mRNA Poly(A) tail: a 3' Enhancer of Translational Initiation: a Thesis

David Munroe
University of Massachusetts Medical School
mRNA POLY(A) TAIL: A 3' ENHANCER OF TRANSLATIONAL INITIATION

A thesis presented
by
David Munroe

Submitted to the faculty of the
University of Massachusetts Medical School in partial
fullfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN
MOLECULAR GENETICS AND MICROBIOLOGY
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David Munroe

Approved as to style and content by:

Janet Stavnezer, Chairman of Committee
Neal Brown, Member of Committee
Paul Dobner, Member of Committee
David Housman, Member of Committee
David Parker, Member of Committee
Robert Singer, Member of Committee

Allan Jacobson, Thesis Advisor
Thomas Miller, Dean of Graduate School of Biomedical Sciences
"Sir", this thesis is dedicated to you.

I would like to thank the following - all for different reasons - listed in no order whatsoever: DH, my mother, PES, EG, Dr. Cox, JTG, Mr. Purple, BCM, SM, SS, AS, S, PD, TC, D.O., RP, big O, LJMM, my committee (JS, PD, DP, NB, RS, and DH), CM, SK, KB, C, AS, SWM, and last but not least AJ.
ABSTRACT

mRNA POLY(A) TAIL: A 3' ENHANCER OF TRANSLATIONAL INITIATION

Most eukaryotic mRNAs have a sequence of polyadenylic acid [poly(A)] at their 3'-termini. Although it has been almost two decades since the discovery of these poly(A) tracts, their function(s) have yet to be clarified. Earlier results from our laboratory 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 poly(A)$^+$, but not poly(A)$^-$, mRNAs. The results of several different experimental approaches have provided evidence which indirectly supports this hypothesis. These results include: 1) the correlation of specific changes in mRNA poly(A) tail length with translational efficiency in vivo and in vitro; 2) correlations between the abundance and stability of PABPs and the rate of translational initiation in vivo and in vitro; and 3) 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.

To evaluate the hypothesis that the 3'-poly(A) tract of mRNA plays a role in translational initiation, we have 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 are translated, degraded, recruited into polysomes, and assembled into mRNPs or intermediates in the translational initiation pathway. Relative to mRNAs which are polyadenylated, we find that poly(A)$^-$ mRNAs have a reduced translational capacity which is not due to an increase in their decay rates, but
is attributable to a reduction in their efficiency of recruitment into polysomes. The defect in poly(A)$^-$ mRNAs affects a late step in translational initiation, is distinct from the phenotype associated with cap-deficient mRNAs, and results in a reduced ability to form 80S initiation complexes. Moreover, poly(A) added in trans inhibits translation from capped poly(A)$^+$ mRNAs, but stimulates translation from capped poly(A)$^-$ mRNAs. We suggest that poly(A) is the formal equivalent of a transcriptional enhancer, i.e., that poly(A)-binding protein (PABP) bound at the 3'-end of mRNA may facilitate the binding of an initiation factor or ribosomal subunit at the mRNA 5'-end.
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CHAPTER 1
INTRODUCTION
This thesis describes a set of experiments designed to provide a direct test of the hypothesis that the 3'-poly(A) tract of mRNA plays a role in eukaryotic translational initiation. Chapter 1 contains the Introduction, which serves as a review of the data relevant to this hypothesis in particular and poly(A) in general. Chapter 2 contains both the Results and Discussion sections. The Results section details the experimental system which I have chosen and describes experiments which establish the role played by poly(A) in the initiation of translation. Other experiments explore the role of poly(A) in mRNP formation and begin to dissect the apparent signalling/recognition events observed between the mRNA 5' and 3'-termini during the initiation of translation. The Discussion section serves mainly as a forum for the discussion of a model for the role of poly(A) in the initiation of translation. This model evolved as a result of discussions between myself, Dr. Jacobson, and coworkers during the course of this work. The Appendix describes: 1) the isolation of an E. coli HB101 mutant used in the replication of pSP65A plasmids and their derivatives; and 2) the experiments designed to determine the translational elongation rate along synthetic poly(A)+ and poly(A)− VSV N mRNA. Chapter 2 contains a manuscript, recently submitted for publication, presented unchanged and in its entirety.

Nuclear polyadenylation

Formation of the 3'-termini of most eukaryotic mRNAs involves a sequence-directed endonucleolytic cleavage of pre-mRNA followed by the addition of a long poly(A) tail. This cleavage-polyadenylation reaction occurs in the nucleus, and in most mammalian cells, results in the addition of approximately 200-250 3'-adenylate residues (Brawerman, 1981). The first data
which suggested that specific nucleotide sequences were involved in mRNA 3'-end formation came from a comparison of the sequences found at the 3'-end of 6 different mRNAs (Proudfoot and Brownlee, 1976). All 6 mRNAs were found to contain the hexanucleotide sequence, AAUAAA, located approximately 20 bases upstream of the beginning of the poly(A) tail. Since this time, an AAUAAA-like sequence has been found in virtually every polyadenylated mRNA examined. These sequences are usually found between 10 and 30 bases upstream of the mRNA 3'-end (Berget, 1984; Wickens and Stephenson, 1984). The only invariant nucleotide in this sequence is the U at position 3 with the most common alternative sequence being AUUAAA (Berget, 1984; Wickens and Stephenson, 1984). Studies analyzing deletions of the AAUAAA sequence (Fitzgerald and Shenk, 1981; Cole and Santangelo, 1983) have established that this sequence is necessary for both the cleavage and polyadenylation reactions while the fact that AAUAAA sequences are found within coding regions, and apparently not utilized, shows this hexanucleotide alone is not sufficient. Indeed, a number of other sequence elements have been identified which seem to be involved in mRNA 3'-end formation. For example, the site of polyadenylation is almost always preceded by a CA dinucleotide (Fitzgerald and Shenk, 1981; McLauchlan et al., 1985). Other important sequences include: 1) a YGUGUUYY (Y=pyrimidine) or GU-rich domain located 5 to 30 nucleotides downstream of the site of polyadenylation; such a sequence has been found in over 60% of genes surveyed (Taya et al., 1982; McLauchlan et al., 1985); 2) a U-rich sequence found in most genes lacking a GU-rich sequence (Conway and Wickens, 1985; McLauchlan et al., 1985); and 3) the consensus sequence CAYUG, which has been found to lie either just 5' or just 3' to the site of poly(A) addition in many mRNAs (Benoist et al., 1980; Berget, 1984). Further
dissection of the reaction(s) involved in eukaryotic mRNA 3'-end formation have been aided by the development of in vitro systems that faithfully duplicate the in vivo processes. Such systems have been useful in demonstrating that the cleavage and polyadenylation reactions are not obligatorily linked (Manley, 1983; Moore and Sharp, 1984; 1985), and that the cleavage reaction involves a single endonucleolytic cut just 3' to the CA dinucleotide preceding the poly(A) addition site (Moore and Sharp, 1985; Sheets et al., 1987). Furthermore, analysis of the complexes formed specifically between pre-mRNAs (with an intact AAUAAA sequence) and components of various in vitro cleavage-polyadenylation extracts have led to the identification of proteins and snRNPs presumed to be involved in the reaction(s) responsible for mRNA 3'-end formation (Moore and Sharp, 1984; 1985; Zarkower and Wickens, 1987; Zhang and Cole, 1987; Christofori and Keller, 1988; Moore et al., 1988a; 1988b; Stefano and Adams, 1988; Wilusz and Shenk; 1988).

**Poly(A)^- mRNAs**

The majority of known eukaryotic mRNAs are polyadenylated. However, there also exists a population of mRNA which lacks poly(A). Early attempts at quantitating the proportion of mRNA which truly lacks poly(A) have estimated that up to 30% of cellular mRNA is poly(A)^- (Milcarek et al., 1974; Greenberg, 1977; Grady, 1978). In these experiments, all mRNAs which failed to bind oligo(dT)-cellulose were considered to be poly(A)^-. It should be noted, however, that mRNAs with short poly(A) tracts (up to 30 nucleotides) may not be retained by oligo(dT)-cellulose and can therefore be mistaken as poly(A)^- (Jacobson, 1987). In fact, Bergmann and Brawerman (1980) have shown that, at
most, only 10% of carefully isolated cellular mRNA is not bound by poly(U)-Sepharose. In other experiments, total HeLa cell mRNA was isolated, oligo(dT)-cellulose fractionated, and translated in vitro. The resulting translation products, from both the bound and unbound fractions, were then analyzed electrophoretically, on two-dimensional gels (Kaufmann et al., 1977). Most of the proteins encoded by the "poly(A)−" mRNA were also found among the poly(A)+ polypeptides, indicating that the two mRNA populations had similar coding capacity. Together, these data are consistent with the vast majority of mRNAs being poly(A)+ and suggest that many mRNAs, previously thought to lack poly(A), are in fact derived from the poly(A)+ population.

To be sure, bona fide poly(A)− mRNAs have been identified. The list of authentic poly(A)− mRNAs include: 1) histone mRNA (Adesnik and Darnell, 1972; Greenberg and Perry, 1972; Grunstein and Schedl, 1976); 2) frog virus 3 mRNA (Willis and Granoff, 1976); 3) reovirus mRNA (Banerjee et al., 1971; Both et al., 1975); 4) a complex population of brain mRNA (Chikaraishi, 1979; Van Ness et al., 1979); and 5) a subpopulation of actin mRNAs (Geoghegan and McCoy, 1986). While the functional significance of mRNA lacking poly(A) is unclear, it is interesting to note that, like poly(A)− histone mRNA, the poly(A)− form of actin message is produced exclusively during the S-phase of the cell cycle (Geoghegan and McCoy, 1986).

