Overexpression of poly(A)-binding protein down-regulates the translation or the abundance of its own mRNA

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Abstract Poly(A)-binding protein (PABP) mRNA is subject to autoregulation through a 61 nucleotides long A-rich sequence in its 5′ untranslated region (UTR). Here, we show that this mode of regulation is exerted in a cell type-specific manner. Thus, overexpression of PABP in mouse NIH 3T3 fibroblasts represses the translation of the respective endogenous mRNA or that of a chimeric mRNA containing just the 5′ UTR of PABP mRNA. In contrast, ectopic expression of PABP in human embryonic kidney 293 cells down-regulates the abundance of the endogenous PABP mRNA, rather than affecting its translational efficiency. Transfection experiments with chimeric constructs suggest that the lack of translational autoregulation of endogenous PABP mRNA in these cells appears to reflect the presence of an overriding regulatory element outside the A-rich region.

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Key words: Translational regulation; Poly(A)-binding protein; Polysome; Autoregulation

1. Introduction

Poly(A)-binding protein (PABP) is a major eukaryotic RNA-binding protein that exhibits a preferential affinity for poly(A) stretches. This highly conserved protein has been implicated in initiation of translation [1], mRNA stability [2], regulation of poly(A) tail length during the polyadenylation reaction [3,4] or poly(A) shortening [5].

The pivotal role played by PABP at multiple levels of post-transcriptional regulation of gene expression has prompted us to isolate the human gene and to study the control mechanism of its own expression [6]. Structural and functional analyses have established PABP mRNA as a new member of the TOP mRNA family. mRNAs of this family are known to encode components of the translational apparatus like ribosomal proteins (rps) and elongation factors and to contain an oligopyrimidine tract at the 5′ terminus (5′ TOP). This motif mediates their translational control in a growth-dependent manner [7]. Indeed, the translational efficiency of PABP mRNA tightly correlates with the growth status of cultured fibroblasts as well as rat liver [6].

In addition, human PABP mRNA contains an A-rich region spanning position 73–133, which is evolutionarily conserved throughout the eukaryotes ([8] and references therein). The identification of this motif led to the hypothesis that PABP mRNA is autogenously regulated at the translational level through binding of the resulting protein to the 5′ untranslated region (UTR) [9]. Indeed, in vitro experiments have shown that addition of PABP to a cell free translation system selectively inhibits the translation of PABP mRNA and that this repression is mediated through binding to the A-rich region [8,10]. Likewise, it has recently been shown that ectopic expression of PABP in HeLa cells decreases the translational efficiency of endogenous PABP mRNA or of a chimeric mRNA containing the A-rich region and short flanking sequences [11].

In the present report, we demonstrate that the PABP mRNA is subject to cell type-specific autoregulation. Thus, ectopic expression of PABP leads to translational repression of endogenous PABP mRNA in NIH 3T3 cells, but to a reduced abundance of this mRNA in 293 cells.

2. Materials and methods

2.1. Cell culture and DNA transfection

NIH 3T3 mouse fibroblasts were grown and transiently transfected as described [12]. Human embryonic kidney 293 cells were grown as described [13]. These cells were transiently transfected by DNA-calcium phosphate co-precipitation essentially as described [14], except for using HEPES-buffered saline pH 7.15 instead of *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline. Assessment of the transfection efficiency was carried out by inclusion of 1.7 μg pEGFP-C1 (Clontech) in the transfection mixture and 20 h later, monitoring the relative distribution of cells expressing the green fluorescence protein under a fluorescence microscope.

2.2. Polysomal fractionation and RNA analysis

Four 100 mm plates of monolayer cultures were used for polysomal analysis. Harvesting and lysis of cells as well as size fractionation of polysomes by sedimentation through sucrose gradients were performed as described [15]. Gradients were divided into two fractions: polysomal, which includes mRNAs loaded with two (disomes) or more ribosomes, and subpolysomal, which contains monosomes, ribosomal subunits and mRNA ribonucleoproteins. RNA was extracted from each fraction by Ultraspec RNA (Biotecx Laboratories, Houston, TX, USA) or EZ-RNA (Biological Industries, Kibbuz Beit Haemek, Israel), according to the suppliers' instructions and poly(A)$^+$ mRNA was isolated by an oligo(dT) column as described [16]. RNA (Northern) blot analysis was performed as described [17]. Quantification of the radioactive signals on the blots was carried out by a Bio Imaging Analyzer (Fujix BAS 1000, Fuji, Japan).

