The mechanism of translation initiation on Aichivirus RNA mediated by a novel type of picornavirus IRES

Yingpu Yu¹, Trevor R Sweeney¹,
Panagiota Kafasla², Richard J Jackson²,
Tatyana V Pestova¹ and Christopher
UT Hellen^{1,*}

¹Department of Cell Biology, SUNY Downstate Medical Center,
Brooklyn, NY, USA and ²Department of Biochemistry,
University of Cambridge, Cambridge, UK

Picornavirus mRNAs contain IRESs that sustain their translation during infection, when host protein synthesis is shut off. The major classes of picornavirus IRESs (Types 1 and 2) have distinct structures and sequences, but initiation on both is determined by their specific interaction with eIF4G. We report here that Aichivirus (AV), a member of the Kobuvirus genus of Picornaviridae, contains an IRES that differs structurally from Type 1 and Type 2 IRESs. Its function similarly involves interaction with eIF4G, but its eIF4G-interacting domain is structurally distinct, although it contains an apical eIF4G-interacting motif similar to that in Type 2 IRESs. Like Type 1 and Type 2 IRESs, AV IRES function is enhanced by pyrimidine tract-binding protein (PTB), but the pattern of PTB's interaction with each of these IRESs is distinct. Unlike all known IRESs, the AV IRES is absolutely dependent on DHX29, a requirement imposed by sequestration of its initiation codon in a stable hairpin.

The EMBO Journal (2011) 30, 4423–4436. doi:10.1038/emboj.2011.306; Published online 26 August 2011

Subject Categories: proteins; microbiology & pathogens
Keywords: Aichivirus; DHX29; eIF4G; IRES; PTB

Introduction

The first stage in eukaryotic translation initiation is assembly of the 48S initiation complex on the initiation codon of mRNA. On most mRNAs, it occurs by the scanning mechanism (Jackson *et al*, 2010). The first step is formation of a 43S preinitiation complex comprising a 40S subunit, an eIF2/GTP/Met-tRNA_i^{Met} ternary complex, eIF3, eIF1, eIF1A and eIF5. 43S complexes attach to the 5'-proximal region of mRNA and scan along the 5'-untranslated region (5'-UTR) to the initiation codon where they stop and form 48S complexes. Attachment of 43S complexes is mediated by eIFs 4F, 4A and 4B. eIF4F consists of three subunits: eIF4E (cap-binding

protein), eIF4A (a DEAD-box RNA helicase, whose activity is enhanced by eIF4G and eIF4B) and eIF4G (a scaffold for eIF4E and eIF4A, which also binds eIF3). eIF4F/4A/4B cooperatively unwind the cap-proximal region of mRNA allowing 43S complexes to bind, and likely promote binding via the eIF4G–eIF3 interaction. eIF4F/4A/4B also assist 43S complexes during scanning, but scanning through highly structured 5'-UTRs requires an additional DExH-box protein, DHX29, which binds directly to 40S subunits (Pisareva *et al*, 2008; Abaeva *et al*, 2011). After a 43S complex locates the initiation codon, eIF5 and eIF5B promote hydrolysis of eIF2-bound GTP, release of eIFs from the 40S subunit and joining of a 60S subunit to form an 80S ribosome.

Viruses rely on the translational apparatus of host cells, and have developed sophisticated mechanisms to suppress translation of cellular mRNAs while ensuring translation of their own. The genomes of Picornaviruses and some other positive-strand RNA viruses contain internal ribosomal entry sites (IRESs), which function with fewer initiation factors than canonical mRNAs. This enables viruses to sustain translation during infection, when the canonical mechanism of initiation is suppressed, for example, by cleavage of eIF4G by viral proteases into an N-terminal fragment that binds eIF4E and a C-terminal fragment that binds eIF4A and eIF3, and by sequestration of eIF4E (Roberts *et al*, 2009). Different classes of viral IRESes use different mechanisms to recruit the 40S subunit, but they are all based on non-canonical interactions of the IRES with canonical components of the translation apparatus. Thus, initiation on two distinct groups of IRES, epitomized by Hepatitis C virus (HCV) and Cricket paralysis virus (CrPV), is based on their specific interaction with the 40S subunit, resulting in its recruitment directly to the initiation codon (Jackson *et al*, 2010). The mechanism of initiation on the two other principal IRES groups, Type 1 and Type 2 picornavirus IRESs, which are epitomized by poliovirus (PV) and encephalomyocarditis virus (EMCV), respectively, is determined by their specific interaction with the central domain of eIF4G, which is stimulated by eIF4A (Pestova *et al*, 1996a, b; Lomakin *et al*, 2000; Pilipenko *et al*, 2000; de Breyne *et al*, 2009). After binding, the eIF4G/eIF4A complex is thought to restructure the IRES to promote attachment of a 43S complex (Kolupaeva *et al*, 2003; de Breyne *et al*, 2009).

Type 1 and Type 2 picornavirus IRESs are ~450 nt long and both consists of five principal domains (designated II–VI in Type 1 and H–L in Type 2 IRESs), but they have distinct structures and their sequences are unrelated except for a few common motifs. These include a Yn-Xm-AUG motif at their 3'-border, which consists of a Yn pyrimidine tract ($n = 8–10$ nt) separated by a spacer ($m = 18–20$ nt) from an AUG triplet. This motif is considered to be the point of entry for 43S complexes onto Type 1 and Type 2 IRESs. However, whereas the AUG triplet of this motif in Type 2 IRESs is the

*Corresponding author. Department of Cell Biology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Box 44, Brooklyn, NY 11203-2098, USA. Tel.: +1 718 221 1034; Fax: +1 718 270 2656; E-mail: christopher.hellen@downstate.edu

Received: 1 June 2011; accepted: 1 August 2011; published online: 26 August 2011

initiation codon, it is silent in Type 1 IRESs, and initiation occurs ~30–150 nt downstream (Jackson, 2005). In both Type 1 and Type 2 IRESs, the Yn-Xm-AUG motifs are preceded by domains (domain V in Type 1 IRESs and domains J–K in Type 2 IRESs) that interact specifically with eIF4G (Pestova *et al*, 1996b; de Breyne *et al*, 2009). However, the eIF4G-interacting domains of these IRESs are not homologous, and although a conserved eIF4G-binding motif occurs in domain J in all Type 2 IRESs (Clark *et al*, 2003; Bassili *et al*, 2004), no equivalent has been confirmed yet for Type 1 IRESs. The eIF4G-binding domains are preceded by the large domain IV in Type 1 IRESs and domain I in Type 2 IRESs, which are essential for IRES function, but whose roles remain unknown. These domains both contain an important apical GNRA tetraloop motif (Kaminski *et al*, 1994; López de Quinto and Martínez-Salas, 1997; Robertson *et al*, 1999). Such motifs commonly stabilize RNA structures by engaging in intramolecular packing interactions with helical RNA ‘receptors’ (Geary *et al*, 2008), but no binding target for the tetraloop of Type 1 and Type 2 IRESs has been identified yet.

Importantly, in addition to canonical eIFs, all Type 1 and Type 2 IRESs also depend, albeit to different extents, on cellular IRES *trans*-acting factors (ITAFs). ITAFs usually contain multiple RNA-binding domains (RBDs) that interact specifically with IRESs. The requirement for ITAFs differs between and even within groups of IRESs. Thus, *unr* and poly(rC) binding protein 2 (PCBP2) are specific for Type 1 IRESs (Jackson, 2005), whereas ITAF₄₅ is specific for only one member of Type 2 IRESs, the foot-and-mouth disease virus (FMDV) IRES (Pilipenko *et al*, 2000). Interestingly, one ITAF, pyrimidine tract-binding protein (PTB), is common to both Type 1 and Type 2 IRESs, but even then, its modes of interaction with these two types of IRESs differ. PTB’s interaction with the Type 1 PV IRES primarily involves localized contacts of its RBDs 1 and 2 with the base of domain V, whereas its interaction with the Type 2 EMCV IRES is characterized by multiple dispersed contacts including those of RBDs 1 and 2 with the apex of domain K and of RBD3/RBD4 with domain H and the base of domains I and L (Kafasla *et al*, 2009, 2010). A long-standing hypothesis is that ITAFs function by stabilizing the optimal IRES conformation for efficient ribosomal recruitment, and consistently, recent studies revealed that cognate ITAFs promote common conformational changes in Type 2 IRESs (Yu *et al*, 2011).