**Cytoplasmic poly(A) metabolism**

Following transport of mRNA to the cytoplasm, 3′-poly(A) tracts are gradually shortened, resulting in a heterogenous population of poly(A) tail lengths (Mendecki et al., 1972; Sheiness and Darnell, 1973; Adams and Jeffery, 1978; Palatnik et al., 1979; Jeffery et al., 1981) which range from that of
newly synthesized mRNA [200-250 adenylate residues (Brawerman, 1981)] to a minimum of about 30 nucleotides (Sheiness and Darnell, 1973; Palatnik et al., 1979; Shapiro et al., 1988). This shortening process appears to be most rapid during the first 2-3 hours following transport (Brawerman and Diez, 1975; Merkel et al., 1976; Mercer and Wake, 1985). The steady state distribution of poly(A) tail lengths may be unique for each individual mRNA species (Ahlquist and Kaesberg, 1979; Krowezynska et al., 1985; Mercer and Wake, 1985; Muschel et al., 1986; Steel and Jacobson, 1987), but generally ranges from 50 to 70 adenylate residues in most mammalian cells (Jeffery and Brawerman, 1974; Ahlquist and Kaesberg, 1979; Mercer and Wake, 1985). The characteristic poly(A) tail lengths associated with each mRNA seem to be dependent upon the relative contributions of: 1) a nonspecific cytoplasmic poly(A) shortening activity; 2) a specific cytoplasmic deadenylation activity; 3) a specific cytoplasmic polyadenylation activity; and 4) the decay rate of each mRNA. These phenomena are discussed below.

A) Poly(A) shortening.

Data which support the existence of a uniform, nonspecific poly(A) shortening mechanism includes the following: 1) the average length of 3'-poly(A) associated with total mRNA has been shown to decrease as a function of time in a variety of eukaryotic cells (Sheiness and Darnell, 1973; Jeffery and Brawerman, 1974; Brawerman and Diez, 1975; Merkel et al., 1976; Adams and Jeffery, 1978; Palatnik et al., 1979; 1980; Jeffery et al., 1981); 2) the average poly(A) tail length of specific eukaryotic mRNAs has also been found to decrease as a function of time (Palatnik et al., 1981; Mercer and Wake, 1985; Restifo and Guild, 1986; Rubin and Halim, 1987; Bernstein et al., 1989);
and 3) the average poly(A) tail lengths of stable mRNAs have been found to be, in general, shorter than the average poly(A) tail length of unstable mRNAs in *Dictyostelium discoideum* (Palatnik et al., 1980; Steel and Jacobson, 1987; Manrow et al., 1988; Shapiro et al., 1988) and yeast (Santiago et al., 1987). Furthermore, the spectra of 3'-poly(A) tail lengths associated with several individual, hybrid-selected mRNAs has been accurately determined (Ahlquist and Kaesberg, 1979; Steel and Jacobson, 1987). Purified mRNAs were 3'-end labeled, digested to completion with RNase A and/or T1, and the remaining poly(A) tracts sized on high resolution polyacrylamide gels. The results of these experiments demonstrate that these mRNAs are associated with a complete ladder of poly(A) tail lengths with single base resolution. These data are consistent with poly(A) shortening occurring in a processive manner, i.e., by sequential removal of adenylate residues, one base at a time.

At present, little is known about the biochemistry of poly(A) shortening. Originally, it was reported that, in HeLa cells, poly(A) shortening was not affected by prior treatment with emetine, an inhibitor of translational elongation (Sheiness and Darnell, 1973). Although these results were subsequently confirmed by Merkel et al. (1976), it was later reported that emetine treatment significantly slowed poly(A) shortening in HeLa cells (Sheiness et al., 1975). Likewise, in wheat germ extracts, the inhibition of protein synthesis by a variety of means was found to correlate with decreased rates of poly(A) shortening (Rubin and Halim, 1987). Recently, Sachs and Davis (1989) have demonstrated that depletion of the yeast poly(A)-binding protein (PABP) *in vivo*, by deletion or promoter inactivation, results in a reduction in the rate of translational initiation and in the inhibition of poly(A) shortening, suggesting that this protein may be involved in removal of 3'-adenylate
residues. Conflicting data have been reported by Bernstein et al. (1989); they found that depletion of the PABP actually hastens the rate of poly(A) shortening in vitro. However, these in vitro data can also be interpreted in terms of PABP protecting 3'-poly(A) tracts from attack by nonspecific nucleases which may be present in the in vitro extract. It would not be unreasonable, therefore, to hypothesize that cytoplasmic poly(A) shortening is dependent upon translational activity and may be mediated by the PABP.

B) Cytoplasmic mRNA deadenylation activity.

In addition to poly(A) shortening, some mRNAs may also be subject to poly(A) removal or deadenylation. Poly(A) removal is characterized by the rapid and abrupt deadenylation of specific mRNAs, usually in response to developmental stimuli (Adams and Jeffery, 1978; Jeffery et al., 1981; Colot and Roshbash, 1982; Rosenthal et al., 1983; Palatnik et al., 1984; Restifo and Guild, 1986; Paris et al., 1988; Wilson and Treisman, 1988). The specificity and rapidity of this activity distinguish it from the apparently universal poly(A) shortening activity detailed above. Although both events result in the loss of adenylate residues, and require ongoing translation (discussed below), other similarities, if any, are unknown. Recently, Wilson and Treisman (1988) have investigated the deadenylation of c-fos mRNA in serum stimulated NIH3T3 cells. They found that this deadenylation was inhibited by cycloheximide and required the presence of an AU-rich sequence within the 3'-untranslated region of the c-fos mRNA. Deletion or replacement of this sequence, with a portion of an SV40 mRNA 3'-untranslated region, resulted in an inhibition of deadenylation. These data are consistent with deadenylation (at least in this example) requiring ongoing translation and the recognition of a specific sequence within the 3'-
C) Cytoplasmic mRNA adenylation activity.

In addition to shortening and removal, mRNA poly(A)-tails may also be elongated following transport to the cytoplasm. Cytoplasmic polyadenylation was first observed to accompany sea urchin embryogenesis (Slater et al., 1972; 1973; 1974; Slater and Slater, 1974) and the cytoplasmic replication of mammalian viruses such as vaccinia (Kates, 1970), polio (Yogo and Wimmer, 1972) and vesicular stomatitis virus (Soria and Huang, 1973). Since this time, examples of cytoplasmic polyadenylation have been reported for a great number of mRNAs in a wide variety of cell types (Diez and Brawerman, 1974; Corti et al., 1977; Be'dard and Bradhorst, 1986; Carrazana et al., 1988; Robinson et al., Zingg et al., 1988) and is often associated with the activation of "stored" maternal mRNAs during oogenesis in sea urchins (Slater et al., 1972; 1973; 1974; 1978; Slater and Slater, 1974), Spisula (Rosenthal et al., 1983; Rosenthal and Ruderman, 1987), Urechis caupo (Rosenthal and Wilt, 1986), Xenopus laevis (Dworkin and Dworkin-Rastl, 1985; Colot and Roshbash, 1982; Paris et al., 1988; McGrew et al., 1989), and mice (Huarte et al., 1987; Strickland et al., 1988). Recently, McGrew et al. (1989) have identified a 50 base sequence which apparently directs the polyadenylation of G10 mRNA during Xenopus laevis oogenesis. They found that deletion of this sequence resulted in an inhibition of G10 mRNA adenylation while insertion of this sequence within the 3'-untranslated region of globin mRNA resulted in its adenylation during oogenesis. Other experiments have revealed that this sequence must retain both an AAUAAA hexamer, and an upstream U-rich sequence to direct regulated polyadenylation. The similarity of these sequences with other, well-defined,
sequences required for nuclear polyadenylation (Berget, 1984; Wickens and Stephenson, 1984; Conway and Wickens, 1985; McLauchlan et al., 1985) are intriguing. Although cytoplasmic poly(A) polymerase activity has been isolated in crude preparations from a wide variety of cell types, (reviewed by Tsiapalis, 1987), not much is known about the biochemistry of the enzyme(s) themselves, the biological signals which regulate their behavior, or their relationship (if any) with nuclear poly(A) polymerase activity.

D) Cytoplasmic mRNA adenylation/deadenylation activities are tightly regulated and highly specific.

Although little is known about the signals which target individual mRNAs for adenylation or deadenylation, it is clear that these activities are tightly regulated. The evidence for the existence of such specific regulation include: 1) the adenylation/ deadenylation of individual mRNAs in response to well-defined biological signals (reviewed above); 2) the adenylation of one set of mRNAs simultaneous with the deadenylation of a second set of mRNAs in the same cell at the same time (Colot and Roshbash, 1982; Rosenthal et al., 1983; Dworkin and Dworkin-Rastl, 1985; Rosenthal and Ruderman, 1987, 1987; Paris et al., 1988); and 3) the description of mRNA sequences which are both necessary (Wilson and Treisman, 1988; McGrew et al., 1989) and sufficient (McGrew et al., 1989) for specific, cytoplasmic adenylation or deadenylation reactions. The existence of such intricate regulation of mRNA poly(A) tail length is an indication of the importance of the poly(A) tail.

