Free Poly(A) Stimulates Capped mRNA Translation in Vitro through the eIF4G-Poly(A)-binding Protein Interaction*

The 5’ cap and 3’ poly(A) tail of classical eukaryotic mRNAs functionally communicate to synergistically enhance translation initiation. Synergy has been proposed to result in part from facilitated ribosome recapture on circularized mRNAs. Here, we demonstrate that this is not the case. In poly(A)-dependent, ribosome-depleted rabbit reticulocyte lysates, the addition of exogenous poly(A) chains of physiological length dramatically enhanced translation initiation. In mechanistic terms, the interaction of wheat germ PABP with eIF4F has been shown to increase the affinity of the eIF4F complex for cap analogue by some 40-fold. Similarly, the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped 5’ end of polyadenylated mRNAs in mammalian extracts. Furthermore, the affinity of eIF4F-complexed plant PABP for poly(A) is significantly greater than that of free PABP. Thus, it seems probable that mRNA 5’–3’ end cross-talk enhances translation, at least in part, by stimulating the formation of initiation factor-mRNA complexes. Because this process only requires a series of RNA-protein and protein-protein interactions, it could occur, theoretically, in trans.

Several further potential translational advantages could be envisaged to result from mRNA circularization. For instance, the PABP-eIF4G interaction would provide a means of tethering the eIF4F complex to a mRNA (albeit at the 3’ end), preventing its dissociation from the message after each initiation event and maintaining an elevated local concentration of eIF4F near the mRNA 5’ end. Noncovalent linking of mRNA ends could also facilitate the recapture of ribosomes that have terminated translation and dissociated from the RNA but remain in its proximity. Obviously, these additional potential advantages of mRNA functional circularization would require that the effects of PABP-bound poly(A) be exclusively intramolecular. In support of cis-stimulation by poly(A), several studies have shown that the addition of exogenous poly(A) to in vitro translation extracts inhibited translation of all mRNAs, regardless of the nature of the 5’ and 3’ ends (20, 21). In contrast, one study reported that whereas capped polyadenylated mRNA translation was inhibited by exogenous poly(A), that of capped nonpolyadenylated mRNA was slightly stimulated (~1.5-fold) at certain poly(A) concentrations (5). These previous analyses were performed in nuclease-treated rabbit reticulocyte lysates (RRL) or in wheat germ extracts, which are far from physiological in reproducing the positive effects of poly(A) on trans-

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1 The abbreviations used are: eIF, eukaryotic initiation factor; PABP, poly(A)-binding protein; RRL, rabbit reticulocyte lysate; UTR, untranslated region.
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laction. Indeed, in such extracts, poly(A) dependence is rather low and the combined effects of cap and poly(A) on translation are at best additive (17).

We recently described a RRL-based system that faithfully reproduces the synergistic cooperation between the cap and poly(A) for translation initiation by mimicking the competitive environment of the intact cell. Because a competitive environment is assured by rendering translation components physically limiting, rather than by adding excess competitor mRNAs, this system allows the molecular dissection of the functional interactions between mRNA 5′ and 3′ ends (17, 19, 22). Here we describe the use of this system for a detailed study of the effect of exogenous poly(A) on translation of mRNAs carrying various combinations of 5′ cap and 3′ poly(A) tail. We demonstrate that poly(A) chains added in trans can dramatically enhance translation of either capped, nonpolyadenylated mRNAs or capped polyadenylated mRNAs carrying poly(A) tails of suboptimal length. As described previously for cap-poly(A) synergy, trans stimulation requires the integrity of the eIF4G-PABP interaction and results at least in part from an increased functional affinity of eIF4E for the 5′ ends of capped nonpolyadenylated mRNAs.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions and In Vitro Transcription**—The construction of the pOp24 plasmids with and without an A 

 poly(A) tract inserted at the unique EcoRI site, 24 nucleotides downstream of the authentic polyadenylation signal has been described elsewhere (17). The pOp24 plasmids contain, under the control of the T7 promoter, a short oligonucleotide-derived 5′ UTR, followed by the region coding for the human immunodeficiency virus (HIV-1 

 \( \text{p}24 \) protein and the influenza virus NS 3′ UTR. Versions of these plasmids containing A 

 poly(A) tracts were constructed by linearization of plasmids carrying A 

 poly(A) tracts with EcoRI, and insertion of a second copy of the original annealed A 

 poly(A) oligonucleotides (17). In vitro transcriptions and quantification and purification of capped and uncapped in vitro transcripts were performed as described (17) using pOp24 plasmids that had been linearized by EcoRI.

