

Conversion of 48S translation preinitiation complexes into 80S initiation complexes as revealed by toeprinting

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Abstract A method of analysis of translation initiation complexes by toeprinting has recently acquired a wide application to investigate molecular mechanisms of translation initiation in eukaryotes. So far, this very fruitful approach was used when researchers did not aim to discriminate between patterns of toeprints for 48S and 80S translation initiation complexes. Here, using cap-dependent and internal ribosomal entry site (IRES)-dependent mRNAs, we show that the toeprint patterns for 48S and 80S complexes are distinct whether the complexes are assembled in rabbit reticulocyte lysate or from fully purified individual components. This observation allowed us to demonstrate for the first time a delay in the conversion of the 48S complex into the 80S complex for β -globin and encephalomyocarditis virus (EMCV) RNAs, and to assess the potential of some 80S antibiotics to block polypeptide elongation. Besides, additional selection of the authentic initiation codon among three consecutive AUGs that follow the EMCV IRES was revealed at steps subsequent to the location of the initiation codon by the 40S ribosomal subunit.

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1. Introduction

Two major steps are known to be involved in the translation initiation in eukaryotes. At the first step, an mRNA assisted by initiation factors eIF4F, eIF4A, eIF4B and probably eIF4H binds the 40S ribosomal subunit carrying ternary complex Met-tRNA^{Met}·eIF2·GTP and factors eIF1A, eIF1, eIF3, and eIF5. As a result, the intermediate, termed 48S complex, is formed (see [1]). At the second step, the 60S subunit binds the 48S complex with participation of factors eIF5 and eIF5B [2] resulting in the formation of an 80S initiation complex. The formation of these complexes may be monitored by two different approaches: sucrose gradient sedimentation and toeprinting. Unlike sucrose gradient sedimentation, the toeprinting technique [3,4] not only reveals a complex of the ribosome with the mRNA but also directly identifies the position of the 40S ribosomal subunit on the mRNA chain. The

technique is based on the primer extension inhibition of reverse transcription from an oligodeoxynucleotide, which is hybridized 3' to the initiation codon of an mRNA. The arrest of reverse transcriptase always occurs at the same positions, +16 to +18 nt 3' of the A in the AUG initiation codon. However, the arrest is only observed when the aminoacylated tRNA forms a codon–anticodon interaction with the mRNA in the P-site of the 40S ribosomal subunit or 80S ribosome [3–5].

Previous reports where the toeprint assay was employed were concentrated mostly on 48S complex formation [5–9]. In those reports where the formation of 80S initiation complexes was analyzed [4,10–12] the authors either did not aim to compare toeprints for 48S and 80S complexes or the low resolution of the gel did not allow such a comparison. As a result, no difference in the corresponding patterns was noted in these publications.

Here, we demonstrate that the 48S and 80S translation initiation complexes have different toeprint patterns. Based on this observation, we analyzed the efficiency of the formation of 48S complexes and their conversion into 80S initiation complexes for three different mRNAs harboring initiation codons in different nucleotide contexts.

2. Materials and methods

2.1. Plasmid constructs

β -Globin cDNA was obtained by reverse transcription-PCR of total poly(A)⁺ mRNA isolated from rabbit reticulocytes using primers 5'-ACACTTGCTTTTGACACAAC-3' and 5'-TTACGAGCTCAAGGGGCTTCATG-3'. The T7 promoter was then added by PCR to the 5'-end of the β -globin sequence and the PCR product was inserted into pUC18 at the *Kpn*I–*Ecl*136II sites resulting in plasmid pbG. Plasmid pTE17 that results in the transcript containing the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) was described previously [13,14]. The cDNA of Hsp70 was a kind gift of R.I. Morimoto. With the help of PCR and proper primers, a construct, pHSP70, was obtained where the 5' untranslated region (UTR) of Hsp70 mRNA was preceded by a T7 promoter and the complete coding sequence was flanked at the 3'-end by a *Hind*III site. Plasmid pHSP70bG represented a fusion of the fragment comprising the 5' UTR and the first 50 nt of the coding sequence of Hsp70 cDNA followed by the complete β -globin coding sequence derived from pbG.

The presence of the β -globin coding sequence downstream from the 5'-terminal fragment of Hsp70 mRNA allowed us to use the toeprint primers (see below) that were tested in the laboratory and showed excellent annealing to β -globin mRNA without raising the temperature above 30°C.

