mRNA Poly(A) Tail, a 3' Enhancer of Translational Initiation

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Received 2 February 1990/Accepted 4 April 1990

To evaluate the hypothesis that the 3' poly(A) tract of mRNA plays a role in translational initiation, we constructed derivatives of pSP65 which direct the *in vitro* synthesis of mRNAs with different poly(A) tail lengths and compared, in reticulocyte extracts, the relative efficiencies with which such mRNAs were translated, degraded, recruited into polysomes, and assembled into messenger ribonucleoproteins or intermediates in the translational initiation pathway. Relative to mRNAs which were polyadenylated, we found that nonpolyadenylated [poly(A)⁻] mRNAs had a reduced translational capacity which was not due to an increase in their decay rates, but was attributable to a reduction in their efficiency of recruitment into polysomes. The defect in poly(A)⁻ mRNAs affected a late step in translational initiation, was distinct from the phenotype associated with cap-deficient mRNAs, and resulted in a reduced ability to form 80S initiation complexes. Moreover, poly(A) added *in trans* inhibited translation from capped polyadenylated mRNAs but stimulated translation from capped poly(A)⁻ mRNAs. We suggest that the presence of a 3' poly(A) tail may facilitate the binding of an initiation factor or ribosomal subunit at the mRNA 5' end.

Most eucaryotic mRNAs have a sequence of polyadenylic acid [poly(A)] at their 3' termini (14, 19, 39, 48, 50). These poly(A) "tails" are added post-transcriptionally in the nucleus, with an initial length, in mammalian cells, of approximately 200 to 250 adenylate residues (9). Following transport of mRNA to the cytoplasm, poly(A) tracts are gradually shortened so that, in steady state, poly(A) tail lengths are heterogeneous, ranging from 50 to 70 A's (2, 8, 10, 38, 59, 80). Although it has been almost two decades since the discovery of these poly(A) tracts, their function(s) has yet to be clarified.

Earlier results from our laboratory (37, 53, 54, 61, 79) led us to propose that poly(A) has a role in translation. More specifically, we proposed that an interaction of the cytoplasmic poly(A)-binding protein (PABP) with a critical minimum length of poly(A) facilitates the initiation of translation of polyadenylated [poly(A)⁺] but not nonpolyadenylated [poly(A)⁻] mRNAs. The results of several different experimental approaches have provided evidence which indirectly supports this hypothesis. These results include (i) the correlation of specific changes in mRNA poly(A) tail length with translational efficiency following fertilization in *Spisula* oocytes (72), during early development in *Dictyostelium discoideum* (61), oocyte maturation in *Xenopus laevis* (34, 55), oocyte activation in mice (33, 84, 86), salivary gland development in *Drosophila melanogaster* (67), and in response to physiologic stimuli in the rat hypothalamus (11, 69, 89); (ii) the higher translational activity of poly(A)⁺ as opposed to poly(A)⁻ mRNAs *in vitro*, as demonstrated in systems as diverse as cell extracts derived from rabbit reticulocytes (16, 73), Ehrlich ascites tumor cells (31), and wheat germ (73), as well as in microinjected *Xenopus* oocytes (15, 21, 34) and electroporated tobacco protoplasts (22); (iii) a correlation between the abundance and stability of PABPs and the rate of translational initiation in developing or heat-shocked *Dictyostelium discoideum* amoebae (53,

54); (iv) the demonstration that exogenous poly(A) is a potent and specific inhibitor of the *in vitro* translation of poly(A)⁺ but not poly(A)⁻ mRNAs in rabbit reticulocyte (4, 28, 37), wheat germ (4), L-cell (49), and pea seed (81) extracts; and (v) the demonstration that purified PABP can stimulate translation *in vitro* (81).

The experiments in this study were designed to provide a direct test of our model. We have constructed a set of plasmids which direct the *in vitro* synthesis of a set of mRNAs differing only in their respective poly(A) tail lengths and compared the efficiencies with which such synthetic mRNAs were translated, degraded, recruited into polysomes, and assembled into messenger ribonucleoproteins (mRNPs) or intermediates in the translational initiation pathway in a reticulocyte cell-free translation system. In addition, we have reevaluated the effects of exogenous competitor poly(A) *in vitro* by using mRNAs that differ either in cap or poly(A) tail status or both. Our results demonstrate that poly(A) is involved in a late step in the initiation of translation and lead us to suggest that poly(A) is the formal equivalent of a transcriptional enhancer, i.e., that PABP bound at the 3' end of mRNA may facilitate the binding of an initiation factor or ribosomal subunit at the mRNA 5' end.

MATERIALS AND METHODS

Plasmid constructions. A series of vectors for the *in vitro* transcription of polyadenylated RNAs, designated pSP65A_n, were derived by insertion of different lengths of poly(dA:dT) within the polylinker of pSP65 (56). These vectors were constructed by conventional techniques (52) as follows (Fig. 1). pSP65 DNA was linearized by digestion with *Pst*I, tailed to various extents with dATP and terminal transferase (Ratcliff Biochemicals), digested with *Hind*III, and separated from the low-molecular-weight products of digestion by preparative electrophoresis in low-melting-point agarose. A 7-base oligonucleotide, including part of a *Hind*III recognition site (5'-AGCTTTT-3'), was phosphorylated at its 5' terminus with ATP and polynucleotide kinase, tailed to various extents with dTTP and terminal transferase, purified

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by DEAE-cellulose chromatography, and ligated to the gel-purified vector molecules.