The cytoplasmic poly(A)-binding protein (PABP)

The mRNAs of every eukaryotic cell are found to be intimately associated
with up to 20 different polypeptide species as ribonucleoprotein complexes (mRNPs) (Irwin et al., 1975; Jeffery, 1977; Mirkes, 1977; Jain and Sarkar, 1979; Greenberg, 1980; Adams et al., 1981; Setyono and Greenberg, 1981; Moon, 1983; Manrow and Jacobson, 1986; 1987). By far, the most widely studied of the mRNP proteins are those that associate with the poly(A) tract of mRNA, the poly(A)-binding proteins (PABPs). First described by Blobel (1973), PABPs have been found in every eukaryotic cell examined thus far. The molecular weight of these PABPs range from 56-89 kD in birds and mammals, with a single 72-80 kD polypeptide being the most prevalent (Blobel, 1973; Irwin et al., 1975; Kish and Pederson, 1976; Jeffery, 1979; Van Venrooij et al., 1977; Mazur and Schweiger, 1978; Jain and Sarkar, 1979; Greenberg, 1980; Grange et al., 1987; Zelus et al., 1989), to 18-68 kD in lower eukaryotes (Mirkes, 1977; Adams et al., De Herdt et al., 1984; Adam et al., 1986; Manrow and Jacobson, 1986; 1987; Sachs et al., 1986). Mammalian PABP has been shown to form a repeating, nucleosome-like structure on mRNA poly(A) tails in the cytoplasm (Baer and Kornberg, 1980; 1983). Monomers, dimers, trimers, and tetramers of PABP complexed with poly(A) have been resolved on sucrose gradients with each monomer consisting of one 75 kD PABP and one 27-residue stretch of poly(A) (Baer and Kornberg, 1980; 1983). Although a nuclear poly(A)-RNP has been detected, it seems to lack the nucleosome-like organization of the cytoplasmic PABP and most likely represents a distinct polypeptide (Setyono and Greenberg, 1981; Baer and Kornberg, 1980; 1983; Sachs and Kornberg, 1985). Subsequent reports have revealed that, at least in yeast, the nuclear PABP (53 kD) is derived from the cytoplasmic PABP (68 kD) by proteolytic cleavage (Sachs and Kornberg, 1985; Sachs et al., 1986). The gene encoding the yeast PABP has been cloned and sequenced revealing the following features of the mRNA and
its encoded polypeptide: 1) an adenosine-rich tract in the mRNA (42 out of 55 nucleotides) immediately 5' of the translation start site; 2) an N-terminal region made up of 4 tandemly arranged, homologous domains, each of which contains an octapeptide consensus sequence found to be conserved among RNA-binding proteins (reviewed by Bandziulis et al., 1989); 3) a C-terminal region which is not included in the nuclear form of PABP; and 4) a proline-rich "hinge" region connecting the N and C-terminal regions (Adam et al., 1986; Sachs et al., 1986). These structural features are conserved in both the human (Grange et al., 1987) and *Xenopus laevis* (Zelus et al., 1989) PABPs. Biochemical analysis of purified yeast PABP has established that: 1) the packing density of PABP is approximately 1 molecule/25 adenylate residues (in striking agreement with the size of poly(A) protected from nuclease digestion by PABP (Baer and Kornberg, 1980; 1983)); 2) the minimum length of poly(A) required for efficient binding to PABP is 12 residues; and 3) PABP can bind to poly(A)_{180-220} and the adenosine-rich domain of the PABP 5'-untranslated region with equal efficiency (Sachs et al., 1986; 1987). Other experiments have demonstrated that the poly(A)-binding domain of PABP resides within each of the 4 N-terminal repeats and that the binding of poly(A) by one domain of PABP promotes dissociation from other domains within the same molecule, suggesting that PABP may move between poly(A) tracts via interstrand exchange (Sachs et al., 1986; 1987). Finally, deletion analyses have shown that the PABP is essential for cell viability and that a 66 amino acid peptide, corresponding to one half of an N-terminal repeat, is sufficient for cell growth (Sachs et al., 1986; 1987). This peptide contained neither the conserved RNA-binding protein octapeptide sequence nor the C-terminal domain of PABP, suggesting that these regions are not required for PABP function. Recent
experiments by Munroe and Jacobson (1989) and Sachs and Davis (1989) have revealed that the PABP is involved in poly(A) shortening and in a late step in translational initiation (discussed below and in Chapter 2).

Translational initiation

Translation in eukaryotes can be divided into 3 distinct phases; initiation, elongation, and termination (reviewed by Austin and Kay, 1982; Kozak, 1983; 1989; Ochoa, 1983; Moldave, 1985; Pain, 1986; Proud, 1986; Rhoads, 1988). Lately, the importance of gene regulation at the level of translational initiation has become more apparent. Translational initiation is a complex multi-step process which, for the purpose of this review, I have subdivided into 4 stages: A) formation of an mRNP, B) formation of the 43S preinitiation complex, C) formation of the 48S preinitiation complex, and D) formation of the 80S initiation complex.

A) Formation of an mRNP.

The mRNAs of every eukaryotic cell are found to be intimately associated with up to 20 different polypeptide species as ribonucleoprotein complexes (mRNPs) (Irwin et al., 1975; Jeffery, 1977; Mirkes, 1977; Jain and Sarkar, 1979; Greenberg, 1980; Adams et al., 1981; Setyano and Greenberg, 1981; Moon, 1983; Manrow and Jacobson, 1986; 1987). Newly formed mRNAs are assembled into mRNP complexes in the cytoplasm (Greenberg, 1981; Greenberg and Carroll, 1985). mRNPs are dynamic structures whose proteins have been shown to freely exchange, in the cytoplasm, with a pool of similar, unassociated proteins (Greenberg, 1981; Greenberg and Carroll, 1985). mRNP complexes, rather than "naked" mRNA, are thought to be the functional form of mRNA in the cell
(Dreyfuss, 1986; Richter and Smith, 1984; Richter, 1988). More specifically, proteins which associate with the mRNA cap or poly(A) tail are known to be required for the efficient initiation of translation (discussed below and in Chapter 2).

B) Formation of a 43S preinitiation complex.

The 43S preinitiation complex consists of ternary complex (eIF-2, GTP, and MET-tRNAf) bound to a 40S ribosomal subunit containing at least 2 additional initiation factors, eIF-3 and eIF-4C (Rhoads, 1988).

Ternary complex formation itself is regulated by another factor, GDP exchange factor (GEF), which consists of 5 polypeptides (Siekierka et al., 1982). GEF is believed to function by converting eIF-2 from its inactive form (eIF-2:GDP) to an active form (eIF-2:GTP) by the displacement of bound GDP and subsequent replacement with GTP (Siekierka et al., 1982). Once "activated" the eIF-2:GTP binary complex is free to interact with MET-tRNAf (Siekierka et al., 1982; Salimans et al., 1984).

The interaction of the ternary complex with the 40S ribosomal subunit is dependent upon the prior association of the 40S subunit with eIF-3 and eIF-4C. These factors are believed to function as ribosome disassociation factors, which prevent the association of 40 and 60S ribosomal subunits and allow the association of the ternary complex with 40S ribosomal subunits (Goumans et al., 1980; Seal et al., 1983).

C) Formation of a 48S preinitiation complex.

The 48S preinitiation complex includes mRNA, the 43S preinitiation complex, ATP, and at least 4 other initiation factors. The association of the
43S preinitiation complex with mRNA requires eIF-4A, eIF-4B, eIF-4E, a 220 kD polypeptide termed p220, and ATP (Rhoads, 1988). eIF-4E, the cap-binding protein, is the only factor known to have specific affinity for mRNA 5'-CAP structures in the absence of ATP (Sonenberg, 1981). In addition, eIF-4E has also been shown to bind to caps even in the presence of excessive secondary structure close to the mRNA 5'-CAP (Pelletier and Sonenberg, 1985). In contrast, eIF-4A can bind caps only in the presence of ATP, p220, and eIF-4E (Sononberg, 1981; Ederg et al., 1983; Grifo et al., 1983). eIF-4A is believed to be an ATP-dependent mRNA unwinding protein. The evidence which supports this conclusion is as follows: 1) eIF-4A can be UV-crosslinked to ATP (Sarkar et al., 1985); 2) eIF-4A has demonstrated ATPase activity (Grifo et al., 1984); 3) eIF-4A has been shown to promote structural changes in reovirus mRNA that result in increased nuclease sensitivity in the presence of ATP (Ray et al., 1985); and 4) eIF-4A has been shown to bind single stranded mRNA in a sequence nonspecific manner (Abramson et al., 1987). The function of p220 is unknown. However, it is known to be required for translation (Tahara et al., 1981; Etchison et al., 1985) and has shown the ability to associate, at least transiently, with both eIF-4A and eIF-4E as a multiprotein complex also known as eIF-4F (Ederg et al., 1983; Grifo et al., 1983). It has been suggested that p220 functions to physically align eIF-4E and eIF-4A, thus promoting cap recognition and RNA unwinding activity respectively (Ray et al., 1985). Other experiments have demonstrated that eIF-4B binds to the eIF-4F/mRNA complex, promoting the release of p220 and allowing the movement of the mRNA relative to the 40S ribosomal subunit in an ATP dependent manner (Kozak, 1980; Ray et al., 1986). These data suggest the following scheme: 1) eIF-4E binds the mRNA 5'-cap; 2) eIF-4A, through its p220-mediated association with
eIF-4E, melts mRNA secondary structure in the vicinity of the cap, allowing the 43S preinitiation complex to bind near the mRNA 5'-end; and 3) eIF-4B associates with the eIF-4F/mRNA complex, causing the release of p220 and allowing the migration of the 40S ribosomal subunit relative to the mRNA.

Following its binding at or near the 5'-cap, the 40S ribosomal subunit is believed to "scan" the mRNA for the appropriate translation start site. First proposed by Sherman et al. (1980), the scanning model predicts that the 40S ribosomal subunit, initially bound at the mRNA 5'-end, migrates along the mRNA and stops at the first AUG in a favorable context for translational initiation (Kozak, 1981; 1984; 1986; 1987a; 1987b; 1989). The predictions of the scanning hypothesis are not, however, absolute. Recent documentation of internal initiation of translation in a eukaryotic system (Pellitier and Sonenberg, 1989) has led to debate over modification of the scanning hypothesis (Kozak, 1989). In any case, 48S preinitiation complex formation is complete when the 40S ribosomal subunit (along with appropriate initiation factors) becomes situated at the "proper" AUG codon.

D) Formation of the 80S initiation complex.

