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A common function for mRNA 5′ and 3′ ends in translation initiation in yeast

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The mRNA poly(A) tail and its associated poly(A) binding protein (Pab1p) are ubiquitous in eukaryotes. The function of the poly(A) tail is to stabilize mRNA and to stimulate its translation. The development of a poly(A)- and cap-dependent yeast in vitro translation system has allowed us to understand how poly(A) stimulates translation. We find that Pab1p but not the cap binding protein eIF-4E is required for poly(A) tail-dependent translation, and that the Pab1p-poly(A) tail complex functions to recruit the 40S ribosomal subunit to the mRNA. These data introduce a new step into the pathway of translation initiation and merge the translational functions of the two ends of mRNA.

[*Key Words*: Translation initiation; mRNA; ribosome; yeast; poly(A)]

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Current models of mRNA translation initiation in eukaryotes postulate that the cap structure on the 5′ end of the mRNA is bound by the cap binding protein complex eIF-4F (for review, see Hershey 1991; Merrick 1992). The cap binding protein eIF-4E is part of this complex and is thought to confer specificity to this binding. Subsequent to eIF-4F binding, the RNA helicase eIF-4A and the mRNA binding protein eIF-4B are loaded onto the 5′-untranslated region (5′ UTR), thereby creating an unstructured region for the incoming 43S ribosomal subunit complex, consisting of the 40S small ribosomal subunit bound to several initiation factors and the methionyl-tRNA. Ribosome binding to the mRNA could be enhanced by an interaction between the eIF-4F complex and the eIF-3 initiation factor associated with the small ribosomal subunit (Trachsel et al. 1980). After ribosomal subunit binding, scanning, and recognition of the initiator methionine codon, the 48S mRNA–ribosomal subunit complex is stimulated by the initiation factor eIF-5 and the 60S large ribosomal subunit to hydrolyze a bound GTP molecule. This allows for the joining of the 60S subunit to the 40S and the completion of the translation initiation process.

Some mRNAs do not have an absolute requirement for the cap structure for their translation, presumably because they have unstructured 5′ UTRs that are already competent for ribosome binding (e.g., see Gehrke et al. 1983). Other mRNAs do not have a cap requirement for their translation because of the presence of an internal ribosome entry site in the 5′ UTR (for review, see McBratney et al. 1993). However, the majority of mRNAs in the cell are thought to initiate their translation through a cap-dependent mechanism.

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Eukaryotic mRNA poly(A) tails have been recognized to be enhancers of mRNA translation initiation (for review, see Jackson and Standart 1990; Sachs and Wahle 1993). None of the current models for translation initiation adequately provide a mechanism by which this can occur. Furthermore, many 3′ UTRs confer mRNA translational regulation without affecting the length of their poly(A) tails (Curtis et al. 1995); none of the current models can provide an explanation for this phenomenon either.

The importance of poly(A) for translation has been underscored by a variety of in vivo and in vitro studies. For example, mRNA polyadenylation is necessary for translational recruitment during early development (e.g., Sheets et al. 1995). The poly(A) tail is presumed to exert its effect in translation through its associated poly(A) binding protein (Pab1p). Evidence for this comes from genetic analyses of *pab1* mutations (Sachs and Davis 1989, 1990). For instance, studies of bypass suppressor mutants capable of growth in the absence of the essential *PAB1* gene in yeast revealed that alterations in the large ribosomal subunit made the normally essential Pab1p dispensable. Although these data emphasize the functional linkage between the poly(A) tail and translation, they do not provide a mechanistic explanation for how Pab1p and poly(A) are needed for translation. Transient expression studies of mRNA have also shown that the cap and the poly(A) tail are required for efficient mRNA translation in both animal and plant cells (Gallie 1991). As with the genetic analysis, these data provide more evidence for the use of the poly(A) tail in translation initiation without providing mechanistic information about the process. Finally, a careful in vitro study analyzing how poly(A) tails stimulated mRNAs to be translated 2- to 2.5-fold better in reticuloctye lysates revealed

that the rate of the 60S subunit joining step was enhanced (Munroe and Jacobson 1990). Although these in vitro data help to formulate a working model of poly(A) and Pab1p function, they are limited in that the extracts used for the study were only mildly dependent on the poly(A) tail for translation.

Because an understanding of the molecular details by which the 3′ end of the mRNA can stimulate translation will be central to an understanding of how mRNA expression is regulated in many cell types, we have undertaken a biochemical analysis of the role of the poly(A) tail and Pab1p in this process. Using a recently developed in vitro translation extract from yeast, which is stimulated between 20- and 50-fold by the cap and the poly(A) tail on mRNA (Iizuka et al. 1994), we have been able to show that Pab1p mediates the poly(A) tail's stimulation of translation. Furthermore, by analyzing translation initiation intermediates through sucrose gradient sedimentation analysis, we have found that Pab1p and the poly(A) tail stimulate the binding of the 40S ribosomal subunit to the mRNA. These data provide new information about how translation initiation occurs in cells and also provide a framework for understanding how the 3′ UTRs of mRNA can regulate an mRNA's translation.

Results

Yeast extracts containing the L-A double-stranded RNA exhibit cap and poly(A) tail-dependent translation

Recently Iizuka et al. (1994) reported that extracts from *Saccharomyces cerevisiae* deficient for the L-A double-stranded RNA (dsRNA) molecule (Wickner 1986) were capable of cap and poly(A) tail-dependent translation. This deficiency allowed for an examination of protein synthesis in S30 extracts of exogenous mRNA using [^{35}S]methionine incorporation and SDS-PAGE. To increase the range of yeast mutants whose extracts could be studied using this technique, we decided to test whether this dsRNA deficiency was required for the cap- and poly(A) tail-dependent translation of an mRNA whose protein product could be assayed enzymatically. Accordingly, we programmed nuclease-treated extracts with an mRNA encoding the firefly luciferase (LUC) protein. Crude S30 extracts prepared from yeast cells containing or lacking the L-A dsRNA molecule each show comparable translation of LUC mRNA when it is either capped (capLUC) or polyadenylated (LUCpA) (Fig. 1A). The presence of the cap or the poly(A) tail stimulated translation of LUC mRNA at least 20-fold in extracts containing the dsRNA (Fig. 1D). The presence of the cap and the poly(A) tail (capLUCpA) lead to a synergistic interaction that typically ranges between 2.5- and 8-fold (Fig 1A,D). The variability in the degree of synergism that is observed could be attributable to slight differences in the length of time the extracts are treated with micrococcal nuclease before being programmed with mRNA. Previous attempts to have L-A dsRNA containing yeast extracts translate in a poly(A) tail-dependent manner probably failed because these assays measured ^{35}S-labeled protein synthesis by gel analysis, which cannot be done because of the high background translation of the dsRNA fragments (Iizuka et al. 1994).

