2198–2205 Nucleic Acids Research, 1995, Vol. 23, No. 12

Autoregulation of poly(A)-binding protein synthesis *in vitro*

Osvaldo P. de Melo Neto[1,2], Nancy Standart[1,*] and Cezar Martins de Sa[2]

[1]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK and
[2]Dept. de Biologia Celular, Universidade de Brasilia, Brasilia DF 70000, Brazil

Received February 22, 1995; Revised and Accepted April 28, 1995

ABSTRACT

The poly(A)-binding protein (PABP), in a complex with the 3′ poly(A) tail of eukaryotic mRNAs, plays important roles in the control of translation and message stability. All known examples of PABP mRNAs contain an extensive A-rich sequence in their 5′ untranslated regions. Studies in mammalian cells undergoing growth stimulation or terminal differentiation indicate that PABP expression is regulated at the translational level. Here we examine the hypothesis that synthesis of the PABP is autogenously controlled. We show that the endogenous inactive PABP mRNA in rabbit reticulocytes can be specifically stimulated by addition of low concentrations of poly(A) and that this stimulation is also observed with *in vitro* transcribed human PABP mRNA. By deleting the A-rich region from the leader of human PABP mRNA and adding it upstream of the initiator AUG in a reporter mRNA we show that the adenylate tract is sufficient and necessary for mRNA repression and poly(A)-mediated activation in the reticulocyte cell-free system. UV cross-linking experiments demonstrate that the leader adenylate tract binds PABP. Furthermore, addition of recombinant GST–PABP to the cell-free system represses translation of mRNAs containing the A-rich sequence in their 5′ UTR, but has no effect on control mRNA. We thus conclude that *in vitro* PABP binding to the A-rich sequence in the 5′ UTR of PABP mRNA represses its own synthesis.

INTRODUCTION

The principal protein associated with eukaryotic mRNA is the poly(A)-binding protein (PABP), a protein of ~70–72 kDa which selectively binds the 3′ poly(A) tail and which is essential for viability in yeast (1). Complex formation between the 3′ poly(A) tail and PABP has been implicated in enhancing translational re-initiation (2–4), as well as in determining mRNA stability (5,6). Accumulating evidence indicates that PABP [or the poly(A)–PABP complex] facilitates 80S initiation complex formation (7–10). The most direct support for this role is the finding that yeast cells lacking PABP have decreased levels of polysomes and several suppressor mutations that allow yeast cells to grow in the absence of PABP affect biogenesis of 60S ribosomal subunits (8). Further support for the role of PABP in initiation comes from the observed synergism between the 5′ cap and a poly(A) tail in promoting translation *in vivo* (11). How PABP regulates mRNA degradation is less well understood. Although initial experiments suggested that PABP protected poly(A) from exonucleases *in vitro* (12), PABP depletion in yeast cells resulted in abnormally long poly(A) tails, due to lack of activation of a PABP-dependent poly(A)-specific nuclease, PAN (10). Since PAN, like PABP, is required for translational initiation, it has been proposed that an interaction between PAN and PABP is important for both poly(A) shortening and translation initiation.

The sequence of PABP, from various organisms ranging from yeast to man, shows the conservation of four N-terminal tandem RNA recognition (RRM) domains followed by a variable C-terminus, believed to promote protein–protein interactions (11–18). The RRM, the most widely found and best characterised RNA binding motif, is composed of 80–90 amino acids and is present in one or more copies in proteins that bind pre-mRNA, mRNA, rRNA and snRNA (13,14). Truncated PABPs, containing only one or two of these RRM domains, are still capable of specific poly(A) binding *in vivo* and *in vitro* (1,15,16), suggesting that the multiplicity of domains affords contact between poly(A)-containing molecules or between poly(A) and a non-polyadenylated part of mRNA (1). Although the minimal binding site for PABP is only 12 A residues, the observed packing density corresponds to about one PABP per 25–27 A residues, supporting the notion that more than one domain binds the poly(A) tail (1).

PABP expression is regulated at the translational level. When quiescent animal cells in culture are stimulated to proliferate by serum a number of specific changes in the pattern of protein synthesis are observed, including an increase in PABP synthesis in the absence of any change in PABP mRNA levels (17). In contrast, in terminally differentiated reticulocytes, which are transcriptionally inert and contain stores of PABP, PABP mRNA is largely repressed (18,19).

All PABP mRNAs cloned to date contain a 50–70 nt long tract rich in A residues in their 5′ untranslated region (UTR) (15,16,20–23) which is capable of binding PABP (20,24). The existence of these motifs has suggested an autoregulatory translational control mechanism in which PABP synthesised in

* To whom correspondence should be addressed

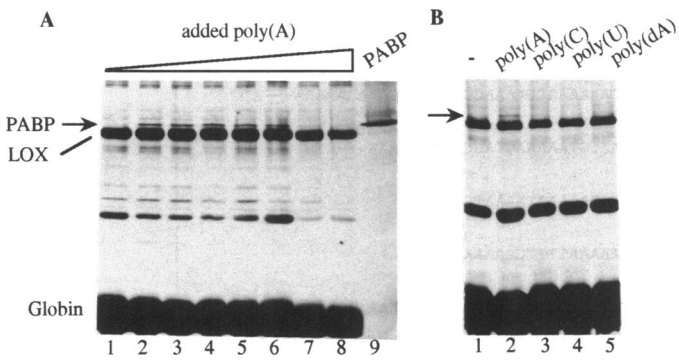

Figure 1. Activation of PABP mRNA translation in the untreated rabbit reticulocyte lysate by low concentrations of poly(A). Rabbit reticulocyte lysate (not nuclease-treated) was incubated with various concentrations of homopolynucleotides and assayed for protein synthesis with [^{35}S]methionine and analysis of translation products by SDS–PAGE and autoradiography. (**A**) Lanes 1–8, poly(A) added at 0, 1, 2, 4, 8, 16, 32 and 64 μg/ml. Lane 9, human PABP made *in vitro* from a T7 RNA polymerase transcript, translated in the nuclease-treated rabbit reticulocyte lysate. The position of the poly(A) binding protein (PABP), lipoxygenase (LOX) and globin are indicated. The assignment of LOX and globin was based on polypeptide size and abundance. (**B**) Rabbit reticulocyte lysate was incubated with different polynucleotides, as indicated, at a final concentration of 8 μg/ml lysate and analysed as above. Lane 1, no added polynucleotide; lane 2, poly(A); lane 3, poly(C); lane 4, poly(U); lane 5, poly(dA). The position of the poly(A) binding protein is indicated by an arrow.

excess over the available 3′ poly(A) tail binding sites may associate with the 5′ UTR of its own mRNA, with somewhat lower affinity than its binding to poly(A), and repress further PABP synthesis. This repression would then be relieved if there was an increase in intracellular poly(A)$^+$ mRNA or an increase in the length of pre-existing poly(A) tails (20,21).

The experiments reported in this study were designed to test the model that PABP can bind the 5′ UTR adenylate stretch and by doing so inhibit its own translation. We found that the A-rich region in the leader of an mRNA was necessary and sufficient for PABP-mediated translational repression, thus providing direct evidence in support of the autoregulatory model.

