Negative Control of the Poly(A)-binding Protein mRNA Translation Is Mediated by the Adenine-rich Region of Its 5′-Untranslated Region*

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Translation of the mRNA for the poly(A)-binding protein (PABP) may be autoregulated by the binding of PABP to the A-rich segment of its 5′-untranslated region (UTR). To test this hypothesis, we examined the effect of different fragments of the 5′-UTR from human PABP cDNA on the translation of the β-galactosidase (β-Gal) reporter gene. Presence of the A-rich sequence from the 5′-UTR of PABP mRNA inhibited expression of the chimeric β-Gal gene in transfected HeLa cells. The differences in expression of β-Gal polyopeptide was due to the translational repression of β-Gal mRNA containing the A-rich 5′-UTR of PABP mRNA. The A-rich region of the 5′-UTR located within nucleotides 58–146 of PABP mRNA was sufficient to mediate translational control of this mRNA expression. We also examined the effect of overexpression of PABP mRNA in HeLa cells. The ectopic PABP mRNA without the A-rich 5′-UTR region was translated efficiently, whereas the translation of the endogenous PABP mRNA was substantially inhibited in the transfected cells. In contrast, the ectopic PABP mRNA containing the A-rich 5′-UTR region did not show similar effect on the translation of the endogenous PABP mRNA in these cells. These results suggest that feedback control of mRNA translation is involved in regulating PABP expression in HeLa cells.

Cellular mRNAs are often complexed with a group of RNA-binding proteins. One of the best studied RNA-binding proteins is the 72-kDa poly(A)-binding protein (PABP),1 that shows specific interaction with the 3′ poly(A) region of all eukaryotic mRNAs (1–3). PABP is ubiquitous and highly conserved in eukaryotic cells. The poly(A)-binding region contains 90 amino acid domains (1–3). Plants and mammals both contain several related polypeptides with specific affinity toward the poly(A) region of the mRNA. These PABP-related polypeptides show tissue-specific expression and may be regulated by growth conditions (4, 5). PABP is essential for viability of eukaryotic cells and is involved in regulating mRNA translation and stability. Results of both in vitro (6) and in vivo (7) studies suggest that the complex of PABP and poly(A) tail plays an important role in mRNA translation. It is believed that the poly(A)-PABP complex is involved in joining the 60 S subunit to the 48 S preinitiation complex (7). PABP also stimulates the binding of 40 S subunits to the mRNA (8). The ability of PABP to interact with the eIF4G and eIF4B is important for its role in the initiation of mRNA translation (9–13). The interactions between PABP and these initiation factors also take place in absence of poly(A) (11). Therefore, it is not clear whether the free PABP or PABP-poly(A) complex is involved in mRNA translation in vivo. In addition, PABP is also involved in poly(A) tail shortening probably through its interaction with a poly(A)-specific 3′-exonuclease (12, 14).

Several studies indicated that PABP expression is regulated at the post-transcriptional level during the cell cycle (15–20). In resting cells, the majority of cellular PABP mRNA was found in the non-translated state. Following stimulation of growth by serum, a rapid transition of the mRNA to the translated state occurred in mouse fibroblasts (19, 20). In contrast, during differentiation of murine myoblasts growth arrest resulted in the migration of repressed PABP mRNA to the translated state. This process was also accompanied by reduced stability of the PABP mRNA in differentiated myotubes resulting in little change in PABP synthesis (18).

Translation of a specific mRNA is often regulated by the interaction of regulatory proteins with defined sequences of the mRNA. For instance, feedback control of ribosomal protein L10 synthesis by the level of free L10 protein is mediated by its binding to the 5′-UTR of the L10 mRNA (21, 22). Similarly, synthesis of ferritin is regulated in vertebrates by the iron level (23). The inhibition of ferritin mRNA translation requires the binding of a 98-kDa translation repressor polypeptide to the conserved 25-nucleotide-long sequence of its 5′-UTR (24, 25). In addition the 3′-UTR of some mRNAs is involved in regulating translation. The translations of protamine (26) and myocyte enhancer factor-2a mRNAs are controlled by RNA-binding proteins that interact with sequences located within the 3′-UTR of these mRNA (27). How the 3′-end of these mRNAs influence ribosome binding at the 5′-end is unknown. It is possible that 3′-UTR binding protein(s) may interfere with the function of poly(A)-PABP complex.