Preparation and Fractionation of Exogenous Poly(A)—Poly(A) (ICN), poly(U) (Sigma), and poly(dA) (Invitrogen) chain length was determined by dephosphorylation of the homopolymer and rephosphorylation of reduced poly(A) in the presence of \( \gamma^{32} \text{P} \) ATP as described (23), followed by electrophoresis of a known weight of homopolymer on 6% sequencing gels with radiolabeled RNA molecular weight standards. Size distribution of poly(A) chains in the starting pool was evaluated by densitometric scanning, which revealed a virtually equimolar distribution of poly(A) chains ranging from 15 to 600 residues. Because the concentrations of different-length chains differed, the average chain length of the nonfractionated poly(A) pool was used to calculate the molar concentration. This radiolabeled pool of poly(A) was also fractionated on denaturing gels, and populations of different lengths of poly(A) were excised from the gels and eluted into water. The absolute yield of recovered poly(A) was calculated on the basis of radiolabel recovery. The actual size distribution of the fractions was then assessed by remigration of recovered poly(A) on denaturing gels and densitometric scanning of the autoradiographs of the dried gels. The number of poly(A) chains of each length was then estimated by an “area under the curve” method.

In Vitro Translation—Nucleic-acid-treated RRL (Promega) was partially depleted of ribosomes and ribosome-associated translation factors by ultracentrifugation in a Beckman TL-100 bench-top ultracentrifuge essentially as described previously (17), except that 1.5 ml volumes of lysate were centrifuged for 30 min at 90,000 rpm at 4 °C. In vitro translation reactions were performed in the presence of \( \gamma^{32} \text{P} \) methionine. Reactions contained 50% by volume of flexi-reticulocyte lysate (Promega) or ribosome-depleted RRL and 30% by volume of H1100 buffer (10 mM HEPES-KOH, pH 7.5, 100 mM KC1, 1 mM MgCl2, 0.1 mM EDTA, and 7 mM β-mercaptoethanol). Reactions were performed with 2.5 or 5 μg/ml of in vitro transcribed mRNAs. Final concentrations, respectively, of added KC1 and MgCl2 were 125 and 0.6 mM in all reactions. In certain experiments, cap analogue (Ambion Inc.), diluted in H1100 buffer or a fragment of recombinant NSP3 encompassing amino acids 163–313 (which had been over-expressed in Escherichia coli and purified exactly as described previously (13); a gift from Dr. D. Fournet) were added to reactions. For NSPS, reactions were incubated on ice in the presence of protein for 10 min prior to the addition of programming RNA. Cap analogue was added immediately after programming RNAs, poly(A), diluted in H1100 buffer, was added to reactions at various concentrations after programming mRNA. Assays supplemented with poly(A) received additional MgCl2 at 1.0 mol/mol of A residues to counteract the potential chelating effect of poly(A). Translations were performed for 90 min at 30 °C, and the translation products were analyzed by SDS-PAGE as described previously (24), using gels containing 23% (w/v) acrylamide. Dried gels were exposed to Biomax MR film (Kodak) for 2 to 14 days depending on the particular experiment. Densitometric quantification of translation products was described previously (17) using multiple exposures of each gel to ensure that the linear response range of the film was respected and that low levels of translation could be accurately quantified.

