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REPORT

The bacterial toxin RelE induces specific mRNA cleavage in the A site of the eukaryote ribosome

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ABSTRACT

RelE/RelB is a well-characterized toxin–anti-toxin pair involved in nutritional stress responses in Bacteria and Archae. RelE lacks any eukaryote homolog, but we demonstrate here that it efficiently and specifically cleaves mRNA in the A site of the eukaryote ribosome. The cleavage mechanism is similar to that in bacteria, showing the feasibility of A-site cleavage of mRNA for regulatory purposes also in eukaryotes. RelE cleavage in the A-site codon of a stalled eukaryote ribosome is precise and easily monitored, making "RelE printing" a useful complement to toeprinting to determine the exact mRNA location on the eukaryote ribosome and to probe the occupancy of its A site.

Keywords: bacterial toxin RelE; eukaryotic ribosomes; mRNA cleavage; initiation complex assembly; toeprinting; RelE printing

INTRODUCTION

RelE/RelB is a well-characterized toxin (RelE)–anti-toxin (RelB) pair, expressed from the *relBE*-operon (Bech et al. 1985) and suggested to be part of the nutritional stress response of Bacteria and Archaea (for reviews, see Gerdes 2000; Gerdes et al. 2005). While RelE is a stable protein, RelB is rapidly hydrolyzed by the Lon protease. Accordingly, when RelE/RelB synthesis stops in the cell, RelB disappears rapidly, which liberates RelE from its complex with RelB and activates its toxic action (Christensen et al. 2001). The RelE toxin does not cleave free mRNA but targets ribosome-bound mRNA and induces hydrolytic cleavage of the A-site-exposed mRNA sequence (Pedersen et al. 2003). This reaction is stimulated not only by the A site of the 70S ribosome but also by the partial A site of the 30S subunit (Pedersen et al. 2003; Zavialov et al. 2005a,b). The exact positioning of ribosome-bound RelE has remained obscure. However, mRNA cleavage by RelE is inhibited by tRNA or protein factor binding to the A site, making it likely that RelE binds in the ribosomal A site or in an A-site-overlapping site. The size and overall shape of RelE and the A-site binding domain 4 of elongation factor G are similar, supporting the view that RelE is located to the ribosomal A site itself (Takagi et al. 2005).

When a eukaryote ribosome is stalled by a stable stem–loop in the translated mRNA, this leads to endonucleolytic cleavage of the mRNA in the "no-go decay" pathway (Doma and Parker 2006), by a mechanism reminiscent of the action of RelE. Although no RelE homolog has so far been identified in eukaryotes, this does not preclude the existence of a eukaryotic toxin/anti-toxin pair with similar action as that of RelE/RelB. The eukaryotic pair could have originated independently, and furthermore, there is little sequence conservation among RNA targeting toxins although their general structure is conserved. Interestingly, expression of bacterial RelE in yeast restricts cell growth (Kristoffersen et al. 2000) and RelE expression in mammalian cell lines can induce apoptosis (Yamamoto et al. 2002), suggesting that RelE targets the translating eukaryotic ribosome and cleaves its mRNA as in Bacteria and Archae.

Here we show that, indeed, the bacterial toxin RelE induces mRNA cleavage in the A site of both 48S and 80S eukaryotic translation initiation complexes assembled in vitro. Moreover, RelE induces cleavage of a stop codon positioned in the A site of a pretermination complex assembled from purified components (Andreev et al. 2006). The hydrolytic action of RelE requires a vacant A site and appears to require correct accommodation of the mRNA in the mRNA-binding channel of the ribosome. From these data we infer that the mechanism by which

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RelE cleaves mRNA on ribosomes is similar in all three kingdoms. Our observations emphasize that the A site is universally conserved, and highlight the possibility of mRNA cleavage on stalled ribosomes in Eukarya according to principles similar to those at work in Bacteria and Archaea.

Since, finally, RelE cleavage in the A-site codon of a stalled eukaryote ribosome is precise and easily monitored, we suggest "RelE printing" as a useful complement to toeprinting to probe the exact mRNA location on the eukaryote ribosome and the occupancy of its A site.