A structurally distinct picornavirus IRES, designated Type 3, occurs in the 5′-UTR of another, hepatitis A virus (Brown *et al*, 1991). It consists of two major domains, has no obvious homology to Type 1 and Type 2 IRESs except for a Yn-Xm-AUG motif and, in contrast to all other picornavirus IRESs, is dependent on the integrity of the eIF4F complex (Ali *et al*, 2001). Otherwise, it remains poorly characterized.

The existence of structurally and mechanistically distinct classes of IRES raises the question of whether other unrelated IRESs are still to be identified. Here, we report that Aichivirus (AV), a member of the Kobuvirus genus of *Picornaviridae* that infects humans, usually subclinically, but that can lead to acute gastroenteritis (Reuter *et al*, 2011), contains an IRES that is structurally distinct from Type 1, Type 2 and Type 3 IRESs. *In vitro* reconstitution of initiation on this IRES revealed that it has some characteristics in common with Type 1 and Type 2 IRESs, and others that are unique.

Results

The AV IRES differs from Type 1 and Type 2 picornavirus IRESs

A model of the AV 5′-UTR downstream of the previously described domains A–D (nts 1–116) (Sasaki and Taniguchi, 2003; Nagashima *et al*, 2005) was derived using complementary bioinformatic approaches. Probabilistic structures of domains E–Ib, Jb, Jc, K and L were obtained by applying an explicit evolutionary model (Knudsen and Hein, 2003) to aligned AV 5′-UTR sequences. A posterior decoding approach (Sato *et al*, 2009) confirmed these elements and identified most of the remainder of domain J. The resulting model (Figure 1A) was tested and refined by free energy minimization (e.g., Zuker, 2003). It was consistent with the pattern of cleavage of AV nts 270–800 by RNase T1 (which is specific for unpaired G residues), modification by 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate (CMCT) which reacts with unpaired U and G residues, and of cleavage by RNase V1, which is specific for base-paired RNA (Figure 1A–D). The AV 5′-UTR, therefore, contains eight major secondary structure elements, designated E–L, downstream of domains A–D.

Picornavirus 5′-UTRs are modular, comprising elements that are required for RNA replication, followed by an IRES. To determine the 5′ border of the AV IRES, we assayed translation in rabbit reticulocyte lysate (RRL) of dicistronic mRNAs consisting of various AV 5′-UTR fragments inserted between a truncated cyclin cistron (Δ XL) and part of the AV polyprotein (Figure 2A). Deleting domains A–H (nts 1–336) reduced IRES function by only ~25%, but its activity was decreased by over 90% by deletion of domains A–I (nts 1–430) and was abrogated by extending deletion into domain J (nts 1–450) (Figure 2B). Thus, domains I, J and K were essential for IRES function, and further analysis therefore focused on this core region and the downstream domain L containing the initiation codon.

The structure of the AV IRES (nts 337–744) differs in many respects from those of Type 1, 2 and 3 IRESs (Brown *et al*, 1991; Bailey and Tapprich, 2007; Kapoor *et al*, 2008). Thus, AV domain I is not related to elements in any of these IRESs. Domain J consists of a long interrupted basal helix and an apical four-way helical junction (Figure 1A), similar to but smaller than domain IV in Type 1 IRESs. Its apical subdomain (Jb) also includes a GNRA tetraloop (Figure 2C), which is essential for the function of Type 1 and Type 2 IRESs. Thus, substitution of the residue at the fourth position of the GNRA motif reduced the activity of PV (Type 1) and EMCV and FMDV (Type 2) IRESs 20-fold, as did purine-to-pyrimidine substitutions at the first and third positions in Type 2 IRESs (Kaminski *et al*, 1994; López de Quinto and Martínez-Salas, 1997; Robertson *et al*, 1999). Although the apex of AV domain K contains an element identical to an apical motif in domain J of Type 2 IRESs (Figure 2D) that is essential for specific interaction with eIF4G (Clark *et al*, 2003; Bassili *et al*, 2004), these domains are otherwise unrelated, and the AV IRES also lacks an equivalent of domain K of Type 2 IRESs. Finally, while the AV initiation codon, AUG₇₄₅, is preceded by a Yn motif like in Type 1/2 IRESs, in contrast to them, it is sequestered in the 5′ strand of a long, stable hairpin (Domain L).

We first investigated the importance of the GNRA tetraloop-containing subdomain Jb and of the putative

Figure 1 Structure of the Aichivirus IRES. **(A)** Model of the secondary structure of the AV IRES (Genbank Acc. AB040749), derived as described in the text, and indicating the positions of nucleotides cleaved by RNases V1 (blue arrows) and T1 (red lozenges), or modified by CMCT (black arrows) based on data shown in **(B–D)**. Domains are labelled E–L. The initiation codon AUG₇₄₅ is circled. **(B–D)** Chemical (CMCT) and enzymatic (RNases V1 and T1) probing of the AV IRES in the presence and absence of PTB. Separation of lanes by white lines indicates that they were juxtaposed from the same gels. The positions of cleaved/modified nucleotides are indicated on the right using symbols as in **(A)**. PTB-dependent changes in cleavage/modification are indicated by arrows beside the numbers. Figure source data can be found with the Supplementary Information.

Figure 2 The 5'-border of the Aichivirus IRES and functional importance of its conserved motifs. (A) Schematic representation of dicistronic AV mRNAs containing AV 5'-UTR fragments, 5'-terminally truncated as indicated by arrows on the IRES model and inserted between truncated cyclin (Δ XL) and AV LVP0 reporter cistrons. (B) The effect of 5'-terminal truncations (as shown in A) on the activity of the AV 5'-UTR assayed by translation in RRL. (C) Structures of the apices of domain J of the AV IRES and of domain V of the Type 1 Coxsackievirus B3 IRES, showing the GNRA tetraloop and the positions of internal deletions made in the AV IRES. (D) Structures of the apices of domain K of the AV IRES and of domain J of the EMCV IRES, showing the conserved motif (red letters) that is required for interaction of the EMCV IRES with eIF4G. The destabilizing AGGU $\rightarrow$ UCCA mutation in the motif of the AV IRES is indicated. (E–H) The activity of monocistronic (MC) AV mRNAs comprising the entire 5'-UTR and LVP0 coding region and containing (E) the AGGU $\rightarrow$ UCCA mutation in the K domain conserved motif, (F) deletions in domain J as indicated in (C), and (G, H) substitutions in the GNRA tetraloop, assayed by translation in RRL. (I) Summary of the efficiency of translation of AV mRNAs with tetraloop substitutions.

eIF4G-interacting motif for AV IRES function. Disruption of the eIF4G-interacting motif almost abrogated AV IRES activity (Figure 2E), indicating that it is as dependent on this motif as are Type 2 IRESs. Deletion of nts 519–541 and 508–551 at the apex of domain Jb (Figure 2C) almost abrogated translation of AV mRNA (Figure 2F, lanes 4 and 5). However, the AV IRES retained $\sim$ 20% activity following deletion of the GNRA-containing hairpin alone (nts 526–539) (Figure 2F, lane 3). Moreover, substitution of individual nucleotides at any position in the GUGA tetraloop surprisingly did not impair, and in some instances even enhanced IRES function (Figure 2G–I). Even a GUGA $\rightarrow$ CCUA mutation, which mimics an inactivating mutation in the EMCV IRES (Robertson *et al*, 1999), did not affect AV IRES function (Figure 2G, lane 6). Thus, although the Jb subdomain is essential for the activity of

the AV IRES, its apical tetraloop does not have the same functional importance as in Type 1/2 IRESs.