2.2. In vitro transcription

Plasmids bG and pHSP70bG were linearized prior to transcription

Abbreviations: RRL, rabbit reticulocyte lysate; EMCV, encephalomyocarditis virus; IRES, internal ribosomal entry site; eIF, eukaryotic initiation factor; GMPPNP, guanylimidodiphosphate

by digestion at the *EcoRI* site located in the 3'-terminal part of β -globin coding sequence which is present in both constructs. The transcription was carried out using the methods for the production of capped RNA described previously [15,16]. The EMCV IRES-containing transcript (nt 377–1155 of the EMCV RNA) was prepared as described in [13].

2.3. Preparation of factors, ribosomal subunits, and Met-tRNA_i^{Met}

40S and 60S ribosomal subunits, Met-tRNA_i^{Met}, and native eIF2, eIF3, eIF4A, eIF4B, eIF5B were prepared as previously described [17]. eIF4F was purified as in [18]. Recombinant eIF1 and eIF1A (eIF1rec and eIF1Arec, respectively) were prepared as described in [7]. Preparation of recombinant eIF4B (eIF4Brec) was performed as suggested in [5]. Recombinant eIF5B (eIF5Brec) was expressed as its C-terminal 2/3 fragment using plasmid pET32a (Novagen) containing the eIF5B open reading frame from nt 571 to 1220. This expression vector was kindly provided by S.A. Wilson [19]. After isolation of eIF5Brec on a Ni-agarose column (Qiagen), the factor was additionally purified by FPLC (Pharmacia) using the chromatographic MonoQ column and a KCl gradient from 100 to 500 mM. Recombinant eIF5 (eIF5rec) was expressed from pET21a-eIF5 kindly provided by C. Proud. This factor was isolated from the extract of transformed *Escherichia coli* BL21(DE3) by ammonium sulfate precipitation (50–70%), followed by chromatography on a PC-11 (Whatman) column and FPLC chromatography on a MonoQ column using a 100–500 mM gradient of KCl.

2.4. Assembly and toeprinting of 48S and 80S complexes in nuclease-treated rabbit reticulocyte lysate (RRL)

To assemble the translation initiation complexes, a nuclease-treated RRL from Promega was employed. First, a master mix containing 7.14 μ l of RRL, 0.25 μ l of human placental ribonuclease inhibitor (40 U/ μ l), and 13 mM MgAc₂ was prepared. To assemble 48S and 80S initiation complexes, 1.25 μ l of 16 mM guanylimidodiphosphate (GMPPNP) and 0.36 μ l of 56 mM MgAc₂ or 0.61 μ l of water and 1 μ l of water solution of cycloheximide (Sigma, 10 mg/ml) were added to this master mix (for hygromycin B and anisomycin, the concentrations are indicated in the appropriate figure legend).

The mixtures were incubated for 5 min at 30°C, followed by addition of 0.5 μ l of mRNA (1 pmol/ μ l) and incubation for another 5 min at the same temperature. Then, 1 μ l of ³²P toeprint primer (5 pmol), 1 μ l of cycloheximide (10 mg/ml) or H₂O to 80S or 48S reconstitution mixtures, respectively, 8 μ l of RT-Mix (0.6 μ l of 320 mM MgAc₂, 2 μ l of 5 mM dNTPs, 1 μ l of 10 \times buffer for reconstitution (0.2 M Tris-HCl, pH 7.5, 1 M KCl, 25 mM MgAc₂, 10 mM dithiothreitol (DTT))), 4.1 μ l of water, and 0.3 μ l of AMV reverse transcriptase (Roche, 24 U/ μ l) per each assay were added and the samples were incubated for 15 min at 30°C. The mixtures were carefully phenol-purified, cDNA products were precipitated with ethanol overnight and finally analyzed using a 6% sequencing gel.

Primers, 5'-TCACCACCAACTTCTCCAC-3', complementary to nt 114–133, and 5'-CACATTCATTCACCTTGC-3', complementary to nt 103–120 of the rabbit β -globin mRNA sequence, were employed to produce toeprints from the β -globin and Hsp70 mRNA initiation regions, respectively. The toeprints for transcripts with the EMCV IRES were obtained with primer 5'-GTAGAGCAGAGCATTTTGGG-3' complementary to positions 890–909 in the EMCV RNA sequence. cDNA products were compared with a dideoxynucleotide sequence ladder obtained by using the same primers and corresponding plasmid DNAs. Sucrose gradient centrifugation analysis of the assembled initiation complexes was performed as previously described [5].