The stimulation by the cap and the poly(A) tail was not attributable to differences in mRNA stability in the dsRNA-containing extracts, as the functional half-life of the mRNA, as determined by the inactivation rate of the mRNA's translation, was nearly identical for the LUC, capLUC, or LUCpA mRNAs (Fig. 1B). The capLUCpA is no greater than twofold more stable than the other transcripts in these assays. The concentration range of mRNA used in these experiments falls into the linear range for programmed translation by the extracts (Fig. 1D), thereby assuring that slight changes in mRNA translation were detectable. Identical chemical half-lives of the capLUCpA and the LUC transcripts were found when measured by the rate at which nucleotides become TCA soluble (Fig. 1C) or by the rate at which the intact mRNA becomes smaller following separation on formaldehyde–agarose gels (data not shown). Because functional and not chemical half-lives are the best measure of translatable mRNA degradation in the system, these chemical half-life measurements only provide an indirect measure of how quickly the translating mRNA is being degraded. They cannot be used to determine the degradation rate of the functional mRNA in the extract. The time lag before translation is observed in the extracts (Fig. 1B) is highly reproducible and could be attributable to a rate-limiting step in messenger ribonucleoprotein (mRNP)–ribosome complex formation.

Poly(A)-dependent translation does not require active cap binding protein

The efficient translation of LUCpA mRNA is not adequately explained by current models of translation initiation, which impart the cap structure and its associated cap binding protein complex eIF-4F with the unique role of recruiting the small 40S ribosomal subunit to the mRNA. Two different methods were used to investigate whether eIF-4E (the cap binding protein in this complex) was needed for poly(A)-dependent translation. First, inhibition of translation with the cap analog ^{7m}GpppG, which competitively inhibits binding of eIF-4E to mRNA, revealed the expected inhibition of translation of the capLUC mRNA (Fig. 2A). In contrast, no inhibition of LUCpA translation was seen at concentrations of analog giving >80% inhibition of capLUC mRNA translation. The twofold stimulation of LUCpA mRNA translation at low concentrations of analog was reproducibly observed (also see Iizuka et al. 1994), although we have not established why this occurs. The inhibition of LUCpA translation at high concentrations of cap analog is not attributable to a specific blockage of eIF-4E, as the nonmethylated analog GpppG, which is bound much less well by eIF-4E, showed comparable degrees of translation inhibition at concentrations >0.5 mM (data not shown). The capLUCpA mRNA's sensitivity to the cap

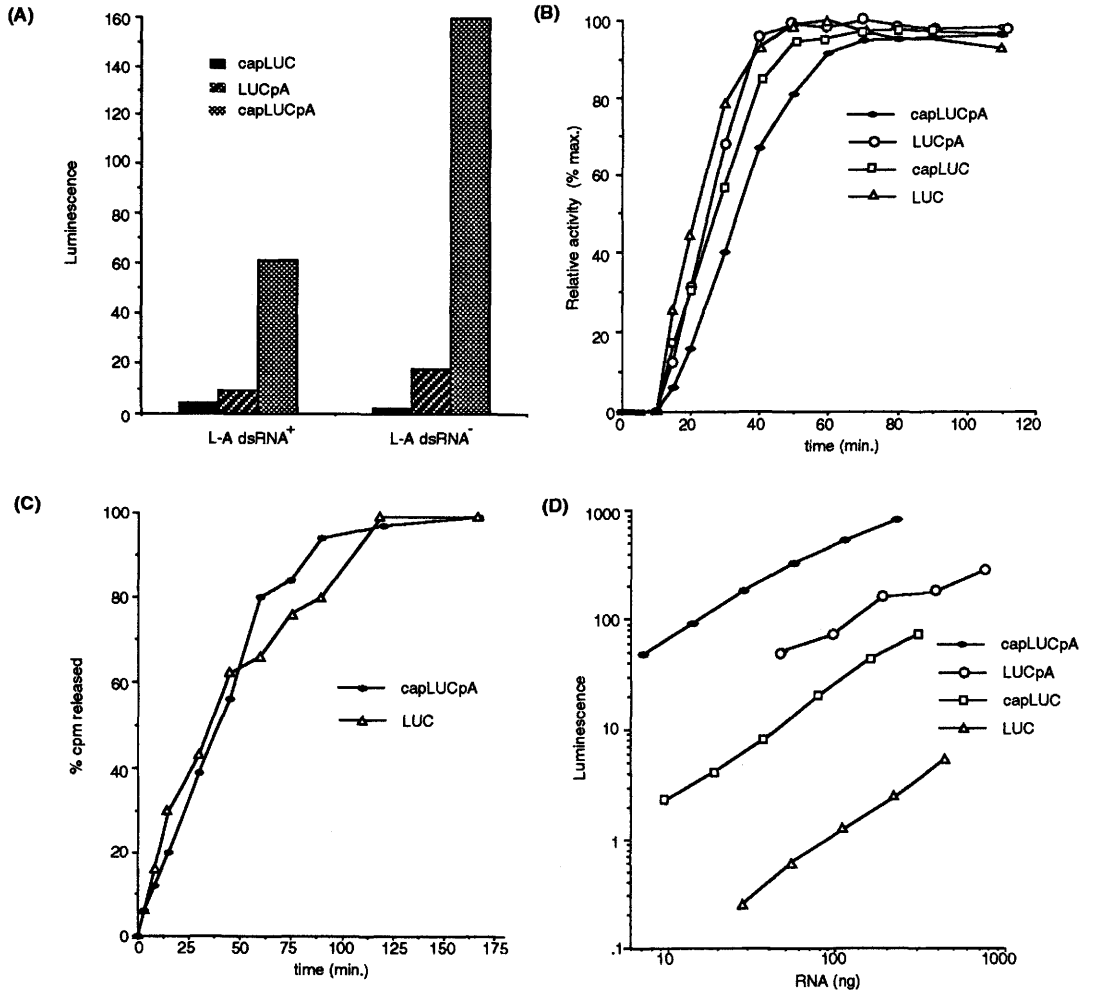

Figure 1. Cap- and poly(A) tail-dependent translation in L-A dsRNA containing yeast extracts is not attributble to differential mRNA degradation. (*A*) Extracts containing or lacking the L-A dsRNA molecule exhibit cap- and poly(A) tail-dependent translation. Crude yeast S30 extracts were prepared from strains YAS 306 (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1* L-A$^+$) and strain MBS [(kindly provided by P. Sarnow (Iizuka et al. 1994)] (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1* L-A°). In vitro translation products from mRNA encoding the LUC protein containing a cap structure (capLUC), a poly(A) tail (LUCpA), or both a cap and a poly(A) tail (capLUCpA) were analyzed by luminescence. (*B*) The functional stabilities of the various mRNAs in the extract are nearly identical. In vitro translation extracts programmed with each of the four mRNAs shown were analyzed for LUC protein synthesis by luminescence as a function of time. The data are replotted as percent of total LUC protein made at the 110 min time point. The luminescence measurements for this point were LUC (0.207), LUCpA (30.05), capLUC (5.43), and capLUCpA (99.1). (*C*) The chemical stabilities of the mRNAs in the extract are nearly identical. Aliquots from in vitro translation extracts programmed with ~100 ng of radiolabeled LUC or capLUCpA mRNA were withdrawn as a function of time, and trichloroacetic acid soluble counts (Lowell et al. 1992) were measured to determine the extent of mRNA destruction. Data are plotted as a percent of total radioactivity released, where the 100% point is that amount found after 175 min of incubation. (*D*) The in vitro translation extracts respond linearly to increases in mRNA concentrations. LUC protein production from in vitro translation extracts programmed with the indicated amount of mRNA was determined by luminescence.

analog (Fig. 2A) reveals a pattern that is consistent with an inactivation of both the cap stimulation and the synergy between the cap and the poly(A) tail, but not an inactivation of the poly(A) tail's stimulation.

