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Coordinate translational regulation in the syntheses of elongation factor 1α and ribosomal proteins in *Xenopus laevis*

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ABSTRACT

The regulation of the synthesis of elongation factor 1α (EF-1α) in *Xenopus laevis* has been analyzed from the point of view of translational control. The 5′ end of EF-1α mRNA, examined by primer extension, revealed the presence of a terminal pyrimidine tract that is characteristic of ribosomal protein mRNAs (rp-mRNAs). We have then compared the translation pattern of EF-1α and rp-mRNAs during *Xenopus* embryogenesis and in *Xenopus* cultured cells during growth rate changes. In *Xenopus* embryos EF-1α transcripts, that appear after midblastula transition, are initially mostly localized on mRNP and translationally inactive. Only later in embryogenesis, together with rp-mRNAs, they are gradually recruited on polysomes. Also in *Xenopus* cells B 3.2, EF-1α mRNA shows a distribution change similar to an rp-mRNA: part of it moves from polysomes to mRNP during serum deprivation and goes back on polysomes after restitution of serum to the culture. Moreover EF-1α mRNA, similarly to rp-mRNAs, is always localized on mRNP or fully loaded on polysomes but never on small polysomes. Therefore EF-1α mRNA for structural features and translation behavior can be included in the 'regulatory' group of rp-mRNAs.

INTRODUCTION

Regulation of the synthesis of several translation factors has been shown to occur primarily at pre-translational levels. In hormone-treated lymphosarcoma cells the decreased synthesis of translation initiation factor (IF) 4A, 4D, and 2α is proportional to the respective mRNA levels (1). Similarly, the synthesis of IF-4A and 4E during rat liver development is regulated at transcriptional level (2). Elongation factor 1α (EF-1α) represents the only example of translation factor sometime reported to be translationally regulated. In fact it has been shown that in mouse fibroblasts after mitogenic stimulation EF-1α synthesis increases whereas its mRNA level remains unchanged (3). Moreover translational regulation of EF-1α has been recently evidenced also in human skin fibroblasts and in mouse lymphosarcoma cells (4). These observations seem interesting in that they suggest a correlation between the syntheses of EF-1α and ribosomal proteins. In fact in eukaryotic cells with the exception of yeast, ribosomal proteins are known to have an important regulation at the translational level. A number of studies carried out in *Dictyostelium* (5), *Drosophila* (6), *Xenopus* (7, 8), and various mammalian systems (9, 10, 11) have shown that the fraction of rp-mRNA recruited on polysomes, and thus engaged in translation, changes according to the cellular requirement for ribosome synthesis in different developmental stages or growth conditions.

In the case of EF-1α however, other data in the literature seem to suggest that its translational control mechanism is different from that of ribosomal proteins or even that it is not translationally controlled at all. For instance a study of EF-1α synthesis in Friend erythroleukemia cells has been carried out by analyzing the distribution of EF-1α mRNA between polysomes and subpolysomal mRNP in different growth conditions (12). After treatment of a stationary culture with fresh medium there is a rapid increase of polysomal EF-1α mRNA associated to degradation of part of the messenger and to an increase of polysomal size. On the contrary experiments in various systems had shown that the change of distribution of ribosomal protein mRNA (rp-mRNA) occurs without changes of polysomal size or mRNA turnover (9, 13). Moreover an analysis of the expression of EF-1α in *Xenopus laevis* has been carried out during embryogenesis and it has been suggested that, at variance with ribosomal proteins, EF-1α mRNA is not controlled at translational level in this context (14).

The here reported structural analysis of the mRNA for *Xenopus* EF-1α (see below), showed the presence of a 5′UTR known to be characteristic of all vertebrate rp-mRNAs up to now analyzed. This type of 5′UTR, short and starting with a sequence of 8–12 pyrimidines, has been demonstrated to be the cis-acting sequence responsible for the translational behavior of rp-mRNAs (4, 15). If this is true the presence of this type of UTR at the 5′ of EF-1α mRNA should make it translationally regulated like rp-mRNAs, which as mentioned had been reported not to be the case. The purpose of the present paper is to define this point solving the contradictory information found in the literature. The results presented show that, from the point of view of translational

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regulation and mRNA stability, EF-1α and ribosomal proteins appear to be coordinated.