2.5. Reconstitution of 48S and 80S translation initiation complexes from purified translational components

48S and 80S complexes were assembled as previously described [5,20] with some modifications. Briefly, the 80S complexes were assembled by incubating 0.5 pmol of mRNA for 10 min at 30°C in a 20- μ l reaction volume that contained the reconstitution buffer (20 mM Tris-HCl, pH 7.5, 110–130 mM KAc, 2.5 mM MgAc₂, 0.1 mM EDTA, 1 mM DTT), 1 mM ATP, 0.4 mM GTP, 0.25 mM spermidine-HCl, 1.5 pmol of Met-tRNA_i^{Met}, eIF1 (0.5 μ g), eIF1A (0.5 μ g), eIF2 (2 μ g), eIF3 (3 μ g), eIF4A (0.5 μ g), eIF4B (0.5 μ g), eIF4F (0.5 μ g), 40S ribosomal subunits (0.15 A260), 60S subunits (0.3 A260), eIF5B (native – 0.2 μ g, recombinant – 1 μ g), eIF5rec (0.6 μ g). The

mixture was incubated for 10 min at 30°C. Then, the primer extension was done as described in [5] and cDNAs purified with phenol-chloroform (1:1) were analyzed using 6% polyacrylamide sequencing gels. The reconstitution of 48S complexes was carried out in a similar way, except that eIF5, eIF5B, and 60S ribosomal subunits were omitted and GMPPNP was added to the assembly mixture instead of GTP.

3. Results and discussion

3.1. Formation in RRL of 48S and 80S translation initiation complexes with cap-dependent mRNAs as revealed by toeprint assay

A necessary prerequisite to obtain good resolution of toeprint bands is the use of a toeprint primer that anneals to a region of the mRNA that is less than 100 nt 3' of the initiation triplet and the use of a 6% sequencing gel. A toeprint primer annealing further downstream or the use of a higher acrylamide concentration in the gel results in a poorer resolution of the bands. Fig. 1 shows the primer extension inhibition by the 48S and 80S complexes assembled in RRL on β -globin and Hsp70 mRNAs. The 48S complex was assembled in the presence of the non-hydrolyzable GTP analogue, GMPPNP, an inhibitor of ribosomal subunit joining. The 48S toeprint for β -globin mRNA (Fig. 1a, lane 3) is characterized by three bands of approximately similar intensity (see also [4,7,21]) at positions +16 to +18 to the A in the initiation codon. Under these selected conditions, this pattern is exclusively accounted for by the 48S complex. The contribution of the 80S complex to the toeprint appears to be negligible as follows from analysis of a similar reaction mixture supplied with the ³²P-labeled mRNA by sucrose gradient sedimentation. No 80S peak was present in the gradient (data not shown). Conversely, when the formation of polysomes was blocked by cycloheximide, and GMPPNP was omitted, only the 80S peak was observed. The toeprint for the 80S complex is characterized by disappearance of the upper band of the triplet characteristic of the 48S toeprint and by a large increase of intensity of the middle band corresponding to position +17 (Fig. 1a, lanes 4 and 5). We interpret these differences to be a more defined and fixed positioning of the sequence downstream from the AUG codon in 80S initiation complexes as compared to 48S intermediates. Similar pictures were obtained for the 48S and 80S initiation complexes assembled in RRL with Hsp70 mRNA (Fig. 1b, lanes 2 and 3).

For both mRNAs, one more band (position +20, see Fig. 1) was also observed. It was separated from the main band of the 80S toeprint by exactly 3 nt (Fig. 1a, lane 5 and Fig. 1b, lane 3). We ascribe this 3-nt 'jump' to an 80S complex where one translocation step has already occurred, due to an incomplete block by cycloheximide even at the conventionally used concentration of 1 mg/ml. The kinetics of formation of the 'jump' (Fig. 2, lanes 2–6) at a lower concentration of cycloheximide (0.2 mg/ml) support this conclusion: the amount of the complex corresponding to the 'jump' slowly increased with time at the expense of non-translocated 80S complexes. In addition to this band, a second 3-nt 'jump' occasionally emerges (positions +22 to +24) which presumably corresponds to the next round of translocation. As expected, on increasing the concentration of cycloheximide, the formation of these translocated 80S complexes was inhibited (Fig. 2, lanes 7 and 8). This observation may additionally facilitate