An extract containing a defective eIF-4E protein was also investigated. This mutant protein has a low affinity for the cap structure and is expressed at 34% of wild-type levels in cells (Altmann et al. 1989; Lavoie et al. 1994). As reported previously (Altmann et al. 1989), we find that this mutant extract is deficient for cap-dependent translation (Fig. 2B). However, these extracts show no decrease in the efficiency of LUCpA mRNA translation. Furthermore, the translation of the capLUCpA mRNA was nearly identical to that of the LUCpA mRNA. These data also indicate that loss of eIF-4E function ablates both cap-dependent translation and the cap and poly(A) tail synergism, but not the stimulation of translation by the poly(A) tail. On the basis of these data, we ruled out the model that poly(A) tails stimulated translation initiation through an eIF-4E-dependent pathway.

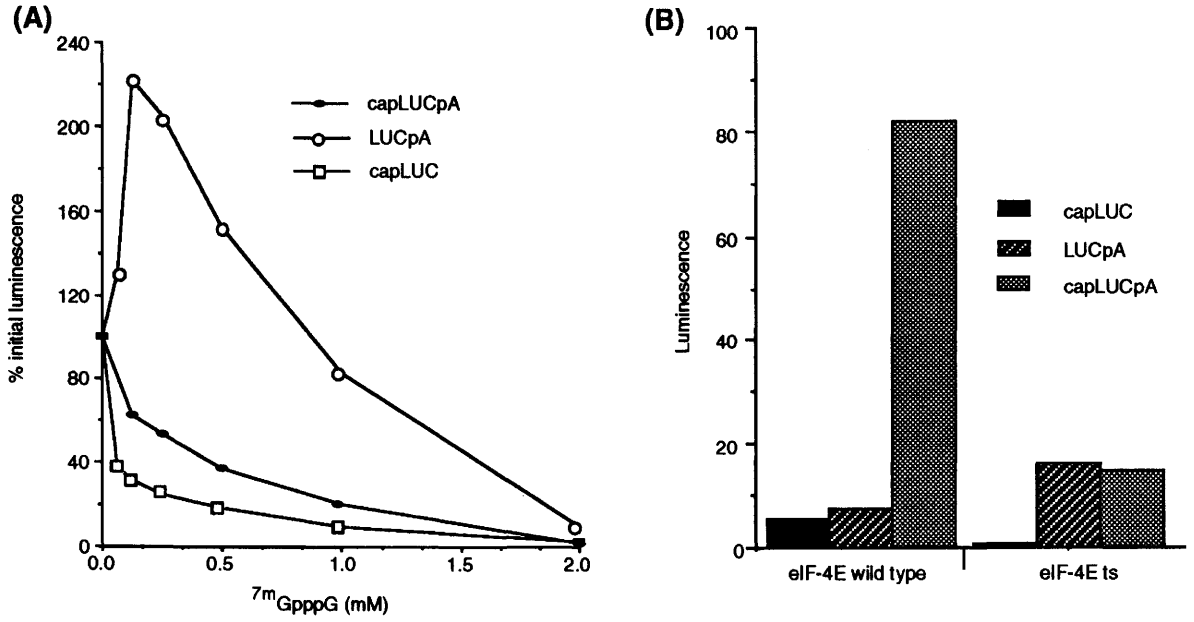

Figure 2. The stimulation of translation by the poly(A) tail does not require the cap binding protein eIF-4E. (*A*) The cap analog ^{7m}GpppG does not affect translational stimulation by poly(A) at low concentrations. LUC protein production from in vitro translation extracts containing the indicated amounts of ^{7m}GpppG and programmed with the indicated mRNAs was determined by luminescence. Data are expressed as percent of LUC production obtained in the absence of the analog. (*B*) Yeast extracts deficient for eIF-4E activity show normal levels of poly(A) tail-stimulated translation. Extracts from the wild-type yeast strain T93C[4E-wt] (*MATa eIF-4E::LEU2 ura3 trp1 leu2 peIF4E TRP CEN*) or the eIF-4E temperature-sensitive strain T93C[4E-2] (*MATa eIF-4E::LEU2 ura3 trp1 leu2 peIF4E-2 TRP CEN*) [kindly provided by M. Altmann (Altmann et al. 1989)] grown at 26°C were assayed for their ability to translate the indicated mRNAs. Because the eIF-4E mutant's extract was >90% inhibited for cap-dependent translation at the time of its preparation, no heating of the extract was required before the assay.

Poly(A)-dependent translation requires the poly(A) binding protein

Like the cap structure, the mRNA poly(A) tail is specifically bound by an RNA-binding protein, Pab1p (for review, see Sachs 1990). We found that translation of LUCpA mRNA was abolished by the addition of Pab1p monoclonal antibodies (Anderson et al. 1993) (Fig. 3A). As a control to show that inhibition was not attributable to a nonspecific effect of adding antibodies, we observed no inhibition when equivalent amounts of monoclonal antibodies against the yeast Pub1 protein (Anderson et al. 1993) were used (data not shown). These Pub1p antibodies were created using the same procedures and cell lines as those for the Pab1p antibodies (Anderson et al. 1993). The inhibition of capLUC translation at high concentrations of the Pab1p antibodies was a nonspecific effect, as the Pub1p antibodies exhibited similar degrees of inhibition (data not shown). Translation was also abolished if Pab1p was immunodepleted from the extracts (Fig. 3B). In these experiments, Pab1p was >90% immunodepleted, as determined by semiquantitative Western analysis of the residual supernatant (data not shown). Translation of the capLUC mRNA was affected only mildly by these treatments. The pattern of inhibition of capLUCpA translation suggested that poly(A) tail-dependent translation and the synergism between the cap and the poly(A) tail were lost. Consistent with this interpretation, the amount of luciferase produced from the capLUCpA mRNA was nearly equal to that of the capLUC mRNA at high antibody concentrations (the absolute value of luciferase production is derived from these figures by multiplying the percent inhibition by the 100% value listed in the legend to Fig. 3). That the amount of luciferase produced at high antibody concentrations was nearly equal for these different transcripts also rules out the possibility that the presence of the antibodies or inactivation of Pab1p leads to enhanced degradation of polyadenylated mRNA in the extracts.