MATERIALS AND METHODS

Biological materials

Xenopus laevis adults were purchased from Nasco (Wisconsin, USA). Embryos were obtained by in vitro fertilization and staged according to Nieuwkoop and Faber (16).

Cell culture

B 3.2 *X.laevis* kidney cells were cultured as previously described (13). They were grown at room temperature (22−26°C) in tissue culture flasks in medium containing 75% Leibovitz L-15, 10% fetal bovine serum, 15% H_2O, supplemented with 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. For the induction of downshift cells were detached, transferred in dishes containing serum-free medium and incubated for various times. For the upshift the medium in the dishes was replaced after 3 hours of downshift and the cells were incubated in serum-containing medium for the indicated times.

Polysome isolation

Cytoplasmic extracts from *Xenopus* embryos and B 3.2 cells were prepared as previously described (13, 17). Briefly embryos and cells were lysed in 10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris−HCl pH 7.5, 1% NaDeoxycholate, 0.2 U/ml RNase inhibitor (Boehringer), 1 mM Dithiotreitol and, respectively, 0.5% and 1% Triton-X100. After few min 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 optical density at 254 nm and precipitated with ethanol overnight at −20°C.

Extraction and analysis of RNA

Total RNA was extracted from cells or gradient fraction pellets 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 carried out essentially according to the manufacturer. Probes for EF-1α (14), rpL14, rpS19 (19), calmodulin (20), histone H3 (21), ferritin (22), clone 3-88 (23), were prepared by the random primer technique (18). Autoradiographs were analyzed with an LKB Ultroscan XL laser densitometer. Quantitation standards were included in some dot blots to establish the linear range response of the experiments.

Primer extension

A synthetic oligonucleotide primer was labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. About 10^5 c.p.m. of the primer were added to 10 μg of total RNA in 30 μl of 50 mM Tris−HCl pH 8.3, 125 mM KCl, 3mM $MgCl_2$ (RT buffer). The mix was incubated for 5 min at 90°C and then for 1 hour at 37°C. After the addition of 5 μl of 1 mM deoxynucleotide triphosphates in RT buffer, half of the annealing mix was incubated with 100 units of M-MLV Reverse Transcriptase (BRL) for 1 hour at 37°C. Samples were then treated with Ribonuclease A (50 μg/ml), purified with phenol-chloroform extraction, ethanol precipitated and analyzed on a polyacrylamide sequencing gel.

RESULTS

EF-1α transcriptional start site

The 5′ untranslated region of rp-mRNAs has been shown to be involved in the translational control of this class of messengers (24, 25). In particular the pyrimidine stretch at the beginning of mRNA, highly conserved among vertebrate rp-genes, has been indicated as an element necessary for translational control (4, 15). Data available for *Xenopus* somatic EF-1α included an incomplete cDNA sequence (14) and the entire gene sequence (26) but not the determination of transcriptional start site. For this reason we decided to clearly define EF-1α mRNA 5′ end through primer extension experiments. On the basis of the cDNA sequence we synthesized the oligonucleotide 5′-GATTA-GATGTTTGGCGGT-3′ complementary to mRNA sequence from position −5 to −22 with respect to the AUG. The oligo was annealed to total RNA from cultured cells and embryos and extended with reverse transcriptase (figure 1). By comparing the length of the extension product with the EF1α genomic sequence (26) we deduced the transcriptional start site located, as indicated in figure 1, within a sequence of 11 pyrimidines at 61 nt from the AUG. The 5′ end pyrimidine stretch and the short 5′ untranslated region make EF-1α mRNA structurally similar to the rp-mRNAs.

Translational regulation during embryogenesis

The translational control of rp-mRNA during early *Xenopus* embryogenesis has been well studied. Transcription of this class of genes starts at stage 8 (midblastula transition) but mRNAs are initially mostly stored as mRNP. Only at later stages (around 26) rp-mRNAs begin to be loaded on polysomes (17). The percentage of mRNA on polysomes of different ribosomal proteins at the various stages is qualitatively reproducible with some variability due in part to the laborious experimental

