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Circularization of mRNA by Eukaryotic Translation Initiation Factors

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Summary

Communication between the 5′ cap structure and 3′ poly(A) tail of eukaryotic mRNA results in the synergistic enhancement of translation. The cap and poly(A) tail binding proteins, eIF4E and Pab1p, mediate this effect in the yeast *S. cerevisiae* through their interactions with different parts of the translation factor eIF4G. Here, we demonstrate the reconstitution of an eIF4E/eIF4G/Pab1p complex with recombinant proteins, and show by atomic force microscopy that the complex can circularize capped, polyadenylated RNA. Our results suggest that formation of circular mRNA by translation factors could contribute to the control of mRNA expression in the eukaryotic cell.

Introduction

Efficient mRNA translation requires a series of protein–mRNA and protein–protein interactions. Structural elements of the eukaryotic mRNA, including the 5′ cap and 3′ poly(A) tail, are important determinants of these interactions. All eukaryotic mRNAs are modified at the 5′ end with a cap structure, which is recognized during translation initiation by the cap-binding protein eIF4E. A second factor, eIF4G, is associated with the mRNA as part of a complex with eIF4E and functions to recruit the 40S ribosome subunit through an interaction with eIF3 (Merrick and Hershey, 1996). Cap-dependent recruitment of the 40S ribosomal subunit to mRNA is subject to several cellular regulatory mechanisms. Phosphorylation of eIF4E can increase its affinity for the cap structure (Sonenberg, 1996). Binding of the 4E-binding proteins (4E-BPs) can inhibit cap-dependent translation by preventing eIF4G binding to eIF4E (Haghighat et al., 1995; Altmann et al., 1997). Cleavage of eIF4G by viral proteases can also inhibit cap-dependent translation (Morley et al., 1997).

Most eukaryotic mRNAs are also posttranscriptionally modified by the addition of a 3′ poly(A) tail. The poly(A) tail is known to be an important regulator of gene expression. This has been most clearly shown in studies of meiotic maturation in frog oocytes, where regulated polyadenylation of cytoplasmic mRNAs leads to their translation (Sheets et al., 1995). A number of other studies have also demonstrated a role for the poly(A) tail in translation (Sachs and Davis, 1989; Munroe and Jacobson, 1990; Gallie, 1991; Tarun and Sachs, 1995). When both a cap and a poly(A) tail are present on an mRNA, they function together to induce a synergistic enhancement of translation (Gallie, 1991; Tarun and Sachs, 1995).

Both cap- and poly(A) tail–dependent translation in *Saccharomyces cerevisiae* requires the translation initiation factor eIF4G. There are two functionally homologous eIF4G proteins in yeast, eIF4G1 (*TIF4631*) and eIF4G2 (*TIF4632*) (Goyer et al., 1993). Both of these proteins contain a highly conserved binding site for eIF4E (Mader et al., 1995; Tarun and Sachs, 1997) and a recently identified binding site for the poly(A) binding protein, Pab1p (Tarun and Sachs, 1996; Tarun et al., 1997). In addition, both proteins have putative binding sites for eIF3 (Lamphear et al., 1995) and RNA (Tarun and Sachs, 1996), the latter of which may span a region within the C-terminal domain that has homology to other RNA-binding proteins (Goyer et al., 1993). We have previously shown that the synergistic activation of translation by the cap and the poly(A) tail can be destroyed in vitro by mutations that disrupt either the interaction between eIF4E and eIF4G (Tarun and Sachs, 1995; Tarun and Sachs, 1997), or between Pab1p and eIF4G (Tarun et al., 1997; Kessler and Sachs, 1998).

