Translation Initiation on Mammalian mRNAs with Structured 5′UTRs Requires DExH-Box Protein DHX29

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SUMMARY

Eukaryotic protein synthesis begins with assembly of 48S initiation complexes at the initiation codon of mRNA, which requires at least seven initiation factors (eIFs). First, 43S preinitiation complexes comprising 40S ribosomal subunits, eIFs 3, 2, 1, and 1A, and tRNA^Met attach to the 5′-proximal region of mRNA and then scan along the 5′ untranslated region (5′UTR) to the initiation codon. Attachment of 43S complexes is mediated by three other eIFs, 4F, 4A, and 4B, which cooperatively unwind the cap-proximal region of mRNA and later also assist 43S complexes during scanning. We now report that these seven eIFs are not sufficient for efficient 48S complex formation on mRNAs with highly structured 5′UTRs, and that this process requires the DExH-box protein DHX29. DHX29 binds 40S subunits and hydrolyzes ATP, GTP, UTP, and CTP. NTP hydrolysis by DHX29 is strongly stimulated by 43S complexes and is required for DHX29’s activity in promoting 48S complex formation.

INTRODUCTION

Eukaryotic protein synthesis begins with assembly of 48S initiation complexes, in which initiator tRNA (Met-tRNA^Met) is base-paired with the initiation codon of mRNA in the P site of the 40S subunit. 48S complex formation on most cellular mRNAs occurs by the scanning mechanism and requires at least seven initiation factors (eIFs) (Pestova et al., 2007). First, 43S complexes comprising 40S subunits, eIF2/GTP/Met-tRNA^Met, ternary complexes (TCs), eIF3, eIF1, and eIF1A attach to the 5′-proximal region of mRNA and then scan along the 5′ untranslated region (5′UTR) to the initiation codon where they stop, forming 48S complexes.

Attachment of 43S complexes to mRNA is mediated by eIFs 4F, 4A, and 4B. eIF4F comprises eIF4E (cap-binding protein), eIF4A (a DEAD-box RNA helicase, whose activity is enhanced by eIF4G and eIF4B), and eIF4G (which binds eIF4E, eIF4A, and also 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. The molecular mechanism by which mRNA enters the mRNA-binding cleft of the 40S subunit (e.g., by threading through this entire channel starting from its entrance, or by direct placement of the cap-proximal mRNA segment into the mRNA-binding cleft) is unknown.

Ribosomal scanning consists of two linked processes: unwinding of secondary structure in the 5′UTR and ribosomal movement along it. During scanning, 43S complexes must be able to reject potential mismatches between the Met-tRNA^Met and non- and near-cognate codons, but also to recognize the correct initiation triplet. The key role in ensuring accurate initiation codon selection belongs to eIF1, which enables 43S complexes to discriminate against 48S complex formation on non-AUG triplets and on AUG triplets in suboptimal context (Pestova and Kolupaeva, 2002; Pisarev et al., 2006). eIF1 binds to the interface surface of the 40S subunit between the platform and Met-tRNA^Met (Lomakin et al., 2003), and it has been suggested that it performs its monitoring function indirectly, by influencing the conformation of ribosomal complexes. Consistently, binding of eIF1 and eIF1A to yeast 40S subunits induces conformational changes that consist of opening of the entry channel “latch” formed between helix (h) 18 in the body and h34 and ribosomal protein (rp) S3 in the neck and establishment of a new head-body connection likely mediated by h16 and rpS3 (Passmore et al., 2007). But what is the role of different factors in ribosomal movement per se? 43S complexes containing TCs, eIF3, eIF1, and eIF1A can bind to the 5′ end of an unstructured 5′UTR and scan to the initiation codon without ATP or factors associated with ATP hydrolysis and RNA unwinding, revealing the intrinsic ability of 43S complexes to move along mRNA (Pestova and Kolupaeva, 2002). Importantly, omission of eIF1A greatly reduces the ability of 43S complexes to form 48S complexes in the absence of eIF4A/4G/4B, and omission of eIF1 almost abrogates it. Although eIF3 is indispensable for 48S complex formation, it is difficult to separate its role in scanning from functions in recruitment of TCs to 40S subunits and initial attachment of 43S complexes. Scanning on 5′UTRs containing even weak internal secondary structure, on the other hand, requires ATP.
and eIF4A/4G/4B, and the requirement for ATP and eIF4A is proportional to the degree of secondary structure in the 5' UTR (Pestova and Kolupaeva, 2002; Jackson, 1991; Svitkin et al., 2001). Continued association of eIF4G with ribosomal complexes (Pöyry et al., 2004) ensures eIF4A's processivity and couples mRNA unwinding with ribosomal movement. It is unknown whether eIF4F/4A/4B bind at the 40S subunit's leading edge and unwind mRNA before it enters the mRNA-binding cleft, or at the trailing edge near the E site and assist scanning by helix-mediated “pulling” of mRNA through the mRNA-binding channel and/or preventing backward movement. eIF4A/4G/4B also stimulate 48S complex formation on mRNAs with unstructured 5'UTRs and make this process less dependent on eIF1/1A (Pestova and Kolupaeva, 2002). Thus, it is clear that scanning requires ATP-dependent unwinding of RNA secondary structure by eIF4A/4G/4B and induction by eIF1/1A of the scanning-competent conformation of 43S complexes.

The efficiency of translation of different mRNAs in vivo and in vitro depends on the degree of secondary structure in their 5'UTRs. Thus, stems of \( \Delta G = -30 \) kcal/mol located distally in the 5'UTR inhibit but do not abolish initiation, whereas stems of \( \Delta G = -60 \) kcal/mol abrogate translation by obstructing ribosomal movement to the initiation codon (Kozak, 1991).