MATERIALS AND METHODS

Plasmid construction and transcription

PABP mRNAs. The human PABP cDNA in the transcription vector pGEM1 (21) linearized with *Bam*HI gave rise to the 2.9 kb long mRNA, which contains 502 nt in its 5′ UTR, followed by the coding region and a 447 nt long 3′ UTR. To obtain a PABP cDNA without the A-rich tract (located between residues 71 and 131), the 2.6 kb *Stu*I–*Bam*HI fragment corresponding to the coding region and 3′ UTR, as well as the 239 nt immediately upstream of the initiator AUG, was subcloned into the *Sma*I and *Bam*HI sites of pGEM1 (Promega). This cDNA yielded Δ(1–263) PABP mRNA. In order to replace the first 263 nt of PABP mRNA with the adenylate sequence alone, complementary oligonucleotides were made which corresponded to the 61 nt long A-rich leader sequence of human PABP mRNA (see Fig. 2) and the same length of pure poly(A). These two sequences are distinguished in the text as A-rich or pure A tracts, respectively. The 2.6 kb *Stu*I–*Bam*HI fragment of PABP mRNA was subcloned into the *Sph*I site of pTZ18 plasmids (Pharmacia) in which the adenylate oligonucleotides were cloned in the polylinker *Xba*I site and whose *Bam*HI sites were abolished by in-filling. The A-rich oligonucleotide sequence was 5′-CTAGCAGGCCTA$_6$TCCA$_8$TCTA$_7$TCTTTT-A$_6$CCCCA$_7$TTTACA$_6$T-3′, in which the runs of A are in bold type. The pure A oligonucleotide sequence was 5′-CTAGCAGG-CCTA$_{61}$T-3′. The adenylate oligodeoxyribonucleotides contained an internal *Stu*I site on their 5′ end to ease cloning, as well as *Xba*I overhang sites at both 5′ and 3′ ends. Though both ends will anneal with *Xba*I-cut DNA, only the 3′ and not the 5′ end will reconstitute an *Xba*I site. All constructs were checked by sequencing and linearized with *Bam*HI prior to transcription with T7 RNA polymerase in the presence of a cap analogue (25).

Cyclin mRNAs. The control mRNA in this study was the *Xenopus laevis* cyclin A mRNA cloned into the *Eco*RI site of pGEM1 (26). To isolate PABP (1–263)–cyclin A the complete *Eco*RI-flanked cyclin A cDNA was blunt ended by in-filling and ligated into wild-type PABP cDNA which had been restricted with *Stu*I and *Bam*HI (to release the PABP nucleotide 264–2866 insert) and blunt ended by in-filling. To place the adenylate oligonucleotides upstream of cyclin A mRNA the *Eco*RI–*Sal*I fragment of cyclin A (missing 90 nt of the 3′ UTR) was ligated into the two pTZ18 vectors containing the adenylate oligonucleotides in the *Xba*I site and linearized with *Sal*I. After ligation the *Eco*RI and remaining *Sal*I ends were filled in and religated. Insert orientation was checked by sequencing prior to linearization with *Sal*I and transcription with T7 RNA polymerase in the presence of a cap analogue.

In vitro translation

The rabbit reticulocyte cell-free synthesizing system was used either as such or after treatment with micrococcal nuclease to give the mRNA-dependent form (27). Protein synthesis was assayed at 30°C with [^{35}S]methionine (0.5 mCi/ml final isotopic concentration; SJ1515; Amersham International) and the radiolabelled products analysed by 15% SDS–PAGE and autoradiography. *In vitro* transcribed RNAs were translated at final concentrations ranging from ~0.2 to 1.25 μg/ml as indicated. When used, polynucleotides were pre-incubated with the reticulocyte mix for 15 min on ice prior to addition of mRNA, followed by incubation at 30°C for 1 h.

The wheat germ cell-free lysate was purchased from Promega and used as recommended, with a final concentration of 93 mM KOAc and incubation for 2 h at 25°C.

UV cross-linking

The RNA probes used in the experiment shown in Figure 4 were synthesized from linearized cDNA using T7 RNA polymerase and [α-^{32}P]ATP (PB 10160; Amersham) as the labelled nucleotide, as described (28,29). The full-length PABP cDNA in pGEM1(21) was linearized with *Stu*I and *Nco*I to give 5′ non-coding RNA fragments corresponding to nucleotides 1–261 and 1–528, respectively (RNAs 1 and 2). Δ(1–263) PABP in pGEM1 linearized with *Nco*I was used to generate a 5′ non-coding RNA containing nucleotides 264–528 (RNA 3). A-rich tract Δ(1–263) PABP in pGEM1 was also linearized with *Nco*I to generate RNA 4. As a control, frog cyclin A cDNA (Xl4 in pGEM1) was linearized with *Hin*dIII to give an RNA corresponding to nucleotides 1–455 (RNA 5). The RNA probe used in Figures 5 and 6 was run off the plasmid pTZ18 containing the A-rich tract only, linearized with *Xba*I.

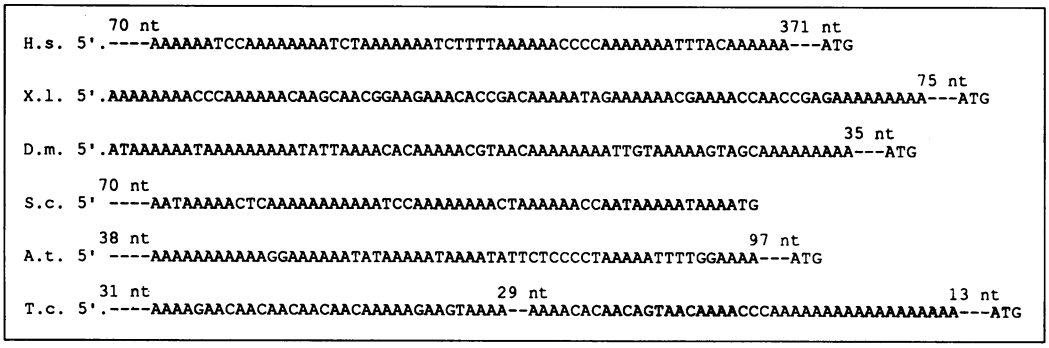

Figure 2. A-rich sequences in PABP mRNA 5′ untranslated regions. The A-rich sequences present in the 5′ UTRs of PABP mRNAs from a wide range of organisms, including human (H.s.; 21), the frog *X.laevis* (X.l.; 15), the fly *Drosophila melanogaster* (D.m.; 22), the yeast *S.cerevisiae* (20), the plant *A.thaliana* (A.t.; 37) and the parasite *T.cruzi* (T.c.; 23), are displayed, as well as the distances in nucleotides (nt) between these sequences and the 5′ cap (where known) and the initiator AUG. Sequences shown as 5′--- indicate that the transcription site has been determined, whereas the ends of those sequences shown as 5′.--- have not been mapped.