These data have led to the prediction that eIF4G can simultaneously bind to Pab1p and eIF4E, and that these interactions result in the formation of a complex that circularizes mRNA (Tarun et al., 1997). These circles may be similar to those seen with membrane-bound polysomes visualized by electron microscopy (Christensen et al., 1987). In support of the prediction that eIF4G interacts with Pab1p and eIF4E, Pab1p has been found to copurify with eIF4E in an eIF4G-dependent manner and coimmunoprecipitate with eIF4E from yeast extracts (Tarun and Sachs, 1996). However, in both of these earlier studies the complexes were purified from crude extracts. Therefore, they did not address the possibility that unidentified factors in addition to eIF4G were required to mediate the eIF4E/Pab1p interaction. These earlier studies also did not directly address the prediction that eIF4G could circularize an mRNA through simultaneous interaction with the 5′ cap/eIF4E and 3′ poly(A) tail/Pab1p complexes. Here, we report the reconstitution of the eIF4E/eIF4G/Pab1p complex using recombinant yeast proteins and the direct visualization of the predicted circular RNA resulting from the interactions of this complex with capped, polyadenylated RNA.

Results

To reconstitute the eIF4E/eIF4G/Pab1p complex, we fused amino acids 188–516 of eIF4G1, which contains only the Pab1p and eIF4E binding sites (Figure 1A, shaded region), to glutathione-S-transferase (GST) to yield GST-4G1$_f$. This fragment is missing the RNA binding domain of eIF4G, and therefore, its use eliminates possible complications in our interpretations that could result from

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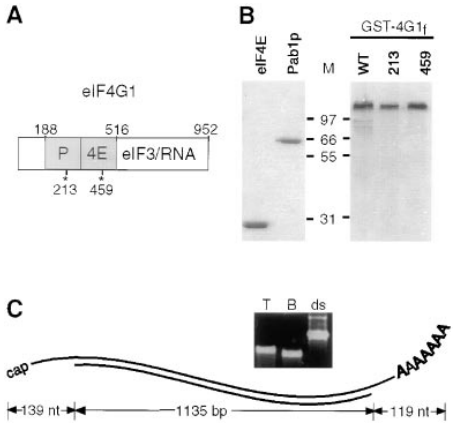

Figure 1. Purified Components Used for Reconstitution of Protein–RNA Complexes

(A) Schematic diagram of yeast eIF4G1. The fragment of eIF4G1 used in these studies as a glutathione-S-transferase fusion protein (GST-4G1$_f$) is shaded. The locations of the binding sites for Pab1p (P), eIF4E (4E), the putative binding sites for eIF3 and RNA (Sachs et al., 1997), and the eIF4G1–213 and −459 mutations (*) are indicated.

(B) Approximately 1 μg of the purified eIF4E, Pab1p, and GST-4G1$_f$ proteins were resolved by SDS–PAGE and visualized by Coomassie brilliant blue staining. The mass of each molecular weight standard in kilodaltons is indicated (M).

(C) Schematic diagram of the dsRNA. The 1392 nucleotide (nt) top strand of the dsRNA species contains a cap structure and a 98 nt poly(A) tail. A complementary bottom strand anneals to this resulting in a 1135 base pair (bp) dsRNA species with 139 and 118 nt single-stranded extensions in the 5′ and 3′ ends, respectively.

(Inset) Purified single- and double-stranded RNA. Approximately 1 μg of the top (T) and bottom (B) single-stranded and 0.5 μg of the dsRNAs were resolved on a 1% agarose-TBE gel and visualized by ethidium bromide staining.

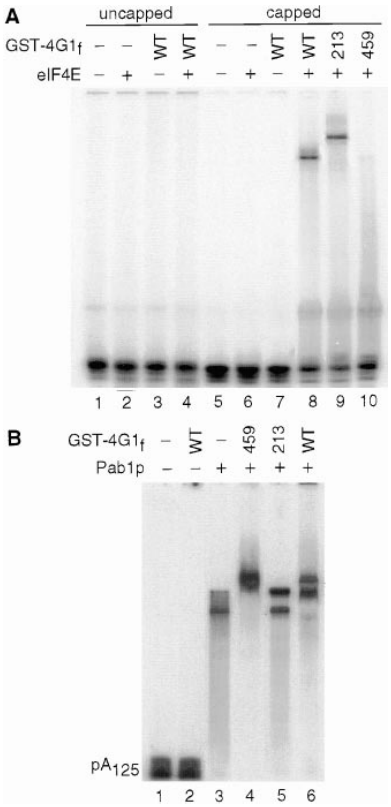

Figure 2. Pab1p and eIF4E Interact with the GST-4G1$_f$ Fragment

(A) Gel mobility shift analysis of capped RNA/eIF4E/GST-4G1$_f$ complexes. Radiolabeled capped RNA was incubated with eIF4E and/or the indicated GST-4G1$_f$ protein. Complexes were resolved by electrophoresis on nondenaturing acrylamide gels and visualized by autoradiography.

