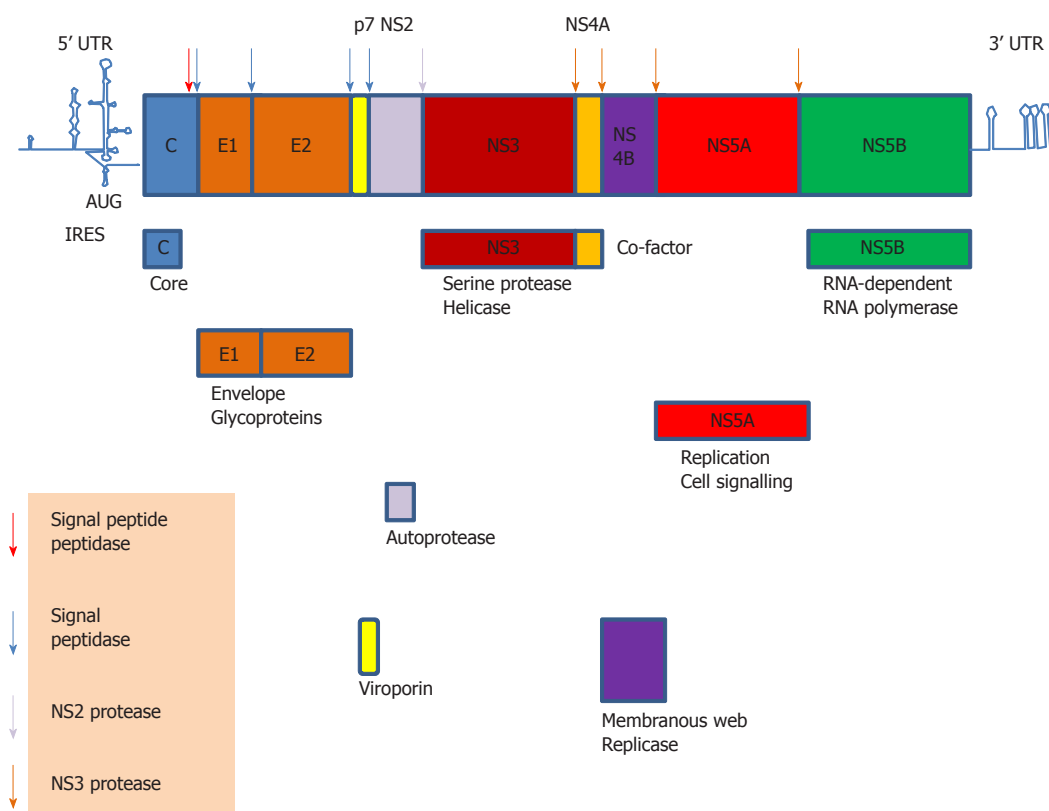
Figure 1 Hepatitis C virus genome. The 9.6 kb hepatitis C virus genome is flanked by highly structured 5' untranslated region (UTR) and 3' UTR which contain regulatory elements. The genomic sequence is translated into a polyprotein *via* internal ribosome entry site (IRES)-mediated translation. The polyprotein is then cleaved into individual structural and non-structural (NS) proteins by cellular signal peptidase and signal peptide peptidase and viral auto protease (NS2) and serine protease (NS3).

most cellular IRES activity[38,39]. As short as a 9-nucleotide sequence from the 5' UTR of the cellular gene Gtx exhibited IRES activity[40,41]. In yeast and Drosophila, strong IRES activity was associated with weak secondary structure[42]. Because IRES-mediated translation is independent of a cap many canonical eIFs are dispensable, however, the requirement for canonical eIFs varies greatly amongst IRESs, ranging from the dependence of the entire set of eIFs in the hepatitis A virus (a picornavirus unrelated to HCV) IRES to none of them in the cricket paralysis virus IRES[31,35,37]. Another characteristic of IRES translation is that it is regulated by a diverse group of proteins known as IRES *trans*-acting factors (ITAFs)[43].

ITAF

Each IRES has a unique set of ITAFs, even within the same group of IRES that shares primary sequence and secondary structure[44]. On the other hand, IRES of diverse origins can share common ITAFs[45]. Many of these ITAFs are RNA chaperone proteins. Most of them facilitate IRES-mediated translation although some are negatively regulating. Common ITAFs include the La autoantigen, polypyrimidine tract binding protein (PTB), heterogeneous nuclear ribonucleoproteins (hnRNPs), poly r(C) binding protein (PCBP), Upstream of N-ras (*u*nr), death-associated protein 5 (DAP5) and the embry-

onic lethal abnormal vision/protein (ELAV/HuR)[30,46-52].