Readdition of recombinant Pab1p (Sachs et al. 1987) to either the neutralized or immunodepleted extracts resulted in nearly complete reconstitution of poly(A) tail-dependent translation and the synergism between the cap and the poly(A) tail (Fig. 3C). The increased amount of Pab1p needed to reconstitute the neutralized extract versus the depleted extract probably reflected the presence of excess antibodies in the neutralized extracts that bound some of the input protein. Semiquantitative Western blots indicated that the amount of endogenous Pab1p present in assayed samples was ~500 ng (0.4% of total protein; data not shown), an amount similar to that needed for reconstitution. These data show that the stimulation of translation by the poly(A) tail and the synergism between the cap and the poly(A) tail requires

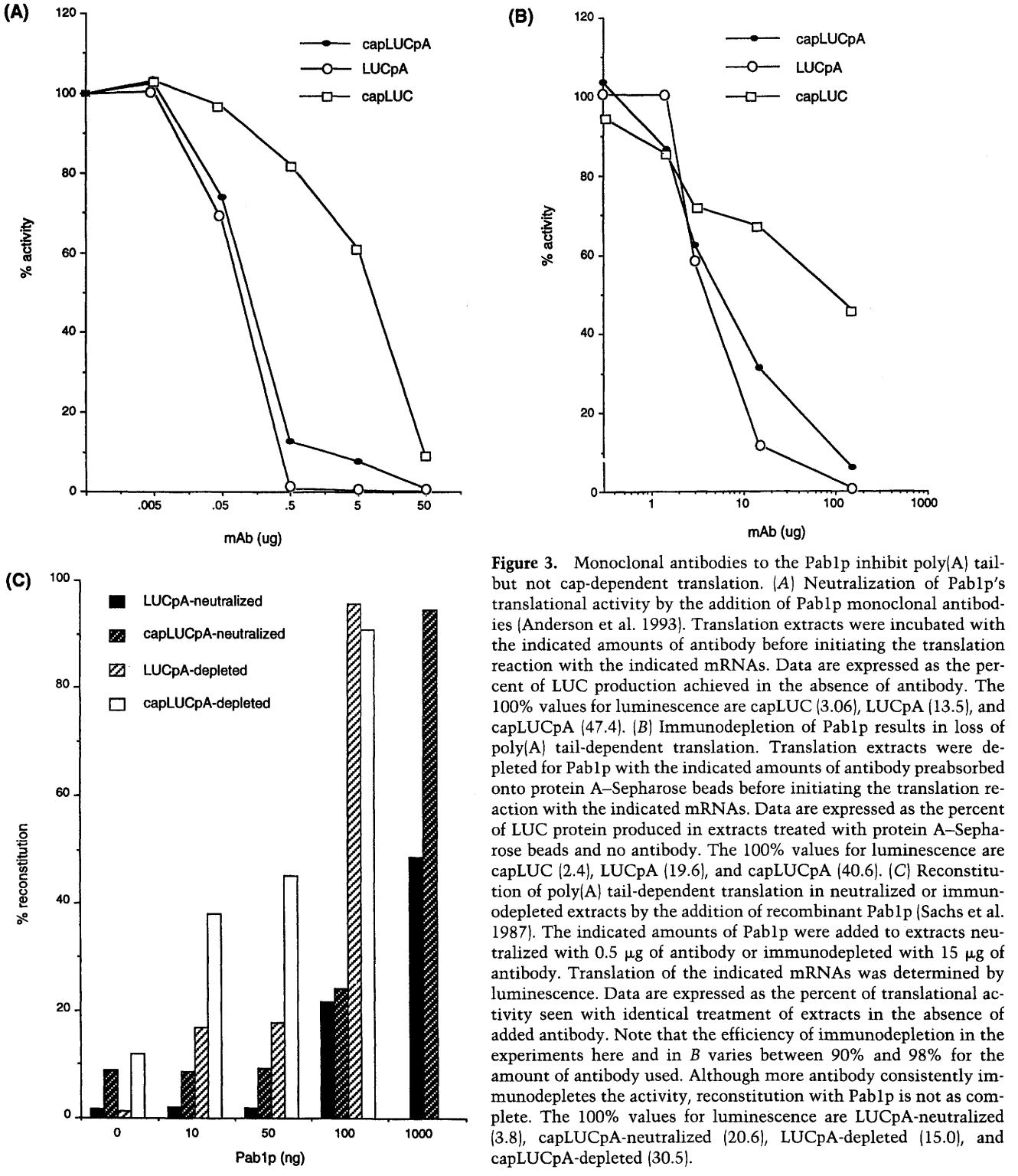

Figure 3. Monoclonal antibodies to the Pab1p inhibit poly(A) tail- but not cap-dependent translation. (*A*) Neutralization of Pab1p's translational activity by the addition of Pab1p monoclonal antibodies (Anderson et al. 1993). Translation extracts were incubated with the indicated amounts of antibody before initiating the translation reaction with the indicated mRNAs. Data are expressed as the percent of LUC production achieved in the absence of antibody. The 100% values for luminescence are capLUC (3.06), LUCpA (13.5), and capLUCpA (47.4). (*B*) Immunodepletion of Pab1p results in loss of poly(A) tail-dependent translation. Translation extracts were depleted for Pab1p with the indicated amounts of antibody preabsorbed onto protein A–Sepharose beads before initiating the translation reaction with the indicated mRNAs. Data are expressed as the percent of LUC protein produced in extracts treated with protein A–Sepharose beads and no antibody. The 100% values for luminescence are capLUC (2.4), LUCpA (19.6), and capLUCpA (40.6). (*C*) Reconstitution of poly(A) tail-dependent translation in neutralized or immunodepleted extracts by the addition of recombinant Pab1p (Sachs et al. 1987). The indicated amounts of Pab1p were added to extracts neutralized with 0.5 μg of antibody or immunodepleted with 15 μg of antibody. Translation of the indicated mRNAs was determined by luminescence. Data are expressed as the percent of translational activity seen with identical treatment of extracts in the absence of added antibody. Note that the efficiency of immunodepletion in the experiments here and in *B* varies between 90% and 98% for the amount of antibody used. Although more antibody consistently immunodepletes the activity, reconstitution with Pab1p is not as complete. The 100% values for luminescence are LUCpA-neutralized (3.8), capLUCpA-neutralized (20.6), LUCpA-depleted (15.0), and capLUCpA-depleted (30.5).

Pab1p, that the inhibition by the antibodies was attributable to loss of Pab1p and not an associated protein, and that the inhibition of poly(A) tail-dependent translation can occur in the absence of inhibition of cap-dependent translation.