(B) Gel mobility shift analysis of poly(A)/Pab1p/GST-4G1$_f$ complexes. Radiolabeled poly(A)$_{125}$ was incubated with Pab1p and/or the indicated GST-4G1$_f$ protein. Complexes were resolved and visualized as in (A).

nonspecific interaction of the full-length protein with RNA. We also constructed eIF4G1 fragments containing point mutations [GST-4G1$_f$-213 (KLRK$_{213–216}$ to AAAA$_{213–216}$) and GST-4G1$_f$-459 (LL$_{459,460}$ to AA$_{459,460}$)] that have been previously shown to diminish either Pab1p binding (Tarun et al., 1997) or eIF4E binding (Tarun and Sachs, 1997) in the full-length eIF4G1, respectively.

The abilities of the various GST-4G1$_f$ proteins to interact with either recombinant eIF4E or Pab1p (Figure 1B) were tested using a gel mobility shift assay (Figure 2). To determine if the different GST-4G1$_f$ proteins could interact with eIF4E, we used either an uncapped (lanes 1–4) or capped (lanes 5–10) labeled RNA (Figure 2A). No complexes were formed with the uncapped transcript (lanes 1–4) or with eIF4E and a capped transcript (lane 6). GST-4G1$_f$ alone also did not bind to the capped RNA probe (Fig 2A, lane 7). We were, however, able to observe an eIF4E-dependent complex in the presence of GST-4G1$_f$ (lane 8). GST-4G1$_f$-213 also formed a complex with eIF4E and the capped RNA (lane 9). The decreased mobility of this complex is possibly the result of the change of 3 charged residues to uncharged residues in the GST-4G1$_f$-213 mutant (Tarun et al., 1997). In contrast, a complex of eIF4E and capped RNA with GST-4G1$_f$-459, which contains mutations in the eIF4E binding domain, was only barely detectable (lane 10). The presence of very low amounts of complex with GST-4G1$_f$-459 (lane 10) indicates that this protein binds very weakly to eIF4E and capped RNA under these assay conditions. We conclude from this assay that the GST-4G1$_f$ fragment interacts with eIF4E in a manner analogous to full-length eIF4G.

Why we were unable to visualize the formation of a complex between eIF4E and the capped RNA by the gel mobility shift assay is unclear. The concentration of eIF4E used in the assay (3 μM) exceeds the predicted K_d (1.0 μM) of its mammalian homolog for binding to capped RNA (Minich et al., 1994). The eIF4E used in our studies is not defective for cap binding as it was purified on a cap-analog column, and maintains the ability to bind the cap structure at the lower concentrations used for visualizing the circular RNA (see below). We conclude that the conditions of the gel shift assay either disrupt or are unable to resolve the eIF4E/cap complex.

To determine if the various GST-4G1$_f$ proteins could interact with the Pab1p/poly(A) RNP, an end-labeled RNA containing 125 adenylate residues was used in the mobility shift assay (Figure 2B). The wild-type GST-4G1$_f$ and the GST-4G1$_f$-459 proteins were both able to supershift the Pab1p/poly(A) complexes (lanes 6 and 4). In contrast, the GST-4G1$_f$-213 protein, which contains