In our in vitro reconstituted initiation system containing eIF2/3/1/1A/4A/4B/4F, 48S complexes did not form efficiently on mRNAs containing GC-rich stems of even moderate stability in their 5'UTRs, although they are translated well in cell-free extracts (Pestova and Kolupaeva, 2002). Moreover, during 48S complex formation on \( \beta \)-globin mRNA, additional toeprints appear +8–9 nt from the AUG codon, equal to as much as 30%–40% of the +15–17 nt toeprint of properly assembled 48S complexes, which most likely represent an initiation complex, in which the 3' portion of mRNA was not properly fixed in the 40S subunit’s mRNA-binding cleft (Battiste et al., 2000). Such aberrant toeprints do not appear when 48S complexes are assembled in cell-free translation extracts. Here we report that we have purified and identified the DExH-box protein DHX29 as a factor that is required for efficient 48S complex formation on mRNAs with highly structured 5'UTRs and that also suppresses the aberrant +8–9 nt toeprint.

**RESULTS**

**Efficient 48S Complex Formation on mRNAs with Structured 5' UTRs Requires DExH-Box Protein DHX29**

Although in an in vitro reconstituted system, eIF2/3/1/1A/4A/4B/4F promoted efficient 48S complex formation on model synthetic mRNAs comprising the \( \beta \)-glucuronidase (GUS) coding region and an unstructured 5'UTR consisting of 19 CAA repeats (CAA-GUS mRNA; Pestova and Kolupaeva, 2002) or 5'UTRs containing GC-rich stems of relatively low stability flanked by CAA repeats (CAA-GUS Stem-1 and Stem-2 mRNAs), yielding intense toeprints +15–17 nt from the AUG codon (Figure 1C, lanes 4, 9, and 14), they did not support high-level 48S complex formation on CAA-GUS Stem-3 and Stem-4 mRNAs containing more stable stems with \( \Delta G = -18.9 \) and \(-27.6 \) kcal/mol, respectively (Figure 1C, lanes 18 and 24), even though they translated efficiently in rabbit reticulocyte lysate (RRL) (data not shown). These eIFs also supported only very weak 48S complex assembly on neutrophil cytosolic factor 2 (NCF2) mRNA containing a 168 nt long 5'UTR (55% GC; \( \Delta G = -54 \) kcal/mol) (Figure 1D, lane 3) and did not promote 48S complex formation at all on CDC25 mRNA containing a 271 nt long 5'UTR (44% GC; \( \Delta G = -120 \) kcal/mol) (Figure 1E, lane 2), even though both mRNAs were relatively efficiently translated in RRL (data not shown). We therefore undertook extensive purification from RRL of a missing factor(s) required for efficient 48S complex formation on mRNAs with structured 5'UTRs. Purification yielded an apparently homogeneous \( \sim 150 \) kDa protein (Figure 1A) that was identified as DHX29 (see Table S1A available online), a putative DExH-box helicase (Figure 1B). DHX29 has a central helicase domain with consensus sequence motifs that are characteristic of DEAH helicases such as DHX9 and the splicing factor Prp2/DHX16 (de la Cruz et al., 1999; Figure 1B) and C-terminally located helicase-associated HA2 and DUF1605 domains of unknown function.

Inclusion of DHX29 in an in vitro reconstituted system strongly (~5–20-fold) increased 48S complex formation on CAA-GUS Stem-3 and Stem-4 mRNAs (Figure 1C, lanes 19 and 25) and on NCF2 mRNA (Figure 1D, lane 4) and allowed 48S complex formation on CDC25 mRNA (Figure 1E, lane 1). DHX29 also slightly (~20%–30%) stimulated the already efficient 48S complex formation on CAA-GUS Stem-1 and Stem-2 mRNAs (Figure 1C, lanes 10 and 15). Toeprints that appeared at intermediate positions on NCF2 and CDC25 5'UTRs in reaction mixtures containing 43S complexes and eIF4A/4B/4F with or without DHX29 (Figure 1D, lanes 3 and 4; Figure 1E, lane 1) likely corresponded to scanning ribosomal complexes arrested upstream of the initiation codon by stable secondary structures. Moderate stimulation of 48S complex formation on stem-containing CAA-GUS mRNAs by DHX29 occurred even in the absence of eIF4A/4B/4F (Figure 1C, lanes 3, 8, 13, 17, and 23), but it was lower than by eIF4A/4B/4F (Figure 1C, lanes 4, 9, 14, 18, and 24). In contrast to CAA-GUS mRNAs, DHX29 did not promote 48S complex formation on NCF2 or CDC25 mRNAs in the absence of eIF4A/4B/4F (Figure 1D, lane 5; Figure 1E, lane 3) and mediated only marginal 48S complex assembly on \( \beta \)-globin mRNA (Figure 2B, lane 6). We speculate that this difference was due to the presence of...
43 unstructured 5'-terminal nt in CAA-GUS mRNAs that can promote eIF4A/4B/4F-independent attachment of 43S complexes (Pestova and Kolupaeva, 2002). If this assumption is correct, then DHX29 likely assists scanning but does not function during attachment of 43S complexes. To verify that 48S complexes assembled with DHX29 were elongation competent, formation of ribosomal complexes was assayed on derivatives of CAA-GUS Stem-3 and Stem-4 mRNAs encoding an MVHC tetrapeptide followed by a UAA stop codon. Addition of 60S subunits, eIF5/5B, elongation factors, and aminoacylated tRNAs to 48S complexes assembled on both mRNAs with DHX29 yielded prominent toeprints +16–17 nt from the UGC Cys codon that occupies the P site of elongating ribosomes arrested at the stop codon (Figure 1F, left panel). As with 48S complexes, substantially more elongation complexes formed on both mRNAs in the presence of DHX29, assayed by toeprinting and sucrose density gradient (SDG) centrifugation (Figure 1F).

