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Oligopyrimidine tract at the 5′ end of mammalian ribosomal protein mRNAs is required for their translational control

(glucocorticoids/lymphosarcoma cells/polyribosomal distribution/human growth hormone)

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ABSTRACT **Mammalian ribosomal protein (rp) mRNAs are subject to translational control, as illustrated by their selective release from polyribosomes in growth-arrested cells and their underrepresentation in polysomes in normally growing cells. In the present experiments, we have examined whether the translational control of rp mRNAs is attributable to the distinctive features of their 5′ untranslated region, in particular to the oligopyrimidine tract adjacent to the cap structure. Murine lymphosarcoma cells were transfected with chimeric genes consisting of selected regions of rp mRNA fused to non-rp mRNA segments, and the translational efficiency of the resulting chimeric mRNAs was assessed in cells that either were growing normally or were growth-arrested by glucocorticoid treatment. We observed that translational control of rpL32 mRNA was abolished when its 5′ untranslated region was replaced by that of β-actin. At the same time, human growth hormone (hGH) mRNA acquired the typical behavior of rp mRNAs when it was preceded by the first 61 nucleotides of rpL30 mRNA or the first 29 nucleotides of rpS16 mRNA. Moreover, the translational control of rpS16-hGH mRNA was abolished by the substitution of purines into the pyrimidine tract or by shortening it from eight to six residues with a concomitant cytidine → uridine change at the 5′ terminus. These results indicate that the 5′-terminal pyrimidine tract plays a critical role in the translational control mechanism. Possible factors that might interact with this translational cis regulatory element are discussed.**

Control at the translational level plays a dominant role in the regulated expression of eukaryotic ribosomal protein (rp) genes under a variety of conditions (1–3). These include: early development of *Dictyostelium discoideum* (4), *Drosophila melanogaster* (5), and *Xenopus laevis* (ref. 6 and references therein); secretory stimulation in the nonmitotic paragonial glands of *D. melanogaster* (7); insulin treatment of chicken embryo fibroblasts (8, 9) and mouse myoblasts (10); and differentiation of mouse myoblasts (11). Selective regulation of rp mRNA translation also occurs in response to growth stimulation or growth arrest of mammalian cells, as has been demonstrated in serum-stimulated mouse fibroblasts (12, 13) and glucocorticoid-repressed mouse P1798 lymphosarcoma cells (14, 15).

The selective nature of the translational control of rp mRNAs suggests that they have some distinctive property that is recognized by the translational apparatus and/or by proteins of the messenger ribonucleoprotein (mRNP) particles. One common feature noted in all sequenced vertebrate rp mRNAs is an oligopyrimidine tract at the 5′ terminus. This element usually consists of a cytidine residue at the cap site followed by an uninterrupted sequence of 7–13 pyrimidine nucleotides (16–25). Indeed, about half of all mRNAs with a 5′-terminal cytidine residue may be rp mRNAs (26, 27).

Two types of evidence lead one to suspect that the oligopyrimidine element might be implicated in the translational control mechanism. First, three mRNAs that are known to alternate between translationally active and repressed states in response to changes in cellular growth status have a pyrimidine tract at their 5′ end; yet only one of them is an rp mRNA (28–31). Second, a 35-nucleotide segment of the 5′ untranslated region (UTR) of *Xenopus* rpS19 mRNA, which contains a typical C-Y_n element (Y = an unspecified pyrimidine) is sufficient to enable a chimeric mRNA to be translationally regulated during *Xenopus* embryo development (17).

In the experiments described below, we have evaluated the importance of the 5′-terminal pyrimidine tract for the translational control of mammalian rp mRNA in mouse lymphosarcoma cells that either were growing normally or were growth-arrested by treatment with the glucocorticoid dexamethasone. Our results indicate that this element plays a critical role in the translational regulation of rp mRNAs.

