Molecular Biology, Vol. 37, No. 3, 2003, pp. 421–428. Translated from Molekulyarnaya Biologiya, Vol. 37, No. 3, 2003, pp. 494–503.

MOLECULAR MECHANISMS OF BIOLOGICAL PROCESSES

UDC 577.217.52

Minor Secondary-Structure Variation in the 5'-Untranslated Region of the β-Globin mRNA Changes the Concentration Requirements for eIF2

S. E. Dmitriev, I. M. Terenin, M. P. Rubtsova, and I. N. Shatsky

Belozerskii Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899 Russia; E-mail: shatsky@libro.genebee.msu.su

Received September 24, 2002

Abstract—Nucleotide sequence changes increasing the number of paired bases without producing stable secondary structure in the 5'-untranslated region (5'-UTR) of the β-globin mRNA had a slight effect on its translation in rabbit reticulocyte lysate at low mRNA concentration and dramatically decreased translation efficiency at a high concentration. The removal of paired regions restored translation. Addition of purified eIF2 to the lysate resulted in equal translation efficiencies of templates differing in structure of 5'-UTR. A similar effect was observed for p50, a major mRNP protein. Other mRNA-binding initiation factors, eIF4F and eIF4B, had no effect on the dependence of translation efficiency on mRNA concentration. Analysis of the assembly of the 48S initiation complex from its purified components showed that less eIF2 is required for translation initiation on the β-globin mRNA than on its derivative containing minor secondary structure elements in 5'-UTR. According to a model proposed, eIF2 not only delivers Met-tRNA, but it also stabilizes the interaction of the 40S ribosome subunit with 5'-UTR, which is of particular importance for translation initiation on templates with structured 5'-UTR.

Key words: translation initiation, β-globin mRNA, mRNA secondary structure, 5'-untranslated region, initiation factor eIF2, reconstruction of the 48S initiation complex, mammals

INTRODUCTION

The binding of the 40S ribosome small subunit with initiator Met-tRNA and its landing on mRNA are the two key steps in translation initiation. In eukaryotes, the first step is mediated by three-subunit eIF2, which forms ternary complex eIF2·GTP·Met-tRNA. The other step is catalyzed by mRNA-binding eIF4F, eIF4A, and eIF4B, which unwind secondary structure elements in the mRNA leader (or 5'-untranslated region, 5'-UTR) and, possibly, ensure the progress of the 40S particle from the 5' end to the AUG codon. Although the structure and function have been studied in detail for each of the above proteins, a general model of translation initiation (e.g., see [1, 2] for review) leaves many questions open. Thus little is still known on the interrelationships of the two initiation steps, e.g., on whether the steps are independent of each other or take a certain order, and whether their order is strongly determined. There is evidence [3, 4] that the ternary complex interacts with the 40S subunit by association–dissociation, and that their complex is stabilized only when the scanning ribosome reaches AUG and these bases are paired with the tRNA anticodon. Most likely, the interaction of the 40S subunit with mRNA is also reversible, the stability of their complex depending inversely on that of secondary structure elements in 5'-UTR [5]. According to this model of dynamic interactions of the initiation components, it is possible to assume that templates with more structured 5'-UTR require higher concentrations of initiation factors and are sensitive to their decrease. Since many factors have a high mRNA-binding activity [6–8], their effective concentrations decrease with increasing mRNA amount in the system, and translation initiation on highly structured templates becomes less likely; in other words, mRNA starts suppressing its own translation. Translation suppression by a template has been observed rather long ago [9, 10] and explained by "titration" of initiation factor(s) soon afterwards [10, 11]. However, since total mRNA was used in those works, differences among individual templates could not be detected. In addition, they failed to identify the translation system component a decrease in which results in suppression, and to propose a mechanism of this effect.

In this work, we demonstrated that β-globin mRNA derivatives with minor variations in the 5'-UTR secondary structure strikingly differ in ability to suppress their own translation, and that the difference may be eliminated by increasing eIF2 concentration. Reconstructing the 48S translation initiation complex from its purified components, we showed

0026-8933/03/3703-0421$25.00

that templates with secondary structure elements in 5-UTR require a higher eIF2 concentration.

EXPERIMENTAL

Plasmid construction employed *Escherichia coli* JM109 and pUC18-related vectors. Manipulations with plasmids and gene-engineering procedures followed the standard protocols [12]. The β-globin cDNA was obtained by reverse transcription–polymerase chain reaction (RT–PCR) with total poly(A)$^+$ mRNA, which was isolated from rabbit reticulocytes [13]; primers ACACTTGCTTTTGACACAAC and TTACGAGCTCAAGGGGCTTCATG; AMV reverse transcriptase (Promega); and *Taq* DNA polymerase (Fermentas). By PCR, the promoter for T7 RNA polymerase was introduced upstream of the region corresponding to 5'-UTR of the β-globin mRNA. The amplified product was ligated in the *Kpn*I–*Ecl*136II sites of pUC18. Then the *Bal*I–*Ecl*136II fragment of the β-globin cDNA was substituted with a sequence coding for full-length green fluorescent protein (GFP). The sequence was obtained by PCR with pGFP-C2 (Clontech) and primers CCATGGGTAAAGGAG and CTCTTACTTGTATAGTTCATCCA. The resulting construct bG was used to obtain the corresponding mRNA. Thus, the bG mRNA (1192 nt) contained 5'-UTR, the first 415 nt of the β-globin mRNA coding region, and the GFP-coding sequence. The translation product of the bG mRNA was about 42 kDa, which is convenient for electrophoretic analysis. Constructs TbG and bTbG were derived from bG by oligonucleotide-directed mutagenesis with a GeneEditor kit (Promega) and oligonucleotides GTAATACGACTCACTATAGGACTCTCTTCGCAT C GCTGTCTGCGAGGGCCAGAGCTTACTTGCAA TCCC and GCTTTTGACACAACTGTGTACTCTCTTCGCATCGCTGTCTGCGAGGGCCAGAGCTTA CTTGCAATCC, respectively. Likewise, constructs Td1bG and Td2bG were obtained with TbG and primers CTTCGCATCGCTAAGCTTACTTGCAATCCC and GTCTGCGAGGGCCAGAATGGTGCATCTGTCC, respectively.

