Adenosine-rich elements present in the 5′-untranslated region of PABP mRNA can selectively reduce the abundance and translation of CAT mRNAs in vivo

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Abstract The poly(A)-binding protein (PABP) is a highly conserved eukaryotic protein whose synthesis is regulated at the post-transcriptional level. The binding of PABP to the poly(A)-rich element found in the 5′-untranslated region (5′UTR) of PABP mRNA specifically inhibits its own translation. In this report, we show that similar adenosine-rich elements in the 5′UTR of the chloramphenicol acetyl-transferase (CAT) gene can significantly reduce the reporter mRNA abundance and translation in human 293 cells. The reduction in mRNA level, but not CAT expression, is dependent on the size of the 5′UTR poly(A) element. Furthermore, one 5′UTR-tethered PABP molecule is enough to inhibit CAT expression without affecting its mRNA level. We propose that the control of PABP synthesis may involve mRNA decay and the repression of translation.
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Key words: PABP; Post-transcriptional control; mRNA stability; 5′-untranslated region; Poly(A)-rich element and eukaryote

1. Introduction

Messenger RNA translation and decay are tightly coupled events [1]. However, the specific correlation between translation inhibition in *cis* and mRNA decay is more controversial [2–4]. Current data show that mRNA deadenylation precedes decapping in mammalian cells [5] through changes in the interactions between proteins that bring together the 5′ and 3′ ends of mRNA [6]. Cross-talk between the mRNA 5′ and 3′ ends is involved in the access of cap to the decapping complex [7,8]. The involvement of the poly(A)-binding protein (PABP) in mRNA circularization [9,10] and the initiation of translation [11–13] corroborates the findings that PABP plays a key role in mRNA decay [14,15].

PABP expression is regulated by a translational repression control mechanism that was first proposed by Sachs et al. [16]. The basis of the repression mechanism was confirmed in vitro [17,18] and in vivo [19], and the binding of PABP to the poly(A)-rich element (pARE) present within the 5′-untranslated region (5′UTR) of its own mRNA was shown to be responsible for stalling the 40S ribosomal subunit during mRNA scanning [20]. In the presence of fetal calf serum (FCS), PABP translation increases in mouse cells [21,22], and the cells apparently respond to this stimulus through a 5′-terminal oligopyrimidine tract motif present in PABP mRNA [23]. A third post-transcriptional mechanism controlling PABP expression may regulate the abundance of PABP mRNA and depends on the cell type studied. In rat L6 myoblasts, differentiation to myotubes decreases the steady-state level of PABP mRNA [24], and in human 293 cells overexpression of ectopic PABP down-regulates the abundance of its endogenous mRNA [25]. However, no mechanisms have been proposed to explain the role of mRNA stability in the regulation of PABP synthesis.

In this study, we show that the presence of the pARE found within the 5′UTR of human PABP mRNA, or an equivalent tract of contiguous adenosine residues placed in the 5′UTR of the chloramphenicol acetyl-transferase (CAT) gene, can significantly reduce the abundance of reporter transcripts and prevent their translation in human 293 cells. Moreover, the length of the 5′UTR poly(A) elements can independently determine the level of reporter transcripts and their susceptibility to translation repression.

2. Materials and methods

2.1. Plasmids

The oligonucleotide primers pARE (CTAGCAGGCCTA$_6$TCCA$_8$-TCTA$_7$TCTTTTA$_6$CCCCA$_7$TTTACA$_6$T) and 61As (CTAGCAGG-CCTA$_{61}$T) were originally cloned in the vector pTZ18R [17]. The 61As tract was substituted for the 20As and 10As tracts by digestion with *Stu*I and *Xba*I and ligation with one of two pairs of oligonucleotide primers (CCTA$_{20}$T and CCTA$_{10}$T), respectively (Melo et al., submitted). pTZ18R-derived construct fragments were extracted with *Sma*I–*Hin*dIII and inserted in the *Hin*dIII site of pSV2-CAT [26] to produce pSV-pARE, pSV-61As, pSV-20As, and pSV-10As. The recombinant plasmids pSV-pARE and pSV-61A were selected for the two possible orientations to produce constructs with uridines

