Poly(A)-tail-promoted translation in yeast: implications for translational control

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Poly(A)-tail-promoted translation in yeast: Implications for translational control

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ABSTRACT

The cap structure and the poly(A) tail synergistically activate mRNA translation in vivo. Recent work using *Saccharomyces cerevisiae* spheroplasts and a yeast cell-free translation system revealed that the poly(A) tail can function as an independent promotor for ribosome recruitment, to internal initiation sites within an mRNA. This raises the question of how regulatory upstream open reading frames and translational repressor proteins binding to the 5′ UTR can function, as well as how regulated polyadenylation can support faithful activation of protein synthesis. We investigated the function of the regulatory upstream open reading frame 4 from the yeast GCN 4 gene and the effect of IRP-1 binding to an iron-responsive element introduced into the 5′ UTR of reporter mRNAs. Both manipulations effectively block cap-dependent translation, whereas ribosome recruitment promoted by the poly(A) tail under non-competitive conditions can efficiently bypass both blocks. We show that the synergistic use of both, the cap structure and the poly-A tail enforced by mRNA competition reinstates the full extent of translational control by both types of 5′ UTR regulatory elements. With a view towards regulated polyadenylation, we studied the function of poly(A) tails of defined length on the translation of capped mRNAs. We find that poly(A) tail elongation increases translational efficiency, particularly under competitive conditions. Our results integrate recent findings on the function of the poly(A) tail into an understanding of translational control.

Keywords: 3′ untranslated regions; 5′ untranslated regions; adenylation; deadenylation; initiation of translation; mRNA metabolism; repressor proteins; upstream AUG codons

INTRODUCTION

Almost all eukaryotic mRNAs receive a 5′ cap structure (m^7GpppN) and a 3′ poly(A) tail as post-transcriptional modifications in the nucleus before being exported to the cytoplasm. In the cytoplasm, the cap structure promotes translation initiation by recruiting the small ribosomal subunit and associated factors to the mRNA (Merrick & Hershey, 1996). The poly(A) tail is also capable of stimulating mRNA translation (Munroe & Jacobson, 1990). A series of biochemical and genetic experiments in yeast have identified a physical interaction between the poly(A)-binding protein (Pab1p) and the translation initiation factor eIF4G (Tarun & Sachs, 1996; Tarun et al., 1997). eIF4G is the subunit of the cap-binding complex eIF4F which interacts with the cap-recognition subunit eIF4E (Lamphear et al., 1995; Mader et al., 1995). Thus, a simultaneous interaction of eIF4G with eIF4E and Pab1p may serve to approximate the two ends of yeast mRNAs. This may be important to mediate the stimulatory effect of both mRNA ends on translation (Hentze, 1997; Sachs et al., 1997), because eIF4G can also form a bridge to the general translation apparatus by interaction with the multisubunit factor eIF3 that binds to the small ribosomal subunit (Lamphear et al., 1995).

RNA electroporation experiments using animal, plant, and yeast cells have demonstrated that the poly(A) tail stimulates translation synergistically with the cap structure in vivo (Gallie, 1991). More recently, this synergism was also observed in cell-free translation reactions based on yeast extracts (Iizuka et al., 1994). Further analysis in this system showed that the poly(A) tail can deliver ribosomes to uncapped mRNAs in a 5′ end-independent fashion. As a consequence, translation in this mode leads to frequent initiation at internal AUG codons. The cap structure co-promotes ribosome recruitment together with the poly(A) tail and, importantly, helps to direct ribosomes recruited in this way to the 5′ end. Furthermore, this combined mode of ribosome recruitment possesses a strong competitive advantage in the presence of a full complement of capped and polyadenylated cellular mRNAs. Thus, the functional interactions between the mRNA cap structure and poly(A) tail predominate under conditions of mRNA

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competition and ensure correct, 5′ end-dependent initiation codon choice (Preiss & Hentze, 1998).

Translational control is a common means of gene regulation that is exerted most frequently through regulatory elements located in the 5′ or 3′ untranslated regions (UTRs) of mRNAs. Ferritin and several other cellular mRNAs are translationally regulated by interactions between iron-responsive elements (IREs), located in proximity of the cap structure within the 5′ UTR and iron-regulatory proteins (IRP) -1 and -2 (Hentze & Kühn, 1996; Rouault et al., 1996). IRP binding interferes with the recruitment of the small ribosomal subunit, apparently by steric means (Gray & Hentze, 1994). The IRE/IRP regulatory system is evolutionary confined to metazoan cells, but also functions when heterologously introduced into yeast cells (Oliveira et al., 1993; Paraskeva et al., 1998a). A different type of translational regulatory motif within the 5′ UTR of yeast mRNAs are short open reading frames (uORF) positioned upstream of the main coding sequence (Geballe, 1996). An array of four such uORFs controls the translation of the yeast GCN4 mRNA by a scanning-reinitiation mechanism (Hinnebusch, 1996). Integral to this mechanism is the function of uORF4 as an efficient barrier to downstream reinitiation for ribosomes translating this uORF.

In this study, we investigate the implications of poly(A)-tail-promoted translation for the proper function of 5′ UTR translational regulatory elements. In addition, we analyze the functional role of differences in poly(A) tail length, using CAT reporter mRNAs with a range of defined poly(A) tail lengths. Our results extend recent findings on the function of the poly(A) tail in constitutive translation towards an understanding of translational control.

RESULTS

Translational regulation by an RNA–protein interaction in the 5′ UTR

To investigate the properties of the IRE/IRP-1 regulatory complex in the context of cap-structure-promoted and/or poly(A)-tail-promoted translation, we used CAT reporter mRNAs that harbor an IRE close to the 5′ end (Fig. 1; sequences which fold into the IRE stem-loop are underlined). The 5′ UTR of construct IRE.CAT resembles that of ferritin mRNA with regard to the position and sequence of the IRE. It was previously shown that IRP-1 binding to the IRE in this construct interferes with 40S ribosomal subunit recruitment to a capped mRNA in rabbit reticulocyte lysate (Gray & Hentze, 1994), by disrupting bridging interactions between the cap-binding complex eIF4F and the 40S subunit and its associated initiation factors (Muckenthaler et al., 1998a). The CAT reporter mRNAs used in this study were transcribed in vitro in four distinct versions: either capped (c), possessing an $(A)_{98}$ tail (a), with both (c–a), or neither of these modifications (−). The in vitro transcription protocol has been optimized to allow very high capping efficiencies of ≥95% (Stripecke & Hentze, 1992). The translation of the four differently modified versions of IRE.CAT mRNA was then analyzed in a micrococcal nuclease-treated yeast extract in the presence or absence of recombinant IRP-1. As shown in Figure 2A, IRP-1 profoundly represses cIRE.CAT mRNA translation (lanes 3 and 4), but displays strikingly little effect on IRE.CATa mRNA (lanes 5 and 6). In IRE.CAT mRNAs, the distance of 45 nt between the IRE motif and the CAT start codon might be sufficient to allow internal ribosome binding downstream of the bound repressor protein, near the AUG codon. To test this possibility, we generated an additional mRNA construct in which the IRE is moved into a start-codon-proximal position (IREscp.CAT, Fig. 1) by deletion of 33 of the intervening nucleotides. This modification is inconsequential for the regulation of cIREscp.CAT mRNA translation (Fig. 2A, lanes 10 and 11), but renders IREscp.CATa mRNA strongly responsive to IRP-1 (lanes 12 and 13). The differences between the IRE.CAT and IREscp.CAT constructs were evaluated and confirmed over a range of IRP-1 concentrations, and persist at saturating amounts for cap-promoted translation (2.56 pmol, Fig. 3A).