In contrast to the examples cited above, regulation of PABP mRNA translation appears to be more subtle. Instead of completely blocking PABP mRNA translation, the distribution of this mRNA between translationally active and inactive populations is modulated. Interestingly, analysis of PABP mRNA sequences in various species showed the presence of conserved highly A-rich sequence in its 5′-UTR. This raises the possibility of negative feedback control of PABP mRNA translation. Previous studies from this and other laboratories have shown that PABP is able to bind to the 5′-UTR A-rich regions and inhibit PABP mRNA translation in a rabbit reticulocyte lysate cell-free system (28, 29), and removal of this sequence from the mRNA translation. It is therefore hypothesized that the poly(A)-PABP complex is involved in joining the 60 S subunit to the 48 S preinitiation complex (7). PABP also stimulates the binding of 40 S subunits to the mRNA (8). The ability of PABP to interact with the eIF4G and eIF4B is important for its role in the initiation of mRNA translation (9–13). The interactions between PABP and these initiation factors also take place in absence of poly(A) (11). Therefore, it is not clear whether the free PABP or PABP-poly(A) complex is involved in mRNA translation in vivo. In addition, PABP is also involved in poly(A) tail shortening probably through its interaction with a poly(A)-specific 3′-exonuclease (12, 14).

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1 The abbreviations used are: PABP, poly(A)-binding protein; eIF, eukaryotic initiation factor; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β-Gal, β-galactosidase; RT-PCR, reverse transcription-polymerase chain reaction; bp, reverse transcription; ARS, auto-regulatory sequence; MOPS, 4-morpholinopropanesulfonic acid.

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PABP mRNA enhances its translation in vitro. We have also shown that the translation of full-length PABP mRNA, but not the truncated mRNA from which the A-rich region of the 5' UTR was removed, can be inhibited in a cell-free system by adding purified human PABP. Furthermore, by using UV-mediated cross-linking of RNA and proteins, we have shown that PABP binds to the first 224 nucleotides of the 5'-UTR of PABP mRNA, and this may repress its own expression (28). Therefore in the present study, we extended these investigations to determine whether similar inhibition of mRNA translation occurs in vivo by the A-rich 5'-UTR of PABP mRNA. We have also examined if PABP synthesis was regulated in HeLa cells when the attempt was made to increase PABP synthesis by ectopic expression of this mRNA.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The parent plasmid pHu73 is a full-length cDNA clone of human PABP mRNA (2). The 487-bp EcoRI/BglII fragment of the 5'-UTR of this clone was used in PCR reactions with appropriate primers to produce amplified DNA consisting of different regions of the PABP mRNA 5'-UTR as shown in Fig. 1 (panel A). The PCR primers used in our studies also contained additional sequences to provide the NcoI and EcoRI sites at 5'- and 3'-ends, respectively, of the amplified products. The PCR reactions were carried out in the buffer containing 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, and 200 μM dNTPs for 35 cycles with 2 units of Taq DNA polymerase (Life Technologies, Inc.). Each cycle was at 94 °C 15 s, 50 °C 30 s, and 72 °C 1 min, and the final cycle was at 72 °C for 5 min. The amplified product of the correct size was purified from an agarose gel using the GeneClean kit (BIO/CAN, Montreal, Canada) and was digested with NcoI and EcoRI before cloning. The PCMV-SPORT-β-Gal (Life Technologies, Inc.) vector was also digested with NcoI and EcoRI, and the 368-bp fragment was removed. The 5'-UTR of PABP mRNA was then ligated to the 7485-bp-long vector fragment that contained all the sequences needed for the expression of the reporter β-Gal gene. Additional cloning was performed by digesting the vector by the enzyme with SunI to generate a 285-bp deletion in the 3'-coding region to express a truncated β-Gal polypeptide as internal controls. The sequences of the 5'-UTR regions of all clones were examined by automated DNA sequencing.

For ectopic expression of human PABP cDNA, two plasmids were constructed. One of these plasmids (pcMVΔPABP) was designed to lack the adenine-rich region from the 5'-UTR of human PABP cDNA clone pHu73. The second plasmid (pcMV983ΔPABP) was constructed by adding an 89-bp PCR-generated fragment (Fig. 1, panel A) containing the A-rich 5'-UTR region to the first construct, the pcMVΔPABP. The PCMV-SPORT-β-Gal (Life Technologies, Inc.) was digested with BamHI and the plasmid backbone was used for the entire β-Gal-truncated region was isolated and ligated to the SunI and BamHI fragment of pHu73 (Fig. 1, panel B).

**Transfection of Cells**—Approximately 1 × 10^6 HeLa W5 cells (ATCC) grown on a 12-well dish in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.) were used for transfection. Subconfluent cultures were transfected in Opti-MEM medium (Life Technologies, Inc.) without serum and antibiotics. For transfection, 1.5 μg of the reporter plasmid DNA was incubated with 10 μg of LipofectAMINE in 400 μl of Opti-MEM medium at 20°C for 30 min before being added to the cells. Cells were incubated with the DNA/polysome mixture for 5 h at 37°C, and then the DNA-containing medium was removed, and transfected cells were allowed to grow for 24 h in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. In some experiments, 0.5 μg of a second plasmid DNA coding for a truncated β-Gal polypeptide (Fig. 1) was used as an internal control to monitor the transfection efficiencies between experiments.

