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Internal translation initiation and eIF4F/ ATP-independent scanning of mRNA by eukaryotic ribosomal particles

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The recombinant mRNAs with 5'-untranslated region, called omega leader, of tobacco mosaic virus RNA are known to be well translated in eukaryotic cell-free systems, even if deprived of cap structure. Using the method of primer extension inhibition (toe-printing), the ribosomal particles were shown to initiate translation at uncapped omega leader when its 5'-end was blocked by a stable RNA-DNA double helix, thus providing evidence for internal initiation. The scanning of the leader sequence and the formation of ribosomal 48S initiation complexes at the initiation AUG codon occurred in the absence of ATP-dependent initiation factor eIF4F, as well as without ATP. The latter results implied the ability of ribosomal initiation complexes for ATP-independent, diffusional wandering (also called bi-directional movement) along the leader sequence during scanning.

The initiation of translation of most eukaryotic cellular mRNAs begins with the binding of ribosomal 43S pre-initiation complex (the ribosomal 40S subunit associated with initiator Met-tRNA_i and several proteins, which serve as initiation factors) to the cap structure at 5'-end of the messenger polyribonucleotide (reviewed in ref. 1). Following the binding, the 43S initiation complex moves along the 5'-untranslated region (5'-UTR) of the mRNA chain until it finds the initiation AUG codon in a proper context^{2,3}. The scanning of the 5'-UTR of mRNA by the ribosomal initiation complex was found to be ATP-dependent⁴. The scanning stops at the initiation AUG codon, where the ribosomal complex is fixed and becomes the 48S initiation complex.

The alternative mechanism, which is characteristic mainly of animal virus RNAs, is the binding of the host ribosomal pre-initiation complex directly to a species-specific well-structured RNA module, called "internal ribosome entry site" (IRES), within the viral RNA sequence upstream of the coding region (reviewed in ref. 5). The existence of true IRESes in cellular mRNAs is still under debates⁶⁻⁸. The RNAs of plant viruses also seem to lack typical IRESes, but often contain special sequences located either within 5'-UTR or 3'-UTR, the so-called "cap-independent translation elements" (CITEs)^{8,9}. CITE sequences were proposed to have an affinity for the initiating ribosomal particles or some of the initiation factors and thus facilitate internal initiation of viral RNA translation. Such a mechanism of cap-independent internal initiation may also be supposed for some cellular mRNAs of eukaryotes^{7,8}.

A common feature of the above-mentioned mechanisms of translation initiation is that they involve ATP and ATP-binding initiation factors, namely eIF4A and eIF4F. The cap-dependent initiation starts with the recognition of the cap structure at the 5'-end of mRNA by the eIF4E subunit of the large heterotrimeric protein eIF4F (eIF4G·eIF4E·eIF4A) associated with the initiating 43S ribosomal particle via eIF3. The binding of the 43S particle-associated eIF4F to the cap is followed by the ATP-dependent scanning of 5'-proximal section of mRNA. The eIF4A is the only ATP-binding and ATP-hydrolyzing subunit of the eIF4F; therefore, it is the eIF4A subunit of the 43S particle-bound eIF4F that supplies energy for the process of *unidirectional* motion of the ribosomal scanning particle along polyribonucleotide chain through alternating binding and hydrolysis of ATP^{10,11}. As to the IRES-directed or CITE-directed initiation of viral RNA translation, the participation of eIF4F, or eIF4G with eIF4A, accompanied by ATP hydrolysis, may be required for supporting unidirectionality of some critical shifting during the interaction of the initiating ribosomal complex with the mRNA structural module.

At the same time, experiments using eukaryotic cell-free translation systems have shown that some naturally capped 5'-UTRs can provide a fairly high level of translation initiation when they are used as uncapped leaders in mRNA constructs. Among them are the representatives of animal viral, plant viral and cellular mRNAs, such as the poly(A) leader sequence of pox virus mRNAs¹², the omega leader of TMV RNA¹³ and the 5'-UTR of mRNA encoding for obelin (a light-emitting protein of hydroid polyp *Obelia longissima*)¹⁴. When the uncapped



sequences mentioned above were used as 5'-UTRs in recombinant mRNAs, similar translation activities could be attained as with using their capped versions, but at somewhat higher mRNA concentrations of the uncapped messages. The most significant observation was that the initiation of translation at the uncapped poly(A) leader and the following recognition of the initiation codon did not require the presence of eIF4F, thus implying an ATP-independent scanning of the leader sequence¹². Earlier the eIF4F/ATP-independent scanning and selection of the initiation codon was shown for uncapped synthetic (CAA)_n leader, and it was reported to result from 5'-terminal initiation, rather than due to internal ribosomal entry¹⁵.