Aliquots (2 µl) of the rabbit reticulocyte lysate were pre-incubated with competitor polynucleotides or water for 5 min on ice followed by incubation with the ^{32}P-labelled RNA probes (~1–1.5 ng, 100 000 c.p.m.) for 15 min at room temperature in binding buffer (10 mM HEPES, pH 7.2, 100 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.1 mg/ml *Escherichia coli* rRNA) in a final volume of 10 µl. After addition of heparin to 5 mg/ml final concentration the samples were subjected to UV irradiation in a Hybaid UV cross-linker (Spectrolinker XL-1500) as recommended, followed by digestion with RNAse A (final concentration 250 µg/ml) and cobra venom ribonuclease (3 U/ml final concentration; Pharmacia) and addition of sample buffer (28,30). The ^{32}P-labelled proteins were analysed by 15% SDS–PAGE and autoradiography.

Cloning and purification of GST–PABP

The *Nco*I–*Bam*HI fragment of human PABP (nucleotides 528–2866, missing the first nine amino acids) was in-filled and ligated into an in-filled, *Bam*HI-cut pGEX2T vector (Pharmacia). Cloned GST–PTB was a kind gift from Jim Patton (31). Cultures (200 ml) of transformed BL21(DE3) cells were grown to ~0.5 OD_{600} before being induced with 0.5 mM IPTG for 3–4 h at 37°C. The cell pellets, resuspended in phosphate-buffered saline supplemented with protease inhibitors (10 µg/ml soybean trypsin inhibitor, 2 µg/ml leupeptin and aprotinin, 1 mM PMSF), were sonicated and the supernatant obtained after centrifugation applied to glutathione–Sepharose (Pharmacia), as recommended by the manufacturer. The proteins eluted with 20 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0, were dialysed against 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl and quantitated by comparison with known amounts of BSA by gel electrophoresis.

To make His_6–PABP, the same cloning strategy as above was used with the pQE30 vector (Qiagen). In this case the DNAs were transformed into *E.coli* strain M15[pREP4] and chromatography was on Ni–NTA (Qiagen).

When the recombinant proteins were tested in *in vitro* translation systems, *E.coli* rRNA (2.5 µg/ml final concentration) and poly(G) (2 µg/ml final concentration) were added to the assays to help reduce any non-specific nuclease activity present in the purified protein preparations.

RESULTS

Poly(A) specifically activates PABP synthesis in untreated rabbit reticulocyte lysate

Previous work demonstrated that PABP mRNA is present in an inactive form in mature reticulocyte cells, which contain a large pool of PABP protein (18,19). We reasoned that if this repression was due to excess PABP binding to the 5′ UTR of PABP mRNA, then addition of poly(A) should rescue PABP synthesis. In the experiment shown in Figure 1 poly(A) was added to the untreated rabbit reticulocyte cell-free extract at a range of concentrations, from 1 to 64 µg/ml, prior to starting the translation assay with [^{35}S]methionine. Strikingly, at low concentrations of added poly(A) the synthesis of a 72 kDa polypeptide is specifically enhanced; the labelling of other proteins is essentially unaffected (Fig. 1A). This 72 kDa band co-migrates with human PABP synthesized *in vitro* from a T7 RNA polymerase transcript (Fig. 1A), as well as in two-dimensional gel electrophoresis (data not shown). Thus the endogenous inactive rabbit PABP mRNA can be specifically activated by the addition of poly(A) <16 µg/ml. Increased PABP synthesis is only seen when poly(A) (8 µg/ml) is added; other polynucleotides tested [including poly(U), poly(C) and poly(dA)] have no effect at this concentration [Fig. 1B; poly(G) not shown]. Poly(A)-mediated stimulation of PABP synthesis was also observed when poly(A) was added 15 min after the start of incubation (data not shown), strongly supporting the model that its effect on PABP mRNA was exerted at the level of translation, rather than stability, and in agreement with previous work demonstrating a pool of repressed PABP in these cells (18,19). We also noted that the maximal level of PABP synthesis seen in reticulocyte reactions stimulated with poly(A) was very similar to those observed when phenol-extracted total rabbit reticulocyte RNA was translated in the nuclease-treated reticulocyte lysate (relative to the same signals of major polypeptides in both cases; data not shown), suggesting that low concentrations of poly(A) completely activate most, if not all, of the repressed endogenous PABP mRNA.

At higher levels of poly(A) the synthesis of all reticulocyte polypeptides was inhibited (Fig. 1A); this repression is due to a total depletion of lysate PABP, apparently required for re-initiation of mRNA (7,9). As has been shown previously, addition of purified PABP protein reverses this inhibition (7). A more recent

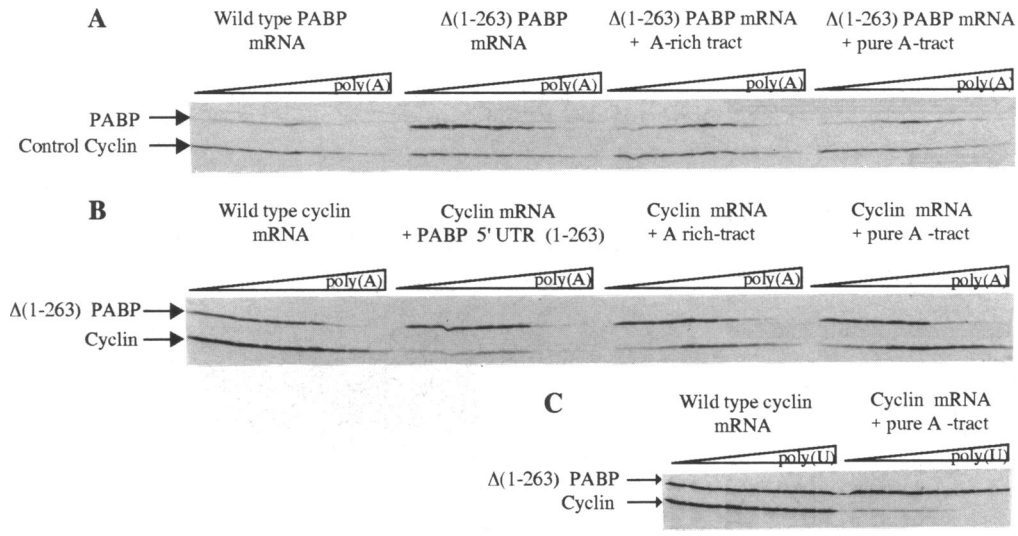

Figure 3. Role of the A-rich tract on translation stimulation by poly(A) of *in vitro* transcribed mRNAs. (**A**) PABP mRNAs. Wild-type PABP as well as various mutant PABP mRNAs were translated *in vitro* in the nuclease-treated rabbit reticulocyte lysate, alongside control *X.laevis* cyclin A mRNA in the presence of added poly(A). Constructs were made where the first 263 nt of the 5′ UTR from the human PABP cDNA were either deleted [Δ(1–263) PABP mRNA] or replaced by oligonucleotides containing only the 61 nt long A-rich tract [Δ(1–263) + A-rich tract PABP mRNA; see Fig. 2] or a pure adenylate stretch of the same size [Δ(1–263) PABP mRNA + pure A tract]. The resulting plasmids were all transcribed *in vitro* with T7 RNA polymerase and the capped mRNAs (~0.5 μg/ml PABP mRNAs and 0.16 μg/ml control cyclin mRNAs; final concentrations) used in translation assays with increasing concentrations of added poly(A) (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/ml). (**B**) Cyclin mRNAs. The PABP coding region of the constructs shown in (A) was replaced by that of *Xenopus* cyclin A cDNA (see Materials and Methods for details). The resulting clones were transcribed and the capped mRNAs translated (~0.5 μg/ml cyclin mRNAs and 0.16 μg/ml PABP control mRNA) in the presence of increasing concentrations of poly(A), as in (A). (**C**) Cyclin mRNA with the pure upstream A tract was used in translation assays to which poly(U) was added at 0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/ml. In (B) and (C) Δ(1–263) PABP mRNA missing the first 263 nt of its 5′ UTR, including the adenylate tract, was translated together with wild-type and A tract-containing cyclin mRNA as an internal control.

study indicated that high levels of exogenous poly(A) may also preferentially inhibit the translation of uncapped mRNAs in this lysate; possibly by sequestering cap binding factors (32).