**Measurement of the β-Gal Protein Level**—Before harvesting, the transfected cells were washed three times with phosphate-buffered saline (PBS, 150 mM NaCl, 16 mM NaH2PO4, 4 mM Na2HPO4, pH 7.2) and lysed directly with 200 μl of a buffer containing 25 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (30). The separated polypeptides were transferred to a nitrocellulose membrane, and levels of β-galactosidase polypeptide were determined by Western blotting technique using mouse anti-β-Gal antibody as described previously (14).

**Measurement of mRNA Levels**—The transfected cells were washed three times with PBS and then lysed in 4 μl guanidine thiocyanate (Fulka, Switzerland). Total RNA from the cells was isolated by phenol/chloroform extraction of the lysate as described previously (31). The specific mRNAs were reverse transcribed with M-MLV reverse transcriptase (Life Technologies, Inc.) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl2 containing 0.5 mM each of dNTPs. The reaction was performed at 37°C for 1 h. The cDNA was amplified by the PCR technique with Taq DNA polymerase for 25 cycles with specific primers (Fig. 1, panel A) as described earlier. The conditions used for RT-PCR gave linear increase in the amplified products with 0.25 to 2 μg input of total cellular RNA. In some reactions additional bands were seen; however, their presence did not affect the linear response.

The level of β-Gal mRNA was also measured in some experiments by RNase protection (32) using the HybSpeed RPA Kit (Ambion; Austin, TX). The PCMV-SPORT-β-Gal vector was linearized by digesting with Bplu11021 enzyme, and the 181 nucleotides long antisense RNA complementary to the 3'-UTR of the β-Gal mRNA was transcribed in vitro by using T7 polymerase. Similarly, a 374-nucleotide-long antisense RNA to the human GAPDH mRNA was produced by the T7 polymerase transcription of a commercially available clone (Ambion, TX). This antisense RNA was used in the RNase protection assay, and due to the presence of genetically engineered additional sequences, produced a 316-nucleotide-long RNAse protected fragment. Approximately 5 μg of RNA was hybridized with 5 × 10^7 cpm (approximately 5 ng) of the antisense RNA, and RNA-protected samples were analyzed by 2% agarose gel electrophoresis (33). The levels of ectopic and endogenous PABP mRNA were measured by S1 nuclease protection of specific oligodeoxynucleotide probes using the multi NPA kit from Ambion. For ectopic PABP mRNA detection a 36-mer oligonucleotide complementary to the vector-derived unique 3'-UTR of the ectopic PABP mRNA with 5 nucleotides mismatched at its 5'-end was used. The sequence of this oligomer is (5'-gcggccgaggtgggctcctgagactct-3'), complementary to nucleotides 680–710 of the vector (Life Technologies, Inc.). For detecting endogenous PABP mRNA, a 30-mer oligonucleotide complementary to the 3'-UTR region of the endogenous PABP mRNA with 5 nucleotides mismatched at its 5'-ends was used. The selected 5'-UTR was deleted from the ectopic mRNA and therefore was specific for the endogenous mRNA. The sequence of this oligomer (5'-ggccgcttgagggccgtgtcctgcggacccgtgactaa-3') is complementary to nucleotides 196–220 of human PABP mRNA. The lowercase letters of the oligomer represent unmatched bases. The levels of human GAPDH mRNA were measured in these studies using a commercially available 44-mer (Ambion) which gives a 30-mer S1 nuclease-protected fragment. These probes were 5'-end-labeled using γ-[32P]ATP and T4 polynucleotide kinase (33). The radiolabeled probe (5 ng = 5 × 10^6 cpm) was hybridized to 5 μg of cellular RNA and subjected to S1 nuclease treatment (31). The S1 nuclease protected oligomers were analyzed by 12% polyacrylamide, 8 M urea gel electrophoresis.

**Subcellular Fractionation**—Following transfection, the cells were lysed in 200 μl of polysomal buffer (10 mM MOPS, pH 7.2, 250 mM NaCl, 2.5 mM MgOAc, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 200 μg/ml heparin, 50 μg/ml cycloheximide). After removing the nuclei and cell debris by centrifugation at 12,000 × g for 10 min, the polysomes were pelleted by ultracentrifugation at 100,000 × g for 1 h in a 75 Ti rotor (Beckman) at 4°C and resuspended in 200 μl of polysomal buffer (34). The supernatant was used as the source for post-polysomal material. The RNA from both fractions was isolated by phenol/chloroform extraction of the lysate as described previously (31). Samples corresponding to equal numbers of cells were used for analysis. In some experiments, the 12,000 × g supernatant of the cytoplasmic fraction was centrifuged in a 10-ml 10–50% sucrose gradient, containing 25 mM HEPES, pH 7.0, 50 mM KCl, 2 mM MgOAc, 50 μg/ml cycloheximide, 15 mM 2-mercaptoethanol at 40,000 rpm in a Beckman SW 41Ti rotor for 2.5 h (35). Gradient fractions were collected using a Buchler Auto Densi-Flow IIC apparatus (Buchler Instruments). Total RNA from each fraction was isolated as described before and precipitated with ethanol using 5 μg of yeast tRNA as the carrier.