The ATP-independent motion of non-translating ribosomal particles along mRNA chains is known to occur in prokaryotes. Thus, bacterial ribosomes, terminated either at a nonsense mutation codon, or at a stop codon between cistrons in a polycistronic mRNA, are capable of randomly sliding along the mRNA chain and reinitiating at a downstream (or upstream) AUG triplet. The model of diffusional (*bidirectional*) scanning-like movements of non-translating bacterial ribosomal particles along the RNA chain, termed "phaseless wandering", was proposed¹⁶ and then experimentally substantiated¹⁷. As mentioned above, in some cases a similar primitive ATP-independent scanning-like process can be carried out by eukaryotic ribosomal initiating complexes as well^{12,15}. Two questions arise: (1) Can the eIF4F/ATP-independent scanning mechanism work in eukaryotic systems only in special cases of regular, presumably low-structured leader sequences, such as poly(A) and (CAA)_n, or can it also be realized at uncapped natural leader sequences with more complicated structures? (2) Does the eIF4F/ATP-independent scanning in eukaryotic systems require the 5'-end to start, or does it also begin from an inner section of 5'-UTR, thus being the case of the 5'-end-independent internal initiation?

To answer these questions, we used the omega leader because it has a number of advantages for such a study. First, it provides a high level

of translation being an enhancer of various foreign coding sequences when used as 5'-UTR of recombinant mRNA constructs both in plant and animal translation systems^{18,19}. Moreover, under optimized conditions the uncapped omega sequence was shown to support translation at the level comparable with that of the capped omega¹³. Second, it lacks evident IRES-like motifs along its nucleotide sequence^{20,21} and is in principle too short (just *ca* 70 nt in length) to organize large well-structured modules of the IRES type. Third, although the omega RNA does not form stable stem-loop structures, it cannot be considered as an unstructured sequence, as a number of physical tests provide evidence for its cooperatively melted, compact conformation²². In the present work we demonstrate that the omega leader deprived of cap structure, being either blocked at 5'-end by a stable untranslatable double helix, or remaining with free 5'-end, can provide effective internal initiation followed by eIF4F/ATP-independent scanning of the 5'-UTR. This result suggests the possibility of a diffusional, so that bi-directional, scanning mechanism for searching the initiation codon in leader sequences of eukaryotic mRNAs.

Results

Synthesis and translation of mRNA with 5'-blocked leader. As outlined above, there were several reasons for our choice of the omega sequence as UTR in mRNA constructs for translation and toe-printing experiments. The starting non-blocked mRNA consisted of 254 nt; it comprised an 84 nt long 5'-UTR, including 67 nt long omega sequence, followed by 170 nt long fragment of RNA encoding the N-terminal part of firefly luciferase. The attachment of 55 nt long oligodeoxyribonucleotide to the 5'-end of the mRNA by the ligation formed a chimeric polydeoxyribo-polyribonucleotide product of 309 nt in length. The design of the mRNA blockage at the 5'-end by the oligodeoxyribo-oligoribonucleotide is illustrated in Fig. 1a. The sequence of the blocking oligodeoxyribo-

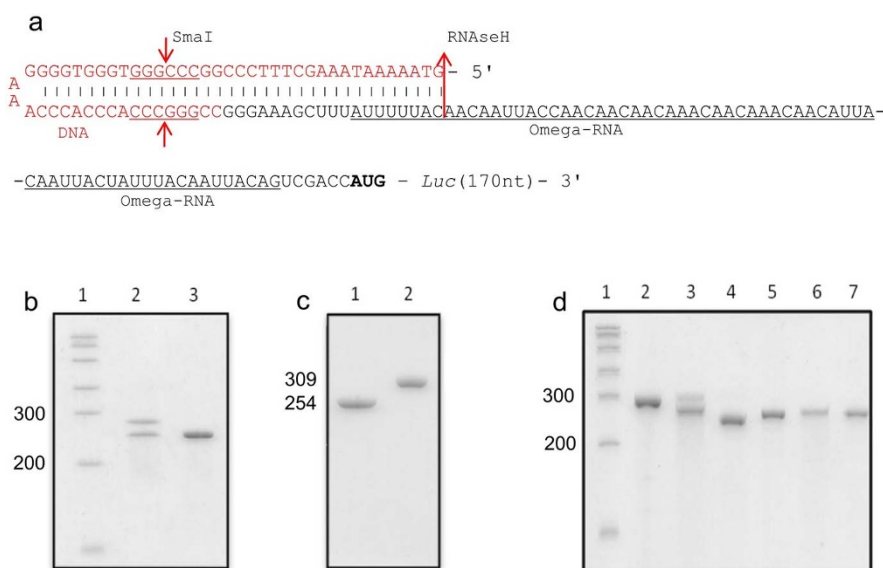


Figure 1 | Blocking of the omega leader RNA and its experimental verification. (a) Scheme of 5'-terminal blockade of the omega leader RNA with the synthetic oligodeoxyribonucleotide (see the text), which forms a stable untranslatable hairpin involving the 5'-end of the RNA. Black letters: the polyribonucleotide (RNA) comprising 67 nt long omega leader sequence (underlined); red letters: the blocking 55 nt long oligodeoxyribonucleotide containing SmaI restriction site (underlined). The arrows indicate the targets for restriction endonuclease SmaI and RNase H. The right-hand arrow at the end of the UTR sequence shows the start of the 170 nt long RNA fragment encoding for the N-terminal part of firefly luciferase. The non-blocked mRNA construct consists of 254 nt and the ligation procedure yields a mixed polydeoxyribo-polyribonucleotide product of 309 nt in length.