Figure 2. DHX29 Suppresses the Aberrant Toeprint +8–9 nt from the AUG Codon
Toeprinting analysis of 48S complex assembly on (A and B) β-globin mRNA, (C) mRNA containing two AUG triplets, and (D) CAA-GUS Stem-1 mRNA in RRL (A), lane 3 and in an in vitro reconstituted initiation system (A–D) with eIFs as indicated. Initiation codons and positions of assembled ribosomal complexes are indicated. Lanes C/T/A/G depict corresponding DNA sequences.

Taken together, these data indicate that eIF4A/4B/4F and DHX29 synergistically promote efficient 48S complex formation on mRNAs with structured 5’UTRs.

**DHX29 Suppresses the Aberrant Toeprint +8–9 nt Downstream of the AUG Codon**
Although eIF2/3/1A/4A/4B/4F ensured efficient 48S complex formation on native capped β-globin mRNA, we have noted additional toeprints +8–9 nt from the AUG codon at up to 30%–40% of the level of the +15–17 nt toeprints that correspond to properly assembled 48S complexes (Figure 2A, lane 3; Battiste et al., 2000). The +8–9 toeprints were apparent on some other mRNAs, for example, on the first AUG codon of mRNA containing two AUG triplets flanked by CAA repeats (Figure 2C, lanes 2 and 4). In contrast, 48S complexes assembled on β-globin or other mRNAs in RRL yielded toeprints exclusively at +15–17 positions (e.g., Figure 2A, lane 3). Appearance of the +8–9 nt toeprint...

required 40S subunits, Met-tRNAMet, eIFs, and an AUG codon, suggesting that it corresponds to a 48S complex in which the 3′ portion of mRNA is not fixed in the 40S subunit's mRNA-binding cleft, thus allowing reverse transcriptase to penetrate further. Formation of the +8–9 nt toeprint was eIF1 dependent: almost no such toeprint was observed on the first AUG codon of mRNA with two AUG triplets in reaction mixtures lacking eIF1 (Figure 2C, compare lanes 2 and 4 with lanes 6 and 8). The +8–9 nt toeprint was also exacerbated by some eIF1A mutants (Battiste et al., 2000). Although DHX29 did not influence the overall yield of 48S complex formation on β-globin mRNA, it suppressed this aberrant toeprint (Figure 2B, lane 3). Importantly, DHX29 had the same effect on the +8–9 toeprint upon its delayed addition to preformed 48S complexes (Figure 2C, lane 4). DHX29 also suppressed the aberrant +8–9 nt toeprint on other mRNAs, including the mRNA with two AUG triplets (Figure 2C, lanes 1 and 3). These data suggest that binding of DHX29 to ribosomal complexes induces conformational changes near the mRNA-binding cleft that influence accommodation of the 3′ portion of mRNA.

DHX29 also increased leaky scanning, enhancing 48S complex formation on the second AUG codon of mRNA with two AUG triplets, irrespective of the presence of eIF1 or eIF1A (Figure 2C, lanes 1, 3, 5, and 7). This increase in leaky scanning is consistent with enhanced processivity of ribosomal complexes.

In reaction mixtures lacking eIF4F/4A/4B, DHX29 promoted low-level 48S complex formation on CAA-GUS Stem-1 even without eIF1/1A (Figure 2D, lane 3). However eIF1, particularly with eIF1A, substantially increased initiation (Figure 2D, lanes 5 and 6).

**DHX29 Specifically Binds to 40S Subunits**

Experiments done to identify interactions between DHX29 and translational components revealed that it bound stably to 40S subunits, but not 60S subunits or 80S ribosomes, and remained associated with them during SDG centrifugation (Figure 3A, lanes 4, 5, and 7). Importantly, DHX29 associated only with 40S monomers, but not the dimers (Figure 3A, lanes 6 and 7) that always occur in mammalian 40S subunit preparations (Unbehaun et al., 2004). DHX29 also bound stably and stoichiometrically to 40S/eIF3 complexes formed with (CUUU)9 RNA (Kolupaeva et al., 2005), to 43S complexes (Figure 3A, lanes 8 and 9), and to yeast 40S subunits, indicating that it associated with a conserved region of 40S subunits (Figure 3B). DHX29's ribosomal binding was nucleotide independent (Figure 3C).

Some DHX29 preparations contained an ~90–95 kDa band (Figure 3D, left panel) that we identified as truncated DHX29 (Table S1B). One of its tryptic peptides corresponded to amino acids 98–106, indicating that ΔDHX29 cannot lack more than 96 N-terminal amino acids and must thus be significantly truncated at its C terminus. If the lower band on the western blot of 40S/DHX29 ribosomal complexes obtained with such preparations of DHX29 (Figure 3D, right panel) corresponds to C-terminally truncated ΔDHX29, the region of DHX29 responsible for ribosomal binding is likely located in the N-terminal two thirds of the protein. Consistently, DHX29 in RRL was bound to 40S-containing ribosomal complexes, but not to 60S subunits or 80S ribosomes (Figure 3E). About 10% of 40S-ribosomal complexes were associated with DHX29, and all of the DHX29 was involved in this interaction (Figure 3F).

To obtain insights into the ribosomal position of DHX29, we compared chemical/enzymatic footprinting of 18S rRNA in 43S and 43S/DHX29 complexes. DHX29 strongly protected CUCG27–625 and UUUG30–552 in h16 from RNase V1 cleavage and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho- p-toluene sulfate (CMCT) modification, respectively (Figure 4A, lanes 7 and 8; Figure 4B, lanes 3 and 4; Figure 4C) and weakly protected A526 from dimethyl sulfate (DMS) modification (Figure 4B, lanes 7 and 8), but did not protect G934 on the opposite strand of the stem from RNase T1 cleavage (Figure 4A, lanes 1 and 2). If the observed protections resulted from direct interaction between h16 and DHX29, rather than from induced conformational changes, then DHX29 likely binds to the 40S subunit near the mRNA entrance (Figure 4D).