It is formally possible that IRP-1 is displaced during translation initiation promoted by the poly(A) tail and that this could occur more readily on IRE.CATa mRNA than on IREscp.CATa mRNA. Based on such an as-

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IRE.CAT       GGGCGAAUUCGAGCUCGGUACCCGGGGAUCC-----UGCUUCAACAGUGCUUGGAC
IREscp.CAT    GGGCGAAUUCGAGCUCGGUACCCGGGGAUCC-----UGCUUCAACAGUGCUUGGAC
IRE/AUG.CAT   GGGCGAAUUCGAGCUCGGUACCCGGGGAUCCAUCGUUGCUUCAACAGUGCUUGGAC

              -----GGAUCUUCUAGAGUCAGCUUCGACGAGAUUUUCAGGAGCUAAGGAAGCUAAAAUG
              -----GGAUCUUCUAGA----------------------------------AGCUAAAAUG
              ACGAUGGAUCU---AGAGUCAGCUUCGACGAGAUUUUCAGGAGCUAAGGAAGCUAAAAUG
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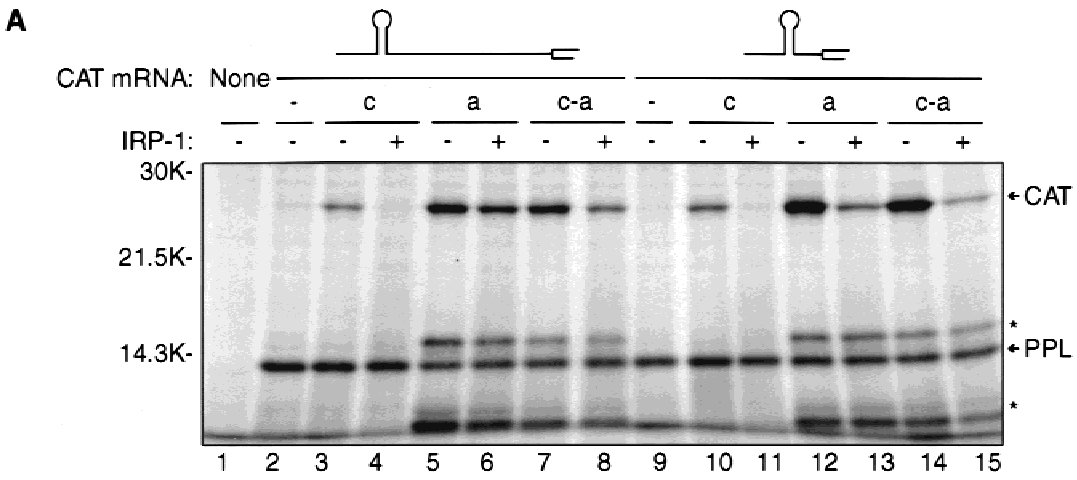
FIGURE 1. Comparison of the IRE-containing CAT mRNA 5′ UTRs used in this study. The sequences folding into the IRE motif are underlined and translation start codons are boxed.



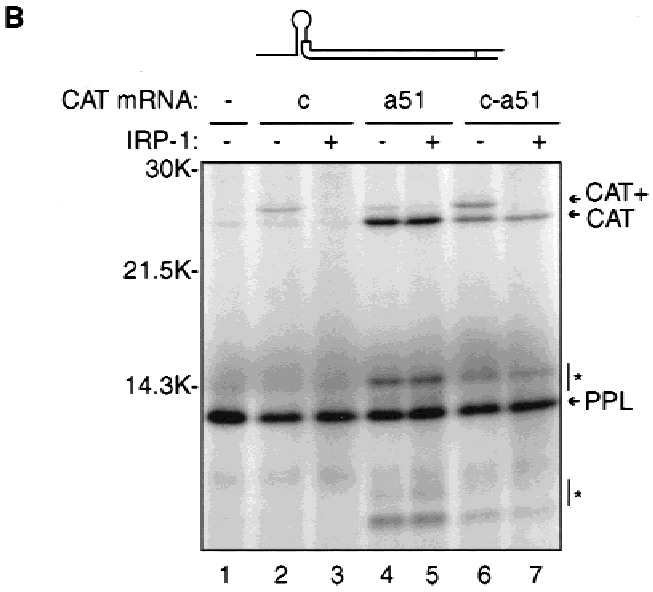
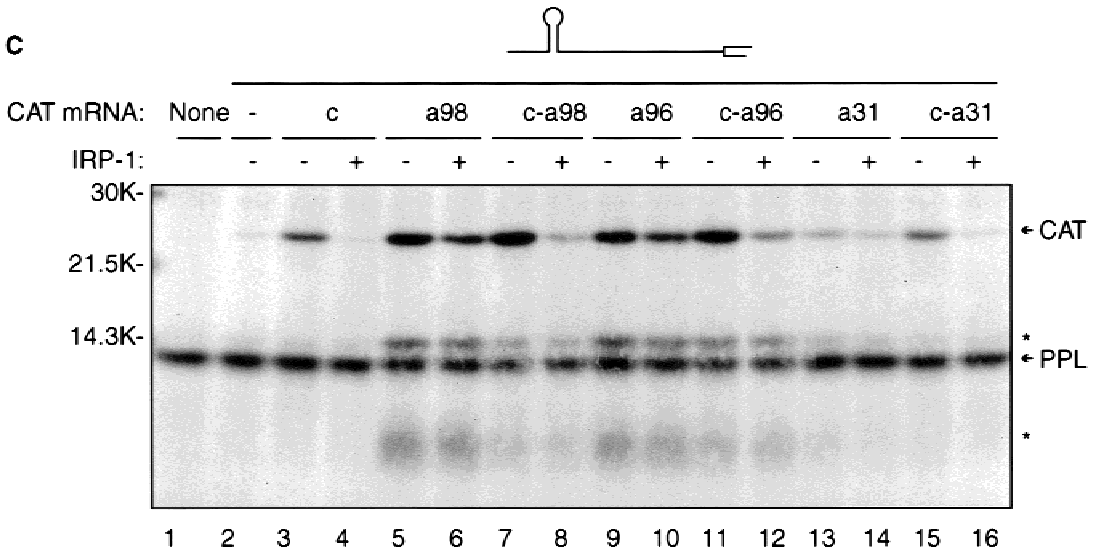