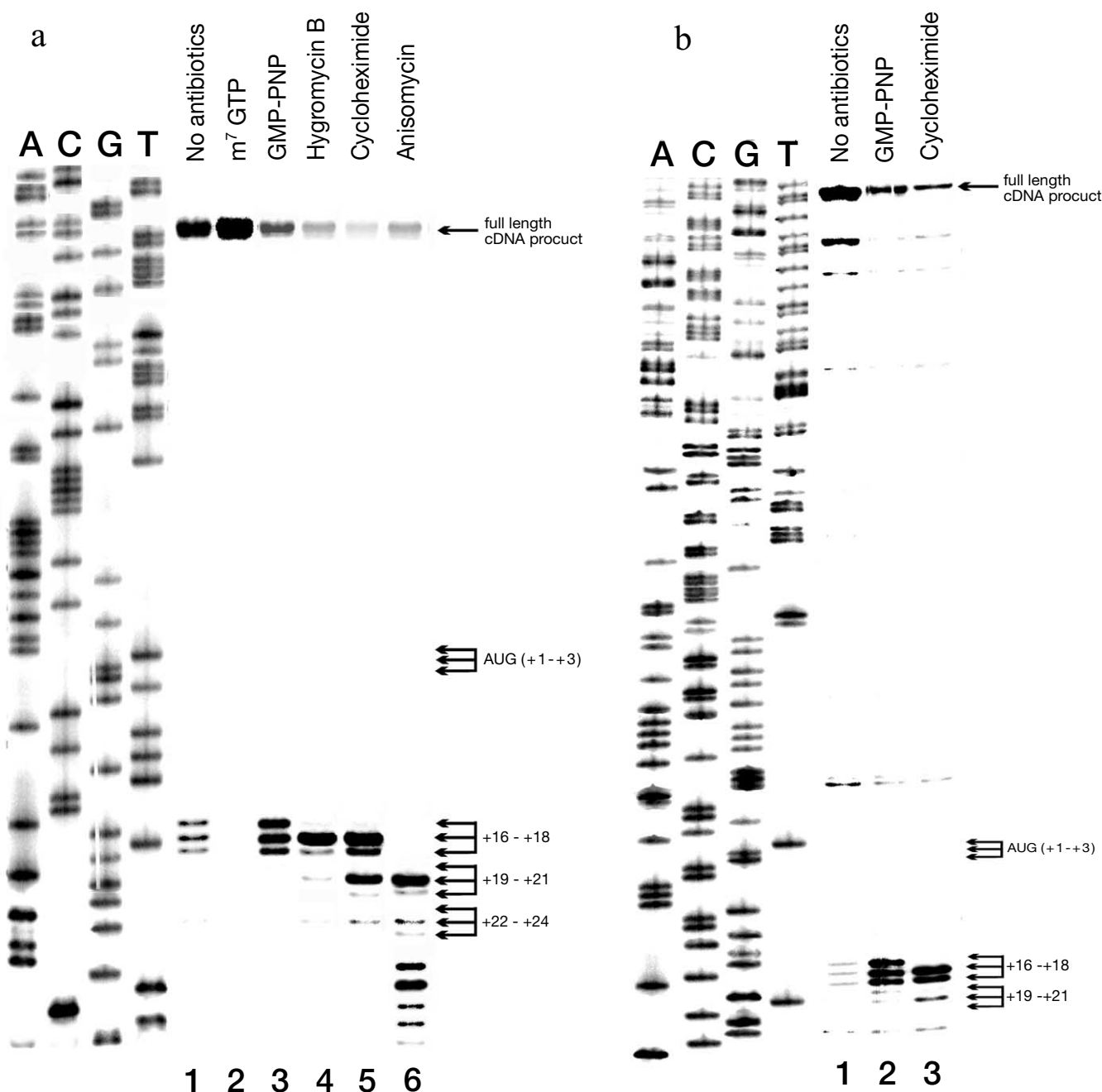


Fig. 1. Toeprint assay of 48S and 80S initiation complexes formation in RRL with β -globin (a) and Hsp70 (b) mRNAs. Positions of the initiation AUG codon and toeprint bands are denoted by arrows. A dideoxynucleotide sequence generated with the same primer (shown to the left) was run in parallel. Concentration of m^7 GTP – 2 mM. The antibiotics were used in the following concentrations: hygromycin B – 2 mg/ml, cycloheximide – 1 mg/ml, and anisomycin – 0.5 mg/ml. For other explanations see text.