RNA was obtained by T7 transcription with cotranscriptional capping. The reaction mixture (20 μl) contained 80 mM HEPES-KOH (pH 7.4); 12 mM $Mg(CH_3COO)_2$; 2 mM spermidine; 10 mM DTT; 10 mM each of ATP, UTP, and CTP; 5 mM m^{7}GpppGTP (Pharmacia); 5 μg of plasmid DNA linearized with *Ecl*136II; 20 units of ribonuclease inhibitor HPRI (Fermentas); and 20 units of T7 RNA polymerase (Fermentas). The mixture was pre-incubated at 37°C for 5 min, supplemented with 0.5 mM GTP, and incubated for 1 h. DNA was eliminated by treatment with 3 μl of RQ DNase (Promega) for 5 min. After phenol deproteinization, the mixture was purified from salts and nucleotides by gel filtration through Sephadex G50. RNA was precipitated with ethanol and dissolved in water.

Translation was carried out with a nuclease-treated rabbit reticulocyte lysate (Promega). The reaction mixture contained 4.3 μl of the lysate, which was supplemented with an amino acid mixture and [^{35}S]methionine as recommended by Amersham, and 1.7 μl of an mRNA solution varying in concentration. The translation product was analyzed by PAGE in 12% gel according to Laemmli [12].

Reconstruction of 48S initiation complexes was carried out according to [14] with minor modification. To obtain Met-tRNA, artificial initiator tRNA^{Met} was synthesized by T7 transcription with pTRM1 (see [15]) and used in place of native total tRNA. The concentration of 40S ribosome subunits was 250 nM. Recombinant eIF1 and eIF1A were isolated from *E. coli* on Ni-NTA agarose as recommended by Qiagen; eIF2, eIF3, eIF4A, and eIF4B were isolated from rabbit reticulocyte lysate as in [16]; eIF4F was isolated from HeLa cell lysate [17]. To completely remove m^{7}GTP, the eIF4F preparation obtained by chromatography on m^{7}G-Sepharose was dialyzed against a buffer containing 1M KCl. The isolated factors were all functional, since the absence of any did not allow the assembly of the initiation complex (data not shown). A p50 preparation was kindly provided by L.P. Ovchinnikov. Reverse transcription was carried out with AMV reverse transcriptase (Promega) and primer Shin18 (TCACCACCAACTTCTTCCAC), which is complementary to sequence 61–80 of the β-globin mRNA coding region. Signal intensity on gel autoradiographs was estimated using the ImageQuant 5.0 program.

Probing of the mRNA secondary structure was performed under conditions similar to those of the assembly of 48S initiation complexes, in a buffer containing 20 mM Tris-HCl (pH 7.4), 80 mM KCH_3COO, 2.5 mM $Mg(CH_3COO)_2$, 0.1 mM EDTA, 1 mM DTT, 0.25 mM spermidine, 1 mM ATP, 0.4 mM GTP. After 1 pmol of mRNA was incubated in 20 μl of the buffer for 10 min at 30°C, the mixture was combined with 1 μl of fresh 10% dimethyl sulfate (DMS, ethanol solution), or with 10 μl of a fresh 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) solution in the buffer, or with 0.1 units of T1 RNase, or with 0.3 units of T2 RNase (Pharmacia). The mixture was incubated for 5 min with DMS or for 20 min with another reagent, and then combined with 1/4 volumes of solution SP (1.5 mM CH_3COONa, pH 5.0, 1 M β-mercaptoethanol, 0.1 mM EDTA) and 5 μg of 18S rRNA (a precipitant). RNA was extracted with phenol–chloroform and precipitated with three volumes of ethanol. The precipitate was dissolved in 10 μl of a mixture containing 5 pmol of ^{32}P-labeled primer Shin18, 5 mM each dNTP, 5 units of HPRI, 40 units of M-MLV reverse transcriptase (Helicon), and 2 μl of 5X M-MLV buffer. The mixture was incubated for 30 min

at 30°C. RNA was extracted with phenol–chloroform, precipitated with ethanol, and dissolved in 4 μl of the stop solution from a USB sequencing kit.

DNA sequencing with the USB kit followed Sanger's procedure [12]. Denaturing electrophoresis of DNA was carried out in 6% PAG with 7M urea.