The 5′ UTR adenylate tract is required for poly(A)-mediated stimulation of translation

Imperfect adenylate tracts are found upstream of the initiator AUG in all PABP mRNAs analysed to date (Fig. 2), from the parasite *Trypanosome cruzi* to man. In general, they extend up to 60–70 nt in length, with stretches of six to eight A residues interrupted with C or T residues; there is a marked paucity of G residues in this A-rich region (see Fig. 2 for references).

To test the role of the 5′ adenylate tract in regulating PABP synthesis, we examined the translation behaviour of *in vitro* transcribed mRNAs generated by T7 RNA polymerase transcription of the full-length as well as of truncated human PABP cDNAs. In these experiments, poly(A) was added at various concentrations to the nuclease-treated rabbit reticulocyte lysate containing PABP mRNA and an internal control mRNA, that encoding *X.laevis* cyclin A. *Xenopus* cyclin A mRNA does not contain an A-rich region in its short (54 nt) 5′ UTR (26).

Poly(A)-mediated stimulation of PABP synthesis was observed with the full-length human PABP mRNA, which contains an unusually long leader of 502 nt (21). In the absence of poly(A), the translation of PABP mRNA was barely detectable at the RNA concentrations used and was significantly enhanced by the addition of low concentrations of poly(A), between 0.5 and 2 μg/ml; cyclin synthesis was not affected at these doses (Fig. 3A). However, as seen previously in the untreated rabbit reticulocyte lysate (Fig. 1A), the translation of both mRNAs is severely reduced at higher concentrations of poly(A). The importance of the A-rich tract for poly(A)-mediated stimulation of translation was confirmed when the translation of a truncated PABP mRNA, transcribed from a cDNA from which the first 263 nt had been deleted, was analysed. The loss of this 5′ segment, which includes the A-rich tract (21), results in a significant increase in PABP synthesis in the absence of poly(A) and addition of low concentrations of poly(A) does not lead to any further increase; thus PABP Δ(1–263) mRNA behaves exactly like the control cyclin A mRNA. In order to test whether the difference between the full-length and truncated PABP mRNAs was indeed due to the adenylate tract or other sequences present in the first 263 nt, PABP cDNAs were constructed in which the 263 nt segment was replaced by a 61 nt oligonucleotide containing only the wild-type human A-rich sequence or a pure adenylate tract of the same size (see Fig. 2 and Materials and Methods). The resulting mRNAs suffered the same decrease in translation efficiency as the wild-type mRNA in the absence of added polynucleotide and were activated by poly(A), showing that the A-rich tract was indeed responsible (Fig. 3A). Interestingly, the stimulation effect seen when exogenous poly(A) was added was somewhat intensified, with the highest translation efficiency [at 2 μg/ml poly(A)] approaching that of PABP Δ(1–263). This difference may indicate that the full-length leader in PABP mRNA contains sequences inhibitory to translation. It may, however, result from the difference between the two mRNAs with respect to the location of the adenylate tract (see Discussion).

To assess whether, in addition to an adenylate tract in the 5′ UTR, poly(A)-mediated stimulation of translation required other sequences in PABP mRNA, we tested the effect of cloning the 263 nt

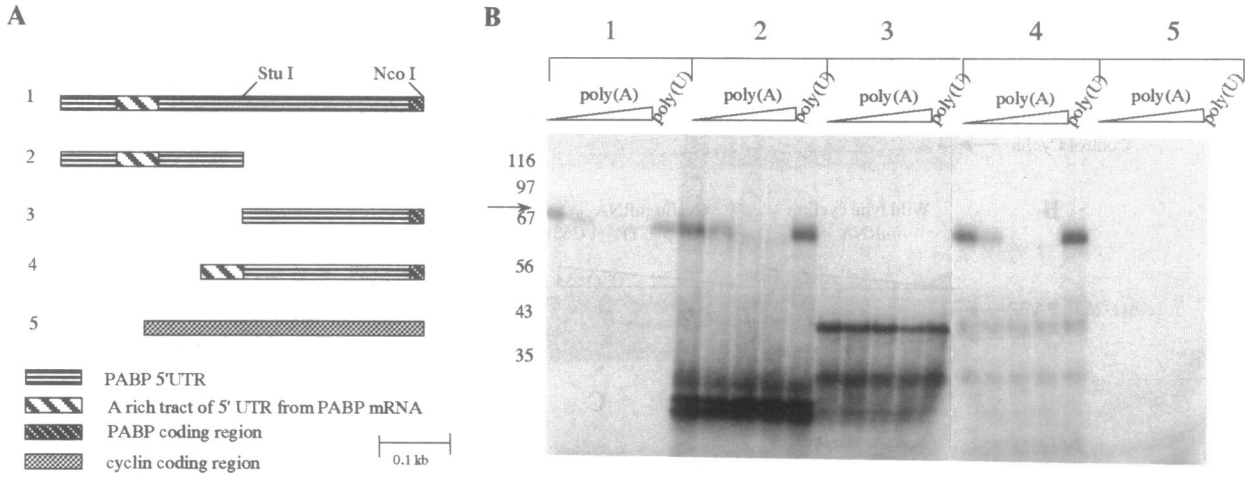

Figure 4. The A-rich tract in the 5′ untranslated region of PABP mRNA can bind PABP in the rabbit reticulocyte lysate. Aliquots of rabbit reticulocyte lysate were mixed with different ^{32}P-labelled RNA probes containing the complete PABP mRNA 5′ UTR or fragments with and without the A-rich tract. The samples were then submitted to UV cross-linking, RNAse A digestion and SDS gel electrophoresis. (**A**) Scheme of the RNA probes indicating their origin. (**B**) Autoradiogram of the UV cross-linking experiment. Each RNA probe was incubated with the lysate in the presence of water and poly(A) at either 1, 4 or 16 μg/ml or poly(U) at 4 μg/ml. On the left are indicated sizes in kDa of protein molecular weight markers. PABP is indicated with an arrow.

human PABP 5′ UTR segment and the two adenylate oligodeoxyribonucleotides upstream of the reporter frog cyclin A mRNA. These cyclin mRNAs were then translated in the presence of increasing concentrations of poly(A) with an internal control mRNA, PABP Δ(1–263) (Fig. 3B). All three cyclin mRNAs containing upstream adenylate tracts showed an overall loss of translation efficiency, compared with the wild-type cyclin A mRNA, in the absence of poly(A). Significantly, however, they gained the ability to respond to low concentrations of poly(A) addition. As seen with PABP mRNAs (Fig. 3A), the mRNAs containing just the adenylate tract, rather than the first 263 nt sequence of PABP 5′ UTR, were more efficiently translated. Although the effects with the wild-type PABP 5′ UTR are relatively small (especially when upstream of cyclin), they were highly reproducible. The maximal stimulation by poly(A) using these constructs was estimated to be 2–3-fold by PhosphoImager analysis.