MATERIALS AND METHODS

Standard protocols were used for recombinant DNA construction (32). The chimeric construct pAct-L32 is a derivative of the rpL32 gene lacking introns 2 and 3 (Δ23 in ref. 33) in which a 675-base-pair (bp) *Acc* I–*Hin*dIII fragment (positions −159 to 516; ref. 18), containing the promoter, first exon, and the 5′ end of intron 1, was replaced by a 1.7-kilobase-pair (kbp) *Xba* I–*Hin*dIII fragment containing a corresponding region of the rat β-actin gene (−1260 to +442; ref. 34). The pAct-GH chimera was constructed by inserting a 2.1-kbp *Bam*HI–*Eco*RI human growth hormone (hGH) gene fragment, derived from pOCH (35) and lacking promoter sequences, between the *Bam*HI and *Eco*RI sites of the polylinker sequence of pAct3 (21). The construction of the pL30-GH and pS16-GH chimeras followed a similar strategy, which involved digestion of pL30CAT (construct g in ref. 36) and pS16CAT (construct c-CAT in ref. 37) with *Eco*RI and *Bam*HI to remove the bacterial chloramphenicol acetyltransferase (CAT) gene and simian virus 40 (SV40) sequences and insertion of the 2.1-kbp *Bam*HI–*Eco*RI hGH gene fragment into the corresponding sites in the vector, immediately downstream of the respective rp gene promoters. For pS16CM5-GH and pS16CM3-GH, the rpS16 sequences spanning positions +30 to +2050 were excised from the rpS16 mutants CM-5 and CM-3 (38), respectively, by digestion with *Eco*RI and *Sac* I. The ends were made blunt by T4 DNA polymerase and ligated with the 2.1-kbp *Bam*HI–*Eco*RI hGH

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Abbreviations: GH, growth hormone; hGH, human GH; mRNP, messenger ribonucleoprotein; rp, ribosomal protein; TLRE, translational regulatory element; UTR, untranslated region.

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gene fragment, which had been made blunt by filling in with Klenow enzyme.

P1798.C7 mouse lymphosarcoma cells were grown as suspension cultures in RPMI medium containing 5% fetal calf serum as described (39). Cells (2×10^7) at a density of 5×10^5 cells per ml were used for DEAE-dextran-mediated DNA transfection (40). Briefly, cells were incubated at room temperature with 3 μg of DNA per 10^6 cells in 0.3 ml containing 0.5 μg of DEAE-dextran per ml of growth medium. The DNA/DEAE- dextran mixture was removed after 1 hr, and the cells were treated for 1 hr at 37°C with growth medium containing 0.1 mM chloroquine diphosphate. The DNAs in these experiments were in the form of circular and linearized plasmids when used for transient and stable transfections, respectively. In the latter case, the chimeric constructs were cotransfected with pActneo (21), keeping the ratio of the test to selective genes at 10:1. Selection for G418 resistance was started 48 hr after transfection, and the resistant cells were grown as a polyclonal culture. When transiently transfected, the cells were split at 24 hr into two halves, one of which was treated with 0.1 μM dexamethasone. Both treated and untreated cells were harvested 24 hr later. For determinations of the amount of secreted hGH, the regular growth medium (RPMI 1640) was replaced 3 hr prior to cell harvesting by fresh Dulbecco's modified Eagle's medium containing or lacking hormone. The RIA of hGH was performed with a commercial kit (St. Nichols, San Diego, CA) according to the supplier's instructions. Lysis of P1798 cells, size fractionation of polysomes by sedimentation through sucrose gradients, extraction of the RNA from the polysomal and the subpolysomal fractions, isolation of the poly(A)$^+$ mRNA fractions, and quantitative RNA (Northern) blot analysis, were performed as described by Meyuhas *et al.* (14). The probes used for hybridization were as follows: GH, a hGH cDNA (provided by T. Fogel) for the GH mRNA reporter segments; Act, a mouse β-actin cDNA (41) for endogenous actin mRNA; L5, a rat rpL5 cDNA (42) for endogenous rpL5 mRNA; 5′ Act, a 440-bp *Bst*NI fragment spanning positions −370 to +74 of the rat β-actin gene (34) for the pAct-L32 chimers.

RESULTS

It was previously observed that in exponentially growing P1798 mouse lymphosarcoma cells, the proportion of rp mRNA actively engaged in protein synthesis—i.e., the proportion incorporated into polyribosomes—is significantly lower than that characteristic of other mRNAs such as those encoding actin, nucleolin, various enzymes, and translational initiation factors (14, 15, 43). On average, only 63 ± 6% of rp mRNA was engaged with ribosomes compared with 91 ± 4% for the other mRNA species. This selective repression of rp mRNA translation was even more pronounced in cells that were growth-arrested by treatment with the glucocorticoid hormone dexamethasone. Under these conditions, only 25 ± 5% of the rp mRNA remained in polyribosomes compared with 83 ± 6% for non-rp mRNAs. In the present experiments, we used this system to evaluate the importance of the 5′ untranslated region (UTR) and, in particular, the ubiquitous oligopyrimidine tract, for the translational control of rp mRNA.