RESULTS AND DISCUSSION

Template-dependent translational suppression was studied with five mRNAs differing only in the 5'-proximal 5'-UTR fragment, which accounted for no more than 3% of the total mRNA length. The coding region and the 28-nt leader sequence adjacent to the AUG codon were identical in all but one mRNA (the only exception was Td2bG, see below). Thus, a difference in their translation could be attributed only to varying efficiency of translation initiation. Since the start codon was always in one context, we actually analyzed the early initiation, that is, the primary binding of the 40S ribosome subunit with the 5' end of mRNA and other events taking place before the subunit reaches the AUG codon region. We assumed that stability of the final 48S complex, kinetics of the binding with the ribosome large subunit, elongation, and termination would be the same with all templates.

The mRNA nucleotide sequences are shown in Fig. 1b. Construct bG contained 5'-UTR of the β-globin mRNA. TbG was the same but the first 25 nt were substituted with the 35-nt 5'-terminal sequence of the late adenoviral mRNA tripartite leader (TPL), which has this sequence single-stranded [18]. In bTbG, the same TPL fragment was inserted at position 25 of the β-globin mRNA 5'-UTR. Thus, bTbG differed from TbG in the 5' end and from bG in the central part of 5'-UTR. In Td1bG, the first 25 nt of the β-globin mRNA were substituted with the 17 5'-terminal nucleotides of TPL. In Td2bG, the β-globin mRNA leader was substituted with the 35-nt 5'-terminal region of TPL. Thus, the five mRNAs differed in structure and, to a lesser extent, in size of 5'-UTR.

Used at various concentrations, all mRNAs were translated in nuclease-treated rabbit reticulocyte lysate supplemented with [^{35}S]methionine. After translation, the reaction mixtures were resolved by SDS-PAGE, gels autoradiographed, and translation efficiencies estimated with a phosphorimager. Though varying only slightly in primary structure, the templates differently behaved in translation (Fig. 2). Translation of bG and Td1bG was efficient even at their highest tested concentrations (up to 170 μg/ml). With the other mRNAs, translation had a distinct peak at 25 μg/ml mRNA and decreased beyond this threshold.

On evidence of primary structure comparisons, the effect could not be associated with the 5'-UTR length (since Td1bG and Td2bG had equally short leaders, and the others insignificantly differed in this parameter) or with the 5'-terminal sequence (which was identical in bG and bTbG or in Td1bG, TbG, and Td2bG). Hence, the difference in translation might be explained by a variation in secondary structure of 5'-UTR. To check this assumption, we chemically and enzymically probed the secondary structure of the 5'-proximal region of bG, TbG, bTbG, and Td1bG. We used T1 RNase, which cleaves single-stranded regions after G; T2 RNase, which nucleotide-nonspecifically cleaves single-stranded regions; and modifying agents DMS and CMCT, which react with nonpaired A or U, respectively. After the reaction, a ^{32}P-labeled primer was annealed to an mRNA, reverse transcription performed, and its product analyzed in a sequencing gel. The results obtained with TbG and bTbG are shown in Fig. 1a. The accessibility of individual nucleotides for modification is characterized in Fig. 1b. It is seen that bG and Td1bG had single-stranded leaders (block UUUU in bG 5'-UTR was also probably single-stranded, but its attack appeared to be hindered by unknown factors; accessibility of the first two nucleotides (GG) was difficult to estimate with all mRNAs, because signals from GG and from mRNA are indistinguishable on autoradiographs). In the other mRNAs, some bases were paired in 5'-UTR. In bTbG, the 5'-proximal part of the leader was single-stranded, and secondary structure elements were only in its central part. However, computer modeling did not reveal any extended double-stranded regions in 5'-UTR of TbG, bTbG, and Td2bG. The inaccessibility of some bases might be due to unstable short hairpins of no more than 7 nt in length and no less than –10 kcal/mol in total free energy. Such hairpins, at least those located in the central part of 5'-UTR (as is the case with bTbG), could not substantially hinder the progress of the scanning ribosome [19, 20]. Indeed, the five templates differed no more than twice in maximal translational efficiency (Fig. 2). Yet these minor secondary structure elements did dramatically decrease translation at a higher mRNA concentration.

To explain the observed effect, an mRNA excess may be assumed to bind a lysate component necessary for translation initiation, a demand for which is low (or negligible) in the case of bG or Td1bG and high in the case of TbG, bTbG, or Td2bG. Apparently, this component (most likely, a protein) must have a mRNA-binding activity and, to initiate translation, must interact with 5'-UTR. When mRNA is in an excess, the interaction ceases to be specific, and nonspecific RNA binding inactivates the component. Alternatively, there are more than one components involved, which are spread (scattered) through excessive templates so that the amount of templates bound with all necessary components reduces substantially.