(b, c, d) Electrophoretic analyses of the polynucleotide preparations in denaturing 6% PAGE. (b) Analysis of the ligation reaction products: 1, standard RNA markers (100–1,000 nt); 2, the result of the ligation reaction of the RNA with the blocking oligodeoxyribonucleotide (the upper band corresponds to the blocked RNA); 3, the initial unblocked RNA. (c) Analysis of the blocked RNA after purification in PAGE: 1, unblocked RNA; 2, purified blocked RNA ligated with oligodeoxyribonucleotide. (d) Analysis of specificity of the ligation reaction product: 1, RNA markers (the same as in B); 2, the RNA blocked with the oligodeoxyribonucleotide; 3, the blocked RNA after treatment with endonuclease SmaI; 4, the blocked RNA after treatment with RNase H; 5, the unblocked RNA; 6, the unblocked RNA after treatment with endonuclease SmaI; 7, the unblocked RNA after treatment with RNase H.



oligoribonucleotide was constructed in such a way that formation of a long (35 base pairs, including 20 G-C pairings) stem-tetraloop structure at the 5'-end of mRNA, involving both ribo- and deoxyribo-nucleotides in the stable GC-rich double helix, must reliably preclude terminal initiation of translation.

Fig. 1, panels b–d, present the results of electrophoretic analyses of the polynucleotides under investigation. Panel 1b shows the electrophoretic pattern of polynucleotides in the reaction mixture after incubation with T4 DNA ligase. The bands of the original mRNA (254 nt) and the isolated product of its ligation with 55 nt long oligodeoxyribonucleotide (resulting in the 309 nt long 5'-blocked mRNA) are compared in panel 1c. The specificity of the ligation was verified by the tests illustrated in panel 1d.

Fig. 2 shows the time-courses of protein synthesis in cell-free translation systems based on wheat germ extract (WGE) (panel 2a) and rabbit reticulocyte lysate (RRL) (panel 2b). It is seen that in both systems the translation time-course of the mRNA with 5'-blocked end (5'DNA₅₅-UTR_Ω-Luc₁₇₀3') was virtually identical to that of the unmodified mRNA (5'UTR_Ω-Luc₁₇₀3'). Therefore, translation of both constructs occurred with the same efficiency, and thus no damage from the blocking procedure has been caused to the message activity. Moreover, the differences at the 5'-end of the two constructs were not reflected in the translation curve shape, including the initial stage of translation, implying the same 5'-end-independent, therefore *internal*, initiation in both cases.

It should again be emphasized that the *ca* 70 nt long omega leader sequence is too short to form an IRES element. The 5'-UTRs that contain typical IRESes are much longer, and each IRES species is to be formed as a ribosome-matched three-dimensional module with well developed secondary and tertiary structures (reviewed in ref. 5). For the omega leader, the detailed deletion studies showed that the

repeating (CAA)_n motifs in the omega sequence are somehow involved in the enhancement phenomenon²⁰. All of these considerations allow us to state that the internal initiation of translation observed in the case of uncapped omega leader is both cap- and IRES-independent.

Formation of the ribosomal 48S initiation complex at initiation AUG codon. After binding to an mRNA, either at the 5'-end (terminal initiation) or somewhere inside the 5'-UTR (internal initiation), the ribosomal 43S initiation complex (see Introduction) slides along the polynucleotide chain until it becomes fixed on the initiation AUG codon at the start of the coding region. The mRNA-fixed ribosomal complex is termed ribosomal 48S initiation complex, and the process of achieving this state is designated as scanning. Thus, investigation of the 48S complex formation at the initiation codon provides direct information about factors and energy requirements of the scanning process.

In order to explore the features of initiation in our case, we applied the technique of extension inhibition analysis of translation initiation complexes, also known as primer extension inhibition technique, or toe-printing²³. This technique was successfully adapted to studies on the role of individual translation initiation factors in ribosomal scanning and initiation codon selection¹⁵. In the present study we used a modification of the technique where 5'-ends of the primers were labeled with a fluorescent dye; the analysis of the fluorescent products was carried out by capillary electrophoresis with optical recording of the electrophoretic patterns²⁴ (see also ref. 12).