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Abbreviations: PABP, poly(A)-binding protein; UTR, untranslated region; β-gal, β-galactosidase; CAT, chloramphenicol acetyl-transferase; IRE, iron-responsive element; IRP, iron regulatory protein; GH, human growth hormone; MS2, viral MS2 coat protein; pARE, poly(A)-rich element; pURE, poly(U)-rich element; RRM, RNA recognition motif

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in their 5′UTR (pSV-pURE (poly(U)-rich element) and pSV-61Us). For the construction of MSC-15 and MSA-15, the human growth hormone (GH) gene of the plasmids MSC-GH and MSA-GH [27] was first replaced by the CAT gene through digestion with *Xba*I–*Hin*dIII and ligation to the *Bam*HI–*Hin*dIII fragment from pSV2-CAT. To extend the distance between the MS2 (a viral coat protein) binding site and cap, we inserted a 76 nt *Bam*HI–*Xba*I fragment from the polylinker region of plasmid pcDNA3 (Invitrogen) into the *Bam*HI site of MSC-15, producing the MSC-91 vector. The effector plasmids were constructed by cloning the in-filled *Nco*I–*Sma*I fragment from GST-PABP [17] into the in-filled *Xho*I site of the vectors pET15-b (Novagen) and pET-MS2 [14]. The MS2, PABP and MS2/PABP genes were subsequently extracted with *Xba*I–*Eco*RV and the in-filled fragment cloned into the *Eco*RV site of pcDNA3 to produce the plasmids pCMV-MS2, pCMV-PABP and pCMV-MS2/PABP, respectively.

2.2. Cell culture and DNA transfection

Approximately 1×10^6 or 2×10^5 293-EBNA cells were grown in six- or 24-well plates with Dulbecco's modified Eagle's medium supplemented with 10% FCS (Life Technologies) and antibiotics, respectively. Sub-confluent cultures were transfected with LipofectAMINE (Life Technologies), as recommended by the manufacturer. The six-well transfection of pSV-CAT constructs was done using 1.1 μg of reporter plasmids and 0.1 μg of pCMVβ (Clontech). The 24-well transfection of pSV-CAT constructs was done with 0.3 μg of reporter plasmids and 0.03 μg of pCMVβ. The six-well transfection of the MSC-91 and MS2 constructs was done with 1.05 μg of reporter plasmid, 0.05 μg of effector plasmid or pCDNA3 as a negative control, and 0.1 μg of pCMVβ. The 24-well transfection of the reporters containing the MS2 binding site and MS2 constructs was done with 0.25 μg of reporter vector, 0.025 μg of pCMVβ, and 0.25 μg of pCDNA3, or with 0.25 μg of reporter vector, 0.025 μg of pCMVβ and 0.025 μg of effector plasmid.

2.3. Reporter enzyme assays, RNA isolation and Northern blotting

20 h after the transfection began, the cells of the 24-well assays were washed twice in phosphate-buffered saline (PBS), harvested and lysed for the measurement of CAT and β-galactosidase (β-gal) as described in [28]. The cells of the six-well assays were washed twice in PBS after 20 h of transfection and the total RNA was extracted with 800 μl of Trizol (Life Technologies), as recommended by the manufacturer. The precipitated total RNA was re-suspended in 10 μl of 1× DNase buffer (Pharmacia) and digested with 7 U of RNase-free DNase I (Pharmacia) for 15 min at room temperature. The RNA (10 μg) was loaded into 1.2% formaldehyde-agarose gels, and Northern blotting was done as previously described [29,30].

2.4. Molecular probes and measurement of mRNA levels

The isolated fragments used as probes in Northern blot analysis were a 0.7 kb *Xba*I–*Hin*dIII fragment of pET-MS2 containing MS2, a 1.6 kb *Hin*dIII–*Bam*HI fragment of pSV2-CAT containing CAT, a 2.4 kb *Nco*I–*Sma*I fragment of GST-PABP containing the human PABP cDNA, and a 3.5 kb *Sca*I–*Not*I fragment of pCMVβ containing β-gal. Approximately 50 ng of each purified probe was labeled with $[\alpha\text{-}^{32}\text{P}]$dCTP using the Megaprime kit (Pharmacia) and hybridization was done as described [30]. The sample pixel intensity of the scanned radiograms was measured with Zero-Dscan software (Scanalytics).