Two explanations are possible for the mRNA variation in requirements for this unknown component. First, a specific mRNA-binding protein might be

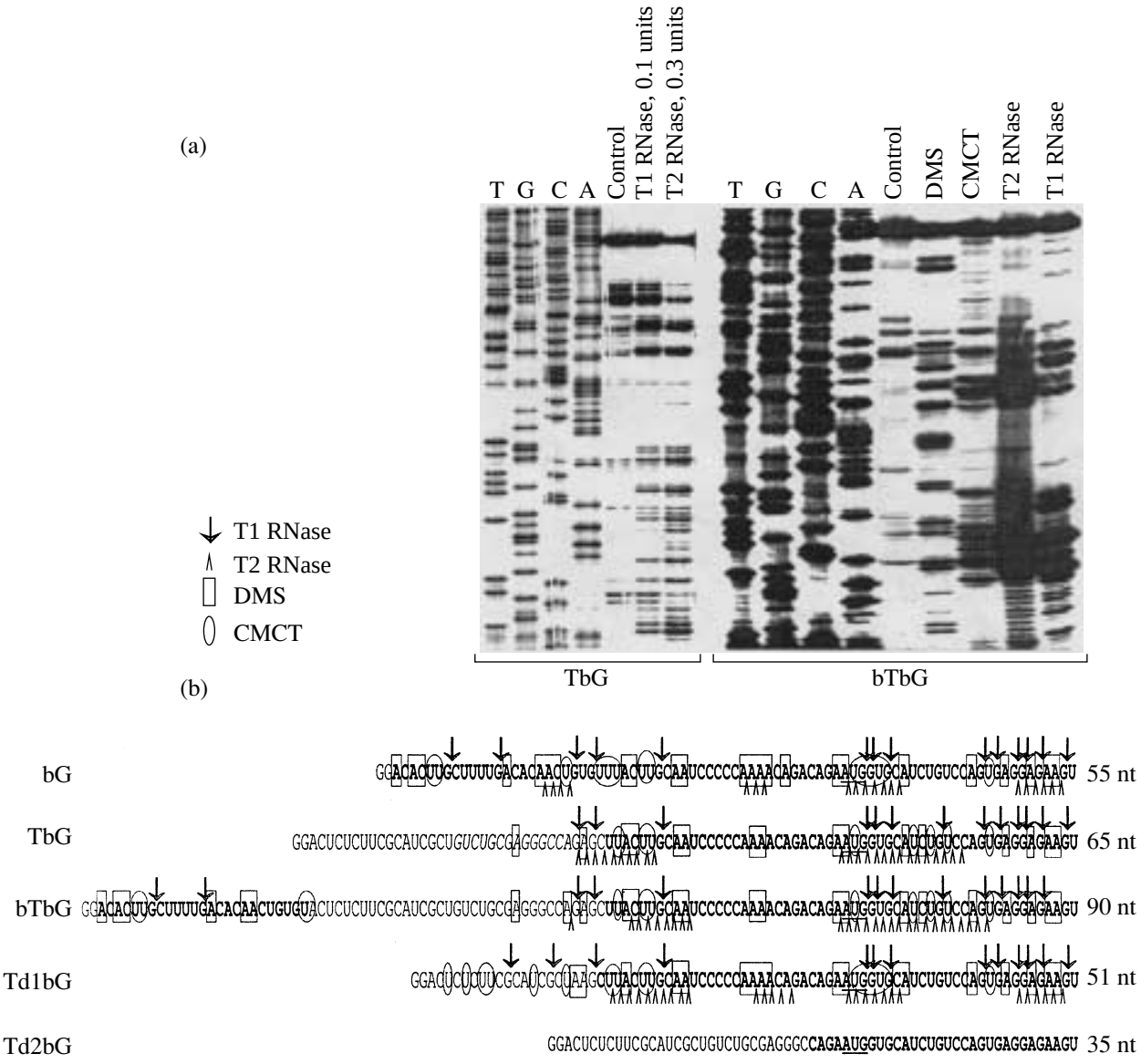

Fig. 1. Chemical and enzymic probing of the 5'-UTR structure with modifying agents DMS (adenine) and CMCT (uridine) and with guanine-specific T1 RNase and nonspecific T2 RNase, which cleave single-stranded RNA. Cytosines were not tested for accessibility. Autoradiographs (a) and nucleotide sequences of 5'-proximal regions (b) are shown. Regions corresponding to the β-globin mRNA are in bold. Initiation AUG codons are underlined. A TbG region differing from the corresponding region of Td1bG is in italics. The size of 5'-UTR is indicated on the right for each mRNA.

involved in translation initiation on TbG, bTbG, and Td2bG. However, this seems unlikely, because these mRNAs only slightly differ in structure from the other two (bG and Td1bG). Indeed, identical protein components were observed for mRNPs of these mRNAs by UV crosslinking and protein affinity chromatography on RNA-Sepharose (data not shown). Another explanation is that secondary structure elements of 5'-UTR increase the requirements for one or more canonical initiation factors, the amount of which is limited in a lysate. The most probable candidates are mRNA-binding eIF4F, eIF4B, and eIF2, the latter having the highest RNA-binding activity. It was also of interest to study the effect of p50, the major mRNP protein, which nonspecifically binds to the mRNA sugar–phosphate backbone, thereby prevents nonspecific binding of initiation factors [21, 22], and may be assumed to reduce the titration of the factor of interest. We isolated and purified to homogeneity eIF4F of HeLa cells and eIF2, eIF4B, and p50 of rabbit reticulocytes. The proteins were individually added to a nuclease-treated rabbit reticulocyte lysate, and the mixtures were used to translate TbG (25 and 150 μg/ml). According to the estimates of factor con-