ITAF modification by stress signals is an important aspect in the regulation of IRES activity under stress conditions, using mechanisms such as nuclear-cytoplasmic shuttling, protein cleavage, phosphorylation and increased protein expression[53-58]. Many of the ITAFs are abundant nuclear proteins, thus nuclear-cytoplasmic shuttling presents an effective means of a fast response[59]. hnRNP A1 shuttled to the cytoplasm during osmotic shock to downregulate translation from the X-linked inhibitor of apoptosis protein (XIAP) IRES but upregulate translation from the fibroblast growth factor-2 IRES[53]. hnRNP A1 also shuttled to the cytoplasm in rhinovirus-2-infected and UVC-irradiated cells to enhance translation from the rhinovirus IRES but limit translation from the apoptotic peptidase activating factor 1 IRES[60]. Proteolysis also plays an important part in regulating IRES activity, either by directly conferring novel function to the truncated protein or by causing protein shuttling after the removal of the nuclear localization signal (NLS), or both. Caspase cleavage of DAP5 during endoplasmic reticulum (ER) stress released an active fragment with a novel ITAF function to activate the cellular inhibitor of apoptosis protein (HIAP2) IRES[54]. Cleavage of the La protein and PTB by the poliovirus serine protease released truncated fragments devoid of NLS to shuttle to the cytoplasm to either activate or repress the poliovirus IRES[55,56]. Phos-
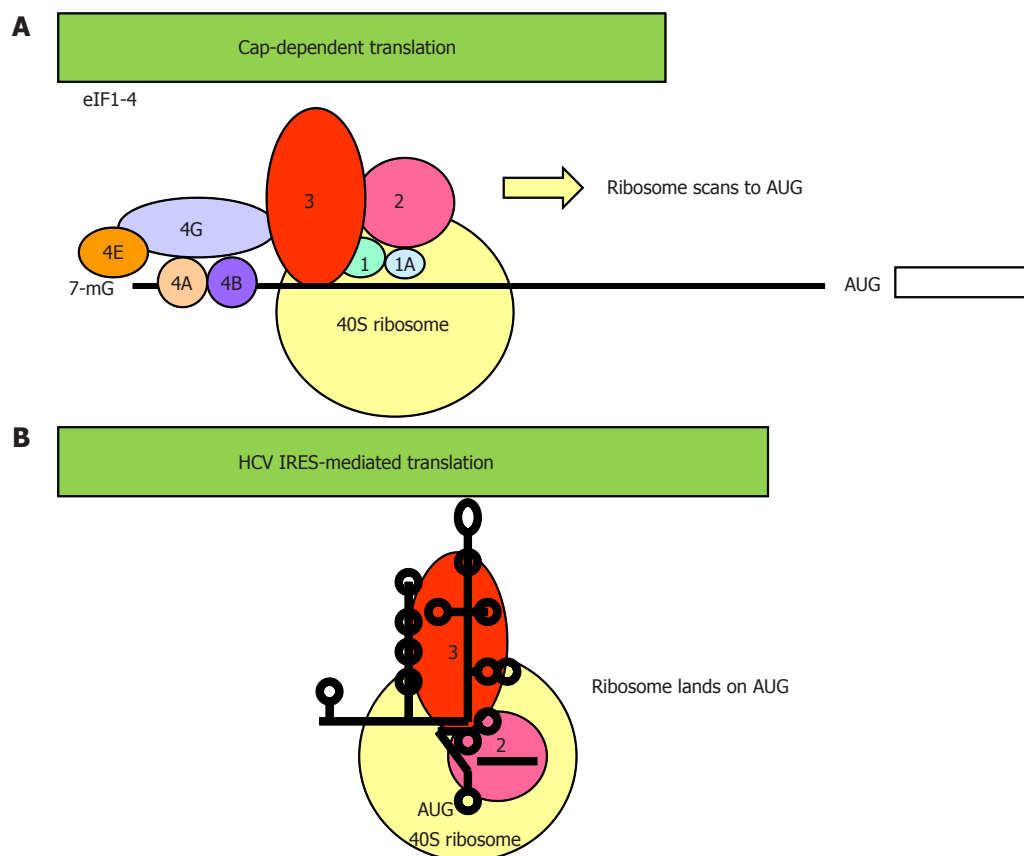
Figure 2 Two different modes of translational initiation. A: Cap-dependent translation. Initiation of translation of most cellular genes requires a 7-methyl guanosine cap-structure at its 5' end and a full set of canonical eukaryotic translation factors (eIFs) for ribosome binding and scanning to the AUG start codon[29]. The cap-binding complex eIF4F consists of the cap-binding eIF4E, the scaffold eIF4G and the helicase eIF4A. eIF4B promotes the RNA unwinding activity of eIF4A. Recruitment of the 40S ribosomal subunit-eIF3-eIF2 pre-initiation complex to the 5' end of the mRNA is *via* protein-protein interaction between eIF4G and eIF3. The ribosomal complex, primed by eIF1/1A, then scans a short distance (50-100 nucleotides) to (usually) the nearest AUG triplet to initiate translation; B: Internal ribosome entry site (IRES)-mediated translation. The viral IRES element spans a considerably longer 5' untranslated region (UTR) (180-450 nucleotides) that folds into a higher order structure and is interspersed with multiple AUG triplets[37]. The requirement for canonical eIFs varies greatly amongst IRESs, ranging from the dependence of the entire set of eIFs in the picornavirus hepatitis A virus IRES to none of them in the cricket paralysis virus IRES. The hepatitis C virus (HCV) IRES has the second simplest require-ment, only needing eIF2 and eIF3. In reminiscent of the prokaryotic ribosomal binding of the Shine-Dalgarno sequence, the HCV IRES can directly recruit the 40S ribosome which lands on the authentic AUG initiator codon[35,63,65]. In some other IRESs, the ribosome lands on an upstream AUG and then scans or shunts to the initiator AUG[37]. An important feature about IRES-mediated translation is that its activity is regulated by IRES *trans*-acting factors[43].

phorylation of ITAFs is also commonly used to modify ITAF function. Phosphorylation of the hnRNP C protein during differentiation stimulated translation from the IRES of c-*sis*[57]. In some cases, over-expression of ITAF is sufficient to promote IRES translation. Elevated expression of *u*nr stimulated translation from the IRES of PITSLRE cyclin-dependent protein kinase during G_2/M phase of the cell cycle[58].