2.3. Construction of plasmids

pAct(polyA)-GH and pAct(polyU)-GH were constructed by 5′ end phosphorylation with polynucleotide kinase and annealing of two complementary oligonucleotides: PABP-50 (gatccAAAAAATCCAAAAAAAATCTAAAAAAAATCTTTTAAAAAACCCCAAAAAAATTTACAAAAAA) and PABP-51 (gatcTTTTTTGTAAATTTTTTTGGGGTTTTTTAAAAGATTTTTTTAGATTTTTTTTGGATTTTTTg). Lowercase letters correspond to the protruding ends of the *Bam*HI site and uppercase letters correspond to nucleotides +73–+133, or +133–+73, of the human PABP gene. The resulting double-stranded oligonucleotide was inserted into the *Bam*HI site of pAct(28)-GH [12] in sense and antisense orientations, respectively.

pFLAG-PABP was constructed by inserting a 1991 bp *Bgl*I (trimmed by T4 DNA polymerase)-*Ssp*I fragment from human

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PABP cDNA [18] containing the entire coding sequence in between *Hin*dIII (filled in by Klenow enzyme) and *Eco*RV sites, within the multicloning site of pFLAG-CMV-2 (Eastman Kodak Company, Cat. number 8275604).

2.4. Molecular probes

The isolated fragment probes used in the Northern blot analysis were a 1.15 kb *Pst*I fragment containing mouse α-actin cDNA [19], 1.07 and 1.78 kb *Eco*RI fragments containing human PABP cDNA [18], a 0.5 kb *Eco*RI-*Bgl*I fragment corresponding to the 5′ UTR of human PABP mRNA (PABP 5′ UTR probe), a 1.4 kb *Eco*RI fragment containing human iPABP cDNA [20], a 0.97 kb fragment bearing the rpL32 processed gene 4A joined to the 5′ and 3′ flanks of 3A [21], a 1.5 kbp *Xba*I fragment containing mouse *S*-adenosylmethionine decarboxylase cDNA [22] and a 0.8 kb *Hin*dIII fragment containing a hGH cDNA (kindly provided by T. Fogel, Bio-Technology General).

3. Results

3.1. PABP mRNA is translationally autoregulated in NIH 3T3 cells

Recent experiments with HeLa cells have shown that PABP mRNA is subject to translational autoregulation [11], but not to growth-dependent translational control (Hornstein et al., manuscript in preparation). Hence, we set out to examine the possibility that both modes of regulation would be incompatible in the same cell. To this end, we transiently co-transfected NIH 3T3 cells with a vector expressing human PABP and a plasmid encoding chimeric GH mRNA, which either contained the poly(A)-rich sequence (PABP-GH2) or lacked this element (PABP-GH1) (Fig. 1). It should be noted that the exogenous PABP transcript lacks the 5′ TOP motif as well as the poly(A)-rich sequence and is therefore refractory to regulation mediated through either of these motifs. Moreover, it is about 800 nucleotides (nt) shorter than the endogenous PABP mRNA and forms a readily distinguishable hybridization band. The translational efficiency of the chimeric PABP-GH mRNAs was assessed by their distribution between polysomal and subpolysomal fractions. The results presented in Fig. 1 demonstrate that overexpression of exogenous PABP led to a remarkable unloading of PABP-GH2 mRNA from polysomes in comparison with its polysomal association when cells were co-transfected with empty vector (26 versus 69% in polysomes, respectively). Ectopic expression of PABP, however, lacked such an effect on PABP-GH1 mRNA (92 versus 84% in polysomes, respectively). The fact that another TOP mRNA, encoding ribosomal protein L32, is mostly in polysomes under all transfection conditions excludes the possibility that the repression of endogenous PABP mRNA resulted from translational repression caused by growth arrest.

Interestingly, overexpression of exogenous PABP represses the translation of the endogenous PABP mRNA as well (Fig. 1). It should be noted, however, that the apparent inhibitory effect on endogenous PABP mRNA is under-represented, as the transfection efficiency (judged by the proportion of cells expressing the green fluorescence protein) was about 30%.