Templates for transcription of polyadenylated rabbit β -globin mRNAs were constructed by using pGEM1R β G and p β G1 (gifts from Argiris Efstratiadis). The 0.34-kilobase-pair (kbp) *AccI*-*BglI* fragment from p β G1 (20) was gel purified and ligated with the gel-purified 3.1-kbp *AccI*-*Bam*HI fragment from pGEM1R β G to produce pDMR β G. The 0.95-kbp *SphI*-*SmaI* fragment from pDMR β G was then gel purified and ligated with the gel-purified 3-kbp *SphI*-*SmaI* fragment from selected pSP65A vectors. After linearization with *HindIII*, transcripts from these templates include 7 nucleotides of 5' noncoding sequence derived from the vector, 10 nucleotides of rabbit β -globin 5' untranslated flanking sequence, the entire rabbit β -globin 5' untranslated region and coding region, 9 out of 95 nucleotides of 3' untranslated region, 11 nucleotides derived from the vector, and a poly(A) tail of specified length.

Templates for transcription of polyadenylated vesicular stomatitis virus N (VSV.N) mRNAs were constructed by using plasmid JS223 (82) (a gift from Jack Rose). The 1.3-kbp *XhoI* fragment from JS223, containing the VSV.N cDNA, was gel purified and ligated into the *SalI* site of pSP65, producing AS65N. AS65N was digested with *SmaI* and *NdeI*, fragment ends were made blunt by filling in with DNA polymerase, and the 1.3-kbp fragment was gel purified and ligated into *SmaI*-cut, phosphatase-treated pSP65A vectors. After linearization with *HindIII*, transcripts from these templates include 16 nucleotides of 5' noncoding sequence derived from the vector, the entire VSV.N 5' untranslated region, coding region, and 3' untranslated region, 15 nucleotides derived from the vector, and a poly(A) tail of specified length.

Poly(A) tail length determinations. All plasmid templates were initially screened to determine the length of the poly(dA:dT) insert, located just 5' of the *HindIII* site in the polylinker, by digestion with *HindIII* and *EcoRI* (pSP65A plasmids and rabbit β -globin constructs) or *HindIII* and *BglI* (VSV.N gene constructs). Restriction digests of plasmids containing poly(dA:dT) inserts were directly compared with otherwise identical plasmids without such inserts on 5% polyacrylamide gels (in TBE [52]). Because replication in bacterial hosts often caused deletions within the poly(dA:dT) insert [resulting in plasmid preparations with heterogeneous poly(dA:dT) lengths; for example, see Fig. 1B], plasmids were prepared in a mutant host (HB101A) which allows plasmid replication without deletion (D. Munroe, Ph.D. thesis, University of Massachusetts Medical School, Worcester, Mass., 1989).

To measure poly(A) tail lengths, RNA was transcribed in vitro (with [α - 32 P]ATP as the label), and 5×10^5 cpm of each transcript was digested either with RNases A and T_1 (100 μ g/ml each) or T_2 (5.0 U/ml) in $2 \times$ SSC (0.3 M NaCl, 0.03 M sodium citrate) at 37°C for 30 to 60 min. Digestion products were purified by an oligo(dT)-cellulose batch procedure (36) and analyzed on 5% polyacrylamide gels run in the presence of 8 M urea and TBE (52).

In vitro transcription and capping of synthetic mRNAs. In vitro transcription and cotranscriptional capping, directed

by SP6 RNA polymerase (Boehringer), were done as described previously (43, 56). After transcription, DNA templates were removed by digestion with RQ1 DNase (1 U/ μ g of DNA; Promega). Transcripts were further purified by two phenol-CHCl₃ (1:1) extractions, CHCl₃ extraction, Sephadex G-50 spin chromatography, and ethanol precipitation. The integrity of all RNA samples was verified by electrophoresis in 4 to 6% polyacrylamide (acrylamide-bisacrylamide, 20:1) gels containing 8 M urea and buffered with TBE (52).

Analysis of RNA 5' termini. Transcripts labeled in vitro with [α - 32 P]ATP (10⁶ cpm) were digested with RNases A (100 μ g/ml), T_1 (100 μ g/ml), and T_2 (5.0 U/ml) in $2 \times$ SSC at 37°C for 30 to 60 min. Reaction products were separated by two-dimensional thin-layer chromatography on cellulose plates (Analtech) in isobutyric acid-concentrated NH₄OH-H₂O (pH 4.3) (577:38:385) in the first dimension and saturated (NH₄)₂SO₄-1 M sodium acetate-isopropanol (80:18:2) in the second dimension as described previously (43).

In vitro protein synthesis. mRNA-dependent translation extracts were prepared from commercial rabbit reticulocyte lysate (Promega) as described previously (59). Reaction conditions for in vitro protein synthesis and procedures for analyzing the translation products of individual mRNAs have been described (37, 59).

Measurement of RNA decay rates in reticulocyte lysates. Synthetic RNAs (<0.2 μ g) labeled with [α - 32 P]ATP (8×10^6 cpm/ μ g) were incubated in 50 μ l of reticulocyte translation extract at 37°C. Samples (5 μ l) were removed after 0, 5, 10, 20, 30, and 60 min of incubation and diluted in 225 μ l of ice-cold buffer B (25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.0], 20 mM EDTA) containing a different 32 P-labeled in vitro transcript (to monitor the recovery of total RNA). Samples were extracted once with phenol (65°C) and once with CHCl₃ and then analyzed on 5% polyacrylamide gels (run in the presence of 8 M urea and TBE). The resulting autoradiographs were quantitated by densitometry.

