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Translation factors promote formation of two states of the closed loop mRNP

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Abstract

Efficient translation initiation and optimal stability of most eukaryotic mRNAs depends on the formation of a closed loop structure and the resulting synergistic interplay between the 5' m⁷G cap and the 3' poly(A) tail^{1,2}. Evidence of eIF4G and Pab1p interaction supports the notion of a closed loop mRNP³, but the mechanistic events that lead to its formation and maintenance are still unknown. Here we have used toeprinting and polysome profiling assays to delineate ribosome positioning at initiator AUG codons and ribosome:mRNA association, respectively, and find that two distinct stable (cap analog resistant) closed loop structures are formed during initiation in yeast cell-free extracts. The integrity of both forms requires the mRNA cap and poly(A) tail, as well as eIF4E, eIF4G, Pab1p, and eIF3, and is dependent on the length of both the mRNA and the poly(A) tail. Formation of the first structure requires the 48S ribosomal complex whereas the second requires an 80S ribosome and the termination factors eRF3/Sup35p and eRF1/Sup45p. Surprisingly, the involvement of the termination factors is independent of a termination event.

Keywords

Yeast; mRNA circularization; translation initiation; termination factors

In vitro translation reactions utilized synthetic mRNAs derived from yeast transcripts, extracts that recapitulate cap/poly(A) tail synergy (Supplementary Fig. 1a), competitive inhibition of translation initiation by m⁷GpppG (cap analog), and analyses of ribosome positioning or mRNA association by toeprinting and sucrose gradient sedimentation. Addition of the elongation inhibitor, cycloheximide (CHX), to translation reactions programmed by the 2135nt AAA and UAA mRNAs containing long or short ORFs, respectively, (Fig. 1a) allowed detection of CHX-dependent initiator AUG toeprints that reflect 80S ribosomes protecting 16–18 nt 3' of the AUG^{4–6} (Fig. 1b, top and middle panels, lanes 1 and 3). These toeprints were dependent on initiation codon recognition, the presence of yeast extract, and concurrent mRNA translation (Supplementary Fig. 1b and c). Supporting the latter conclusion, toeprints were almost completely eliminated by 2.7mM cap analog, a concentration that distinguished *bona fide* toeprints from background bands (Fig. 1b, top and middle panels, lanes 2 and 4). Lower cap analog concentrations also inhibited AUG toeprint accumulation, with 70% and 96% sensitivity obtained at 0.05mM and 0.5mM, respectively (Fig. 1b, lower panel). A shorter mRNA (miniUAA1, 488nt, Fig. 1a) also yielded the AUG toeprint (Fig. 1c, upper panel, lane 1), but this band was resistant to 2.7mM cap analog (lane 2) and only manifested sensitivity at higher concentrations (Fig. 1c, lower panel). Thus, in wild-type extracts, the short capped (see

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Supplementary Fig. 1d) and polyadenylated miniUAA1 mRNA is ~160-fold more resistant to cap analog than the longer AAA mRNA. The miniADE2 (485nt) and ADE2 (2070nt) mRNAs, whose respective sizes (but not sequences) are comparable to those of the miniUAA1 and AAA transcripts, also show the same results (Supplementary Fig. 2a). Consistent with the apparent mRNA size dependence of cap analog resistance *in vitro*, mRNAs with intermediate sizes (from 488nt to 2135nt), but with the same stability (Supplementary Fig. 2b), showed intermediate phenotypes, with approximately 40% sensitivity to 2.7mM cap analog obtained with an 882nt mRNA, 60% sensitivity with an 1105nt mRNA, and near maximal sensitivity reached with a 1336nt mRNA (Fig. 1d).

To rule out cap-independent translation as the basis for cap analog resistance, we translated and toeprinted a polyadenylated miniUAA1 mRNA with no 5' cap. This mRNA, which is stable during translation (Supplementary Fig 2c), initiates inefficiently in wild-type extracts (Fig. 2a, lane 1), strongly suggesting that translation of our reporter mRNAs is cap-dependent. As reported previously^{7,8} the addition of cap analog stimulates translation of uncapped mRNA (Fig. 2a, lane 2), possibly because it titrates other inhibitors. Collectively, these data suggest that interactions between the eIF4F cap-binding complex and the 5' m⁷G cap of the mini mRNAs are much stronger than those occurring with the long mRNAs.