3. Results

3.1. pAREs in the 5′UTR of CAT mRNAs reduce the reporter activity regardless of its length and structure

To examine the effect of putative 5′UTR PABP-binding sites on CAT translation, 293-EBNA cells were transiently transfected with the parental reporter pSV2-CAT, or pSV2-CAT containing the 5′UTR-regulatory elements: the human wild-type pARE (as shown in Fig. 4), 61As, 20As, and 10As, as well as the pURE and 61Us constructs (negative controls) (Fig. 1A). From the transcription start site, these constructs are located either 86 nucleotides from the beginning of the

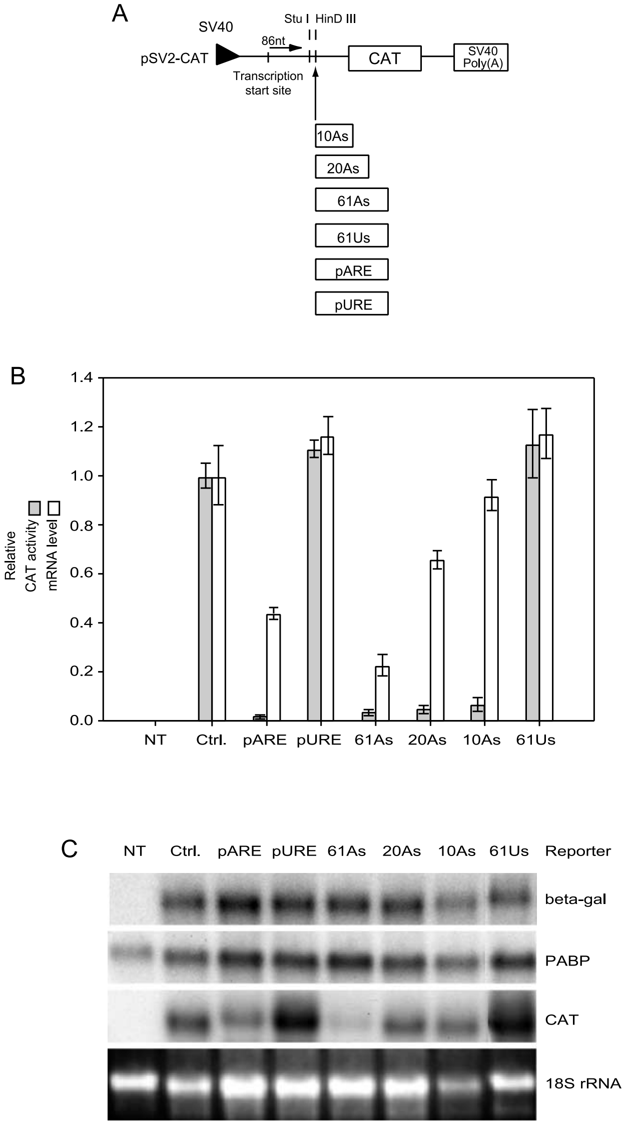

Fig. 1. The effect of 5′UTR-polyadenosine on the translation and abundance of CAT mRNA. A: Diagram of the reporter CAT constructs containing the predicted 5′UTR PABP-binding sites (pARE, 61As, 20As and 10As), or the polyuridine negative controls (pURE and 61Us), under control of the SV-40 promoter. The relevant restriction sites, nucleotide distances and nomenclatures are indicated. B: Human kidney 293-EBNA cells (2×10^5/well of 24-well plates) were transfected with CAT constructs containing the putative PABP binding site in its 5′UTR (described in panel A), or with the negative controls (pURE and 61Us), as well as the transfection efficiency reporter plasmid pCMVβ. Three transfection reactions were done for each experimental condition and the mean values and standard deviations were determined (gray bars). The ratio of CAT to the β-gal activity was set to 1 for control cells transfected with the parental pSV2-CAT vector (Ctrl. lane). NT lane indicates the non-transfected 293-EBNA cells. C: 293-EBNA cells (1×10^6/well of six-well plates) were transfected with the same constructs described in panel B and Northern blot analysis of the reporter mRNAs was done using the β-gal and CAT probes. The endogenous PABP mRNA was probed as an internal control. The CAT mRNA levels were normalized to those of β-gal and PABP mRNAs (or ethidium bromide-stained 18S rRNA). The Northern blot assays were done in triplicate and the means of the normalized mRNA measurements are shown in panel B (white bars), together with the respective CAT activities.