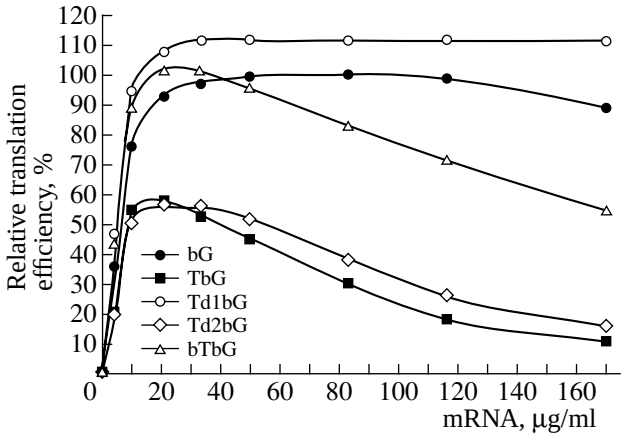

Fig. 2. Translation of several mRNAs in nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Here and in Fig. 4: Signal intensity from the translation products on gel autoradiographs was estimated with a phosphorimager. Maximal signal intensity observed with bG was taken as 100%.

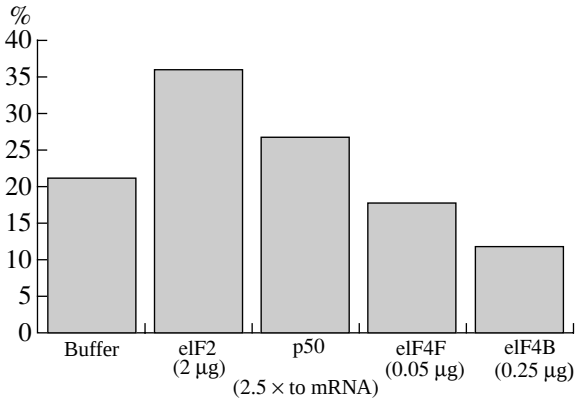

Fig. 3. Effects of translation initiation factors and p50 on translation autoinhibition by TbG. The template (25 or 150 μg/ml) was translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Ordinate, ratio of signal intensities from translation products obtained with 150 and 25 μg/ml mRNA, %. Abscissa, factors added into the translation system.

tents in the cell [23–25], each factor was added in the amount 3–4 times higher than its content in the lysate. Molar ratio p50:mRNA was 2.5:1 as in [21]. Translation efficiency was estimated as above. The ratio of efficiencies observed at higher and lower mRNA concentration is shown in Fig. 3. Of all factors tested, only eIF2 increased this ratio (e.g., it prevented a decrease in translation at a higher mRNA concentration). As expected, p50 also exerted this effect, but to a lesser extent than eIF2. The two other factors, eIF4F and eIF4B, enhanced translation of TbG used at 25 μg/ml, whereas its translation at a higher concentration was unchanged or even suppressed (data not shown). As a result, the ratio of translation efficiencies decreased (Fig. 3). When mRNA was used at 25 and 75 μg/ml, the positive effect of eIF2 was greater, while the effect of the other factors was again negative (data not shown).

To study the translational effect of a higher eIF2 concentration in more detail, bG and TbG used at various concentrations were translated in rabbit reticulocyte lysate supplemented with purified eIF2 (2 μg per 6 μl of the reaction mixture). The results obtained under these conditions (Fig. 4) strikingly differed from those obtained with the standard lysate (Fig. 2). Translation efficiency grew with TbG concentration increasing up to 70 μg/ml, and translation efficiency–mRNA concentration plots of bG and TbG were similar (although, like in the above experiments, translation of TbG was half as efficient as that of bG). Thus, it was a lack of eIF2 that accounted for a dramatic decrease in translation at a higher TbG concentration. Most likely, this is true for other templates having minor secondary structure elements in 5'-UTR.

Our data suggest that mRNA with a single-stranded leader have lower concentration requirements for eIF2. To check this assumption, 48S initiation complexes were reconstructed from purified components on bG and TbG at various eIF2 concentrations. A mixture of mRNA, 40S ribosome particles, Met-tRNA, guanosine 5'-[β,γ-imido]triphosphate (GMPPNP), ATP, eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, and eIF4F was incubated for 5 min at 30°C (see Experimental). The 48S complex was visualized by toe-printing (reverse transcription with a primer directed to the mRNA coding region). After deproteinization, the mixture was applied on a sequencing

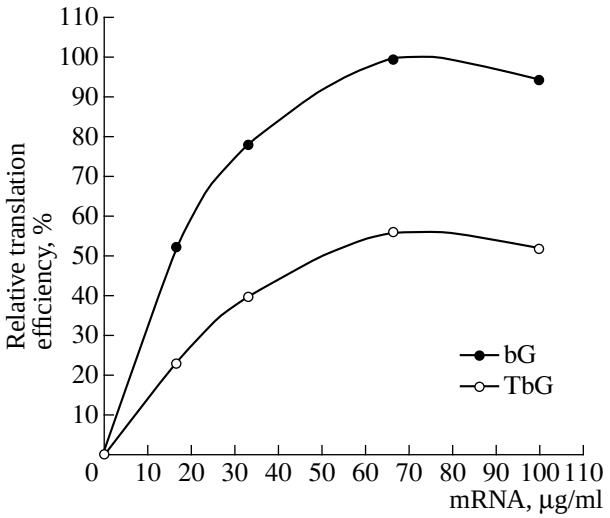

Fig. 4. Translation of bG and TbG in rabbit reticulocyte lysate supplemented with eIF2 (2 μg per sample).