RESULTS AND DISCUSSION

RelE cleaves mRNA in ribosomal 48S initiation complexes in rabbit reticulocyte lysate

To check whether RelE can cleave an mRNA bound to the eukaryotic ribosome, 48S initiation complexes on capped β-globin mRNA were formed in rabbit reticulocyte lysate (RRL) and treated with the toxin. The reverse transcription (RT) reaction was then carried out on deproteinized mRNA samples. RelE treatment resulted in the appearance of two bands at positions +5 and +6 relative to the A of the AUG triplet (Fig. 1, lane 5). This "RelE print" was, in other words, localized to the partial A-site codon of the 48S complex. The toeprint assay, conducted in parallel, confirmed formation of the 48S complex (Fig. 1, lane 2).

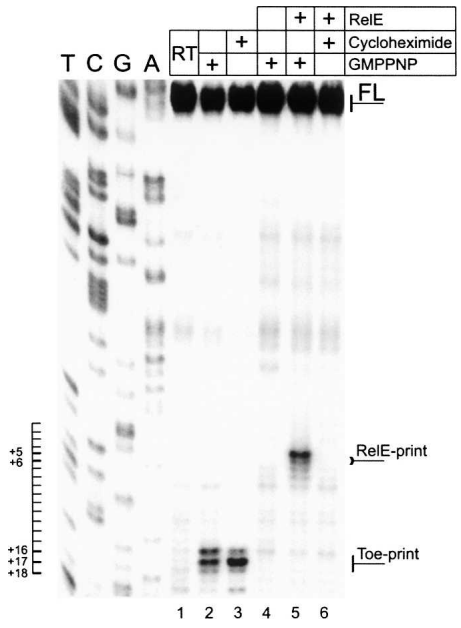

FIGURE 1. RelE induces cleavage of β-globin mRNA within 48S initiation complexes formed in RRL Positions of toeprint and RelE-print bands are shown on the *right* of the gel. A dideoxynucleotide sequence generated with the same primer was run in parallel (shown on the *left* of the gel). The full-length product of primer extension is denoted as "FL." Positions of bands with respect to the A-residue of the AUG initiation codon are shown on the *right* of the gel. Lane *1* corresponds to the negative control (Mg^{2+} was added to final concentration 30 mM prior to addition of mRNA to prevent initiation complex formation).

We also formed 80S ribosomal complexes with the same mRNA in RRL supplied with the cycloheximide, an antibiotic drug known to block the elongation step of protein synthesis (Obrig et al. 1971). After incubation, the 80S complexes were subjected to RelE action and toeprinting analysis. The latter confirmed formation of the mRNA-containing 80S complex (Fig. 1, lane 3), but there was no RelE cleavage of the mRNA (Fig. 1, lane 6). This could mean that although RelE could cleave mRNA in the 48S initiation complex, the toxin was unable cleave in the eukaryotic 80S ribosome. Alternatively, the A site of the 80S ribosome could in this experiment have been occupied by a tRNA or an elongation factor by the action of cycloheximide. Such factor binding to the A site would, in analogy with previous results obtained for the *Escherichia coli* system (Pedersen et al. 2003; Zavialov et al. 2005a,b), have inhibited the hydrolytic action of RelE. To sort out these issues, we turned to our eukaryote in vitro system for assembly of translation initiation complexes from purified components (Pestova et al. 1996, 1998a; Dmitriev et al. 2003; Andreev et al. 2006); as will be described next.

RelE-dependent cleavage of mRNA in the 80S ribosome requires a vacant A site

The requirements for RelE-induced cleavage of mRNA on the bacterial 70S ribosome have been extensively characterized with the help of an in vitro system for protein synthesis with components of high purity, and they are (1) a vacant A site and (2) an mRNA anchored in its track on the 30S subunit (Pedersen et al. 2003; Zavialov et al. 2005a,b).