Sucrose gradient analysis of translation intermediates in reticulocyte lysates. 3 H- and 32 P-labeled mRNAs were mixed in equimolar amounts and used in the following assays at final concentrations of ≤ 4 μ g/ml. Polysomal distribution was monitored in 25 to 50 μ l of reticulocyte translation extract. Following a 15-min incubation at 37°C, samples were diluted 1:10 in ice-cold buffer A (25 mM HEPES [pH 7.0], 50 mM KCl, 2 mM magnesium acetate) containing cycloheximide (50 μ g/ml) and fractionated on gradients of 15 to 50% sucrose in buffer A. Gradients were centrifuged at $175,000 \times g$ for 110 min at 4°C in an SW41 rotor. Formation of 48S preinitiation complex was monitored in 25 to 50 μ l of reticulocyte lysate including edeine (10 μ M; a generous gift of Peter Walter). Following a 5-min incubation at 37°C, samples were diluted 1:10 in ice-cold buffer C (25 mM HEPES [pH 7.0], 50 mM KCl, 20 mM EDTA) and fractionated on 10 to 30% sucrose gradients in buffer C (SW41 rotor, $200,000 \times g$, 180 min, 4°C). mRNPs were assembled in 25 to 50 μ l of reticulocyte lysate containing 20 mM EDTA. Following a 5-min incubation at 37°C, samples were diluted 1:10 in ice-cold

T_1 . Lanes 1 and 2, RBG mRNA with 0 A's; lanes 3 and 4, RBG mRNA with 23 A's; lanes 5 and 6, RBG mRNA with 32 A's; and lanes 7 and 8, RBG mRNA with 68 A's. Lane M, size markers (in nucleotides). (D) Efficiency of in vitro capping. To assess the extent of in vitro capping, a short (10-nucleotide) RNA was transcribed from pSP65 digested (within the polylinker) with *EcoRI*. Transcription reaction mixes included [α - 32 P]ATP and 7 MeGpppG. Transcripts were digested with RNases A, T_1 , and T_2 , and digestion products were separated by two-dimensional thin-layer chromatography.

buffer C and fractionated on 5 to 20% sucrose gradients in buffer C (SW41 rotor, 200,000 $\times$ g, 360 min, 4°C).

For the analysis of all sucrose gradients, fractions were collected from the bottom, transferred directly into ice-cold 5% trichloroacetic acid (TCA), and filtered through GF/C disks, which were subsequently washed with 5% TCA–95% ethanol. Radioactivity in each sample was determined by scintillation spectrometry and correction of ^3H counts for ^{32}P spillover. The respective sums of the ^3H and ^{32}P counts per minute were determined for each gradient, and the data are presented as the percentage of total counts present in each fraction. Sedimentation coefficients were determined relative to ribosomal particles of known value.

Determination of translational elongation rates. The rates of translational elongation on VSV.N mRNAs were measured by a modification of the method described previously (62). Standard reticulocyte translation extract (100 μl) containing [^{35}S]methionine was incubated with synthetic, capped VSV.N mRNA (4 $\mu\text{g}/\text{ml}$) at 37°C. At the indicated times, samples (13 μl) were removed and pipetted into 387 μl of ice-cold buffer A containing cycloheximide (50 $\mu\text{g}/\text{ml}$). Isotopic incorporation into total VSV.N polypeptide was monitored by acetone precipitation of 175 μl from each sample. In parallel, isotopic incorporation into released VSV.N polypeptide was monitored by pelleting polysomes from an additional 200 μl of each sample (centrifugation for 2 h, 24 lb/in 2 , 4°C, in a Beckman Airfuge) followed by acetone precipitation of 175 μl of the resulting supernatant (600 μl of acetone, –20°C, 16 h). Following acetone precipitation, each sample was washed once with an additional 600 μl of acetone, dried, and suspended in 35 μl of sample buffer (46). Samples were fractionated on standard 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (46), which were subsequently analyzed by quantitative fluorography (47).

RESULTS

Synthesis and characterization of in vitro transcripts. To test the hypothesis that poly(A) may be involved in protein synthesis, we compared the relative efficiency with which synthetic mRNAs differing only in poly(A) tail length were recruited into polysomes and translated in a cell-free rabbit reticulocyte extract. Synthesis of mRNAs which differed in poly(A) tail length required the construction of derivatives of plasmid pSP65 (56) which contained poly(dA:dT) insertions of different length between the *Pst*I and *Hind*III sites of the polylinker (referred to as pSP65A vectors; Fig. 1A). The length of dA:dT tract carried by each pSP65A plasmid was assessed by digestion with restriction enzymes at sites which bracket the dA:dT region, followed by analysis on 5% polyacrylamide gels (Fig. 1B). Because plasmid replication in conventional bacterial hosts (such as *Escherichia coli* HB101) often resulted in deletions within the dA:dT region (Fig. 1B, lane 6), we isolated a mutant bacterial host, HB101A, which allows the replication of plasmids containing long dA:dT tracts without deletion (Munroe, thesis). cDNAs encoding rabbit β -globin (RBG) mRNA or VSV.N mRNA were cloned within the polylinker regions of pSP65A vectors containing 0 to 68 dA:dT residues. Linearization of these vectors with *Hind*III, followed by in vitro transcription, led to the synthesis of RBG or VSV.N mRNAs which terminated in specific lengths of poly(A) (as defined by gel electrophoretic analysis of the RNase A and T_1 digestion products from each corresponding transcript; Fig. 1C). In part, the validity of conclusions drawn from experiments with such in vitro transcripts rests upon the structural

integrity of these mRNAs. Thus, we analyzed each RNA preparation by gel electrophoresis and measured the extent of polyadenylation by binding to oligo(dT)–cellulose. We found that virtually all of the RBG mRNA containing a 68-nucleotide poly(A) tail was retained by oligo(dT)–cellulose, while its poly(A) $^-$ counterpart was not, and that each RNA species was represented by a single band on denaturing polyacrylamide gels (data not shown).