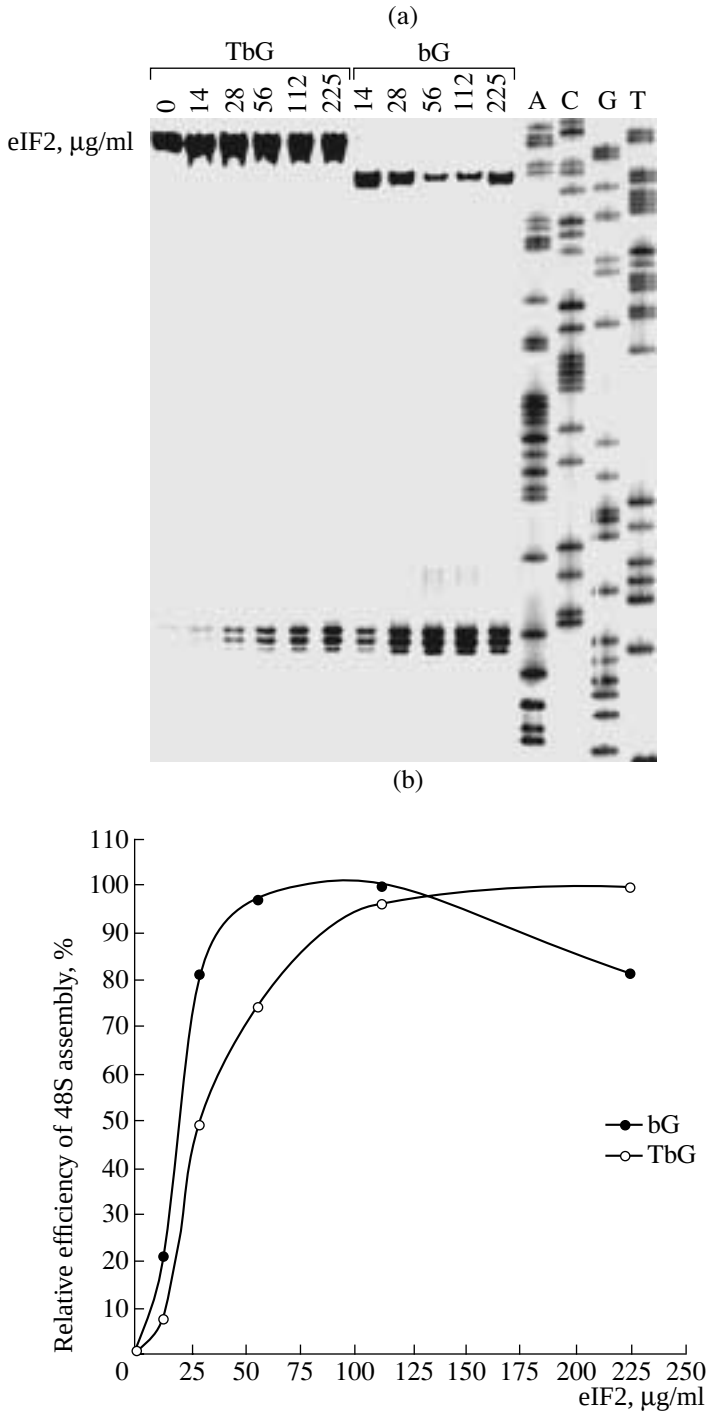

Fig. 5. Reconstruction of 48S initiation complexes from purified components at various eIF2 concentrations by toe-printing. Gels with toe-prints were autoradiographed (a) and signal intensities estimated with a phosphorimager (b). Maximal intensity observed with each template was taken as 100%.

gel along with sequencing products obtained with the same primer. Gels were autoradiographed (Fig. 5a), and signal intensities estimated with a Molecular Dynamics PhosphorImager (Fig. 5b). With increasing eIF2, efficiency of 48S complex assembly on bG increased more rapidly than on TbG, reaching its maximum at 50 μg/ml eIF2. With TbG, the maximum was observed at approximately 125 μg/ml eIF2. This clearly showed that the two mRNAs differed almost twice in necessary eIF2 concentration, which was probably due to minor secondary structure elements present in 5'-UTR of TbG but not of bG. Interestingly,

assembly of the 48S complex on bG decreased slightly at higher-than-optimal concentrations of eIF2 (Fig. 5b). Possibly, at a high concentration, eIF2 competes with the 40S subunit or factors of the eIF4 group for nonstructured 5'-UTR and thereby prevents their binding to mRNA. The effect was not observed in the case of TbG and the same eIF2 concentrations, yet further increase in eIF2 (up to 500 μg/ml) did slightly inhibit the assembly of 48S complexes (data not shown).

