Translational control of terminal oligopyrimidine mRNAs requires a specific regulator

Fabrizio Loreni, Francesco Amaldi*

Dipartimento di Biologia, Universitá "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma, Italy

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Abstract Terminal oligopyrimidine (TOP) mRNAs are a group of messengers translationally regulated according to the growth status of the cell. Two hypotheses have been proposed for the mechanism of the regulation: (i) there is a specific translational regulator which can reversibly alter TOP-mRNA structure, (ii) a component of the general translational apparatus can specifically affect the translation of TOP-mRNAs. To verify one of the two hypotheses we induced a partial inhibition of translation initiation in *Xenopus* cultured cells and analyzed the effect on TOP-mRNA translation. Our results suggest that a specific regulator is necessary to explain the translational control of these of mRNAs.

Key words: Translational control; Translation inhibitor; Ribosomal protein mRNA

1. Introduction

The presence of a terminal oligopyrimidine tract (TOP) at the 5′ end is the common structural feature of a group of mRNAs in vertebrates. The pyrimidine tract starts with a cytosine residue (cap site) and extends for 5–14 nucleotides. TOP-mRNAs include all ribosomal protein (rp) mRNAs plus few other messengers coding for products related to the protein synthesis apparatus, like for instance elongation factor (eEF)1α and eEF2. Their expression is specifically and coordinately regulated at the translational level according, in general, to the growth status of the cell. Translational control of TOP-mRNAs and in particular of rp-mRNAs has been studied in detail for more than ten years in many experimental systems. Translational activity is usually monitored by analyzing the distribution of mRNAs between active polysomes and inactive subpolysomal mRNPs. It has been found that in actively growing cells TOP-mRNAs are mostly localized on polysomes whereas in quiescent or growth-inhibited cells they are on mRNPs (for review see [1,2]). The principal characteristics of TOP-mRNA translational control can be summarized as follows: (i) the regulation is specific (i.e. it does not involve other non-TOP-mRNAs), (ii) TOP-mRNAs move between polysomes and mRNPs without any irreversible modification, (iii) localization change does not require ongoing transcription, (iv) the distribution of TOP-mRNA between polysomes and mRNPs is bimodal, that is the mRNAs are either fully loaded on polysomes or are on mRNPs. Sequence elements essential for translational control have been identified in the 5′ UTR [3–5]. In particular, Meyuhas and collaborators showed that a single mutation of a cytosine into alanine in the TOP region as well as change of position of the pyrimidines are sufficient to abolish translational control [6]. Although putative translational regulators which bind the 5′ UTR of TOP-mRNAs have been identified in two laboratories [7,8], clear evidence that any of them is involved in translational control has not yet been obtained.

The lack of a good candidate as translational regulator keeps still open the issue of whether such an activity exists. In fact it has been proposed more than twenty years ago by H. Lodish that selective translational control can be obtained on the basis of differential affinity of mRNA for a limiting component of the translational apparatus [9]. The decrease of the amount (or activity) of the limiting factor would cause selective exclusion from polysomes of low affinity mRNAs. The model is attractive for its simplicity and its application to TOP-mRNA translational regulation has been recently hypothesized [10]. Prime candidate as regulatory component is initiation factor (eIF)4E, whose activity is dependent on phosphorylation and interaction with regulatory proteins [11]. Although there is a correlation between eIF4E activity and TOP-mRNA translation, some data argue against its role in the regulation [1]. Also rpS6 phosphorylation has been shown to correlate with TOP-mRNA translational activation [12] and p70 S6 kinase has been proven to be necessary for the regulation [13]. Again, however, formal demonstration that any of the components of the general translation apparatus plays an active role in TOP-mRNA translational regulation is lacking. Therefore the only evidence for a regulation based on a general component is the so far unsuccessful search for a translational regulator, whereas the arguments for a specific mechanism can be defined as follows: (i) in vitro translation experiments failed to show that TOP-mRNAs have low affinity for the translational apparatus [1], (ii) the distribution of TOP-mRNA between polysomes and mRNPs is bimodal whereas in the case of an affinity-based regulation it would be expected a statistical decrease in the size of polysomes during translational repression. However this last argument, which we believe is the most convincing, still does not exclude the possibility that the undetection of intermediate size polysomes is due to the low sensitivity of experimental analysis.