These results show that the coding region and 3′ UTR of PABP mRNA do not play any part in poly(A)-mediated stimulation of translation; the presence of an upstream adenylate tract is both necessary and sufficient to down-regulate expression of mRNA in the absence of added poly(A) and for its stimulation at low concentrations of poly(A). The stimulatory effect is specific for poly(A), since addition of poly(U) at all concentrations tested (1–32 μg/ml) did not lead to any significant difference in the behaviour of wild-type or adenylate tract-bearing cyclin mRNAs (Fig. 3C).

PABP binds the 5′ UTR adenylate tracts in PABP mRNA

So far we have shown that PABP mRNA or reporter mRNAs with an adenylate tract in their leaders are not efficiently translated *in vitro* unless low concentrations of poly(A) are added. We postulated that this polynucleotide acted as an activator by titrating the PABP bound to the A-rich 5′ UTRs, which normally impede translation. To address this issue, we first used UV cross-linking to examine the ability of various RNAs, with or without the adenylate tract, to bind reticulocyte PABP. In this experiment ^{32}P-labelled RNA was added to reticulocyte lysate pre-incubated with water and poly(A) or poly(U) and the samples subjected to UV irradiation, RNAse A digestion and SDS–PAGE (Fig. 4). Under these conditions RNA binding proteins acquire covalently linked short stubs of labelled RNA and can be visualized by autoradiography (30). Non-specific binding is largely reduced by inclusion of *E.coli* rRNA in the binding reaction and addition of heparin after the incubation. All the RNA probes (nos 1, 2 and 4) containing the adenylate tract bound to a major reticulocyte protein of ~72 kDa; whereas RNAs of similar length lacking the A-rich tract did not (nos 3 and 5; see Fig. 4A for scheme of RNA probes). On the basis of its size and sequence binding specificity we concluded that this protein was PABP. In confirmation, we observed that the 72 kDa protein was not able to cross-link to the labelled A-rich RNA probes in lysates pre-incubated with poly(A), whereas similar treatment with poly(U) did not affect its binding (Fig. 4B). Thus the A-rich tract in the 5′ UTR of human PABP mRNA is capable of binding PABP. This observation is consistent with similar experiments using the nitrocellulose filter binding assay and UV cross-linking in yeast (1) and human (24) cell extracts.

PABP specifically represses translation of mRNAs bearing 5′ adenylate tracts

To directly demonstrate the role of PABP in regulating the expression of mRNAs bearing an A-rich leader, a recombinant human GST–PABP fusion protein was overexpressed and purified on glutathione–Sepharose. As a control, we also overexpressed and purified GST–PTB. PTB (polypyrimidine tract binding protein) is an RNA binding protein with, as its name suggests, affinity for pyrimidine-rich sequences which has been implicated not only in pre-mRNA splicing (31,33), but also in internal initiation of translation (34,35). First, both GST fusion proteins (Fig. 5A) were tested for poly(A) binding activity by UV cross-linking with a ^{32}P-labelled A-rich tract RNA probe (Fig. 5B). Initial results indicated that the cross-linked recombinant proteins would not enter the polyacrylamide gel unless mixed

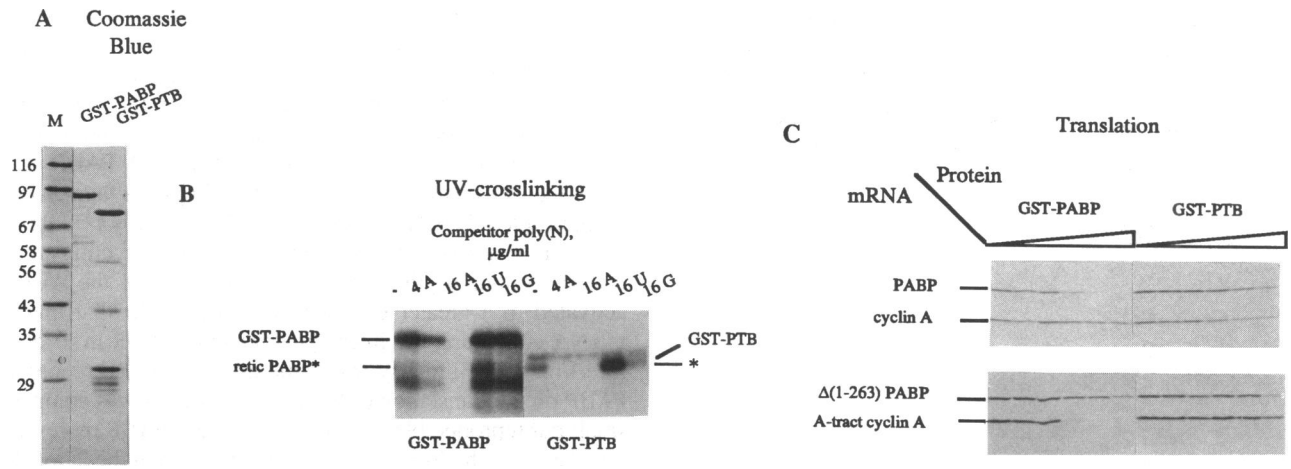

Figure 5. Recombinant human PABP represses translation of mRNAs bearing A-rich tracts in their 5′ non-coding regions. Recombinant, GST-tagged PABP and the control PTB proteins were purified from *E.coli* by glutathione–Sepharose chromatography, tested for RNA binding activity by UV cross-linking and for translational repression in the nuclease-treated rabbit reticulocyte lysate. (**A**) Coomassie blue stained SDS–polyacrylamide gel of purified GST–PABP and GST–PTB. Lane M; protein molecular weight markers with their sizes in kDa indicated. (**B**) UV cross-linking assays. GST–PABP and GST–PTB were mixed with reticulocyte lysate and submitted to UV cross-linking with a ^{32}P-labelled A-rich tract RNA probe in the presence of water and poly(A) at either 4 or 16 µg/ml, poly(U) at 16 µg/ml or poly(G) at 16 µg/ml. The positions of GST–PABP and PTB are indicated; * indicates the position of the rabbit reticulocyte PABP. (**C**) GST–PABP, but not GST–PTB, specifically represses translation of mRNAs bearing A-rich tracts in their 5′ non-coding regions. (Upper panel) Wild-type PABP (1.25 µg/ml) and cyclin mRNAs (0.17 µg/ml) were translated in the nuclease-treated rabbit reticulocyte lysate in the presence of increasing concentrations of recombinant protein (0, 2.8, 5.6, 11.2, 22.4 and 45 µg/ml). (Lower panel) Δ(1–263) PABP (0.33 µg/ml) and pure A tract cyclin (1.25 µg/ml) mRNAs were translated in the presence of increasing concentrations of recombinant protein as above.

with carrier proteins such as BSA or reticulocyte lysate, so for these assays the recombinant proteins were mixed with reticulocyte lysate prior to UV irradiation. GST–PABP was efficiently cross-linked to the RNA probe and this binding was competed out by adding poly(A), but not poly(U) or poly(G). On the other hand, the very much weaker binding of GST–PTB to the probe was not reduced by any competitor RNA, implying that this protein did not have high or specific affinity for poly(A) (Fig. 5B). In both cases the slightly smaller rabbit reticulocyte PABP was observed to cross-link the adenylate RNA probe and to respond to added polynucleotides as expected (Fig. 5B; see also Fig. 4).