**RESULTS**

Many of the mechanisms proposed to underlie cap-poly(A) synergy require that the positive effects of poly(A) tails on translation are restricted to the mRNAs which carry these 3′ ends, that is that translation stimulation by poly(A) is exclusively a cis event. The aim of the current study was to test this hypothesis. The recently described nucleic-acid-treated, ribosome-depleted RRL translation system is ideal for such a study, because poly(A)-dependence results from physically limiting concentrations of ribosomes and ribosome-associated translation factors rather than the presence of intact, functional competitor mRNAs (19). Effectively, functional analyses of the mechanism of poly(A) action are hindered in competitor-based systems by the fact that poly(A)-dependence of the extract is lost as soon as translation conditions are altered (7, 17, 19). The pOp24 plasmids used for the present study have been described elsewhere (17). Briefly, the plasmids contain a short oligonucleotide-derived 5′ UTR followed by the region coding for the human immunodeficiency virus (HIV-1 

 \( \text{p}24 \) protein and the influenza virus NS 3′ UTR. Three versions of the plasmid differ only by the absence or presence of an A 

 poly(A) tract downstream of the 3′ UTR (see “Experimental Procedures”).

As previously demonstrated (17, 19), the effects of capping and polyadenylation on translation of mRNAs derived from the pOp24 plasmids in a standard RRL assay system are at best additive, i.e. the cumulative stimulatory effects of cap and poly(A) are not dramatically greater than the effects of the sum of each modification alone (Table I). However, when the same mRNAs are translated in a ribosome-depleted RRL extract under optimal conditions (Table I; Ref. 19), significant cap-poly(A) synergy is observed. This synergy is typically greater than 5-fold with poly(A) tails of 50 residues and exceeds 10-fold

| Cap/poly(A) contexta | Standard RRL | Depleted RRLb |
|----------------------|--------------|---------------|
| b+50                 | 1.8          | 2.8           |
| b+100                | 2.9          | 4.3           |
| b+150                | 23 (1.4×)    | 166 (10.8×)   |

a RNAs were transcribed with or without cap /with or without a poly(A) tail of 50 or 100 A residues.

b Cap-poly(A) synergy calculated as: stimulation upon capping and polyadenylation divided by (stimulation upon capping added to stimulation upon polyadenylation) is given in parentheses alongside each value for translation efficiency on +/− mRNA in depleted RRL. For comparison, the same calculation is shown for translation in standard RRL. Final concentrations in mRNAs were 5 μg/ml.
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Efficient Stimulation of Translation by Free Poly(A) in Trans—To examine the potential of polyadenylic acid to stimulate translation efficiency in trans, we employed a commercial poly(A) preparation consisting of poly(A) chains in the approximate range of 15 to 600 residues, present in roughly equimolar proportions (Fig. 1a, Load lane). This total poly(A) preparation was added at a range of concentrations to translation extracts programmed with Op24-derived mRNAs carrying a cap, a poly(A<sub>100</sub>) tail or both (Fig. 1b). Added poly(A) significantly inhibited translation of uncapped polyadenylated mRNA even at low concentrations (−/+ lanes). Similarly, translation of capped mRNA carrying an A<sub>100</sub> tail was reduced 2–3-fold at low poly(A) concentrations (molar ratios of poly(A):mRNA <1:1) and then slightly but reproducibly stimulated at higher poly(A) to mRNA ratios to return to and even exceed the efficiency observed in the absence of poly(A) addition (Fig. 1b, +/+ lanes). However, at the highest poly(A) concentration tested, translation efficiency was again suppressed with respect to the control lane (Fig. 1b, +/− lanes). Finally, although capped, nonpolyadenylated mRNA translation was unaffected by low concentrations of poly(A), it was dramatically stimulated even to exceed slightly that of +/+ mRNA translated in the absence of added poly(A) when the molar concentration of poly(A) to mRNA exceeded 1:1 (5–8-fold stimulation at poly(A):mRNA ratios of 2.5–10; Fig. 1b, +/− lanes). Even when the molar ratio of poly(A) to +/− mRNA exceeded 10, although translation stimulation was significantly reduced, absolute efficiency still exceeded that observed in the absence of poly(A) addition.