FIGURE 2. Translational repression by IRP-1 binding to an IRE element in the 5′ UTR. **A:** Reactions with nuclease-treated extract were programmed with 100 fmol IRE.CAT (lanes 2–8) or IREscp.CAT mRNAs (lanes 9–15; the 5′ UTR structure is indicated by the pictograms above the panel) and 2 fmol PPL mRNA. CAT mRNAs were used in four versions: either capped (c), possessing an $(A)_{98}$ tail (a), with both (c–a), or neither of these modifications (–). Translation was assayed in the presence or absence of 1.28 pmol recombinant IRP-1 (indicated by + or – directly above the lanes). The reaction in lane 1 contains no added mRNA and the positions of protein molecular mass markers are shown on the left (in Daltons). The positions of CAT and PPL protein are indicated by the arrows on the right. The presence of additional protein products in lanes with polyadenylated CAT mRNA is marked by the asterisks. **B:** Reactions and IRP-1 addition as in **A**, using 167 fmol of IRE/AUG.CAT mRNAs (the 5′ UTR structure is indicated by the pictogram above the panel) and 2 fmol PPL mRNA. **C:** Reactions and IRP-1 addition as in **A**, using 100 fmol IRE.CAT mRNAs carrying different types of poly(A) tail and 2 fmol PPL mRNA. mRNAs transcribed from pIRE.CAT$(A)_{98}$ were trimmed cotranscriptionally (see Materials and Methods) to remove all heterologous nucleotides at the 3′ end, yielding an $(A)_{96}$ tail (a96 and c–a96, lanes 9–12). Transcripts from pIRE.CAT$(A)_{31}$ were also used (a31 and c–a31, lanes 13–16). As controls, the results shown in lanes 2–8 of panel A are recapitulated (lanes 2–8) and lane 1 shows translation of the PPL control mRNA without addition of CAT transcript.



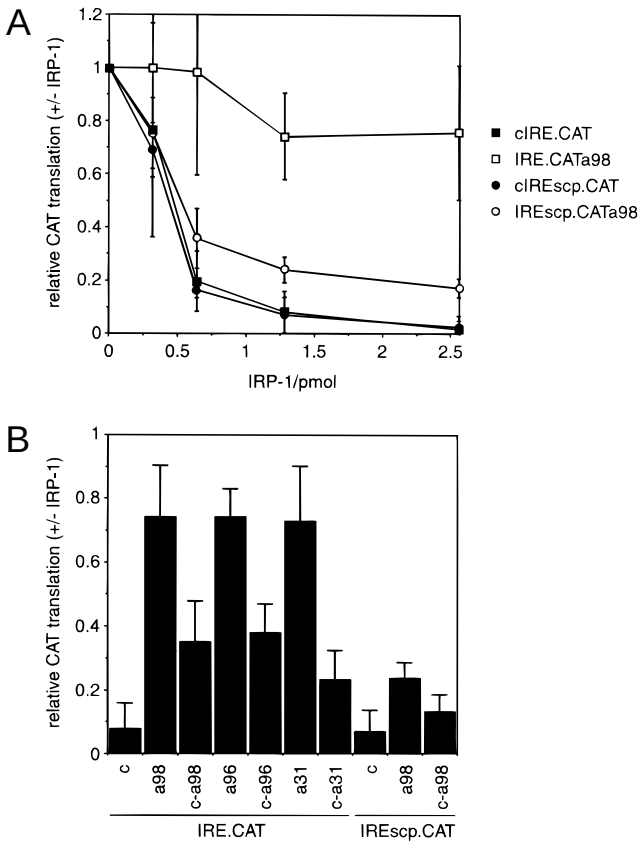


FIGURE 3. Quantitative summary of IRP-1 repression patterns. **A:** Cap- and poly(A)-tail-promoted translation were analyzed as in Fig. 2A, using a range of IRP-1 additions from 0.32–2.56 pmol. The ratio of CAT protein to the internal PPL control was determined and set to 1 for the control experiment without IRP-1 addition. In this way, the graph compares the responses of cIRE.CAT (filled squares) and IRE.CATa (open squares) with those of cIREscp.CAT (filled circles) and IREscp.CATa(open circles). Averaged values from several repeat experiments are given with standard deviation. **B:** The data shown in Fig. 2A,C and several repeat experiments were quantified and averaged. CAT translation in the presence of 1.28 pmol recombinant IRP-1 is expressed as a percentage of the corresponding control reaction in the absence of the repressor with standard deviation.

sumption, the data in Fig. 2A could be explained by a conventional 5′ end-dependent mechanism. To address this, we used another construct, termed IRE/AUG.CAT (Fig. 1; Paraskeva et al., 1998b), which harbors an in-frame AUG codon within the IRE motif. This mRNA allows the simultaneous analysis of both, initiation at an AUG which is precluded directly by IRP-1 binding (yielding an N-terminally extended "CAT+" polypeptide of 27.5 kD) and one which is at a sufficient distance 3′ to the IRE (yielding authentic CAT protein). Capped or uncapped IRE/AUG.CAT mRNA was transcribed either with or without a tail of 51 adenosines (see also below) and analyzed in translations with nuclease-treated extract (Fig. 2B). In the absence of IRP-1 (Fig. 2B, lanes 1, 2, 4, and 6), the translation pattern is similar to that seen previously with the BIG.CAT mRNA construct (Preiss & Hentze, 1998). Note that cIRE/AUG.CAT mRNA predominantly yields the larger CAT+ polypeptide (Fig. 2B, lane 2), and that the poly(A) tail mediates strong (Fig. 2B, lanes 4 and 5) or diminished (Fig. 2B, lanes 6 and 7) initiation at downstream AUG codons. Addition of IRP-1, however, leads to a strong repression of initiation from the IRE-internal AUG codon in all modes of translation tested (Fig. 2B, lanes 3, 5, and 7). This demonstrates that there is no selective displacement of IRP-1 from the IRE structure mediated by the presence of the poly(A) tail. Importantly, initiation at the CAT AUG is again unaffected during poly(A)-dependent translation (Fig. 2B, lanes 4 and 5), thus excluding a displacement/scanning mechanism.