The Pab1p stimulates 40S ribosomal subunit joining

To discern what step of translation initiation was stimulated by Pab1p, sucrose gradient sedimentation of translation extracts programmed with radiolabeled

mRNA containing either a cap, a poly(A) tail, or both a cap and a poly(A) tail were performed (Fig. 4). Similar experiments have been used to understand the mechanism by which the iron response element (IRE) binding protein inhibits translation of IRE-containing mRNAs (Gray and Hentze 1994; details presented in this work establish the validity of the interpretations we derive from our data). The large size (1.9 kb) of the luciferase mRNA precluded its use in this analysis, as it sedimented as a large particle even in the absence of translation. Instead, the 327-nucleotide MFA2 mRNA from yeast was used because its sedimentation in the absence of translation was not overlapping in size with any of the translation initiation intermediates. Radioactive peaks in the gradient were assigned to be different translation initiation complexes based on their drug sensitivity and sedimentation values (Fig. 4A–C). For instance, the 80S monoribosomal peak increased in abundance in the presence of the elongation inhibitor cycloheximide. The 48S preinitiation complex, which contains the 40S subunit and its associated factors bound to the initiator methionine codon, increased in abundance when the nonhydrolyzable analog GMPPNP was added to the translation extract. This increase in abundance reflects the requirement for GTP hydrolysis before 60S subunit joining occurs. The presence of the two peaks in gradients from GMPPNP-treated extracts results from having either one or two 40S subunits bound to the mRNA (see Gray and

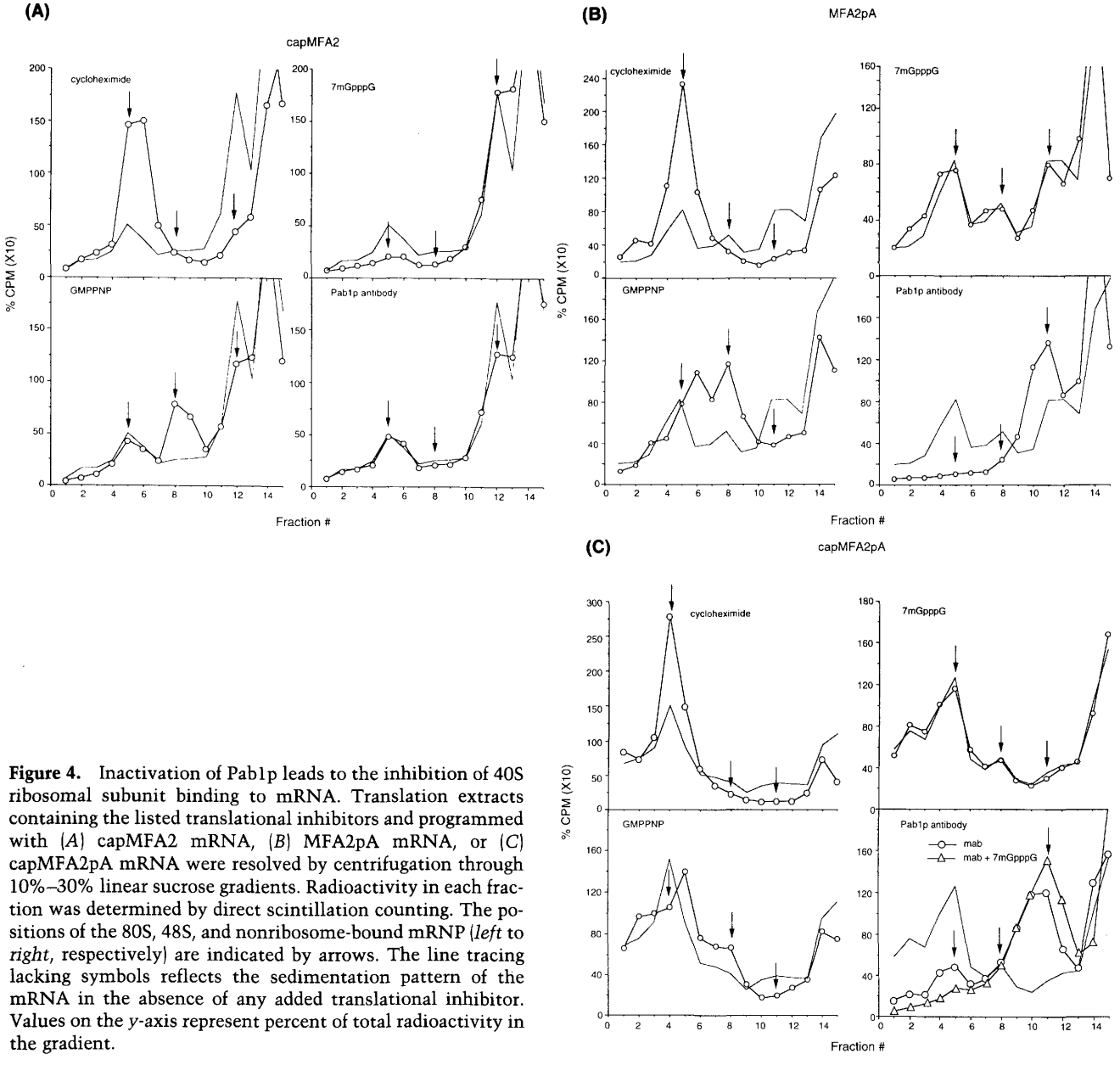

Figure 4. Inactivation of Pab1p leads to the inhibition of 40S ribosomal subunit binding to mRNA. Translation extracts containing the listed translational inhibitors and programmed with (*A*) capMFA2 mRNA, (*B*) MFA2pA mRNA, or (*C*) capMFA2pA mRNA were resolved by centrifugation through 10%–30% linear sucrose gradients. Radioactivity in each fraction was determined by direct scintillation counting. The positions of the 80S, 48S, and nonribosome-bound mRNP (*left* to *right*, respectively) are indicated by arrows. The line tracing lacking symbols reflects the sedimentation pattern of the mRNA in the absence of any added translational inhibitor. Values on the *y*-axis represent percent of total radioactivity in the gradient.

Hentze 1994, and below). The capMFA2 mRNA's sedimentation into the gradient was abolished almost completely in the presence of the cap analog ^{7m}GpppG (Fig. 4A). This inhibition of 40S subunit joining resulted in a change in size of the mRNA to something less dense than the 40S subunit, thereby providing a sedimentation marker for the nontranslating MFA2 mRNA, referred to as the mRNP in Figures 4 and 5.

The sedimentation profile of MFA2pA mRNA was not affected by the addition of ^{7m}GpppG (Fig. 4B). However, addition of the Pab1p antibodies shifted its sedimentation to the position of nontranslating mRNA. In contrast, the sedimentation of the capMFA2 mRNA was not affected by the addition of the Pab1p antibodies (Fig. 4A). These data indicate strongly that neutralization of Pab1p function prevents the joining of the 40S subunit to the MFA2pA mRNA. If 60S subunit joining had been blocked, an accumulation of the 48S precursor would have been observed.

The sedimentation of capMFA2pA mRNA confirms this conclusion (Fig. 4C). The addition of GMPPNP led to the majority of the mRNA sedimenting as the larger of the two accumulating species, indicating that this mRNA joins two 40S subunits more efficiently (Gray and Hentze 1994) than either the capMFA2 or the MFA2pA. Addition of ^{7m}GpppG had only a mild effect on the disappearance of the 80S monosome peak. The addition of the Pab1p antibodies led to a significant disappearance of the 80S peak and the appearance of the nontranslating mRNA peak. The addition of both the antibodies and ^{7m}GpppG eliminated the 80S peak almost completely, shifting the majority of the mRNA into its nontranslating form.