The final step in eukaryotic translational initiation is characterized by the joining of the 60S ribosomal subunit with a complete 48S preinitiation complex. This reaction is initiated by eIF-5, which catalyzes the hydrolysis of eIF-2 associated GTP, causing the release of eIF-2:GDP and eIF-3 from the 40S subunit (Trachsel and Staehelin, 1978; Peterson et al., 1979a; 1979b), thus allowing the association of the 60S and 40S ribosomal subunits. This association is likely to involve the release of a dissociation factor, eIF-6, from 60S ribosomal subunits (Russel and Spremulli, 1979; Valenzuela et al., 1982).
However, it is unknown if this release occurs prior to, or as a consequence of, the joining of the two ribosomal subunits.

Recently, Munroe and Jacobson (1989) and Sachs and Davis (1989) have presented evidence which suggest that the PABP may be involved in the formation of the 80S initiation complex. These data are discussed in Chapter 2.

**Poly(A) function**

Since the discovery of mRNA poly(A) tails, almost two decades ago (Kates, 1970; Lim and Canellakis, 1970; Darnell et al., 1971a; 1971b; Edmonds et al., 1971; Lee et al., 1971), three general hypotheses regarding their function have been proposed. These include a role for poly(A) in: 1) mRNA processing and transport, 2) mRNA stability, and 3) protein synthesis.

**A) Poly(A) and mRNA processing and transport.**

A role for poly(A) in mRNA processing and transport was originally suggested by the results of experiments in 3'-deoxyadenosine (cordycepin) treated cells (Penman et al., 1970; Darnell et al., 1971a; Mendecki et al., 1972). Treatment with cordycepin resulted in the inhibition of nuclear polyadenylation along with a significant reduction in the amount of mature mRNA found in the cytoplasm. However, more recent reports have indicated that mRNAs which have become poly(A)⁺ in cordycepin treated HeLa cells are both correctly spliced (Zeevi et al., 1981; 1982) and effectively transported (Zeevi et al., 1982). In other experiments, poly(A)⁺ in vitro transcripts were found to be efficiently spliced in cell-free HeLa whole-cell extracts (Konarska et al., 1984). In addition, naturally poly(A)⁺mRNAs (such as histone mRNA) are both spliced and transported effectively (Adesnik and Darnell, 1972). Consistent with this,
poly(A)$^+$ and poly(A)$^-$ sea urchin mRNAs have been reported to be transported from the nucleus to the cytoplasm with similar kinetics (Nemer et al., 1975). Finally, while a nuclear role for poly(A) is possible, a cytoplasmic function is strongly suggested by the existence of mammalian viruses which lack nuclear components in their life cycles yet have polyadenylated mRNAs (Armstrong et al., 1972; Ehrenfeld and Summers, 1972; Pridgen and Kingsbury, 1972; Soria and Huang, 1973; Weiss and Bratt, 1974).

B) Poly(A) and mRNA stability.

mRNA poly(A) tail length has also been thought to be a major factor in the determination of mRNA decay rates. Early evidence in support of this hypothesis include experiments in which poly(A)$^+$ and poly(A)$^-$ mRNAs were microinjected into Xenopus oocytes (Huez et al., 1974; Marbaix et al., 1975). These experiments compared the stability of native rabbit globin mRNA to that which had been deadenylated by processive phosphorolysis with polynucleotide phosphorylase. It was found that the native mRNA was significantly more stable than the deadenylated mRNA. These results were subsequently confirmed by other experiments in microinjected oocytes (Nudel et al., 1976; Huez et al., 1977; Drummond et al., 1985) and in cordycepin-treated HeLa cells (Zeevi et al., 1981; 1982). However, similar microinjection experiments with different poly(A)$^+$ and poly(A)$^-$ mRNAs have not supported these data (Sehgal et al., 1978; Deshpande et al., 1979; McCrae and Woodland, 1981; Huez et al., 1983; Galili et al., 1988). Most of the studies suggesting a direct relationship between poly(A) tail length and mRNA stability have made use of normal, poly(A)$^+$ mRNAs which have been rendered poly(A)$^-$ or had their poly(A) tails shortened to well below steady state length. Such an
approach is questionable because: 1) in general, poly(A) deficient mRNAs are a unique species not derived from a poly(A)$^+$ precursor (discussed above) and 2) poly(A) tails do not normally shorten below a length of 30 nucleotides (discussed above). Such "trimming" of poly(A) below this minimum size may affect some other property which, in turn, makes mRNA less stable. Consistent with this, it has been demonstrated that globin mRNA, with a minimum poly(A) tail length of 30 3'-adenylate residues, is just as stable as native globin mRNA when microinjected into *Xenopus* oocytes (Huez et al., 1975; Nudel et al., 1976). Other experiments suggesting a direct relationship between poly(A) tail length and mRNA stability are those demonstrating the functional stabilization of poly(A)$^-$ histone mRNAs by the addition of a 50 nucleotide poly(A) tail (Huez et al., 1978). However, the demonstration by Graves et al. (1987) that histone mRNA degradation requires a 3'-end stem-loop structure, along with a myriad of reports correlating poly(A) tail length with translational efficiency (described below), makes such an interpretation of these results questionable.

Other studies examining the relationship between mRNA poly(A) tail length and stability are just as controversial. For example, increases in the decay rate of several mRNAs have been found to correlate with their deadenylation in vivo (Colot and Roshbash, 1982; Mercer and Wake, 1985; Restifo and Guild, 1986; Wilson and Treisman, 1988; Gallie et al., 1989). These results are consistent with poly(A) shortening proceeding mRNA destabilization. Similarly, it has been reported that the decay of many mRNAs are preceded by deadenylation in cell-free extracts (Brewer and Ross, 1988; Brenstein et al., 1989). However, if poly(A) shortening was directly related to mRNA stability then stable mRNAs should exhibit biphasic decay kinetics. Such a relationship is, in general, not found (Palatnik et al., 1979; 1980; Shapiro et al., 1988; Herrick et al., 1989). In
addition, there have been several in vivo reports documenting: 1) poly(A) tail loss in the absence of mRNA destabilization (Nemer et al., 1975; Krowczynska et al., 1985; Swartwout et al., 1987; Paris et al., 1988) and 2) the destabilization of tissue-type plasminogen activator mRNA following polyadenylation in mouse oocytes (Huarte et al., 1987; Strickland et al., 1988). Finally, while poly(A) tail length may contribute to the stability of some mRNAs, a direct relationship between poly(A) tail length has been ruled out by experiments examining the poly(A) tail length distribution of various stable and unstable mRNAs in Dictyostelium discoideum (Palatnik et al., 1980; Manrow et al., 1988; Shapiro et al., 1988) and yeast (Santiago et al., 1987).

C) Poly(A) and protein synthesis.

Soon after their discovery, the possibility that 3'-poly(A) tails might play a role in translation was extensively tested and "ruled out" by experiments which compared the in vitro translational capacity of adenylated and deadenylated forms of various mRNAs. It was generally concluded that artificial deadenylation (Bard et al., 1974; Sippel et al., 1974; Soreq et al., 1974; Williamson et al., 1974; Spector et al., 1975) or blockage of the poly(A) tail with poly(U) (Munoz and Darnell, 1974) did not significantly reduce the translatability of mRNA. Support for a translational role for poly(A) was subsequently resurrected by Doel and Carey (1976) who noted that the translation systems used in the previous studies were relatively insensitive due to a poor efficiency of reinitiation. Doel and Carey (1976) were able to show that while poly(A)+ ovalbumin mRNA translated more efficiently than its deadenylated counterpart in a highly active reticulocyte extract, no such difference was seen in less active wheat germ extracts. This translational
discrimination was attributed to a reduced rate of initiation on the poly(A)-mRNA since this mRNA was found on smaller polysomes and participated in fewer rounds of translation. Results from a variety of experimental approaches have provided additional evidence which implicate a role for poly(A) in translation. These data can be interpreted in terms of a translational model for poly(A) function (Jacobson and Favreau et al., 1983; Palatnik et al., 1984; Chapter 2) which postulates that: 1) an interaction between the 3'-poly(A) tract of mRNA and a cytoplasmic poly(A)-binding protein (PABP) enhances the efficiency of translational initiation at the mRNA 5'-end; 2) this interaction is directly dependent upon the length of the poly(A) tail such that newly synthesized mRNA with relatively long poly(A) tails has a translational advantage over steady-state mRNA with its relatively short poly(A) tails; 3) this interaction is not essential for translation; and 4) the regulatory mechanisms which ensure the efficient translation of poly(A)+ and poly(A)-mRNAs may be quite different. The data in support of this model are discussed below and include: 1) correlations between the adenylation status of mRNA and its translatability in vitro and in vivo; 2) demonstrations of exogenously added poly(A) as a potent inhibitor of the translation of poly(A)+ but not poly(A)-mRNA in vitro; and 3) correlations between the stability and abundance of PABPs and the rate of translational initiation.

mRNA adenylation status correlates with translational efficiency in vitro and in microinjected Xenopus oocytes