Short mRNAs may be preferentially resistant to cap analog because they form a more stable closed loop mRNP than long mRNAs, possibly by promoting increased affinity of mRNP factors for each other or for mRNA structures such as the 5' cap^{3,9-11}. To test the relationship of the closed loop state to m⁷GpppG resistance, we analyzed the toeprinting of capped, poly(A)⁻ miniUAA1 mRNA. This transcript showed strong sensitivity to cap analog (Fig. 2b, lane 2). Further analyses of miniUAA1 mRNAs having different poly(A) tail lengths (but identical stabilities; Supplementary Fig 2d) showed that miniUAA1 mRNA with a poly(A) tail of 14 or 18As (Figure 2c, lanes 1-4) displayed a phenotype similar to poly(A)-deficient mRNA (>95% sensitivity to 2.7mM cap analog). Increasing poly(A) tail length to 22, 25, or 33 residues yielded an intermediate phenotype (~80% sensitivity to cap analog; lanes 5-10), whereas a miniUAA1 mRNA with 57 As showed no sensitivity to 2.7mM cap analog (lanes 11 and 12). These results correlate cap analog resistance with poly(A) length, a result consistent with closed loop formation. Since Pab1p:poly(A) association requires a minimum of 12 adenosines, and multiple Pab1p molecules can bind the same poly(A) tract in a 27nt repeating unit¹²⁻¹⁴, it appears that at least two Pab1p molecules are required for a stable closed loop structure. Consistent with this conclusion, previous studies showed that an A₁₅ tail did not suffice to stimulate translation in *Drosophila*¹⁵ and mammalian¹⁶ extracts, but that longer poly(A) tails promoted strong translational enhancement.

Toeprinting of miniUAA1 mRNA after translation in Pab1p-deficient (*pab1Δ/pbp1Δ*) extracts reinforced the notion of a critical role for Pab1p in cap analog resistance. Translation initiation in these extracts was highly sensitive to cap analog (Fig. 3a, lanes 3 and 4; compare to extracts from wild-type [lanes 1 and 2] or *pbp1Δ* [a *pab1Δ* suppressor] cells [lanes 5 and 6]), and the apparent 80-fold increase in cap analog sensitivity (Fig. 1c, lower panel, and Supplementary Fig. 5b, upper panel) was directly attributable to the absence of Pab1p because supplementation with 15pmole of recombinant Pab1p, but not the same amount of BSA, restored cap analog resistance to wild-type levels (Supplementary Fig. 5a). While Pab1p is essential for cap analog resistance, its level must be well balanced since excess Pab1p, i.e., the addition of 15pmole Pab1p to wild-type extracts (lanes 3 and 4) or 38pmole Pab1p to *pab1Δ/pbp1Δ* extracts (data not shown), reduces the extent of cap analog resistance.

To further evaluate the relationship between cap analog resistance and closed loop formation, we determined whether the same regions of Pab1p were required for both phenomena. Extracts derived from *pab1-134* cells, where Pab1p has wild-type affinity for eIF4G1 but reduced

affinity for eIF4G2¹⁷ (yeast has two eIF4G isoforms encoded by the functionally redundant *TIF4631* and *TIF4632* genes, respectively¹⁸), showed a wild-type phenotype (Fig. 3a, lanes 7 and 8). However, toeprinting analyses in *pab1-184* extracts, in which Pab1p does not bind to eIF4G1 or eIF4G2¹⁷, revealed a strong sensitivity to cap analog (Fig. 3a, lanes 9 and 10). Similarly, extracts of *pab1-ΔRRM1* cells, in which Pab1p has lost the ability to interact with the poly(A) tail, and probably with either isoform of eIF4G as well¹⁹, also exhibit strong sensitivity to cap analog (Fig. 3a lanes 11 and 12), but not to the same concentration of GTP (Supplementary Fig. 2e). Both *pab1-184* and *pab1-ΔRRM1* extracts show about 10-fold more sensitivity to cap analog than those obtained from cells lacking the *PAB1* gene (Supplementary Fig. 5b), possibly indicating dominant-negative effects of the mutant proteins. To determine whether Pab1p interaction with the termination factor eRF3/Sup35p²⁰ is also required for cap analog resistance, we analyzed extracts of *pab1-ΔC-term* cells. Figure 3a (lanes 13 and 14) shows that this mutation also confers cap analog sensitivity, albeit 2.5-fold less sensitivity than that observed in *pab1Δpbp1Δ* extracts (Supplementary Figs. 5b,d). In addition, the pattern of toeprint inhibition with the *pab1-ΔC-term* extract appears different from that seen with other *pab1* mutants (Supplementary Figs. 5b,d), suggesting that the Pab1p C-terminal domain may be involved in a step distinct from that involving the other domains. All of the *pab1* mutant extracts show full sensitivity to cap analog when programmed with poly(A)⁻ miniUAA1 mRNA (Supplementary Fig. 2f), but recombinant Pab1p could not complement the phenotypes of these extracts, further suggesting dominant-negative effects of the mutated proteins (data not shown).