Because of their importance in translation, the 5'-terminal structures of the synthetic transcripts were also analyzed. Thin-layer chromatography analysis of the RNase A, T_1 , and T_2 digestion products of a truncated transcript (Fig. 1D) indicated that capping of in vitro transcripts occurred with over 95% efficiency. Similar experiments have been performed with full-length RBG transcripts and have yielded similar results (data not shown). These data indicate that each preparation of mRNA is homogeneous and full length, carries a poly(A) tail of specific size, and, when capped, has the expected 5'-terminal structure.

Previous experiments have also utilized purified or synthetic mRNAs to evaluate a possible role for poly(A) in translation (15–17, 21). Our experiments differ from these earlier studies in that we used synthetic mRNAs which closely resemble their in vivo counterparts, i.e., (i) the poly(A) $^+$ mRNAs used here contain homogeneous poly(A) tracts equivalent to steady-state lengths, (ii) the poly(A) $^+$ and poly(A) $^-$ mRNAs used here differ only in poly(A) sequences and not other mRNA sequences, and (iii) both the poly(A) $^+$ and the poly(A) $^-$ mRNAs used in this study lack additional [non-poly(A)] homopolymeric tracts.

Poly(A) $^+$ mRNAs translate with greater efficiency than poly(A) $^-$ mRNAs. The effects of differences in 3' polyadenylation status and/or 5' cap structure on the relative translational capacity of otherwise identical VSV.N mRNAs were determined by monitoring the incorporation of [^{35}S]methionine into VSV.N polypeptide in rabbit reticulocyte lysates. Over a broad range of mRNA concentrations, capped poly(A) $^+$ (61 A's) VSV.N mRNA had 1.4 to 2.5 times the translational activity of capped poly(A) $^-$ VSV.N mRNA, 1.4 to 1.9 times the activity of uncapped poly(A) $^+$ (61 A's) VSV.N mRNA, and 3.3 to 4.0 times the activity of uncapped poly(A) $^-$ VSV.N mRNA (Fig. 2A). These data show that (i) poly(A) $^-$ mRNAs and uncapped mRNAs have comparable reductions in translational capacity (approximately twofold) and (ii) the translational deficiencies associated with the absence of a cap or a poly(A) tail are additive.

To ensure that the differences observed in the translational activities of poly(A) $^+$ and poly(A) $^-$ mRNAs were not due to differential mRNA stabilities, we compared the decay rates of capped poly(A) $^+$ and poly(A) $^-$ VSV.N and RBG synthetic transcripts in reticulocyte extracts. Over the course of a 60-min incubation, we found the poly(A) $^+$ and poly(A) $^-$ forms of each mRNA to be equally stable (Fig. 2B). Therefore, we can attribute the observed differential formation of protein product by poly(A) $^+$ and poly(A) $^-$ mRNAs solely to differences in their respective translational efficiencies.

Poly(A)-deficient mRNAs are defective in translational initiation. The data in Fig. 2 demonstrate that the 3' poly(A) tail of mRNA is an important determinant of translational efficiency, i.e., its contribution to in vitro translation is of the same magnitude as that of the 5' cap. In an effort to identify the particular step of translation (i.e., initiation, elongation, or termination) affected by poly(A), the ability of capped poly(A) $^+$ mRNAs to form polysomes in vitro was compared with that of otherwise identical mRNAs lacking caps and/or poly(A) tails. ^{32}P -labeled, capped and polyadenylated

FIG. 2. Relative translational efficiency and stability of synthetic mRNAs differing in poly(A) tail length and/or 5' cap structure. (A) Relative translational efficiency. VSV.N in vitro transcripts, of indicated structure [e.g., with (C+, A+) and without (C-, A-) 5' caps and poly(A) tracts] and concentration, were incubated for 30 min at 37°C in an mRNA-dependent, cell-free rabbit reticulocyte translation extract supplemented with [35 S]methionine. Synthesis of VSV.N polypeptide in equal volumes of each translation reaction was analyzed by SDS-polyacrylamide gel electrophoresis and quantitative fluorography. The data presented are the averages of the densitometric quantitation of VSV.N polypeptide in two independent experiments. (B) Relative mRNA stability. The percent of mRNA level at time 0 is shown on the y axis. Capped, 32 P-labeled RBG or VSV.N in vitro transcripts, containing either 68 or 0 3'

mRNA was mixed with an equal amount of 3 H-labeled mRNA which lacked either caps or poly(A) tails or both, and the mixture was incubated in reticulocyte lysates at 37°C for 15 min. The polysomal distribution of each combination of mRNAs was then analyzed on 15 to 50% sucrose gradients (Fig. 3). To facilitate comparison of different mRNAs, the data are plotted as the percentage of total mRNA present in each fraction. Control experiments demonstrated that the results were not affected by the choice of 3 H or 32 P labeling, since labeling the C-RBG-68A mRNA [capped RBG mRNA with a 68-nucleotide poly(A) tail] with 3 H and the competitor transcript with 32 P yielded similar results (data not shown), and mixing 32 P-labeled C-RBG-68A mRNA with 3 H-labeled C-RBG-68A mRNA yielded superimposable polysome profiles (Fig. 3A). Poly(A)⁺ RBG and VSV.N mRNAs were both recruited into polysomes to a greater extent than their poly(A)⁻ counterparts (Fig. 3B and C). A comparison of the ratio of poly(A)⁺ to poly(A)⁻ mRNA across the polysomal region of these gradients (insets, Fig. 3B and C) revealed that those poly(A)⁻ mRNAs which did form polysomes were associated with a smaller average number of ribosomes than poly(A)⁺ mRNAs. Figure 3 also shows that the polysomal distribution of poly(A)-deficient mRNAs was comparable to that observed with cap-deficient mRNA (compare Fig. 3B and C with D). Collectively, these data indicate that, like uncapped mRNAs, poly(A)⁻ mRNAs have a reduced rate of translational initiation.