identification of the toeprint pattern resulting from the 80S complex. The ‘jump’ was not observed when hygromycin B was used instead of cycloheximide (Fig. 1a, lane 4). The former antibiotic is thought to be a more powerful inhibitor of translocation of the peptidyl-tRNA from the A- to the P-site of the ribosome [22]. Unlike hygromycin and cycloheximide, the block of polypeptide elongation produced by anisomycin was very leaky (Fig. 1a, lane 6) in agreement with previous reports [4,10]. Only the toeprints corresponding to translocated 80S complexes were observed even at the high concentration of the drug (0.5 mg/ml or 2 mM).

3.2. Toeprint assay of 80S translation initiation complexes reconstituted from fully purified components with β -globin mRNA

Earlier, 48S translation initiation complexes with β -globin mRNA have been successfully reconstituted from individual translational components, the reconstitution being monitored by toeprinting [7]. The formation of 80S complexes with β -globin mRNA has also been performed [15]. However, the assembly of 80S complexes was analyzed only by sucrose gradient sedimentation [15]. As the approach of reconstitution of initiation complexes from purified components combined with

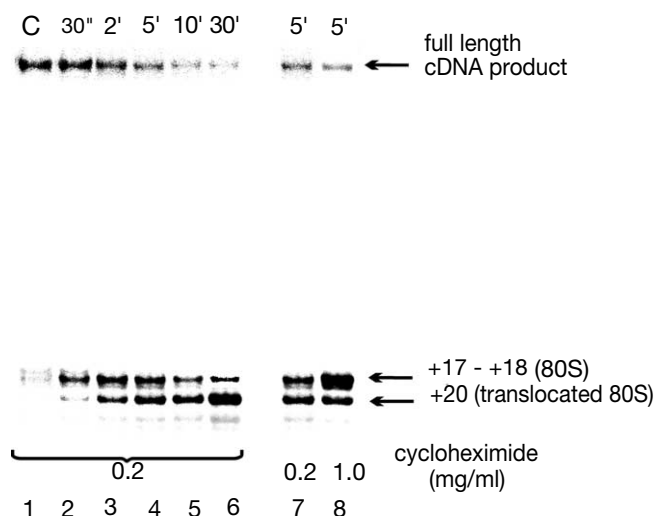


Fig. 2. Inhibition of the polypeptide elongation at different concentrations of cycloheximide as revealed by toeprint assay in RRL: lane 1 – no cycloheximide; lanes 2–6 – kinetics of formation of 80S complexes that already passed through one translocated step at 0.2 mg/ml of the drug; lanes 7 and 8 – demonstration of the enhanced block of translocation at a higher (1 mg/ml) cycloheximide concentration.

toeprinting may find a wider application, it was important to confirm whether in this case the toeprints show patterns similar to those obtained in RRL. As seen in Fig. 3 (lanes 2 and 3), the toeprints for 48S and 80S complexes reconstituted from purified components with β -globin mRNA are identical to those obtained in RRL (compare with Fig. 1a, lanes 3 and 4) with one expected exception: as our reconstitution mixture does not contain elongation tRNAs and factors, the band corresponding to the translocation ‘jump’ (see above) is absent in the pattern of 80S complexes.

Finally, to gain additional support that we correctly ascribed the toeprint patterns to 48S and 80S complexes assembled from individual components, the complexes were purified by sucrose gradient sedimentation (data not shown) and aliquots from the 48S or 80S peaks were analyzed by toeprinting as described before [4]. The toeprints were found identical to those obtained directly for the unfractionated reconstitution mixture (Fig. 3, compare lanes 2 and 3 with lanes 4 and 5, respectively).

3.3. The existence of a delay in conversion of the 48S initiation complex formed with β -globin mRNA into the 80S complex

In the experiments described above, the transition of 48S complexes into 80S complexes and migration of 80S complexes along the mRNA chain in the 3'-end direction were prevented by addition of GMPPNP or cycloheximide, respectively. Fig. 1a, lane 1 shows the results of toeprinting for β -globin mRNA when the blocking reagents were omitted and the system contained GTP. One might expect in this case not to observe toeprint bands at all. Surprisingly, the toeprint bands were present and the pattern was typical for the 48S complex (note the presence in lane 1 of three bands of approximately equal intensity characteristic of the 48S complex (see e.g. lane 3) vs the single major band typical for the 80S complex as exemplified in lane 4). That the bands belonged to the toeprint was confirmed by their disappearance on addition of m^7 GTP (Fig. 1a, lane 2). This suggests the existence, at least in vitro, of a delay in conversion of the 48S intermediate into the 80S complex.