Factor requirements for initiation on the AV IRES

We next identified initiation factors required for 48S complex formation on the AV IRES. Assembly of 48S complexes was monitored by appearance of characteristic toe-prints 15–21 nt downstream of the initiation codon, depending on the mRNA (e.g., Pestova *et al*, 1996a). Although efficient 48S complex formation on the AV IRES occurred in RRL in the presence of GMPPNP (Figure 3A), no 48S complexes formed in an *in vitro* reconstituted translation system in the presence of 40S subunits, Met-tRNA^{Met} and eIFs 1, 1A, 2, 3, 4A, 4B and 4F (Figure 3B). Thus, in contrast to the Type 2 EMCV IRES (Pestova *et al*, 1996a), canonical eIFs were not sufficient for

The activity(s) that was able to complement eIFs 1/1A/2/3/4A/4B/4F, 40S subunits and Met-tRNA^{Met} in promoting efficient 48S complex formation on the AV IRES, was present

in the 0–40% ammonium sulphate (AS) fraction of the ribosomal salt wash (RSW). During further purification, it eluted in the 100-mM KCl flow-through fraction from DEAE, and then in the 300 mM KCl fraction from phosphocellulose (data not shown). After subsequent FPLC on MonoS, the activity eluted at 300 mM KCl (data not shown). However, after FPLC on MonoQ, none of the individual fractions exhibited full activity, and efficient 48S complex formation occurred only if the flow-through fraction and the ~250-mM KCl elution fraction were combined (data not shown). Further chromatography of the 250-mM KCl MonoQ fraction on a Hydroxyapatite column identified its active constituent as DHX29. Together with the MonoQ flow-through fraction, recombinant DHX29 promoted efficient 48S complex formation (Figure 3D) and was therefore used in all subsequent experiments. Gel filtration on Superdex 200 of the MonoQ flow-through yielded an active fraction (Figure 3E, lanes 1–3) that contained proteins that were identified as Argonaute 2 (AGO2), RNA-activated protein kinase PKR, isoforms of PTB and elongation factor eEF1 α (Figure 3F; Supplementary Tables 1–4). Recombinant PTB1 replaced the active gel-filtration fraction without loss of activity in 48S complex formation on the AV IRES when included with 40S subunits, Met-tRNA_i^{Met}, DHX29 and eIFs 1, 1A, 2, 3, 4A, 4B and 4F, whereas recombinant AGO2 and fractions containing eEF1 α or PKR without PTB did not (Figure 3E, lanes 4 and 5; data not shown). Although DHX29 and PTB synergistically stimulated 48S complex formation on the IRES, the individual stimulatory activity of DHX29 was much greater than that of PTB (Figure 3G). Importantly, this is the first time that DHX29 has been reported to be required for internal ribosomal entry, and contrasts with its negative influence on the unrelated CrPV- and HCV-like IRESs (Pisareva *et al*, 2008).

Systematic omission experiments revealed that eIF2, eIF3, eIF4A and the eIF4A-interacting central domain of eIF4G (eIF4G_{736–1115} designated as ‘eIF4Gm’) were essential for 48S complex formation on the AV IRES, eIF1 and eIF4B had a moderate stimulatory effect, whereas eIF1A and eIF4E were not required (Figure 3H). Consistent with an absolute requirement for eIF4A and eIF4Gm for 48S complex formation, AV IRES-mediated translation in RRL was very efficiently inhibited by the eIF4A^{R362Q} dominant-negative mutant (Pause *et al*, 1994), whereas in control reactions, initiation on the CSFV IRES was insensitive to eIF4A^{R362Q} (Figure 3I). Incubation with 60S subunits, eIF5, eIF5B, eEF1H, eEF2 and aa-tRNAs of 48S complexes formed on mutant AV mRNA containing a stop codon three triplets downstream of the initiation codon (Figure 3J, upper panel) in the presence of DHX29, PTB and eIFs 2/3/4A/4Gm (Figure 3J, lane 1) yielded pretermination complexes characterized by toe-prints 16–17 nt downstream of the GCA triplet preceding the stop codon (Figure 3J, lane 2), indicating that 48S complexes assembled in this way could form elongation-competent 80S ribosomes.

The requirement for DHX29 results from sequestration of the initiation codon in a stable hairpin

The AV initiation codon AUG₇₄₅ is sequestered in a stable hairpin, domain L (Figures 1A and 4A). To investigate whether this distinctive feature of the AV IRES could account for its specific requirement for DHX29, we investigated the effect of disrupting domain L by deletion or substitution

(Figure 4B) on IRES activity. Disruption of domain L enhanced the activity of the IRES during *in vitro* translation in RRL (Figure 4C; data not shown). Moreover, in contrast to the *wt* IRES, which was strictly dependent on DHX29 (Figure 4D, lanes 1 and 2), mutant IRESs with destabilizing mutations in domain L did not require DHX29 for efficient 48S complex formation (Figure 4D, lanes 4, 5, 7, 8, 10 and 11). The AV IRES’ requirement for DHX29 was, therefore, determined by the need to unwind domain L.

Specific functional interaction of eIF4G/eIF4A with the AV IRES

Initiation on Type 1 and Type 2 IRESs is based on specific interaction of these IRESs with eIF4Gm, which is enhanced by eIF4A (Pestova *et al*, 1996b; Lomakin *et al*, 2000; de Breyne *et al*, 2009). Domain K of the AV IRES contains an element identical to a conserved element in domain J of Type 2 IRESs (Figure 2D) that determines their specific interaction with eIF4Gm. Disruption of this motif strongly impaired AV IRES-mediated translation (Figure 2E, lanes 2 and 3), indicating that it is similarly important for AV IRES function. We, therefore, tested whether the AV IRES also specifically interacts with eIF4Gm and whether this interaction is stimulated by eIF4A, using the directed hydroxyl radical cleavage technique, in which locally generated hydroxyl radicals cleave the IRES in the vicinity of Fe(II) tethered to unique cysteines on the surface of eIF4G via 1-(*p*-bromoacetamidobenzyl)-EDTA (BABE), after which cleavage sites are determined by primer extension. eIF4Gm consists of five pairs of α -helices (‘HEAT-repeats’) (Marcotrigiano *et al*, 2001; Figure 5A). We employed six single-cysteine mutants and a cysteine-less (Cys-less) eIF4Gm variant (Figure 5A) that were fully active in 48S complex formation on the EMCV IRES and that we previously used to investigate interaction of eIF4G with Type 1 and 2 IRESs (Kolupaeva *et al*, 2003; de Breyne *et al*, 2009; Yu *et al*, 2011). Numbering of residues in eIF4G is based on its revised sequence (NM_182917), and the cysteine residue in the D₉₂₈→D₉₂₈C insertion mutant is designated as C929. Hydroxyl radicals generated from two positions (shown as coloured spheres in Figure 5A) cleaved the AV IRES in eIF4G/IRES complexes (summarized in Figure 5F). Hydroxyl radicals generated from C829, located between helices 2b and 3a, cleaved strongly at nts 690–693, moderately at nts 663–664, and weakly at nts 651–652 and 705–710, whereas C929, located after helix 4b, cleaved moderately at nts 678–680 and weakly at nts 675–676 (Figure 5C, lanes 1–3). Inclusion of eIF4A strongly enhanced cleavage of the AV IRES from both positions on the surface of eIF4Gm (Figure 5C, lanes 7–9). These data indicate that as in the case of Type 2 IRESs, eIF4Gm binds specifically to domain K of the AV IRES, with its N-terminus directed towards its base and its C-terminus directed towards its apex, which could be consistent with enhancement of this interaction by eIF4A.

As in the case of Type 2 IRESs (Kolupaeva *et al*, 2003; Yu *et al*, 2011), the strongest eIF4G cleavage sites in the AV IRES (Figure 5F) overlapped with a conserved motif at the apex of domain K (Figure 2D), which is essential for efficient AV IRES-mediated translation (Figure 2E). Disruption of this motif abrogated cleavage of the IRES by Fe(II)-eIF4Gm (Figure 5D), suggesting that this mutant IRES’s loss of function is due to loss of its ability to bind stably to eIF4G.

Figure 4 Conditional requirement of the Aichivirus IRES for DHX29. **(A)** The model of the AV IRES, with domain L in bold. **(B)** Structures of the L domain of the *wt* AV IRES and AV IRES mutants containing destabilizing deletion and substitutions in domain L. **(C)** The activity of MC AV mRNAs containing destabilizing substitutions in domain L **(B)**, assayed by translation in RRL. **(D)** 48S complex formation on the *wt* AV IRES and the L domain AV IRES mutants **(B)** depending on the presence of DHX29, assayed by toe-printing. Black lozenges indicate toe-prints in the area of the initiation codon region of the L domain mutants.