This conclusion was confirmed by experiments which showed that, in vitro, the translational elongation rate on capped VSV.N mRNA with a 61-nucleotide poly(A) tail was identical to that of poly(A)⁻ capped VSV.N mRNA (2.9 amino acids per s at 37°C) (Munroe, thesis). mRNA lacking both a poly(A) tail and a 5' cap showed a polysome profile (Fig. 3E) indicative of a drastic reduction in translational initiation. This observation is consistent with the additivity of the effects of poly(A) deficiency and cap deficiency observed in the experiments shown in Fig. 2A.

Figure 3F summarizes a series of experiments in which we compared the extent of polysome formation of capped RBG mRNAs differing only in poly(A) tail length with that of a capped RBG transcript containing 68 3' adenylate residues. The data indicate that the efficiency of polysome formation increases as a function of poly(A) tail length, with the most pronounced effect occurring between poly(A) lengths of >5 to ≤32 adenylate residues. There must be a minimal length of poly(A) required for maximum recruitment of mRNA into polysomes, since mRNAs with poly(A) tails containing 5 adenylate residues were equivalent to poly(A)⁻ mRNAs in this assay.

Poly(A)⁻ mRNAs are deficient in 80S initiation complex formation. The results of the experiments shown in Fig. 3 demonstrate that poly(A) functions in the initiation of translation. Next, we wished to determine the stage of translational initiation at which poly(A) is involved. Eucaryotic translational initiation can be divided into four stages, three of which are mRNA dependent: (i) formation of an mRNP, (ii) the association of the mRNP with the 43S preinitiation complex (40S ribosomal subunit, Met-tRNA_f, GTP, and various initiation factors) to form a 48S preinitiation complex, and (iii) formation of an 80S initiation complex by the

adenylate residues, were incubated at 37°C in a reticulocyte translation extract. Samples were withdrawn at the indicated times, and RNA levels were quantitated by autoradiography and densitometry as described in Materials and Methods.

C-RBG-68A vs C-RBG-68A

C-RBG-68A vs C-RBG-0A

FIG. 3.—Continued.

C-VSV.N-61A vs C-VSV.N-0A

C-RBG-68A vs UC-RBG-68A

FIG. 3.—Continued.

C-RBG-68A vs UC-RBG-0A

FIG. 3. Polysomal distribution of synthetic mRNAs differing in poly(A) tail lengths and/or 5' cap structure. (A to E) Equal amounts of a ^{32}P -labeled, capped RBG or VSV.N in vitro transcript containing 68 3' adenylate residues ($\square$) and a ^3H -labeled RBG or VSV.N in vitro transcript of indicated structure (+) were mixed and incubated for 15 min at 37°C in a reticulocyte translation extract. After incubation, translation reaction mixes were fractionated on 15 to 50% sucrose gradients. Fractions were collected from the bottom of the gradients directly into ice-cold 5% TCA and filtered. Insets present the ratio of ^{32}P to ^3H counts per minute across the polysomal regions of the gradients. Arrows indicate the position of the 80S peak. (A) Capped poly(A) $^+$ RBG (68 A's) versus capped poly(A) $^+$ RBG (68 A's); (B) capped poly(A) $^+$ RBG (68 A's) versus capped poly(A) $^-$ RBG (0 A's); (C) capped poly(A) $^+$ VSV.N (61 A's) versus capped poly(A) $^-$ VSV.N (0 A's); (D) capped poly(A) $^+$ RBG (68 A's) versus uncapped poly(A) $^+$ RBG (68 A's); (E) capped poly(A) $^+$ RBG (68 A's) versus uncapped poly(A) $^-$ RBG (0 A's). (F) In experiments analogous to those shown in panels A to E, the percentage of ^{32}P -labeled, capped RBG mRNA with 68 A's found on polysomes was compared with that of ^3H -labeled, capped RBG mRNA of indicated poly(A) tail length. All fractions sedimenting faster than 80S were considered polysomal. Data were normalized so that the amount of ^{32}P -labeled RBG mRNA with 68 A's present on polysomes was considered 100%.

binding of the 60S ribosomal subunit to the 48S preinitiation complex. We used sucrose gradient fractionation and various inhibitors of eucaryotic translation to compare the mRNPs, 48S preinitiation complexes, and 80S initiation complexes formed by poly(A) $^+$ and poly(A) $^-$ RBG mRNAs (Fig. 4).

EDTA is a Mg^{2+} -chelating agent which dissociates ribosomes and inhibits their association with mRNA, allowing only the formation of mRNPs. mRNPs formed in EDTA-treated reticulocyte lysates by adenylated RBG mRNAs (with 68 A's) sedimented faster in sucrose gradients than their unadenylated counterparts (Fig. 4A). Comparable dif-

ferences have been observed in the sedimentation rates of mRNPs formed by poly(A)⁺ and poly(A)⁻ VSV.N mRNAs (unpublished observations). These results suggest that poly(A)⁺ mRNAs form mRNPs that have a greater protein content and/or different secondary structure than those formed by poly(A)⁻ mRNAs, a conclusion consistent with previous measurements of the buoyant densities of L-cell histone and nonhistone mRNPs (24).