As evident from Figs. 2 and 6, translation of polyadenylated CAT mRNAs in nuclease-treated yeast extract results in the appearance of smaller translation products (marked in the figures by an asterisk). We interpret these peptides as deriving from initiation events at AUG codons located within the CAT open reading frame (Preiss & Hentze, 1998). Taking into account all possible reading frames, such putative translation products of significant size can be calculated to range from 17.8–16.6 kDa and 8.9–4 kDa. These size predictions are consistent with the bands seen here, given the limited resolution of small peptides by conventional SDS-PAGE. Consistent with our interpretation of internal ribosome binding downstream of an IRE/IRP-1 complex, the synthesis of these smaller products is insensitive to IRP-1 binding even in the case of IREscp.CATa or IRE/AUG.CATa51 mRNA (Figs. 2A, lanes 12 and 13 and 2B, lanes 4 and 5). Taken together, these data demonstrate that the IRE/IRP-1 complex can be bypassed during poly(A)-tail-promoted translation, provided that the region upstream of the translational start site(s) is accessible.

The observed 5′ end-independence of poly(A)-tail-promoted translation was characterized further by manipulating the exact nature of the mRNA 3′ end in two ways. First, a shorter poly(A) tail of 31 adenosines in a sequence context which was identical to the 98-adenosine construct was used. Translation of IRE.CATa31 mRNA shows that the ability of the shorter poly(A) tail to bypass the IRE/IRP-1 complex is qualitatively preserved (Fig. 2C, lanes 13 and 14), although shortening of the poly(A) tail to this length results in an overall reduction of translation (see also below). Second, the in vitro transcribed CAT mRNAs used in this study contain 25 nt of heterologous sequence 3′ of the poly(A) segment. To eliminate a possible influence of these nucleotides on the interpretation of our results, these nucleotides were specifically removed together with the two 3′ terminal adenosines by oligonucleotide-mediated digestion with RNase H (see Materials and Methods). The efficacy of this trimming was confirmed by denaturing PAGE (data not shown). Translation analysis of IRE.CATa96 mRNA, which lacks the heterologous nu-

cleotides present in IRE.CATa98 mRNA, demonstrated that neither the translational efficiency nor the resistance to IRP-1 addition were affected by this treatment (Fig. 2C, compare lanes 5 and 6 with lanes 9 and 10).

The data presented in Fig. 2A,C and several repeat experiments were quantified and are summarized in Fig. 3B. The data show that the translation of the c–a (capped and polyadenylated) versions of IRE.CAT mRNA display an intermediate response to IRP-1 addition. Most likely, the nonrepressable component of translation represents internal initiation events resulting from the cap-uncoupled activity of the poly(A) tail which is prevalent in nuclease-treated extract (Preiss & Hentze, 1998). Consistent with this notion, the poly(A)-tail-dependent smaller translation products (see above) are still detected using the (c–a) versions of the CAT mRNAs but are reduced in intensity compared to the corresponding (a) version (Figs. 2 and 6). This suggests that the combined presence of both translational promoters is not sufficient to ensure stringent IRE/IRP-1 control under conditions where mRNAs are limiting, i.e. the noncompetitive conditions in micrococcal nuclease-treated extracts. Therefore, IRP-1 mediated translational regulation was compared in nuclease-treated (recapitulating the situation in Fig. 2A, lanes 1–8) versus untreated extracts (Fig. 4). The latter still contains a full complement of cellular mRNAs that compete with the CAT reporter mRNAs for limiting components of the translation machinery, thus also necessitating an immunoprecipitation step with CAT monoclonal antibody to isolate the CAT polypeptide from the background of products arising from the translation of the cellular mRNAs (Preiss & Hentze, 1998). Several interesting aspects emerge from this comparison. First, only the cIRE.CATa mRNA in the absence of IRP-1 yields substantial CAT translation products under competitive conditions (Fig. 4, lane 7). This is in striking contrast to the nuclease-treated extract, where the IRE.CATa mRNA in particular is well translated irrespective of the presence of IRP-1 (Fig. 4, lanes 12,13). Second, both cIRE.CAT and cIRE.CATa mRNAs are highly responsive to IRP-1 regulation under competitive conditions (Fig. 4, lanes 3,4 and 7,8). Third, the very low level of poly(A)-tail-promoted translation of IRE.CATa mRNA also efficiently bypasses the IRE/IRP-1 block under competitive conditions (Fig. 4, lanes 5,6), indicating that this mode of translation initiation lacks competitiveness, but does not fundamentally change in untreated extracts. We conclude that only the translation of a capped and polyadenylated mRNA under competitive conditions reflects the physiological situation of efficient translation in the absence, and efficient repression in the presence of the regulatory protein.

Translational regulation by a uORF

Next, we tested the function of a small regulatory 5′ UTR open reading frame, using the uORF 4 sequences (including the 3′ stretch of 10 nt required for its strong inhibitory action on reinitiation at downstream AUGs) from the translationally regulated GCN 4 mRNA (uORF.CAT) (Hinnebusch, 1996). By introducing a point mutation into the uORF4 start codon, the control construct xORF.CAT was generated. Comparison of uORF-with xORF.CAT mRNA translation allows us to assess the specific effects of uORF 4 on the different modes of translation (Fig. 5). Cap structure-promoted translation of CAT mRNA is strongly suppressed by the uORF 4, irrespec-