We next determined the orientation of eIF4A in AV IRES/eIF4G/eIF4A complexes, again using directed hydroxyl radical cleavage. eIF4A consists of two RecA domains joined by a linker (Figure 5B). The eIF4A-NTD binds to the C-terminal helix of eIF4G's central domain, whereas the eIF4A-CTD binds to its N-terminal two HEAT-repeats (e.g., Marintchev *et al*, 2009). We employed nine single-cysteine eIF4A mutants (Figure 5B) that have previously been used to determine the position of eIF4A in the IRES/eIF4G/eIF4A complexes assembled on Type 1 and 2 IRESs (de Breyne *et al*, 2009; Yu *et al*, 2011). Hydroxyl radicals generated from three positions (shown as coloured spheres in Figure 5B) cleaved the AV IRES in IRES/eIF4G/eIF4A complexes (summarized in Figure 5G). Hydroxyl radicals from C33 and C42 in the eIF4A-NTD induced strong cleavage at nts 675–679 at the apex of domain K, and C42 also induced weaker cleavage nearby at nts 668–671 (Figure 5E, lanes 2 and 3). The overlap between the sites of cleavage induced by eIF4G-C929, eIF4A-C33 and eIF4A-C42 (Figure 5F and G) is consistent with the proximity of these residues in eIF4G/

eIF4A complexes (Marintchev *et al*, 2009). C351, which is close to the mRNA-binding surface of eIF4A, induced cleavage in the pyrimidine tract of the Yn-Xm-AUG motif of the AV IRES, at nts 731–742 (Figure 5E, lane 4), as in the case of the Type 2 EMCV IRES (Yu *et al*, 2011).

Taken together, these observations suggest that eIF4G and eIF4A likely bind to the structurally distinct AV and Type 2 IRESs in a functionally analogous manner.

The interaction of PTB with the AV IRES

The requirement for PTB for efficient initiation on the AV IRES parallels the stimulatory effect of PTB on the activity of Type 1 and Type 2 IRESs (Pestova *et al*, 1996a; Hunt and Jackson, 1999; Pilipenko *et al*, 2000). PTB has four RBDs (Figure 6A). RBDs 1 and 2 are independent domains connected by flexible linkers, whereas RBDs 3 and 4 have a fixed orientation relative to each other (Oberstrass *et al*, 2005; Figure 6B). Although PTB stimulates both Type 1 and Type 2 IRESs, its modes of interaction with them are distinct. Thus, the EMCV IRES bound PTB at dispersed sites with RBDs 1

Figure 5 Interaction of the Aichivirus IRES with eIF4G/eIF4A. (A, B) Ribbon diagrams of (A) the HEAT-1 domain of eIF4G (PDB: 1HU3) and (B) eIF4A in the closed ATP/RNA-bound conformation (PDB: 3EX7), with spheres indicating newly introduced cysteines. (C–E) Primer extension analysis of directed hydroxyl radical cleavage of (C–E) *wt* and (D) AGGU → UCCA mutant AV IRESs from (C) Fe(II)-tethered eIF4Gm, in the presence/absence of eIF4A and PTB, as indicated, (D) Fe(II)-tethered eIF4Gm in the presence of eIF4A and (E) Fe(II)-tethered eIF4A in the presence of eIF4Gm. (F, G) Sites of hydroxyl radical cleavage from positions on (F) eIF4Gm and (G) eIF4A, mapped onto the secondary structures of domain K of the AV IRES. Cleavage sites are shown in colours that match the colours of corresponding spheres in (A) and (B). Sites of strong cleavage are indicated by thick edging. The insert panel shows a model of the AV IRES, with domain K in bold.

and 2 interacting with domain K near the 3'-end of the IRES, and RBDs 3 and 4 interacting with domain H and the basal part of domain I near the 5'-end of the core IRES (Kafasla

et al, 2009), whereas the PV IRES binds PTB in a highly localized manner with RBDs 1 and 2 interacting with the basal part of the domain V, close to the binding site of eIF4G,

Figure 6 Interaction of the AV IRES with PTB. (A) Schematic representation of PTB1 showing the positions of the four RBDs. (B) Ribbon diagrams of PTB RBDs 1, 2 and 3 + 4 (PDB ID codes 2AD9, 2ADB and 2ADC), with spheres indicating the native (C250) and newly introduced cysteines. Cysteines that induced cleavage are coloured green. (C–E) Primer extension analysis of hydroxyl radical cleavage of the AV IRES from PTB-tethered PTB. Sites of hydroxyl radical cleavage are coloured to match domains in (A) and (B). (F) Sites of hydroxyl radical cleavage from PTB, mapped onto the secondary structure of the AV IRES. Cleavage sites are shown in colours that match the colours of domains in (A) and (B). (G) PTB-dependent changes in sensitivity to cleavage/modification (blue—protection, red—enhancement) (Figure 1B and C), mapped onto the secondary structure of the AV IRES.

and RBDs 3 and 4 interacting with the single-stranded regions flanking domain V (Kafasla *et al*, 2010).

To investigate the interaction of PTB with the AV IRES, we employed complimentary footprinting and directed hydroxyl radical cleavage techniques. First, sites of protection of the AV IRES by PTB from modification by CMCT and from cleavage

by RNases T1 and V1 were identified. PTB protected nts 638–640 from CMCT modification, nt 703 from RNase T1 cleavage and nts 380–381, 411–414, 647–648, 657–658 and 690–693 from RNase V1 cleavage (Figure 1B–D). These sites are primarily located in domain K's central region, but some mapped to domain I (Figure 6G). No protection was observed

in domains J or L. Reproducible enhancement of cleavage by RNase V1 occurred at nts 342–343 at the base of domain I, and at nts 668–669 and 686 at the apex of domain K (Figures 1B, C and 6G), which could be due to PTB-mediated stabilization of helices and/or their increased exposure. PTB-dependent enhancement of RNase cleavage at other sites was not reproducibly observed.

For hydroxyl radical cleavage experiments, we employed a panel of single-cysteine PTB mutants that had been used to investigate the interaction of PTB with Type 1 and 2 IRESs (Kafasla *et al*, 2009, 2010; Figure 6B). C139 and C141 in RBD1 induced nearly identical patterns of cleavage (Figure 6C–E, lanes 3 and 4; Figure 6F), most strongly in domain I (nts 366–368, 379–382, 390–394 and 403–406), but also at the apex of domain J (nts 488–489, 502–509, 514–519, 529–531, 544–555 and 563–565) and in domain K (nts 639–648, 652–655 and 704–711). C219, C242 and C250 in RBD2-induced cleavage at overlapping sites in domain K (nts 645–647, 646–651, 695–698 and 709–711) (Figure 6E, lanes 5–7) consistent with their proximity to each other on one surface of RBD2 (Figure 6B), whereas the more distant C284-induced cleavage at distinct sites in this domain (nts 654–658, 686–691 and 699–704) (Figure 6E, lane 8; Figure 6F). C373 and C395 in RBD3 cleaved most strongly in domain I (nts 357–359 and 411–413) (Figure 6C, lanes 9 and 10; Figure 6F) whereas C419 and C432 cleaved most strongly in domain K (nts 636–639, 637–642, 645–651 and 709–711) (Figure 6E, lanes 11 and 12; Figure 6F). C373/C395 and C419/C432 are on opposite sides of RBD3 (Figure 6B), so this pattern of cleavage suggests that RBD3 is situated between domains I and K. No cleavage occurred upstream of domain I, in the *Yn-Xm*-AUG motif or in domain L.

In summary, RBD1 is situated most closely to domain Ib and adjacent unpaired loop residues, but also in proximity to the central region of domain K and to the Jb element of domain J, which suggests a possibility of a kink in domain J that would allow its apex to approach Ib and K domains. RBD2 interacts exclusively with the central region of domain K around the large internal loop (nts 696–703), whereas RBD3 binds to an adjacent, more basal region of domain K and to domain I. RBD4 does not appear to interact with the IRES. This pattern of interaction differs from PTB's interactions with Type 1 and Type 2 IRESs (Kafasla *et al*, 2009, 2010), consistent with the assignment of the AV IRES to a distinct class.