**Stimulation of 48S Complex Formation by DHX29 Requires Its NTPase Activity**

Like other DExH-box proteins (Lee et al., 1997; Tanaka and Schwer, 2005), DHX29 lacked nucleotide specificity and hydrolyzed ATP, GTP, CTP, and UTP (Figure 5A). These proteins all lack the Q motif upstream of the helicase domain that has been implicated in determining the specificity of adenine recognition by the related DEAD-box helicases (Tanner et al., 2003). DHX29's NTPase activity was strongly stimulated by 43S complexes, whereas stimulation by single-stranded (CUUU)9 RNA was low (Figures 5A and 5B; we note that the concentration of DHX29 in experiments shown in Figure 5A was substantially higher than in Figure 5B). 18S rRNA had higher stimulatory activity than (CUUU)9 RNA but lower than 43S complexes (Figure 5B). If the ribosome binding site for DHX29 is formed by 18S rRNA, and is to some extent preserved in naked 18S rRNA, specific binding of DHX29 to 18S rRNA could account for its relatively high stimulatory activity. However, stimulation was greatest in the presence of 43S complexes and (CUUU)9 RNA, and it is tempting to speculate that this combination simulates mRNA-attached or scanning 43S complexes. To verify whether NTP hydrolysis by DHX29 is required for stimulation of 48S complex formation, we investigated eIF4A/4B/4F-independent 48S complex assembly on CAA-GUS Stem-1 mRNA in the presence of DHX29 and different NTPs (Figure 5C). 43S complexes formed with eIF2/3/1A were separated from unincorporated GTP by SDG centrifugation and incubated with DHX29 and mRNA in the presence/absence of GTP, ATP, CTP, UTP, GMPPNP, or AMPPNP. DHX29's stimulatory activity was higher with GTP or ATP than with CTP or UTP (Figure 5C, lanes 4–7). No stimulation occurred without nucleotides or with nonhydrolyzable GMPPNP or AMPPNP (Figure 5C, lanes 3, 8, and 9). NTP hydrolysis by DHX29 was therefore required for its activity in 48S complex formation.

**DHX29 Does Not Possess a Processive Helicase Activity**

To investigate the potential helicase activity of DHX29, we used RNA duplexes comprising overhanging 25 nt long 5′ or 3′ ends and 13 nt or 10 nt long double-stranded regions (ΔG = −21 and −14.6 kcal/mol, respectively), corresponding blunt duplexes, as well as duplexes resembling stems 2, 3, and 4 of
CAA-GUS Stem2-4 mRNAs. DHX29 could not unwind 13 nt long duplexes with overhanging 5' or 3' ends in the presence of any NTP, whereas unwinding by eIF4A/4F was efficient (Figure 6A, left panel; data not shown). Very weak unwinding by isolated 43S/DHX29 complexes (Figure 6A, right panel, lane 2) may in fact be attributable to ribosomal scanning after attachment of 43S complexes to the 5' overhang. Unwinding by DHX29 of 10 nt long duplexes with overhanging ends was marginal (<5%), and blunt duplexes were not unwound (Figure 6B, lanes 2 and 3; data not shown). DHX29 could unwind Stem-2 duplex (Figure 6C, lane 3), although we note that this duplex was intrinsically unstable under experimental conditions, resulting in a noticeable background of RNA monomers (Figure 6C, lane 2). Unwinding of Stem-3 duplex by DHX29 was marginal.

Figure 3. Interaction of DHX29 with 40S Subunits
(A–C) Association of DHX29 with (A) individual 40S and 60S subunits, 80S ribosomes, 40S/eIF3/(CUUU)9 complexes, and 43S complexes containing 40S subunits and eIFs 2/3/1/1A, (B) yeast 40S subunits, and (C) 40S/eIF3/(CUUU)9 complexes in the presence/absence of nucleotides as indicated (lanes 4–7). (D) DHX29 preparation containing a C-terminally truncated fragment resolved by SDS-PAGE (left panel) and its association with 40S subunits (right panel). Ribosomal peak fractions obtained by SDG centrifugation were analyzed by SDS-PAGE and fluorescent SYPRO staining (A–C) and/or western blotting using DHX29 antibodies (A and D).

(E) Association of DHX29 with ribosomal complexes in RRL in the presence of GMPPNP assayed by SDG centrifugation. In addition to optical density, the ribosomal profile of RRL was analyzed by scintillation counting to monitor [35S]Met-tRNAi incorporation. Gradient fractions were analyzed by western blotting using DHX29 antibodies.

(F) Estimation of the proportion of 40S-bound DHX29 relative to free protein (upper panel) and of the ratio of DHX29-bound versus unbound 40S-ribosomal complexes assayed by western blotting using DHX29 antibodies (lower panel).
Stem-4 duplex was not unwound (Figure 6C, lane 9), whereas eIFs 4A/4F unwound 5%-10% of Stem-4 duplex (Figure 6C, lane 10). Thus, like some other DExH-box proteins (Tanaka and Schwer, 2005), DHX29 is not a processive RNA helicase.