HCV IRES

The HCV 5' UTR is divided into four stem-loop domains (Figure 3)[61]. The IRES element is made up of approximately 340 nucleotides and spans domain Ⅱ and extends into the core-coding region encompassing a double pseudoknot fold[13,62-64]. The HCV IRES has the second simplest requirement of eIFs, only needing eIF2 and eIF3. In reminiscent of the prokaryotic ribosomal binding of the Shine-Dalgarno sequence, the HCV IRES can directly recruit the 40S ribosome before association with eIF2

and eIF3 to form the 43S pre-initiation complex[35,63,65]. Structural and biochemical studies have indicated an unusually vast binding site for the ribosome encompassing domains Ⅱ, Ⅲ and Ⅳ and conformational changes in both the 40S ribosomal subunit and the IRES have been observed upon their interaction[66]. Binding between the IRES and the ribosome is thought to be mediated via ribosomal proteins although the role of RNA-RNA interaction cannot be excluded[67-69]. eIF3 can bind both the ribosome and junction domain Ⅲabc and domain Ⅲb of the IRES, thus playing a significant role in stabilizing the ribosome-eIF2 binary complex[70]. The ternary complex eIF2α-GTP-the tRNA for the first methionine (^{Met}tRNAi) does not directly bind the IRES, rather it forms a ternary complex with eIF3 and the 40S ribosomal subunit to position ^{Met}tRNAi directly onto the AUG codon in the P-site of the ribosome[70].

A number of putative ITAFs for the HCV IRES have been identified, including La, PTB, hnRNP D, hnRNP L, HuR, the NS1-associated protein 1 and miR-122 (Fig-
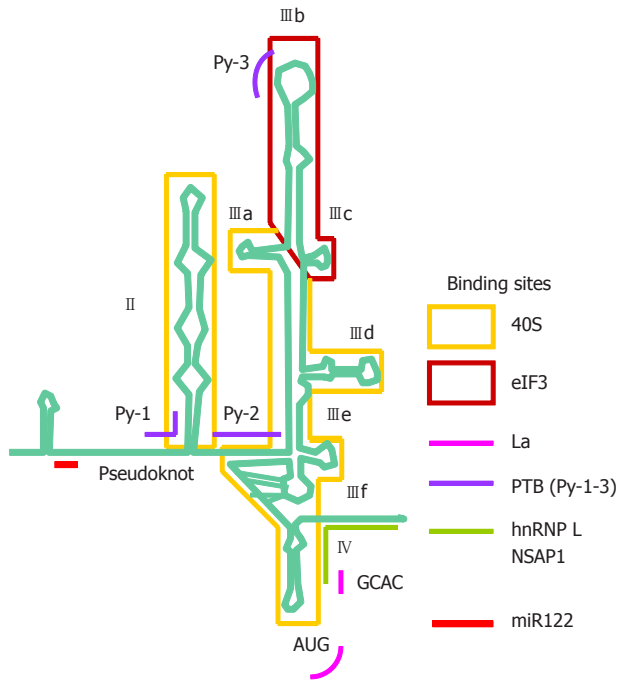
Figure 3 The hepatitis C virus internal ribosome entry site. The hepatitis C virus (HCV) 5' untranslated region (UTR) is divided into four stem-loop domains and sub-domains[61]. The internal ribosome entry site (IRES) element is made up of approximately 340 nucleotides and spans domain Ⅱ and extends into the core-coding region encompassing a double pseudoknot fold[13,62-64]. The stem, loop and unpaired regions are all important in interaction with host factors. The HCV IRES can directly recruit the 40S ribosomal subunit before association with eukaryotic translation factor (eIF)2 and eIF3 to form the 43S pre-initiation complex[35,65]. Structural and biochemical studies have indicated an unusually vast binding site for the ribosome encompassing domains Ⅱ, Ⅲ and Ⅳ and conformational changes in both the 40S ribosomal subunit and the IRES have been observed upon their interaction[66]. Binding between the IRES and the ribosome is thought to be mediated *via* ribosomal proteins although the role of RNA-RNA interaction cannot be excluded[67-69]. eIF3 can bind both the ribosome and junction domain Ⅲabc and domain Ⅲb. The ternary complex eIF2α-guanosine triphosphate (GTP)-the tRNA for the first methionine (Met tRNAi) does not directly bind the IRES, rather it forms a ternary complex with eIF3 and the 40S ribosomal subunit to position Met tRNAi directly onto the AUG codon in the P-site of the ribosome[70]. A number of non-canonical host factors are able to bind the HCV IRES and likely play a facilitating role in IRES translation. However, there is evidence for a critical role of the La autoantigen in IRES translation, by binding to and altering the conformation of the IRES to orchestrate assembly of the ribosomal complex[82,83]. NS: Non-structural.