Our results suggest a new mechanism of translational regulation of eukaryotic mRNA. It is known that the amount of active eIF2 in the cell may greatly vary. Regulation of the eIF2 activity by phosphorylation of its α subunit has been well studied in virus infections, apoptosis, amino acid starvation, heme deficiency, accumulation of misfolded proteins, and other stress conditions (e.g., see [26] for review). The resulting deficiency in active eIF2 differentially changes mRNA translation: templates with high concentration requirements for eIF2 cease to be translated, whereas advantage is acquired by those that allow initiation at a low concentration of the ternary complex. A variation of mRNA requirements for eIF2 is long known [27]. Until cap-binding factors (eIF4 group) were discovered, this property was considered as a major factor determining competition between templates [28]. However, differences between mRNAs were explained by specific affinity of their 5'-UTR for eIF2, and the efficiency of translation initiation was thought to directly depend on the efficiency of mRNA binding with eIF2. On the other hand, there is evidence that, upon binding to mRNA, eIF2 loses its tRNA-binding activity, and formation of the ternary complex is impossible [29]. Our results agree better with this finding. Indeed, inactivation of eIF2 bound with excessive mRNA provides the simplest explanation for a dramatic decrease in translation of TbG and similar templates. According to earlier models [27, 30], eIF2 remains active when bound to its proper site (in the leader region or close to the AUG codon). Moreover, this binding is necessary for translation initiation and occurs independently of the mRNA interaction with the ribosome small subunit or even before it. Inactivation takes place when eIF2 binds to the coding region. From this standpoint, suppressed translation of mRNA with structured 5'-UTR is explained by its lower affinity for eIF2 (as compared with a single-stranded leader). With such mRNAs, eIF2 mostly binds to the coding region and loses its activity. When a template has nonstructured 5'-UTR, eIF2 binding to its proper site is more likely, and the effect of increasing mRNA concentration is lower. Structural preferences of eIF2 in RNA binding are poorly understood. It has been reported that eIF2 affinity for certain mRNAs is even lower than for double-stranded RNA [31]. Yet the relevant data are scarce and hardly enough to conclude that the difference in affinity for eIF2 accounts for different translational efficiency of templates with single-stranded or structured leaders. In addition, this hypothesis disagrees with our finding that formation of the 48S complex on bG decreases at a high eIF2 concentration (Fig. 5b). Hence, we propose the following model.

The 40S ribosome subunit binds with ternary complex eIF2 · Met-tRNA · GTP either before or after its landing on mRNA, the latter usually taking place when eIF2 is lacking [3]. In the resulting complex, eIF2 stabilizes the interaction between the 40S subunit and mRNA owing to its contact with both these components. In the case of a nonstructured leader, mRNA tightly binds with the ribosome even in the absence of the ternary complex, whereas any secondary structure element present in 5'-UTR dramatically weakens their interaction [5]. In other words, in the former case, eIF2 plays a single function in translation initiation: it delivers Met-tRNA to the 40S subunit, which is tightly associated with mRNA and "waits" for the delivery. Apparently, this complex lives long enough for the ternary complex to be bound even at a low eIF2 concentration in the system. Templates with structured leaders utilize eIF2 not only for delivering Met-tRNA, but also for stabilizing their complex with the 40S subunit. In the absence of eIF2, the complex is unstable and rapidly dissociates as Met-tRNA fails to arrive. The probability of the 40S subunit binding to the AUG codon directly depends on the eIF2 concentration. When the concentration is low, initiation on such templates is low-efficient. Possibly, ternary complex eIF2 · Met-tRNA · GTP acts as an accessory factor of nonprocessive helicase eIF4A, because its binding with mRNA prevents unwound duplexes from clapping and thereby further stabilize the mRNA complex with the 40S subunit. This role is commonly ascribed to eIF4B [32], yet the contribution of eIF2 is also possible.

Thus, low initiation rate on mRNA with structured 5'-UTR cannot be explained solely and to the full extent by the time-consuming unwinding of secondary structure elements. More likely, the cause is higher concentration requirements for eIF2, which stabilizes the interaction of the ribosome small subunit with mRNA. We did not intend to study the kinetics of 48S complex binding with structurally differing mRNAs at various eIF2 concentrations in this work, yet such studies are necessary.

ACKNOWLEDGMENTS

We are grateful to Ya.E. Dunaevskii for help in isolating initiation factors, to L.P. Ovchinnikov for a p50 preparation, and to A.V. Pisarev for discussions. This work was supported by the Russian Foundation for Basic Research (project nos. 96-04-55009, 01-04-06503) and by the Russian program Integration (2000-1.5-88).