The naturally capped β-globin mRNA usually serves as a paradigmatic eukaryotic mRNA for *in vitro* studies of translation problems. The previous studies of the initiation factor requirements in the process of 5'-UTR scanning showed that the capped β-globin mRNA strictly required eIF4F for scanning¹⁵ (see also ref. 12). Fig. 3a shows the result of the control test in our experiments. The upper electrophoregram demonstrates the formation of the 48S initiation complexes on the mRNA with capped β-globin mRNA in the presence of full set of initiation factors and ATP. The characteristic “trident” on the right side of the electrophoregram indicates the position of the 48S ribosomal complex at the initiation AUG codon, and the intensity of the trident peaks reflects the quantity of the initiating ribosomal complexes that have reached the initiation codon as a result of scanning. It can be seen that omission of the ATP-dependent factor eIF4F from the incubation mixture abolished the formation of initiation 48S complex at the initiation codon (lower electrophoregram). Evidently, this result reflects the fact that eIF4F is the only ATP-binding and ATP-hydrolyzing initiation factor within the initiating ribosomal complex and thus the only one that is capable of providing the energy-dependent unidirectional scanning.

The results of the omission of eIF4F in the case of the mRNA constructs with the omega leader, either capped, or uncapped, or specially blocked, are presented in Fig. 3b–d. Fig. 3b shows the formation of the 48S initiation complexes on the mRNA with capped omega leader in the presence of full set of initiation factors and ATP (upper electrophoregram) and in the absence of the ATP-dependent factor eIF4F (lower electrophoregram). It is seen that the omission of eIF4F (lower electrophoregram) led to a significant decrease of the amount of the ribosomal initiation complexes fixed at the initiation AUG codon. Thus, in the case of the capped omega leader most of the initiating 48S complexes were formed as a result of eIF4F-dependent scanning, implying the involvement of ATP and, hence, the energy expenditure during the scanning process. At the same time, some amount of the initiating ribosomal complexes was formed at the initiation codon in the absence of eIF4F, providing evidence for some level of energy-independent (diffusional) scanning as well.

Fig. 3c demonstrates that the uncapped omega leader also ensured an efficient formation of the initiating 48S complexes at initiation AUG codons in the presence of the full set of the initiation factors

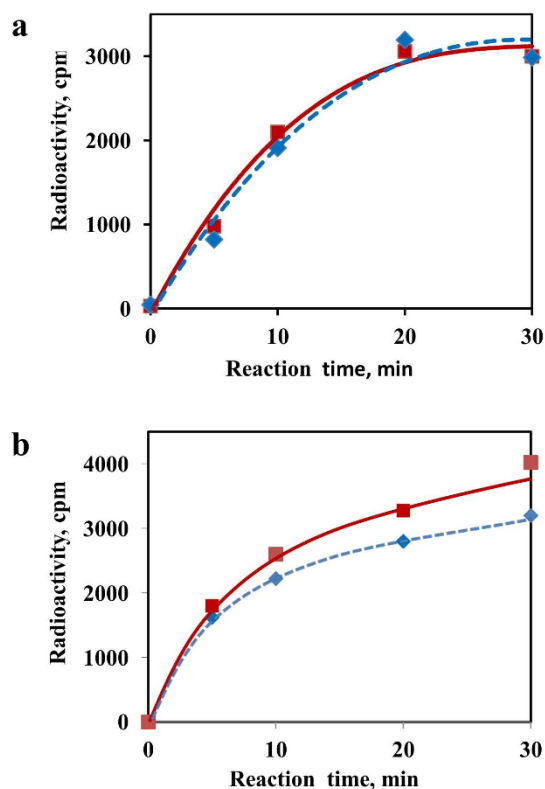


Figure 2 | Time-course curves of protein synthesis in cell-free translation systems based on wheat germ extract (WGE) (a) and rabbit reticulocyte lysate (RRL) (b). Solid red curves: translation of the unblocked mRNA (5'UTR_Ω-Luc₁₇₀3'); dashed blue curves: translation of the 5'-end blocked mRNA (5'DNA₅₅-UTR_Ω-Luc₁₇₀3').