The differences in cap analog sensitivity of the different *pab1* extracts are consistent with the loss of interactions characteristic of specific Pab1p domains²¹ being involved in closed loop mRNA formation and stabilization. Accordingly, we analyzed miniUAA1 mRNA toeprints in extracts from strains harboring mutations in genes encoding different Pab1p-interacting proteins. Extracts of *eIF4G1-ΔN300* cells¹⁷, in which there is no eIF4G2 and Pab1p:eIF4G1 interaction is disrupted, show strong sensitivity to cap analog and correlate well with the cap analog sensitivity phenotype of *pab1-184* extracts (Fig. 3b and Supplementary Fig. 5c). Likewise, extracts harboring mutated eRF3 (*sup35-R419G*)²² or eRF1 (*sup45-2*)²³ termination factors show both sensitivity to cap analog (but not to GTP [Supplementary Fig. 2e]) and toeprint patterns that are strikingly similar to those obtained with the *pab1-ΔC-term* extract (Fig. 3c and Supplementary Fig. 5d), with the exception that they also yield two extra bands 5' of the AUG toeprint that are suggestive of initiation anomalies (see Supplementary Data and Supplementary Fig. 3a). Control reactions demonstrated that the termination mutant extracts were fully sensitive to cap analog when programmed with poly(A)⁻ miniUAA1 mRNA (Supplementary Fig. 2g) and that the addition of extra Mg²⁺ to the different translation reactions (to compensate for a possible titration by cap analog) had no effect on their toeprint phenotypes (Supplementary Fig. 2h). These results, and the observation that toeprinting of miniUAA1 mRNA after translation in extracts defective in eIF3 and eIF4E also exhibited more sensitivity to cap analog than did wild-type extracts (Supplementary Fig. 4), imply that cap analog resistance results from formation of a stable closed loop structure.

Further insights into closed loop formation followed from experiments using extracts supplemented with mutant transcripts or different competitive inhibitors, or independent analytical methods. First, we evaluated whether the role of eIF4G:PABP:eRF3 interactions in closed loop formation (and, possibly, ribosome recycling²⁴), was also dependent on translation termination²⁴. We prepared a mini mRNA with no stop codon and analyzed its response to cap analog in extracts derived from wild-type or termination-defective cells. Translation of nonstopminiUAA1 in wild-type extracts showed strong cap analog resistance (Fig. 3d, lanes 1 and 2), suggesting that conventional termination steps are not required for a stable closed loop mRNP. In contrast, *sup35-R419G* and *sup45-2* extracts programmed with nonstopminiUAA1 RNA exhibit strong sensitivity to cap analog (Fig. 3d, lanes 3-6), indicating

that, while termination *per se* is not required, *de novo* formation of a stable closed loop structure in yeast is dependent on the principal termination factors. Second, since there was no requirement for termination, we considered the possibility that closed loop formation precedes the first round of mRNA translation and thus analyzed the toeprinting of miniUAA1 mRNA after two-minute time courses of translation in wild-type or Pab1p-mutant extracts. In wild-type extracts, cap analog-resistant AUG toeprints were obtained within 30 sec of incubation with miniUAA1 mRNA. Similarly, in *pab1-184* and *pab1-ΔRRM1* extracts, sensitivity to cap analog was observed at the same time point (Figs. 3e,f). Third, to monitor mRNA:ribosome association by an independent method, we utilized sucrose gradient fractionation to analyze the translation of miniUAA1 mRNA. Figure 3g shows that, in the absence of cap analog, 60-70% of miniUAA1 mRNA is associated with the polyribosomal fractions whereas, in the presence of cap analog, most of the miniUAA1 mRNA in the *pab1-ΔRRM1*, *sup35-R419G*, and *sup45-2* extracts associated with the non-polysomal fractions. In contrast, the mRNA translated in the wild-type extract showed the same distribution as in the absence of the drug. Taken together, these data provide additional evidence that translation of capped and polyadenylated miniUAA1 mRNA is resistant to cap analog in wild-type extracts.