Thus, free poly(A) can dramatically stimulate capped, nonpolyadenylated mRNA translation and also moderately improve the translation of capped mRNAs, which carry short poly(A) tails. It should be noted that the order of addition of mRNA and poly(A) to extracts and the omission of extra Mg<sup>2+</sup> ions to compensate for sequestering by poly(A) did not significantly alter the results (data not shown). Additionally, the observed effects were specific to poly(A), as evidenced by the inability of either poly(dA) or poly(U) to effect similar stimulation of translation on either +/− or +/+ mRNAs (Table II). Interestingly, high concentrations of poly(U) dramatically inhibited +/+ mRNA translation, presumably by interacting with poly(A<sub>100</sub>) tails (Table I). Similar poly(A) length-dependent increases in cap-poly(A) cooperativity have been reported in numerous in vitro translation systems (see for example Ref. 25).

Fig. 1. Exogenous poly(A) stimulates capped mRNA translation in ribosome-depleted RRL. a, electrophoretic analysis of poly(A) preparations used. A commercial poly(A) pool, radiolabeled at the 5’ end, was separated electrophoretically. Populations corresponding to different poly(A) chain lengths were excised from the wet gel and eluted into water. Aliquots of both the pool starting material (Load lane, 1 μl loaded) and each population (Pop. lanes, 5 and 2.5 μl loaded, from left to right) were re-migrated through a 6% denaturing polyacrylamide gel. The autoradiograph of the dried gel is shown, and the approximate lengths (in nucleotides) of the different poly(A) populations are indicated on the left. b, effects of pool poly(A) preparation on translation of mRNAs derived from pOp24. Ribosome-depleted RRL translation reactions were programmed with 5 μg/ml (final RNA concentration) of Op24 mRNAs synthesized to contain a cap (+/−), poly(A<sub>100</sub>) tail (−/+), or both (+/+). Reactions were then supplemented with control buffer (0 lanes) or increasing concentrations of poly(A) pool (calculated as the molar excess of poly(A) chains with respect to mRNA) in control buffer. MgCl<sub>2</sub> was added in a 1:1 molar ratio with respect to the added A residues. Identical results were obtained without extra MgCl<sub>2</sub> addition (data not shown). Translation products were separated by SDS-polyacrylamide gel electrophoresis using gels containing 23% (w/v) acrylamide. The autoradiograph of the dried gel is shown. Translation efficiency (in arbitrary units (AU)) was quantified densitometrically as described under “Experimental Procedures.” Black circles, hatched triangles, and open squares correspond, respectively, to +/−, −/+, and +/+ mRNAs. Error bars represent the standard deviation calculated from three independent experiments.
with the mRNA 3' poly(A) tail and effectively sequestering this element. Finally, exogenous poly(A) had no discernible effect on mRNA stability in the depleted RRL system (data not shown), which is perhaps not surprising given that poly(A) tails acting in *cis* stimulate translation in this system without altering mRNA half-life (17, 22).

**Length Dependence of Poly(A) for Trans Stimulation**—To address the length requirements for this *trans* stimulation of translation by free poly(A), the original heterogeneous poly(A) preparation was separated electrophoretically, and three distinct populations of different sizes were excised from the gels and purified: 19–52 residues (Pop. 1), 50–180 residues (Pop. 2), and 150–400 residues (Pop. 3) (Fig. 1a; see “Experimental Procedures”). These fractionated poly(A) populations were then each compared at equivalent molar concentrations (with respect to the number of poly(A) chains) with the nonfractionated original preparation for their effects on capped (+/−) and capped poly(A<sub>pop</sub>) (+/+) mRNA translation (Fig. 2). Extracts were programmed with 2.5 rather than 5 μg/ml pOp24-derived mRNAs to facilitate the addition of molar excess poly(A). Under these conditions, stimulation of +/− and +/+ mRNA translation with the nonfractionated poly(A) preparation was even more dramatic (14- and 2.5-fold stimulation, respectively, with 2.5 μg/ml of +/− and +/+ mRNAs, as opposed to 5- and 1.5-fold using 5 μg/ml of the same RNAs; compare Fig. 1b and left-hand panel, Fig. 2).