ure 3)[71-82]. There is evidence for a critical role of the La autoantigen in IRES translation, by binding to and altering the conformation of the IRES to orchestrate assembly of the ribosomal complex[82,83]. La is one of the best known ITAFs and is pivotal in mediating translation from a number of IRESs[72,82,84-89]. It normally functions in RNA metabolism *e.g.*, nascent polⅢ transcript processing and RNP assembly but is co-opted as an ITAF in IRES translation[90]. Pathologically, La is an autoantigen in a number of autoimmune diseases such as lupus and Sjögren's syndrome[91]. The binding site for the La autoantigen has been mapped to the initiator AUG and the adjacent GCAC motif although additional binding sites may exist[88,92]. The binding sites for some other ITAFs have also been mapped[61,92]. Together with that of the ribosomal subunit and eIF3, these binding sites offer attractive targets for antiviral intervention because of (1)

the highly conserved nature of the IRES[93]; (2) the distinctive mode of translation making it likely to produce an anti-viral with a high therapeutic index[94]; and (3) the use of cellular targets making it less ready to select for resistant mutants.

HCV IRES activity can also be modulated by the viral proteins core, NS2/3, NS3 and NS5A and the 3' UTR[95-100]. A long range interaction between the IRES and the 3' UTR is thought to be essential for IRES activity[101]. However, it is still unclear what constitutes the *bona fide* ITAFs and how they regulate HCV IRES activity, in particular under stress.

OXIDATIVE STRESS IN HEPATITIS C

Accumulation of reactive oxygen species (ROS) and the generation of oxidative stress are implicated in the development of a number of inflammatory diseases, including viral hepatitis[102]. Chronic hepatitis C patients present elevated blood and hepatic levels of pro-oxidants, reduced anti-oxidants levels, iron overload with increased lipid peroxidation, decreased hepatic glutathione and increased oxidative DNA damage[103-108]. Proteomic and microarray analysis of liver biopsies revealed increased oxidative stress in hepatitis C samples[109,110].

Important ROS include superoxide anion $O_2^{\cdot-}$, H_2O_2 and hydroxyl radical $\cdot OH$. ROS exist in every cell as part of the by-products of active respiration in the mitochondria (Figure 4)[111]. ROS are harmful to cells as they will cause oxidative damage to intracellular macromolecules and are eliminated by anti-oxidant enzymes such as superoxide dismutase, catalase and the glutathione system to maintain redox balance (Figure 4). A low level of ROS, in particular H_2O_2, is however, important mediator of cellular signal transduction pathways[111,112]. A high level of ROS is important in fighting infections[113]. Immune recognition of infected cells triggers the release of ROS from sequestered phagocytes and activated macrophages. Endogenous ROS are also produced as a direct result of hepatitis C viral replication and interactions of a number of hepatitis C viral proteins with the host cell, as evidenced by studying infected cultured cells and ectopically expressed viral proteins (core, NS3 or NS5A) in cultured hepatocytes, monocytes and isolated mitochondria[6-12,114,115]. This is supported by data from *in vivo* studies. Transgenic mice carrying the structural proteins exhibited elevated levels of ROS and were more susceptible to oxidant injury[10]. Infection of a *SCID/Alb/uPA* chimeric mouse (mouse with chimeric human and mouse liver) also revealed increased oxidative stress in infected hepatocytes[116]. It has recently been shown that the NAD(P)H oxidases, Nox1 and Nox4, are two of the endogenous ROS sources in HCV-infected cultured cells and liver samples[117,118].

EVASION OF OXIDATIVE DEFENCE-HCV AND OTHERS

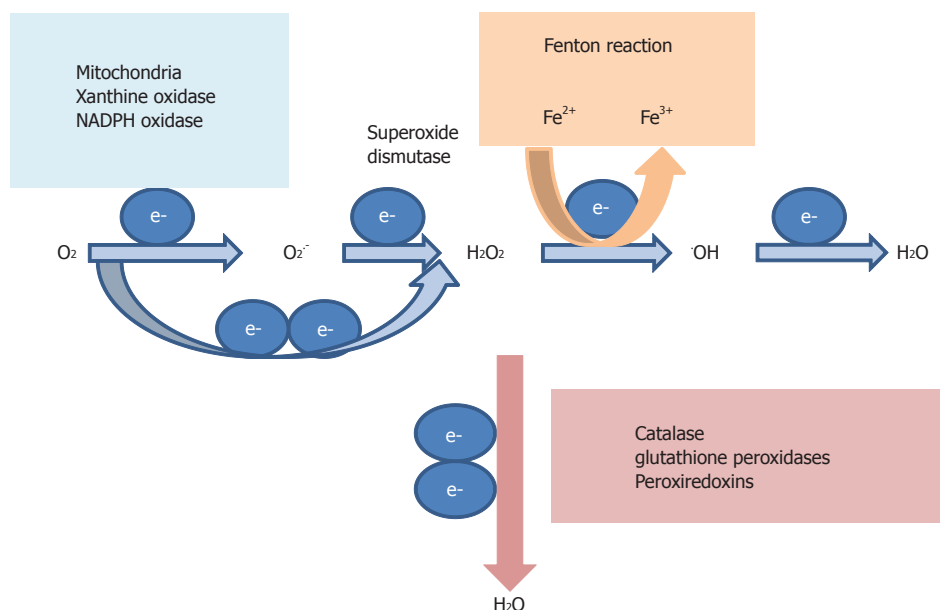ROS are lethal to pathogens. How do pathogens coun-