The previous observations are consistent with the notion that interactions between the mRNP 5' and 3' ends are established early in the translation process (Figs. 3d-f). Hence, we sought to identify the step of translation initiation associated with closed loop formation. The toeprinting assays described above utilized CHX to stabilize the 80S ribosome on the initiator AUG⁴ and thus monitored a late step in initiation. To determine whether closed loop mRNP formation occurred earlier, we analyzed mRNA toeprints in extracts supplemented with the non-hydrolyzable GTP analog, GMP-PNP⁴, a drug that blocks 48S to 80S conversion *in vitro*. In both wild-type and *pbp1Δ* extracts, translation of miniUAA1 mRNA in the presence of GMP-PNP gave the same results observed with CHX, namely, full resistance to 2.7mM cap analog (Fig. 4a, lanes 1, 2 and 5, 6). This result implies that steps essential to establishing the closed loop phenotype occur prior to or during formation of the 48S complex.

As observed with CHX toeprinting, cap analog sensitivity of miniUAA1 mRNA in the presence of GMP-PNP was dependent on a poly(A) tail (data not shown) and the availability of functional Pab1p and eIF4G (Figs. 4a [lanes 3, 4, 7-16] and 4b [lanes 5-8]). However, alterations in termination factor activity yielded different toeprint phenotypes. Extracts of termination factor mutants supplemented with GMP-PNP either at the beginning of the reaction (data not shown), or after 4 min of translation, were fully resistant to cap analog and lacked the upstream toeprints detected with CHX (Fig. 4c). Consistent with these results, sucrose gradient analyses of mRNA:ribosome association in extracts supplemented with GMP-PNP at the onset of the reaction showed no differences between the wild-type and the termination mutant extracts in the presence or absence of cap analog while the *pab1-ΔRRM1* extract manifested a phenotype characteristic of cap analog inhibition of translation initiation (Supplementary Fig. 5f). Thus, while eRF1 and eRF3 do not appear to affect the rate of translation initiation (Supplementary Fig. 3d) or the formation of the closed loop mRNP that includes only the 48S complex, they are required upon 80S formation to generate a second state of the closed loop structure.

The 5' cap and 3' poly(A) tail act synergistically to promote the stability and translatability of an mRNP²¹. Here we demonstrate that these mRNA appendages communicate as the first round of translation commences, establishing interactions involving at least 2 molecules of poly(A)-associated Pab1p, the initiation factors eIF4G, eIF4E, and eIF3, and the termination factors eRF1 and eRF3. Two forms of the stable closed loop structure can be distinguished during initiation on short but not long mRNAs *in vitro*. The first requires the preinitiation 48S complex but not eRF1 and eRF3, whereas the second is formed after 60S joining and requires the two eRFs as well as all the other components of the first structure. The restriction of efficient

in vitro closed loop formation to short mRNAs suggests that other factors facilitating this process may be absent or inactive in our cell-free system.

METHODS SUMMARY

Synthetic mRNAs of different sizes or sequence were translated in cell-free extracts derived from wild-type yeast cells or from mutant strains lacking the activity of specific translation factors. The elongation inhibitor, cycloheximide, or the non-hydrolyzable GTP analog, GMP-PNP, were used to trap 80S or 40S ribosomes at initiator AUG codons, and primer extension inhibition (toeprinting) assays were used to delineate the specific positions of those ribosomes on the different mRNAs. Polysome profiling assays, employing sucrose gradient fractionation of translation reactions and subsequent northern blotting of gradient fractions, were used to assess ribosome:mRNA association. The cap analog, m⁷GpppG, was used in all experiments as a competitive inhibitor of translation initiation.