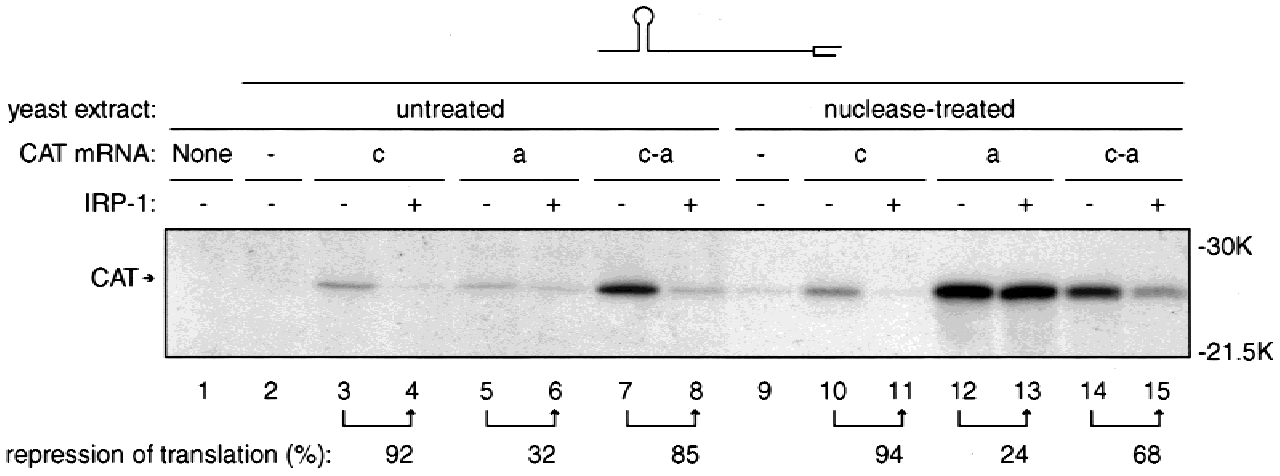


FIGURE 4. IRP-1-mediated repression of translation under competitive and noncompetitive conditions. Translation reactions were programmed with 100 fmol of IRE.CAT mRNAs using either micrococcal nuclease-treated (lanes 9–15) or untreated extract (lanes 1–8). The effect of IRP-1 addition was assayed as in Fig. 2. CAT translation was analyzed by quantitative immunoprecipitation. Translational repression by IRP-1 addition (in percent) is stated below the figure (averaged values from two experiments are given). Lanes with added IRP-1 are linked to the corresponding control lane in the absence of repressor by the arrows.

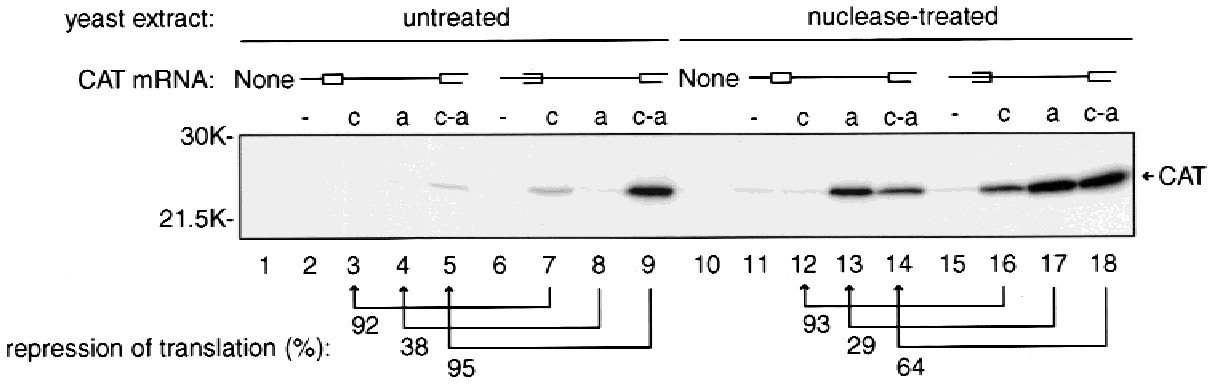


FIGURE 5. Upstream-ORF-mediated repression of translation under competitive and noncompetitive conditions. Translation reactions were programmed with 167 fmol of uORF.CAT (lanes 2–5 and 11–14) or xORF.CAT (lanes 6–9 and 15–18; pictograms above the panel display their 5′ UTR features) mRNAs using either micrococcal nuclease-treated (lanes 10–18) or untreated extract (lanes 1–9). CAT translation was analyzed by quantitative immunoprecipitation and the repressive effects of a functional uORF on translation are stated below the figure (averaged values from two experiments are given). Lanes with uORFCAT mRNA and the corresponding xORF.CAT control are linked by the arrows.

tive of whether mRNA competition is established or not (Fig. 5, lanes 3,7 and 12,16). By contrast, poly(A)-tail-promoted translation efficiently bypasses this "genetic block" (Fig. 5, lanes 4,8 and 13,17), but nonetheless yields little protein under competitive conditions (Fig. 5, lane 4). While the uORF 4 in cuORF.CATa mRNA is substantially bypassed in nuclease-treated extract (Fig. 5, lanes 14,18), it functions as an efficient (95%) translational barrier under competitive conditions (Fig. 5, lanes 5,9). These data agree with the conclusions drawn from experiments with the IRE/IRP-1 regulatory system (Fig. 4), in that they underscore the importance of mRNA competition to secure the physiological function of 5′ UTR regulatory mechanisms acting on capped and polyadenylated mRNAs.

Previous work has established that reporter mRNAs with different end modifications are similarly stable in the cell-free translation system used here (Iizuka et al., 1994; Tarun & Sachs, 1995; Preiss & Hentze, 1998). However, we wanted to exclude that the presence of an upstream ORF could trigger an accelerated mRNA degradation in our translation reactions, perhaps by an upf-mediated pathway (Peltz et al., 1994). Therefore, we compared the physical stability of the cuORF.CATa and cxORF.CATa mRNAs in untreated yeast extract. Under these conditions, the two mRNAs yield vastly different amounts of CAT protein (see Fig. 5, lanes 5,9). We found that both mRNAs are similarly stable with half lives exceeding the time of the translation assay (in the range of 100 min, data not shown), confirming that the observed CAT protein yields largely reflect changes in translation efficiency.

Effects of variations in poly(A) tail length on translation

As already observed in an earlier experiment (Fig. 2C), translation of otherwise identical mRNAs bearing poly(A) tails of 98/96 and 31 adenosines, respectively, displays differences in the nuclease-treated yeast extracts. It had been previously observed that changes in the length of the 3′ UTR of mRNAs affect their translatability when transfected into mammalian cells (Tanguay & Gallie, 1996). To test a possible influence of 3′ UTR length on translation in our assay, we obtained an IRE.CAT mRNA set which was derived from a plasmid template carrying the poly(A/T)$_{98}$ segment insertion in the reverse orientation, thus yielding mRNA with a poly(U) tail. As shown in Fig. 6, the poly(U) tail fails to significantly stimulate translation of either a capped (lane 5) or un-