To check whether RelE-induced cleavage of mRNA on the mammalian ribosome have the same requirements, we prepared 48S initiation complexes, which contained β-globin mRNA, eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, eIF4F, 40S subunits, and initiator Met-tRNA ($tRNA_i^{Met}$). The formation of the 48S complex was verified by toeprinting (Fig. 2A, lane 6). Incubation of the 48S complex with RelE resulted in a clear RelE print (Fig. 2A, lane 2) as in RRL (Fig. 1, lane 5).

When eIF2 and $tRNA_i^{Met}$ were excluded from the 48S complex preparation, there was neither a toeprint nor an RelE print (Fig. 2A, lanes 7,3, respectively), showing that lack of 48S complex formation prevented mRNA-cleavage by RelE. Substitution of ATP, essential for the helicase activity of eIF4A, by its nonhydrolysable analog AMPPNP strongly suppressed the RelE prints and toeprints (Fig. 2A, lanes 5,1, respectively).

To form the 80S initiation complex, eIF5, eIF5B, and 60S subunits were added. This resulted in toeprints and RelE prints (Fig. 2B, lanes 6,2) of about equal strength, as in the 48S complex case (Fig. 2A, lanes 6,2, respectively). Analysis

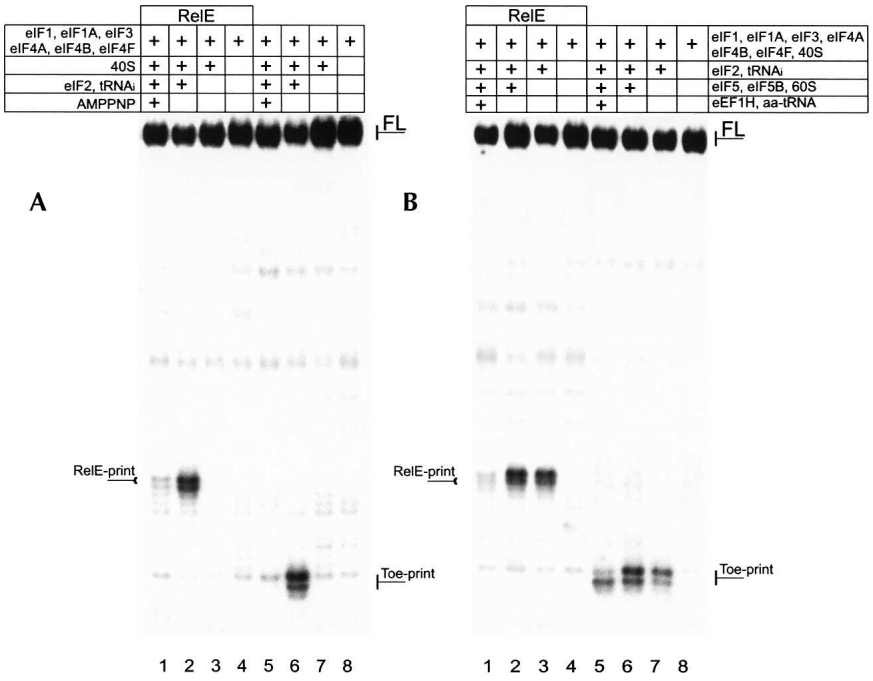

FIGURE 2. RelE induces cleavage of β-globin mRNA in the 48S (*A*) and 80S (*B*) initiation complexes generated from purified components. Positions of toeprint and RelE-print bands are indicated at the opposite sides of each gel. The full-length product of primer extension is denoted as "FL."

of this reconstitution mixture by sucrose gradient centrifugation showed incomplete (~70%–80%) conversion of 48S into 80S complexes, even at a molar excess of 60S ribosomal subunits (data not shown). Therefore, there was some contribution of 48S complexes to the patterns of toeprinting and RelE printing shown in Figure 2B. However, when aa-tRNAs and eEF1H were added to such an 80S reconstitution mixture, the subsequent RelE print became much weaker, while the toeprint remained strong (Fig. 2B, lanes 5,1). From this, we conclude that RelE *could* induce cleavage of mRNA both in the 48S and 80S initiation complexes and that the 80S ribosome reaction required a vacant A site, in analogy with mRNA cleavage by RelE on the bacterial ribosome (Pedersen et al. 2003).