**RESULTS**

**Effect of PABP 5'-UTR on β-Gal Expression**—Earlier studies (28, 29) using in vitro transcribed mRNAs have shown that PABP could bind to the 5'-UTR of its mRNA and inhibits its translation in vitro. However, the mRNA transcribed in vitro
differed from its in vitro counterpart in terms of capping and methylation of the 5'-end and polyadenylation of the 3'-end, which may be important for RNA-protein interactions. Presence of additional intracellular RNA-binding proteins may also influence the binding of PABP to the 5'-UTR of its mRNA. Therefore, it was necessary to test the autoregulation of PABP mRNA translation in a cellular environment. This was performed by expressing chimeric β-Gal mRNA consisting of different lengths of the PABP mRNA 5'-UTR upstream of the β-Gal open reading frame in HeLa cells.

The constructs used in these studies are shown in Fig. 1, panel B. Results of transient expression of β-Gal polypeptide by these various plasmids are shown in Fig. 2. Western blot analyses of the transfected cell extracts show that among the different chimeric constructs used for transfection, maximum level of β-Gal polypeptide was present in the p264-Gal-transfected cells (lane b). This construct lacked the putative PABP-binding site at the 5'-UTR. In contrast, presence of most of the 5'-UTR (p487-Gal, lane a), or oligo(A)-rich half of the 5'-UTR (p223-Gal, lane c), or as little as the predominantly A-rich region between nucleotides 58 and 146 (p89-Gal, lanes d and e) caused significant reduction of the β-Gal level. Maximum (80%) reduction in β-Gal level was observed with the shortest PABP 5'-UTR construct p89-Gal (lanes d and e).

It should be noted that the level of β-Gal polypeptide was significantly lower in cells transfected with the chimeric plasmids than that with the parent plasmid (pCMV-SPORT-β-Gal) (Fig. 2, compare lanes f and b). Measurement of β-Gal mRNA levels in these cells showed that this was primarily due to a higher ectopic mRNA level in cells transfected with pCMV-SPORT-β-Gal (Fig. 3). The reason for these differences is not clear but may be due to the presence of additional transcriptional modulating sequences in the parent plasmid which was removed during the subcloning procedures. In all experiments presented here the difference in transfection efficiencies between experiments was monitored by co-transfecting cells with a second plasmid containing a truncated β-Gal gene. The results show that there were no significant differences in the levels of the truncated β-Gal polypeptide between different transfection experiments (Fig. 2, Δβ-Gal). Similar results were observed in three independent experiments.

In order to determine whether translational control of the chimeric β-Gal mRNA was responsible for the differential expression of the β-Gal polypeptide in transfected cells, we measured β-Gal mRNA steady-state levels (Fig. 3) by RNase protection analyses. The mRNAs produced by all constructs used in these studies had common 3'-ends; therefore, we used antisense RNA synthesized from Bpu1102I-digested pCMV-SPORT-β-Gal plasmid to measure mRNA levels. As internal controls we also measured the level of GAPDH mRNA using the same RNA samples. The antisense GAPDH RNA was transcribed in vitro from commercially available clone from Ambion and produced a slightly shorter RNase-protected fragment due to engineered mismatches in the RNA sequence. The chimeric β-Gal mRNA levels were normalized, using the GAPDH mRNA levels, and the results (Fig. 3) show that there was little difference in the β-Gal mRNA levels in cells transfected with p89-Gal p223-Gal, p264-Gal, and p487-Gal. However, as discussed earlier compared with these constructs there were approximately 3 times more β-Gal mRNA present in cells transfected with the parent CMV-SPORT-β-Gal plasmid. Comparison of the β-Gal polypeptide (Fig. 2) and mRNA levels (Fig. 3) therefore suggests that the p89-Gal, p223-Gal, and p487-Gal mRNAs were less efficiently translated than the p264-Gal and pCMV-SPORT-β-Gal-derived mRNAs. These results suggest that the presence of the short, A-rich regions of the PABP mRNA 5'-UTR inhibited mRNA translation. Furthermore, the polypeptide levels in p487-Gal and PCMV-SPORT-β-Gal-transfected cells were proportional to their mRNA levels. These results suggest that apart from the A-rich segment between nucleotides 58 and 146 of the PABP mRNA 5'-UTR other regions of this UTR had a minimal effect on mRNA translation.
The GAPDH mRNA as controls. Concentrations of the undigested probes were adjusted and the undegraded probe before RNase treatment (lanes a–c) in cells transfected with 3'-UTR containing p89-Gal mRNA containing the 5'-UTR region proximal to the initiation site (Fig. 2, panel A). In all transfections, no detectable difference was observed in the stability of GAPDH mRNA (Fig. 2, panel A). These results suggest that presence of a potential PABP-binding site at the 5'-UTR had no detectable effect on mRNA stability.