Using *Xenopus* cultured cells as experimental system, we decided to verify if TOP-mRNA translational control can be the effect of decreased activity of a component of translational apparatus (Lodish model) or if it requires a specific translation effector. For this purpose we followed in parallel the kinetics of TOP-mRNA localization change during serum deprivation (control) and during sperimentally induced 'Lodish conditions'. To decrease the efficiency of translation initiation, which is considered the limiting step of protein synthesis, we used two different conditions: administration of

*Corresponding author. Fax: +39 (6) 2023500.
E-mail: amaldi@utovrm.it

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pactamycin and heat shock. Pactamycin is a antibiotic that inhibits translation initiation in eukaryotes by interfering with the attachment of initiator tRNA to the initiation complex [14]. Heat shock, on the other hand, induces complex response which includes a general translation repression. Data from various eukaryotic cells (reviewed in [15]) indicate that raising the temperature of a cell 5–10°C above their normal temperature causes inhibition at the level of translation initiation, as evidenced by disaggregation of polysomes. The molecular mechanism responsible for the repression involves the two initiation factors indicated as key regulators of translation, eIF2 and eIF4E. Both factors are converted into the inactive form during heat shock, by phosphorylation of eIF2α and dephosphorylation of eIF4E causing translation inhibition [15]. Therefore both pactamycin treatment and heat shock cause inhibition of initiation that, according to the affinity model, should induce selective exclusion of TOP-mRNAs from polysomes. Our analysis shows that initiation inhibition is not sufficient to induce a selective TOP-mRNA translational repression and therefore a specific translational regulator is required to explain the mechanism of regulation.

2. Materials and methods

2.1. Cell culture

Xenopus laevis kidney cell line B 3.2 was grown in medium containing 61% Leibovitz L-15, 10% fetal bovine serum (FCS), 29% H_2O, supplemented with 2 mM glutamine, 50 u/ml penicillin and 50 mg/ml streptomycin. Cells were cultured at 24°C. Serum deprivation was done as described [16], by transferring cells into serum-free medium. Heat shock temperature (33°C) was as described for *Xenopus* cell line A6 [17].

2.2. Polysome isolation

$1–2\times10^6$ cells, washed once with buffer A (100 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4), were treated directly on the plate with 300 μl of lysis buffer containing 10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, 1% Triton-X100, 1% NaDeoxycholate, 0.2 u/ml RNase inhibitor (Boehringer), 1 mM Dithiothreitol and transferred to an eppendorf tube. After a few minutes of incubation on ice with occasional vortexing, the extracts were centrifuged for 10 min at 13000 rpm in the cold room. The supernatant was frozen in liquid nitrogen and stored at −70°C or loaded directly on a 15–50% linear sucrose gradient containing 30 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and centrifuged in an SW41 rotor for 110 min at 37000 rpm. Fractions were collected monitoring the absorbance at 260 nm and treated directly with proteinase K.

2.3. Extraction and analysis of RNA

Total RNA was extracted from gradient fractions by proteinase K method [18]. For Northern analysis, RNA was fractionated on formaldehyde-agarose gels and transferred on Gene Screen Plus membrane (NEN). Northern and dot blots were done essentially according to the manual. *Xenopus* rpL18 [19] and calmodulin [20] probes were prepared by the random primer technique. Quantitation of Northern and dot blot analyses was done by PhosphorImager analysis (Molecular Dynamics) or by autoradiography and laser densitometer analysis (LKB Ultroscan XL).

3. Results and discussion

3.1. TOP-mRNA specific translational control

We have previously described that B 3.2 cells transferred into serum-free medium respond by rapidly altering the percentage of TOP-mRNAs loaded on polysomes which, within an hour, changes from 70–80 to 20–30 [16]. Here we used this experimental system as an example of TOP-mRNA translational control showed in many other eukaryotic cells (see Section 1) to analyze the mechanism of the regulation. The protocol we employ to evidence translational control is the following: (1) cytoplasmic extracts from cells grown in various conditions are separated on sucrose gradient; (2) the gradient is collected in 18 fractions while a general polysome profile is obtained by monitoring the absorbance of the gradient at 260 nm; (3) RNA is extracted from the fractions, analyzed by Northern hybridization with different probes, and the quantitation of the hybridizations (as percentage of