As noted above, Doel and Carey (1976) provided the first evidence linking 3'-poly(A) with translational efficiency. They were able to show that native
poly(A)$^+$ ovalbumin mRNA possessed a greater translational capacity than ovalbumin mRNA whose poly(A) tail had been selectively removed with polynucleotide phosphorylase. The polyadenylated ovalbumin mRNA was found on larger polysomes and participated in a greater number of rounds of translation per message than did the deadenylated mRNA in cell-free rabbit reticulocyte lysates. These results were confirmed by Rubin and Halim (1987) who determined that the efficiency of globin polypeptide synthesis, in reticulocyte lysate or wheat germ extract, is directly proportional to mRNA poly(A) tail length. Likewise, the translatability of mRNAs microinjected into Xenopus oocytes or transferred to plant protoplasts by electroporation has been found to correlate with adenylation status. For example, Deshpande et al. (1979) reported that native $\alpha_2u$-globin mRNA [average poly(A) tail length, 175] was translated more efficiently, and reached peak translatability faster, than poly(A)-poor $\alpha_2u$-globin mRNA [average poly(A) tail length, 40] in microinjected Xenopus oocytes. Drummond et al. (1985) found that synthetic poly(A)$^+$ SP6 mRNAs coding for chicken lysozyme, calf preprochymosin, and Xenopus $\beta$-globin were translated 5-20 fold more effectively than poly(A)$^-$ transcripts. Galili et al. (1988) have demonstrated a similar translational discrimination following microinjection of synthetic maize zein or Xenopus $\beta$-globin transcripts into Xenopus oocytes. Furthermore, they reported that the adenylated mRNAs were found on polysomes with a larger average number of ribosomes than their unadenylated counterparts, indicating that the poly(A)$^-$ mRNAs were deficient in translational initiation. In other experiments, synthetic polyadenylated transcripts, encoding ribosomal protein L1, were found to be actively translated following microinjection into stage VI oocytes. Upon oocyte maturation these mRNAs became deadenylated and released from
polysomes, paralleling the deadenylation and translational inactivation of endogenous ribosomal protein mRNAs (Hyman and Wormington, 1988). Recently, Gallie et al. (1989) have investigated the translational role of 3'-poly(A) tracts in plants. They found that a synthetic poly(A)$^+$ (50 adenylate residues) α-glucuronidase mRNA is translated 16-120 fold more effectively than a similar poly(A)$^-$ mRNA in electroporated tobacco, carrot, maize, and rice protoplasts.

mRNA poly(A) tail length correlates with translatability in vivo

There are also many examples of mRNA adenylation status correlating with translational efficiency in vivo. The earliest of these studies involved the separation of polysomal sea urchin nonhistone mRNAs into poly(A)$^+$ and poly(A)$^-$ fractions (Nemer et al., 1975). The results of these experiments indicated that poly(A)$^+$ mRNAs were more fully loaded with ribosomes than poly(A)$^-$ mRNAs. Perhaps the most well characterized in vivo correlation of translational efficiency and polyadenylation status involves the fate of 5 developmentally regulated mRNAs during oogenesis in Spistula (Rosenthal et al., 1983). Four of these mRNAs are poly(A)$^-$ and translationally inactive in the oocyte. Following fertilization, these mRNAs became adenyliated and simultaneously recruited onto polysomes. In contrast, a fifth message, α-tubulin, was found to become deadenylated coincident with its exclusion from polysomes. There are numerous other examples of mRNA poly(A) tail length correlating with translational efficiency in species as diverse as Dictyostelium discoideum and rats. These examples have been summarized in Table 1.

The correlation between poly(A) tail length and translational efficiency in vivo is not perfect. There are also examples of translationally inactive
polyadenylated mRNAs (Raff, 1980; Rosenthal et al., 1983) as well as examples of mRNAs which appear to lose their poly(A) tracts as they become translationally active (Hruby and Roberts, 1977; Iatrou and Dixon, 1977; Kleene, 1989).

**Poly(A) is a competitive inhibitor of translational initiation**

An alternative approach to assessing the role of poly(A) in translation was taken by Jacobson and Favreau (1983). It was reasoned that if 3'-poly(A) tracts did function in protein synthesis then exogenous poly(A) might competitively inhibit the in vitro translation of poly(A)$^+$ mRNAs in much the same way that 5'-CAP structures have been shown to inhibit the in vitro translation of capped mRNAs (Roman et al, 1976; Hickey et al., 1976). They found that exogenous poly(A) inhibited the translation of poly(A)$^+$, but not poly(A)$^-$, mRNAs in reticulocyte lysates and that comparable inhibition was not observed with other ribopolymers (Jacobson and Favreau, 1983). This poly(A)-mediated inhibition was dependent upon the size of the competitor poly(A) and could be overcome by increased mRNA concentrations or by translating mRNPs instead of mRNA. Furthermore, poly(A) mediated inhibition was not found to affect the average size of the polypeptides synthesized, suggesting that this inhibition occurs at the level of translational initiation. These observations have been confirmed by others in reticulocyte lysates (Bablanian and Banerjee, 1986; Grossi de Sa et al., 1988), L-cell lysates (LeMay and Millward, 1986), dry pea seed extracts (Sieliwanowicz, 1987) and in *Xenopus* oocytes (Drummond et al., 1985). The most straightforward and generally accepted interpretation of
these results is that exogenous poly(A) inhibits translation by limiting the availability of unbound PABP. Consistent with this interpretation, the addition of purified PABP has been reported to overcome poly(A)-mediated translational inhibition in reticulocyte lysates (Grossi de Sa et al., 1988).

The abundance and stability of PABPs correlates with translational efficiency in vivo and in vitro

The results of experiments examining the effect of exogenous poly(A) on translation (discussed above) suggest that the role of poly(A) in protein synthesis is mediated by the PABP. Other data supporting this interpretation have been provided by Manrow and Jacobson (1986; 1987). They found that during the first 30 minutes of *Dictyostelium discoideum* development, when translational initiation is sharply reduced (Cardelli et al., 1981), the PABPs are selectively degraded (Manrow and Jacobson, 1986). The turnover of the PABPs was found to increase 20-25 fold at this time, resulting in an 80-90% decrease in their relative abundance. In related experiments (Manrow and Jacobson, 1987), similar reductions in PABP stability and abundance were found to accompany a heat-shock induced reduction in translational initiation. In contrast, the level of PABPs was not affected by reductions in the rate of translational elongation (Manrow and Jacobson, 1987). These results have been confirmed by Sieliwanowiez (1987) who found that the abundance of the 60kD dry pea seed PABP correlates with translational efficiency in an in vitro translation system derived from the embryo axes of dry pea seeds.
Prospectus

Although the data discussed above are consistent with a translational role for poly(A), and its associated protein(s), most of the evidence is indirect. In the following section I describe a set of experiments designed to test directly the hypothesis that poly(A) is involved in translational initiation. I have compared the relative efficiency with which synthetic mRNAs, differing solely in poly(A) tail length, are translated, degraded, recruited into polysomes, and assembled into mRNPs or intermediates in the translational initiation pathway in an mRNA-dependent rabbit reticulocyte cell-free translation system. In addition, I have also re-evaluated the effects of exogenous, competitor poly(A) on the in vitro translation of these mRNAs. My results demonstrate that poly(A) is involved in a late step in translational initiation. The data are consistent with poly(A) being 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.
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*A. Adenylation of mRNA correlating with recruitment onto polysomes. B. Deadenylation of mRNA correlating with exclusion from polysomes.*
**Introduction**

Most eukaryotic mRNAs have a sequence of polyadenylic acid [poly(A)] at their 3'-termini (Kates, 1970; Lim and Canellakis, 1970; Darnell et al., 1971; Edmonds et al., 1971; Lee et al., 1971). These poly(A) "tails" are added post-transcriptionally in the nucleus with an initial length, in mammalian cells, of approximately 200-250 adenylate residues (Brawerman, 1981). Following transport of mRNA to the cytoplasm, poly(A) tracts are gradually shortened such that, in steady-state, poly(A) tail lengths are heterogeneous, ranging from 50-70 A's (Sheiness and Darnell, 1973; Jeffery and Brawerman, 1974; Brawerman and Diez, 1975; Brawerman, 1976; Adams and Jeffery, 1978; Palatnik et al., 1979). Although it has been almost two decades since the discovery of these poly(A) tracts, their function(s) have yet to be clarified. Earlier results from our laboratory (Jacobson and Favreau, 1983; Palatnik et al., 1984; Manrow and Jacobson, 1986, 1987; Shapiro et al, 1988) 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 poly(A)$^+$, but not poly(A)$^-$, mRNAs. The results of several different experimental approaches have provided evidence which indirectly supports this hypothesis. These results include: 1) the correlation of specific changes in mRNA poly(A) tail length with translational efficiency following fertilization in Spisula oocytes (Rosenthal et al., 1983), during early development in Dictyostelium discoideum (Palatnik et al., 1984), oocyte maturation in Xenopus (Hyman and Wormington, 1988; McGrew et al, 1989), oocyte activation in mice (Huarte et al., 1987; Strickland et al., 1988), salivary gland development in Drosophila
melanogaster (Restifo and Guild, 1986), and in response to physiologic stimuli in the rat hypothalamus (Carrazana et al., 1988; Robinson et al., 1988; Zingg et al., 1988); 2) the higher translational activity of poly(A)$^+$, as opposed to poly(A)$^-$, mRNAs in vitro as demonstrated in systems as diverse as cell-free extracts derived from rabbit reticulocytes (Doel and Carey, 1976; Rubin and Halim, 1987) and wheat germ (Rubin and Halim, 1987), as well as in microinjected Xenopus oocytes (Deshpande et al., 1979; Galili et al., 1988; Hyman and Wormington, 1988) and electroporated tobacco protoplasts (Gallie et al., 1989); 3) a correlation between the abundance and stability of PABPs and the rate of translational initiation in developing or heat-shocked Dictyostelium discoideum amoebae (Manrow and Jacobson, 1986, 1987); 4) 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 (Jacobson and Favreau, 1983; Bablanian and Banerjee, 1986; Grossi de Sa et al., 1988), wheat germ (Bablanian and Banerjee, 1986), L-cell (Lemay and Millward, 1986) and pea seed (Sielawanowicz, 1987) extracts; and 5) the demonstration that purified poly(A)-binding protein can stimulate translation in vitro (Sielawanowicz, 1987).

The experiments in this paper 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 are translated, degraded, recruited into polysomes, and assembled into mRNPs or intermediates in the translational initiation pathway in a reticulocyte cell-free translation system. In addition, we have re-evaluated the effects of exogenous, competitor poly(A) in vitro, 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.