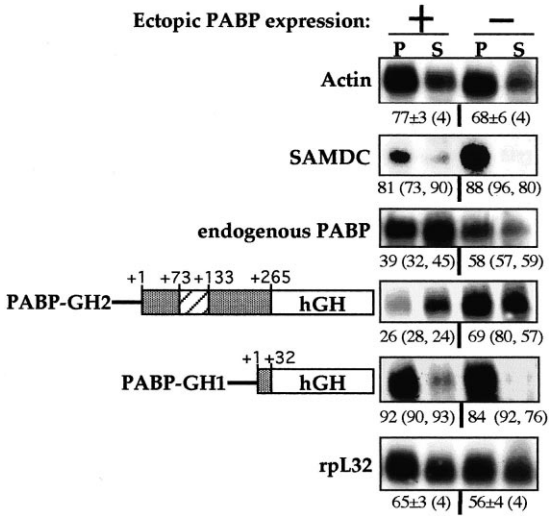

Fig. 1. Overexpression of PABP in NIH 3T3 cells represses the translation of the respective endogenous mRNA or a chimeric mRNA containing just the 5′ UTR of PABP mRNA. NIH 3T3 cells were transiently co-transfected with 3 μg of pPABP-GH1 or pPABP-GH2, 12 μg of pFLAG-PABP expressing exogenous PABP (+) or empty vector (pFLAG-CMV-2) (−) and 1.7 μg of pEGFP-C1. 24 h later, the transfection efficiency was assessed to be 30%, by monitoring the expression of the green fluorescence protein under a fluorescence microscope. Cells were harvested 48 h post-transfection and their cytoplasm was extracted and size-fractionated by sucrose gradient centrifugation. RNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization. The polysomal distribution of the indicated mRNAs was monitored with the respective cDNA probes. pPABP-GH1 and pPABP-GH2 are schematically presented adjacent to the respective autoradiograms. The 5′ flanking sequence and exon 1 of the PABP gene are denoted as a thin line and dotted box, respectively. The poly(A)-rich sequence is presented as a striped box. The relative translational efficiency of each mRNA is numerically presented beneath autoradiograms as percentage of the mRNA engaged in polysomes. These figures are expressed as averages ± S.E.M. of the number of determinations in parentheses or the average with the individual values in parentheses if only two determinations are available.

Furthermore, since we have previously shown that PABP mRNA is translationally regulated in a growth-dependent fashion in this cell line [6], we conclude that autoregulation and growth-dependent translational control can operate in the same cell type.

To test whether mRNAs, besides PABP mRNA, are subject to translational repression by the ectopic expression of PABP, we set out to identify other mRNAs with an A-rich sequence within their 5′ UTR. To this end, we searched the GenBank for conserved eukaryotic mRNAs with a stretch of at least eight A residues (as in human PABP mRNA) within their 5′ UTR. Such a survey has disclosed that the mRNA encoding *S*-adenosylmethionine decarboxylase (SAMDC) from various mammals meets these criteria (Table 1). However, despite the presence of an A-rich sequence (31 A residues out of 41 nt)

Table 1
Poly(A)-rich sequences in the 5′ UTR of SAMDC mRNA from various mammals

Species	Poly(A)-rich sequence within the 5′ UTR	Proportion of (A)s	Accession number
Homo sapiens	AAAAAAAguuAAuAuAAAAuuAuAgcAAAAAAAAAAA-118 nt-AUG	27/37	M21154
Mus musculus	AAAAAAAAguuAAuAuAAAAuuAuAgcAAAAAAAAAAAAAA-118 nt-AUG	31/41	D12780
Rattus norvegicus	AAAAAAAguuAAuAuAAAAuuAuAgcAAAAAAAAAAAAA-118 nt-AUG	29/39	M34464