METHODS

Synthetic mRNAs

Synthetic, capped poly(A)-containing miniUAA1, UAA and AAA mRNAs were transcribed *in vitro* from chimeric genes cloned in a pSP65A vector that included ~65 dT residues for transcription of a poly(A) tail⁵. mRNAs of intermediate lengths originated from truncated DNA constructs derived from pSP65A-CAN1/LUC⁵ by deletion of the LUC ORF from *Pst* I restriction sites (created by site-directed mutagenesis; Stratagene). mRNAs differing in poly(A) tail length were synthesized from *miniUAA1* DNA cloned in pSP65A vectors that contain the corresponding lengths of dT residues¹⁶. The construct utilized for synthesis of long ADE2 mRNA was generated in a one-step PCR amplification, using *EcoR* I site-containing oligonucleotide #165 (which also contains mutations of two upstream AUGs) (5'-CGGAATTCATTATAGAGCATTTCATATATAAATTGGTGCCTAAAATCGTTGGATCTCTC-3') and *BamH* I site-containing oligonucleotide #164 (5'-GAGGATCCAAATTCTTAAAAAAGGACACCTGTAAGCGTTG-3') and cloned into the *EcoR* I and *BamH* I restriction sites of the pSP65A vector. The *miniADE2* DNA construct was generated by deletion of the 1575nt fragment from a *Pst* I restriction site 260-265 nt downstream of the start of the construct and a *Pst* I site immediately 5' to the normal stop codon (both sites were created by site-directed mutagenesis). All plasmid constructions were confirmed by DNA sequencing. Capped and polyadenylated RNAs were synthesized with the SP6 mMessage mMachine kit (Ambion), according to the manufacturer's protocol, from *Hind* III-linearized plasmids for poly(A)-containing mRNA or from *Cla* I linearized plasmids for mRNA lacking the poly(A) tail. At least 80% of *in vitro* transcribed mRNAs were capped, as determined by immunoprecipitation using anti-2,2,7-trimethylguanosine agarose conjugate (Calbiochem). Uncapped mRNA was synthesized using the MEGascript kit (Ambion). RNA yields were quantified by spectrophotometry and their integrities were assessed by agarose gel electrophoresis.

Extracts and translation reactions

Saccharomyces cerevisiae strains²⁶⁻³⁰ (Supplementary Table 1) were used to make extracts for *in vitro* translation by techniques described previously⁶. Wild-type strains from different genetic backgrounds gave similar results. Translation and toeprinting assays were essentially the same as those described before⁵. Unless indicated otherwise, translation reactions were incubated for 4 min with 0.06 pmol of each RNA substrate, and terminated by incubation for 3 min with 2mM cycloheximide or GMP-PNP. Where indicated, translation initiation was subjected to competitive inhibition by 2.7 mM cap analog (m⁷GpppG), a concentration that readily distinguishes initiation complexes that are, respectively, sensitive or resistant to this