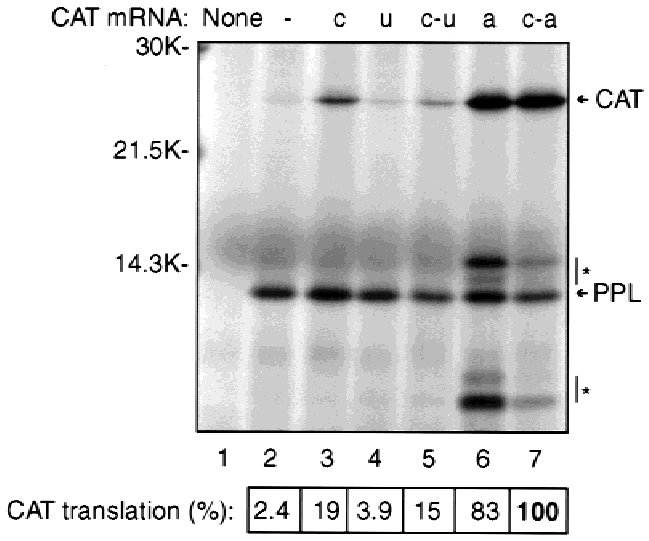


FIGURE 6. A poly(U) tail cannot substitute for a poly(A) tail during translation. Translation reactions using micrococcal nuclease-treated extract were programmed with 167 fmol of IRE.CAT mRNAs and 2 fmol PPL mRNA. In addition to the previously employed variants (−, c, a, c–a) 2 mRNAs carrying a 98 nt poly(U) tail (u, c–u; see Materials and Methods) were evaluated.

capped mRNA (lane 4). These results underscore the specific requirement for a poly(A) tail to promote translation in this assay and exclude the alternative interpretation that stimulation of translation results from the increased length of the 3′ UTR.

We next examined the effect of different poly(A) tail length systematically, by generating a series of capped IRE.CAT mRNAs with poly(A) tails ranging from 15 to 98 adenosines in length. The translation of these mRNAs was quantitatively compared in nuclease-treated and untreated extracts (Fig. 7A). The quantitative analysis of multiple repeat experiments is summarized in Fig. 7B. It is clearly apparent that translation efficiency positively correlates with poly(A) tail length in both types of extract. Under noncompetitive conditions (Fig. 7, black bars), CAT yields begin to increase significantly at and beyond 31 adenosines, and reach an early saturation at 51 adenosines. Under competitive conditions (Fig. 7, white bars), CAT translation increases steadily between 15 and 98 adenosines. Thus, differences in poly(A) tail length modulate the translational output, particularly under competitive conditions.

Changes in the length of the poly(A) tail during the incubation with the extracts could complicate the interpretation of the results shown in Fig. 7A,B. To address this potential caveat, we determined the poly(A) tail status of the cIRE.CATa51 and cIRE.CATa98 mRNAs before and after cell-free translation using the Poly(A) test (PAT) assay (Sallés & Strickland, 1995; Muckenthaler et al., 1997). As shown in Fig. 7C, both mRNAs display largely unchanged poly(A) tail lengths after the incubation compared to the input controls. This is true for mRNAs incubated in nuclease-treated and untreated extracts. The observed differences in translation, therefore, appear to be an accurate reflection of the number of adenosines present on the input mRNAs.

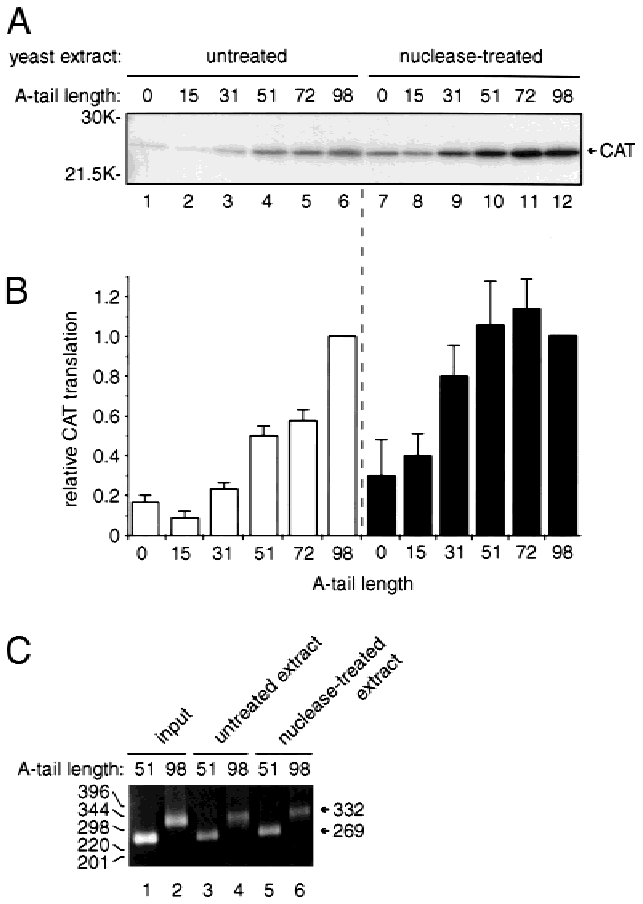


FIGURE 7. Effects of different poly(A) tail lengths on translation. **A:** Capped IRE.CAT mRNAs were transcribed carrying stretches of poly(A) at their 3′ end ranging from 0 to 98 nt (see Materials and Methods). For most accurate determination of their concentrations, these mRNAs were analyzed in parallel by agarose gel electrophoresis and PhosphorImager quantification (data not shown). Translation reactions were programmed with 167 fmol of these mRNAs using either micrococcal nuclease-treated (lanes 7–12) or untreated extract (lanes 1–6). CAT translation was analyzed by quantitative immunoprecipitation. **B:** CAT translation data in nuclease-treated (black bars) and untreated extract (white bars), from **A** and several repeat experiments were averaged and expressed as the fraction of the result obtained with cIRE.CATa98 mRNA (error bars represent the standard deviation). **C:** RNA was extracted after 60 incubation from translation reactions with either cIRE.CATa51 (lanes 3,5) or cIRE.CATa98 mRNA (lanes 4,6) and analyzed for poly(A) tail length by the PCR-based PAT assay (see Materials and Methods). Translation was performed either in nuclease-treated (lanes 5,6) or untreated extract (lanes 3,4). Also shown are control reactions with the corresponding input mRNAs (lanes 1,2). Positions of DNA size markers are indicated on the left (in base pairs) and the arrows on the right point towards the theoretical product sizes for complete poly(A) tails.