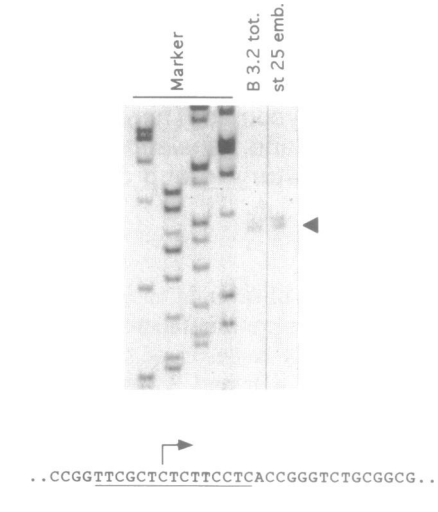

Figure 1. Analysis of EF-1α mRNA 5′ end. A 18mer complementary to EF-1α mRNA was annealed to 10 μg of total RNA from B3.2 cells and 10 μg of total RNA from stage 25 embryos. Half of the annealing mixes were extended with reverse transcriptase and the extension products are indicated in the figure by the arrowhead. Sequence marker was prepared using the same oligonucleotide of the primer extension and the plasmid pXef1 containing EF-1α cDNA. The size of the bands indicate that EF-1α mRNA is 20 nucleotides longer than the cDNA. The resulting transcriptional start site, based on the sequence of the gene, is indicated in the lower part of the figure.

procedure but also to the genetic background of the animals used (27).

At least three EF-1α genes under different developmental control have been identified in *Xenopus*. One, called 42Sp50, is detected only in oocytes; a second form of EF-1α mRNA (EF-1αO) is abundant in oocytes, transiently expressed in early embryos and it is not detectable in adult tissues. A third form (EF-1αS) is transcribed starting from midblastula transition and it is the only EF-1α mRNA present in adult tissues (28). A preliminary analysis during *Xenopus* embryogenesis reported that the distribution of somatic EF-1α mRNA between polysomes and mRNP is different from ribosomal proteins (14).

We have carried out a detailed analysis of the percentage of mRNA on polysomes at different embryo stages using probes specific for somatic EF-1α and rpS19. As control we used a probe from a cDNA clone (3-88) that hybridize to an mRNA mostly loaded on polysomes in early embryogenesis (23). The results are showed in figure 2 and indicate that EF-1α and r-protein mRNAs change distribution in a very similar way. They start with about 10% of mRNA on polysomes at stage 14 and gradually reach 60% of loading at stage 42, while 3-88 mRNA (that is clearly detectable after stage 14) is about 60% on polysomes up to stage 35 and then it decreases the loading to 40% at stage 42.

Translational regulation in cultured cells during nutritional changes

We have previously shown (13) that transferring *Xenopus* B 3.2 cells into serum-free medium (downshift) produces a rapid and coordinate change of the percentage of rp-mRNA on polysomes from 70−80 to 25−35. Restoration of serum content of the medium (upshift) induces a similarly rapid return to the original conditions (70−80% of mRNA on polysomes).

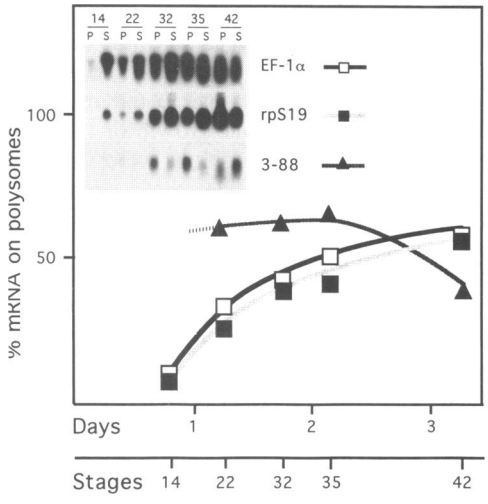

Figure 2. mRNA localization during embryogenesis. Cytoplasmic extract from 10 embryos of various stages were separated on sucrose gradients into two fractions: polysomes (P) and subpolysomal mRNP (S). RNA extracted from each fraction was used in Northern blot analysis with probes for EF-1α, rpS19, and clone 3-88. Densitometric values of the autoradiographs showed in the upper part of the figure are reported in the plot as percentage of mRNA on polysomes at the different stages. Signals of the Northern appear diffused due to a slight degradation of the RNA caused by the polysome preparation procedure. Length heterogeneity can be excluded as total RNA extracted from the same sources gives sharp bands (not shown).