compound. Toeprinting utilized two different primers that were complementary to their respective targets at comparable distances from the AUG of interest. Primer #3029 was used for mRNAs spanning 600nt (miniUAA2) to 2135nt (AAA) and primer #55 was used with miniUAA1 mRNA. Samples from translation reactions programmed with ADE2 and miniADE2 mRNAs were toeprinted with primer #172, complementary to the sequence CACTAAAGAATCTTCAAGTAAAACATCCC, and primer #3238, complementary to the sequence GGACTTCATACATAGAAATCAACG, respectively. For mRNA:ribosome association assays, the equivalent of four translation reactions were fractionated in an 11ml sucrose gradient (7-47%). The gradients were fractionated and scanned at 254 nm, and the resulting absorbance profiles were used to determine the position of the polysomal and non-polysomal fractions²⁵. RNA was extracted from each fraction and analyzed by northern blotting as described previously²⁵.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Jacobson, A. Translational Control. In: Hershey, JW.; Mathews, MB.; Sonenberg, N., editors. 451-480. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 1996.
2. Gallie DR. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev* 1991;5:2108–2116. [PubMed: 1682219]
3. Wells SE, Hillner PE, Vale RD, Sachs AB. Circularization of mRNA by eukaryotic translation initiation factors. *Mol. Cell* 1998;2:135–140. [PubMed: 9702200]
4. Dmitriev SE, Pisarev AV, Rubtsova MP, Dunaevsky YE, Shatsky IN. Conversion of 48S translation preinitiation complexes into 80S initiation complexes as revealed by toeprinting. *FEBS Lett* 2003;533:99–104. [PubMed: 12505166]
5. Amrani N, et al. A *faux* 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. *Nature* 2004;432:112–118. [PubMed: 15525991]
6. Wu C, Amrani N, Jacobson A, Sachs MS. The use of fungal in vitro systems for studying translational regulation. *Methods Enzymol* 2007;429:203–225. [PubMed: 17913625]
7. Tarun SZ Jr. Sachs AB. A common function for mRNA 5' and 3' ends in translation initiation in yeast. *Genes Dev* 1995;9:2997–3007. [PubMed: 7498795]
8. De Gregorio E, Preiss T, Hentze MW. Translational activation of uncapped mRNAs by the central part of human eIF4G is 5' end-dependent. *RNA* 1998;4:828–836. [PubMed: 9671055]
9. Sachs, A. Translational Control of Gene Expression. In: Sonenberg, N.; Hershey, JW.; Mathews, MB., editors. 447-465. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 2000.
10. Karim MM, et al. A mechanism of translational repression by competition of Paip2 with eIF4G for poly(A) binding protein (PABP) binding. *Proc Natl Acad Sci U S A* 2006;103:9494–9499. [PubMed: 16772376]
11. Christensen AK, Kahn LE, Bourne CM. Circular polysomes predominate on the rough endoplasmic reticulum of somatotropes and mammatropes in the rat anterior pituitary. *Am. J. Anat* 1987;178:1–10. [PubMed: 3825959]
12. Baer BW, Kornberg RD. The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. *J. Cell. Biol* 1983;96:717–721. [PubMed: 6833379]

13. Sachs AB, Bond MW, Kornberg RD. A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. *Cell* 1986;45:827–835. [PubMed: 3518950]
14. Sachs AB, Davis RW, Kornberg RD. A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability. *Mol. Cell. Biol* 1987;7:3268–3276. [PubMed: 3313012]
15. Gebauer F, Corona DF, Preiss T, Becker PB, Hentze MW. Translational control of dosage compensation in *Drosophila* by Sex-lethal: cooperative silencing via the 5' and 3' UTRs of msl-2 mRNA is independent of the poly(A) tail. *EMBO J* 1999;18:6146–6154. [PubMed: 10545124]
16. Munroe D, Jacobson A. mRNA poly(A) tail, a 3' enhancer of translational initiation. *Mol. Cell. Biol* 1990;10:3441–3455. [PubMed: 1972543]
17. Otero LJ, Ashe MP, Sachs AB. The yeast poly(A)-binding protein Pab1p stimulates *in vitro* poly(A)-dependent and cap-dependent translation by distinct mechanisms. *EMBO J* 1999;18:3153–3163. [PubMed: 10357826]
18. Goyer C, et al. TIF4631 and TIF4632: two yeast genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an RNA recognition motif-like sequence and carry out an essential function. *Mol Cell Biol* 1993;13:4860–4874. [PubMed: 8336723]
19. Kessler SH, Sachs AB. RNA recognition motif 2 of yeast Pab1p is required for its functional interaction with eukaryotic translation initiation factor 4G. *Mol. Cell. Biol* 1998;18:51–57. [PubMed: 9418852]
20. Cosson B, et al. Poly(A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence *PSI(+)* propagation. *Mol. Cell. Biol* 2002;22:3301–3315. [PubMed: 11971964]
21. Mangus DA, Evans MC, Jacobson A. Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biol* 2003;4:223.1–223.14. [PubMed: 12844354]
22. Salas-Marco J, Bedwell DM. GTP hydrolysis by eRF3 facilitates stop codon decoding during eukaryotic translation termination. *Mol. Cell. Biol* 2004;24:7769–7778. [PubMed: 15314182]
23. Stansfield I, Kushnirov VV, Jones KM, Tuite MF. A conditional-lethal translation termination defect in a *sup45* mutant of the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem* 1997;245:557–563. [PubMed: 9182990]
24. Uchida N, Hoshino S, Imataka H, Sonenberg N, Katada T. A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in cap/poly(A)-dependent translation. *J. Biol. Chem* 2002;277:50286–50292. [PubMed: 12381739]
25. Mangus DA, Jacobson A. Linking mRNA turnover and translation: assessing the polyribosomal association of mRNA decay factors and degradative intermediates. *Methods* 1999;17:28–37. [PubMed: 10075880]
26. Iizuka N, Sarnow P. Translation-competent extracts from *Saccharomyces cerevisiae*: effects of L-A RNA, 5' cap, and 3' poly(A) tail on translational efficiency of mRNAs. *Methods* 1997;11:353–60. [PubMed: 9126550]
27. Mangus DA, Amrani N, Jacobson A. Pbp1p, a factor interacting with *Saccharomyces cerevisiae* poly(A)-binding protein, regulates polyadenylation. *Mol. Cell. Biol* 1998;18:7383–7396. [PubMed: 9819425]
28. Tarun SZ Jr, Wells SE, Deardorff JA, Sachs AB. Translation initiation factor eIF4G mediates *in vitro* poly(A) tail-dependent translation. *Proc. Natl. Acad. Sci. USA* 1997;94:9046–9051. [PubMed: 9256432]
29. Barnes CA, Singer RA, Johnston GC. Yeast *prt1* mutations alter heat-shock gene expression through transcript fragmentation. *EMBO J* 1993;12:3323–3332. [PubMed: 8344268]
30. Nielsen KH, et al. Functions of eIF3 downstream of 48S assembly impact AUG recognition and GCN4 translational control. *EMBO J* 2004;23:1166–1177. [PubMed: 14976554]