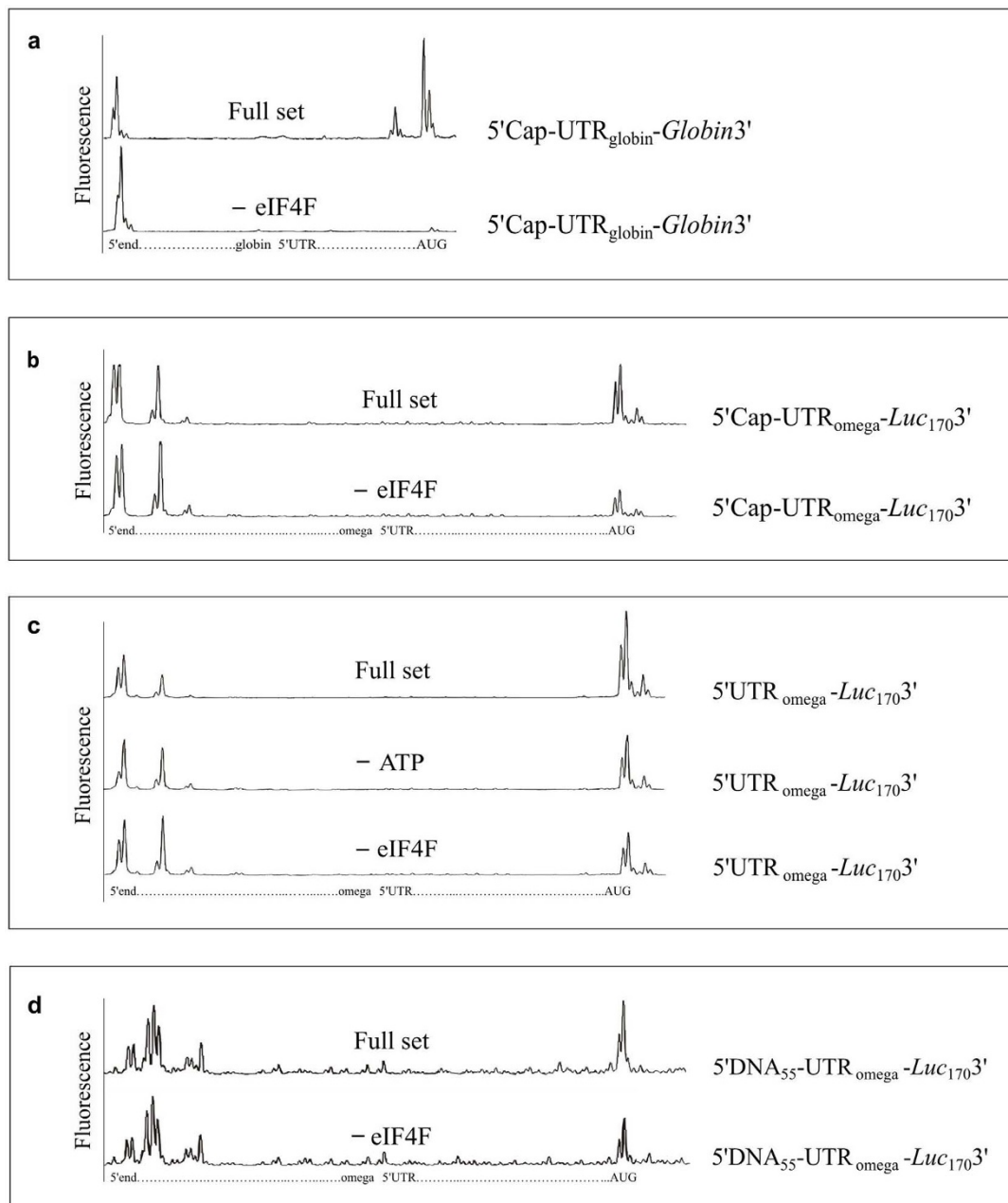


Figure 3 | Formation of ribosomal 48S initiation complexes at initiation AUG codon resulting from scanning of 5'-UTRs (coding regions of the mRNAs are not shown). In each panel (a, b, c and d) the upper electropherogram shows the result of incubation of mRNA with initiating ribosomal particles in the presence of the full set of initiation factors (eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, and eIF4F, together with initiator Met-tRNA_i and ATP), and the lower one demonstrates the result of the omission of eIF4F. The middle electropherogram in panel (c) demonstrates the result of the omission of ATP. (a) Capped β -globin mRNA as a control. (b) Omega leader with capped 5'-end. (c) Omega leader with free (uncapped) 5'-end. (d) Omega leader with blocked 5'-end. Relative fluorescence intensities of cDNA products generated by reversed transcription are plotted against nucleotides of the leader sequences of corresponding mRNAs. On each electropherogram the integral fluorescence of the left-hand major peaks reflects the amount of the full-length product when mRNA was read out by reversed transcription up to the 5'-end without stop. The integral fluorescence of the right-hand major peaks, here described as a “trident”, corresponds to the product of the reversed transcription stopped by the initiation 48S ribosomal complex formed at the initiation AUG codon.

and ATP (upper electropherogram). In this case, however, the omission of eIF4F or ATP did not result in such a significant decrease of the amount of the complexes fixed at AUG (lower electropherograms). This result supports the conclusion that the uncapped omega leader allows a sufficiently high level of productive eIF4F/ATP-independent scanning of the 5'-UTR. A certain effect of the eIF4F or ATP omission may be explained in such a way, that a fraction of pre-initiation ribosomal complexes associated with uncapped 5'-untranslated region of mRNA had acquired.

It is noteworthy that the presence of the cap-structure at the 5'-end was inhibitory for the eIF4F-independent formation of the 48S complexes at the initiation AUG codon. This unexplainable phenomenon reproduced the result of previous experiments with poly(A) leader when efficient scanning of the uncapped leader occurred in the absence of eIF4F¹², whereas the capping was found to inhibit the eIF4F-independent scanning of poly(A) sequence (unpublished).