RelE-induced cleavage of the HCV RNA requires P-site bound tRNA

In order to identify a minimal set of factors that allows for mRNA-induced cleavage by RelE on the eukaryote ribosome, we took advantage of the prokaryote-like hepatitis C virus (HCV) IRES-mediated and the c1 leaderless mRNA eukaryote translation systems (see Pestova et al. 1998b; Andreev et al. 2006). The HCV IRES element forms a stable initiation intermediate with the 40S ribosomal subunit (40S-HCV IRES binary complex henceforth), which in the presence of Met-tRNA$_i$, eIF2, and eIF3 generates a functional 48S initiation complex (Pestova et al. 1998b). Translation initiation directed by the HCV IRES does not involve scanning (Pestova et al. 1998b). It has therefore been suggested that the initiation codon of this mRNA occupies the partial P site already in the 40S-HCV IRES binary complex, but direct experimental evidence for this assertion has been missing. In addition, we do not know whether the HCV mRNA is already correctly positioned in the mRNA binding cleft of the 40S ribosome in such a binary complex.

As is evident from Figure 3A (lanes 4,1), RelE induced cleavage of the mRNA in the 48S complex formed on the HCV IRES but not in the binary 40S·HCV IRES initiation intermediate. Omission of eIF3 from the 48S reconstitution mixture slightly reduced the strength of the RelE print, but omission of eIF2 and tRNA$_i^{Met}$ completely eliminated the RelE-print RNA (Fig. 3A, lanes 2,3). Thus, eIF2 and tRNA$_i^{Met}$ were essential for RelE action in the case of HCV mRNA. These data, together with the well-known difference between toeprint patterns for the HCV·40S binary complex and the 48S complex (Fig. 3A; Pestova et al. 1998b), suggest that in the absence of tRNA$_i^{Met}$ in the P site, the HCV coding region does not occupy a precise position in the mRNA binding channel.

Minimal set of translational components required for RelE-cleavage in the A site of mRNA

The c1 leaderless mRNA can form a functional complex with the 80S ribosome and Met-tRNA$_i^{Met}$ in the absence of any initiation factor (Andreev et al. 2006). In line with this, the mRNA could in this case be efficiently cleaved by RelE (Fig. 3B, lane 1).

To investigate whether RelE can induce cleavage of mRNA in the A site of a pretermination 80S ribosome, such a complex was reconstructed on a leaderless c1(UAA-7) mRNA, in which the seventh triplet of the c1 ORF was a UAA stop codon (Andreev et al. 2006). For this, elongation factors eEF1A, eEF2, and mammalian bulk aminoacyl-tRNA were added to 80S c1 leaderless mRNA initiation complexes (Fig. 3C) in the presence of GTP. The toeprints show the ribosome stalled at the nucleotide triplet preceding the nonsense UAA codon (Fig. 3C, lane 2) and mRNA cleavage exactly at the UAA stop codon was induced by RelE (Fig. 3C, lane 4). When the nonhydrolysable GTP analog GMPPNP replaced GTP, neither a toeprint (at