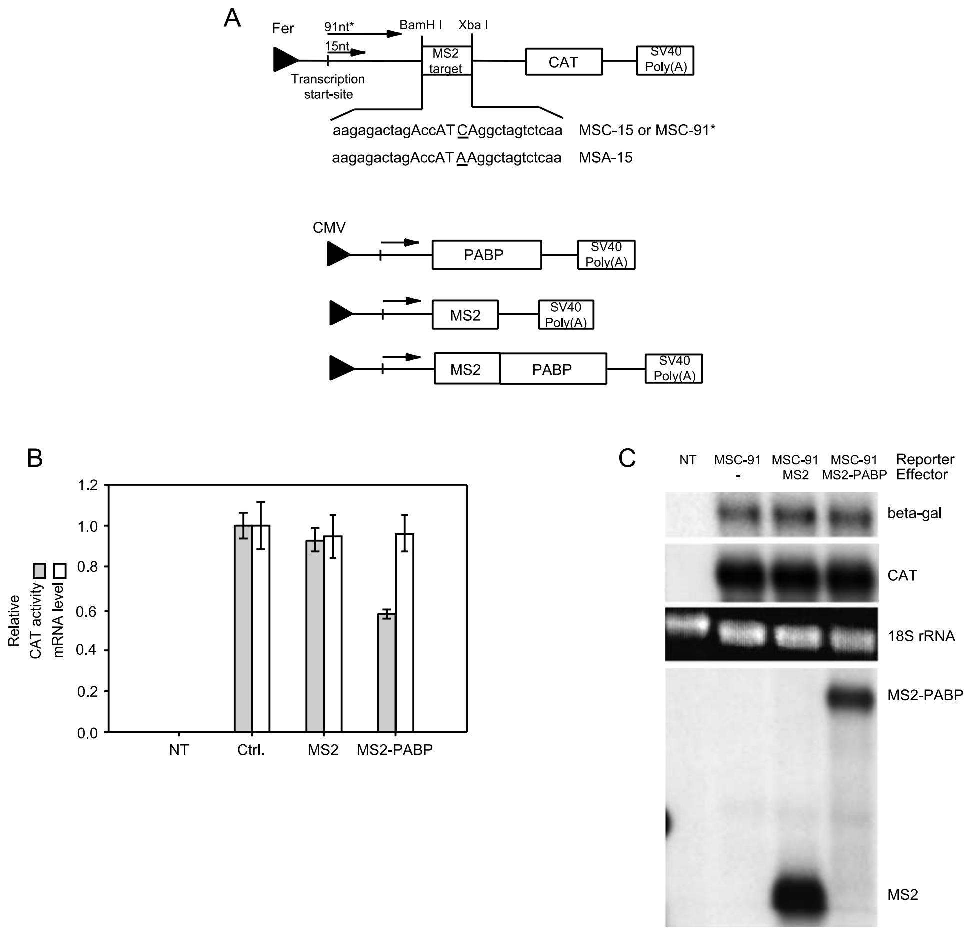

Fig. 2. The effect of a single 5′UTR-tethered MS2–PABP fusion protein on the translation and abundance of CAT mRNA. A: Diagram of the reporter CAT gene containing the MS2-binding site at 15 nt (MSC-15) or 91 nt (MSC-91) from the mRNA 5′ end, and the attenuated MS2-binding site (MSA-15) constructs. All reporter constructs were under control of the ferritin promoter. The MS2-binding stem is represented by small letters and the nucleotide loop that alters the protein binding affinity is underlined (upper panel). Diagram of the effector plasmids containing MS2, PABP, or the fusion protein MS2–PABP genes under control of the CMV promoter (lower panel). B: Human 293-EBNA cells (2×10^5/well of 24-well plates) were co-transfected with MSC-91, pCMVβ and pCDNA3 vectors (Ctrl. lane), or with the MSC-91, pCMVβ and effector plasmids (MS2 and MS2–PABP lanes). The transfections were done in triplicate and the CAT activities were normalized to the β-gal and are shown with their standard deviations. The ratio of CAT to β-gal activity was set to 1 for control cells transfected with no MS2 fusions (gray bars). NT lane indicates the non-transfected 293-EBNA cells. C: For the mRNA measurements, 293-EBNA cells (1×10^6/well of six-well plates) were transfected with the constructs described in panel B. Northern blots with 10 μg of total RNA were done and the CAT and MS2 mRNA levels were normalized to the β-gal mRNA and ethidium bromide-stained 18S rRNA. The Northern blot assays were done in triplicate and the means of the normalized mRNA measurements are shown in panel B (white bars), together with the respective CAT activities.