These experiments show that neutralization of Pab1p function results in loss of 40S ribosomal subunit binding to mRNA. To show that Pab1p stimulates 40S joining to mRNA, the effects of readdition of the recombinant Pab1p protein to crude extracts containing GMPPNP and neutralized Pab1p were examined (Fig. 5). As a control, we found that capMFA2 mRNA did not accumulate as a 48S preinitiation complex when incubated in extracts containing GMPPNP and ^{7m}GpppG (Fig. 5B). The existence of a small 48S peak in this experiment is ascribed to incomplete inhibition of the 40S joining step (see below). MFA2pA mRNA also did not accumulate as a 48S complex when incubated in extracts containing the antibody and GMPPNP (Fig. 5C). When Pab1p was added back to the neutralized extract, a marked increase in the appearance of a 48S peak was observed for MFA2pA mRNA (Fig. 5D), thereby directly showing that Pab1p stimulates 40S ribosomal subunit joining. The reappearence of a 48S peak when Pab1p is added back is not attributable to the sedimentation of an RNP containing Pab1p and no ribosomal subunit, as the 80S peak showed comparable increases when the experiments were performed in the presence of cycloheximide instead of GMPPNP (data not shown).

CapMFA2pA mRNA accumulates as two species when incubated in extracts containing GMPPNP, antibody, and ^{7m}GpppG (Fig. 5E). The larger species represent the 48S complexes, and the smaller one the nontranslating mRNA. The addition of both antibody and ^{7m}GpppG does not completely inhibit the formation of the 48S complexes. An explanation for this is found by a comparison of Figures 4 and 5. The experimental conditions for the results in Figure 4 were such that any 40S joining that occurred in the presence of the cap analog and/or the antibody would have led to the translation of the mRNA and eventually the disappearance of this mRNA from the monosome region of the gradient. The presence of a small 80S peak in the ^{7m}GpppG panel of Figure 4A probably represents this fraction of translation resulting from incomplete inhibition by the cap analog. In Figure 5, however, the experimental conditions are such that any 40S joining that occurs as a result of incomplete inhibition by the cap analog and/or the antibody leads to the irreversible formation of a 48S complex. We would predict that at infinite time all of the input mRNA would end up in 48S complexes because of the lack of equilibrium between this complex and the starting material. As a result, the presence of residual 48S peaks in Figure 5 B,C, and E, are assumed to arise from the incomplete inhibition of the 40S joining step by the different inhibitors.

Readdition of Pab1p stimulated both the disappearance of the nontranslating complex and the appearance of the large complex containing two 40S subunits (Fig. 5F). The level of the 48S complex presumably did not change, as its decrease due to production of the large complex was compensated for by its increase due to the production of 48S complexes from the nontranslating peak. These data confirm that Pab1p stimulates 40S ribosomal subunit joining.

The observation that the addition of ^{7m}GpppG affected 80S monosome formation on the capMFA2pA mRNA only mildly (Fig. 4C) is at odds with the 40% decrease that is predicted from the translation data with capLUCpA (Fig. 2A). Furthermore, the observation that the addition of both ^{7m}GpppG and Pab1p antibodies failed to inhibit 40S subunit joining as well as either did for capMFA2 or MFA2pA mRNA (Fig. 5) is also surprising. One explanation for this is that the cap and the poly(A) tail interact cooperatively for a common target that leads to 40S ribosome subunit recruitment. As a result, higher concentrations of these inhibitors would be needed to effectively compete for this target binding site on the capped and polyadenylated mRNA. Assuming that different mRNAs exhibit different degrees of cooperative binding between their cap and poly(A) tail, we would conclude that the MFA2 mRNA has a more cooperative cap and poly(A) tail interaction than the LUC mRNA.

Discussion

The experiments presented in this paper extend the work by Iizuka et al. (1994) by showing that translation extracts from yeast strains containing the L-A dsRNA are competent for cap- and poly(A) tail-dependent translation. The accuracy of these extracts in mimicking the in