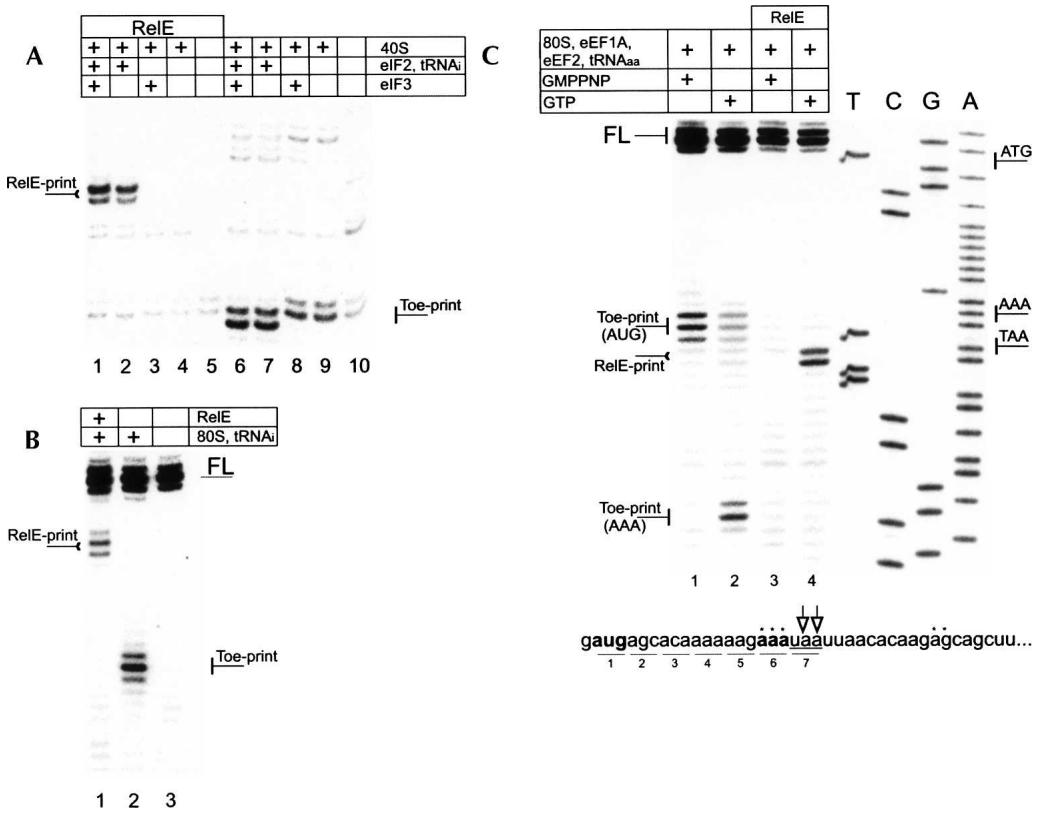

FIGURE 3. RelE printing and toeprinting of 40S·mRNA binary complex and 48S complex formed on the HCV mRNA (*A*), 80S·Met-tRNAi·c1 *lacZ* mRNA complex (*B*), and the pretermination complex formed on c1(UAA-7)*lacZ* mRNA (*C*). For *A* and *B*, the positions of the toeprint and RelE-print bands are indicated on the opposite sides of each gel. For *C*, positions of the toeprint bands originating from complexes at the AUG initiation codon and the triplet (AAA) preceding the termination codon and RelE-print bands are shown on the *left* of the gel. A dideoxynucleotide sequence generated with the same primer was run in parallel (shown on the *right* of the gel). The full-length product of primer extension is denoted FL. The sequence of the mutant cI(UAA-7) mRNA is presented below the autograph. The termination nucleotide triplet is underlined, and the initiation codon and the triplet preceding the termination signal are shown in boldface. Asterisks above the sequence show the positions of the toeprint bands. Empty arrows show the positions of the RelE-induced cleavages.

AAA) nor an RelE print was visible (Fig. 3C, lanes 1,3). Taken together, these results suggest that the minimal set of factors allowing for RelE induction of mRNA cleavage in the A site consists of (1) the 40S subunit or the 80S ribosome, (2) a peptidyl-tRNA or methionyl-tRNA$_i^{Met}$, and (3) the mRNA itself.

RelE printing as a novel approach to study the selection of initiation codons on mRNAs by eukaryotic 40S ribosomal subunits

The high precision of RelE cleavage in the A-site codon of stalled eukaryote ribosomes (Figs. 1–3) and the simplicity of the assay, suggest RelE printing as an alternative or complementary technique to probe the location of mRNA on the 40S ribosomal subunit. Moreover, RelE printing is expected to convey a distinct advantage over toeprinting in some cases due to the fact that (1) RelE printing is not affected by mRNA occlusion by the mRNA binding proteins, while toeprinting is; and (2) RelE printing is not affected by RNase H cleavage of mRNA in mammalian cell extracts, while toeprinting is.