We compared the ability of poly(A)⁺ and poly(A)⁻ mRNAs to form 48S preinitiation complexes in edeine-treated reticulocyte lysates (Fig. 4B). Edeine is a small basic peptide antibiotic that inhibits association of the 60S ribosomal subunit with 48S preinitiation complexes, resulting in the accumulation of 48S preinitiation complexes (44, 58, 77). In an edeine-treated reticulocyte lysate, poly(A)⁺ (68 A's) and poly(A)⁻ RBG mRNAs were capable of forming 48S preinitiation complexes with equal efficiency, although the complex formed by poly(A)⁻ RBG mRNA sedimented slightly more slowly than that formed by poly(A)⁺ mRNA (Fig. 4B). This difference in sedimentation rate most likely reflects the greater protein content of poly(A)⁺ mRNPs (Fig. 4A). Since 48S preinitiation complex formation has been shown previously to be dependent upon cap-binding proteins (68), we examined the polypeptides which can be UV cross-linked to poly(A)⁺ and poly(A)⁻ mRNAs in reticulocyte lysates (with mRNA labeled specifically in the 5' cap). These experiments indicate that RBG and VSV.N mRNAs associate with the same set of cap-binding proteins regardless of adenylation status (Munroe, thesis). Therefore, we conclude that poly(A) is not necessary for the formation of 48S preinitiation complexes.

Next we compared the ability of poly(A)⁺ and poly(A)⁻ RBG mRNAs to form 80S initiation complexes in anisomycin- and cycloheximide-treated reticulocyte lysates. Both of these drugs inhibit peptide bond formation and, when added to an *in vitro* protein synthesis system prior to the addition of mRNA, lead to the accumulation of 80S initiation complexes (71, 77). In reticulocyte lysates treated with anisomycin (Fig. 4C) or cycloheximide (Fig. 4D), poly(A)⁺ RBG mRNAs accumulated as 80S initiation complexes with 2- to 2.5-fold the efficiency of their poly(A)⁻ counterparts. Comparable results were observed with poly(A)⁺ and poly(A)⁻ VSV.N mRNAs (data not shown). These experiments are quantitatively consistent with the differences in translational efficiency and ribosome recruitment shown by poly(A)⁺ and poly(A)⁻ mRNAs in previous experiments (Fig. 2 and 3) and suggest that the most likely cause of these differences is a reduced ability of poly(A)⁻ mRNAs to form 80S initiation complexes. Since 48S preinitiation complex formation is cap dependent, a role for poly(A) in the formation of 80S initiation complexes, but not 48S preinitiation complexes, is consistent with our observations on the additivity of the defects associated with cap and poly(A) deficiencies (Fig. 2 and 3).

Competitive inhibition by exogenous poly(A): differential sensitivity of mRNAs with or without caps or poly(A) tails. Earlier results from our laboratory demonstrated that, in reticulocyte extracts, exogenous poly(A) is a potent and specific inhibitor of the translational initiation of poly(A)⁺ but not poly(A)⁻ mRNAs (37). This poly(A)-mediated inhibition probably occurs by limiting the availability of unbound PABP, in much the same way that purified 5' cap structures have been found to inhibit the *in vitro* translation of capped mRNAs (30, 70). Surprisingly, Jacobson and Favreau (37) found that exogenous poly(A) could also stimulate (40 to 300%) the *in vitro* translation of poly(A)⁻

mRNAs. We have extended these observations by monitoring the effects of exogenous poly(A) and poly(C) on the translation of VSV.N mRNAs differing in 5' cap structure and/or 3' poly(A) tail (Fig. 5).

As expected from previous experiments, translation of capped, polyadenylated (61 A's) VSV.N mRNA was inhibited 50% by exogenous poly(A) at 30 µg/ml (Fig. 5A), while similar concentrations of control polynucleotide [poly(C)] had no effect (Fig. 5B). The absence of a cap on poly(A)⁺ VSV.N mRNA increased its sensitivity to inhibition by added poly(A) significantly (Fig. 5A), but only increased its sensitivity to added poly(C) slightly (Fig. 5B). These data are consistent with the additivity of the initiation defects of cap-deficient and poly(A)-deficient mRNAs observed above (Fig. 2 and 3) since, in the presence of competitor poly(A), an uncapped poly(A)⁺ mRNA probably becomes the formal equivalent of an uncapped poly(A)⁻ mRNA. In contrast, the translation of capped, poly(A)⁻ VSV.N mRNA was stimulated almost 40% by low concentrations (5 to 10 µg/ml) of poly(A) but was not significantly affected by poly(C) (Fig. 5). This suggests that poly(A) may be capable of working in *trans* to augment translational initiation. Such *trans* effects, however, are dependent on the presence of a 5' cap, since the same concentration of poly(A) that gave maximal stimulation of translation on capped unpolysadenylated VSV.N mRNA (10 µg/ml) was found to inhibit, by more than 85%, the translation of uncapped unpolysadenylated mRNA (Fig. 5A). These results imply that an event(s) in translational initiation involves the recognition of both 3' poly(A) and 5' cap structures (and/ or their associated proteins).

DISCUSSION

Poly(A) has a role in translational initiation. The translational activity of poly(A)⁻ VSV.N mRNA is approximately one-half that of the same mRNA containing a 61-nucleotide poly(A) tail (Fig. 2A). Consistent with this observation, poly(A)⁺ VSV.N and RBG mRNAs were found on larger polysomes and recruited into polysomes to a greater extent than their poly(A)⁻ counterparts (Fig. 3). Since the differences in the translational activities and the polysome profiles of poly(A)⁺ and poly(A)⁻ mRNAs cannot be attributed to differences in their stabilities (Fig. 2B), extents of capping (Fig. 1D), or translational elongation rates (Munroe, thesis), we conclude that they differ in their rates of translational initiation. This conclusion is borne out by the twofold difference observed in the formation of 80S initiation complexes by poly(A)⁺ and poly(A)⁻ mRNAs (Fig. 4C and D). The reduced efficiency of 80S initiation complex formation by poly(A)⁻ mRNA is quantitatively consistent with its reduced efficiencies of translation and ribosome recruitment (Fig. 2A and 3) and also explains the additivity of the translational defects associated with the absence of both a cap and a poly(A) tail (Fig. 2A and 3). Our results are also consistent with those of Nelson and Winkler (57), who showed that, in reticulocyte extracts, poly(A)⁻ histone mRNA formed a significantly higher percentage of "half-mers" (polysomes containing bound 40S subunits but lacking 60S subunits) than poly(A)⁺ globin mRNA.