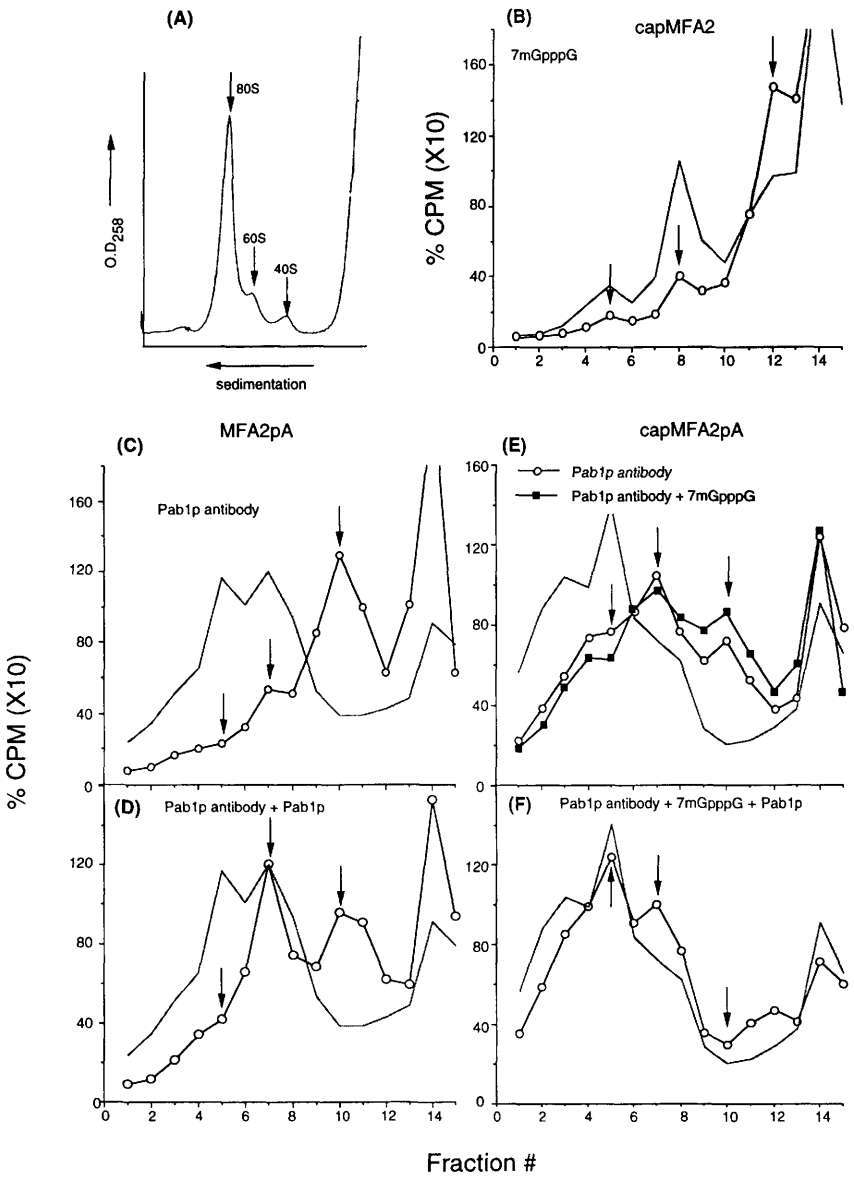

Figure 5. Addition of Pab1p to neutralized translation extracts stimulates 40S ribosomal subunit binding to mRNA. Translation extracts containing GMPPNP and the listed translational inhibitors, programmed with capMFA2 mRNA (*B*), MFA2pA mRNA (*C*) and (*D*), and capMFA2pA mRNA (*E*) and (*F*) were resolved by centrifugation through 10%–30% linear sucrose gradients. Radioactivity in each fraction was determined by scintillation counting. The positions of the mRNA with two 40S subunits bound, the 48S, and the nonribosome-bound mRNP (*left* to *right*, respectively) are indicated by arrows. The line tracing lacking symbols reflects the sedimentation pattern of the mRNA in the presence of only GMPPNP. Values on the *y*-axis represent percent of total radioactivity in the gradient. A representative OD_{258} tracing from one gradient (*A*) is also shown.

vivo requirements for translation is confirmed by their dependence on the cap structure for translation of capped mRNA and their inactivation of capped mRNA translation in the presence of a mutated cap binding protein. Although it is formally possible, we do not believe that our results for the MFA2 and the LUC mRNAs will be unique for them but, instead, will be true for many mRNAs in the cell. The unusual observation that uncapped polyadenylated mRNA is translated as efficiently as capped mRNA in these extracts has been found to be attributable to the ability of the poly(A) tail and the Pab1p to recruit the 40S ribosomal subunit to the mRNA. This ability strongly suggests that the cap and the poly(A) tail on mRNA have a common function in the translation initiation process.

Our data support the hypothesis that the poly(A) tail and the cap structure stimulate translation initiation through the use of different RNA-binding proteins. A biochemical explanation for the synergism between the cap and the poly(A) tail on mRNA is found from an examination of the sizes of the stalled ribosome complexes in the presence of GMPPNP (Figs. 4 and 5). While either the cap or the poly(A) tail on mRNA stimulates one or two ribosomes to bind per mRNA in the time allotted in these experiments, the capped and polyadenylated mRNA exists predominantly as a form with two ribosomes bound. A time course of complex formation on MFA2pA mRNA in the presence of GMPPNP confirms that at early times the predominant peak in the gradient corresponds to the 48S position, with the heavier peak increasing in abundance later in the assembly reaction (A. Sachs, unpubl.). We note that the amount of translation of the LUC mRNAs (Fig. 1) correlates perfectly with the ability of these mRNAs to recruit two 40S subunits.

From these data, it can be concluded that the synergism between the cap and the poly(A) tail is attributable to a heightened ability to recruit the 40S ribosomal subunit to the mRNA. These 40S subunit joining data also provide independent biochemical evidence to support the hypothesis that poly(A) tails stimulate 40S ribosomal subunit joining in the translation cycle.

Previous work examining cap and poly(A) tail synergism in translation has suggested that the poly(A) tail mediates its function by binding to eIF-4E and its associated proteins, thereby increasing their effective concentration so as to allow more efficient cap-dependent translation (Gallie and Tanguay 1994). Although we do see that eIF-4E function is required for the synergism between the cap and the poly(A) tail, we also find that eIF-4E function is not required for poly(A) tail translational stimulation. This negates the hypothesis that the poly(A) tail acts solely as a loading site for eIF-4E, which then binds to the cap structure. However, these data do not rule out the possibility that poly(A)-dependent translation requires one of the other proteins in the eIF-4F complex (Goyer et al. 1989; Lanker et al. 1992).

A study examining translation in reticulocyte lysates proposed that the poly(A) tail stimulated joining of the 60S ribosomal subunit (Munroe and Jacobson 1990). Although this may be true, the failure of these experiments to detect the 40S joining stimulation may have resulted from the use of extracts that are only partially stimulated by the poly(A) tail. The data presented here do not address whether 60S subunit joining is also stimulated by the poly(A) tail, as the inhibition of 40S joining by Pab1p depletion precludes studying the effects of poly(A) and Pab1p on this step.

Our previous work showing that mutations in the 60S ribosomal subunit were able to suppress a loss of Pab1p from yeast (Sachs and Davis 1989, 1990) indicated that Pab1p's target was the translational apparatus. Each of these bypass suppressor strains had in common an excess of free 40S subunits relative to 60S subunits. Given the above data, we propose that one way to suppress a Pab1p deletion might be to compensate for the loss of this 40S ribosome recruitment protein by increasing the concentration of the free 40S ribosomal subunit.

Because poly(A) tails and the cap structure are likely to enhance the same step of translation initiation, its seems likely that those mRNAs that have a low affinity for eIF-4E or that do not require eIF-4E for translation will be more dependent on their poly(A) tails for their efficient expression. This predicts that these mRNAs could have more elaborate regulation of the accessibility or length of their poly(A) tails than most mRNAs. Many potential examples of this already exist in the literature. For instance, the translation of mRNAs encoding heat shock proteins is not as sensitive to the loss of functional eIF-4E as is that of most cellular mRNAs (Panniers 1994). For at least one of these mRNAs, poly(A) tail length regulation has been observed (Dellavalle et al. 1994). During early development, mRNA expression is highly dependent on its poly(A) tail (for review, see Wickens 1992). During this period of development, it has long been recognized that many of the translation initiation factors are limiting (e.g., see Klein and Melton 1994). As a corollary to this, it is interesting to consider the function of the PHAS-I, a negative regulator of eIF-4E in mammalian cells (Lin et al. 1994; Pause et al. 1994). Those mRNAs whose expression is repressed by the presence of this protein could have their expression induced by mitogens that relieve eIF-4E repression or by other cellular factors that alter the status of the mRNA's poly(A) tail. This would provide multiple levels of regulation to the mRNA's expression. Finally, it has been assumed that IRE sites (IRES) on mRNA are functionally equivalent to the cap structure (for review, see McBratney et al. 1993). However, given our findings of an alternative way to recruit 40S subunits to the mRNA, it is possible that these elements and poly(A) tails are more equivalent in function.

The in vitro data presented here make it extremely likely that the 3′ and 5′ ends of the mRNA are functionally interacting to recruit the 40S ribosomal subunit to the mRNA. The binding target of Pab1p that allows this recruitment to occur remains unidentified. This target could be a ribosomal protein or it could be a ribosome-associated protein. These data also provide a mechanistic framework for understanding how 3′-untranslated regions (3′ UTRs) that do not exert their effect on the polyadenylation apparatus could regulate translation. For instance, they could bind to either Pab1p or Pab1p's target to prevent translational activation. Understanding how Pab1p exerts its translation function will be central to understanding both the phenomena of 3′ UTR regulation and the potential regulation of translation by other intracellular proteins. In summary, the finding that the cap and the poly(A) tail have overlapping functions in the translation initiation cycle will require the modification of existing models of translation and will provide new avenues for studying the post-transcriptional control of gene expression.