A similar pause appears to exist in the case of conversion of the 48S complex assembled at the authentic initiation codon of the EMCV RNA into the 80S complex (see Section 3.4). Therefore, at least in some cases, the rate of joining of ribosomal subunits is comparable or even longer than the rate of location of the initiation triplet by the 40S ribosomal subunit. This is not necessarily the case for other mRNAs which may have the initiation codon in distinct nucleotide contexts. Whether this delay is characteristic of the translation in cellular extracts or else may be also observed in vivo remains to be seen. Nevertheless, we speculate that a slow rate of 80S complex formation, at least for some initiation regions, may favor the possibility of translational regulation at the step of ribosomal subunits joining [12]. Certainly, more experimentation with different mRNAs and proper kinetic measurements are needed to support this hypothesis.

3.4. Formation of 48S and 80S translation initiation complexes on the EMCV RNA initiation sequence

The EMCV mRNA is known to contain three consecutive closely spaced AUG triplets separated by a short distance from the upstream positioned IRES: the 10th, 11th (authentic start codon) and 12th from the 5'-end of the viral RNA. The 11th and 12th AUGs are in the same reading frame. Assembly of the 48S complex on the EMCV RNA transcript (nt 377–1155) in RRL resulted in the toeprints for all three AUG codons (Fig. 4), albeit of a different intensity in agreement with previous reports [5,23,24]. The weakest was the toeprint at the 10th AUG, whereas the toeprint triplets at the 11th and 12th AUGs had comparable intensities with some preference for the authentic start site. Strikingly, formation of the 80S complex almost exclusively occurred at the authentic (11th) start codon (Fig. 4, lanes 2 and 4). These data suggest that selection of a particular AUG codon on an mRNA occurs not only during scanning of the mRNA 5' UTR but also at steps following the location of the initiation codon by the 40S ribosomal subunit. Curiously, when GTP was added instead of GMPPNP in the same concentration, the toeprint at the 11th AUG characteristic of 48S complexes still persisted whereas those for the 10th and 12th AUG codons were completely

missing (Fig. 4, lane 2). We speculate that the 48S complex at the 10th AUG is less productive in formation of the 80S complex and undergoes a decay presumably accompanied by GTP hydrolysis [25]. Alternatively, the complex first formed at the 10th AUG occasionally slides further downstream to form a new codon–anticodon pair with the 11th AUG triplet. The 48S complex at the authentic initiation triplet appears to be rather stable since there is a delay in its conversion into the 80S complex (compare the toeprint pattern shown in lane 2 with those in lanes 3 and 4).

This increased stability of the 48S intermediate and its subsequent conversion into the 80S complex may restrain sliding of the 40S ribosomal subunit further downstream, thereby accounting for a strong reduction of the toeprint at the next (12th) AUG triplet when the GTP hydrolysis is not blocked by GMPPNP.

Taking together, these data suggest that the selection of the start site among different AUGs on an mRNA may occur not only at the step of location of the initiation codon by the 40S ribosomal subunit as dictated by the scanning hypothesis but