Figure 4 Oxidant and anti-oxidant systems. During active respiration in the mitochondria, electron leaked from the respiratory chain reacts with oxygen (O_2) to form reactive oxygen species (ROS) superoxide anion O_2^- and pro-oxidant-hydrogen peroxide (H_2O_2). O_2^- is quickly dismuted to H_2O_2 in a reaction catalyzed by superoxide dismutase. Non-mitochondrial sources of O_2^- and H_2O_2 include cytosolic xanthine oxidase and plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. H_2O_2 is decomposed by iron in the Fenton reaction to yield the highly oxidizing hydroxyl radical ^-OH, causing macromolecule damage, DNA damage and lipid peroxidation. H_2O_2 can be reduced to water (H_2O) by catalase, glutathione peroxidases and peroxiredoxins.

teract the damaging effects of ROS? Bacteria do it at the transcriptional level as they normally do. Some bacteria such as *Escherichia coli* and *Salmonella typhimurium* can sense and counteract oxidative stress by inducing transcription of response genes from the OxyR regulon[119,120]. Viruses, being obligatory intracellular parasites with limited genomic capacity, exploit different strategies to suit their life style. The poxvirus molluscum contagiosum virus has a large DNA genome thus is capable of encoding their own anti-oxidant protein to become resistant to the cytotoxic effect of H_2O_2[121,122]. HCV is a small RNA virus with a limited genomic capacity. There is no evidence that HCV encodes an anti-oxidant protein. This has led us to speculate that HCV deploys a different strategy to evade the host oxidative defence. Instead of counteracting oxidative stress, it utilizes oxidative stress to facilitate its own survival. This would be advantageous to the virus because it persists as a chronic infection. Precedence can be found in human immunodeficiency virus (HIV), which also establishes a chronic infection and has been associated with increased oxidative stress in HIV patients[123]. HIV replication was facilitated by ROS via activation of the transcription factors nuclear factor-kappa B and hypoxia inducible factor 1 alpha to stimulate gene expression from the HIV long terminal repeat[124-126]. The effect of ROS on HCV replication is inconclusive, as opposing results were obtained from laboratory studies (most likely due to the use of different pro-oxidants and HCV expression systems) although some clinical studies and anti-oxidants trials do support a stimulatory role of ROS on HCV replication[17,127-137]. Translation is the first step in the replication of a plus strand RNA virus so it would make sense if the virus can exploit the host oxida-

tive defence in facilitating this very first step. Indeed, we have previously shown that H_2O_2 stimulates translation from the HCV IRES[14].

Amongst viruses, HCV and HIV infections are commonly associated with elevated oxidative stress in patients, meaning that the viruses are continuously exposed to oxidative stress[123]. Coincidentally, they both cause chronic infections and translation from their IRESs is both upregulated by H_2O_2, suggesting that the viruses can adapt to and utilize oxidative stress to their own advantage[14,138]. Amongst cellular IRESs, translation from the IRESs of nuclear factor erythroid-2 related factor 2 (Nrf2) and ferritin is stimulated by pro-oxidants[139-141]. Coincidentally, these proteins are both involved in restoring redox balance, suggesting that upregulation of IRES translation could be a homeostatic response to oxidative stress. Nrf2 is the coordinator of the anti-oxidant response to oxidative stress and translation from its IRES was stimulated by H_2O_2[139,141]. Ferritin sequesters excess iron from catalyzing the Fenton reaction that leads to the production of free radicals and translation from the ferritin IRES was activated by iron (Figure 4)[140]. A protective response to oxidative stress was also mediated by IRES translation in a pathological setting of ischaemic insults[142]. A rapid rise in the level of H_2O_2 damaged the neuron but at the same time, conferred neuroprotection to ischaemic insults by stimulating translation from the Sp1 IRES. Altogether these results suggest that one of the adaptive responses to oxidative stress could be at the level of translation and that an IRES is being deployed to achieve this. It is interesting to see whether viruses co-opted a homeostatic cellular IRES in their counter-defence against oxidative stress or *vice versa*.
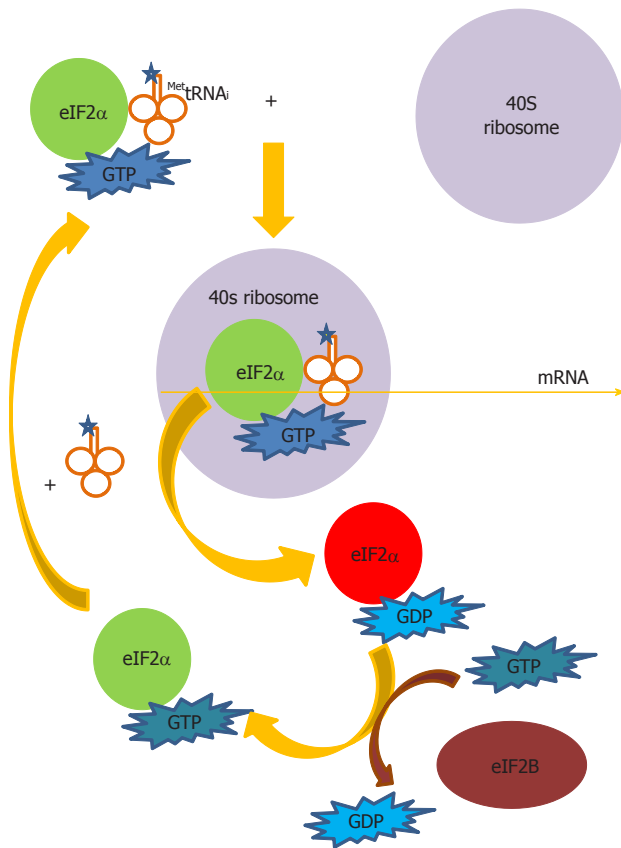
persistent infection.