A-rich tracts or 91 nucleotides from the control U-rich sequences. Transfected cells were allowed to grow for 20 h prior to harvesting and analysis of the CAT and β-gal activities (see Section 2). As shown in Fig. 1B, all constructs containing adenosine stretches in the reporter mRNA 5′UTR significantly repressed the CAT reporter activity to the same level, regardless of the size or structure of the A-containing elements. This inhibitory effect was oligo-A sequence-specific since oligo-U sequences did not block the CAT translation as shown by the pURE and 61Us constructs, which had the same levels of reporter activity as the parental (pSV2-CAT) construct (Fig. 1B). These results are consistent with those previously reported by this group, in which UV cross-linking assays were used to show the ability of reticulocyte PABP and GST-PABP fusions to bind the pARE of human PABP mRNA [17]. Moreover, the specificity for poly(A)-tract binding was mapped to the first and second RNA recognition motifs (RRMs) of PABP, in *Xenopus* [31] and yeast [32,33].

3.2. pAREs in the 5′UTR reduce the levels of CAT mRNA in 293 cells

Since the stability of CAT mRNA is refractory to the effect of 5′UTR translation inhibitory elements [2,4,34], we decided to analyze the CAT mRNA level of the constructs used in the CAT activity assays (Fig. 1A). When compared to wild-type pSV2-CAT, the presence of either the wild-type pARE or the 61 adenosines in the 5′UTR of the CAT message dramatically decreased the mRNA levels (Fig. 1B,C). The effect produced by the pARE sequence was less intense than the pure 61As tract, although both constructs repressed CAT activity to the same extent. In contrast, the 61Us and pURE controls had no effect on the amount of mRNA or on CAT activity when compared with pSV2-CAT mRNA (Figs. 1B,C). The decrease in reporter mRNA levels induced by multiple A tracts was proportional to the tract length. The construct containing 10As produced an equivalent amount of mRNA to that of pSV2-CAT, while the level of pSV-20As mRNA was intermediate to that in the 10As and 61As constructs, despite the fact that all constructs repressed CAT activity to a similar extent (Fig. 1B). These findings, together with the observation that the eight adenosines in tandem formed the biggest poly(A)-pure stretch in the human wild-type pARE (Fig. 4), indicate that potential PABP binding sites in the 5′UTR of mRNAs can regulate the abundance and translation of reporter mRNA. Our results are consistent with the selection/amplification (SELEX) assays in which human PABP [35] or *Xenopus* PABP [36] molecules can bind to RNA containing a minimum tract of 5As within an oligoribonucleotide of at least 20 bases. X-ray structure studies have shown that the PABP can establish electrostatic interactions through its first two RRMs with seven consecutive adenosines (A2–A8) in 11As oligoribonucleotides [37]. These results suggest that one RNA-bound PABP molecule can inhibit translation without affecting mRNA stability. Consequently, the size and structure of the 5′UTR pAREs could independently control the abundance and translation of reporter mRNA.

3.3. *A single RNA-tethered PABP molecule can inhibit translation without affecting mRNA abundance*

The mechanism of translation repression by 5′UTR-bound proteins is well-characterized [38]. In the model system, the iron-responsive element (IRE) stem loop present in the 5′UTR of ferritin mRNA can inhibit the attachment of the ribosomal 40S subunit to mRNA, thereby blocking ferritin translation when the IRE is bound to the iron regulatory protein (IRP) [39,40]. The IRE–IRP steric blockade occurs independently of the IRP sequence [27,34]. However, when the IRE is placed more than 40 nucleotides away from the cap, the inhibitory effect is severely reduced [41,42]. To examine the effect of a single RNA-bound PABP molecule on mRNA translation and abundance when the RNA–protein binding site is placed beyond the limits of steric hindrance, we tethered the MS2–PABP fusion molecule to the 5′UTR of CAT mRNA through the viral MS2-coat protein binding site (Fig. 2A). Previous reports have shown that the MS2–PABP fusion binds to the MS2 target site and retains the properties of native PABP in vivo [12,14].