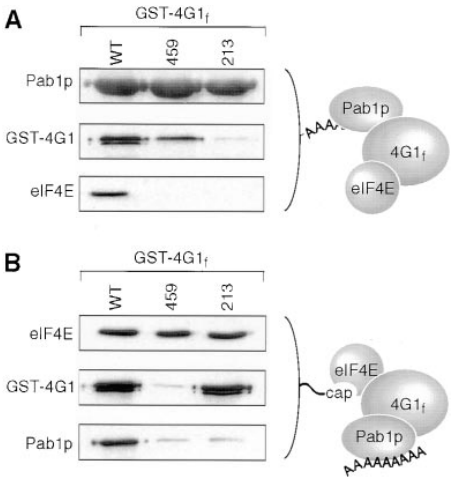

Figure 3. Reconstitution of the eIF4E/GST-4G1$_f$/Pab1p Complex

(A) Reconstitution with Pab1p immobilized on poly(A)-Sepharose resin. Pab1p bound to poly(A)-Sepharose resin was incubated with the indicated GST-4G1$_f$ proteins and eIF4E. Bound proteins were eluted with buffer containing SDS, resolved by SDS–PAGE, and visualized by Western analysis with the appropriate antibodies.

(B) Reconstitution with eIF4E immobilized on the cap-analog resin ^{7m}GDP-agarose. eIF4E bound to the resin was incubated with the indicted GST-4G1$_f$ proteins and Pab1p/poly(A)$_{125}$ complexes. Bound proteins were analyzed as in (A).

mutations in the Pab1p binding domain, did not supershift the mobility of the Pab1p/poly(A) complexes (lane 5). Similar mobility shifts were observed with a poly(A)$_{50}$ probe (data not shown), suggesting that the eIF4G/Pab1p interaction can occur with poly(A) tails of physiologic lengths (Sachs, 1990). In combination with the above mobility shift data using capped RNA, these data support the hypothesis that the GST-4G1$_f$ protein interacts with eIF4E and Pab1p with the same specificity that has been observed for the full-length eIF4G1 protein.

Having established the specificity of the individual interactions, we next sought to determine whether eIF4E and Pab1p could simultaneously interact with GST-4G1$_f$ by using either eIF4E or Pab1p affinity resins. In these experiments, eIF4E bound to the cap analog resin ^{7m}GDP-agarose or Pab1p bound to poly(A)-Sepharose was incubated with each of the various GST-4G1$_f$ proteins and either soluble Pab1p in the presence of poly(A) or eIF4E. Following extensive washing and then elution of the bound proteins from the resin, each protein in the eluate was detected by Western analysis (Figure 3). We found that eIF4E bound to the Pab1p/poly(A) complex when GST-4G1$_f$ was used, but not when either GST-4G1$_f$-213 or GST-4G1$_f$-459 was used (Figure 3A). Similarly, Pab1p bound to the eIF4E/^{7m}GDP complex when GST-4G1$_f$ was used, but not when either GST-4G1$_f$-213 or GST-4G1$_f$-459 was used (Figure 3B). These data support the conclusion that the eIF4E/eIF4G/Pab1p complex can be reconstituted in a specific manner with the purified recombinant proteins. These results also indicate that mutations that disrupt the individual interactions between eIF4E or Pab1p with eIF4G also disrupt the corresponding interaction within the eIF4E/eIF4G/Pab1p complex. Similar results were obtained using each of the eIF4G1 fragments after cleavage with thrombin protease to remove GST (data not shown).

Having demonstrated that GST-4G1$_f$ can bind specifically and simultaneously to Pab1p and eIF4E, we next wanted to test the prediction that these interactions could result in circularization of RNA. Complexes were visualized using atomic force microscopy (AFM), which allows for the imaging of biological samples in near physiological conditions without the need to fix the sample. Visualization of single-stranded RNA in fluid presented several technical problems. These included poor binding of the single-stranded RNA to mica, as has been previously observed (Kasas et al., 1997), and the propensity of single-stranded RNA to form extensive intramolecular structure that results in an observed globular appearance. To overcome these problems and to provide an easily visualized linear RNA molecule for imaging, we constructed a partially double-stranded (ds) RNA with single-stranded overhangs consisting of a capped 5′ end and a polyadenylated 3′ end (Figure 1C). The length of the dsRNA region (1.1 kb) was chosen to optimize the probability of the two ends of the RNA coming in contact with each other (Shore and Baldwin, 1983). The presence of single-stranded RNA overhangs allowed for unobstructed protein binding to the ends of the molecule.