The role of PTB in initiation on the AV IRES

To investigate the mechanism by which PTB stimulates initiation on the AV IRES, we first assayed its influence on the interaction of the IRES with eIF4G/eIF4A using directed hydroxyl radical cleavage. PTB modestly enhanced cleavage of the IRES from eIF4Gm in the absence of eIF4A (Figure 5C, compare lanes 2, 3 with lanes 5, 6), and slightly more so in its presence (Figure 5C, compare lanes 8, 9 with lanes 11, 12). PTB did not change the overall pattern of cleavage of the IRES (although cleavage from C929 at the apex of domain K was enhanced slightly more than cleavage from C829), and the relative stimulatory effect of PTB on cleavage from eIF4Gm was much lower than that of eIF4A. PTB also did not change the pattern of cleavage from eIF4A, and only slightly enhanced the intensity of cleavage in the apex of domain K from C33 and C42 (Figure 5E, compare lanes 2–4 with lanes 6–8). Thus, directed hydroxyl radical cleavage did not reveal

any dramatic effect of PTB on the interaction of the AV IRES with eIF4G/eIF4A.

During identification of factors required for 48S complex formation on the AV IRES, we noticed that inclusion in reaction mixtures of PTB or PTB-containing fractions resulted in the appearance of specific toe-prints at nts 712–713 in the basal half of domain K at the boundary of the PTB binding site identified by directed hydroxyl radical probing (Figure 6F), and also immediately flanking the initiation codon (nts 744, 746, 752, 755, 760 and 764) (Figure 3; summarized in Figure 7B). Some toe-prints around the initiation codon occurred even on mRNAs containing destabilizing deletion or substitutions in domain L (Figure 4D, lanes 4, 5, 7 and 8). Systematic omission of factors revealed that toe-prints at nts 712–713 in domain K were caused by the specific interaction of the IRES with PTB alone (Figure 7A, lane 4). In contrast, toe-prints around the initiation codon were induced by eIF4A and eIF4G, but they were extremely weak in the absence of PTB (Figure 7A, lanes 1 and 2). These toe-prints were not detected on mRNA with the disrupted conserved apical motif in domain K (Figure 7C, compare lanes 3 and 6) that severely impaired binding of eIF4G (Figure 5D), indicating that their appearance required specific interaction of eIF4G/eIF4A with the IRES. On the other hand, PTB caused equally efficient toe-prints at nts 712–713 even if the IRES contained destabilizing mutations that impaired binding of eIF4G (Figure 7C, lane 5).

Importantly, similar toe-prints around the initiation codon, which were caused by eIF4G/eIF4A, were also detected on Type 1 and Type 2 IRESs (Kolupaeva *et al*, 2003; de Breyné *et al*, 2009), and were attributed to induced conformational changes. However, in contrast to the AV IRES, these toe-prints on Type 1 and Type 2 IRESs were prominent even in the absence of ITAFs. If these conformational changes are a prerequisite for attachment of 43S complexes to the IRES, then their enhancement by PTB could account for its role in initiation on the AV IRES.

Discussion

The structure of the AV IRES

Bioinformatic analysis of the IRESs of AV and related viruses such as klassevirus revealed that they have common structures (this report; Sweeney *et al*, in preparation). The predicted structure of the AV IRES (Figure 1A) was validated by chemical and enzymatic footprinting. Its length (~410 nt) is similar to that of Type 1 and Type 2 IRESs. It consists of three major domains (I, J and K) that are typical for all members of this new group of IRESs, as well as the initiation codon-containing hairpin domain L, which is conserved in all known AV isolates, and in klassevirus is only one nucleotide shorter. The organization of domains in the AV IRES is similar to that in Type 1 and Type 2 IRESs. Thus, they all have class-specific domains at their 5'-borders (domains II and III in Type 1 IRESs, domain H in Type 2 IRESs and domain I in the AV IRES) that are followed by structurally related cruciform domains (domain IV in Type 1 IRESs, domain I in Type 2 IRESs and domain J in the AV IRES), then by domains that bind specifically to eIF4G (domain V in Type 1 IRESs, domain J–K in Type 2 IRESs and domain K in the AV IRES) and finally, by the *Yn-Xm*-AUG motif at their 3'-borders. However, the structure of the AV IRES is distinct, with some elements that

Figure 7 Toe-prints induced in the AV IRES by interaction with eIF4Gm/eIF4A and PTB. (A, C) Primer extension was done on (A) *wt* and (C) *wt* and AGGU→UCCA mutant AV MC mRNAs, in the presence of PTB, eIF4A and eIF4Gm, as indicated. Separation of lanes by white lines indicates that they were juxtaposed from the same gels. Toe-prints induced by PTB alone are indicated by arrows. Toe-prints that are caused by eIF4A/eIF4Gm, but strongly stimulated by PTB are indicated by lozenges. (B) The positions of toe-prints caused by PTB and eIF4Gm/eIF4A mapped onto the secondary structure of domains K–L of the AV IRES. Figure source data can be found with the Supplementary Information.

are unique, and others that resemble those that are characteristic of Type 1, or of Type 2 IRESs, or are common to both.

The unique elements of the AV IRES are the functionally important domain I at its 5′-border, and the AUG-containing domain L at its 3′-border. Although disruption of domain L did not result in the loss of IRES function and even enhanced the activity of the IRES during translation in RRL, it would be premature to conclude that it is redundant, given its strict conservation, and also because its influence during viral infection has not yet been assayed.

The structure of the apex of AV IRES's cruciform domain J resembles the apex of the corresponding domain IV in Type 1 IRESs, and, to a lesser extent, of domain I in Type 2 IRESs. Interestingly, although AV domain J also contains an apical GNRA tetraloop, mutation of it did not impair IRES function, in direct contrast to Type 1 and Type 2 IRESs (Kaminski *et al*, 1994; López de Quinto and Martínez-Salas, 1997; Robertson *et al*, 1999). The AV IRES's domain J differs further from domain IV of Type 1 IRESs in that it lacks the apical C-rich binding sites for PCBP2, an ITAF that is specific for Type 1 IRESs (Gamarnik and Andino, 2000). Thus, although AV domain J and domain IV of Type 1 IRESs show some structural similarity, they appear not to contain the same functional motifs.

The major point of similarity between AV and Type 2 IRESs is the eIF4G-binding motif at the apex of domain K in the former (Figure 2D) and at the apex of domain J in the latter (Clark *et al*, 2003; Bassili *et al*, 2004). However, in other respects, these domains are unrelated, and whereas in Type 2 IRESs, domain J has a branch that constitutes domain K, there is no equivalent branching domain in the AV IRES. Domain K of Type 2 IRESs is functionally important, and its truncation or deletion substantially reduces the affinity of

these IRESs for eIF4G and, as a result, translational activity (e.g., Kolupaeva *et al*, 2003).

The presence of a Yn-Xm-AUG motif at the 3′-border of the AV IRES is a feature that is also characteristic of both Type 1 and Type 2 IRESs. As in Type 2 IRESs and in contrast to Type 1 IRESs, the AUG of this motif is a functional initiation codon in the AV IRES. However, unlike Type 2 IRESs, the AUG in the AV IRES is sequestered in a stable hairpin.

The mechanism of initiation on the AV IRES

The common structural elements and sequence motifs that the AV IRES shares with Type 1 and Type 2 IRESs determine basal similarities in the mechanisms of initiation on all of these IRESs, whereas its unique structural characteristics account for some distinctive aspects. 48S complex formation on the AV IRES requires eIF2, eIF3, eIF4A, eIF4Gm and DHX29, and is strongly stimulated by PTB. As in the case of Type 2 IRESs (Pestova *et al*, 1996a, b; Pilipenko *et al*, 2000) and, as far as is currently understood, of Type 1 IRESs (de Breyne *et al*, 2009), initiation on the AV IRES is based on the specific interaction of eIF4Gm with IRES domain K, which is located immediately upstream of the Yn-Xm-AUG motif. Again, as in the case of Type 1 and Type 2 IRESs (Lomakin *et al*, 2000; de Breyne *et al*, 2009), specific binding of eIF4G is likely enhanced by eIF4A. The 'shutoff' of cellular translation in picornavirus-infected cells is commonly associated with dissociation of eIF4E from eIF4F by proteolytic scission of eIF4G and/or sequestration of eIF4E. Although the mechanism responsible for 'shutoff' during AV infection (Sasaki *et al*, 2003) is not known, the ability of its IRES to bind eIF4Gm/eIF4A with high affinity and thus to promote initiation without eIF4E would enable it to compete effectively with cellular mRNAs for access to the translation

apparatus early in infection, when eIF4F is intact, and late, if eIF4E dissociates from eIF4G.