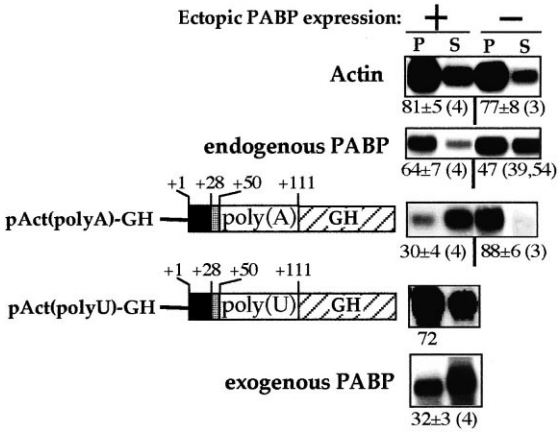

Fig. 2. Overexpression of PABP in 293 cells represses the translation of a chimeric mRNA containing the PABP poly(A)-rich sequence in its 5′ UTR, but lacks such an effect on endogenous PABP mRNA. 293 Cells were transiently co-transfected with 5.5 μg of pAct-(polyA)-GH or pAct(polyU)-GH, 13.5 μg of pFLAG-PABP expressing exogenous PABP (+) or empty vector (pFLAG-CMV-2) (−) and 1.7 μg of pEGFP-C1. The transfection efficiency was estimated to be 90% by monitoring the proportion of cells expressing the green fluorescence protein. Cells were harvested 24 h post-transfection and their cytoplasmic extract was size-fractionated by sucrose gradient centrifugation. RNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization. Actin cDNA and PABP 5′ UTR probes were used for monitoring the polysomal distribution of the respective endogenous mRNAs, whereas the exogenously expressed mRNAs were detected by GH and PABP cDNA. pAct(polyA)-GH and pAct(polyU)-GH are schematically depicted adjacent to the respective autoradiograms. The 5′ flanking sequence and the first transcribed nucleotides of the actin gene are denoted as a thin line and filled box, respectively. A linker sequence is presented as a stippled box. The poly(A) domain is the authentic 61 nt poly(A)-rich sequence from the human PABP gene, whereas the poly(U) domain represents the same sequence that was inversely inserted. The relative translational efficiency of each mRNA is numerically presented beneath autoradiograms as the percentage of the mRNA engaged in polysomes. These figures are expressed as averages ± S.E.M. of the number of determinations in parentheses or the average with the individual values in parentheses if only two determinations are available.

within the 5′ UTR of mouse SAMDC mRNA, its translation was refractory to the ectopic expression of PABP (compare the polysomal association of SAMDC mRNA with that of PABP). It appears, therefore, that overexpression of PABP in NIH 3T3 cells selectively represses the translation of its own mRNA.

3.2. *Translation of endogenous PABP is refractory to overexpression of PABP in 293 cells*

The relatively inefficient transfection of NIH 3T3 cells has limited our ability to precisely quantify the effect of overexpression of PABP on endogenous transcripts. Therefore, we turned to 293 cells which, under our experimental conditions, showed a 70–100% transfection efficiency. Hybridization with a probe, detecting both exogenous and endogenous PABP mRNAs, revealed that the signal of the exogenous PABP mRNA in 293 cells transfected with PABP expression vector was approximately 16-fold stronger (Fig. 3a and data not shown). Nevertheless, the abundance of PABP in these cells was only 2-fold higher than that in cells transfected with empty vector (Fig. 3c). To assess the effect of the overexpression of PABP in these cells, we initially examined the translation of a chimeric hGH mRNA starting with the first 28 nt of rat β-actin mRNA followed by the 61 nt long A-rich sequence from human PABP mRNA (see Act(polyA)-GH in Fig. 2). The translational efficiency of the chimeric transcript, as judged by the polysomal association, was repressed 3-fold in the presence of ectopic expression of PABP (from 88% in polysomes in cells transfected with empty vector to 30% in PABP overexpressing cells). If, however, the A-rich sequence is inversely inserted, as in pAct(polyU)-GH (Fig. 2), the resulting mRNA instead contains a U-rich sequence. This transcript is efficiently translated even in the presence of excessive PABP expression (72% in polysomes). It appears therefore that the 61 nt long A-rich sequence from PABP 5′ UTR is both necessary and sufficient for conferring PABP-dependent translational repression on a heterologous mRNA. Surprisingly, despite the apparent translational repression of Act-(polyA)-GH mRNA and the high transfection efficiency of these cells (approximately 90%), endogenous PABP was refractory to the ectopic expression of PABP (see endogenous PABP in Fig. 2). Thus, under these circumstances, this mRNA remained mostly (64%) associated with polysomes. It should be noted that relatively low polysomal association is a hallmark of all TOP mRNAs even in growing cells (see rpL32 mRNA in Fig. 2 and [7]).