Complexes were assembled using the components described in Figure 1, and images of unfixed nucleic acid–protein complexes were collected in fluid using tapping mode AFM (Hansma et al., 1994). Each image was generated by movement of the probe in a single direction. In some instances, this results in masking of the area where the RNA intersects with the protein as a result of the shadow of the large protein complex. In these cases, it was difficult to see that the circle was complete. To overcome this problem, we generated two separate images for each field of view by scanning in opposite directions. Areas that were in shadow in one image were clearly visible when scanned from the opposite direction.

Circular RNA molecules were visualized with this technique (Figure 4). Imaged molecules were categorized as being either circular or linear by visual inspection of a series of images from two or more experiments (Figure 4 and Table 1). Molecules were counted only if they appeared to contain a single RNA of the correct length. Primarily linear molecules (>93%) were observed in the absence of any of the proteins (Figure 4A), in the presence of just Pab1p and GST-4G1$_f$ (Figure 4B), or in the presence of Pab1p, eIF4E, and GST-4G1$_f$-213 (Figure 4C). In contrast, a high percentage (54%) of circular molecules was observed when Pab1p and eIF4E were mixed with the GST-4G1$_f$ (Figures 4E and 4F). The AFM images show a complex of proteins bound to each circle, although individual protein components could not be discerned. The observed number of circles possibly underestimates the efficiency of circularization for at least two reasons. First, a fraction of the capped transcripts (approximately 30%) are predicted to carry the cap in the inverted orientation (Pasquinelli et al., 1995), and this may render them unavailable for interaction with eIF4E. Second, a small fraction of the RNAs that most

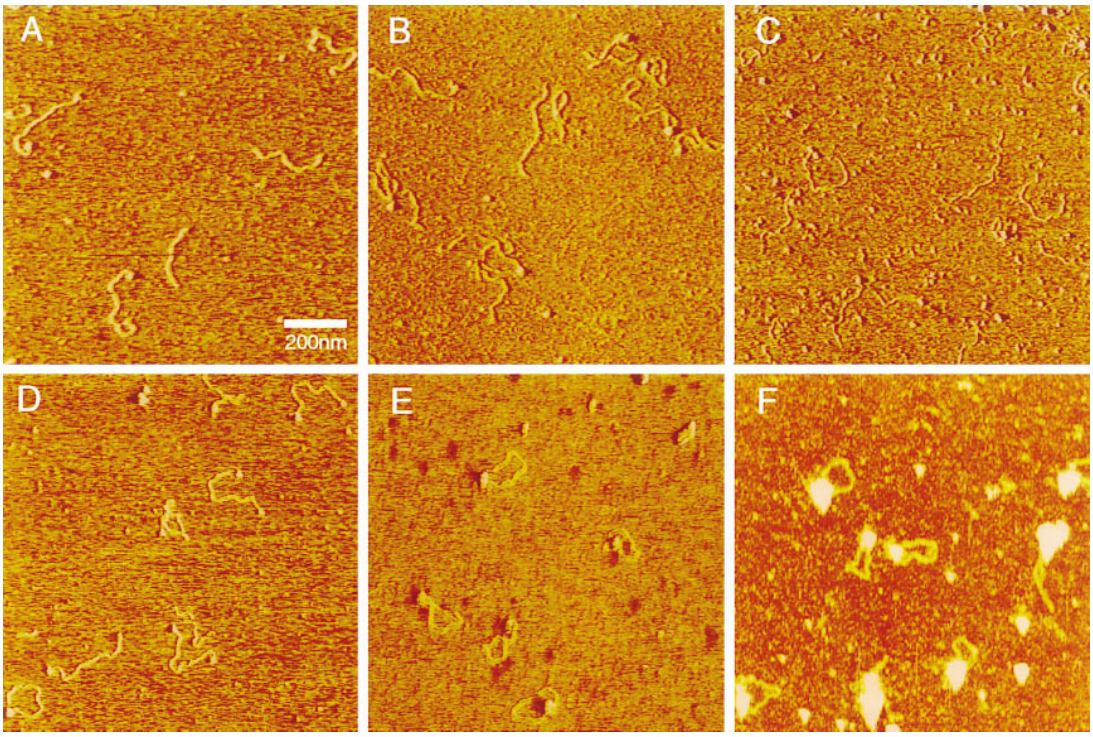

Figure 4. Visualization of Circular RNA/Protein Complexes by Atomic Force Microscopy