Association of eIF4G/eIF4A with AV, Type 1 and Type 2 IRESs also induces specific toe-prints in the vicinity of the AUG codon of the Yn-Xm-AUG motif, which likely represents conformational changes caused by eIF4G/eIF4A (Kolupaeva *et al*, 2003; de Breyne *et al*, 2009; this study). Although it has been suggested that this localized modulation of IRES structure by eIF4G/eIF4A prepares the region around the AUG codon for attachment of 43S complexes, the actual mechanism of their recruitment remains unknown. Thus, the ability of eIF4G deletion mutants that do not interact with eIF3 to promote initiation on the EMCV IRES, albeit at a reduced level (Lomakin *et al*, 2000), suggests that in addition to the eIF3/eIF4G interaction, other contacts between the IRES and components of the 43S complex (e.g., eIF3 or the 40S subunit itself) likely contribute to attachment of 43S complexes.

The fact that initiation on the AV IRES does not require eIF1 and eIF1A, factors that strongly stimulate scanning and monitor the fidelity of initiation codon selection during this process, suggests that the 43S complex bind to the initiation codon directly, without prior scanning. Analogously, initiation on Type 2 EMCV and FMDV IRESs at the AUG codons of their Yn-Xm-AUG motifs also does not require eIF1 or eIF1A (Pestova *et al*, 1996a; Pilipenko *et al*, 2000), and they only become essential for initiation at the second FMDV AUG codon, which is downstream of the Yn-Xm-AUG motif (Andreev *et al*, 2007).

The unique feature of initiation on the AV IRES, which distinguishes it from all previously characterized IRESs, is its absolute dependence on DHX29, which is determined by the sequestration of the initiation codon into a stable hairpin, domain L, whose stability likely renders eIF4G/eIF4A insufficient to prepare a 'landing pad' for the 43S complex. DHX29 was first identified on the basis of its involvement in scanning on highly structured 5'-UTRs (Pisareva *et al*, 2008), and its omission results in bypassing by 43S complexes of stable stems without their inspection (Abaeva *et al*, 2011). However, DHX29 has recently also been found to be required for initiation on Sindbis virus 26S mRNA, which involves direct recruitment of the 40S subunit to an initiation codon that is sequestered in a hairpin, without prior scanning (Skabkin *et al*, 2010). Analogously to Sindbis virus 26S mRNA, DHX29 is likely required for unfolding and correct accommodation of the 3'-terminal region of the AV IRES in the ribosomal mRNA-binding cleft to allow recognition of the initiation codon by the anticodon of the P site Met-tRNA^{Met}. This role of DHX29 is consistent with its dispensability when domain L is destabilized by mutations.

Initiation on the AV IRES is enhanced by PTB, and in this respect resembles Type 1 and some Type 2 IRESs (Jackson, 2005). There is apparently no universal mechanism by which PTB enhances initiation on different IRESs. Toe-printing, footprinting and directed hydroxyl radical cleavage data established that PTB engages most strongly in interaction with AV IRES domains K and I, and is also in proximity to the Jb element of domain J. Although PTB's pattern of interaction with the AV IRES differs significantly from its strongly localized interaction with the base of domain V of the Type 1 PV IRES and its widely distributed interactions with the apex of domain K, domain H and the base of domains I and L of the Type 2 EMCV IRES (Kafasla *et al*, 2009, 2010), it is notable

that in all cases, one major point of contact with PTB involves the IRES's eIF4G-binding domain. The interaction of PTB with domain V of the PV IRES slightly reorients eIF4Gm on this domain (Kafasla *et al*, 2010), and although directed hydroxyl radical cleavage experiments did not provide evidence for similar PTB-induced changes in the orientation of eIF4G on the AV IRES, the strong enhancement by PTB of eIF4G/eIF4A-induced toe-prints around the initiation codon indicates that PTB nevertheless influences the activity of eIF4G/eIF4A on the AV IRES. PTB's role in stimulating initiation on the AV IRES might therefore be to enhance the ability of eIF4G/eIF4A to induce conformational changes in the IRES that are required for productive attachment of 43S complexes.

In conclusion, although the AV and Type 1 and Type 2 IRESs are structurally distinct, there are some apparent important functional similarities in their mechanism of action, which indicates that even elements with distinct sequences and structures can perform functionally related roles.

Materials and methods

Plasmids

Expression vectors used were for His₆-tagged eIF1 and eIF1A (Pestova *et al*, 1998a), wt eIF4A and eIF4B (Pestova *et al*, 1996a), eIF4A^{R362Q} mutant (Pause *et al*, 1994), eIF4A single-cysteine mutants (de Breyne *et al*, 2009), eIF4G^{L736-1115} ('eIF4Gm') wt and single-cysteine mutants (Kolupaeva *et al*, 2003), eIF5 and eIF5B (Pestova *et al*, 2000), DHX29 (Skabkin *et al*, 2010), PTB wt (Hellen *et al*, 1993), PTB single-cysteine mutants (Kafasla *et al*, 2009), *Escherichia coli* methionyl tRNA synthetase (Lomakin *et al*, 2006) and Argonaute 2 (Rivas *et al*, 2005).

Transcription vectors for tRNA^{Met} and dicistronic CSFV IRES-containing mRNA ('DC CSFV') have been described (Pestova *et al*, 1998b; Pestova and Hellen, 2001). A monocistronic AV mRNA ('MC Aichivirus') transcription vector was made (GenScript) by inserting DNA corresponding to a 5'-terminal T7 promoter, two G residues and a variant of AV nts 1-1599 (GenBank AB040749) followed by two UAA stop codons into pUC57. The AV sequence contained substitutions that eliminated a *Bam*HI restriction site at nt 1410 and introduced AUG triplets at positions corresponding to codons 177, 223, 234, 241, 259, 262 and 276 of the 285 amino acid long AV LAVP0 coding sequence. Dicistronic AV transcription vectors ('DC Aichivirus') containing fragments of the AV 5'-UTR between a T7 promoter linked to a variant of nts 2-1369 of *Xenopus laevis* cyclin B2 mRNA (Genbank J03167) (ΔXL) in which nts 1169-1218 had been replaced by the sequence TATGG and a slightly truncated version of the AV coding region described above were made in a similar manner, by inserting synthetic DNA into pUC57. Further mutations were introduced in MC and DC AV vectors by NorClone Biotech Laboratories (London, Ontario). tRNA^{Met} and DC CSFV, MC Aichivirus and DC Aichivirus mRNAs were transcribed using T7 polymerase.

Purification of initiation factors, ribosomal subunits and aminoacylation of tRNA

40S and 60S ribosomal subunits, eIF2, eIF3, eIF4F, eEF1H, eEF2 and total aminoacyl-tRNA synthetases were purified from RRL (Pisarev *et al*, 2007). Recombinant eIF1, eIF1A, eIF4A, eIF4B, eIF4Gm, eIF5, eIF5B, methionyl tRNA synthetase, DHX29, PTB and Argonaute 2 were expressed and purified from *E. coli* (Rivas *et al*, 2005; Pisarev *et al*, 2007; Kafasla *et al*, 2009; Skabkin *et al*, 2010). Native total tRNA (Promega) and *in vitro* transcribed tRNA^{Met} were aminoacylated as described (Pisarev *et al*, 2007).

Purification of additional activities required for initiation on the AV IRES

Additional factors required for initiation on the AV IRES were purified from RRL on the basis of their activity in supporting efficient 48S complex formation on the AV IRES in the *in vitro*

reconstituted system in the presence of 40S subunits and eIFs 2/3/1/1A/4A/4B/4F, which was monitored by toe-printing.