This advantage of the RelE print was illustrated by the experiments, where both RelE-prints and toeprints were formed in the RRL or in the RRL with the addition of 20% (v/v) of S30 extracts from HeLa cells (RRL+HeLa) (Fig. 4A). These two different systems are known to differ drastically in respect to content of RNAse H and mRNA-binding proteins: RRL has low concentration of these components as opposed by extracts from cultured cells. This feature of RRL has great impact on the toeprinting efficiency (see below).

Two mRNAs, where the Fluc coding sequence was linked to the IRES-elements from either human rhinovirus (HRV) or foot-and-mouth disease virus (FMDV) RNAs, were used. The toeprinting worked poorly in RRL+HeLa (Fig. 4A, lanes 3,10), whereas the RelE printing had high quality. As expected, the RelE print was stronger for RRL+HeLa than for RRL in the case of HRV (Fig. 4A, cf. lanes 5 and 6). The opposite situation was observed for FMDV (Fig. 4A,

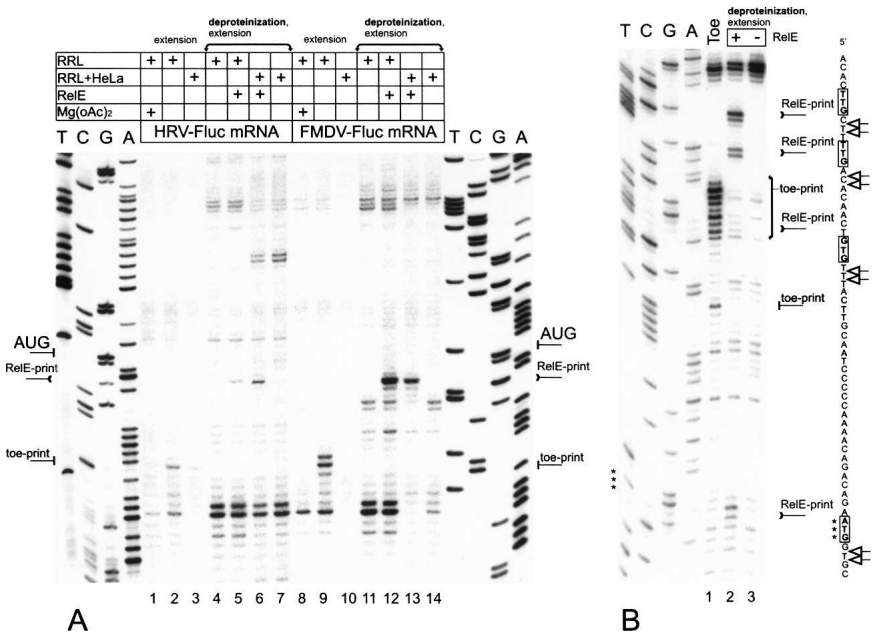

FIGURE 4. (*A*) RelE printing and toeprinting of HRV-Fluc and FMDV-Fluc RNAs in the combined RRL-HeLa extracts. cDNA ladders for these RNAs are shown on the *left* and *right* of the gel, respectively. Groups of lanes marked "extension" show the results of toeprinting, whereas those denoted as "deproteinization extension" correspond to RelE-print assays. For comparison, the assays with RRL only also presented (lanes *1*,*2*,*4*,*5*,*8*,*9*,*11*,*12*). Lanes *1* and *8* are control assays where the reconstitution was blocked by 30 mM of magnesium. (*B*) RelE-induced cleavage of the β-globin mRNA in 48S complexes formed on near-cognate codons in the absence of eIF1. The translation initiation complexes on the β-globin mRNA were assembled under the same conditions and with the same batches of components as in the experiments shown in Fig. 2 except eIF1 was not added to the reconstitution system. For higher resolution of upper bands, a long run gel was required (2 h 10 min instead of regular 1 h 10 min), and therefore, the coding part of the mRNA is not presented in the gel. The positions of toeprint and RelE-print bands as well as the sequence of the β-globin mRNA are shown on the *right* of the gel. The sequence of the β-globin mRNA is also presented on the *right* of the autograph. Lane *1* shows results of toeprinting in the absence of eIF1. Lanes *2* and *3* are the primer extensions on free mRNAs isolated from reconstitution mixtures treated (+) and untreated (−) with RelE. The position of AUG triplet and near-cognate triplets are shown in boxes. Empty arrows show the positions of RelE attack. Asterisks denote the position of the initiation codon.