Results

Synthesis and characterization of in vitro transcripts

To test the hypothesis that poly(A) may be involved in protein synthesis, we have compared the relative efficiency with which synthetic mRNAs, differing only in poly(A) tail length, are recruited into polysomes and translated in a cell-free rabbit reticulocyte extract. Synthesis of mRNAs which differ in poly(A) tail length required the construction of derivatives of plasmid pSP65 (Melton et al., 1984) which contain dA:dT insertions of different length between the Pst I and Hind III sites of the polylinker (see Figure 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 (Figure 1B). Because plasmid replication in conventional bacterial hosts (such as HB101) often resulted in deletions within the dA:dT region (Figure 1B; lane 6), we have isolated a mutant bacterial host, HB101A, which allows the replication of plasmids containing long dA:dT tracts without deletion (DM and AJ, unpublished results). cDNAs encoding rabbit β-globin (RBG) mRNA or vesicular stomatitis virus N (VSV.N) mRNA were cloned within the polylinker regions of pSP65A plasmids containing 0-68 dA:dT residues. Linearization of these
vectors with Hind III, followed by in vitro transcription, leads to the synthesis of RBG or VSV.N mRNAs which terminate in specific lengths of poly(A). In part, the validity of conclusions drawn from experiments using such in vitro transcripts rests upon our ability to demonstrate the structural integrity of these mRNAs. Thus, the length of poly(A) encoded by each template plasmid has been defined by gel electrophoretic analysis of the RNase A and T1 digestion products from each corresponding transcript (Figure 1C) and the fraction of each transcript which contains poly(A) has been assessed by binding to oligo(dT)-cellulose. The data of Figure 1D show that virtually all of the RBG mRNA containing a 68 nucleotide poly(A) tail is retained by oligo(dT)-cellulose while its poly(A)- counterpart is not. Figure 1D also shows that each RNA species is represented by a single band on a denaturing polyacrylamide gel, indicating that these RNA preparations are homogenous and full length representations of their respective DNA templates. Because of their importance in translation, the 5'-terminal structures of the synthetic transcripts were also analyzed. TLC analysis of the RNase A, T1, and T2 digestion products of a truncated transcript (Figure 1E) indicates that capping of in vitro transcripts occurs with over 95% efficiency. Similar experiments have been performed with full length RBG transcripts and have yielded similar results (data not shown). Together, these data indicate that each preparation of mRNA is homogenous 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 (Doel and Carey, 1976; Deshpande et al, 1979; Drummond et al, 1985; Galili et al, 1988). Our experiments differ from these earlier studies in that we have used synthetic
mRNAs which closely resemble their in vivo counterparts, i.e.: a) the poly(A)+ mRNAs used here contain homogeneous poly(A) tracts equivalent to steady-state lengths, b) the poly(A)+ and poly(A)- mRNAs used here only differ in poly(A) sequences and not other mRNA sequences, and c) 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 35S-methionine into VSV.N polypeptide in rabbit reticulocyte lysates. Figures 2A and 2B show that, over a broad range of mRNA concentrations, capped, poly(A)+ (61A’s) VSV.N mRNA has 1.4-2.5 times the translational activity of capped, poly(A)- VSV.N mRNA, 1.4-1.9 times the activity of uncapped, poly(A)+ (61A’s) VSV.N mRNA, and 3.3-4.0 times the activity of uncapped, poly(A)- VSV.N mRNA. These data show that: a) poly(A)- mRNAs have a reduced translational capacity which is comparable to that of uncapped mRNAs and b) 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 (Figure 2C). 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 of Figure 2 demonstrate that the 3'-poly(A) tail of mRNA is an important determinant of translational efficiency. 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 otherwise identical mRNAs lacking caps and/or poly(A) tails. $^{32}P$-labeled, capped and polyadenylated 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 15-50% sucrose gradients (Figure 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: a) labeling the C-RBG-68A mRNA with $^3$H and the competitor transcript with $^{32}P$ yielded similar results (data not shown) and b) mixing $^{32}P$-labeled C-RBG-68A mRNA with $^3$H-labeled C-RBG-68A mRNA yielded superimposable polysome profiles (Figure 3A). Figures 3B and 3C show that poly(A)$^+$ rabbit $\beta$-globin and VSV.N mRNAs are both recruited into polysomes to a greater extent than their poly(A)$^-$ counterparts. A comparison of the ratio of poly(A)$^+$:poly(A)$^-$ mRNA across the polysomal region of these gradients (insets, Figures 3B and 3C) reveals that those poly(A)$^-$ mRNAs which do form polysomes are 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 is comparable to that observed with cap-deficient mRNA (compare Figures 3B and 3C with 3D). Collectively, these data indicate that poly(A)⁻ mRNAs have a reduced rate of translational initiation. This conclusion has been confirmed by experiments which show that, in vitro, the translational elongation rate on capped, VSV.N mRNA with a 61 nucleotide poly(A) tail is identical to that of poly(A)⁺, capped VSV.N mRNA (2.9 aa/sec at 37°C; DM and AJ, unpublished observations). mRNA lacking both a poly(A) tail and a 5’-cap shows a polysome profile (Figure 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 of Figures 2A and 2B and suggests that the 5’-cap and 3’-poly(A) tail affect different events in the translational initiation pathway.

Figure 3F summarizes a series of experiments in which we compared the extent of polysome formation of capped rabbit β-globin mRNAs, differing only in poly(A) tail length, to that of a capped rabbit β-globin transcript containing 68 3’-adenylate residues. The data indicate that a minimal poly(A) length (>5A’s) is required for maximum recruitment of mRNA into polysomes. Beyond this minimal poly(A) length, 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 5-32 adenylate residues.

**Poly(A)⁻ mRNAs are deficient in 80S initiation complex formation**

The results of experiments shown in Figure 3 demonstrate that poly(A) functions in the initiation of translation. Next, we wished to determine the stage of translational initiation that poly(A) was involved in. Eukaryotic
translational initiation can be divided into four stages, three of which are mRNA dependent: 1) formation of an mRNP, 2) the association of the mRNP with the 43S pre-initiation complex (40S ribosomal subunit, met-tRNA<sub>f</sub>, GTP, and various initiation factors) to form a 48S pre-initiation complex, and 3) formation of an 80S initiation complex by the binding of the 60S ribosomal subunit to the 48S pre-initiation complex. In Figures 4 and 5 we use UV cross-linking, sucrose gradient fractionation, and various inhibitors of eukaryotic translation to compare the mRNPs, 48S pre-initiation complexes, and 80S initiation complexes formed by poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RBG mRNAs.

EDTA is a Mg<sup>++</sup>-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 68A's) sediment faster in sucrose gradients than their unadenylated counterparts (Figure 4A). Comparable differences have been observed in the sedimentation rates of mRNPs formed by poly(A)<sup>+</sup> and poly(A)<sup>-</sup> VSV.N mRNAs (DM and AJ unpublished observations). These results suggest that poly(A)<sup>+</sup> mRNAs form mRNPs that have a greater protein content and/or different secondary structure than those formed by poly(A)<sup>-</sup> mRNAs, a conclusion consistent with previous measurements of the buoyant densities of L-cell histone and non-histone mRNPs (Greenberg, 1979). In an effort to characterize poly(A)<sup>+</sup> and poly(A)<sup>-</sup> mRNP structures further, we have utilized a label transfer procedure to identify individual proteins which bind specifically to poly(A)<sup>+</sup> and poly(A)<sup>-</sup> RBG mRNAs. RBG mRNA labeled with α<sup>-</sup>AT<sub>32</sub>p was introduced to lysates and irradiated with UV light to cross-link bound mRNP proteins to the mRNA. Following RNase digestion, the labeled proteins were examined by gel electrophoresis (Figure 5). The results of this
experiment indicate that poly(A)$^+$ mRNPs contain a greater amount of protein and a larger number of different polypeptides than poly(A)$^-$ mRNPs (Figure 5A). The observed differences in protein content between poly(A)$^+$ and poly(A)$^-$ mRNPs can be attributed primarily to polypeptides that associate with poly(A) because labeling of these polypeptides is: 1) not detected following RNase T2 digestion (Figure 5A); 2) not detected in experiments in which the mRNA has been isotopically labeled with GTP (data not shown), CTP or UTP (Figure 5B); 3) found to increase with increasing poly(A) tail length (Figure 5C); and 4) reduced in the presence of exogenous, competitor poly(A) (Figure 5D). Previous studies have indicated that the PABP associated with the poly(A) tracts of rabbit reticulocyte mRNAs has a molecular weight of 78kd (p78; Greenberg and Carroll, 1985). In the experiments of Figures 5A-D we have detected a 78kd polypeptide associated with poly(A), as well as proteins of higher molecular weights. It is likely that the latter represent multimers of PABP bound to the same poly(A) tract (Baer and Kornberg, 1980; 1983).

In Figure 4B we have compared the ability of poly(A)$^+$ and poly(A)$^-$ mRNAs to form 48S pre-initiation complexes in edeine-treated reticulocyte lysates. Edeine is a small basic peptide antibiotic that inhibits association of the 60S ribosomal subunit with 48S pre-initiation complexes resulting in the accumulation of 48S pre-initiation complexex (Obrig et al., 1971; Kozak and Shatkin, 1978; Schneider et al, 1984). In Figure 4B we demonstrate that poly(A)$^+$ (68A's) and poly(A)$^-$ RBG mRNAs are capable of forming 48S pre-initiation complexes with equal efficiency in an edeine-treated reticulocyte lysate (Figure 4B), although the complex formed by poly(A)$^-$ RBG mRNA sediments slightly more slowly than that formed by poly(A)$^+$ mRNA. This
difference in sedimentation rate most likely reflects the greater protein content of poly(A)$^+$ mRNPs observed in Figures 5A-D. 48S pre-initiation complex formation has been shown previously to be dependent upon cap-binding proteins (Rhoads, 1988). Therefore, we have examined the cap-binding proteins which can be UV cross-linked to poly(A)$^+$ and poly(A)$^-$ mRNAs in reticulocyte lysates using mRNA labeled specifically in the 5'$\prime$-cap. The data indicate that RBG (Figure 5E) and VSV.N (DM and AJ, unpublished observations) mRNAs associate with similar sets of cap-binding proteins, regardless of adenylation status. Therefore, the experiments in Figures 4B and 5E establish that poly(A) is not necessary for the formation of 48S pre-initiation complexes.