Materials and methods

Preparation of S30 extracts

Cytoplasmic S30 extracts were prepared essentially as described by Iizuka et al. (1994). Briefly, 600 ml of a chilled $OD_{600} = 1.5$ yeast culture in 2% peptone/1% yeast extract/3% glucose were harvested, washed once in 15 ml of buffer A (30 mM HEPES at pH 7.4, 100 mM KOAc, 2 mM MgOAc, 2 mM dithiothreitol) plus 8.5% mannitol and four times in 10 ml of buffer A plus mannitol. Cells were harvested for 5 min at 3000 rpm in an SS-34 rotor for the first four washes and at 4000 rpm for the last wash. Following weighing of the cell paste (typically between 3 and 4 grams), cells were resuspended in 1.5 times cell weight of buffer A plus mannitol plus 0.5 mM PMSF and six times cell weight of cold, 0.5-mm glass beads (Biospec Products). Cells were lysed in a 38-ml capped centrifuge tube by five cycles of shaking for 1 min over a 50-cm hand path and 1 min of cooling in ice water. Following clarification of the lysate by two spins at 18,000 rpm in the SS-34 rotor, the supernatant was chromatographed by gravity flow on a 2.5×8-cm G-25 Superfine column (Pharmacia) equilibrated in buffer A plus PMSF. Fractions (0.5 ml) containing the peak OD_{260} material and those within 75% of this value

were pooled [typically 2–3 ml total pool volume at 25 mg/ml (OD_{260} = 90)] and frozen directly in liquid N_2 in 100-μl aliquots.

Preparation of mRNA

LUC RNA templates were prepared with 1 μg of *Bam*HI linearized T3LUC and T3LUCpA DNA (Iizuka et al. 1994) in a 20-μl reaction volume using the Ampliscribe T3 transcription kit (Epicentre Technologies). Trace amounts of [^{32}P] UTP were added to each transcription reaction to facilitate calculations of mRNA yield. Radioactive mRNA was prepared by decreasing by 10-fold the amount of UTP, and adding 50 μCi of [^{32}P] UTP to the reaction. Capped mRNA was synthesized with the cap analog ^{7m}GpppG (New England Biolabs). mRNA was purified by direct precipitation in 2.5 M NH_4OAc, followed by resuspension in 40 μl and desalting over a Sephacryl S-200 spin column (Pharmacia). mRNA integrity and concentration were confirmed by electrophoresis in 1.5% formaldehyde–agarose gels.

Radioactive MFA2 mRNA was prepared from *Sac*I [no poly(A)] or *Pst*I [poly(A)] digested pAS225, which contains the entire MFA2 cDNA upstream of a poly(A)$_{100}$ tract in the pSP65 SP6 transcription vector. For mRNA synthesis, nucleotide concentrations were decreased 10-fold from that used in the radioactive T3 transcription system, and 50 μCi of [^{32}P]UTP and 20 units of SP6 RNA polymerase (Promega) were present. mRNA in 2.5 M NH_4OAc was precipitated with an equal volume of ethanol and, following resuspension, in 40 μl of H_2O desalted over a Sephacryl S-200 spin column (Pharmacia). mRNA integrity and purity were confirmed by gel electrophoresis.

In vitro translation

Extracts were prepared for translation by thawing at 4°C, adding $CaCl_2$ to 480 μM, micrococcal nuclease (Pharmacia) to 150 U/ml, and incubation at 26°C for 5 min. Following the addition of EGTA to 2 mM final concentration, extracts were stored on ice until use.

Extract (7.5 μl) was added to 7.5 μl of 2× RNA mix (prepared in the following order: 2.4 μl H_2O, 2.5 μl 6× translation buffer [132 mM HEPES–KOH at pH7.4, 720 mM KOAc, 12 mM MgOAc, 4.5 mM ATP, 0.6 mM GTP, 150 mM creatine phosphate (Boehringher Mannheim), 0.24 mM amino acid–methionine, 10.2 mM DTT], 1 μl of 1 mM methionine, 0.1 μl of RNasin (Promega), 0.5 μl of mRNA, and 1 μl of creatine phosphokinase (4 mg/ml, Sigma) and incubated at 26°C for the indicated times. Reactions were quenched by quick freezing in liquid N_2. After thawing on ice, luminescence was measured by adding 10 μl of the translation mix to 50 μl of LUC assay reagent (Promega) and measuring the emission for 15 sec on a Turner TD-20e luminometer. Unless indicated otherwise, mRNA translation reactions were for 40 min with equal amounts (~50 ng) of each mRNA substrate using extracts from the L-A$^+$ yeast strain YAS 306.

For cap analog inhibition studies, 1.5 μl of a 10× stock dilution of analog in buffer A was added to the 2× RNA mix before the addition of 6.0 μl of extract. For antibody neutralization studies, 1 μl of diluted Pab1p monoclonal antibody in buffer A was incubated with 6.5 μl of extract for 15 min at 4°C before addition of the 2× RNA mix. For immunodepletion, the indicated amounts of antibody were bound to 15 μl of a 50% slurry of protein A–Sepharose beads in a final volume of 23 μl of buffer A for 1 hr at 4°C. Following three washes in 100 μl of buffer A, the pelleted beads were resuspended in 20 μl of crude extract and incubated for 1 hr at 4°C with gentle rocking. Following clearing of the beads by centrifugation, 7.5 μl of the residual supernatant was assayed for translation. For Pab1p reconstitution, the indicated amount of recombinant Pab1p (Sachs et al. 1987) was incubated with the extract for 15 min at 4°C before the addition of the 2× RNA mix. All presented data are representative of at least three independent experiments.

Sucrose gradient analysis

Extract (22.5 μl, with or without 1.5 μg of antibody) was mixed with 22.5 μl of a 2× RNA mix containing ~20,000 cpm (<10 ng) of mRNA and the indicated compounds at a final concentration of 760 μM. These concentrations of cycloheximide and GMPPNP were found to inhibit translation by >99%. Following 20 min at 26°C, reactions were quenched with 100 μl of ice-cold buffer A containing 0.25% glutaraldehyde (Gray and Hentze 1994) and fractionated on linear 10%–30% sucrose gradients by centrifugation at 4°C for 2.75 hr at 40,000 rpm in an SW-41 rotor. All gradients were monitored for absorption at 258 nm during their collection from the bottom to ensure their integrity. Radioactivity in each fraction was determined by scintillation counting in the absence of scintillation fluid. All results are representative of at least three independent experiments. Note that fractions containing the various translation intermediates vary by one fraction between experiments. As a result, the assignment of the location of these intermediates in the gradients was based on the peaks of radioactivity in the control samples and not on the fraction numbers.

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