Fig. 3d answers the question whether the free 5'-end of the uncapped omega leader is required to start the scanning process,

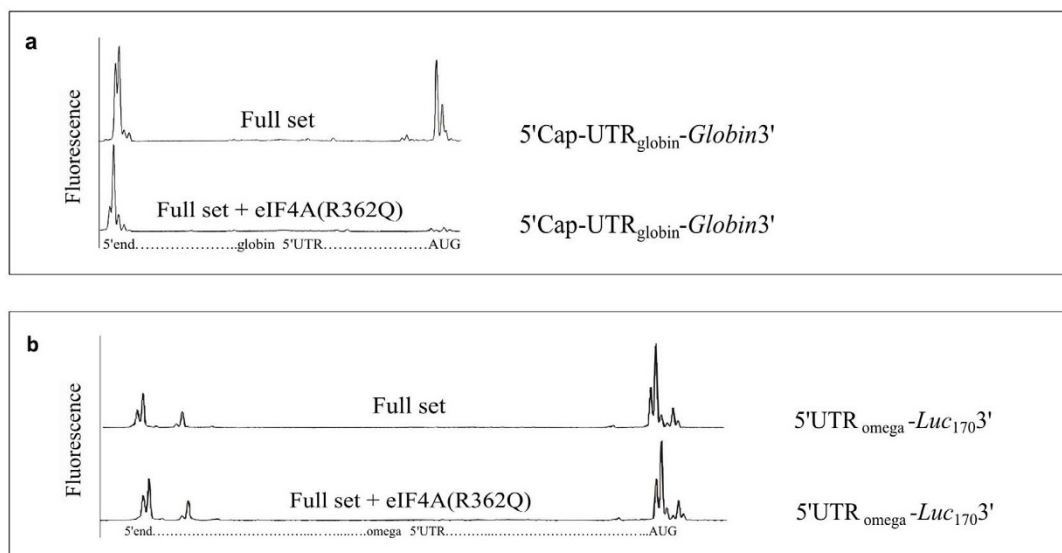


Figure 4 | Effect of the eIF4A (R362Q) mutant protein on the formation of ribosomal 48S initiation complexes at initiation AUG codon. The upper electrophoregram in each panel (a, b) shows the result of incubation of mRNA with ribosomal particles and the full set of initiation factors (for more details see the legend to Fig. 3), and the lower one demonstrates the result of the addition of the eIF4A (R362Q) mutant (0.1 mg/ml). (a) Capped β -globin mRNA as a control. (b) Omega leader with uncapped 5'-end.

or the pre-initiation 43S complexes may bind to inner parts of the omega sequence and start the scanning from there, as should be in the case of internal initiation. It is seen that the 5'-blocked omega leader does allow the 48S complex formation at initiation AUG codon both in the presence (upper electrophoregram) and in the absence of eIF4F (lower electrophoregram).

Incidentally, the presence of the additional toeprints seen near the 5'-end in the case of mRNAs with omega leader may deserve attention. In our practice similar additional toeprints did not appear with other leaders and seem to be omega-sequence-specific. These false toeprints are reproducible in the presence of various initiation factors sets and even in the absence of initiation factors; they do not depend on “functional” toe-printing, inhibitors of scanning, etc. Seemingly, this reflects some structural property of the omega leader terminus.

Effect of eIF4A(R362Q) mutant on scanning and translation of uncapped mRNA. It was reported that the (R362Q) mutation of initiation factor eIF4A results in a specific inhibitor of eukaryotic translation initiation: when added to a cell-free system the mutant

protein substitutes for eIF4A subunit of the factor eIF4F and thus inactivates of eIF4F as ATP-dependent component of the scanning ribosomal complex²⁵. Our toe-printing experiments showed that the addition of the eIF4A(R362Q) mutant to the incubation mixture completely inhibited the formation of the initiation 48S complex on the capped β -globin mRNA (Fig. 4a). At the same time, the eIF4A(R362Q) mutant did not stop the formation of the 48S initiation complexes on the mRNA with the uncapped omega leader (Fig. 4b). These experiments confirmed that the uncapped omega leader can be scanned by the ribosomal initiation complex lack of the ATP-dependent eIF4F component, i.e. by using the eIF4F/ATP-independent scanning mode.

Figure 5 shows the result of the additional experiment where the effect of the eIF4A(R362Q) mutant on translation of mRNA containing the capped β -globin leader and full-length luciferase coding sequence (Fig. 5a), in comparison with the uncapped mRNA with omega leader and the same luciferase coding sequence (Fig. 5b). It can be seen that the eIF4A(R362Q) mutant completely inhibited the translation of the mRNA with capped β -globin leader, this result