To address this problem, we constructed a set of chimeric genes capable of producing mRNAs in which rp and non-rp coding regions were linked in various combinations to the 5′ UTR of an authentic or mutated rp mRNA or to the 5′ UTR of a typical non-rp mRNA—i.e., β-actin (Fig. 1). These chimeric genes were transfected into P1798 cells, and the translational efficiency of the resultant mRNAs was assessed under normal growing conditions and after 24 hr of exposure to dexamethasone, either by the extent of mRNA recruitment into polyribosomes or by a direct measurement of the protein encoded by the chimeric mRNA. For the polyribosome

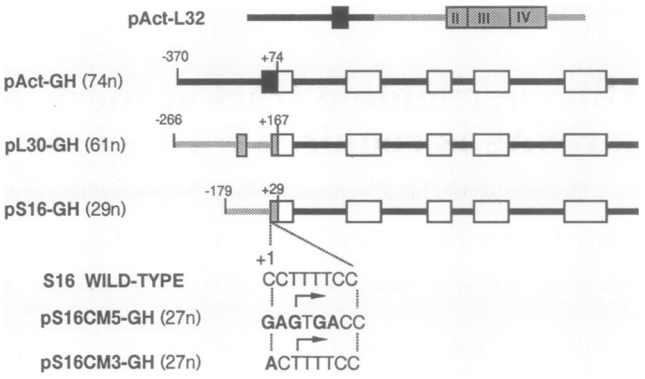

FIG. 1. Schematic representation of rp chimeric genes. The filled, open, and stippled boxes represent exons of rat β-actin, hGH, and mouse rp, respectively. The flanking and intron sequences are denoted as a thick solid line for actin and hGH genes and as a stippled thick line for rp genes. The number of nucleotides (n) derived from the 5′ UTR of the rp mRNAs, which precede the hGH sequences, is indicated in parentheses. The three octanucleotides at the bottom represent the 5′ pyrimidine tract in the wild-type rpS16 (spanning positions 1–8) and the two substitution mutants. Arrows indicate the major transcription start sites in the mutants.

assays, cytoplasmic extracts were fractionated into polyribosomes and subpolysomal particles (free mRNP) by sucrose gradient centrifugation, and the relative proportion of chimeric mRNAs in these fractions was determined by Northern blot analyses. As an internal control for these measurements, we also monitored the mRNAs produced by the endogenous rpL5 and β-actin genes.

The chimeric mRNA pAct-L32, in which the untranslated first exon (46 bp) of the mouse rpL32 gene was replaced by that (75 bp) of rat β-actin (Fig. 1), was translated in stably transfected P1798 cells with equal efficiency in the presence and absence of dexamethasone. Thus, its behavior resembled that of the endogenous actin mRNA rather than that of the endogenous rpL5 mRNA, which was dramatically dissociated from polyribosomes upon hormonal treatment (Act-L32 in Fig. 2). This result indicates that the 5′ UTR of an rp mRNA is essential for its translational control.

To establish whether the 5′ UTR is sufficient to confer translational control on another mRNA, we analyzed chimeras in which the 5′ UTR of either L30 mRNA (61 nucleotides) or S16 mRNA (29 nucleotides) is joined to the coding region of hGH mRNA (L30-GH and S16-GH, respectively, Fig. 1). As a control, we joined the hGH coding region to the 5′ UTR (74 nucleotides) of rat β-actin (Act-GH, Fig. 1). The L30-GH and S16-GH chimeric mRNAs were released from polyribosomes in dexamethasone-treated cells to roughly the same extent as the endogenous L5 mRNA (Fig. 2). In contrast, the Act-GH chimeric mRNA resembled the endogenous actin mRNA in not being dissociated from polyribosomes in response to the hormone treatment. These results, together with that for the Act-L32 chimera, indicate that the translational-regulatory element (TLRE) of the rp mRNAs is confined to their 5′ UTR.