Figure 5 Delivery of ^{Met}tRNAi to the ribosome. Eukaryotic translation factor (eIF)2α forms a ternary complex with guanosine triphosphate (GTP) and the tRNA for the first methionine (^{Met}tRNAi) to deliver ^{Met}tRNAi to the 40S ribosomal subunit. Following hydrolysis of GTP to guanosine diphosphate (GDP), the eIF2α-GDP complex leaves the ribosome. GDP is converted to GTP in an exchange reaction catalyzed by the exchanger eIF2B. eIF2α-GTP can then be recycled for more ternary complex formation with ^{Met}tRNAi to continue translation initiation. When eIF2α is phosphorylated at Serine-51 (red), it inhibits eIF2B. Thus GDP cannot be exchanged for GTP and protein synthesis is arrested at this step.

Will all viruses possessing an IRES element be capable of taking advantage of oxidative stress to increase their replication rate? IRES is present in all members of the *Picornaviridae* family including poliovirus, rhinovirus (common cold), encephalomyocarditis virus, foot-and-mouth disease virus and hepatitis A virus[143]. Picornavirus IRESs are divided into Type I-V based on structural and functional similarity[144]. Type IV IRES is grouped with IRESs from the two genera *Hepacivirus* and *Pestivirus* of the *Flaviviridae* family (here known as HCV-like IRES), leading to the speculation that HCV acquired an IRES element from picornavirus in the distant past by recombination[143,145]. This may explain why IRES is not a common feature of the *Flaviviridae* family and is absent from the genus *Flavivirus*. IRESs have also been found in some retroviruses and DNA viruses establishing chronic/latent infections such as HIV and Kaposi's sarcoma-associated herpesvirus[138,146]. It is interesting to see whether responsiveness to oxidative stress is a function preserved in all HCV-like IRESs regardless of whether they establish an acute or chronic infection or it is a function evolved with

MECHANISMS OF IRES TRANSLATION UNDER OXIDATIVE STRESS CONDITION

How then could an IRES facilitate an adaptive oxidative response? All protein synthesis relies on eIF2α to deliver ^{Met}tRNAi to the 40S ribosomal subunit by forming a ternary complex eIF2α-GTP-^{Met}tRNAi (Figure 5)[147]. Following hydrolysis of GTP to GDP, the eIF2α-GDP complex leaves the ribosome. GDP is converted to GTP in an exchange reaction catalyzed by the exchanger eIF2B, allowing eIF2α-GTP to be recycled for more complex formation with ^{Met}tRNAi to continue translation initiation. Phosphorylation of eIF2α at Serine-51 (Ser-51) inhibits eIF2B, thus arresting protein synthesis at the step of GDP-GTP exchange. To date four mammalian eIF2α kinases have been known. They are the RNA-activated protein kinase (PKR), PKR-like ER eIF2α kinase (PERK), heme-regulated inhibitor of translation (HRI) and the mammalian homologue of yeast eIF2α kinase general control nonderepressible 2 (GCN2)[148-151]. These kinases share similarity in their kinase domains but differ in their regulatory domains allowing them to respond to distinct stress stimuli whilst phosphorylating eIF2α at the identical residue Ser-51. PKR is specifically activated by ds-RNA during virus infections; PERK is specifically activated by ER stress; HRI is specifically activated by heme deficiency in erythroid cells primarily involved in the regulation of haemoglobin synthesis whereas GCN2 is specifically activated by amino acid starvation. We have shown that H_2O_2 is also a stress signal to induce phosphorylation of eIF2α although currently we do not have evidence to suggest which of the four mammalian kinases is operating in our system[14]. All of the four kinases have been shown to be the eIF2α kinase under different oxidative stress conditions and in different cell types: HRI in arsenite-induced oxidative stress; GCN2 in UV-irradiation-induced oxidative stress and PKR and PERK in H_2O_2-stimulated osteoblastoma and HEK293 cells[152-156]. It is equally possible that oxidative stress-induced eIF2α phosphorylation is a result of inhibition of a phosphatase rather than activation of a kinase[157].