Previous studies have also demonstrated a reduced efficiency of translation for poly(A)⁻ mRNAs (16, 21, 34, 72). In other studies the ratios of the translational activities of poly(A)⁺ and poly(A)⁻ mRNAs have varied from the 2-fold effect seen here (16, 31) to significantly larger effects (5- to 120-fold [17, 22, 34; unpublished observations]). The latter occur in *in vivo* systems with significantly higher overall

FIG. 4.—Continued.

FIG. 4. Formation of mRNPs, 48S preinitiation complexes, and 80S initiation complexes by poly(A)⁺ and poly(A)⁻ mRNAs. Formation of mRNPs and initiation and preinitiation complexes was monitored by incubating equal amounts of a ³²P-labeled, capped poly(A)⁺ RBG mRNA (with 68 A's) (□) with a ³H-labeled, capped poly(A)⁻ RBG mRNA (+) in reticulocyte extracts supplemented, incubated, and fractionated as indicated for each panel. (A) mRNP formation in a reticulocyte lysate containing 20 mM EDTA. Following incubation for 5 min at 37°C, the reaction mix was fractionated on a 5 to 20% sucrose gradient. (B) 48S preinitiation complex formation in a reticulocyte extract containing 10 μM edeine. After 5 min at 37°C, the translation reaction mix was fractionated on a 10 to 30% sucrose gradient. The 48S peak, which was only marginally apparent in untreated lysates (data not shown), is indicated by an arrow. (C) 80S initiation complex formation in a reticulocyte extract supplemented with anisomycin (1 mg/ml). The 80S peak, which accumulated in anisomycin-treated but not untreated extracts (data not shown), is indicated by an arrow. (D) 80S initiation complex formation in a reticulocyte extract supplemented with cycloheximide (75 μg/ml). The 80S peak, which accumulated in cycloheximide-treated but not untreated extracts (data not shown), is indicated by an arrow.

levels of translational initiation than occur in reticulocyte extracts and give support to the notion that the extent of the difference between poly(A)⁺ and poly(A)⁻ mRNAs will be dependent on the extent of reinitiation (15, 16, 21, 61). Although the differences between poly(A)⁺ and poly(A)⁻ mRNAs are substantially smaller *in vitro* than they are in some *in vivo* systems, we are confident that the differences are significant because (i) the magnitude of the poly(A) effect *in vitro* is equivalent to that obtained by differences in mRNA capping (Fig. 2 and 3), (ii) the effects of removing poly(A) tails and caps are additive (Fig. 2 and 3), (iii) differences in the translational efficiencies of poly(A)⁺ and poly(A)⁻ mRNAs have been established in double-label mixing experiments which allowed direct comparison of the two types of mRNA, (iv) progressive differences are evident, e.g., the effects of lengthening the poly(A) tail on ribosome recruitment or the ratio of poly(A)⁺ to poly(A)⁻ mRNAs across polysome gradients (Fig. 3), and (v) the twofold extent of the differences is observed at all levels, i.e., translation activity, recruitment into polysomes, and formation of 80S initiation intermediates.

Although a role for poly(A) in translational initiation is supported by the studies reported here (as well as those discussed in the Introduction), it should be noted that the presence of a poly(A) tail is not necessary or sufficient for translational activity. There are examples of translationally inactive polyadenylated mRNAs (66, 72) as well as examples of mRNAs which appear to lose their poly(A) tracts when going from translationally inactive to active forms (35, 41, 42). Likewise, since individual poly(A)⁻ mRNAs are known to be translated *in vivo* (3, 27), the presence of a poly(A) tail is not essential and the mechanisms which ensure the efficient translational initiation of poly(A)⁺ and poly(A)⁻ mRNAs may be quite different.

Effect of poly(A) on translation is directly related to its length. The experiments shown in Fig. 3 demonstrate that the percentage of a given mRNA which is recruited into polysomes is dependent on the length of the poly(A) tracts associated with that mRNA. Maximal recruitment of mRNA into polysomes requires a poly(A) tail of greater than 5 adenylate residues. Beyond this size, the efficiency of polysome formation increases as a function of poly(A) tail length, with the most pronounced effect occurring between poly(A) lengths of >5 and ≤32 adenylate residues. This is consistent with previous results which demonstrated that the activity of poly(A) as a competitive inhibitor of translation was inversely related to its length (37). The minimal size required for either activity (ribosome recruitment or competitive inhibition) is similar to that protected by a monomer of PABP (5, 75), suggesting a dependence on specific poly(A)-PABP interactions (see below).