Surprisingly, although lacking the 5′ TOP and the A-rich sequences of PABP mRNA, the exogenous PABP transcript is translationally repressed to the same extent as a transcript containing only the 61 nt A-rich sequence (exogenous PABP in Fig. 2b). One plausible explanation is that inherent struc-

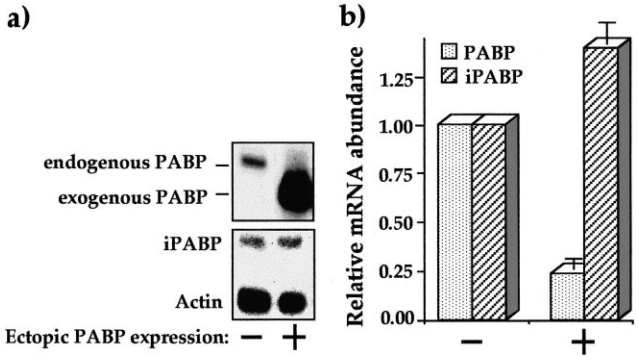

Fig. 3. Overexpression of PABP in 293 cells reduces the abundance of endogenous PABP mRNA. (a) 293 cells were transiently co-transfected with 13.5 μg of pFLAG-PABP expressing exogenous PABP (+) or empty vector (pFLAG-CMV-2) (−) and 2.8 μg of pEGFP-C1. The transfection efficiency was estimated to be 90% by monitoring the proportion of cells expressing the green fluorescence protein. Cells were harvested 24 h post-transfection, poly(A)$^+$ mRNA was extracted and aliquots representing a similar number of transfected cells were subjected to Northern blot analysis with cDNA probes for iPAPB, actin and PABP. The distinct band of endogenous PABP mRNA has readily been detected in both lanes of this experiment when a specific probe directed against the 5′ UTR was used (data not shown). (b) The abundance of mRNAs encoding endogenous PABP, iPABP or actin was assessed by phosphorimaging of the radioactive signals obtained in experiments like that presented in (a) and Fig. 2. The relative abundance of PABP or iPABP mRNAs is presented as the ratio of their abundance to that of actin. The relative abundance of PABP or iPABP mRNAs in cells transfected with pFLAG-CMV-2 was arbitrarily set at one and the relative abundance of the corresponding mRNAs in cells transfected with pFLAG-PABP was normalized to this value. The results are presented as an average ± S.E.M. of four experiments.

tural properties of the chimeric mRNA encoding exogenous PABP render it inefficiently accessible to the translational apparatus of 293 cells, irrespective of the nature of its product.

3.3. The relative abundance of PABP mRNA is autogenously regulated in 293 cells

Simultaneously with the demonstration that endogenous PABP mRNA is translationally refractory to overexpression of PABP in 293 cells, we have reproducibly observed a decline in the abundance of this mRNA relative to that of actin mRNA. We have assumed that PABP mRNA might be autoregulated in this cell line by modulating its abundance rather than its translation. To directly examine this possibility, we quantified the overall autoradiographic signals (in polysomal and subpolysomal fractions) of mRNAs encoding endogenous PABP, iPABP and actin in 293 cells transfected with PABP expression vector or empty vector. Our results indicated that ectopic expression of PABP led to a 4-fold decrease in the abundance of endogenous PABP mRNA relative to that of actin mRNA (Fig. 3a and b). The selectivity of this effect is underscored by the fact that the relative abundance of iPABP mRNA, which exhibits 74% homology with PABP mRNA [20], was not reduced under these circumstances (Fig. 3a and b). The template-product relationships between exogenous PABP mRNA and its encoded protein do not enable us to unequivocally determine whether the abundance of this mRNA is also repressed in the presence of excessive amounts of PABP. Nevertheless, it appears that endogenous PABP gene expression can be autoregulated through its mRNA abundance (as in 293 cells) and its mRNA utilization (as in NIH 3T3 cells (Fig. 1) and HeLa cells [11]).

4. Discussion

Translational *cis*-autoregulatory elements of PABP mRNA have previously been shown to reside within 89 nt [11]. However, we have now delimited this element precisely to the 61 nt A-rich sequence spanning positions 73–133. This sequence can confer translational autoregulation on a heterologous transcript even in 293 cells, where endogenous PABP is refractory to this mode of regulation.