DHX29 Can Participate in Multiple Rounds of 48S Complex Formation

DHX29 stimulated 48S complex formation most strongly when it was present in substoichiometric amounts relative to 43S complexes. Thus, SDG-purified 43S/DHX29 complexes with a 43S:DHX29 ratio of 10:1 (Figure 6D, lane 3) were most active in 48S complex assembly on CAA-GUS mRNA (Figure 6E, lane 2), whereas complexes with 43S:DHX29 ratios of 2:1 and 1:1 (Figure 6D, lanes 4 and 5) were progressively less active (Figure 6E, lanes 3 and 4). Importantly, a mixture of DHX29-free and DHX29-saturated 43S complexes that individually had the lowest activities (Figure 6E, lanes 4 and 5) together promoted very efficient 48S complex formation (Figure 6E, lane 6). These results suggest that a proportion of DHX29 might be inactive, but that DHX29 from active 43S/DHX29 complexes could dissociate from ribosomal complexes and participate in new rounds of initiation. Alternatively, stimulation of 48S complex formation by DHX29 might require its dissociation from the 40S subunit at some point in the process before the 48S complex is formed, in which case the excess of free 43S complexes would ensure rebinding of dissociated DHX29 to a new 43S complex. To investigate this possibility, DHX29-saturated 43S complexes were mixed with purified 40S/eIF3/(CUUU)_9 complexes, which themselves could not participate in 48S complex formation but could potentially provide a “trap” for dissociated DHX29, thereby stimulating 48S complex formation by 43S/DHX29 complexes. 40S/eIF3/(CUUU)_9 complexes did not stimulate 48S complex formation by 43S/DHX29 complexes (Figure 6F, compare lanes 3 and 5). Although this could be because DHX29’s affinity to 40S/eIF3/(CUUU)_9 complexes is lower than to 43S complexes, the possibility that a proportion of DHX29 might be inactive cannot be excluded. The stage at which DHX29 dissociates from ribosomal complexes is not known, but we note that consistently less DHX29 was bound to 48S than to 43S complexes (Figure 6G).

The Influence of DHX29 on 48S Complex Formation during IRES-Mediated Initiation

The genomes of several families of RNA viruses contain internal ribosomal entry sites (IRESs), which mediate end-independent initiation, enabling viral mRNAs to bypass the canonical cap-dependent mechanism. IRESs are classified into structurally unrelated groups that mediate initiation by distinct mechanisms that require fewer eIFs than canonical initiation. Three mechanisms of IRES-mediated initiation have been identified, and they are all based on specific noncanonical interactions of IRESs with canonical components of the translation apparatus. The first is exemplified by type 2 picornavirus IRESs (e.g., encephalomyocarditis virus; EMCV), which promote initiation at their 3’ border by a mechanism that relies on specific interaction of the IRESs’ J-K domain upstream of the initiation codon with eIF4G.
and involves direct attachment of the 43S complex to the initiation codon, which is likely mediated by interaction of 43S-bound eIF3 with IRES-bound eIF4G (Pestova et al., 1996). Initiation on the IRESs of hepatitis C virus (HCV) and classical swine fever virus (CSFV) is determined by their ability to bind directly and independently to 40S subunits and eIF3 (Pestova et al., 1998b). These interactions enable 43S complexes to attach directly to the initiation codon of HCV-like IRESs without scanning or local unwinding of mRNA. Initiation on the intracistronic region (IGR) IRESs of dicistroviruses (e.g., cricket paralysis virus; CrPV) is also determined by their ability to bind directly to 40S subunits, but unlike HCV-like IRESs, does not use eIFs or initiator tRNA: the ribosomal P site is occupied by a domain of the IRES, which mimics the codon-anticodon interaction (Wilson et al., 2000).

Figure 5. Stimulation of 48S Complex Formation by DHX29 Requires Its NTPase Activity

(A) Thin-layer chromatography analysis of DHX29’s NTPase activity in the presence/absence of SDG-purified 43S complexes comprising 40S subunits and eIF2/3/1/1A. Ten microliter reaction mixtures containing 1 pmol DHX29, 1 pmol 43S complexes, and 6.7 μM [α-32P]ATP, [α-32P]GTP, [α-32P]UTP, or [α-32P]CTP, as indicated, were incubated at 37°C for 40 min. The positions of [32P]NDPs are indicated.

(B) Time courses of ATP hydrolysis by DHX29 in the presence/absence of (CUUU)_9 RNA, 18S rRNA, 43S complexes, or 43S/(CUUU)_9, assayed by thin-layer chromatography (lower panels). The upper panel shows direct quantification of results shown in the lower panels. Ten microliter reaction mixtures containing 0.3 pmol DHX29, 6.7 μM [γ-32P]ATP, and 20 pmol (CUUU)_9 RNA, 0.3 pmol 18S rRNA, 0.3 pmol 43S complexes, or 0.3 pmol 43S complexes with 20 pmol (CUUU)_9 RNA, as indicated, were incubated at 37°C. Aliquots were removed after 2–30 min. This experiment is representative of the data collected with this assay.

(C) Toeprinting analysis of 48S complexes assembled on CAA-GUS Stem-1 mRNA in the presence of SDG-purified 43S complexes, DHX29, and NTPs or nonhydrolyzable NTP analogs, as indicated. The position of 48S complexes is indicated.
the CSFV IRES to 40S subunits also yields two sets of toeprints: at UUU387–389, corresponding to the leading edge of the 40S subunit +15–17 nt from the P site AUG codon, and at C334, corresponding to a contact of the 40S subunit with the pseudoknot of the IRES (Pestova et al., 1998b). As with the CrPV IRES, DHX29 strongly reduced the toeprints at UUU387–389 in 40S/CSFV IRES complexes irrespective of when it was added (Figure 7D, lanes 2–4). Interestingly, for both IRESs, DHX29 had less effect on toeprints corresponding to 40S/IRES contacts outside the mRNA-binding cleft (AA6161–6162 and C334 toeprints) than on toeprints at the leading edge of the bound 40S subunit (Figures 7A, 7B, and 7D). Moreover, when assayed by SDG centrifugation, 40S/IRES complex formation in the presence of DHX29 was reduced by only ~30% for both IRESs (data not

Figure 6. Helicase Activity of DHX29
(A–C) Nondenaturing PAGE showing unwinding of (A) 13 bp and (B) 10 bp RNA duplexes with 25 nt long single-stranded overhanging 5′ regions, and (C) RNA duplexes resembling Stems 2–4 by DHX29, 43S complexes, 43S/DHX29 complexes, and eIF4A/eIF4F, as indicated. Duplex (1 nM) was incubated with 0.15 μM DHX29, 50 nM 43S complexes, 50 nM 43S/DHX29 complexes, or 0.15 μM eIF4A/eIF4F and 0.2 mM NTPs, as indicated, at 37°C for 40 min. Mobilities of duplex and single-stranded RNAs are indicated schematically on the left. The control for denatured strands is represented by 95°C.