Representative AFM images of complexes formed on the capped, polyadenylated dsRNA with the indicated proteins. (A) The dsRNA in the absence of protein appears as 300 nm elongated structures. In the presence of GST-4G1$_f$ and Pab1p (B) or GST-4G1$_f$-213, Pab1p, and eIF4E (C), the dsRNA also appears elongated. If the dsRNA is incubated with GST-4G1$_f$-459, Pab1p, and eIF4E (D), most of the dsRNA is linear although some circles are observed (lower left corner). In the presence of GST-4G1$_f$, Pab1p, and eIF4E (E and F), much of the dsRNA is in a circular conformation with a protein complex positioned where the ends of the RNA meet. Differences in the apparent width of the RNA result from variation in the tip and imaging force used to generate each image. (A)–(E) were generated using deflection mode; (F) was generated using height mode.

likely represented collapsed circles could not be accurately scored as positives.

Interestingly, circularization was also observed in almost 25% of the molecules when the GST-4G1$_f$-459 mutant was used (Figure 4D). While we did not observe any interaction between this mutant and eIF4E with the affinity resin (Figure 3), this mutant did exhibit partial complex formation in the gel mobility shift assay (Figure 2A). This may indicate differences in the stringency of the assays. Previous characterization of this mutant also argues that it is partially functional in vivo and in vitro. Yeast strains carrying this mutant eIF4G have a temperature-sensitive growth phenotype that can be suppressed by overexpression of eIF4E (Tarun and Sachs, 1997).

Table 1. Quantification of Circular Complexes Formed on dsRNA by Various Combinations of Proteins and Visualized by AFM

eIF4E	Pab1p	GST-4G1$_f$	Circular (%)	Linear (%)	Total
–	–	–	5.8	94.2	139
–	+	WT	6.6	93.4	166
+	+	213	3.4	96.6	183
+	+	459	24.4	75.6	131
+	+	WT	53.8	46.2	210

Individual molecules were assigned as being either linear or circular by visual inspection. Molecules were counted only if they appeared to contain a single RNA of the correct length.

Deficiencies in extracts containing this mutant protein can also be rescued by the addition of excess recombinant eIF4E (Tarun and Sachs, 1997). These data indicate that the interaction of GST-4G1$_f$-459 with eIF4E is weakened but probably not completely destroyed. In summary, these data show that AFM can be used to visualize RNA–protein complexes in solution. More specifically, the high frequency of circular RNA observed in the presence of the eIF4E/GST-4G1$_f$/Pab1p complex, but not in the presence of a subset of these proteins or with mutant GST-4G1$_f$ proteins, lends strong support to the conclusion that this complex of translation initiation factors can circularize capped, polyadenylated mRNA.

Discussion

We have demonstrated the reconstitution of the eIF4E/GST-4G1$_f$/Pab1p complex and have shown that it is sensitive to mutations that disrupt the individual interactions between eIF4G and eIF4E or Pab1p. We have also visualized circular RNAs that are induced by this complex in solution using AFM. These data provide strong physical evidence for the hypothesis that mRNA can be circularized by translation initiation factors.

Our reconstitution of RNA circles with the eIF4E/eIF4G1/Pab1p complex does not rule out the possibility

that other proteins contribute to the formation or stabilization of circular mRNA in vivo. Additional proteins may stabilize the interactions between eIF4E, eIF4G, and Pab1p. There may also be other RNA-binding proteins that stabilize interactions between either end of the mRNA and the eIF4E/eIF4G/Pab1p complex. Such interlocking interactions could account for why mutations that diminish the interactions within the eIF4E/eIF4G/Pab1p complex in vitro do not lead to cell lethality (Tarun and Sachs, 1997; Tarun et al., 1997). It also remains formally possible that mRNA circularization is not an essential feature of translation. The ability of these interactions to circularize mRNA in vivo remains to be addressed.