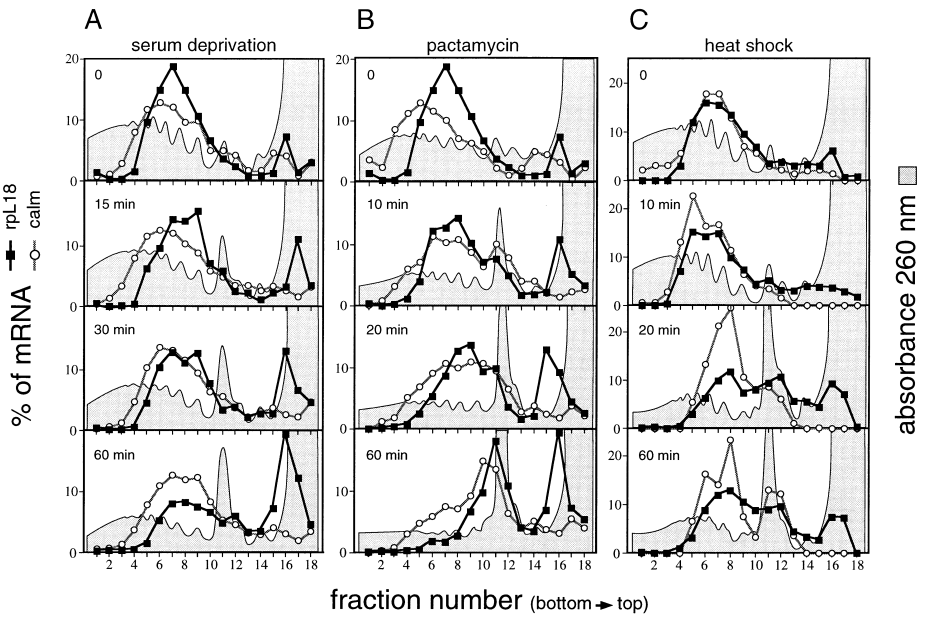

Fig. 1. mRNA localization in B 3.2 cells during serum deprivation, pactamycin treatment, and heat shock. Cytoplasmic extracts were separated on sucrose gradients and collected while monitoring absorbance at 260 nm. RNA extracted from each fraction was analyzed on Northern blot with rpL18 and calmodulin probes. Quantitation of the signals is reported as percentage of mRNA in each fraction with the first fraction at the bottom of the gradient. The grey areas in the background represent a proportional reproduction of the absorbance profile of the gradients.

RNA in the fractions) is superimposed to the polysome profile. This procedure gives essentially two kinds of information: the localization of the probed mRNA within the polysome profile, and the status of the polysomes in the cell. Cells were subjected to three different treatments: (i) serum deprivation, (ii) administration of 0.01 μg/ml of pactamycin, (iii) heat shock at 33°C. According to preliminary dose-response experiments, 0.01 μg/ml of pactamycin cause about 60% inhibition of amino acid incorporation (data not shown). After drug or heat shock treatments cells were analyzed according to the above described protocol and Northern blots were probed with at least one TOP-mRNA and at least one non-TOP-mRNA. TOP-mRNA probes were *Xenopus* rpL18, rpL4, rpS25, whereas control non-TOP were *Xenopus* calmodulin, histone H3 and β-actin. As already known, all TOP-mRNAs showed the same hybridization pattern and also the various control mRNAs gave similar results. Therefore only hybridizations with rpL18 and calmodulin probes are reported Fig. 1 as an example. Serum deprivation experiment (Fig. 1A) confirmed previously described [16] results: (i) within 1 h most of rpL18-mRNA moves from polysomes to subpolysomal mRNPs, (ii) the relocation does not involve passage of rpL18-mRNA on smaller size polysomes, (iii) calmodulin mRNA does not show any change of localization, (iv) in the general polysome profile 80S monomer increases gradually and polysome amount decreases maintaining approximately the same size.

Pactamycin treatment (Fig. 1B) produces a clearly different result. In fact rpL18-mRNA moves gradually from polysomes to mRNPs through an evident decrease of polysome size. A similar pattern is followed by calmodulin mRNA that however is never accumulated in the mRNP fractions. The general polysome profile, at variance with serum deprivation, shows a gradual size decrease of polysomes which after 1 h are mostly dimers and 80S monomers.