(D) SDG-purified 43S complexes containing different amounts of DHX29 and analyzed by SDS-PAGE and fluorescent SYPRO staining.

(E and F) Toeprinting analysis of 48S complex formation on CAA-GUS Stem-1 mRNA in the presence of SDG-purified free 43S complexes and 43S complexes containing different amounts of DHX29 (shown in [D]). The positions of the initiation codon and assembled 48S complexes are indicated. Lanes C/T/A/G depict corresponding DNA sequences.

(G) Association of DHX29 (and eIF2, as a loading control) with 43S complexes and 48S complexes assembled on native globin mRNA assayed by SDG centrifugation and western blotting.
shown). Thus, binding of DHX29 to 40S subunits strongly affects fixation of the IRES in the area of the mRNA-binding cleft, but has a weaker effect on the overall affinity of IRESs to 40S subunits.

Importantly, even upon delayed addition, DHX29 abrogated toeprints corresponding to 48S complexes assembled on the CSFV IRES in the presence of eIFs as indicated. Initiation codons and positions of assembled ribosomal complexes are indicated. Lanes C/T/A/G depict corresponding DNA sequences.

Figure 7. Influence of DHX29 on 48S Complex Formation on Viral IRESs
Toeprinting analysis of 40S/IRES binary and 48S complexes assembled on (A and B) CrPV, (C) SPV9, (D) wild-type and ΔDomain II CSFV, and (E) EMCV IRESs in the presence of eIFs as indicated. Initiation codons and positions of assembled ribosomal complexes are indicated. Lanes C/T/A/G depict corresponding DNA sequences.

(Pestova et al., 2008). Deletion of IRES domain II, which is responsible for conformational changes induced in 40S subunits by IRES binding (Spahn et al., 2001), eliminates the sensitivity of 48S complexes to dissociation by eIF1 (Pestova et al., 2008). Although deletion of domain II did not completely suppress the dissociating effect of DHX29, 48S complexes assembled on the IRES lacking domain II were nevertheless less sensitive to DHX29 than complexes assembled on the wild-type IRES.
48S complexes assembled on the HCV-like IRES of Simian picornavirus type 9 (SPV9), which are much more resistant to dissociation by elf1 (de Breyn et al., 2008), were also resistant to dissociation by DHX29 (Figure 7C). It is likely relevant that the predicted structure of the SPV9 IRES domain II differs significantly from that of HCV and CSFV IRESs.

Initiation on the EMCV IRES occurs predominantly at AUG$_{834}$ and infrequently at AUG$_{826}$ (Kaminski et al., 1990). Although DHX29 did not affect the overall level of 48S complex formation on this IRES, in the presence of elf1, it significantly increased the proportion of 48S complexes formed on AUG$_{826}$ even upon its delayed addition (Figure 7E, lanes 5–7).

Such distinct effects of DHX29 on 48S complex formation on different IRESs are consistent with DHX29 causing conformational changes in 40S subunits, which can or cannot be tolerated by IRES-bound complexes. We note that elf1, which induces conformational changes in 40S subunits (Passmore et al., 2007), also has distinct effects on 48S complex formation on different IRESs (de Breyn et al., 2008; Pestova et al., 1998a, 2004, 2008).

**DISCUSSION**

We have identified the DEXH-box protein DHX29 as a factor that is required for efficient initiation on mammalian mRNAs with structured 5′UTRs, which typically encode regulatory proteins. The extent of the requirement for DHX29 correlated with the stability of the secondary structure elements in the 5′UTRs of mRNAs. Although isolated internal stems of $\Delta G = -13.1$ kcal/mol or less could be efficiently overcome by scanning complexes in the presence of only elf4A/4G/4B, efficient ribosomal movement through stems of $\Delta G > -19$ kcal/mol required DHX29. Ribosomal scanning relies on the ATP-dependent helicase activity of elf4A/4G/4B and is influenced by the conformation of scanning 43S complexes, which is modified by elf1/1A. How does DHX29 stimulate 48S complex formation on mRNAs with structured 5′UTRs? Does it participate directly in unwinding of mRNA or does it remodel 43S complexes to increase their scanning processivity? The answer to this question is linked to the mechanism by which elf4A/4G/4B assist scanning, and elucidation of this requires knowledge of their location in ribosomal complexes. If elf4A/4G/4B bind at the leading edge and unwind mRNA before it enters the 40S subunit, it is unlikely that another helicase, DHX29, would participate directly in the same process. In this case, DHX29 could enhance the processivity of ribosomal movement by remodeling ribosomal complexes to ensure correct entry into and/or fixation of mRNA in the mRNA-binding cleft. But if elf4A/4G/4B, as suggested (Siridechadilok et al., 2005), bind at the trailing edge near the E site and assist scanning by helicase-mediated “ratcheting” of mRNA through the mRNA-binding channel, then DHX29 might directly unwind mRNA before it enters the 40S subunit. Although this possibility seems unlikely because DHX29 is not a processive helicase, it cannot be strictly excluded that binding of DHX29 to ribosomal complexes might enhance its helicase activity. However, even if elf4A/4G/4B act at the trailing edge, DHX29 could still assist scanning not by direct unwinding of mRNA but by remodeling ribosomal complexes and influencing accommodation of mRNA in the mRNA-binding channel, in which case mRNA secondary structure would be unwound by the scanning 40S subunit itself. In this hypothetical situation, correct positioning of mRNA at the entrance to the mRNA-binding channel would be particularly important. We note that the bacterial ribosome has helicase activity, which involves ribosomal proteins S3, S4, and likely S5 (Takayar et al., 2005).