Even though a tolerant bacterial host was used, the upper limit of poly(A) lengths examined in this study (68 residues) was dictated by the instability of plasmids harboring longer dA:dT regions (Munroe, thesis). Results from other experimental approaches [e.g., enzymatic addition of poly(A) tails] suggest that poly(A) tails longer than those used in this study will have significantly larger effects (17). Since mRNA in mammalian cells is initially synthesized with a poly(A) tail of approximately 200 adenylate residues (which is subsequently shortened to a steady-state length of 50 to 70 residues [2, 8, 10, 38, 60, 80]) it is likely that, *in vivo*, newly synthesized mRNA has a translational advantage over pre-existing mRNA. Evidence for such translational discrimination has been obtained in *Dictyostelium discoideum* (61, 79). We have previously noted that such results suggest that by simply changing the adenylation status of individual mRNAs or mRNA populations, large changes in protein-synthetic

FIG. 5. Competitive inhibition and translational stimulation by exogenous poly(A). Reticulocyte translation lysate containing [35 S] methionine was incubated for 5 min at 37°C in the presence of either poly(A) or poly(C) at the indicated concentrations. Synthetic VSV.N mRNAs of the indicated structures (See Fig. 2 legend) were then added to a final concentration of 0.25 μ g/ml. Following incubation for 30 min at 37°C, equal volumes of each sample were analyzed by SDS-polyacrylamide gel electrophoresis and quantitative fluorography. Data are expressed relative to control samples lacking added homopolymer. (A) Translation in the presence of exogenous poly(A). (B) Translation in the presence of exogenous poly(C).

patterns can be accomplished without significant mRNA synthesis or turnover (61).

Likely mediator of the translational function of poly(A) is the cytoplasmic PABP. Previous experiments with a variety of eucaryotic cells have utilized UV cross-linking to identify cytoplasmic PABPs (1, 24, 25, 52). Greenberg and Carroll (26) have shown that the only polypeptide which can be UV cross-linked to poly(A) in reticulocyte extracts has a molecular mass of 78 kilodaltons (kDa). In other experiments we have used UV cross-linking and label transfer to characterize the proteins bound in vitro to mRNAs of different poly(A) length (Munroe, thesis; unpublished data). These experiments showed that the major difference in the mRNP proteins associated with poly(A)⁺ and poly(A)⁻ mRNAs was due to proteins associated with poly(A). The p78 PABP monomer was identified in these experiments, but putative PABP dimers and trimers bound to the same molecule of poly(A) were also detected (5, 6; Munroe, thesis; unpublished data). The appearance of such putative dimers and trimers increased as a function of poly(A) tail length in a manner consistent with expectations based on binding site sizes (75) and in parallel with increases in translational efficiency. These results suggest that the likely mediator of the poly(A)-related effects on translational initiation is the PABP. This interpretation is supported by three independent observations: (i) in *D. discoideum*, we have shown that there is a correlation between the abundance and stability of the PABPs and the rate of translational initiation (53, 54); (ii) Sieliwanowicz (81) has shown that the PABP isolated from germinated pea embryo axes stimulates translation from poly(A)⁺ mRNAs in vitro; and (iii) Sachs and Davis (76) have shown that depletion or thermal inactivation of the PABP in *Saccharomyces cerevisiae* leads to the inhibition of translational initiation.

Poly(A) is an enhancer of translational initiation. We propose that poly(A) acts as the formal equivalent of a transcriptional enhancer. In much the same way that complexes between transcriptional enhancers and their binding proteins are thought to stimulate transcriptional events (64, 65), we suggest that the 3' poly(A)-PABP complex stimulates the

formation of 80S initiation complexes at the mRNA 5' end. Current models for the stimulation of transcription by enhancers predict the looping out of DNA located between the enhancer and promoter sequences (64, 65). Similarly, we predict that factors bound to the 3' and 5' ends of mRNA interact during the formation of 80S initiation complexes. Evidence in support of such an arrangement includes (i) secondary structure maps of rabbit α -globin (29) and murine β -globin (51) mRNAs which suggest that the 5' and 3' domains of these mRNAs are in close proximity; (ii) electron micrographs of circular or "folded-back" polysomes (13, 18, 32, 45, 87); (iii) suppression of a PABP mutation in *Saccharomyces cerevisiae* by a mutation in ribosomal protein L46 (76); and (iv) our finding that exogenous poly(A) may be capable of working in *trans* to stimulate the translation of capped poly(A)⁻ mRNA (Fig. 5A).

Transcriptional enhancers are known to work both 3' and 5' to their respective promoter elements. Although we have not tested the effect of a 5' poly(A) tail on translational initiation, there are several naturally occurring mRNAs which contain 5' poly(A) sequences capable of binding PABP. These include poxvirus mRNAs (7, 63, 78), the yeast PABP mRNA (1, 74), and the human PABP mRNA (23). The apparent conservation of this domain between yeast and human PABP mRNAs suggests that it affords some advantage. In addition, the finding that poly(A) will stimulate translation in *trans* (Fig. 5A) implies that (i) the orientation of poly(A) with respect to the 5' cap is unimportant and (ii) the oligo(A) present in reovirus particles (12, 83) may be of significance to the translational regulation which occurs during reovirus infection.

If the PABP normally has a stimulatory role in translation, then changes in the cellular levels of this protein (54, 81) or structural variants of this protein could have inhibitory activity. With regard to the latter point, a protein which retained poly(A)-binding activity but lost stimulatory activity could, in principle, be a translational inhibitor. In this regard it is interesting that an oocyte-specific PABP (85) appears to act as a translational repressor (40). Thus, the polyadenylation of stored mRNAs upon fertilization in *Xe-*

nopus (and other organisms) (33, 34, 55, 72, 84, 86) may reflect a need to provide a vacant binding site for the "stimulatory form" of the PABP.

ACKNOWLEDGMENTS

We thank Jack Rose and Arg Efstratiadis for gifts of plasmid DNAs, Laura Steel for advice on the construction of pSP65A vectors, and Neal Brown, Paul Dobner, Roy Parker, Laura Steel, and Michael Rosbash for comments on our manuscript.

This work was supported by Public Health Service grant (GM27757) to A.J. from the National Institutes of Health.

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