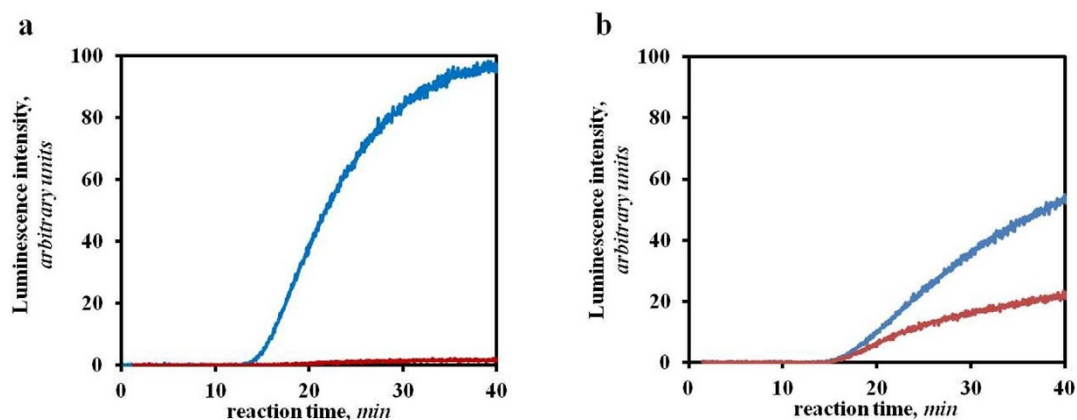


Figure 5 | Effect of the eIF4A (R362Q) mutant on the time-course curves of luciferase (Luc) synthesis in cell-free translation system based on rabbit reticulocyte lysate (RRL). (a): Translation of Luc-mRNA with capped β -globin leader. (b): Translation of Luc-mRNA with uncapped omega leader. Blue curves: control experiments (no eIF4A (R362Q) is added); red curves: translation in the presence of the eIF4A (R362Q) mutant (0.1 mg/ml). The luciferase synthesis was carried out and recorded in a luminometer cell in the presence of 0.2 mM luciferin and the luminescence was recorded online³².



being in agreement with the expectation that in this system only the eIF4F/ATP-dependent scanning could provide for the delivery of the initiation 43S complexes to the initiator codons of translated mRNA. At the same time, the translation of the uncapped omega-leader-containing luciferase mRNA in the presence of the eIF4A(R362Q) mutant, where the scanning of the leader sequence was “de-energized” and only eIF4F/ATP-independent scanning mode could be expected, was also inhibited, but partly. Earlier, the partial inhibition of translation of the mRNA with uncapped synthetic regular (CAA)_n leader in the presence of the eIF4A(R362Q) mutant protein was observed¹⁵. Evidently, in both cases the inhibition was due to inactivation of the energy-dependent eIF4F present in the translation mixture, whereas uninhibited scanning of the uncapped leader sequences by the 43S ribosomal initiation complex was performed via the low-efficient eIF4F-independent diffusional movement. It seems natural that the rate of the delivery of the ribosomal initiation complexes to the initiation codon via random wandering (diffusional movement) along a leader sequence must be slower, as compared with the unidirectional movement driven by ATP-charged eIF4F.

Discussion

One can believe that the uncapped omega leader, together with the uncapped poly(A) leader of pox viral mRNAs¹², may be the specific examples of the most primitive (either relic or viral-specific) 5'-UTRs. On the other hand, it can be found that these examples are not unique among eukaryotic mRNAs. In any case, these examples provide evidence for the existence of a primitive mechanism of eukaryotic translation initiation, namely *internal initiation* with subsequent *energy-independent scanning*, which can be realized in cell-free translation systems with uncapped mRNAs, and possibly *in vivo* in some special cases.

It is this way of initiation that can be used *in vivo* in the situations where terminating eukaryotic ribosomes have to re-initiate translation of downstream (or upstream) open reading frames (ORF) (see, e.g., refs. 26–28). This is a typical situation when a regulatory short ORF is located upstream of the main coding region of a eukaryotic mRNA (reviewed in ref. 29). At the same time the rarer cases were also mentioned when reinitiation at the nearest AUG codon occurred after termination of translation of the main coding sequence²⁹. The eIF4/ATP-independent, apparently diffusional (bidirectional) character of scanning in the cases of post-termination reinitiation at the same mRNA was reported²⁸.

In connection with the result of the present work and the recent reports cited in the previous paragraph the observations made earlier in our laboratory³⁰ should be reminded here. The studies on eukaryotic polyribosomes formed in cell-free translation systems showed that they can acquire the double-row configuration with circular topology of mRNA (see also ref. 31) and perform circular translation, when ribosomes terminate at 3'-proximal termination codon and reinitiate at 5'-proximal initiation codon of the same mRNA chain³⁰. The reinitiation was not inhibited by AMP-PNP and thus seemed to be ATP-independent. At that time it was supposed that the polysomal ribosomes reinitiate translation within the circularized polyribosomes without scanning of 5'-UTR sequence, but rather via direct jumping of terminating ribosomes to initiation site. Now, after having the results reported here, we incline to the mechanism of the ATP-independent, i.e., diffusional (bidirectional) scanning of 5'-UTR within a circularized polyribosome. Indeed, in the circular polyribosome the 5'-end of mRNA is not free, but is somehow involved in the interaction with the 3'-terminal region of the same mRNA. Thus, in such a case the circular polysomal mRNA may be considered as if blocked at the 5'-end by its own 3'-terminal region. Therefore, the situation with the post-termination reinitiation in circularized polyribosomes³⁰ may be similar to that described here for the 5'-end-independent (internal) initiation with subsequent

eIF4F/ATP-independent (diffusional, or bidirectional) scanning of the 5'-UTR.