Short poly(A) chains (19–52 residues) had no significant effect on the translation of +/− mRNAs but moderately inhibited +/+ mRNA translation at all of the poly(A) concentrations tested (Fig. 2, Pop 1). Conversely, the addition of poly(A) chains of between 50 and 180 residues affected the translation of both +/− and +/+ mRNAs in a manner similar to that observed with the nonfractionated poly(A) preparation (Pop 2 and Total lanes, Fig. 2), with the exception that +/− mRNA translation was stimulated less efficiently with population 2 poly(A) than with nonfractionated poly(A) at equivalent molar concentrations. Finally, poly(A) chains of 150–400 residues stimulated both +/− and +/+ mRNA translation, albeit less dramatically than the intermediate length chains (Pop 3 and Pop 2 lanes, Fig. 2). However, stimulation of +/− and +/+ mRNA translation was observed at poly(A) to mRNA ratios of less than 1, suggesting that these long poly(A) chains could simultaneously trans-activate the translation of several mRNA molecules. It should also be noted that although the estimated added molar excesses of poly(A) for the different populations are approxima-

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**Fig. 2.** *Trans* stimulation of translation by poly(A) is poly(A) chain length-dependent. Ribosome-depleted RRL translation reactions were programmed with 2.5 μg/ml (final RNA concentration) Op24 mRNAs synthesized to contain a cap (+/−) or cap and poly(A<sub>pop</sub>) tail (+/+). Reactions were then supplemented with buffer (0 lanes) or the indicated molar ratios (with respect to programming RNA) of poly(A) pool (Total lanes) or purified poly(A) populations 1, 2, or 3 (see Fig. 1A, Pop. lanes). MgCl<sub>2</sub> was added in a 1:1 molar ratio with respect to the added A residues. Analysis of translation products was exactly as described in the legend for Fig. 1B; open squares and filled circles correspond to +/+ and +/− mRNAs, respectively.

**Fig. 3.** Effects of exogenous poly(A) on the dose responses of mRNAs translated in depleted RRL. Ribosome-depleted RRL translation reactions were programmed with (from left to right) 12, 6, 3, or 1.5 μg/ml of Op24 mRNAs synthesized to contain a cap (+/−) or cap and poly(A<sub>pop</sub>) tail (+/+), as indicated. Reactions then received H100 buffer (+ panels) or a constant 4-fold molar excess (with respect to mRNA) of population 2 poly(A) chains in H100 buffer (+ panels). MgCl<sub>2</sub> was added in a 1:1 molar ratio with respect to the added A residues in the poly(A) population. Analysis of translation efficiency was exactly as described in the legend for Fig. 1; translation efficiency (in arbitrary units) is plotted below each panel. Open and filled circles (+/− mRNA) and squares (+/+ mRNA) correspond, respectively, to reactions without and with exogenous poly(A). The stimulation of +/− and +/+ mRNA translation by exogenous poly(A) is also shown at each mRNA concentration (triangles and dashed lines).
Translation efficiency is expressed relative to that observed with the same mRNA in the absence of homopolymer addition, which was arbitrarily set as 1.0 for each mRNA. In absolute terms, translation of +/− mRNA was 8-fold more efficient than that of −/− mRNA under the experimental conditions used (final mRNA concentrations of 5 μg/ml).

Table II
Effects of different homopolymers of equivalent length on translation efficiency

| Molar ratio of polymer to mRNA | +/− mRNA translation with: | −/− mRNA translation with: |
|-------------------------------|--------------------------|--------------------------|
|                               | Poly(A) | Poly(U) | Poly(A) | Poly(U) | Poly(U) |
| 0.2                           | 1.27    | 0.87    | 1.39    | 0.38    | 0.79    |
| 0.4                           | 1.82    | 1.01    | 1.90    | 0.39    | 0.20    |
| 0.8                           | 2.11    | 0.97    | 0.86    | 0.42    | 0.52    |
| 2.5                           | 5.46    | 1.06    | 0.74    | 0.97    | 0.70    |
| 3.0                           | 4.01    | 1.50    | 0.54    | 1.77    | 0.88    |