To address the question of whether the pyrimidine tract at the extreme 5′ end of the UTR is necessary for the translational regulation of rp mRNA, we used rpS16 mutant genes in which one or more of these pyrimidines is substituted by purines (38). In one mutant, CM-5, five of the eight pyrimidines are substituted with purines (Fig. 1). High-resolution S1 nuclease protection analysis of the mRNA produced by this gene in transfected COS.7 cells indicated that transcription is initiated with the guanine residue at the +3 position (38). Thus, in contrast to S16-GH mRNA, which initiates transcription with the sequence CCUUUUCC, the otherwise identical mRNA produced by S16CM5-GH genes should

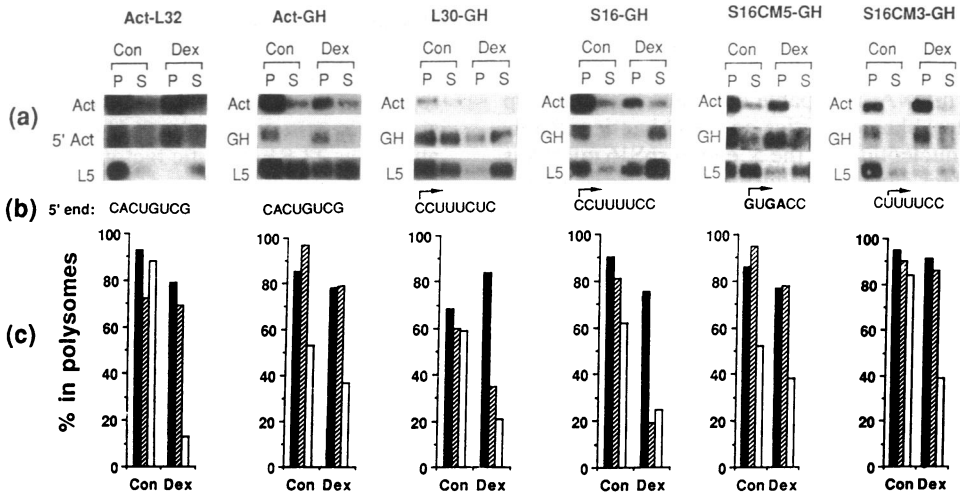

FIG. 2. Polyribosome–subpolyribosome distribution of various chimeric mRNAs. (*a*) P1798 cells were stably transfected with pAct-L32 or transiently transfected with the hGH chimeric genes indicated at the top of each column. Cytoplasmic extracts from untreated (lanes Con) or 24-hr dexamethasone-treated (lanes Dex) cells were centrifuged through sucrose gradients and separated into polyribosomal (lanes P) and subpolyribosomal (lanes S) fractions. Poly(A)$^+$ mRNA from equivalent aliquots of these fractions was analyzed by Northern blot hybridization with the probes indicated to the left of each autoradiogram. (*b*) The sequence at the 5′ end of the chimeric mRNAs. Arrows indicate the major transcription start sites in the wild-type and mutant rp genes, deduced from high-resolution S1 nuclease protection analyses (38); the C preceding the arrow in S16CM3-GH is a minor start site. The start site for the rat β-actin gene was localized to the triplet CAC; by analogy to the human β-actin gene (44), it is likely to be the A residue. (*c*) The percentage of the various RNAs in the polyribosomal fractions was determined by densitometric scanning of the autoradiograms in *a*. The filled and open bars represent the endogenous actin and L5 mRNAs, respectively. The hatched bar represents the mRNA produced by the chimeric transfected gene.

initiate transcription with the sequence GUGACC (Fig. 2*b*). The polyribosome analyses (Fig. 2*c*) showed that the translational characteristics of S16CM5-GH mRNA are more similar to actin mRNA than to rp mRNA. Particularly striking was the lack of translational repression in dexamethasone-treated cells (compare S16-GH Dex lanes vs. S16CM5-GH Dex lanes in Fig. 2). These results suggest that the integrity of the 5′-terminal pyrimidine tract is necessary for the translational regulation of rp mRNA.

To investigate the importance of length and composition of the pyrimidine tract for the functions of the TLRE, we used the mutant CM-3, which contains a single cytidine → adenosine change at the cap site (Fig. 1). Studies of the transcription of this mutant in transfected COS cells (38) and in cell-free nuclear extracts prepared from HeLa cells (44) have indicated that initiation occurs primarily at position +3 and to a lesser extent at +2. Thus, most of the S16CM3-GH mRNA should initiate transcription with the sequence UUUUCC, and a minor fraction (about 25%), with CUUUUCC (Fig. 2*b*). Somewhat surprisingly, we observed that this subtly altered mRNA also lacked the translational characteristics of rp mRNA (Fig. 2). This result could be interpreted to mean either that a minimum length of seven or eight pyrimidines is essential for an effective TLRE or, if the contribution of the minor fraction is insignificant, that a cytidine residue at the 5′ terminus is required or that both of these features are critical.