Methods

Plasmid. Plasmid pTZ10ΩLuc containing firefly luciferase coding region with omega leader sequence under control of the T7 promoter was constructed in our laboratory³². The plasmid was cleaved by restriction endonuclease Bsp119I (Fermentas) in the region of the luciferase gene, 167 nt downstream of the AUG start codon.

In vitro transcription. Transcription was performed as described earlier¹³, with some modifications. The reaction mixture contained 80 mM Tris-OAc (pH 7.5), 10 mM KCl, 22.2 mM Mg(OAc)₂, 20 mM DTT, 2 mM spermidine, 0.01% Triton X-100 (vol/vol), 0.2 mM EDTA, 4 mM ribonucleoside triphosphates (ATP, GTP, CTP, and UTP each), 40 mM guanosine monophosphate (GMP), 80 mg/ml polyethylene glycol 8000, 0.05 mg/ml DNA template, 0.8 units/μl RNase inhibitor RiboLock (Fermentas) and 12 units/ml T7 RNA polymerase (Fermentas). The excess GMP was added to provide the presence of the monophosphate group at the 5'-end of the transcript, as required for the subsequent ligation reaction.

Ligation and purification of modified RNA. The mixture for the ligation reaction contained 40 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP, 50 μg of the RNA transcript, 10 μg of the oligodeoxyribonucleotide and 50 units of T4 DNA ligase (Fermentas) in 50 μl total volume. The reaction mixture was incubated for 20 h at 16°C, then extracted by phenol (pH 5.5), and the nucleic acids were precipitated with ethanol. The precipitated nucleic acids were collected by low-speed centrifugation; the pellet was dissolved in 90% formamide with TBE buffer and heated for 1 min at 90°C. To purify the modified RNA from non-ligated products, the mixture was run on preparative electrophoresis in denaturing 6% PAAG containing 7 M urea. The gel was stained by toluidine blue and the upper band was excised from the gel. The crushed pieces of the gel were transferred into a mini tube, then supplemented with equal volume of the solution containing 40 mM Tris-OAc (pH 7.5), 4 M NH₄-OAc, 2 mM EDTA and double volume of phenol (pH 8.0). The mini tube was shaken intensively overnight at 4°C and centrifuged. The upper aqueous phase was collected and the RNA was precipitated with ethanol.

Cell-free translation systems. The cell-free translation system derived from wheat germ extract (WGE) was described previously¹³ and used here with minor modifications. The translation mixture based on WGE contained 20 mM HEPES-KOH (pH 7.6), 5.33 mM Mg(OAc)₂, 40 mM K-OAc, 2 mM DTT, 0.5 mM spermidine, 5 mM ATP, 0.2 mM GTP, 16 mM phosphocreatine, 0.1 μg/μl phosphocreatine kinase (Boehringer-Mannheim), 1 unit/μl RNase inhibitor RiboLock (Fermentas), 37 μg/μl total tRNA (Novagen), 0.1 mM amino acids (Sigma) each with exception Phe and Leu, 0.1 mM [¹⁴C]Phe, 487 mCi/mmol (PerkinElmer), 0.1 mM [¹⁴C]Leu, 342 mCi/mmol (PerkinElmer), 250 pmoles/ml mRNA, 30% WGE. The translation reaction was carried out at 25°C.

The cell-free translation in rabbit reticulocyte lysate (RRL) was performed using Flexi® Rabbit Reticulocyte Lysate System (Promega), according to the protocol recommended by the company. The reaction mixture was supplemented with 0.01 mM [³⁵S]Met, 800 Ci/mmol (PerkinElmer).

In situ monitoring of luciferase synthesis. The detailed method was described previously³². A microcentrifuge tube with the sample containing 0.2 mM luciferin was put to the temperature-controlled cell of a luminometer, and the intensity of light emission was measured continuously by collecting the streaming data with the computer as a kinetic curve. The reaction was carried out at 30°C.

Toe-printing assay. Formation of the ribosomal initiation complexes, the primer extension inhibition assay (toe-printing) and the analysis of the products of the primer extension reaction were performed as described in ref. 12. The assembly of the ribosomal 48S initiation complexes was done from individual purified components of the translation apparatus, namely 40S ribosomal subunits, mRNA, Met-tRNA_i, initiation factors eIF1, eIF2, eIF3, eIF4A, eIF4B and eIF4F, as well as ATP and GMP-PNP (the latter was added in order to block translation after reaching the initiation codon); the mixture was incubated at 37°C during 15 min. The primer extension reaction was performed using DNA primer with fluorescent label. The cDNAs formed in the primer extension reaction were analyzed by capillary gel electrophoresis. The collected data were processed with GeneMarker 1.5 software (SoftGenetics). Fluorescence intensities corresponding to each cDNA peak were measured to determine the amount of reverse transcription products.

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Author contributions

A.S.S. and S.Ch.A. designed the experiments, analyzed data and wrote the manuscript; P.A.S., D.Kh.F. and E.A.S. performed the experiments.

Additional information

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