FIG. 4 Trans stimulation of translation by free poly(A) requires the eIF4G-PABP interaction. A, ribosome-depleted RRL translation reactions were preincubated for 15 min at 4 °C with NSP3 (10 μg/ml) or buffer before being programmed with +/− or +/− poly(A) alone (open squares) or +/− mRNAs (circles). B, ribosome-depleted RRL translation reactions were preincubated with NSP3 (10 μg/ml) or buffer prior to being programmed with 2.5 μg/ml poly(A)−24-derived capped, nonpolyadenylated mRNA. Reactions were then supplemented with a 2.5-fold molar excess (with respect to mRNA) of population 2 poly(A) as indicated. Analysis of translation efficiency was exactly as described in the legend for Fig. 1. Open and filled symbols correspond, respectively, to reactions treated with NSP3 or buffer and programmed with +/− (squares) or +/− mRNAs (circles). B, ribosome-depleted RRL translation reactions were preincubated with NSP3 (10 μg/ml) or control buffer prior to being programmed with 2.5 μg/ml poly(A)−24-derived capped, nonpolyadenylated mRNA. Reactions were then supplemented with a 2.5-fold molar excess (with respect to mRNA) of population 2 poly(A) chains in H100 buffer or H100 buffer alone. Finally, reactions received increasing concentrations of m7GpppG cap analogue. MgCl2 was added in a 1:1 molar ratio with respect to the added cap analogue and A residues in the poly(A) population. Analysis of translation efficiency was exactly as described in the legend for Fig. 1. Translation efficiency for +/− mRNA translated in the presence of buffer alone is indicated (open circles); population 2 poly(A) alone (closed circles) or NSP3 plus population 2 poly(A) (filled squares) is plotted against cap analogue concentration.

Effects of Exogenous Poly(A) Chains on the Dose Responses of mRNAs Translated in Depleted RRL—One of the features of translation in the ribosome-depleted RRL system is that the translation extracts are much more rapidly saturated by capped, polyadenylated than by capped, nonpolyadenylated mRNAs (Ref. 17; Fig. 3). Effectively, a poly(A) tail acting in cis causes a displacement in the dose response of a capped mRNA, without actually increasing the yield of translation product obtained from the RNA under saturating conditions. Thus, the effects of exogenous poly(A) chains on the dose responses of capped, nonpolyadenylated and capped, polyadenylated mRNAs were determined. Exogenous poly(A) chains did not increase the absolute yield of translation product from either +/− or +/− mRNAs, but rather they behaved like a poly(A) tail acting in cis, provoking saturation of the translation extracts at significantly reduced mRNA concentrations (Fig. 3). Indeed, translation of +/− mRNA at the highest RNA concentrations tested was actually inhibited by the addition of exogenous poly(A), presumably because the exogenous poly(A) was acting to render the concentration of mRNA saturating.

Trans Stimulation by Poly(A) Requires the eIF4G-PABP Interaction and Increases the Functional Affinity of eIF4E for Cap—We previously demonstrated that 5′-cap-3′poly(A) cooperative translation stimulation required the integrity of the eIF4G-PABP interaction. Cap-poly(A) synergy was abolished when extracts were incubated with a fragment of the rotavirus nonstructural protein NSP3, which interacts with eIF4G and displaces PABP from the eIF4F complex (17). Thus, we evaluated whether trans stimulation of translation by poly(A) also depended on the eIF4G-PABP interaction. To this end, translation extracts were pretreated with NSP3 fragment or with control buffer, programmed with +/− or +/− poly(A)−24 mRNAs and then supplemented with different amounts of 50–180 residue poly(A) chains. Inclusion of NSP3 fragment at 10 μg/ml, a concentration previously determined to be sufficient to disrupt the eIF4G-PABP interaction (Ref. 17; data not shown), abolished cap-poly(A) synergy on +/− mRNA as expected and moreover totally inhibited the stimulatory effects of exogenous poly(A) on +/− and +/− mRNA translation at all of the concentrations of poly(A) tested (Fig. 4A). However, NSP3 did not neutralize the inhibitory effects of low concentrations of poly(A) on +/− mRNA translation.