A preliminary analysis on Northern blot showed that EF-1α mRNA exhibits a similar distribution change: it is localized mostly on polysomes during rapid growth and mostly on mRNP after the downshift (figure 3). The localization change is specific for ribosomal protein and EF-1α mRNAs since control messengers like calmodulin and ferritin do not show any modification of the distribution pattern. Then we performed a more detailed analysis of the kinetics of the distribution change both during the downshift and the upshift. Sucrose gradients of cytoplasmic extracts were collected into three fractions (polysomes, monomer, and mRNP) and the RNA extracted from the fractions was analyzed by dot blot hybridization with probes for EF-1α, rpL14 and histone H3. The signals were evaluated by laser densitometry and the results are reported on figure 4 as percentage of mRNA on polysomes. The kinetics of the distribution changes of EF-1α and rpL14 mRNAs are analogous, indicating that both messengers respond coordinately to translational control mechanisms.

As mentioned in the introduction, a change of polysomal size has never been observed in the analysis of rp-mRNA translational

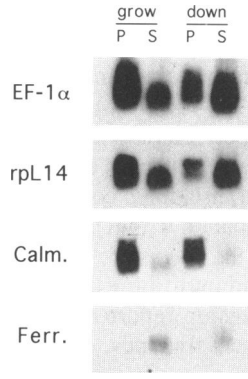

Figure 3. mRNA localization during growth rate change in cultured cells. Cytoplasmic extracts were prepared from cells during exponential growth (grow) and after 3 hours of serum-deprivation (down). Extracts were separated on sucrose gradients into two fractions: polysomes (P) and subpolysomal mRNP (S). RNA extracted from each fraction was used in Northern blot analysis with probes for EF-1α, rpL14, calmodulin and ferritin.

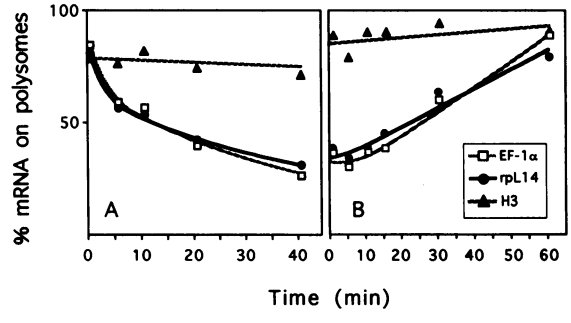

Figure 4. Kinetics of mRNA distribution changes. Cytoplasmic extracts were prepared from cells at various times of downshift **(A)** and upshift **(B)**. Extracts were separated on sucrose gradients into three fractions: polysomes (1), monomer (2) and subpolysomal RNP (3). Total RNA was extracted from the fractions and used in dot blot analysis with probes for EF-1α, rpL14 and histone H3. Densitometric values are reported on the figure as percentage of mRNA on polysomes (fraction 1/fraction 1+fraction 2+fraction 3×100).

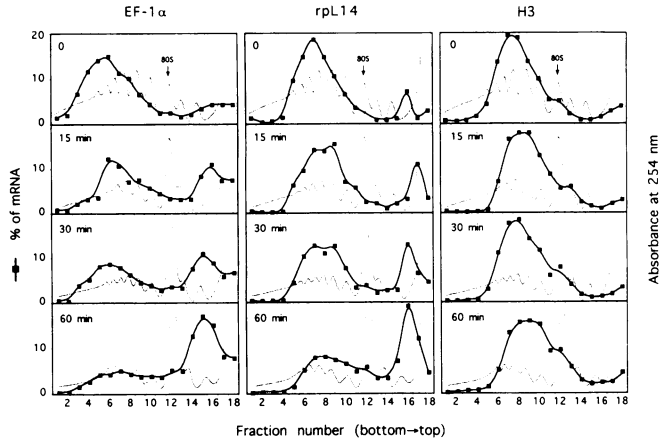

Figure 5. Bimodal distribution of EF-1α and rpL14 mRNAs during downshift. Cytoplasmic extracts were prepared from cells at various times of downshift and separated on sucrose gradients. Eighteen fractions were collected and RNA extracted from each fraction was used in dot blot analysis with probes for EF-1α, rpL14, and histone H3. Densitometric values are reported on the figure as percentage of mRNA in the various fractions. The absorbance pattern of the sucrose gradients are drawn in dotted line in the corresponding plots.