Fig. 1.

Toeprint analyses of initiation on long and short mRNAs in the presence of CHX in wild-type extracts. **(a)** General schematic of the miniUAA1, UAA, and AAA mRNAs. Sizes (in nt) listed under each construct respectively refer to the length of the 5' UTR, the coding region, the 3' UTR, and the poly(A) tail. **(b)** Addition of cap analog to AAA and UAA mRNAs inhibits accumulation of the AUG toeprint bands. **(c)** miniUAA1 mRNA AUG toeprints are resistant to 2.7mM cap analog and become sensitive only at higher concentrations. **(d)** Sensitivity to 2.7 mM cap analog is mRNA size dependent. The values on the graph are the average of two independent experiments. Positions of the toeprints are indicated with arrows. The left portions

of panels **b** and **c** show dideoxynucleotide sequencing reactions for the AAA or miniUAA1 templates (with 5' to 3' sequence reading from the top to the bottom).

Fig. 2. Cap analog resistance of the miniUAA1 mRNA is cap and poly(A) dependent in wild-type extracts and suggests formation of a stable closed loop structure. (a) AUG toeprint analyses of uncapped mRNA. (b) Poly(A)-deficient mRNAs are highly sensitive to cap analog. (c) Resistance to cap analog is poly(A) size dependent.

Fig. 3.

Formation of a stable closed-loop structure on a capped and polyadenylated mRNA in the presence of an 80S complex requires Pab1p interactions with eIF4G, mRNA, and Sup35p. (a) Toeprinting analyses of miniUAA1 mRNA in Pab1p-defective, wild-type, or *pbp1Δ* extracts. (b) Sensitivity to cap analog in an eIF4G1 mutant incapable of Pab1p interaction. (c) *sup35-R419G* and *sup45-2* mutants show sensitivity to cap analog and additional toeprint bands upstream of the initiator AUG. (d) Sensitivity to cap analog is independent of the termination event. (e, f) Cap analog resistance or sensitivity of miniUAA1 mRNA appears at the onset of translation. (g) Sucrose gradient fractionation of miniUAA1 mRNA translated in wild-type and mutant extracts in the absence and presence of cap analog, and in the presence of CHX. In panel g, the values above the horizontal line depict fraction numbers and those below the line denote the respective percentages of mRNA associated with polysomal or non-polysomal fractions.

Fig. 4. Stabilization of the closed-loop structure on a capped and polyadenylated mRNA in the presence of a 48S complex requires Pab1p interactions with eIF4G and mRNA. **(a)** Extracts from Pab1p- and eIF4G1-deficient strains are cap analog sensitive in the presence of GMP-PNP. **(b)** Addition of recombinant Pab1p restores resistance to cap analog in *pab1Δpbp1Δ* extracts in the presence of GMP-PNP. **(c)** Extracts from the *sup35-R419G* and *sup45-2* termination mutants are cap analog resistant in the presence of GTP analog.