In addition to the indirect assessment of the translational efficiencies by analysis of the relative polyribosomal distribution of the hGH chimeric mRNAs, we also used a RIA to measure directly the synthesis of the hGH. Since this polypeptide is secreted from the cells, we determined its accumulation in the culture medium during the last 3 hr of the 24-hr incubation period, either in the presence or the absence of dexamethasone. To assess the net effect of the hormone on the translation of the hGH mRNA, the amount of hGH obtained by the RIA was divided by the content of hGH mRNA in each sample of transfected cells. The mRNA values were determined by Northern blot analysis of poly(A)$^+$ mRNA from roughly equivalent amounts of hormonally treated or untreated cells. The ratio of hGH produced by dexamethasone-treated and control cells was compared for three different chimeric constructs: S16-GH, S16CM5-GH, and S16CM3-GH (Table 1). The results of this analysis were in good agreement with those obtained by the polyribosome assay. For S16-GH, the amount of hGH synthesized by the hormone-treated cells was about one-quarter of that synthesized by control cells, whereas for S16CM5-GH and S16CM3-GH, there was no significant difference in the amount of hGH synthesized by control and dexamethasone-treated cells. Hence, both assays suggest that the selective repression of the translation of S16 mRNA by glucocorticoids requires an uninterrupted stretch of more than six pyrimidines at the 5′ end of the mRNA and possibly a cytidine residue at the cap site.

DISCUSSION

The foregoing experiments show that the translational control of rp mRNAs depends on the sequence features of their extreme 5′ end. In agreement with the results of similar

Table 1. Synthesis of hGH by chimeric mRNAs containing alterations in the 5′-terminal pyrimidine tract

Exp.	hGH measurement	Ratio Dex-treated/control		
		S16-GH	S16CM5-GH	S16CM3-GH
1	Protein (RIA)	0.49	0.86	0.58
	mRNA	2.6	0.83	0.64
	Protein/mRNA	0.19	1.04	0.90
2	Protein (RIA)	0.41	0.77	1.0
	mRNA	1.24	0.66	1.0
	Protein/mRNA	0.33	1.17	1.0
Mean	Protein/mRNA	0.26	1.1	0.95

P1798 lymphosarcoma cells were transfected with each of the indicated chimeric genes and grown for 24 hr in normal medium and for an additional 24 hr in the presence or absence (control) of dexamethasone (Dex) with a change of medium 3 hr prior to harvesting. The amount of hGH protein in equivalent volumes of medium at the time of harvest was assayed by RIA and expressed as a ratio of hormone-treated to control cells. These values were divided by the relative amount of hGH mRNA in equivalent samples of hormone-treated and control cells to give a measure of the translational efficiency of each chimeric mRNA.

investigations in other systems (ref. 17; L. Bowman, personal communication), we have observed that a segment of the 5′ UTR of rpL30 or rpS16, when joined to the coding region of an hGH reporter mRNA, is capable of conferring typical rp translational control characteristics on the chimeric mRNA. No more than 29 nucleotides of the 5′ end of rpS16 mRNA is needed for this effect, thus localizing the TLRE to a relatively short segment with no significant base-pairing potential.

The pyrimidine tract adjacent to the cap site appears to be an essential component of the TLRE, as judged by the fact that mRNA produced by mutant S16-hGH genes with one or several purine substitutions in this 5′ terminal segment does not exhibit translational control. Previous studies of the *in vivo* (38) and *in vitro* (45) transcription of mutant rpS16 genes bearing these same substitutions indicated that these mutations cause the major transcriptional initiation site to be displaced two nucleotides downstream from that of the wild-type S16 gene. Since all of the known rpS16 promoter elements are present in the S16-hGH constructs, it seems reasonable to suppose that the same displacement occurs in the transcription of the chimeric genes. Thus, it would appear that a change in the 5′ terminal segment from CCUUUUCC to GUGACC (in S16CM5) or to UUUUCC (in S16CM3) is sufficient to abolish the function of the TLRE.

A comparison of the 5′-terminal sequences of 12 vertebrate rp genes, for which the cap site was determined by S1 nuclease protection analysis or primer extension (Table 2), indicates (*i*) a strong preference for cytidine as the initiating residue, (*ii*) that no 5′-terminal pyrimidine tract is less than eight nucleotides in length, and (*iii*) that the particular sequence of pyrimidines within the tract is variable. Consistent with these properties, our results with the rpS16 mutants suggest that length and/or the 5′-terminal cytidine residue may be functionally critical features of the pyrimidine tract. There also may be important structural information in the sequences immediately downstream of the pyrimidine tract. Although there are no obvious common sequence motifs in this region, there conceivably could be subtle patterns of nucleotides that contribute importantly to the overall structure of the extreme 5′ end. Further experiments with additional mutant mRNAs will be required to verify these suppositions and to establish the minimum requirements for TLRE function.