It is worth noting that although the abundance of exogenous PABP mRNA is 16-fold that of the endogenous transcript in 293 cells, the actual increase in the PABP synthesis capacity is much lower. Thus, overexpression of PABP is accompanied by a 4-fold decrease in the relative abundance of the endogenous PABP mRNA (Fig. 3a and b), which means an increase by a factor of only four in the overall abundance of PABP mRNA. Furthermore, only one-third of the exogenous PABP mRNA is engaged in translation (Fig. 2), in comparison with the two-thirds of the endogenous mRNA. Western blot analysis has disclosed that this 2-fold increase in overall PABP synthesis potential is indeed reflected in a similar enhancement in the steady state level of PABP (data not shown). It appears, therefore, that autoregulation is attained with merely doubling the abundance of PABP in 293 cells.

Autoregulation at the translational level has previously been demonstrated for several mRNAs. Thus, synthesis of several ribosomal proteins is repressed by interaction of the protein products with their own transcript in *Escherichia coli* [23] and the yeast [24,25]. Likewise, the ability of specific mammalian proteins to bind their own mRNAs and to repress their own translation in a cell free translation system has been reported for thymidylate synthase [26,27], dihydrofolate reductase [28,29], p53 [30] and PABP [8,10]. Nonetheless, evidence that a translational autoregulatory mechanism indeed operates in mammalian cells has been documented so far only for mRNAs encoding α-tubulin [31], protein phosphatase type 2A [32] and PABP (Fig. 1 and [11])

Apparently, overexpression of PABP in 293 cells reduces the abundance of endogenous PABP through transcriptional and/or post-transcriptional mechanisms, rather than repressing its translation. It should be noted, however, that the inefficient transfection of NIH 3T3 cells does not allow us to unambiguously determine whether, in addition to the translational repression, a decline in the abundance of endogenous PABP occurs in these cells. Hence, it is not clear whether 293 cells have lost the translational autoregulation but have maintained autoregulation at the abundance level of PABP mRNA or have undergone a switch from one mode of autoregulation to the other. Significantly, overexpression of yeast ribosomal proteins L32 leads to feedback inhibition of both the splicing of the respective primary transcript and translation of the mature mRNA [24,25]. Post-transcriptional autoregulation has been demonstrated for mRNAs encoding several other ribosomal proteins like yeast CRY2 (rpS14) [33,34] and rpL2 [35,36], *Xenopus* rpL1 [37,38], as well as transformer-2 transcript from *Drosophila* [39]. Transcriptional autoregulation has been shown for genes encoding mammalian rpS14 [40], interleukin-6 [41] as well as for circadian oscillators in both plant [42] and *Drosophila* [43]. Characterization of the level at which the abundance of PABP mRNA is autoregulated, and delineation of the respective *cis*-regulatory element(s), should await further studies.

The differential effect of the 61 nt long A-rich sequence on mRNAs encoding endogenous PABP or Act(polyA)-GH mRNA in 293 cells is reminiscent of the differential effect of the 5′ TOP on mRNAs encoding endogenous EF2 and PABP and the corresponding chimeric constructs ([13] and manuscript in preparation). The A-rich sequence resides within the first exon of the human gene [44] and therefore, its selective elimination from PABP mRNA in 293 cells by differential splicing is highly unlikely. Instead, we assume that sequences within the native PABP mRNA can override the translational autoregulatory properties of the 61 nt long A-rich sequence in a cell type-specific manner. Conceivably, translational autoregulation of endogenous PABP mRNA is excluded in 293 cells because these cells differentially express a protein(s) that binds to a sequence outside the A-rich region. Presumably, binding of this protein enables continuous scanning of the 5′ UTR by the 40S ribosomal subunit, despite the binding of PABP to this region. However, in the absence of the binding sequence of this putative protein, as in the case of Act-(polyA)-GH mRNA, it can no longer eliminate the translational repression exerted by excess PABP. It should be noted that a precedent for failure to demonstrate autoregulation of PABP synthesis has been set in yeast with an inactivated poly(A) polymerase. The loss of poly(A) and, consequently, the increase in the ratio of PABP to poly(A) in these cells had only a minor effect on the total level of PABP [45].

Being a determinant in multiple mechanisms controlling the translation of mRNA, its stability and the poly(A) tail length seems to justify a fine-tuning of PABP accumulation. It appears, therefore, that the combination of growth-dependent

translational control and autoregulation ensures that the production of PABP meets precisely the changing requirements to prevent excessive amounts of free protein. Indeed, it has been shown that overexpression of PABP in *Xenopus* oocytes leads to interference in the stage-specific program of protein synthesis during oocyte maturation [46].

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