Suppression of the aberrant +8–9 nt toeprint, which most likely represents a 48S complex with the 3′ portion of mRNA not firmly fixed in the mRNA-binding cleft of the 40S subunit, by DHX29 even on delayed addition to preassembled 48S complexes indicates that DHX29 does induce conformational changes in these complexes that influence ribosomal accommodation of the 3′ portion of mRNA. The appearance of aberrant +8–9 nt toeprints depended on the presence of elf1/1A. Binding of elf1/1A to yeast 40S subunits causes the entry “latch” between h18 in the body and h34/rpS5 in the neck to open and establishes a new connection between rpS3 and h16 (Passmore et al., 2007). Such opening of the entry latch might weaken fixation of the 3′ portion of mRNA in the mRNA-binding cleft that could account for appearance of the +8–9 nt toeprint. It is likely that the conformation of the 40S subunit with the open latch is more conducive to attachment of 43S complexes to mRNA, whereas processive scanning might require firm fixation of mRNA in the mRNA-binding cleft. In this case, the conformation of ribosomal complexes would require further modification, which could be promoted by DHX29.

Another indication that DHX29 causes conformational changes in 40S subunits comes from its influence on ribosomal complexes assembled on viral IRESs. Thus, even on delayed addition, DHX29 affected 40S-ribosomal binding and proper fixation in the mRNA-binding cleft of CrPV and CSFV IRESs. The CrPV- and CSFV-like HCV IRESs both induce similar conformational changes in 40S subunits, which were suggested to facilitate fixation of these IRESs in the mRNA-binding cleft (Spahn et al., 2004). It is therefore likely that binding of DHX29 to 40S subunits does not allow such IRES-induced changes to occur and/or causes other conformational changes in 40S subunits that are not compatible with binding and proper positioning of the IRESs on 40S subunits. Moreover, DHX29 dissociated 48S complexes assembled on the CSFV IRES and influenced the ratio of 48S complexes assembled on AUG$_{826}$ and AUG$_{834}$ of the EMCV IRES. Although the dissociating effect of DHX29 on 48S complexes is similar to that reported for elf1 (Pestova et al., 2008), the conformational changes induced in 40S subunits by elf1 (Passmore et al., 2007) and the potential conformational changes induced by DHX29 are likely not identical because these factors have opposite effects on 40S/CrPV IRES complexes (Pestova et al., 2004; this study) and on the ratio of 48S complexes assembled on two AUGs of the EMCV IRES (Pestova et al., 1998a; this study).

Footprinting experiments revealed that in 43S complexes, DHX29 protects h16 of 18S rRNA. We cannot conclude unambiguously whether such protection is caused by direct contact of DHX29 with h16 or reflects conformational changes in the 40S subunit induced by DHX29. If DHX29 indeed binds h16 near the mRNA entrance, this position of DHX29 would be...
consistent with both hypothetical modes of action (remodeling of 43S complexes or mRNA unwinding). However, if the observed protections correspond to conformational changes in ribosomal complexes, then the region of such changes is entirely consistent with remodeling of 40S subunits near the mRNA entrance, which would likely affect accommodation of the 3′ portion of mRNA in the mRNA-binding cleft. Moreover, it is exactly the area of the 40S subunit that undergoes conformational changes upon binding of elf1 and elf1A. It is not known whether stable secondary structures in 5′UTRs only slow ribosomal scanning or also increase dropoff of 43S complexes. If dropoff can occur, then proper fixation of mRNA in the mRNA-binding cleft would also stabilize ribosomal association with mRNA and increase the processivity of scanning complexes. Moreover, the potential influence of the conformation of ribosomal complexes on the processivity of the ribosome-bound elf4A/4G/4B helicase complex could strictly also not be excluded. Although we are not yet in a position to discriminate between the remodeling and unwinding mechanisms by which DHX29 might stimulate 48S complex formation, it is worth noting that it has become apparent that many DExH/DB proteins function primarily in remodeling of RNA and RNP complexes rather than in processive unwinding of RNA duplexes (reviewed by Pyle, 2008). Thus, many DExH/D proteins have additional RNA-binding domains that contribute to strong ATP-independent RNA annealing activity, which in conjunction with their ATP-dependent unwinding activity suggest that such proteins can induce alternating conformational rearrangements in RNA and RNP complexes upon their transition between ATP-bound and ATP-free states. In addition, DExH/D proteins can also function as RNPases, displacing proteins from RNA in an ATP-dependent manner.

Interestingly, although DHX29 and elf4F/4A/4B acted synergistically in 48S complex formation on mRNAs with 5′UTRs containing stable hairpins, DHX29 alone also promoted relatively efficient 48S complex formation on mRNAs with 5′UTRs containing less stable stems and unstructured 5′-terminal regions that could promote elf4F/4A/4B-dependent attachment of 43S complexes and even mediated low-level 48S complex formation on mRNAs with 5′UTRs containing stems of high stability. DHX29 might therefore be responsible for translation of at least a subclass of mRNAs in conditions when elf4G is depleted (Ramirez-Valle et al., 2008).

In RRL, DHX29 was wholly associated with 40S-ribosomal complexes, but DHX29-bound 40S-ribosomal complexes nevertheless constitute only ~10% of all 40S-ribosomal complexes. In this respect, it is particularly important that DHX29 can participate in multiple rounds of 48S complex formation. Although it would be most logical to suggest that DHX29 remains associated with ribosomal complexes during the entire scanning process and dissociates from assembled 48S complexes as a result of conformational changes that likely occur upon establishment of codon-anticodon base-pairing, we cannot exclude that DHX29 might dissociate earlier, particularly if it functions by remodeling ribosomal complexes rather than by unwinding mRNA. Our experiments also indicate that a proportion of purified DHX29 might be inactive in stimulating 48S complex formation, even though it could still bind ribosomal complexes. Although DHX29 might have been partially inactivated during purification, phosphorylation of human DHX29 at Ser192, Ser200, Tyr811, and Tyr826 (http://www.phosphosite.org/) could also influence its activity.