We have shown by direct visualization that the eIF4E/GST-4G1$_f$/Pab1p complex has the ability to circularize a capped, polyadenylated RNA. The unique characteristics of a circular structure could lead to novel functional consequences. Circularization could induce changes within some of the translation factors that increase their ability to stimulate subsequent steps in the initiation process. For instance, the eIF4E/eIF4G/Pab1p complex may have a higher affinity than eIF4G alone for the 40S ribosomal subunit. It may also have a greater capacity to stimulate the activity of other translation factors such as eIF4A. Circularization could also function as a proofreading mechanism that enhances the recognition of intact and correctly processed mRNA by the translation machinery, since only mRNAs with both a 5′ cap and 3′ poly(A) tail would form circles. Additional regulatory complexity may also be introduced to translation by circularization. Regulatory proteins bound to the 5′ or 3′ untranslated regions (UTR) of mRNA could act by disrupting or enhancing circularization. Recycling of translation factors may also occur more efficiently when mRNA is circularized. Since the proteins involved in RNA circularization in this study are found in all animals and plants, this feature of RNA topology could serve highly conserved functions in translation initiation in all eukaryotes.

Experimental Procedures

Protein Purification

Fragments of the *TIF4631,tif4631–213* or *tif4631–459* genes (Tarun and Sachs, 1997; Tarun et al., 1997) encoding amino acids 188–516 were amplified with the polymerase chain reaction (PCR), subcloned as BamH1/EcoR1 fragments into pGEX2T (Pharmacia) to produce pAS570, pAS571, and pAS572, respectively, and placed into bacterial strain BL21 to yield strains BAS3252, BAS3253, and BAS3254. The resulting proteins are fused at their N termini to GST and contain at their C termini a hexahistidine tag. IPTG-induced fusion proteins from bacterial extracts prepared in buffer A [phosphate-buffered saline (PBS), 0.1% Triton X-100] from 500 ml of cell culture were batch absorbed to 1 ml of ProBond nickel-agarose (Invitrogen) preequilibrated with buffer A containing 5 mM imidazole. Following washing with two 5 ml aliquots of buffer A containing 20 mM imidazole, and again with buffer A containing 40 mM imidazole, proteins were eluted in 5 ml of buffer A containing 250 mM imidazole, 1 mM PMSF. Eluted proteins were diluted to 10 ml with buffer A and then batch absorbed to 1 ml of glutathione-Sepharose 4B resin (Pharmacia) preequilibrated in buffer A. Following washing with 10 ml of buffer A, proteins were eluted with 2 ml of 20 mM reduced glutathione, 100 mM Tris (pH 8.0), 150 mM KCl, 10% glycerol. After dialysis against buffer B [100 mM KCl, 10 mM HEPES (pH 7.4), 0.2 mM EDTA, 1 mM PMSF], proteins were stored at −70°C. Recombinant Pab1p and eIF4E were purified as previously described (Sachs et al., 1987; Edery et al., 1988; Vasilescu et al., 1996), with the exception that the eIF4E was dialyzed against 1 M KCl, 20 mM HEPES (pH 7.4), 0.2 mM EDTA prior to final dialysis against buffer B.

RNA Synthesis

RNAs were transcribed with T3 RNA polymerase under conditions described by the manufacturer (Epicentre Technology) using either pAS575 linearized with BamH1 to synthesize the capped, polyadenylated 1392 nucleotide top strand or pAS576 linearized with Sph1 to synthesize the 1186 nucleotide complementary bottom strand. The transcripts were annealed to form a partially double-stranded RNA species by mixing equimolar amounts of RNA in buffer B and heating to 95°C for 2 min, and were then slow cooled to room temperature.