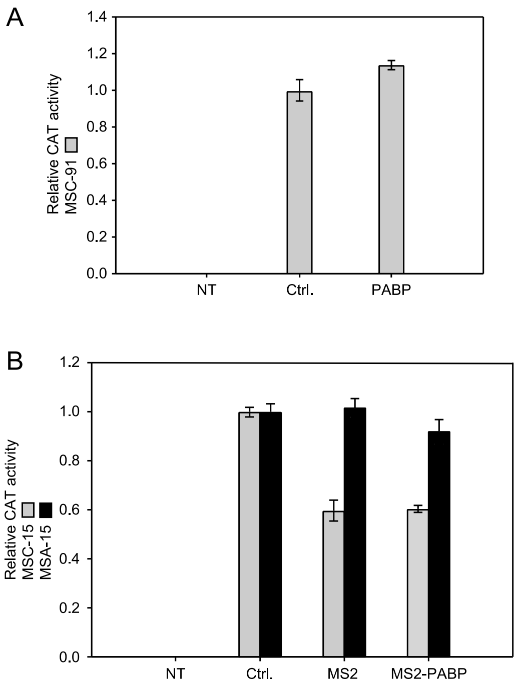

Fig. 3. The inhibition of CAT translation by PABP is dependent of the *cis* elements and is abrogated by a single mutation. Plasmids encoding different reporter mRNAs, as well as the MS2–PABP fusion proteins, were co-transfected in 293-EBNA cells and 20 h later the cells were harvested and assayed for CAT and β-gal activities. Three transfection reactions were done for each experimental condition and the mean values and standard deviations were determined. The ratio of CAT to β-gal activity was set to 1 for control cells transfected with no MS2 fusions. A: 293-EBNA cells (2×10^5/well of 24-well plates) were co-transfected with MSC-91, pCMVβ and pCDNA3 vectors (Ctrl. lane), or with MSC-91, pCMVβ and pCMV-PABP plasmids (PABP lane). B: 293 cells (2×10^5/well of 24-well plates) were co-transfected with the reporter plasmid MSC-15 (gray bars), or MSA-15 (black bars), together with pCMVβ plus the pCDNA3 vector (Ctrl. lane), or with pCMVβ and effector plasmids (MS2 and MS2–PABP lanes). NT lanes indicate the non-transfected 293-EBNA cells.

We co-transfected the CAT reporter and effector plasmids expressing MS2, PABP, or the MS2–PABP fusion protein. The CAT assays showed that the presence of the MS2-binding stem loop had no effect on reporter translation when the effector protein was absent. However, CAT mRNA translation was inhibited by MS2–PABP binding to its 5′UTR at 91 nucleotides from the mRNA 5′ end (Fig. 2B). Neither MS2 alone (Fig. 2B), nor untethered ectopically expressed PABP (Fig. 3A) were able to block the reporter synthesis. The MS2 construct blocked CAT expression only when the MS2-binding site was inserted 15 nt downstream from the mRNA 5′ end (Fig. 3B). The effect of MS2–PABP on reporter trans-

H.s. tttattatt**aaaaaatccaaaaaaaatctaaaaaaatcttttaaaaaaccccaaaaaaatttacaaaaaa**tccgcgtctcccccgccggagactttatttttttttcttcctcttttataaaataa

M.m. tttattattaaaaaatccaaaaaaaatctaaaaaatcctttgaaaaaacccaaaaaaaatttacaaaaaaatccgcgtctcccccgccggagactttatttttttttcttcctcttttataaaataa

P.m. tttttttttttttttttttttttttgtttt...70nt...aaaacaaaaaaaaacctcaaaaaaataaaaaacaacaaaaaaaatagcaaaaccccccgaataaaaacataaaaattt

D.m. tataaaaaaataaaaaaaaatattaaaacacaaaaacgtaacaaaaaaaaattgtaaaaagtagcaaaaaaaaaatcgagcgtgtttgag

C.e. ggcacgagaaactgctcaaaaacccataaaaatttaaaaaaccctaaaaatcaccaaaaaaattattctttg

S.c. ctttttattcaataaaaactcaaaaaaaaaaatccaaaaaaaaactaaaaaaccaataaaaataaaat

A.t. tttcttaccctatttcatcttccctctcctctgatatacccat aaaaacacaaaaaaaacgaaaagataaaaactttggctttagttttgttgattttgtcattcgattcgatttctttttcttctttc