The results obtained with the heat shock (Fig. 1C) are essentially similar. The treatment induces a gradual decrease in polysome size both of rpL18 and calmodulin mRNAs. In this case the amount of rpL18-mRNA relocalized from polysomes to mRNPs is smaller compared to pactamycin treatment. This could be due to a different kinetics or to a different degree of translational inhibition. Also the effect of heat shock on the general polysomal profile is less drastic compared to pactamycin treatment but the accumulation of 'halfmers' (i.e. polysomes with extra small subunits on mRNA) is more evident.

As mentioned in Section 1, two hypotheses have been proposed for the mechanism that regulates the translation of TOP-mRNAs. One postulates the existence of a translational regulator able to reversibly modify the structure of TOP-mRNAs [1,7]. According to the circumstances the regulator, positive or negative, can render the mRNA either translatable or untranslatable. The other hypothesis states that TOP-mRNAs have low affinity for a limiting component of the translational apparatus. Lowering the activity of such component would be sufficient to cause a selective translational repression of TOP-mRNAs [9]. The essential difference between the two hypotheses is that in the first model the decision 'translate or not translate' is made at the level of mRNA, whereas in the second one it depends on the ribosome (or its accessory factors). As a consequence, during translational repression we can expect the following alternatives: (i) a certain amount of TOP-mRNAs became untranslatable (and localized on mRNPs) but the rest of the messengers is normally translated (fully loaded on polysomes); (ii) a certain amount of ribosomes became incompetent to translate TOP-mRNAs and we observe a gradual decrease of the number of ribosomes per messenger, that is a reduction of polysome size.

To verify one of the hypotheses we asked a simple question: if we induce a decrease of translation initiation, is the result a selective translational repression of TOP-mRNA, similar to what is observed during serum deprivation? Both pactamycin treatment and heat shock gave a negative answer. In fact even at early time points the TOP-mRNA translational repression is not selective. Moreover the repression follows a different pattern compared to the translational control by serum deprivation: there is a clear decrease of polysome size instead of the bimodal distribution. These results suggest that to obtain the TOP-mRNA translational control observed during serum deprivation is necessary that a specific regulator modifies translation availability of TOP-mRNAs, possibly by binding the 5′ UTR. However a specific factor does not rule out the participation of some component of the general translation apparatus to the regulatory mechanism. For instance the well established involvement of p70 S6 kinase in TOP-mRNA translational control suggests a role of phosphorylated rpS6 in the regulation. The function of rpS6 could be to interact positively or negatively with TOP-mRNAs and/or the specific regulator and make the regulation dependent on different signals. However any complex regulatory mechanism to be consistent with our results as well as with the other published data, must involve some reversible structural modification of TOP-mRNAs.

3.2. General translational control

The data presented in Fig. 1 evidence also the change of general polysome profile in the different treatments. It is generally assumed that the rate-limiting step of protein synthesis under physiological conditions is the initiation phase. For this reason the average polysomal density of one ribosome per 80–100 nucleotides is not the maximum possible reached for instance at pausing sites [21]. Any decrease of initiation activity would cause a consequent reduction of polysome size. In fact this is what we observe during pactamycin treatment and, to a minor extent, during heat shock. This is also what has been observed by other groups during heat shock [15], mitosis [22] and some cases of serum deprivation [23]. However the decrease of polysome size is not observed in our example of serum deprivation as well as in dexamethasone treated mouse lymphosarcoma cells [24] or differentiating mouse myoblasts [25]. In these latter examples, together with TOP-mRNA translational repression, there is a more or less pronounced decrease of protein synthesis activity and the amount of polysomes decreases. Polysome size, however, does not change indicating that initiation activity remains constant relatively to mRNA amount or that there is a parallel decrease of initiation and elongation. A possible explanation is that a protein synthesis decrease without changes in polysome size is a kind of 'first level' response of cell to extracellular signals (growth factor decrease, differentiation signals, etc.). At this stage there is a parallel decrease of initiation activity as well as of mRNA available for translation because of degradation or modification as in TOP-mRNAs. When the perturbation of the equilibrium is more severe, like for instance during heat shock or pactamycin treatment, initiation inhibition became

visible at the level of polysome size. In this scenario the specific TOP-mRNA translational regulation is an early cellular response and could be considered a very sensitive indicator of the growth conditions of the cell. Although speculative, we believe that this model could be useful in the analysis of the complexity of translation regulation.

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