DISCUSSION

These studies were prompted by recent insights into biochemical and functional properties of the poly(A) tail, particularly in the yeast *Saccharomyces cerevisiae*. Following the establishment of a cell-free translation system that recapitulates the synergism between the cap structure and the poly(A) tail (Iizuka et al., 1994), it was shown that the poly(A) tail can mediate the recruitment of a 43S ribosomal initiation complex to an uncapped mRNA (Tarun & Sachs, 1995). As expected, the cap structure was shown to suffice for promotion of protein synthesis in a yeast extract from which the endogenous (capped and polyadenylated) mRNAs had been removed by treatment with micrococcal nuclease. More surprisingly, the poly(A) tail was also found to function as a self-sufficient promoter of translation under these conditions. By contrast, both the cap structure or the poly(A) tail alone fail to efficiently promote translation in vivo (yeast spheroplasts) or in yeast extracts that retain their endogenous mRNAs (Preiss & Hentze, 1998). The major difference between translation in vivo/untreated extracts and nuclease-treated extracts results from mRNA competition in the former, and lack of competition in the latter systems (Preiss & Hentze, 1998). Poly(A)-tail-promoted translation can reach the correct as well as internal initiation codons. The presence of a cap structure on a polyadenylated

mRNA redirects ribosome recruitment to the 5′ end. This process, however, is only partially effective in nuclease-treated extract. To achieve a high fidelity of initiation, it additionally requires the competitive environment in untreated extracts which suppresses the independent activity of the poly(A) tail (Preiss & Hentze, 1998; see also Introduction).

These findings regarding mechanisms that direct "constitutive translation" pose questions concerning "regulated translation" by mRNA elements. We aimed to directly test predictions made by the above findings, and to integrate our knowledge concerning the physiological in vivo functions of three regulatory modules into currently available information on the properties of the cap structure and the poly(A) tail in translation initiation. To this end, the role of (1) regulatory 5′ UTR RNA-protein interactions, (2) upstream ORFs, and (3) variations in poly(A) tail length were examined. Even if the IRE/IRP system appears to be phylogenetically restricted to metazoan cells (Rothenberger et al., 1990; Muckenthaler et al., 1998b), it was chosen as a model system for regulatory 5′ UTR RNA-protein interactions, because the IRE/IRP system functions in yeast (Oliveira et al., 1993; Paraskeva et al., 1998a) and is one of the best understood examples of this type of translational control. Likewise, the effects of variations in poly(A) tail length were analyzed in the yeast extract in spite of a present lack of examples of regulated polyadenylation in yeast. To date, yeast extracts are the only available cell-free systems that reflect the functional synergism between the poly(A) tail and the cap structure that governs translation in most (if not all) eukaryotic cells.

Our experiments with the IRE/IRP system show that translation of IRE.CATa mRNA, carrying the IRE in a cap-proximal but start codon-distal position, escapes the regulatory influence of IRP-1 binding under noncompetitive conditions. Ribosomes that are stalled at a translation initiation codon protect a region of approximately 35 nt (nucleotide position −20 to +15 with respect to the AUG; Jackson, 1996). We hypothesized that translational repression could be restored to such an uncapped, polyadenylated mRNA by direct interference with ribosome binding to the targeted start codon. This was indeed demonstrated after placing the IRE stem-loop only 12 nt upstream of the translation initiation codon of IREscp.CAT mRNA or by incorporating a start codon into the IRE itself in IRE/AUG.CAT. These results suggest that "internal" translation initiation promoted by the poly(A) tail fails to overcome the inhibitory effect of a high affinity RNA-protein complex at or near a translation initiation codon. Even under noncompetitive conditions, the presence of a cap structure (cIRE.CATa) helps to direct ribosome recruitment to the 5′ end, and thus to increase the regulatory effect of IRP binding (Fig. 2A,C, compare lanes 7 and 8 with lanes 5 and 6, and Fig. 3B). In combination with the competitive conditions of untreated yeast extract, the required synergy strongly favors translation initiation events in a 5′ end-dependent fashion, resulting in effective repression of cIRE.CATa mRNA by IRP-1 (Fig. 4, lanes 7 and 8), as was previously seen in living yeast (Oliveira et al., 1993) and mammalian (Goossen & Hentze, 1992) cells. However, extrapolation of these findings to IRP-1-mediated regulation in metazoan cells requires caution, given the emerging mechanistic differences of the poly(A) tail effect on translation between yeast and animal cells. In the latter case, one possible link between the poly(A) tail and the translation machinery could be established through interaction of the poly(A) binding protein with a novel protein termed PAIP (for poly(A) interacting protein). PAIP displays homology to the central region of mammalian eIF4G and interacts with eIF4A, but apparently not with the cap-binding protein eIF4E or the 40S ribosomal subunit associated eIF3 (Craig et al., 1998). Furthermore, different or additional mechanisms to direct translation initiation to the 5′ end may operate in mammalian cells, because even uncapped mRNAs are translated in a 5′ end-dependent fashion both in vitro (De Gregorio et al., 1998) and in vivo (Gunnery et al., 1997). Our analysis of the differently modified versions of the uORF.CAT mRNA shows that uORF 4, just like an inhibitory IRE/IRP complex, is bypassed by poly(A) tail promoted translation under noncompetitive conditions. Thus, the proper function of both types of regulatory elements is ensured by mRNA competition.

Investigating the length dependence of poly(A)-promoted translation in micrococcal nuclease-treated and untreated extracts demonstrates that changing the length of a poly(A) tail on an mRNA in the typical range (in yeast) of 15–80 nt leads to a strong quantitative effect on translation efficiency. Our results predict that changes in poly(A) tail length cause quantitatively different effects on translational regulation, depending on the ratio between the limiting components of the translational machinery and other actively translated (and competing) mRNAs. A previous study has looked at the relationship between the efficiency of polysome formation and the poly(A) tail length in the noncompetitive environment of micrococcal nuclease-treated rabbit reticulocyte lysate (Munroe & Jacobson, 1990). In the range tested (0–68 adenosines), the stimulation was already approaching its modest maximal level of ~1.5-fold when using a 32-nt poly(A) tail. This is reminiscent of our results obtained under noncompetitive conditions (approximately threefold maximal stimulation, saturation occurs between 31–51 nt of poly(A) tail; see Fig. 7), underscoring the importance of the context of (competing) cellular mRNA. A study using a conditional mutation in the yeast poly(A) polymerase gene (*pap-1*) which accumulates poly(A)-deficient and poly(A)$^-$ mRNA also points towards increased competitiveness as part of the effect of the poly(A) tail on translation. A

decrease in the cellular abundance of either small or large ribosomal subunits in the *pap-1* background led to a differential shift of deadenylated mRNAs into smaller polyribosomes (Proweller & Butler, 1997). Further, evidence has been presented for poly(A)-tail shortening during "mRNA aging" (Lowell et al., 1992; Jacobson, 1996). Our findings are consistent with the notion that such age-related poly(A)-tail shortening could serve to favor the translation of newly synthesized mRNAs.