lanes 12,13), in agreement with data reported by Borman et al. (1995). It is very well known that the HRV RNA, unlike the FMDV RNA, is translated inefficiently in RRL, which is very poor in auxiliary factors essential for the IRESs from RNAs of entero- and rhinoviruses (Borman et al. 1995 and references cited therein; Belsham and Jackson 2000).

Complementation of toeprints with RelE printing can be a fruitful approach in studying ribosome–mRNA interactions both in terms of mRNA accommodation in the A site and the ribosome positioning on the mRNA as such. To illustrate this, we analyzed the complex formed near the 5′ end of β-globin mRNA in the absence of initiation factor eIF1, which is essential for proper scanning selection of the initiator triplet in the 48S initiation complex (Pestova et al. 1998a; Pestova and Kolupaeva 2002; Lomakin et al. 2006; Cheung et al. 2007). In the absence of eIF1, the small ribosomal subunit stalls near the 5′ end of β-globin mRNA (~20–24 nucleotides [nt] from the 5′ end) and forms a 48S complex, referred to as "complex 1" (Pestova et al. 1998a). It has remained unclear whether the polynucleotide chain was correctly accommodated in the mRNA binding channel of the 40S subunit in this aberrant 48S complex and, furthermore, which nucleotide triplets were positioned in the P and A sites of the 40S subunit. By itself, toeprinting cannot identify the A- and P-site codons of a stalled ribosome complex, since it only probes the position of the 3′ border of ribosome-covered mRNA. We therefore complemented toeprinting with RelE printing in order to identify the P- and A-site codons of the aberrant 48S complex formed in the absence of eIF1.

A high-resolution toeprint analysis of 48S complexes assembled on the β-globin mRNA in the absence of eIF1 reveals several distinct bands near the 5′ end (Fig. 4B, lane 1), rather than the previously observed single broad band (Pestova et al. 1998a). The A-site codons were identified from the RelE cleavage experiment performed in parallel, showing 48S complexes stalled at initiator-like codon triplets, i.e., at UUG_5, UUG_{11}, and GUG_{22} (Fig. 4B, lane 2). This result shows that the complex 1 (Pestova et al. 1998a) is in fact a set of 48S complexes stalled at P-site codons which are near-cognate to the anti-codon of the initiator $tRNA_i^{Met}$. Since RelE cleavage was observed, we suggest that the mRNA was properly placed in the small subunit track.

CONCLUSIONS

We have demonstrated RelE-induced mRNA cleavage in the vacant A site of the mammalian ribosome or the partial A site of its small subunit, under conditions when the mRNA was properly positioned in its track on the 40S subunit. Our results demonstrate identical modes of action of RelE on mRNAs bound to bacterial and mammalian ribosomes, suggesting that there may exist mRNA degradation pathways via A-site mRNA cleavage in Eukarya similar to those in Bacteria. This is in line with an earlier proposal based on in silico comparative analyses of *relE*-like motifs in genomes from all tree domains of life

(Anantharaman and Aravind 2003). In conjunction with previous data from bacterial systems (Pedersen et al. 2003; Zavialov et al. 2005a,b), the present findings support the view that the structural organization of the A site of the small ribosomal subunit is very similar in bacteria and mammals.