Presence of mRNA in the Translationally Repressed Fraction— Previous studies from several laboratories showed the efficiency of translation of a specific mRNA can be monitored by examining the distribution of the mRNA between the polysomal and post-polysomal fractions (36). A simple way to test this is to fractionate the cytoplasmic extract by centrifugation at 100,000 × g for 1 h to remove the polysomes from the extract. The pellet recovered after centrifugation consists of materials with sedimentation coefficients ≥120 S. This represents translationally active mRNAs that are bound to more than one 80 S ribosome. The supernatant fraction therefore contains most of the non-ribosome and monosome-bound mRNAs (36). To examine whether fusion of different regions of the PABP mRNA 5'-UTR affects the subcellular distribution of the chimeric β-Gal mRNA, we measured the steady-state levels of these mRNAs in subcellular fractions. As internal controls we also analyzed the distribution of GAPDH mRNA between polysomal and post-polysomal fractions. For these analyses we have used RT-PCR reactions which showed values comparable to that of nuclease protection assays (see Fig. 6). This offered a quick comparison of the translational status of mRNAs from different constructs. The results (Fig. 5) clearly showed a preferential distribution of the adenine-rich 5'-UTR containing β-Gal mRNA (p89-Gal, compares lanes d and i) in the post-polysomal fraction. Nearly 90% of cytoplasmic p89-Gal mRNA was present in the non-translated population. This distribution pattern was not markedly different when the adenine-rich sequence was located further downstream from the initiation codon of β-Gal mRNA as was the case with the p223-Gal and p487-Gal mRNAs (lanes f and h). In these chimeric transcripts, the putative PABP-binding site of the 5'-UTR of PABP mRNA was located at different distances from the initiation codon ranging from 24 to 31 nt.

Influence of the 5’-UTR on mRNA Stability— Complexes involving PABP and 3’ poly(A) of mRNAs are known to play a role in regulating mRNA stability. Therefore, the presence of a potential PABP-binding site at the 5’-UTR of the chimeric β-Gal mRNA could influence the interaction of PABP with the 3’ poly(A) region of these mRNAs and affect mRNA stability. We therefore investigated the half-life of different chimeric β-Gal mRNAs in transfected cells. This was carried out by treating the cells 24 h after transfection with actinomycin D to block RNA synthesis. The steady-state levels of the mRNA was measured by RT-PCR at different times following inhibition of RNA synthesis. For each set of experiments, we also measured the level of GAPDH mRNA as internal controls. The results indicate that the half-lives of chimeric β-Gal mRNAs containing different regions of PABP mRNA 5'-UTR were similar (Fig. 3). The p89-Gal mRNA containing the shortest 5'-UTR decayed at approximately the same rate as the p264-Gal mRNA containing the 5'-UTR region proximal to the initiation site (Fig. 4, panel B). In all transfections, no detectable difference was observed in the stability of GAPDH mRNA (Fig. 4, panel A). These results suggest that presence of a potential PABP-binding site at the 5'-UTR had no detectable effect on mRNA stability.

FIG. 2. β-Gal protein levels in cells transfected with different plasmids. HeLa cell monolayers were transfected with 1.5 μg of PABP-CMV-β-Gal plasmids and 0.5 μg of a pCMV-β-Gal plasmid expressing a truncated form of β-Gal polypeptide (Fig. 1B, SunI) included as an internal control, according to the procedures described under “Experimental Procedures.” Twenty four hours after transfection, cells were lysed, and equal amounts of total proteins were separated by SDS-polyacrylamide gel electrophoresis. The polypeptides were transferred to a nitrocellulose membrane, and the β-Gal expression was detected by Western immunoblotting as described under “Experimental Procedures.” The β-Gal and Δβ-Gal polypeptides were expressed in the PABP-β-Gal (lanes a–e), pCMV-SPORTβ-Gal (lane f), and mock (lane g)-transfected cells are shown. The normalized levels of β-Gal mRNA before RNase treatment were measured by RT-PCR at different times following inhibition of RNA synthesis. For each set of experiments, we also measured the level of GAPDH mRNA as internal controls. The results indicate that the half-lives of chimeric β-Gal mRNAs containing different regions of PABP mRNA 5'-UTR were similar (Fig. 4). The p89-Gal mRNA containing the shortest 5'-UTR decayed at approximately the same rate as the p264-Gal mRNA containing the 5'-UTR region proximal to the initiation site (Fig. 4, panel B). In all transfections, no detectable difference was observed in the stability of GAPDH mRNA (Fig. 4, panel A). These results suggest that presence of a potential PABP-binding site at the 5'-UTR had no detectable effect on mRNA stability.