The requirements for Pab1p binding introduce an additional reference point of interest. A homopolymer of 12 adenosines represents a minimal binding site for Pab1p, whereas the binding of multiple Pab1p molecules to a longer homopolymer favors a packing density of 25 nt per molecule of Pab1p (Sachs et al., 1987). We show here that a poly(A) tail of 15 adenosines displays no significant stimulatory effect on the translation of a capped mRNA. This suggests that one bound Pab1p molecule per mRNA is not sufficient for a cooperative interaction with the cap structure. On the other hand, 15 adenosines do suffice for a moderate stimulation of translation when appended to an uncapped version of the reporter (data not shown), suggesting that the requirement for the binding of multiple Pab1p molecules to promote translation is not absolute. Furthermore, complex formation between a poly$(A)_{50}$ probe and the purified wheat germ initiation factors eIF4F, eIF(iso4F), and eIF4B was found in gel shift experiments (Gallie & Tanguay, 1994). It will be interesting to use the yeast cell-free translation system to quantitatively correlate the translation efficiency of an mRNA determined by the length of its poly(A) tail with the binding of Pab1p and/or initiation factor molecules.

In summary, we have confirmed the ability of the poly(A) tail to promote translation under noncompetitive conditions, and the role of the cap structure in directing ribosome recruitment to the 5′ end. We show here that mRNA competition and the resulting requirement for both the cap structure and the poly(A) tail to synergistically promote translation is essential for the proper function of 5′ UTR regulatory elements. Furthermore, we provide evidence that differences in poly(A) tail length affect translation particularly effectively when mRNAs have to compete for limiting translation components.

MATERIALS AND METHODS

Plasmid constructs

Plasmid pIRE.CAT is identical to the previously described pI12-CAT (Gray et al., 1993), except for a C to T substitution at position 57 (with reference to the transcription start site) in the 5′ UTR. In pIREscp.CAT, positions 64–96 were deleted (see Fig. 1) whereas in pIRE/AUG.CAT the 3′ region of the IRE was changed to include an AUG in-frame with the downstream CAT coding region (the construction of this plasmid is detailed in Paraskeva et al., 1998b). The plasmids puORF.CAT and pxORF.CAT were described before (Preiss & Hentze, 1998). Each construct (except pIRE/AUG.CAT) was also prepared carrying an $(A/T)_{98}$ segment in the 3′ UTR, inserted into the *Pst* 1 site (Preiss & Hentze, 1998). A plasmid pIRE.CAT$(U)_{98}$ was also isolated, carrying the $(A/T)_{98}$ segment in the reverse orientation. A spontaneous deletion event during bacterial growth of pIRE.CAT$(A)_{98}$ allowed the isolation of a further plasmid with a shortened (A/T) segment, pIRE.CAT$(A)_{31}$. Furthermore, oligonucleotides of the sequences G$(A)_n$TGCATA and AGCTTATGCA$(T)_n$CTGCA (n = 15, 51, and 150) were annealed pairwise, followed by ligation between the *Pst* I and *Hin*d III sites of pIRE.CAT. This resulted in the plasmids pIRE.CAT$(A)_{15}$ and pIRE.CAT$(A)_{51}$. The construct with the $(A/T)_{150}$ proved too unstable for isolation in pure form but allowed the isolation of another spontaneous deletion clone, pIRE.CAT$(A)_{72}$. A plasmid pIRE/AUG.CAT$(A)_{51}$ was also obtained by replacing the region between the *Msc* I/ *Hin*d III sites with an *Msc* I/ *Hin*d III fragment from pIRE.CAT$(A)_{51}$. All 5′ and 3′ UTR alterations were confirmed by sequencing and each preparation of $(A/T)_n$-containing plasmids was checked for deletions in the (A/T) segment by flanking restriction enzyme digestion. pPAT3 (previously named p51A (Muckenthaler et al., 1997)) encodes a truncated form of preprolactin (PPL) protein and includes an $(A/T)_{50}$ segment at the 3′ end.

In vitro transcription

All CAT mRNAs were transcribed in the presence or absence of m^7GpppG from the corresponding *Hin*d III-linearized plasmids using T7 RNA polymerase and accurately quantified by trace-labeling and agarose gel electrophoresis as previously detailed (Preiss & Hentze, 1998). The transcription protocol used here is an adaption of a published procedure (Dasso & Jackson, 1989) and was previously tested to yield ≥95% capping efficiency (Stripecke & Hentze, 1992). Prepared in this way, the mRNAs with an $(A)_{98}$ or $(A)_{31}$ tail possess 25 nt, and the mRNAs with $(A)_{72}$, $(A)_{51}$, or $(A)_{15}$ 10 nt of heterologous nucleotides downstream of the A-segment, respectively. To eliminate these nucleotides (and the last two adenosines), transcription of pIRE.CAT$(A)_{98}$ was also performed in the presence of oligonucleotide A-clip (CCTAGAGGATCCC CTT) and RNase H for cotranscriptional digestion as described (Chabot, 1994). Complete clipping of the RNA was confirmed by 4% denaturing PAGE. Capped PPL mRNA was transcribed from *Nsi* I-linearized pPAT3 with Sp6 RNA polymerase.

Cell-free translation

Extract preparations from the yeast strain MBS, micrococcal nuclease treatment, and translation reactions were performed following established procedures (Iizuka et al., 1994; Tarun & Sachs, 1995; Preiss & Hentze, 1998). When comparing nuclease-treated with untreated extracts, the latter were subjected to a parallel mock pretreatment, omitting only the nuclease. For these comparisons, all reactions were subjected to quantitative CAT immunoprecipitation as described (Preiss & Hentze, 1998), prior to analysis by 15% SDS-PAGE and fluorography. Note that the smaller CAT translation products

(marked in the Figs. 2 and 6 by an asterisk) are not recognized by the monoclonal anti-CAT antibody. Quantification of protein products was done with a PhosphorImager (Molecular Dynamics).

Recombinant, His-tagged IRP-1 protein was produced and purified as previously described (Gray et al., 1993), and added directly to the translation reactions.

Poly(A) test

The polymerase chain reaction (PCR)-based PAT assay (Sallés & Strickland, 1995; Muckenthaler et al., 1997) was used to measure poly(A) tail length. RNA was extracted from translation reactions after 60 min incubation using the RNA clean reagent (AGS, Heidelberg). Aliquots of the isolated RNA or an equivalent amount of input mRNA were then analyzed essentially as previously described (Muckenthaler et al., 1997), except that the PCR products were resolved on native 2% agarose gels. The mRNA-specific PCR primer CAT-X (ATC ATGCCGTTTGTGATGG) corresponds to nucleotide positions 678–696 in the coding region of the IRE.CAT mRNA. The resulting PCR products thus derive from an upstream region (190 bp for cIRE.CATa51 mRNA and 206 bp for cIRE.CATa98 mRNA), a poly(A) segment, and 18 bp contributed by the oligo (dT)-anchor. The PAT assay has an inherent variability of approximately $\pm$10 bp.

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