In Figures 4C-E we have compared the ability of poly(A)$^+$ and poly(A)$^-$ RBG and VSV.N mRNAs to form 80S initiation complexes in anisomycin- or 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 (Rose and Lodish, 1976; Schneider et al., 1984). In reticulocyte lysates treated with anisomycin (Figure 4C) or cycloheximide (Figures 4D and E) poly(A)$^+$ RBG or VSV.N mRNAs accumulated as 80S initiation complexes with over twice the efficiency of their poly(A)$^-$ counterparts. 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 (Figures 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. Moreover, since 48S pre-initiation complex formation is cap-dependent, a role for poly(A) in the formation of 80S initiation complexes, but not 48S pre-
initiation complexes, is consistent with our observations on the additivity of cap and poly(A) associated defects (Figures 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 (Jacobson and Favreau, 1983). This poly(A)-mediated inhibition is likely to occur 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 (Hickey et al, 1976; Roman et al, 1976). Surprisingly, Jacobson and Favreau (1983) found that exogenous poly(A) could also stimulate (140-300%) the in vitro translation of poly(A)$^-$ mRNAs. We have extended these observations by monitoring the effects of exogenous poly(A) or poly(C) on the translation of VSV.N mRNAs differing in 5'-cap structure and/or 3'-poly(A) tail (Figure 6). As expected from previous experiments, translation of capped, polyadenylated (61A's) VSV.N mRNA was inhibited 50% by exogenous poly(A) at 30ug/ml (Figure 6A) while similar concentrations of control polynucleotide [poly(C)] had no effect (Figure 6B). The absence of a cap on poly(A)$^+$ VSV.N mRNA increased its sensitivity to inhibition by added poly(A) significantly (Figure 6A), but only increased its sensitivity to added poly(C) slightly (Figure 6B). In contrast, the translation of capped, poly(A)$^-$ VSV.N mRNA was stimulated almost 40% by low concentrations (5-10µg/ml) of poly(A), but not significantly affected by poly(C) (Figure 6). The same concentration of poly(A) that gives maximal stimulation of translation on capped, unadenylated VSV.N
mRNA (10μg/ml) was found to inhibit, by more than 85%, the translation of uncapped, unadenylated mRNA (Figure 6A). These data are consistent with the additivity of the initiation defects of cap-deficient and poly(A)-deficient mRNAs observed above (Figures 2 and 3) and also suggest that, in the case of poly(A)+ mRNAs that are capped, poly(A) may be capable of working in trans to augment translational initiation. Moreover, these results imply that event(s) in translational initiation involve the recognition of both 3'-poly(A) and 5'-cap structures (and/or their associated proteins).

Discussion

We have postulated that the 3'-poly(A) tail of eukaryotic mRNA facilitates translational initiation through an interaction with the cytoplasmic poly(A)-binding protein (PABP) (Jacobson and Favreau, 1983; Palatnik et al., 1984; Manrow and Jacobson, 1987). The data to support this hypothesis have been mostly indirect, including: a correlation between the adenylation status of individual mRNAs and their translatability in vivo or in vitro (Humphries et al., 1974; Doel and Carey, 1976; Deshpande et al., 1979; Rosenthal et al., 1983; Palatnik et al., 1984; Bird et al., 1985; Drummond et al., 1985; Wells and Kedes, 1985; Restifo and Guild, 1986; Huarte et al., 1987; Rubin and Halim, 1987; Sieliwanowicz, 1987; Carranza et al., 1988; Galili et al., 1988; Robinson et al., 1988; Shapiro et al., 1988; Strickland et al., 1988; Hyman and Wormington, 1988; Zingg et al., 1988; McGrew et al., 1989), the demonstration that exogenously added poly(A) is a potent competitive inhibitor of the translation of poly(A)+, but not poly(A)- mRNAs in vitro (Jacobson and Favreau, 1983; Bablanian and Banerjee, 1986; Lemay and Millward, 1986; Grossi
de Sa et al., 1988), and a correlation between the abundance and stability of the Dictyostelium poly(A)-binding proteins and the rate of translational initiation in vivo (Manrow and Jacobson, 1986; 1987). To test our hypothesis directly, we have constructed a set of plasmids which serve as templates for the synthesis of synthetic rabbit β-globin or VSV N mRNAs which differ only in their respective poly(A) tail lengths. Using highly active reticulocyte lysates, such mRNAs were then compared with respect to their efficiencies of translation, rates of decay, and recruitment into polysomes, mRNPs, or intermediates in the translational initiation pathway.

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 (Figure 2A). Consistent with this observation, poly(A)+ VSV.N and rabbit β-globin mRNAs are found on larger polysomes and recruited into polysomes to a greater extent than their poly(A)− counterparts (Figure 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 (Figure 2B), extents of capping (Figure 1E), or translational elongation rates (data not shown), we conclude that they differ in their respective 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)− RBG or VSV.N mRNAs (Figures 4C-E). The reduced efficiency of 80S initiation complex formation by poly(A)− mRNA is
quantitatively consistent with its reduced efficiencies of translation and ribosome recruitment observed in Figures 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 (Figures 2A and 3). Our results are also consistent with those of Nelson and Winkler (1987) 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 (Doel and Carey, 1976; Rosenthal et al., 1983; Galili et al., 1988; Hyman and Wormington, 1988). In other studies the ratios of the translational activities of poly(A)+ and poly(A)− mRNAs have varied from the twofold effect seen here (Doel and Carey, 1976) to significantly larger effects (5-120-fold; Drummond et al., 1985; Gallie et al., 1989; Hyman and Wormington, 1988; DM and AJ, unpublished observations). The latter occur in in vivo systems with significantly higher overall 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 re-initiation (Doel and Carey, 1976; Deshpande et al., 1979; Palatnik et al., 1984; Galili et al., 1988).

Although a role for poly(A) in translational initiation is supported by the studies reported here (as well as those discussed above), 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 (Raff, 1980; Rosenthal et al., 1983) as well as examples of mRNAs which appear to lose their poly(A) tracts when going from
translationally inactive to active forms (Iatrou and Dixon, 1976; Kleene and Flynn, 1987; Kleene, 1989). Likewise, since individual poly(A)$^-$ mRNAs are known to be translated in vivo (Adesnik and Darnell, 1972; Greenberg and Perry, 1972), 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.

The effect of poly(A) on translation is directly related to its length

The experiments of Figure 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. Efficient recruitment of mRNA into polysomes requires a poly(A) tail of greater than 5 adenylate residues and increases most substantially between poly(A) lengths of 5 to 32 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 size (Jacobson and Favreau, 1983). The minimal size required for either activity (ribosome recruitment or competitive inhibition) is similar to that protected by a monomer of poly(A)-binding protein (PABP) (Baer and Kornberg, 1980; Sachs et al., 1987), 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. 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 (Drummond et al., 1985). 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 50-70 nucleotides [Sheiness and Darnell, 1973; Jeffery and Brawerman, 1974; Brawerman and Diez, 1975; Brawerman, 1976; Adams and Jeffery, 1978; Palatnik et al., 1980]) 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 (Palatnik et al., 1984; Shapiro et al., 1988). 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 patterns can be accomplished without significant mRNA synthesis or turnover (Palatnik et al., 1984).

The translational function of poly(A) is mediated by the cytoplasmic PABP

Previous experiments using a variety of eukaryotic cells have utilized UV-crosslinking to identify cytoplasmic poly(A)-binding proteins (PABPs) (Greenberg, 1979; 1980; Adam et al., 1986; Manrow and Jacobson, 1986) which, in mammalian cells, have molecular weights of 73-78 kd (Blobel, 1973; Greenberg, 1980). In the experiments of Figure 5, UV-crosslinking and label transfer was used to characterize the proteins bound in vitro to mRNAs of different poly(A) length. These experiments showed that the major difference in the RNP proteins associated with poly(A)+ or poly(A)- mRNAs was due to proteins associated with poly(A). Although proteins larger than PABP monomers were identified in these experiments, their molecular weights are suggestive of PABP dimers and trimers (Baer and Kornberg, 1980; 1983). 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 (Sachs et al., 1987) 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: a) in Dictyostelium discoideum, we have shown that there is a correlation between the abundance and stability of the poly(A)-binding proteins and the rate of translational initiation (Manrow and Jacobson, 1986, 1987); b) Sieliwanowicz (1987) has shown that the poly(A)-binding protein isolated from germinated pea embryo axes stimulates translation from poly(A)+ mRNAs in vitro; and c) Sachs and Davis (1989) have shown that depletion or thermal inactivation of the PABP in yeast leads to the inhibition of translation.

**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 (Ptashne, 1986; 1988), 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 (Ptashne, 1986; 1988). Similarly, we predict that the 3' and 5'-ends of mRNA interact during the formation of 80S initiation complexes. Evidence in support of such an arrangement includes: 1) secondary structure maps of rabbit α-globin (Henndell et al., 1978) and murine β-globin (Lockard et al., 1986) mRNAs which suggest that the 5' and 3' domains of these mRNAs are in close proximity; 2) electron micrographs of circular or "folded-back" polysomes
(Warner et al., 1962; Dubochet et al., 1973; Hsu and Coca-Prados, 1979; Ladhoff et al., 1981; Christensen et al., 1987); 3) suppression of a PABP mutation in Saccharomyces cerevisiae by a mutation in ribosomal protein L46 (Sachs and Davis, 1989); and 4) our finding that exogenous poly(A) may be capable of working in trans to stimulate the translation of capped, poly(A)- mRNA (Figure 6A).