FIG. 3. The β-Gal mRNA levels. Equal amounts of RNA from transfected cells were used for RNase protection assay to quantify the β-Gal mRNA levels as described under “Experimental Procedures.” Electrophoresis of the samples was performed in 2% agarose gels, and the autoradiographs were developed after 2 days of exposure using Kodak X-Omat film. The levels of different chimeric β-Gal mRNAs (panel A, lanes c–g) and GAPDH mRNA (panel B, lanes c–g) in cells transfected with p487, p264, p223, p89-β-Gal, and pCMV-SPORT-β-Gal plasmids are shown. Complete digestions of the probe in absence of RNA (lane b) and the undegraded probe before RNase treatment (lane a) are shown as controls. Concentrations of the undigested probes were adjusted before loading to give equivalent band intensities. The GAPDH mRNA probe was designed to give a shorter Rnase-protected fragment. The normalized levels of β-Gal mRNA were calculated by using the GAPDH mRNA level.
from approximately 100 in p89-Gal to approximately 500 in p487-Gal. The results of mRNA distribution studies therefore confirm that the distance between AUG and the translational regulatory region had no significant effect on the ability of this adenine-rich sequence to inhibit mRNA translation. In contrast to those mRNAs containing the A-rich domain, p264-Gal was found predominantly in the translated polysomal fraction (Fig. 5, lane b). Simultaneous analyses of the GAPDH mRNA distribution between translationally active and inactive populations showed no variation in cells transfected with different constructs. In all cases more than 90% of GAPDH mRNA was present in the translationally active polysomal fraction (Fig. 5, lanes a–e). The cytoplasmic distribution of GAPDH mRNA also suggested that the observed translational repression was not due to unexpected loss of mRNA from the polysomal population during lysis and subcellular fractionation of the cells. The distribution of chimeric mRNAs between the polysome and post-polysome fractions was in general agreement with the levels of β-Gal polypeptide (Figs. 2 and 5). However some discrepancy was noticed because despite any detectable difference in the distribution profile between p487 and p89-Gal mRNAs there was almost 2-fold difference in β-Gal polypeptide levels in cells transfected with these constructs. The reason for the discrepancy is not clear but is most likely due to the difference in the amount of monosome-bound slowly translated mRNAs in the post-polysomal fractions. This could also explain
ventitious dissociation of polyribosomes or simply the consequence of false negatives in RNase protection reactions, the same samples were analyzed for the distribution of GAPDH mRNA. The results (Fig. 6, panel C) show that the polysomal distribution profile of the GAPDH mRNA was similar to that of the p264-Gal mRNA. Thus our results suggest that formation of pre-initiation complex between mRNA and 40 S ribosomal subunits was not completely inhibited by the presence of PABP at the 5'UTR. It is however likely that scanning of the mRNA by the ribosomal subunit was hindered by 5'UTR-bound PABP resulting in much slower joining of the 60 S ribosomal subunit to form the initiation complex.