The preceding discussion is based on the assumption that elf4A is the only DEAD-box RNA helicase involved in initiation. However, biochemical and genetic analyses have implicated other DEAD/DExH-box proteins in initiation, including Ded1p and the homologous mammalian proteins DDX3/PL10, Drosophila Vasa (Chuang et al., 1997; de la Cruz et al., 1997; Hartman et al., 2006; Johnstone and Lasko, 2004; Lee et al., 2008). The mechanisms by which Ded1p, DDX3, RHA, and Vasa act in the initiation process are incompletely characterized but are likely distinct. Ded1p has been characterized in the greatest detail: it is a more processive helicase than elf4A (Marsden et al., 2006), its function is not redundant with that of elf4A, and mutations in Ded1p are synthetic lethal with mutations in elf4A and deletion of TIF1 (elf4A) and cdc33 (elf4E) and deletion of TIF4631 (elf4G) or STM1/TIF3 (elf4B) (Chuang et al., 1997; de la Cruz et al., 1997). This has led to suggestions that elf4A may, as a subunit of elf4F, function in promoting recruitment of 43S complexes to the cap-proximal region of mRNA, whereas Ded1p assists ribosomal complexes during scanning, particularly on mRNAs with long 5′UTRs (e.g., Marsden et al., 2006). The molecular interactions that could couple Ded1p with scanning ribosomes are not known. It remains to be seen whether mammalian Ded1p homologs like DDX3 also function during scanning, after ribosomal loading, in which case they would likely unwind mRNA before it enters the mRNA-binding cleft, near its entrance. If this is indeed the case, it is even more likely that DHX29 functions in remodeling the 40S/mRNA elf4F complex rather than in unwinding mRNA during initiation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

See Supplemental Data.

**Purification of Initiation Factors, Ribosomal Subunits, and Aminoacylation of tRNA**

40S and 60S subunits, elfs 2/3/4F, elf1H, elf2F, and total aminoacyl-tRNA synthetases were purified from RRL or HeLa cells, recombinant elfs 1/1A/4A/4B/5/5B, pyrimidine tract-binding protein (PTB), and Escherichia coli methionyl-tRNA synthetase were expressed and purified from E. coli, and native total tRNA (Novagen) and in vitro transcribed IRNA§§ were aminoacylated as described (Pisarev et al., 2007).

**DHX29 Purification**

DHX29 was purified from RRL on the basis of activity in supporting 48S complex formation in the in vitro reconstituted system on mRNAs with structured 5′UTRs, which was monitored by toeprinting. Purification involved preparation of ribosomal salt wash, fractionation by ammonium sulfate precipitation, chromatography on DEAE cellulose and on phosphocellulose, and FPLC on MonoS, MonoQ, and hydroxyapatite columns. DHX29 was identified by mass spectrometry of tryptic peptides.

**Assembly and Analysis of Initiation Complexes**

48S complexes were assembled on capped in vitro transcribed (CAA)-GUS mRNA, its derivatives containing stems or two AUG codons, (CAA)-Stem-MVHC-STOP mRNAs, NCF2 mRNA, CDC25 mRNA, and native β-globin mRNA, CrPV, SPV9, EMVC, and CSFV IRESs, and analyzed by primer extension using avian myeloblastosis virus reverse transcriptase (AMV-RT) and 32P-labeled primers as described (Pisarev et al., 2007). To assay elongation
on (CAA)-Stem-MVHC-STOP mRNAs, 48S complexes were supplemented with eIF5, eIF5B, 60S subunits, tRNA aminoacylated with Met, Val, His, and [35S]Cys, eEF1H, and eEF2, and incubated at 37°C for 20 min. Elongation complexes were assayed by toeprinting, or by centrifugation through 10%–30% SDGs in a Beckman SW55 rotor at 53,000 rpm for 75 min with subsequent monitoring of [35S]MVHC formation.

To investigate the requirement for NTP hydrolysis by DHX29 for its activity in stimulating 48S complex formation and the ability of DHX29 to participate in multiple rounds of initiation, 48S complexes were assembled on CAA-GUS Stem-1 mRNA using SDG-purified 43S complexes.

For toeprinting analysis of 48S complexes assembled on β-globin mRNA in RRL, globin mRNA was incubated in RRL (Promega) in the presence of 2 mM GMPNP for 10 min at 30°C.

Analysis of Ribosomal Binding of DHX29
DHX29 was incubated with 40S subunits, 60S subunits, 80S ribosomes, 40S/eIF3/(CUUU)9, or 43S complexes in the presence/absence of nucleotides and subjected to centrifugation through 10%–30% SDGs. Fractions that corresponded to ribosomal complexes were analyzed by SDS-PAGE with subsequent fluorescent SYPRO staining or western blotting using DHX29 antibodies. To investigate the ribosomal association of DHX29 in RRL, RRL (Promega) was incubated with 1 mM GMPNP and subjected to SDG centrifugation. Gradient fractions were analyzed by western blotting.

Chemical and Enzymatic Footprinting
Ribosomal complexes were assembled by incubating 40S subunits, eIFs 2/3/1/1A, and Met-RNA[A]P with or without DHX29, then enzymatically digested by incubation with RNase V1 or RNase T1, or modified by incubation with CMCT or DMS. Cleavage/modification sites in 18S rRNA were identified by primer extension using AMV-RT.

NTPase Assay

Helicase Assay
Short RNA oligonucleotides (Dharmacon) were 32P labeled with T4 polynucleotide kinase and annealed with complementary long RNA oligonucleotides. RNA duplexes were purified on Superdex 75, incubated with DHX29, 43S complexes, 43S/DHX29 complexes, eIF4A/eIF4F, and NTPs, and analyzed in 16% nondenaturing gels.

SUPPLEMENTAL DATA
Supplemental Data include one table and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01374-3.

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