In all, 0.5 M KCl RSW was prepared from 1.21 RRL (Green Hectares) and fractionated by AS precipitation (Pisarev *et al*, 2007). The active 0–40% AS fraction was dialysed against buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.1 mM EDTA) + 100 mM KCl and applied to a DEAE (DE52) column equilibrated with buffer A + 100 mM KCl. The active flow-through fraction was applied to a phosphocellulose (P11) column equilibrated with buffer A + 100 mM KCl. Step elution was done with buffer A containing 100, 200, 300, 400 and 500 mM KCl. The activity eluted at 300 mM KCl. This fraction was dialysed against buffer B (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT, 0.1 mM EDTA) + 100 mM KCl and applied to a FPLC MonoS HR 5/5 column. Fractions were collected across a 100–500-mM KCl gradient. The activity eluted at ~300 mM KCl. This fraction was dialysed against buffer A + 50 mM KCl and applied to a FPLC MonoQ HR 5/5 column. None of individual fractions exhibited full activity, and efficient 48S complex formation occurred only if the flow-through fraction and the fraction eluted at ~250 mM KCl were combined. Further chromatography of the 250-mM KCl MonoQ fraction on a Hydroxyapatite column identified DHX29 as its active constituent. To purify the active component of the MonoQ flow-through fraction, it was concentrated and transferred into buffer A + 200 mM KCl using Microcon YM30 centrifugal filtration devices, and then applied to a Superdex 200 gel-filtration column. The active gel-filtration fraction contained AGO2, PKR, PTB and eEF1 α that were identified by LC-nanospray tandem mass spectrometry of peptides derived by in-gel tryptic digestion at the Rockefeller University Proteomics Resource Center. Recombinant PTB was able to substitute this fraction in 48S complex formation on the AV IRES.

In vitro translation

DC Aichivirus, MC Aichivirus and DC CSFV mRNAs (0.1 μ g) were translated using the Flexi RRL System (Promega) (20 μ l reaction volume) supplemented with 0.5 mCi/ml [³⁵S]methionine (43.5 TBq/mmol) for 60 min at 37°C. Translation products were analysed by electrophoresis using NuPAGE 4–12% Bis-Tris-Gel (Invitrogen), followed by autoradiography.

Assembly and analysis of ribosomal complexes

To assemble 48S complexes, 1.5 pmol *wt* or mutant MC AV mRNAs were incubated with 2 pmol 40S subunits, 4 pmol Met-tRNA^{Met}, and indicated combinations of 4 pmol eIF2, 3 pmol eIF3, 10 pmol eIF4A, 5 pmol eIF4B, 10 pmol eIF4Gm, 2.5 pmol eIF4F, 10 pmol eIF1, 10 pmol eIF1A, 0.2 pmol recombinant DHX29, 5 pmol PTB, 5 pmol AGO2 and different RSW fractions were incubated for 10 min at 37°C in 20 μ l buffer C (20 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, 0.25 mM spermidine) supplemented with 1 mM ATP and 0.4 mM GTP. Assembled 48S complexes were analysed by toe-printing using avian myeloblastosis virus reverse transcriptase (AMV RT) and ³²P-labelled primers as described (Pisarev *et al*, 2007). cDNA products were resolved in 6% polyacrylamide sequencing gels. To assay elongation on AV-MAA-STOP mRNA, 48S complexes were supplemented with 3 pmol 60S subunits, 5 pmol eIF5, 5 pmol eIF5B, 15 mg total native aa-tRNA, 5 pmol eEF1H and 5 pmol eEF2 and incubated at 37°C for an additional 15 min and analysed by toe-printing.

Toe-printing analysis of 48S complex formation in RRL in the presence of GMPPNP was done as described (Pilipenko *et al*, 2000).

Chemical and enzymatic footprinting

In all, 5 pmol of free AV mRNA (nts 1–1599) or AV mRNA that was preincubated with 15 pmol PTB were enzymatically digested by incubation with RNase T1 (0.04 U/ μ l) or RNase V1 (0.0006 U/ μ l), or modified by incubation with CMCT (126 mg/ml) for 10 min at 30°C

in 50 μ l buffer C (Kolupaeva *et al*, 2007). Cleaved or modified sites were identified by primer extension, using AMV RT and primers complementary to AV nts 841–822, nts 586–567 and nts 504–485.

Directed hydroxyl radical cleavage

eIF4Gm, eIF4A and PTB single-cysteine mutants were derivatized with Fe(II)-BABE as described (Kolupaeva *et al*, 2003) by incubating 3000 pmol of a protein with 1 mM Fe(II)-BABE in 100 μ l buffer containing 80 mM HEPES (pH 7.5), 300 mM KCl and 10% glycerol for 30 min at 37°C. Derivatized proteins were separated from unincorporated reagent by buffer exchange on Microcon YM-30 filter units and stored at –80°C.

To investigate hydroxyl radical cleavage, 5 pmol *wt* or mutant AV mRNA (nts 1–1599) was incubated at 37°C for 10 min in 50 μ l buffer D (20 mM HEPES (pH 7.6), 100 mM KCl, 2.5 mM MgCl₂ and 5% glycerol) with 10 pmol [Fe(II)-BABE]-eIF4Gm (in the presence/absence of 10 pmol unmodified eIF4A and/or PTB), or with 10 pmol [Fe(II)-BABE]-eIF4A (in the presence/absence of 10 pmol unmodified eIF4Gm and PTB) or with 10 pmol [Fe(II)-BABE]-PTB. To generate hydroxyl radicals, reaction mixtures were supplemented with 0.05% H₂O₂ and 5 mM ascorbic acid and incubated on ice for 10 min. Reactions were quenched by adding 20 mM thiourea. Sites of hydroxyl radical cleavage were determined by primer extension using AMV RT and appropriate [³²P]-labelled primers. cDNA products were resolved in a 6% sequencing gel.

Toe-printing analysis of the interaction of the AV IRES with PTB, eIF4Gm and eIF4A

In all, 2 pmol of *wt* or mutant AV mRNA (nts 1–1599) was incubated with indicated combinations of eIF4Gm (4 pmol), eIF4A (10 pmol) and PTB (8 pmol) for 10 min at 37°C in 20 μ l buffer C supplemented with 1 mM ATP, and then analysed by primer extension using AMV RT and the [³²P]-labelled primer. cDNA products were resolved in a 6% sequencing gel.

Bioinformatic analysis

AV sequences (Genbank accession #AB040749, AB010145, AY74 7174, GQ927710, FJ890523, DQ028632, GQ927704, GQ927705, GQ927706, GQ927707, GQ927708, GQ927709, GQ927711 and GQ927712) were aligned with Clustalw2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) using default parameters. Secondary structure elements were modelled using probabilistic (Pfold: <http://www.daimi.au.dk/~compbio/pfold/>; Knudsen and Hein, 2003) and posterior decoding approaches (Centroidfold: <http://www.ncrna.org/centroidfold/>; Sato *et al*, 2009) and were verified and refined by free energy minimization using Mfold (<http://mfold.rna.albany.edu/?q=mfold>; Zuker, 2003) and RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>; Gruber *et al*, 2008), in all instances using default parameters.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank L Joshua-Tor for the AGO2 plasmid. This work was supported by NIH Grant AI51340 to CUTH, NIH Grant GM59660 to TVP and BBSRC Project Grant BB/E004857 to RJJ.

Author contributions: YY, TRS, TVP and CUTH conceived and designed the experiments. YY, TRS and PK performed the experiments. CH performed the bioinformatic analyses. YY, TRS, RJJ, TVP and CUTH analysed the data and RJJ, TVP and CUTH wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Abaeva IS, Marintchev A, Pisareva VP, Hellen CU, Pestova TV (2011) Bypassing of stems versus linear base-by-base inspection of mammalian mRNAs during ribosomal scanning. *EMBO J* **30**: 115–129
- Ali IK, McKendrick L, Morley SJ, Jackson RJ (2001) Activity of the hepatitis A virus IRES requires association between the cap-binding translation initiation factor (eIF4E) and eIF4G. *J Virol* **75**: 7854–7863
- Andreev DE, Fernandez-Miragall O, Ramajo J, Dmitriev SE, Terenin IM, Martinez-Salas E, Shatsky IN (2007) Differential factor requirement to assemble translation initiation complexes at the alternative start codons of foot-and-mouth disease virus RNA. *RNA* **13**: 1366–1374
- Bailey JM, Tappich WE (2007) Structure of the 5' nontranslated region of the Coxsackievirus B3 genome: chemical modification and comparative sequence analysis. *J Virol* **81**: 650–668