Finally, we have demonstrated RelE printing as a feasible and precise experimental approach to monitor the position of mRNA on eukaryote ribosomes under all conditions when the A site is free. Toeprinting in combination with RelE printing can be used to monitor the mRNA location on the ribosome and to determine whether or not the A site is occupied.

MATERIALS AND METHODS

RelE expression and purification

RelE was expressed and purified according to the method of Pedersen et al. (2002) with some modifications to the purification procedure. These modifications were limited to the final refolding step, which accounted for the large losses of yield in the original protocol. In the modified procedure, pure denatured RelE was precipitated by dialysis against buffer lacking any denaturing agent (50 mM NaCl, 20 mM Tris at pH 7.5, 2 mM β-mercaptoethanol). The precipitate was harvested and resuspended in buffer containing 10 M guanidinium·HCl, 20 mM Tris (pH 7.5), 20% glycerol, and 1 mg/mL BSA. Dissolved protein was shock-dialyzed against polimix buffer (Jelenc and Kurland 1979) supplemented with 20% glycerol and stored at −20°C.

In vitro transcription

Plasmid pbG coding for β-globin mRNA was described earlier (Dmitriev et al. 2003). Prior to T7 transcription, pbG was digested with Ecl136II. Plasmid pHCV(40–372) (see Pestova et al. 1998b) digested with EcoRI was used to obtain the mRNA containing the HCV IRES-element. The HRV-Fluc RNA was produced from a PCR product prepared on the base of the corresponding dicistronic plasmid (Dmitriev et al. 2007). The construct FMDV-Luc with Fluc linked to the second AUG codon of FMDV RNA was described by Andreev et al. (2007). The c1 and c1(TAA-7) mRNAs were obtained by T7 transcription from pc1 and pc1(TAA-7) plasmids (Andreev et al. 2006) respectively, following digestion with AwrII. Both capped and uncapped mRNAs were transcribed using a RiboMax kit (Promega).

Preparation of ribosomes, initiation and elongation factors, Met-tRNAi, and total aminoacylated tRNA

Translation initiation factors, 40S, and 60S ribosomal subunits were obtained as described (Pestova et al. 1996, 1998a; Dmitriev et al. 2003). 80S ribosomes, Met-tRNAi, and total aminoacylated tRNA were obtained as described previously (Andreev et al. 2006). eEF1A and eEF2 were kind gifts from B.S. Negrutskii (Institute of Molecular Biology, Kiev) and L.P. Ovchinnikov (Institute of Protein Research, Moscow Region), respectively.

Toeprinting and RelE-printing assays

48S and 80S complex assembly on the capped β-globin mRNA in RRL (Promega) was performed as described previously (Dmitriev et al. 2003). The RelE printing and toeprinting in the mixture of 80% of RRL and 20% of S30 extract from HeLa cells (v/v) was performed in a similar way.

Reconstitution of 48S and 80S complexes on the capped β-globin mRNA from purified components (Dmitriev et al. 2003) was performed as described before with one modification; i.e., natural prokaryotic $tRNA_i^{Met}$ was used instead of T7-transcribed Met-tRNAi. The former was previously shown to be a functional substitute for the mammalian Met-tRNAi in 40S and 80S translation initiation complexes assembly (Andreev et al. 2006). 80S complex assembly on the c1 *lacZ* leaderless mRNA and subsequent reconstruction of the polypeptide elongation step from purified components were performed as described previously (Andreev et al. 2006) with a minor modification—eEF1H was replaced with eEF1A.

To perform RelE-printing in parallel to toeprinting, the volume of each reaction mix was doubled and the mix was incubated as described above, and then one aliquot was used for a RT reaction for toeprinting (Dmitriev et al. 2003). Another aliquot of the reaction mix was incubated with RelE (final concentration of 2 μM) or with the equivalent volume of buffer as a negative control for 10 min at 37°C. The RNA was purified by phenol/chloroform extraction and precipitated with ethanol, and then RT was carried out with the same primer as for toeprinting. cDNA products were analyzed on 6% sequencing gel and compared with a dideoxynucleotide sequence ladder obtained by using the same primers and corresponding plasmid DNAs.

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