Although the A-rich 5'UTR region of PABP mRNA could confer repression of translation of the reporter β-Gal mRNA, it was not evident whether overexpression of PABP in HeLa cells is prevented through this sequence. To investigate this we reasoned that if overexpression of PABP beyond a threshold level is detrimental to cells, attempts to overexpress it in cells by transfection with PABP cDNA driven by a strong promoter would result in silencing of the endogenous PABP expression. This will be particularly evident if the construct for ectopic PABP expression lacks the presumptive autoregulatory A-rich 5'UTR. We therefore developed two constructs. The first one lacks the 1-223-nucleotide-long region containing the A-rich sequence of the 5'UTR. The second plasmid was created by joining a short fragment containing the A-rich segment of the 5'UTR with this construct (Fig. 1, panel B). Transfections of cells with these constructs were carried out, and then cytoplasmic distribution of endogenous and ectopic PABP mRNAs was measured. The mRNA levels were measured by S1 nuclease protection of oligodeoxynucleotides specific for either the ectopic PABP or the endogenous PABP mRNA. We took advantage of the 5'UTR deletion and the presence of unique 3'UTR in the ectopic PABP mRNA in designing specific oligomers. Results (Fig. 7) show that there was no detectable nuclease-protected oligomer in non-transfected cells when the primers specific for ectopic PABP mRNA were used (Fig. 7, panel A, lanes c and a). Analysis of the subcellular distribution of these mRNAs shows that the ectopic PCMVAPABP mRNA was efficiently translated, and nearly 90% of this mRNA was present in the polysomal fraction (Fig. 7, panel A, lanes e and f). In contrast 90% of the PCMV89ΔPABP mRNA was found in the repressed post-polysomal fraction (lanes g and h). Most interesting, however, was the effect on the translation of the endogenous PABP mRNA. In untreated control cells, 70% of the endogenous mRNA was found in the translated polysomal fraction (Fig. 7, panel B, lanes c and d). A dramatic change in its translation was observed in PCMVΔPABP-transfected cells (lanes e and f). The majority of the endogenous PABP mRNA was found in the translationally repressed state following expression of the ΔPABP mRNA. These results show that efficient translation of the truncated unregulated PABP mRNA could force the PABP mRNA with the autoregulatory sequence to the non-translated state, thus preventing overexpression of the polypeptide. This shift to the non-translated state was unique to PABP mRNA translation and was not observed for the GAPDH mRNA translation. In mock-treated control, PCMVAPABP-, and PCMV89ΔPABP-transfected cells a similar distribution of GAPDH mRNA between polysomal and non-polysomal fractions was observed. In all circumstances more than 80-90% of GAPDH mRNA was present in the polysomal fraction (Fig. 7, panel C, lanes c, e, and f). It should also be noted that more endogenous PABP mRNA was present in polysomes than the chimeric β-Gal mRNAs and the ectopic PABP mRNA with the regulatory sequence at their 5'UTR (Figs. 5, panel B, and 7, panel B). The reason for this is not known but
isolated and analyzed by S1 nuclease protection of 32P-labeled oligomer.

Cells were also mock-transfected with LipofectAMINE but without any pCMV89 D PABP (Fig. 1) as described under “Experimental Procedures.”

Panels A–C, distribution of ectopic (PABPEc) and endogenous PABP mRNAs (PABP恩) in post-polysomal free (F) and polysomal (P) fractions. HeLa cells were transfected with either pCMVΔPABP or pCMV89ΔPABP (Fig. 1) as described under “Experimental Procedures.” Cells were also mock-transfected with LipofectAMINE but without any DNA. The polysome and post-polyosomal RNA from these cells were isolated and analyzed by S1 nuclease protection of 32P-labeled oligomer probes as described under “Experimental Procedures.” Digestion of probes in absence of RNA (panels A–C; lane b) were used as controls. The shorter S1 nuclease-protected probes for ectopic (panel A) and endogenous (panel B) PABP mRNAs and GAPDH mRNA (panel C) are shown for both polyribosomal (P) and post-polyribosomal (F) fractions by arrows on the right, and the undigested oligomers (lane a, panels A–C) are shown by arrows on the left. The intensity of bands was measured by scanning as described previously. Panel D, analysis of the PABP level in transfected cells. Following transfection of cells with different plasmids the level of PABP was examined by Western immunoblotting technique as described under “Experimental Procedures.” The human PABP antibody was a gift from Dr. G. Dreyfuss (University of Pennsylvania School of Medicine). PABP levels in PCMV D PABP-transfected cells. This re-Examples did not rule out the possibility of other regulatory sequences existing within the 5′-UTR of PABP mRNA. PABP mRNA translation appeared to be differently regulated in different cells (5, 16–18). It is therefore possible that its translation can be regulated by sequence(s) other than the putative PABP-binding sequence in other cell lines or in a tissue-specific manner. It is conceivable that the presence of cell-specific RNA-binding proteins may be involved in differential regulation of translation of PABP mRNA. However, our major concern was to test the possibility of autoregulation and the potential role of the oligo(A)-rich sequence in mRNA translation in vivo. To test further this model, we examined the effect of ectopic expression of this mRNA on the translation of endogenous mRNA. Our results showed that translation of the endogenous PABP mRNA was repressed at attempts were made to overexpress PABP. These results suggest that the level of PABP and its mRNA may determine how much of the cytoplasmic pool of the PABP mRNA is translated. This checkpoint in PABP expression depends on the presence of the A-rich region of its 5′-UTR.

In cells transfected with the PABP expression vectors, the mRNA levels were increased by more than 2-fold. But only a modest increase (25%) in the PABP level was found (Fig. 7). In the cells expressing the unregulated ectopic mRNA, this was achieved by almost completely blocking the translation of endogenous mRNA and translating the ectopic mRNA. On the other hand when the ectopic mRNA had the auto-regulatory sequence (ARS) both endogenous and ectopic mRNAs translation were controlled. However, the translation of the ectopic PABP mRNA with a shorter 5′-UTR and the ARS positioned closer to the initiation codon than in the endogenous mRNA was repressed more than that of the endogenous mRNA. The reason for this difference is not clear but may reflect the influence of other regulatory sequences. As a result of nearly complete inhibition of the ectopic mRNA translation and a small shift of the endogenous PABP mRNA to the non-translated state, the levels of PABP in these cells were reduced by 50%.