- Bassili G, Tzima E, Song Y, Saleh L, Ochs K, Niepmann M (2004) Sequence and secondary structure requirements in a highly conserved element for foot-and-mouth disease virus internal ribosome entry site activity and eIF4G binding. *J Gen Virol* **85**: 2555–2565
- Brown EA, Day SP, Jansen RW, Lemon SM (1991) The 5' nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation *in vitro*. *J Virol* **65**: 5828–5838
- Clark AT, Robertson ME, Conn GL, Belsham GJ (2003) Conserved nucleotides within the J domain of the encephalomyocarditis virus internal ribosome entry site are required for activity and for interaction with eIF4G. *J Virol* **77**: 12441–12449
- de Breyne S, Yu Y, Unbehauen A, Pestova TV, Hellen CU (2009) Direct functional interaction of initiation factor eIF4G with type 1 internal ribosomal entry sites. *Proc Natl Acad Sci USA* **106**: 9197–9202
- Garnarik AV, Andino R (2000) Interactions of viral protein 3CD and poly(rC) binding protein with the 5' untranslated region of the poliovirus genome. *J Virol* **74**: 2219–2226
- Geary C, Baudrey S, Jaeger L (2008) Comprehensive features of natural and *in vitro* selected GNRA tetraloop-binding receptors. *Nucleic Acids Res* **36**: 1138–1152
- Gruber AR, Lorenz R, Bernhart SH, Neuböck R, Hofacker IL (2008) The Vienna RNA website. *Nucleic Acids Res* **36**: W70–W74
- Hellen CU, Witherell GW, Schmid M, Shin SH, Pestova TV, Gil A, Wimmer E (1993) A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc Natl Acad Sci USA* **90**: 7642–7646
- Hunt SL, Jackson RJ (1999) Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. *RNA* **5**: 344–359
- Jackson RJ (2005) Alternative mechanisms of initiating translation of mammalian mRNAs. *Biochem Soc Trans* **33**: 1231–1241
- Jackson RJ, Hellen CU, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* **11**: 113–127
- Kafasla P, Morgner N, Pöyry TA, Curry S, Robinson CV, Jackson RJ (2009) Polypyrimidine tract binding protein stabilizes the encephalomyocarditis virus IRES structure via binding multiple sites in a unique orientation. *Mol Cell* **34**: 556–568
- Kafasla P, Morgner N, Robinson CV, Jackson RJ (2010) Polypyrimidine tract-binding protein stimulates the poliovirus IRES by modulating eIF4G binding. *EMBO J* **29**: 3710–3722
- Kaminski A, Hunt SL, Gibbs CL, Jackson RJ (1994) Internal initiation of mRNA translation in eukaryote. In: *Genetic Engineering*, Setlow JK (ed) vol. 16, pp 115–155. New York: Plenum Press
- Kapoor A, Victoria J, Simmonds P, Slikas E, Chiochansin T, Naem A, Shaukat S, Sharif S, Alam MM, Angez M, Wang C, Shafer RW, Zaidi S, Delwart E (2008) A highly prevalent and genetically diversified Picornaviridae genus in South Asian children. *Proc Natl Acad Sci USA* **105**: 20482–20487
- Knudsen B, Hein J (2003) Pfold: RNA secondary structure prediction using stochastic context-free grammars. *Nucleic Acids Res* **31**: 3423–3428
- Kolupaeva VG, de Breyne S, Pestova TV, Hellen CU (2007) *In vitro* reconstitution and biochemical characterization of translation initiation by internal ribosomal entry. *Methods Enzymol* **430**: 409–439
- Kolupaeva VG, Lomakin IB, Pestova TV, Hellen CU (2003) Eukaryotic initiation factors 4G and 4A mediate conformational changes downstream of the initiation codon of the encephalomyocarditis virus internal ribosomal entry site. *Mol Cell Biol* **23**: 687–698
- Lomakin IB, Hellen CU, Pestova TV (2000) Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. *Mol Cell Biol* **20**: 6019–6029
- Lomakin IB, Shirokikh NE, Yusupov MM, Hellen CU, Pestova TV (2006) The fidelity of translation initiation: reciprocal activities of eIF1, IF3 and YciH. *EMBO J* **25**: 196–210
- López de Quinto S, Martínez-Salas E (1997) Conserved structural motifs located in distal loops of aphthovirus internal ribosome entry site domain 3 are required for internal initiation of translation. *J Virol* **71**: 4171–4175
- Marcotrigiano J, Lomakin IB, Sonenberg N, Pestova TV, Hellen CU, Burley SK (2001) A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Mol Cell* **7**: 193–203
- Marintchev A, Edmonds KA, Marintcheva B, Hendrickson E, Oberer M, Suzuki C, Herdy B, Sonenberg N, Wagner G (2009) Topology and regulation of the human eIF4A/4G/4H helicase complex in translation initiation. *Cell* **136**: 447–460
- Nagashima S, Sasaki J, Taniguchi K (2005) The 5'-terminal region of the Aichi virus genome encodes cis-acting replication elements required for positive- and negative-strand RNA synthesis. *J Virol* **79**: 6918–6931
- Oberstrass FC, Auweter SD, Erat M, Hargous Y, Henning A, Wenter P, Raymond L, Amir-Ahmady B, Pitsch S, Black DL, Allain FH (2005) Structure of PTB bound to RNA: specific binding and implications for splicing regulation. *Science* **309**: 2054–2057
- Pause A, Méthot N, Svitkin Y, Merrick WC, Sonenberg N (1994) Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J* **13**: 1205–1215
- Pestova TV, Borukhov SI, Hellen CU (1998a) Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* **394**: 854–859
- Pestova TV, Hellen CU (2001) Preparation and activity of synthetic unmodified mammalian tRNAi(Met) in initiation of translation *in vitro*. *RNA* **7**: 1496–1505
- Pestova TV, Hellen CU, Shatsky IN (1996a) Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol Cell Biol* **16**: 6859–6869
- Pestova TV, Lomakin IB, Lee JH, Choi SK, Dever TE, Hellen CU (2000) The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* **403**: 332–335
- Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CU (1998b) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* **12**: 67–83
- Pestova TV, Shatsky IN, Hellen CU (1996b) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol Cell Biol* **16**: 6870–6878
- Pilipenko EV, Pestova TV, Kolupaeva VG, Khitrina EV, Poperechnaya AN, Agol VI, Hellen CU (2000) A cell cycle-dependent protein serves as a template-specific translation initiation factor. *Genes Dev* **14**: 2028–2045
- Pisarev AV, Unbehauen A, Hellen CU, Pestova TV (2007) Assembly and analysis of eukaryotic translation initiation complexes. *Methods Enzymol* **430**: 147–177
- Pisareva VP, Pisarev AV, Komar AA, Hellen CU, Pestova TV (2008) Translation initiation on mammalian mRNAs with structured 5'UTRs requires DEXH-box protein DHX29. *Cell* **135**: 1237–1250
- Reuter G, Boros A, Pankovics P (2011) Kobuviruses – a comprehensive review. *Rev Med Virol* **21**: 32–41
- Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L (2005) Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol* **12**: 340–349
- Roberts LO, Jopling CL, Jackson RJ, Willis AE (2009) Viral strategies to subvert the mammalian translation machinery. *Prog Mol Biol Transl Sci* **90**: 313–367
- Robertson ME, Seamons RA, Belsham GJ (1999) A selection system for functional internal ribosome entry site (IRES) elements: analysis of the requirement for a conserved GNRA tetraloop in the encephalomyocarditis virus IRES. *RNA* **5**: 1167–1179
- Sasaki J, Nagashima S, Taniguchi K (2003) Aichi virus leader protein is involved in viral RNA replication and encapsidation. *J Virol* **77**: 10799–10807
- Sasaki J, Taniguchi K (2003) The 5'-end sequence of the genome of Aichi virus, a picornavirus, contains an element critical for viral RNA encapsidation. *J Virol* **77**: 3542–3548
- Sato K, Hamada M, Asai K, Mituyama T (2009) CENTROIDFOLD: a web server for RNA secondary structure prediction. *Nucleic Acids Res* **37**: W277–W280
- Skabkin MA, Skabkina OV, Dhote V, Komar AA, Hellen CU, Pestova TV (2010) Activities of Ligatin and MCT-1/DENR in eukaryotic translation initiation and ribosomal recycling. *Genes Dev* **24**: 1787–1801
- Yu Y, Abaeva IS, Marintchev A, Pestova TV, Hellen CU (2011) Common conformational changes induced in type 2 picornavirus IRESs by cognate trans-acting factors. *Nucleic Acids Res* **39**: 4851–4865
- Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**: 3406–3415