REFERENCES

1. Dever T.E. 2002. Gene-specific regulation by general translation factors. *Cell*. **108**, 545–556.
2. Pestova T.V., Kolupaeva V.G., Lomakin I.B., Pilipenko E.V., Shatsky I.N., Agol V.I., Hellen C.U. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci. USA*. **98**, 7029–7036.
3. Dasso M.C., Milburn S.C., Hershey J.W., Jackson R.J. 1990. Selection of the 5'-proximal translation initiation site is influenced by mRNA and eIF-2 concentrations. *Eur. J. Biochem*. **187**, 361–371.
4. Hinnebusch A.G. 1993. Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. *Mol. Microbiol*. **10**, 215–223.
5. Seal S.N., Schmidt A., Marcus A. 1989. Ribosome binding to inosine-substituted mRNAs in the absence of ATP and mRNA factors. *J. Biol. Chem*. **264**, 7363–7368.
6. Barrieux A., Rosenfeld M.G. 1977. Characterization of GTP-dependent Met-tRNAf binding protein. *J. Biol. Chem*. **252**, 3843–3847.
7. Abramson R.D., Dever T.E., Lawson T.G., Ray B.K., Thach R.E., Merrick W.C. 1987. The ATP-dependent interaction of eukaryotic initiation factors with mRNA. *J. Biol. Chem*. **262**, 3826–3832.
8. Hughes D.L., Dever T.E., Merrick W.C. 1993. Further biochemical characterization of rabbit reticulocyte eIF-4B. *Arch. Biochem. Biophys*. **301**, 311–319.
9. Gozes I., Schmitt H., Littauer U.Z. 1975. Translation *in vitro* of rat brain messenger RNA coding for tubulin and actin. *Proc. Natl. Acad. Sci. USA*. **72**, 701–705.
10. Wettenhall R.E., Slobbe A. 1976. Inhibitory action of different RNA fractions on the translation of globin mRNA *in vitro*. *Biochim. Biophys. Acta*. **432**, 323–328.
11. Minich W.B., Korneyeva N.L., Ovchinnikov L.P. 1989. Translational active mRNPs from rabbit reticulocytes are qualitatively different from free mRNA in their translatability in cell-free system. *FEBS Lett*. **257**, 257–259.
12. Sambrook J., Russell D. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd Ed. N.Y.: Cold Spring Harbor Lab. Press.
13. Gielen J., Aviv H., Leder P. 1974. Characteristics of rabbit globin mRNA purification by oligo(dT) cellulose chromatography. *Arch. Biochem. Biophys*. **163**, 146–154.
14. Shatskii I.N. 2001. *In vitro* reconstruction of initiation complexes to study the molecular mechanisms of translation initiation in mammals. *Mol. Biol*. **35**, 628–637.
15. Pestova T.V., Hellen C.U. 2001. Preparation and activity of synthetic unmodified mammalian tRNAi(Met) in initiation of translation *in vitro*. *RNA*. **7**, 1496–1505.
16. Merrick W.C. 1979. Purification of protein synthesis initiation factors from rabbit reticulocytes. *Methods Enzymol*. **60**, 101–108.
17. Grifo J.A., Tahara S.M., Morgan M.A., Shatkin A.J., Merrick W.C. 1983. New initiation factor activity required for globin mRNA translation. *J. Biol. Chem*. **258**, 5804–5810.
18. Zhang Y., Dolph P.J., Schneider R.J. 1989. Secondary structure analysis of adenovirus tripartite leader. *J. Biol. Chem*. **264**, 10679–10684.
19. Kozak M. 1989. Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Mol. Cell Biol*. **9**, 5134–5142.
20. Vega Laso M.R., Zhu D., Sagliocco F., Brown A.J., Tuite M.F., McCarthy J.E. 1993. Inhibition of translational initiation in the yeast *Saccharomyces cerevisiae* as a function of the stability and position of hairpin structures in the mRNA leader. *J. Biol. Chem*. **268**, 6453–6462.
21. Minich W.B., Ovchinnikov L.P. 1992. Role of cytoplasmic mRNP proteins in translation. *Biochimie*. **74**, 477–483.
22. Pisarev A.V., Skabkin M.A., Thomas A.A., Merrick W.C., Ovchinnikov L.P., Shatsky I.N. 2002. Positive and negative effects of the major mammalian messenger ribonucleoprotein p50 on binding of 40S ribosomal subunits to the initiation codon of beta-globin mRNA. *J. Biol. Chem*. **277**, 15445–15451.
23. Safer B., Kemper W., Jagus R. 1979. The use of [^{14}C]eukaryotic initiation factor 2 to measure the endogenous pool size of eukaryotic initiation factor 2 in rabbit reticulocyte lysate. *J. Biol. Chem*. **254**, 8091–8094.
24. Duncan R., Hershey J.W. 1983. Identification and quantitation of levels of protein synthesis initiation factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem*. **258**, 7228–7235.
25. Rau M., Ohlmann T., Morley S.J., Pain V.M. 1996. A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. *J. Biol. Chem*. **271**, 8983–8990.
26. Clemens M.J. 2001. Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis. *Progress Mol. Subcell. Biol*. **27**, 57–89.
27. Rosen H., Di Segni G., Kaempfer R. 1982. Translational control by messenger RNA competition for eukaryotic initiation factor 2. *J. Biol. Chem*. **257**, 946–952.
28. Di Segni G., Rosen H., Kaempfer R. 1979. Competition between alpha- and beta-globin messenger ribonucleic acids for eukaryotic initiation factor 2. *Biochemistry*. **18**, 2847–2854.
29. Barrieux A., Rosenfeld M.G. 1978. mRNA-induced dissociation of initiation factor 2. *J. Biol. Chem*. **253**, 6311–6314.
30. Thomas A.A., Scheper G.C., Voorma H.O. 1992. Hypothesis: is eukaryotic initiation factor 2 the scanning factor? *New Biol*. **4**, 404–407.
31. Rosen H., Knoller S., Kaempfer R. 1981. Messenger ribonucleic acid specificity in the inhibition of eukaryotic translation by double-stranded ribonucleic acid. *Biochemistry*. **20**, 3011–3020.
32. Jaramillo M., Dever T.E., Merrick W.C., Sonenberg N. 1991. RNA unwinding in translation: assembly of helicase complex intermediates comprising eukaryotic initiation factors eIF-4F and eIF-4B. *Mol. Cell Biol*. **11**, 5992–5997.