Beyond a precise definition of the cis-acting TLRE, it is also important to identify the trans-acting factor that determines the activity of this regulatory element. Conceivably there might be a factor that specifically binds to the TLRE and prevents the mRNA elements from interacting with ribosomes and other components that are essential for protein synthesis. An example of such a factor is the protein that represses the translation of ferritin mRNA by binding to a stem–loop structure in the 5′ UTR of this mRNA (46). If an analogous TLRE-specific factor exists, it presumably would be quantitatively regulated or qualitatively modified in response to changes in cellular growth rate. An interaction of protein(s) with the TLRE of mRNA that is sequestered in translationally inactive mRNP particles was suggested by a study of mouse P21 mRNA (47). This mRNA, although encoding a nonribosomal protein, shares with rp mRNAs similar structural and regulatory features—namely, a 5′-terminal oligopyrimidine tract and growth-dependent translational control (28, 31). Treatment of P21 mRNA with RNase T1 revealed that the guanine residues immediately downstream of the 5′ pyrimidines are much more accessible when the mRNA is engaged in polyribosomes than when packaged in mRNP particles. Additional evidence for the involvement of such mRNP proteins in translational control has been recently reviewed by Scherrer (48).

In addition to a TLRE-specific factor, translational control may involve a factor that is part of the general protein-synthesizing machinery. Because the rp mRNAs normally exist in a delicate balance between translationally active and inactive states (14, 15), they should be especially sensitive to changes in the activity of a critical translational initiation factor. If such a factor had a particularly low affinity for rp mRNA, glucocorticoids could exert their effect on translational repression by decreasing its activity or content. The fact that dexamethasone reduces the abundance of the mRNAs encoding the eukaryotic translational initiation factors eIF-4A, eIF-2α, and eIF-4D by 60–70% in P1798 cells (43) is consistent with this idea. Moreover, an increase in the translational efficiency of rp mRNAs during transition of Swiss 3T3 cells from nongrowing to growing states occurs simultaneously with enhanced phosphorylation of the eukaryotic initiation factor eIF-4E (13). This factor, which is also known as the cap-binding protein, constitutes, together with eIF-4A and a 220-kDa protein, the eIF-4F complex. Due to the relatively low abundance of eIF-4E (49), the entire eIF-4F complex is a limiting component in the binding of eukaryotic mRNAs to the ribosome. Since this step is generally considered to be the overall limiting step in translation (50), eIF-4F is a prime candidate for modulating translation efficiency. Indeed, the latter has been implicated in discrimination between weak and strong mRNAs (reviewed in refs. 51 and 52). Since rp mRNAs apparently have normal cap structures, as inferred from the heterogeneous products observed in S1 nuclease protection analyses (20, 38, 45), they should be able to bind eIF-4F. If indeed, this or another initiation factor is involved in the specific translational control of rp mRNAs, we would presume that its interaction with these mRNAs is affected by the unique structure at their 5′ end.

Clearly, isolation and subsequent structural and functional characterization of the relevant trans-acting factor should resolve whether it is of a general or specific nature and whether it acts as a repressor or an activator.

Table 2. 5′ Oligopyrimidine tracts in vertebrate rp mRNAs

rp mRNA	5′-Terminal sequence	Consecutive pyrimidines, no.	Ref.
mS16	CCUUUUCCG	8	20
mL32	CUUCUUCCUCG	10	18
mL30	CCUUUCUCG	8	19
mL7	CUCUCUUCUUUUCCG	14	21
mL7a	*C*UUU*CUUUCUCCA	8/12	22
hS14	†CUCUUUCCG	8	24
hS17	CCUCUUUUA	8	25
XL1	CCUUUUCUCUUCG	12	16
XL14	CCUUUCCUCCCCG	12	16
XS19	CCUUUCCUUCG	10	16
XS8	CCUCUUCUA	8	16
XL32	CCUUUUCCUCCA	11	44

m, h, and X refer to mouse, human, and *Xenopus laevis* mRNAs, respectively.
*Two start sites detected.
†Center of a diffuse starting region.

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