T.b. tatattgtctttgtccagcctctaaaaaaaaataacaaaaataaaaataaatatagtagctaacaaaaaaaacacaaaggtaaggaagagcaaacaaaaggtatccagtgccacttagtt

Fig. 4. The presence of pARE at the 5′UTR of eukaryote PABP transcripts. The pARE context, including neighboring pyrimidine-rich sequences, is conserved in different PABP cDNAs. H.s., *Homo sapiens* (Y00345); M.m., *Mus musculus* (AK005009); P.m., *Petromyzon marinus* (AF032896); D.m., *Drosophila melanogaster* (L05109); C.e., *Caenorhabditis elegans* (U24123); S.c., *Saccharomyces cerevisiae* (M12780); A.t., *Arabidopsis thaliana* (L19418); and T.b., *Trypanosoma brucei* (AF042190). The potential auto-regulatory PABP binding sites are underlined and the human pARE sequence used in this study is in bold type.

lation was attenuated when a mutation which severely reduced the affinity of MS2 for its mRNA target site was introduced in the reporter plasmid (Fig. 3B). These results showed that the negative effect of MS2 on translation occurred only when the *cis* element is present and that it was site-specific. There was no difference in the amount of mRNA between assays in which the reporter was transfected alone and assays in which the reporter was co-transfected with the MS2 constructs (Fig. 2C). This finding was consistent with the pSV-10As transfection data (Fig. 1B) and indicated that one PABP binding site in the 5′UTR was enough to inhibit reporter translation but did not mediate the reduction in reporter mRNA levels.

4. Discussion

In HeLa and NIH 3T3 cells, the ectopic expression of PABP leads to a shift in endogenous PABP mRNA from the polysomal to the post-polysomal fraction with no effect on its abundance [20,25]. In contrast, in 293 cells, overexpression of PABP does not produce a similar shift in endogenous PABP mRNA but selectively reduces its abundance [25]. In addition, as shown here, the presence of A-rich elements in the 5′UTR of an otherwise stable mRNA selectively reduced its abundance in 293-EBNA cells, and this reduction was proportional to the number of putative PABP binding sites in the A-rich element. In support of these observations, the conservation of pARE within the 5′UTR of the PABP mRNAs among different eukaryotes (Fig. 4) was indicative of a relevant regulatory feature that cannot be explained solely by its ability to repress translation, especially considering that just 10As were enough to inhibit translation to the same extent as 61As or pARE (Fig. 1B). A further observation was that the reduced translation efficiency of reporter mRNAs containing 5′UTR A-rich elements were maintained even when the number of possible PABP binding sites was reduced to one, or when a single MS2–PABP fusion protein was tethered to the 5′ end of the mRNA, without a decrease in the amount of reporter transcript (Figs. 2B and C). Interestingly, despite the equivalent CAT repression produced by the wild-type 5′UTR pARE, or the artificial 61As stretch, their effects on the level of CAT mRNA were distinct (Fig. 1B,C). This discrepancy may reflect differences in the behavior of the PABP multimeric-induced binding to pure adenosine and the pARE element (Melo et al., submitted). Thus, the structure and size of the putative 5′UTR PABP-binding sites can influence the reporter mRNA level, whereas the repression of translation is independent of the reduction in mRNA abundance.

These findings suggest that the presence of multiple PABP molecules bound to its own 5′UTR mRNA may change the conventional cross-talk between the poly(A) tail–PABP complex, the decapping apparatus and the cap-binding complex (eIF-4F), and lead to commitment to the mRNA decay pathway. This hypothesis is strengthened by reports showing that communication between eIF-4F, the decapping complex (Dcp1p and Dcp2p), the decapping activation factors (Lsm1p–7p) and Pab1p is the major determinant of mRNA stability in yeast [15,43,44]. Since the autogenous repression of PABP translation is not absolute (Fig. 1B [19,20,25]), mRNA decay may reduce the number of PABP transcripts in the cytoplasm, thereby avoiding the residual translation caused by continuous PABP transcription.

In conclusion, we propose the existence of a two-way mechanism for controlling PABP synthesis in human 293 cells in which mRNA translation and stability are regulated to provide the appropriate cytoplasmic level of PABP molecules. The existence of a similar mechanism operating in other cell types, as well as the factors which mediate this type of expression control, remain to be determined.

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