control whereas after growth stimulation of Friend erythroleukemia cells part of EF-1α mRNA has been reported to move from small to heavier polysomes. Modification of polysomal size during mRNA localization changes could be an important point for the discussion on translational control mechanism, therefore we looked at the distribution within the polysomes of EF-1α and rpL14 mRNAs during the downshift. For this analysis the sucrose gradients were collected into 18 fractions and the values obtained from the hybridizations are reported in figure 5 as percentage of mRNA in the different fractions. The results show that in the first hour of downshift polysomal mRNA decrease from 80 to 40% for rpL14 and from 80 to 30% for EF-1α, whereas histone H3 mRNA does not show any change of distribution (80% on polysomes). The change of distribution of both rpL14 and EF-1α mRNAs occurs without appreciable variation of polysomal size showing again the similarity of translational regulation of the two messengers.

EF-1α mRNA stability in cultured cells

To further extend the comparison we have analyzed the stability of EF-1α and ribosomal protein mRNAs in cultured cells. For this purpose we have treated B 3.2 cells for various time with the transcriptional inhibitor actinomycin D. Then we have analyzed the decrease of mRNA amount on Northern blot using probes for EF-1α, rpS19, and histone H3 as a control. The results, showed in figure 6A, indicates that EF-1α and ribosomal protein mRNAs have a similarly high stability, as both messengers maintain the same level even after 24 hours of transcription inhibition, whereas H3 mRNA shows a clear decrease.

Analysis of EF-1α mRNA level in Friend erythroleukemia cells showed that after growth stimulation there is a rapid degradation of about 60% of the messenger and a distribution change (12). We have previously shown that, in *Xenopus* cultured cells, rp-mRNAs do not exhibit any alteration of turnover during growth rate changes (13). To analyze EF-1α mRNA behavior in our system we have examined the messenger level during the downshift and the upshift both in the presence or absence of

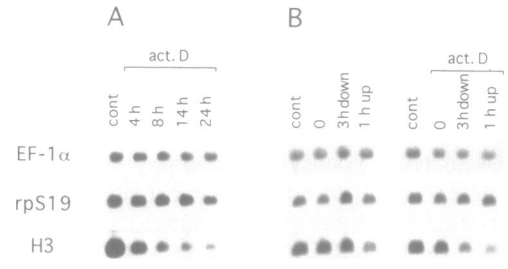

Figure 6. Northern blot analysis of mRNA stability. Five μg of total RNA from cells was loaded on gel and after transfer on membrane was hybridized with EF-1α, rpL14 and histone H3 probes as indicated. Equal loading of the lanes and complete transfer was controlled by ethidium bromide staining. **A**: growing cells (cont), cells treated for 4, 8, 14, 24 hours with actinomycin D. **B**: growing cells (cont), cells transferred into serum-free medium at zero time (0), and after 3 hours of incubation (3h down), cells incubated 3 hours in serum-free medium and 1 hour in serum-rich medium (1 h up), same four samples but incubated in presence of actinomycin D starting 1 hour before downshift (excluded control).

transcriptional inhibitor. Northern blot analysis reported in figure 6B shows that EF-1α mRNA level is not altered during growth rate changes even in the absence of transcription. This result indicates that, similarly to rp-mRNAs, the above described distribution change of EF-1α mRNA during the downshift and the upshift is not associated to turnover variations.

DISCUSSION

In this report we have analyzed the translational regulation of EF-1α synthesis in *Xenopus laevis* comparing it with the already known translational regulation of ribosomal protein synthesis.

Regulation at the translational level has been shown to play an important role in the synthesis of ribosomal protein in *Xenopus* and probably in all eukaryotes excluding yeast (see introduction). The percentage of rp-mRNA loaded on polysomes changes in response to variations in the growth status of the cell or to follow established developmental plans. In general the amount of rp-mRNA on polysomes is proportional to the requirement for ribosome production. Slow cellular growth or the presence of stored maternal ribosomes are conditions were active ribosome production is not required and under these circumstances rp-mRNAs are mostly non-polysomal (mRNP). Conversely during rapid growth or when maternal ribosomes decrease, ribosomal protein messengers are largely loaded on polysomes. This translational control is mediated by elements present in the 5′ UTR of rp-mRNAs (15, 24, 25). Analysis with gene constructs and specific mutagenesis showed in fact that the presence and the localization of a pyrimidine tract at the 5′ end of rp-mRNAs is necessary for regulation (4).