Little is known of how ^{Met}tRNAi is delivered to maintain IRES translation under oxidative stress condition, when eIF2α is phosphorylated. So the question will be under this condition what is used to deliver ^{Met}tRNAi? Although many eIFs are dispensable for IRES-mediated translation, almost all still rely on eIF2α to deliver ^{Met}tRNAi, thus are sensitive to the inhibitory effect of phospho-eIF2α. The HCV IRES is no exception. Under non-stressed condition, translation from the HCV IRES is still dependent on eIF2α to deliver ^{Met}tRNAi[158-160]. However, some IRESs can evade this critical step of translational control and allow them to maintain translation under conditions that would otherwise inhibit protein synthesis. First, instead of downregulation by phospho-eIF2α, translation from select viral and cellular

IRESs is actually upregulated by phospho-eIF2α[161-163]. The exact mechanism of how phospho-eIF2α upregulates select IRES translation is unclear. Regarding HCV, there is no evidence that translation from the HCV IRES is upregulated by phospho-eIF2α, either under stress conditions that induce phosphorylation of eIF2α or by ectopic expression of a phospho-mimetic eIF2α-SD (substitution of Ser-51 with Aspartate-51 which mimics the structure of phospho-eIF2α)[14,164,165]. Secondly, a minority of IRESs does not require any eIFs for translation. The cricket paralysis virus intergenic IRES simply folds to mimic the function of ^{Met}tRNAi[166]. The HCV IRES can also operate without eIF. However, this "factor-less" translation was performed under *in vitro* condition, using a non-physiological high concentration of Mg^{2+}[167]. It is not known whether the HCV IRES can operate in an eIF-less mode of translation *in vivo*. Thirdly, IRES translation can switch from eIF2-dependent to eIF2-independent mode of translation under stress conditions or during virus infections that induce phosphorylation of eIF2α. Translation from the poliovirus IRES during early phase of infection was dependent on eIF2α but was independent of eIF2α during late phase of infection and this eIF2-independence was assisted by the viral 2A protease[168]. HCV infection also induces phosphorylation of eIF2α[169]. Translation from the HCV, classical swine fever virus (CSFV) and the cellular XIAP IRESs was resistant to the inhibitory effect of eIF2α phosphorylation by switching from eIF2-dependent to eIF2-independent mode of translation, using alternative eIF such as eIF5B, eIF2A or eIF2D/ligatin to deliver ^{Met}tRNAi[158-160,170-173]. It remains to be seen which mechanisms operate to deliver ^{Met}tRNAi in IRES translation under oxidative stress condition.

However, the use of an eIF2-independent mode of translation simply allows translation to operate at a lower efficiency when the more efficient canonical eIF2α-dependent pathway is inhibited[158,160]. The HCV IRES behaves in a very different way under oxidative stress condition in that translation is not only maintained, but is actually upregulated, suggesting a different or additional way of regulation under oxidative stress condition[14,174]. This is similar to the HIV and Nrf2 IRESs, in which translation is stimulated by oxidative stress[138-139,141]. Thus far two mechanisms have been proposed by which oxidative stress stimulates IRES translation, both of which involve ITAF, stressing the importance of ITAF in translational regulation during oxidative stress.

A positive regulatory mechanism
A positive regulatory mechanism in which oxidative stress stimulates IRES translation by increasing cytoplasmic level of ITAF, either by promoting its cytoplasmic shuttling or by one of the mechanisms mentioned above. An example can be seen in the Nrf2 IRES. H_2O_2 stimulated Nrf2 IRES translation by increasing shuttling of its ITAF, La, to the cytoplasm[141]. In this case, the H_2O_2-responsive element has been mapped to a region responsible for both basal and H_2O_2-induced IRES activity[139].

A derepression mechanism
This is similar to the positive regulatory mechanism in which oxidative stress stimulates IRES translation by increasing cytoplasmic level of ITAF. However, in this case, the IRES activity is normally repressed by being locked into a weakly active conformation by a repressor protein. Oxidative stress induces eIF2α phosphorylation to shut down global protein synthesis including that of the repressor. As the repressor level drops, oxidative stress increases the cytoplasmic level of an activator ITAF, either by promoting its cytoplasmic shuttling or by one of the mechanisms mentioned above. The release of the repressor allows binding of the activator ITAF to induce a conformational change in the IRES to activate translation. In this case, the H_2O_2-responsive element has been mapped to a negatively regulating domain that inhibits basal IRES translation. An example can be found in the HIV IRES, although in this case the repressor and activator ITAFs have yet to be identified to support this derepression hypothesis[138].

As for HCV, we currently do not have evidence to suggest how H_2O_2 activates IRES translation. However, others have found that iron stimulates translation from the HCV IRES, *via* upregulation of eIF3 and La mRNAs[175,176]. Iron catalyzes the Fenton reaction in the conversion of H_2O_2 into the highly oxidizing and damaging ·OH (Figure 4)[111]. Thus iron promotes oxidative stress and iron overload is frequent in HCV patients[105]. Although these studies did not show a direct correlation between oxidative stress and IRES translation, they provide an indication of how this might work and the similarity with the two proposed mechanisms in that they all involve an ITAF. Further work will be required to dissect the mechanisms of how H_2O_2 activates translation from the HCV IRES.

Still exactly how oxidative stress stimulates IRES translation is far from clear. Despite collectively known as IRES, each IRES is unique in terms of sequence, structure, use of eIF and ITAF, mechanism of translation and response to stress. Cellular IRESs are distinctly different from viral IRESs in that they are naturally capped, flatter and for most, depend on short motif rather than overall structure to function[38,39]. HIV-a retrovirus-has a capped mRNA which is translated by a cap-dependent mechanism under normal circumstances[177]. For HIV and some cellular genes, IRES-mediated translation serves as an alternative mechanism of translation under stress conditions[138]. In contrast, when IRES-mediated translation represents the main (sole) mechanism of translation in RNA viruses such as picornavirus and HCV the mRNAs are uncapped[38]. Thus it is anticipated that the mechanisms used to respond to oxidative stress would be as diverse as the IRES itself.