Next we examined the role of PABP in poly (A)-mediated activation of translation. Indeed, preliminary assays showed that adding recombinant PABP to a translation assay resulted in loss of activation mediated by poly(A) (not shown). We reasoned, however, that addition of PABP together with poly(A) to an *in vitro* translation system may be operationally equivalent to not adding poly(A). We thus tested the effect of GST–PABP on translation of mRNAs with and without adenylate leader sequences in the absence of added poly(A). As shown in Figure 5C, we found that addition of increasing amounts of recombinant PABP to cell-free systems containing wild-type PABP mRNA and the control cyclin mRNA resulted in striking preferential repression of PABP mRNA (upper panel). Similarly, when cyclin A mRNA with the pure A tract in its 5′ UTR and the internal control Δ(1–263) PABP mRNA were co-translated in the presence of GST–PABP, the translation of the mRNA bearing a 5′ adenylate tract was specifically inhibited (lower panel). We estimate that complete and specific inhibition was achieved in assays in which the recombinant protein was ~50 molar in excess over the *in vitro* transcribed mRNA; in other words, in ~20–25 molar excess over RNA binding sites, assuming a packing density of ~25 residues (20). That this effect was due to PABP itself, rather than the GST moiety of the recombinant protein, was demonstrated in control assays with GST–PTB, which did not significantly inhibit the translation of any of these messages at any dose tested (Fig. 5C). Furthermore, purified His_6-tagged PABP behaved in an identical manner to GST–PABP (data not shown). We noted, in independent experiments, that the translation of wild-type PABP mRNA was somewhat less sensitive to added PABP than the pure A tract cyclin mRNA (Fig. 5C and see Discussion).

Human PABP also represses translation of mRNAs bearing 5′ adenylate tracts in a wheat germ extract

Finally, in order to extrapolate our findings beyond the reticulocyte system, we examined the effect of added PABP in wheat germ cell-free extracts. First, using the UV cross-linking assay we analysed the plant lysate for specific poly(A) binding proteins. We found, as shown in Figure 6A, that a protein of ~62 kDa bound an A-rich ^{32}P-labelled RNA probe in the presence of competitor poly(U) or poly(G). Since its cross-linking was abolished by added poly(A), it seemed very likely that this protein was the wheat PABP. Interestingly, in two other plants, the pea (36) and *Arabidopsis thaliana* (37), the PABPs are slightly shorter than their vertebrate counterparts (~60 and 65 kDa, respectively). Since a poly(A) tail enhances the translation of mRNA in electroporated plant cells (38), it was very likely that poly(A) and the poly(A)–PABP complex play similar roles in plants as they do in higher eukaryotes. Moreover, as shown in Figure 2, *A.thaliana* PABP mRNA contains an A-rich leader sequence, implying that plant PABPs may also be autogenously regulated (35). In a wheat germ *in vitro* translation system containing the control Δ(1–263) PABP mRNA and the pure A tract cyclin mRNA the synthesis of cyclin was significantly more sensitive to inhibition by added GST–PABP than the control (Fig. 6B), providing evidence that the inhibition of adenylate-tract leader mRNAs by PABP is not restricted to vertebrate systems.

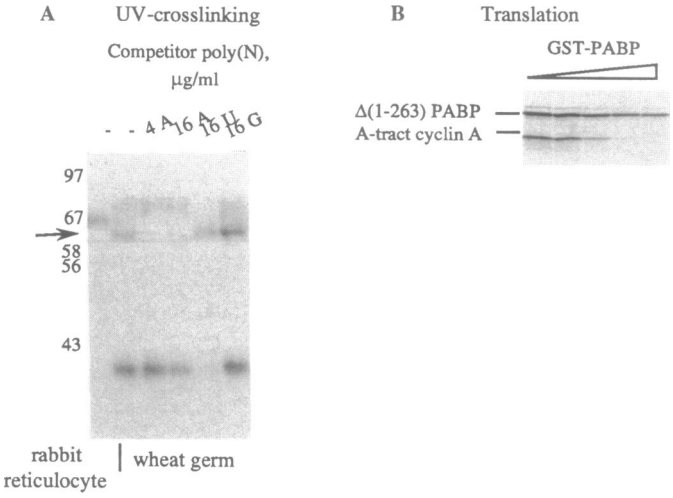

Figure 6. Adenylate tract mRNA is also repressed by PABP in the wheat germ cell-free lysate. (**A**) UV cross-linking of the wheat germ lysate with a ^{32}P-labelled A-rich tract RNA probe in the presence of water and poly(A) at either 4 or 16 μg/ml, poly(U) at 16 μg/ml or poly(G) at 16 μg/ml. The samples were submitted to UV cross-linking, RNAse A digestion and SDS gel electrophoresis. A control lane with rabbit reticulocyte lysate is included. The arrow indicates the position of the wheat germ PABP. (**B**) Δ(1–263) PABP (1 μg/ml) and pure A tract cyclin mRNAs (1.25 μg/ml) were translated in the wheat germ lysate in the presence of increasing amounts of GST–PABP (0, 2.8, 5.6, 11.2 and 22.4 μg/ml).

DISCUSSION

Poly(A)-binding protein mRNA joins a recently growing list of eukaryotic mRNAs which rely on negative autogenous control to modulate their translation and which include those encoding human thymidylate synthase (39,40) and dihydrofolate reductase (41), as well as yeast L32 ribosomal protein (42). sn RNP U1A protein is another example of an RNA binding protein which regulates its own levels, but in this case the U1A protein binds to a site in the 3′ UTR of its mRNA and prevents polyadenylation via a direct interaction with poly(A) polymerase (43).

The levels of PABP are tightly controlled, reflecting the cellular needs for this ubiquitous mRNA binding protein with facilitating roles in both mRNA stability and the initiation phase of protein synthesis (see Introduction for references). In the terminal stages of erythropoiesis the synthesis of PABP is repressed (19) and in quiescent cells PABP mRNA is largely in the untranslated state, but its translation is strongly activated by serum or growth factors. Interestingly, serum activation of PABP synthesis is prevented by actinomycin D treatment, though PABP mRNA levels are unaffected (17). These examples clearly support the notion that in the absence of active transcription of poly(A)$^+$ mRNA, excess PABP down-regulates its expression. Our findings provide strong evidence that this down-regulation is due to PABP binding to the adenylate-rich leader sequence present in its mRNA, which prevents translation.