It has also been demonstrated previously that the eIF4G-PABP interaction on capped, polyadenylated mRNAs increases the functional affinity of eIF4E for the capped mRNA 5′ end (18, 19). Indeed, 8–10-fold higher concentrations of cap analogue were required to inhibit the translation of +/− as compared with +/− mRNA in ribosome-depleted RRL (19). However, this increased resistance of +/− mRNA translation to cap analogue inhibition was abrogated upon interruption of the eIF4G-PABP interaction by NSP3. Thus, we assessed whether poly(A) in trans also alters the affinity of the eIF4E-cap interaction. Capped, nonpolyadenylated mRNA was translated in the presence of buffer or a 2.5-fold molar excess of 50–180-residue poly(A) chains with or without pretreatment of extracts with NSP3 fragment and in the presence of increasing concentrations of cap analogue. In the absence of NSP3, +/− mRNA translation was stimulated ~3–4-fold by the exogenous poly(A) employed and showed significantly reduced sensitivity to cap analogue inhibition as compared with +/− mRNA translated without added poly(A) (Fig. 4B; minimum inhibitory concentration (MIC50) of 66 and 12 μM with and without added poly(A), respectively). However, in the presence of NSP3, exogenous poly(A) did not alter the sensitivity of +/− mRNA trans-
In this study we have shown that exogenous poly(A) chains are extremely efficient in stimulating translation of capped, nonpolyadenylated mRNAs in ribosome-depleted, poly(A)-dependent reticulocyte lysates. Several lines of evidence strongly suggest that trans stimulation by poly(A) is not the result of some general, nonspecific effect of the added homopolymer on the translation machinery. Trans stimulation was effected only by poly(A) among the homopolymers tested, was specific to translation of mRNAs carrying a cap (free poly(A) was inhibitory to translation of uncapped mRNAs), required poly(A) chains of physiological length (equivalent concentrations of short poly(A) chains had no stimulatory effect on translation), and was only observed when near equimolar ratios of poly(A) to mRNA were employed. Of more importance, by the criteria examined to date, trans stimulation by poly(A) is mechanistically indistinguishable from cap-poly(A) synergy: (a) respond quantitatively to increases in poly(A) length, (b) require the eIF4G-PABP interaction, and (c) correlate with an increased functional affinity of eIF4E for the capped mRNA 5' end (as measured by the resistance of mRNA translation to cap analogue inhibition). In the present study, the absolute degree of trans stimulation by exogenous poly(A) was at least as great as that observed with poly(A50) or poly(A100) tails. Thus, a central conclusion from these results is that mRNA circularization per se is dispensable for poly(A) stimulation of capped mRNA translation at least in vitro. Moreover, because the mRNA the 5' and 3' termini are not brought together by the cap-eIF4E-eIF4G-PABP-exogenous poly(A) interaction, it seems reasonable to conclude that the stimulatory effects of poly(A) on capped mRNA translation in this system are not due to facilitated ribosome recycling promoted by proximity of the mRNA 5' and 3' ends (see model, Fig. 5). Likewise, one can exclude tethering of eIF4F via PABP to the poly(A) tail (and preventing its dissociation from the mRNA after each round of initiation) as a molecular mechanism underlying synergy.

Poly(A) chains of physiological length also significantly stimulated in trans the translation of capped mRNAs carrying short poly(A50) tails in an eIF4G-PABP-dependent manner. It remains to be determined whether the longer, added poly(A) chains were displacing the A50 tails from the eIF4G-PABP complex (and thus breaking the mRNA circles) or rather that stimulation was due to an artificial elongation of the existing poly(A) tails mediated via PABP multimerization (see Fig. 5). However, it should be noted that virtually identical profiles of stimulation by exogenous poly(A) were observed using capped
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mRNAs carrying longer (\(A_{100}\)) tracts (data not shown), arguing in favor of elongation rather than displacement.