"QUASISPECIES"/INTRA-HOST IRES VARIANTS IMPACT ON OXIDATIVE RESPONSIVENESS AND PERSISTENCE
HCV genome exhibits a high degree of sequence varia-

tion, with > 30% difference between genotypes and 20%-25% between sub-types[178]. Due to structural constraint, the 5' UTR (which contains the IRES element) is the most conserved region, but substitutions along the IRES region are common amongst genotypes, sub-types and even "quasispecies"/intra-host variants[93,179]. Substitutions have been mapped to the stem, loop and unpaired regions[36,180,181]. Most of the substitutions in the stem regions are co-variants thus preserving the structural integrity of the IRES element. A minority of substitutions in the stem regions results in loss of base-pairing and alteration in IRES structure and hence function. Substitutions mapped to the loop or unpaired regions are important as well, as they may contain binding sites for the ribosomal subunit, eIF3 and ITAFs[36,182,183]. Therefore, despite being a highly conserved region, slight alteration in the IRES sequence can have a profound effect on basal IRES translation and responsiveness to stresses. The efficiency of genotypic IRESs has been compared in various studies. IRESs from some genotypes or sub-types were more efficient in mediating basal translation (*i.e.*, under non-stressed condition), however, the results are not consistent across studies, most probably due to the use of different IRES regions in their studies and the existence of intra-genotypic variation in the IRES sequences used in different studies[184-186]. Indeed, substitutions are commonly found in closely related IRES sequences isolated from a single patient and some of these substitutions impacted a substantial change in the translational efficiency, highlighting the fact that HCV exists as a swarm of variants each with slightly different IRES sequence and structure hence efficiency in basal translation and responsiveness to stresses/IFN[187]. It is well known that genotype is a determining factor in patients' response to IFN treatment. Comparison of IRESs from six genotypes did not reveal any differences in their translational responsiveness to IFN, however, IRESs isolated from sustained responders of genotype 3a patients had lower translation efficiencies than that from non-responders and were more prone to IFN inhibition[188,189]. Other studies also identified marked differences in the distribution of substitutions between sustained responder and non-responder IRESs and between pre-treatment and post-treatment IRESs in non-responders, regardless of genotypes[190-193]. These results further emphasize the significance of intra-host IRES variants in determining stress/IFN responsiveness.

Variation in IRES sequence can also have an effect on virus replication *via* two mechanisms. First, as many of the translated proteins are required for virus replication, a change in the translation efficiency can alter the availability of proteins involved in virus replication. Second, the antisense IRES contains the promoter for the plus strand synthesis in virus replication, thus variation in IRES sequence can affect the rate of replication[194,195]. It is therefore interesting to see whether intra-host variation in IRES sequence will also result in a swarm of variants with different degrees of replication efficiencies under oxidative stress condition. This may also explain why

opposing results were obtained regarding the effects of ROS on HCV replication[17,127-137].

Therefore, studies with viruses with high sequence variability such as HIV and HCV have been complicated by the existence of a population of intra-host variants in each patient. The collective response of this population of intra-host variants will ultimately determine the response to oxidative stress and outcome of infection (persistence).

CONCLUSION

HCV establishes a chronic infection[1]. To survive in a harsh environment the virus needs to deploy a number of machinery to evade the host anti-viral responses, one of which is oxidative stress[113]. H_2O_2 induces phosphorylation of eIF2α resulting in inhibition of global (including that of viral) protein synthesis and constitutes an important defence against virus infection[14]. Possession of an IRES element enables some viral and cellular genes to continue protein synthesis when the majority of protein synthesis is inhibited by phosphorylation of eIF2α, by means of (1) a phospho-eIF2α-dependent mechanism[161-163]; (2) an eIF-less mechanism[166,167]; and (3) an eIF2α- independent mechanism[158-160,168,170-173]. At present, there is no evidence to suggest which mechanism is operating to enable translation from the HCV IRES to proceed when eIF2α is phosphorylated by H_2O_2[14]. Thus far studies on other IRESs have led to the proposal of a positive regulatory and a derepression mechanism, both involving H_2O_2-responsive ITAF, such as the La autoantigen, implicating a pivotal role of ITAF in H_2O_2-regulated IRES translation[138-139,141].

HCV IRES appears to belong to a class of IRESs that is translationally upregulated by H_2O_2[14,138-142]. This category of IRESs includes a number of cellular IRESs that orchestrate the anti-oxidants response and IRESs from viruses that establish chronic infections in a highly oxidative environment. It is interesting to see whether viruses co-opted a cellular homeostatic IRES or it is an inherent property of the viral IRES in the facilitation of a persistent infection.

HCV exhibits a high degree of sequence variability[178]. One must therefore take into consideration the collective response of a swarm of intra-host variants, each with different IRES structure and function and hence different translation and replication efficiencies under oxidative stress condition and, as a functional unit, will ultimately determine how well the virus can survive a highly oxidative environment in the process leading to persistence.

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