It seems likely that the poly(A) tails of all cytoplasmic mRNAs in all eukaryotes are associated with PABP, but *Xenopus* oocytes may be an exception. Immunoblotting methods failed to detect PABP in oocytes or early embryos and although more sensitive methods showed that it is actually present in oocytes, the question remains open as to whether there is sufficient PABP to cover all of the mRNA tails (44).

Amongst the class of eukaryotic messages whose translation is autogenously controlled, that coding for PABP is unique in that its mRNA contains two possible binding sites: the poly(A) tail at the 3′ end and the adenylate-rich sequence in the 5′ non-coding region. Regulation of PABP synthesis by the autoregulatory model is predicted if the binding affinity of PABP for the imperfect A-rich tract in its mRNA 5′ UTR is less than that for an uninterrupted 3′ poly(A) tract. Though we did not address this issue directly, we consistently observed that the cyclin A reporter mRNA containing the pure A tract upstream of the AUG was activated to a greater extent by added poly(A) than the same mRNA containing the interrupted adenylate tract in its 5′ UTR (see Fig. 3B). Similarly, when the effect of added recombinant PABP on the translation of these two messages was compared, a small but reproducible difference was seen: PABP repressed the translation of the former to a greater extent than the latter. These results are compatible with a model in which PABP has a lower affinity for its 5′ UTR binding site than for the 3′ poly(A) tail. However, previous direct determination of the binding affinity of yeast PABP to poly(A) and the yeast PABP mRNA leader adenylate tract showed them to be the same (20), so this facet of the autoregulatory model of PABP synthesis is not yet fully clear. The *in vitro* transcribed PABP mRNAs (though not the cyclin mRNAs) used in this work possessed 18 3′ A residues, potentially comprising a binding site for PABP, determined to be a minimum of 12 A residues (1). However, in experiments in which this tract was missing from the PABP mRNAs, by linearizing the cDNA at an upstream site, we observed the same regulatory effect of the 5′ adenylate tract as in the full-length mRNAs (not shown).

Several examples of translationally regulated eukaryotic mRNAs bearing repressor binding sites in their leader sequences have been reported recently, including those encoding ferritin and erythroid 5-aminolevulinate synthase (reviewed in 45), ribosomal proteins (reviewed in 46) and the *Drosophila* spermatocyte Mst87F gene family products (47). The paradigm for this type of control mechanism is ferritin mRNA, whose translation *in vivo* is stimulated by haemin or iron and inhibited by the iron chelator desferal. This regulation is attributable to the 5′ UTR, particularly to a short stem–loop motif known as the IRE (iron response element) and its specific mRNA binding protein known as IRP (iron regulatory protein), a 100 kDa protein which has been identified as the cytoplasmic *cis*-aconitase (45). Interestingly, for efficient regulation of translation by iron/desferal the IRE must be located quite close to the 5′ cap structure, within 40 nt. If it is located more distal from the cap, then regulation *in vivo* and *in vitro* is much less pronounced, even though the binding of IRP is relatively unaffected by the position of the IRE (48,49). Likewise, the translation of vertebrate ribosomal protein mRNAs is controlled by 5′ UTR motifs, consisting of runs of pyrimidines residues, which show a position effect (46,50,51). The expression of a family of seven closely homologous *Drosophila* spermatocyte proteins is also regulated by a 5′ UTR motif in a position-dependent manner (47,52). In fact, a protein binding to a cap-proximal site need not be an authentic translational repressor to interfere with initiation, as suggested by studies of model regulatory systems set up with high affinity specific RNA binding proteins (53,54). Thus it appears that any tight and cap-proximal RNA–protein interaction can inhibit initiation.

These results are very reminiscent of the positional effect for stem–loop structures in 5′ non-coding regions. It is thought that when located near the cap such an inhibitory secondary structure prevents any initial stable interaction of the primed 40S ribosomal

subunit with the 5′ end of the mRNA; when distant from the cap it is presumed that the scanning 40S ribosomal subunit and associated initiation factors are able to unwind the hairpin loop (55). Extrapolation of these ideas to the position effect of the IRE regulatory element implies that IRP bound to an IRE located distal from the cap can easily be displaced by the scanning ribosomes and RNA helicases.

However, not all binding sites for translational repressors are cap-proximal. For example, in contrast to the above examples, the main binding site of the autoregulated thymidylate synthase mRNA (39) for the enzyme has recently been mapped to a 35 nt segment around the initiation codon, which is 94 nt from the 5′ end (with a second binding site in the coding region; 40), a position from which IRP does not actively repress ferritin mRNA. This implies the existence of at least two different mechanisms for ribosome occlusion by RNA–protein interactions in the 5′ UTR. Moreover, some translational repressors function by recognizing motifs located in the 3′ UTR, such as the 48 kDa protein which inhibits the translation of erythroid 15-lipoxygenase mRNA (28).

We note that in at least two PABP mRNAs in which the transcription site has been mapped or an extensive cDNA obtained the adenylate tract binding site is found distal to the cap. Thus in yeast (*Saccharomyces cerevisiae*) PABP mRNA the adenylate tract starts ~70 nt downstream of the cap (20) and in the human PABP cDNA (which is not necessarily full-length) this distance is 70 nt (21). The data reported in this study confirm that the cap-distal adenylate motif, present in wild-type PABP mRNA, is functional *in vitro* (Fig. 5C). We need, therefore, to consider the possibility that as well as the apparently purely steric block imposed by cap-proximal binding sites on ribosome binding (56) there are additional mechanisms operating with cap-distal binding sites (and 3′ UTR binding sites) which prevent the 43S pre-initiation complex binding to mRNA or affect ribosome scanning. We are currently investigating the effect of systematically altering the location of the adenylate stretch with respect to the cap on PABP binding and autogenous repression.

ACKNOWLEDGEMENTS

We gratefully acknowledge stimulating discussions with Richard Jackson, Matt Hentze and Mike Wormington during the course of the experiments and we thank Richard Jackson for critical reading of the manuscript. We also thank Tim Hunt and Jeremy Minshull for cyclin plasmid XlA4 and Jim Patton for the gift of GST–PTB DNA. This work was supported by the MRC, CNPq and the British Council. OPMN received a scholarship from the Brazilian Research Council CNPq and NS holds the Amersham Fellowship in Biochemistry.