Mobility Shift Assays

For the eIF4E/GST-4G1$_f$ gel mobility shift assays, 30 pmol of eIF4E and of each GST-4G1$_f$ protein in a total of 6 μl was incubated with 25 fmol of radiolabeled probe and 2 μl of 5× binding buffer [50 mM Tris (pH 8.0), 3.5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 0.5 mM EDTA, 7.5% polyvinyl alcohol, 100 ng/μl tRNA] for 15 min at room temperature in a final volume of 10 μl. A final concentration of 3 μM was used for both eIF4E and eIF4G based on the association constant measured for nonphosphorylated eIF4E and m^7GpppG (4.3×10^5) (Minich et al., 1994). Complexes were separated on a 1.5 mm, 4% acrylamide-0.5× TBE native gel run for 3 hr at 12 V/cm. The radiolabeled 65 nt RNA probe was produced by in vitro transcription with T3 RNA polymerase using pAS575 polylinker as template in the presence or absence of cap analog followed by gel purification. The Pab1p/4G1 gel mobility shift assays were the same except that 3 pmol of Pab1p was used and the RNA was 5′-end-labeled poly(A)$_{125}$ prepared essentially as described in Deardorff and Sachs (1997). The different complexes observed in the gel mobility shift experiment represent different numbers of Pab1p bound to poly(A)$_{125}$.

Reconstitution of Complexes on Affinity Resins

50 μl of a 25% slurry of poly(A)-Sepharose resin was equilibrated in buffer A and incubated for 30 min with 150 pmol of Pab1p in 250 μl of buffer A. 200 pmol of each GST-4G1$_f$ was added in 10 μl and incubated for 45 min, and the resin was then washed with 5× 500 μl of buffer A. Following resuspension in 200 μl of buffer A with 200 pmol of eIF4E and incubation for 45 min, the resin was again washed with 4× 500 μl of buffer A. 30 μl of a 50% slurry of ^{7m}GDP-agarose resin was equilibrated in buffer A and incubated with 200 pmol of eIF4E in 250 μl of buffer A for 30 min. 200 pmol of each GST-4G1$_f$ was added to 10 μl and incubated for 45 min, and the resin was then washed with 5× 500 μl of buffer A. Following resuspension in 200 μl of buffer A with 125 pmol of Pab1p and 20 pmol of poly(A)$_{125}$ and incubation for 45 min, the resin was again washed with 4× 500 μl of buffer A. All incubations were done at 4°C with end-over-end rotation. Bound proteins in both experiments were detected by Western analysis as in Tarun and Sachs (1996). The antibodies for eIF4E and Pab1p have been previously described (Anderson et al., 1993; Tarun and Sachs, 1997). Rabbit antibodies were raised against GST-4G1$_f$ using standard protocols and used at a dilution of 1:2000.

Microscopy

Protein–dsRNA complexes were formed by mixing 200 pmol of GST-4G1$_f$ and eIF4E with 15 pmol of Pab1p and 5 pmol of dsRNA in a final volume of 50 μl of buffer B. After a 15 min incubation at room temperature, RNA–protein complexes were separated from unbound proteins by passage through a 1 ml Sepharose CL-4B column (8 mm diameter, Bio-Rad) equilibrated in buffer B at room temperature. Approximately 200 μl fractions were collected by gravity, and 10 μl of each was analyzed by electrophoresis on 1% agarose-0.5× TBE gels to determine which contained the dsRNA. Aliquots of these fractions were diluted 10-fold into a buffer to yield a solution containing 50 mM KCl, 10 mM HEPES (pH 7.4), 15 mM $NiCl_2$, 20 μM EDTA, and then 150 μl was immediately applied to mica in a fluid cell. The mica had been pretreated for 30–60 min with a solution of 0.1N HCl, 20 mM $NiCl_2$, rinsed with water, and air dried just prior to binding of the sample. AFM images (nonoverlapping 2 m^2 fields)

were collected with a NanoScope IIIa (Digital Instruments, Santa Barbara, CA) operated in tapping mode with commercial NP-S silicon nitride tips (Digital Instruments). Images were processed by flattening.

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