**DISCUSSION**

The presence of approximately 60% adenine base between nucleotides 71 and 131 of the PABP mRNA 5′-UTR offers potential PABP-binding site(s) for autoregulation of its translation. In support of this autoregulation model it was shown that removal of the first 223 nucleotides of its 5′-UTR sequence in cultured HeLa cells also demonstrated the inhibitory effect of PABP on mRNA translation. The presence of the 58–146 nucleotides from PABP mRNA 5′-UTR resulted in inhibition of translation of the reporter β-Gal mRNA in HeLa cells. Although the adenine-rich region was located between nucleotides 71 and 131, we used a slightly larger region (nucleotides 58–146) of the 5′-UTR in our analyses. Due to the predominance of a single base the A-rich region was not clonable without some flanking sequences. The results of our studies did not rule out the possibility of other regulatory sequences existing within the 5′-UTR of PABP mRNA. PABP mRNA translation appeared to be differently regulated in different cells (5, 16–18). It is therefore possible that its translation can be regulated by sequence(s) other than the putative PABP-binding sequence in other cell lines or in a tissue-specific manner. It is conceivable that the presence of cell-specific RNA-binding proteins may be involved in differential regulation of translation of PABP mRNA. However, our major concern was to test the possibility of autoregulation and the potential role of the oligo(A)-rich sequence in mRNA translation in vivo. To test further this model, we examined the effect of ectopic expression of this mRNA on the translation of endogenous mRNA. Our results showed that translation of the endogenous PABP mRNA was repressed when attempts were made to overexpress PABP. These results suggest that the level of PABP and its mRNA may determine how much of the cytoplasmic pool of the PABP mRNA is translated. This checkpoint in PABP expression depends on the presence of the A-rich region of its 5′-UTR.
Thus, there was over-compensation of PABP expression. The precise mechanism of PABP-mediated feedback control of translation is not known. However, the results suggest that 48 S preinitiation complexes were formed but were not efficiently converted to the initiation complex. It is therefore possible that binding of PABP at the ARS may decrease the rate of the 40 S ribosomal subunit movement along the mRNA. According to this model the distance between the initiation codon and the ARS is unlikely to have a major influence on mRNA translation. The distance of the ARS from the 5'-cap region, however, might have stronger influence than that of the ARS and the AUG codon. This is conceivable since the 3' poly(A)-PABP complex may be involved in the translation initiation through its interaction with the eIF4G. Therefore, formation of a similar complex close to the 5'-capped end may mute their functional interaction. Further studies are required to understand how ARS-PABP complex works.

Feedback control of PABP mRNA translation may serve an important purpose in regulating the cellular level of free PABP. Under normal circumstances, cytoplasmic PABP is present in excess over the availability of the poly(A)-binding sites (37). The reason for the presence of free PABP is not clear. However, some of this may result from degradation or shortening of the poly(A) tail. Presence of multiple poly(A)-binding domains in PABP (38) potentially contributes to the dynamic nature of its interaction with the 3' poly(A) tail (39). This could allow movement of PABP between the 3' poly(A) tail and other lower affinity PABP binding sequences (37) including the ARS at the 5' UTR of PABP mRNA. How efficiently PABP would interact with the lower affinity binding sequences may depend on its cellular level.

Presumably an optimal level of PABP is required for maintaining an equilibrium between its various functions. Our studies have shown that the presence of the putative PABP-binding site at the 5'-UTR of PABP mRNA reduces by approximately 3-fold the translation of this mRNA both in vitro (28) and in vivo (Fig. 3). Although the presence of a certain excess amount of PABP is tolerated and may even be required for normal cellular function, its expression beyond a threshold could be controlled at the mRNA translation level. Excess PABP beyond the threshold level may detrimentally alter the pattern of protein synthesis in the cell, for example by increasing translation of inherently inefficient mRNAs. In addition stability of some unstable mRNAs may also increase if excess PABP is made. Together, these events could have a dramatic effect on the growth regulation and cellular differentiation. This possibility is supported by the previous observation that overexpression of eIF4E results in cellular transformation (41). Since the role of PABP in translation is believed to be mediated through its interaction with the cap structure and eIF4G (of which eIF4E is a component), it will be important to control PABP expression through feedback mechanisms. This is further supported by the observation that overexpression of PABP in Xenopus oocytes influenced the deadenylation and translation of maternal mRNAs (42).

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