REFERENCES

1 Sachs,A.B., Davis,R.W. and Kornberg,R.D. (1987) *Mol. Cell. Biol.*, **7**, 3268–3276.
2 Jackson,R.J. and Standart,N. (1990) *Cell*, **62**, 15–24.
3 Munroe,D. and Jacobson,A. (1990) *Gene*, **91**, 151–158.
4 Sachs,A. (1990) *Curr. Opin. Cell Biol.*, **2**, 1092–1098.
5 Bernstein,P. and Ross,J. (1989) *Trends Biochem. Sci.*, **14**, 373–377.
6 Baker,E.J. (1993) In Belasco,J.G. and Brawerman,G. (eds), *Control of Messenger RNA Stability*. Academic Press, San Diego, CA, pp. 367–415.
7 Grossi de Sa,M.-F., Standart,N., Martins de Sa,C., Akhayat,O., Huesca,M. and Scherrer,K. (1988) *Eur. J. Biochem.*, **176**, 521–526.
8 Sachs,A.B. and Davis,R.W. (1989) *Cell*, **58**, 857–867.
9 Munroe,D. and Jacobson,A. (1990) *Mol. Cell. Biol.*, **10**, 3441–3455.
10 Sachs,A.B. and Deardorff,J.A. (1992) *Cell*, **70**, 961–973.
11 Gallie,D.R. (1991) *Genes Dev.*, **5**, 2108–2116.
12 Bernstein,P., Peltz,S.W. and Ross,J. (1989) *Mol. Cell. Biol.*, **9**, 659–670.
13 Burd,C. and Dreyfuss,G. (1994) *Science*, **265**, 615–621.
14 Birney,E., Kumar,S. and Krainer,A.R. (1993) *Nucleic Acids Res.*, **21**, 5803–5816.
15 Nietfeld,W., Mentzel,H. and Pieler,T. (1990) *EMBO J.*, **9**, 3699–3705.
16 Burd,C.G., Matunis,E.L. and Dreyfuss,G. (1991) *Mol. Cell. Biol.*, **11**, 3419–3424.
17 Thomas,G. and Thomas,G. (1986) *J. Cell Biol.*, **103**, 2137–2144.
18 Akhayat,O., Vincent,A., Goldenberg,S., Person,A. and Scherrer,K. (1983) *FEBS Lett.*, **162**, 25–32.
19 Maundrell,K., Imaizumi-Scherrer,M.T., Maxwell,E.S., Civelli,O. and Scherrer,K. (1983) *J. Biol. Chem.*, **258**, 1387–1390.
20 Sachs,A.B., Bond,M.W. and Kornberg,R.D. (1986) *Cell*, **45**, 827–835.
21 Grange,T., de Sa,C., Oddos,J. and Pictet,R. (1987) *Nucleic Acids Res.*, **15**, 4771–4787.
22 Lefrere,V., Vincent,A. and Amalric,F. (1993) *Gene*, **126**, 295–296.
23 Batista,J., Teixeira,S., Donelson,J., Kirchhoff,L. and Martins de Sá,C. (1994) *Mol. Biochem. Parasitol.*, **67**, 301–312.
24 Berger,L.C., Bag,J. and Sells,B.H. (1992) *Eur. J. Biochem.*, **204**, 733–743.
25 Dasso,M.C. and Jackson,R.J. (1989) *Nucleic Acids Res.*, **17**, 3129–3144.
26 Minshull,J., Golsteyn,R., Hill,C.S. and Hunt,T. (1990) *EMBO J.*, **9**, 2865–2875.
27 Jackson,R.J. and Hunt,T. (1983) *Methods Enzymol.*, **96**, 50–72.
28 Ostareck-Lederer,A., Ostareck,D.H., Standart,N. and Thiele,B.J. (1994) *EMBO J.*, **13**, 1476–1481.
29 Walker,J., Melo Neto,O. and Standart,N. (1995) In Martin,R. (ed.), *Methods in Molecular Biology. Protein Synthesis: Methods and Protocols*. Humana, Totowa, NJ, in press.
30 Leibold,E.A. and Munro,H.N. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2171–2175.
31 Patton,J., Mayer,S., Tempst,P. and Nadal-Ginard,B. (1991) *Genes Dev.*, **5**, 1237–1251.
32 Gallie,D.R. and Tanquay,R. (1994) *J. Biol. Chem.*, **269**, 17166–17173.
33 Gil,A., Sharp,P.A., Jamison,S.F. and Garcia-Blanco,M.A. (1991) *Genes Dev.*, **5**, 1224–1236.
34 Borman,A., Howell,M.T., Patton,J.G. and Jackson,R.J. (1993) *J. Gen. Virol.*, **74**, 1775–1788.
35 Hellen,C.U.T., Witherell,G.W., Schmid,M., Shin,S.H., Pestova,T.V., Gil,A. and Wimmer,E. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 7642–7646.
36 Sieliwanowicz,B. (1987) *Biochim. Biophys. Acta*, **908**, 54–59.
37 Belostotsky,D.A. and Meagher,R.B. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6686–6690.
38 Gallie,D.R., Lucas,W.J. and Walbot,V. (1989) *Plant Cell*, **1**, 301–311.
39 Chu,E., Koeller,D.M., Casey,J.L., Drake,J.C., Chabner,B.A., Elwood,P.C., Zinn,S. and Allegra,C.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8977–8981.
40 Chu,E., Voeller,D., Koeller,D.M., Drake,J.C., Takimoto,C.H., Maley,G.F., Maley,F. and Allegra,C.J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 517–521.
41 Chu,E., Takimoto,C.H., Voeller,D., Grem,J.L. and Allegra,C.J. (1993) *Biochemistry*, **32**, 4756–4760.
42 Dabeva,M.D. and Warner,J.R. (1993) *J. Biol. Chem.*, **268**, 19669–19674.
43 Gunderson,S.I., Beyer,K., Martin,G., Keller,W., Boelens,W.C. and Mattaj,I.W. (1994) *Cell*, **76**, 531–541.
44 Zelus,B.D., Giebelhaus,D.H., Eib,D.W., Kenner,K.A. and Moon,R.T. (1989) *Mol. Cell. Biol.*, **9**, 2756–2760.
45 Melefors,O. and Hentze,M.W. (1993) *BioEssays*, **15**, 85–90.
46 Kaspar,R.L., Morris,D.R. and White,M.W. (1993) In Ilan,J. (ed.), *Translational Regulation of Gene Expression*. Plenum Press, New York, NY, Vol. 2, pp. 335–348.
47 Schäfer,M., Kuhn,R., Bosse,F. and Schäfer,U. (1990) *EMBO J.*, **9**, 4519–4525.
48 Goossen,B., Caughman,S., Harford,J., Klausner,R. and Hentze,M. (1990) *EMBO J.*, **9**, 4127–4133.
49 Goossen,B. and Hentze,M. (1992) *Mol. Cell. Biol.*, **12**, 1959–1966.
50 Hammond,M.L., Merrick,W. and Bowman,L.H. (1991) *Genes Dev.*, **5**, 1723–1736.
51 Avni,D., Shama,S., Loreni,F. and Meyuhas,O. (1994) *Mol. Cell. Biol.*, **14**, 3822–3833.
52 Kempe,E., Muhs,B. and Schäfer,M. (1993) *Dev. Genet.*, **14**, 449–459.
53 Stripecke,R. and Hentze,M.W. (1992) *Nucleic Acids Res.*, **20**, 5555–5564.
54 Stripecke,R., Oliviera,C.C., McCarthy,J.E.G. and Hentze,M.W. (1994) *Mol. Cell. Biol.*, **14**, 5898–5909.
55 Kozak,M. (1992) *Annu. Rev. Cell Biol.*, **8**, 197–225.
56 Gray,N.K. and Hentze,M.W. (1994) *EMBO J.*, **13**, 3882–3891.