As a first step in the characterization of translational regulation of EF-1α synthesis we have analyzed the 5′ end of the messenger. The results showed that EF-1α mRNA share with rp-mRNA the presence of a 5′ end polypyrimidine tract and therefore it is a likely candidate for translationally regulated messenger. Next we have examined the behavior of EF-1α mRNA, in parallel with an rp-mRNA, under those conditions that would evidence translational regulation: early embryogenesis and nutritional changes in cultured cells. In both these experimental situations we have analyzed the distribution of mRNA between polysomes and mRNP looking at different embryonic stages or at different times after change of culture medium. The analysis showed a

clear similarity in the behavior of the two messengers both qualitative and quantitative. In fact the minor differences in the percentage of mRNA on polysomes between EF-1α mRNA and the ribosomal protein mRNA are within the variability observed among different rp-mRNAs (27). Thus EF-1α synthesis is regulated at the translational level according to the requirement for production of protein synthesis machinery (ribosomes, translation factors). After midblastula transition when transcription is activated, *Xenopus* embryo still contains a large supply of maternal ribosomes and probably EF-1α, accumulated during oogenesis (29). In this situation ribosomal protein and EF-1α genes start to be transcribed but mRNAs are mostly not translated and localized on mRNP. At later stages, when maternal reserve decreases, ribosomal protein and EF-1α mRNAs are loaded on polysomes.

In a similar way transferring B 3.2 cells into serum-free medium reduces growth rate and therefore the request for production of protein synthesis machinery. The response to these conditions is a rapid change of localization of EF-1α and ribosomal protein mRNAs (from polysomes to mRNP). Restitution of serum to the culture induces a reversal of the phenomenon with a reloading of EF-1α and ribosomal protein mRNAs on polysomes.

In all the systems analyzed in detail so far, rp-mRNAs are always distributed in a 'bimodal' manner (9, 13). That is rp-mRNAs can be found only on mRNP or fully loaded on polysomes with variations in the amount of the messengers present in the two localizations. The fact that rp-mRNAs are never found in intermediate localizations (lighter polysomes) has been used as an argument in favour of a regulation mechanism based on a specific translational repressor (13). We have looked at the localization of EF-1α mRNA during the downshift with the aim of evidencing the possible 'bimodal' distribution. The results show that within an hour of downshift about 50% of EF-1α mRNA move gradually from mRNP to polysomes. Moreover the localization change occurs maintaining a 'bimodal' distribution, with messengers moving from mRNP directly to heavy polysomes. Therefore EF-1α and ribosomal protein mRNAs, other than being coordinated, are controlled by a similar mechanism probably involving a translational repressor.

Finally the similarity in the behavior of EF-1α and ribosomal protein mRNAs is further evidenced by the analysis of messenger stability. Rp-mRNAs have been shown to be quite stable in mouse fibroblasts with an half life of 8 hours (9). During growth rate changes the steady state level of this class of messengers can show alteration, as for instance in rat liver development (2) and fibroblasts stimulation (9), or it can remain substantially stable, as in the case of growth arrest of mouse lymphosarcoma cells (11). We have previously shown that in *Xenopus* cultured cells rp-mRNAs are stable and their turnover is not altered during growth rate change (13). Here we have compared the level of EF-1α and ribosomal protein mRNAs after prolonged transcription inhibition and during growth rate changes in presence or absence of transcriptional inhibitor. All the analysis showed that also with respect of accumulation pattern, EF-1α and ribosomal protein mRNAs exhibit coordinate regulation.

Our analysis shows that EF-1α mRNA belong to a group of messenger coordinately regulated in the translational activity. This group, that share structural features in the 5′ end involved in their regulation, is composed mostly by rp-mRNAs. It includes also mRNAs of non-ribosomal protein (30, this report) that are however component of the translation machinery and therefore it is not unexpected that they are coordinately regulated. Translational control is a kind of regulation very suitable for rapid and economic adaptations of the cell to growth requirements. Synthesis of translation machinery requires a large amount of cellular energy and translational regulation is surely the right strategy to regulate the production of its components. Due to the complexity of the protein synthesis apparatus we think that other products will be found coordinately regulated at the translational level.

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