Therefore, how does poly(A) (or PABP) stimulate capped mRNA translation? In the present study, we have demonstrated that poly(A) trans stimulation, like cap-poly(A) synergy, can be attributed at least in part to increased initiation factor-RNA affinities. Effectively, trans stimulation by poly(A) correlated with an increased functional affinity of eIF4E for cap, an effect that was abolished upon interruption of the eIF4G-PABP interaction. Indeed, previous studies have demonstrated that the eIF4G-eIF4E interaction increases the affinity of eIF4E for cap (26), as does binding of wheat germ PABP to eIF4F (18). This latter interaction also increases the affinity of PABP for poly(A) (12). Additional indirect evidence for increased initiation factor-RNA affinities derives from the effects of exogenous poly(A) on uncapped polyadenylated mRNAs as described here. Although exogenous poly(A) stimulated the translation of capped mRNAs with poly(A)\(_{100}\) tails, it inhibited the translation of \(-/\)+ mRNAs. A reasonable explanation would be that exogenous poly(A) competes more efficiently with the poly(A) tails of uncapped versus capped mRNAs for PABP binding (Fig. 5), which would again suggest that the affinity of PABP for poly(A) tails depends heavily on the cap status of the mRNA. However, although it is tempting to speculate that cap-poly(A) cooperativity is mainly because of increased affinity of eIF4E or PABP for the mRNA ends, several results suggest that the mechanism of cooperativity is likely to be more complex.

First, capped mRNA translation in Xenopus oocytes can be stimulated by physical tethering of PABP or discrete portions of PABP to the 3’ UTR of a nonpolyadenylated mRNA (27). Similarly, the addition of excess PABP to yeast cell-free extracts stimulates capped, nonpolyadenylated mRNA translation (28). Thus, signaling by poly(A) through PABP is apparently not absolutely essential for PABP to stimulate capped mRNA translation. However, it was not determined whether PABP tethered to the 3’ UTR or added in trans was as efficient as poly(A)-bound PABP in stimulating capped mRNA translation. This is perhaps unlikely because cap-poly(A) synergy (25) and trans stimulation of capped mRNA translation (this study) are both poly(A) length-dependent. Thus, it seems highly probable that more than one PABP molecule per RNA is needed for maximal translation stimulation. It should be noted that several of the PABP domains used in Ref. 27 cannot dimerize, and PABP in general is monomeric in the absence of poly(A) (29).

Second, although tethered RNA recognition motifs 1 and 2 of Xenopus or yeast PABP can stimulate capped mRNA translation via the eIF4G-PABP interaction, translation stimulation by RNA recognition motifs 3 and 4 is apparently independent of this interaction (27). Similarly, trans stimulation of capped translation by free PABP is still effected by PABP mutants with much diminished capacities to interact with eIF4G (28). Thus, additional factors that interact with PABP apparently participate in cap-poly(A) cooperativity in yeast and Xenopus. Although the only known effect of NSP3 on the mammalian translation machinery is the eviction of PABP from eIF4G, we cannot formally exclude that other, as yet unidentified interactions are also interrupted. Indeed, the involvement of additional PABP-protein interactions in cap-poly(A) cooperativity would help explain the apparent requirement for multiple PABP molecules per RNA for optimal translation stimulation.

The data presented here demonstrate that poly(A) can stimulate capped mRNA translation in trans without the need for mRNA circularization. Hence, facilitated recapture of terminating ribosomes due to the proximity of mRNA 5′ and 3′ ends does not apparently contribute significantly to the synergistic stimulation of translation observed upon capping and polyadenylation, at least in vitro. Thus, the role of poly(A) in cap-poly(A) synergy in vitro is to provide a scaffold for the binding of sufficient molecules of PABP to fully activate translation. However, these data do not rule out other possible roles for circularization in the context of an intact cell, such as reversal of Ski2p-mediated repression of nonpolyadenylated mRNA translation and enhanced 60 S subunit joining (30) or increased mRNA stability. The latter effect at least would be undetected in the depleted RRL system, in which polyadenylation and capping of a mRNA do not affect functional mRNA half-life (Ref. 19; data not shown). In addition, we do not believe that our results imply that polyadenylation of one mRNA species within a cell would indiscriminately stimulate the translation on unrelated messages. Indeed, it remains possible that as yet unidentified mechanisms prevent poly(A) tail sharing between intact, translationally active mRNAs. Our future experiments will be designed to address these possibilities.

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