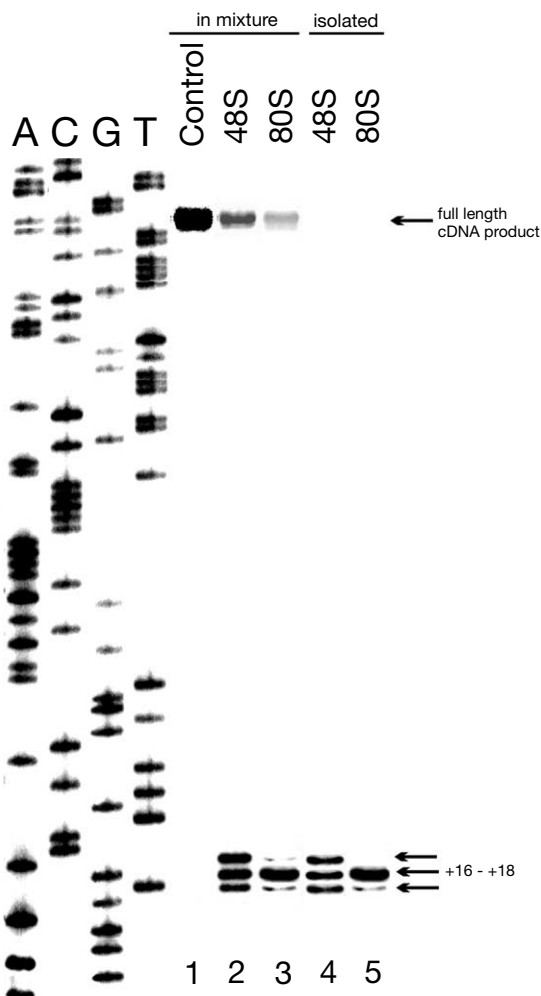
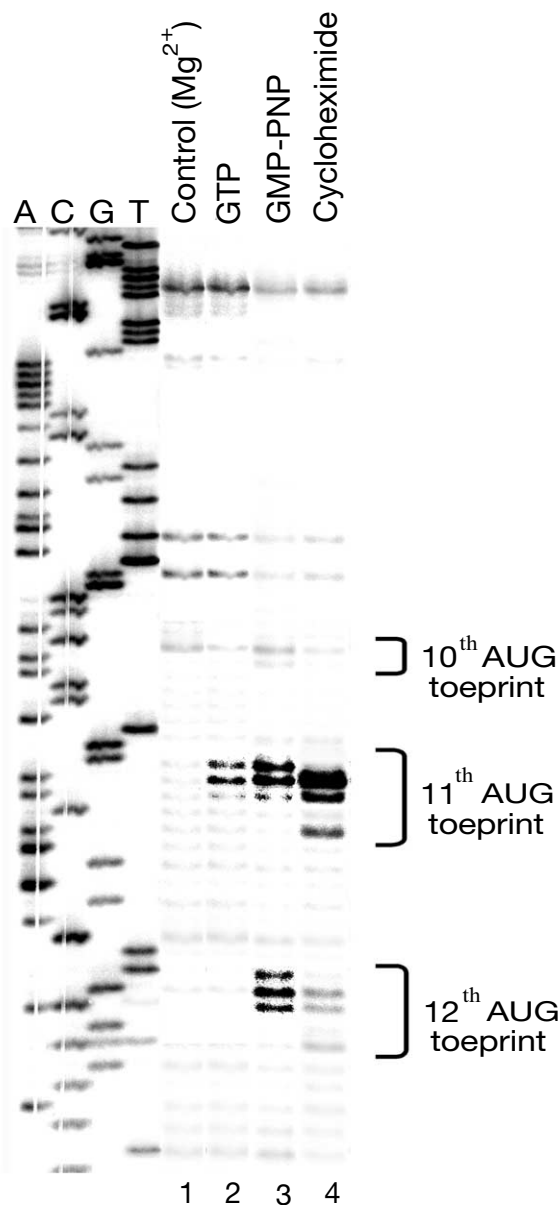


Fig. 3. Toeprint assay of 48S and 80S initiation complexes assembled from purified translation initiation components with β -globin mRNA: lane 1 – control (-Met-tRNA^{Met}); lanes 2 and 3 – 48S and 80S complexes, respectively, directly analyzed in the reconstitution mixture; lanes 4 and 5 – toeprints of 48S and 80S complexes, respectively, isolated by sucrose gradient sedimentation.



820-AACACG[ATG]ATAAT[ATG]GCCACAACC[ATG]GAACAAGAGACTTGCGGCGCACTC

Fig. 4. Toeprint assay of 48S and 80S complexes formed in RRL with the IRES-containing EMCV RNA fragment. The nucleotide sequence immediately following the EMCV IRES with three consecutive AUG codons (boxed) is shown at the bottom of the figure. Lane ‘control (Mg²⁺)’ demonstrates inhibition of the assembly of the initiation complexes at 30 mM of Mg²⁺. For other explanations see text.

also at the step of eIF2-associated GTP hydrolysis and subsequent joining of the 60S subunit to the preformed 48S pre-initiation complex.

We confirm that the process of formation of the 80S complex initiated by GTP hydrolysis may represent one more essential proofreading step in selection of the authentic AUG codon of mRNA in agreement with suggestions discussed in [25]. How the AUG context may affect this proofreading step is unclear. Future experiments in a defined system with purified components may shed light on the molecular mechanism of such a selection.

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