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 (Bertholet et al., 1987; Patel and Pickup, 1987; Schwer et al., 1987), the yeast PABP mRNA (Adam et al., 1986; Sachs et al., 1986), and the human PABP mRNA (Grange et al., 1987). 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 (Figure 6A) implies that a) the orientation of poly(A) with respect to the 5'-cap is unimportant and b) that the oligo(A) present in reovirus particles (Carter et al., 1974; Stotzfus et al., 1974) 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 (Manrow and Jacobson, 1987; Sieliwanowicz, 1987) 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 to note that an oocyte-
specific PABP (Swiderski and Richter, 1988) appears to act as a translational repressor (Kick et al., 1987). Thus, the polyadenylation of stored mRNAs upon fertilization in Xenopus (and other organisms) (Rosenthal et al., 1983; Huarte et al., 1987; Hyman and Wormington, 1988; Strickland et al., 1988; McGrew et al., 1989), may reflect a need to provide a vacant binding site for the "stimulatory form" of the PABP.
Experimental Procedures

Plasmid constructions

A series of vectors for the in vitro transcription of polyadenylated RNAs, designated pSP65A<sub>n</sub>, was derived by insertion of different lengths of poly(dA:dT) within the polylinker of pSP65 (Melton et al., 1984). Construction of these vectors utilized conventional techniques (Maniatis et al., 1982) and proceeded as follows (see Figure 1): pSP65 DNA was linearized by digestion with PstI, tailed to varying extents with dATP and terminal transferase (Ratliff Biochemicals), digested with HindIII, and separated from the low molecular weight products of digestion by preparative electrophoresis in low melt agarose. A 7-base oligonucleotide, comprising part of a HindIII recognition site (5'-AGCTTTT-3'), was phosphorylated at its 5'-terminus with ATP and polynucleotide kinase, tailed to varying extents with dTTP and terminal transferase, purified by DEAE-cellulose chromatography, and ligated to the gel-purified vector molecules. These vectors were constructed by Dr. Allan Jacobson.

Templates for transcription of polyadenylated rabbit β-globin mRNAs were constructed using pGEM1RβG and pβG1 (gifts from Dr. Argiris Efstratiadis). The 0.34 kbp AccI-BglI fragment from pβG1 (Efstratiadis et al., 1977) was gel purified and ligated with the gel purified 3.1 kbp AccI-BamHI fragment from pGEM1RβG to produce pDMRβG. The 0.95 kbp SphI-SmaI fragment from pDMRβG 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'-non-coding sequence derived from the vector, 10 nucleotides of rabbit β-globin 5'
untranscribed 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 using plasmid JS223 (Sprague et al., 1983; a gift from Dr. 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, phosphatased pSP65A vectors. After linearization with HindIII, transcripts from these templates include 16 nucleotides of 5'-non-coding 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 (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 BglII (VSV N gene constructs). Restriction digests of plasmids containing (dA:dT) inserts were directly compared with otherwise identical plasmids, without such inserts, on 5% polyacrylamide gels (in TBE; Maniatis et al., 1982). Because replication in bacterial hosts often caused deletions within the (dA:dT) insert (resulting in plasmid preparations with heterogeneous (dA:dT) lengths; for example, see Figure 1B), plasmids were prepared in a mutant host (HB101A) which allows plasmid replication without

To measure poly(A) tail lengths, RNA was transcribed in vitro (using α-AT\(^{32}\)P as label) and 5\(\times\)\(10^5\) cpm of each transcript was digested either with RNases A and T\(_1\) (100\(\mu\)g/ml each) or T\(_2\) (5.0 U/ml) in 2X SSC (0.3M NaCl, 0.03M sodium citrate) at 37°C for 30 to 60 min. Digestion products were purified by an oligo(dT)-cellulose batch procedure (Jacobson, 1988) and analyzed on 5% polyacrylamide gels run in the presence of 8M urea and TBE (Maniatis et al., 1982).

**In vitro transcription and capping of synthetic mRNAs**

In vitro transcription and co-transcriptional capping, directed by SP6 RNA polymerase (Boehringer), were as described previously (Konarska et al., 1984; Melton et al., 1984). After transcription, DNA templates were removed by digestion with RQ1 DNase (1 U/µg DNA; Promega). Transcripts were further purified by two phenol:CHCl\(_3\) (1:1) extractions, CHCl\(_3\) extraction, Sphedex G-50 spin-chromatography, and ethanol precipitation. The integrity of all RNA samples was verified by electrophoresis in 4-6% polyacrylamide (acrylamide:bis-acrylamide=20:1) gels containing 8M urea and buffered with TBE (Maniatis et al., 1982). For the label transfer experiments described in Figure 5D uncapped synthetic mRNAs were post-transcriptionally capped in the presence of α-GT\(^{32}\)P and guanyl transferase essentially as described by Greenberg and Burn (1988).

**Analysis of RNA 5'-termini**

Transcripts labeled in vitro with α-AT\(^{32}\)P (10\(^6\) cpm) were digested with RNases A (100µg/ml), T\(_1\) (100µg/ml), and T\(_2\) (5.0 U/ml) in 2X 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/conc. NH$_4$OH/H$_2$O, pH 4.3, 577:38:385 in the first dimension and saturated (NH$_4$)$_2$SO$_4$/1M NaOAc/isopropanol, 80:18:2 in the second dimension as described by Konarska et al (1984).

**In vitro protein synthesis**

mRNA-dependent translation extracts were prepared from commercial rabbit reticulocyte lysate (Promega) as described previously (Palatnik et al., 1979). Reaction conditions for in vitro protein synthesis and procedures for analyzing the translation products of individual mRNAs were as described (Palatnik et al, 1979; Jacobson and Favreau, 1983).

**Sucrose gradient analysis of translation intermediates in reticulocyte lysates**

Polysomal distribution was monitored in 25-50µl of reticulocyte translation extract. Following a 15 min incubation at 37°C, samples were diluted 1:10 in ice cold buffer A (25mM Hapes, pH 7.0, 50mM KCl, and 2mM MgOAc) containing 50µg/ml cycloheximide and fractionated on gradients of 15-50% sucrose in buffer A. Gradients were centrifuged at 175,000g for 110 min at 4°C in an SW41 rotor. Formation of 48S pre-initiation complex was monitored in 25-50 µl of reticulocyte lysate including edeine (10µM; a generous gift of Dr. Peter Walter). Following a 5 min incubation at 37°C, samples were diluted 1:10 in ice cold buffer C (25mM Hapes, pH 7.0, 50mM KCl, 20mM EDTA) and fractionated on 10-30% sucrose gradients (in buffer C, SW41 rotor, 200,000g, 180 min, 4°C). mRNPs were assembled in 25-50µl of reticulocyte
lysate containing 20mM EDTA. Following a 5 min incubation at 37°C, samples were diluted 1:10 in ice cold buffer C and fractionated on 5-20% sucrose gradients (in buffer C, SW41 rotor, 200,000g, 360 min, 4°C).

For the analysis of all sucrose gradients, fractions were collected from the bottom, transferred directly into ice cold 5% TCA, and filtered through GF/C discs which were subsequently washed with 5% TCA and 95% ethanol. Radioactivity in each sample was determined by scintillation spectrometry and correction of 3H cpm for 32p spillover. The respective sums of the 3H and 32p cpm were determined for each gradient and the data are presented as the percentage of total cpm present in each fraction. Sedimentation coefficients were determined relative to ribosomal particles of known S value.

**Measurement of RNA decay rates in reticulocyte lysates**

Synthetic RNAs labeled with α-AT32P (10^6cpm) were incubated in 50μl of reticulocyte translation extract at 37°C. Aliquots (5μl) were removed after 0, 5, 10, 20, 30, and 60 min of incubation and diluted in 225μl of ice cold buffer B (25mM Heps, pH 7.0, 20mM EDTA) containing a different 32p-labeled in vitro transcript (to monitor the recovery of total RNA). Samples were extracted once with phenol (65°C) and once with with CHCl3, and then analyzed on 5% polyacrylamide gels (run in the presence of 8M urea and TBE). The resulting autoradiographs were quantitated by densitometry.

**Determination of translational elongation rates**

The rates of translational elongation on VSV N mRNAs were measured by a modification of the method described by Palmiter (1973). Standard reticulocyte translation extract (100μl) containing 35S-methionine was
incubated with synthetic, capped VSV N mRNA (4µg/ml) at 37°C. At the indicated times, aliquots (13µl) were removed and pipetted into 387 µl of ice cold buffer A containing 50µg/ml cycloheximide. Isotopic incorporation into total VSV N polypeptide was monitored by acetone precipitation of 175 µl from each aliquot. In parallel, isotopic incorporation into released VSV N polypeptide was monitored by pelleting polysomes from an additional 200 µl of each aliquot (centrifugation for 2 hr, 24 psi, 4°C, in a Beckman Airfuge) followed by acetone precipitation of 175 µl of the resulting supernatant (600 µl acetone; -20°C; 16 hr). Following acetone precipitation, each sample was washed once with an additional 600 µl acetone, dried, and resuspended in 35 µl of sample buffer (Laemmli, 1970). Samples were fractionated on standard 10% SDS-polyacrylamide gels (Laemmli, 1970) which were subsequently analyzed by quantitative fluorography (Laskey and Mills, 1975).

Identification of mRNP proteins by label transfer

Rabbit reticulocyte lysates supplemented with 32P-labeled synthetic mRNA (4.0µg/ml) were incubated for 5 min at 37°C. mRNP proteins were cross-linked to mRNA by UV-irradiation at ambient temperature (flux=2000uW/cm²; dose=3.6X10⁵ ergs/mm²). Following irradiation, label was "transferred" from mRNA to mRNP proteins by digestion with RNases A, T₁, and T₂ or with RNase A alone (Manrow and Jacobson, 1986).
Figure 1B.
Figure 1C.
Figure 1D.
Figure 1. Construction and characterization of pSP65A vectors and their transcripts.

(A) Summary of construction scheme for pSP65A vectors.

(B) Inserts of (dA:dT)n within the polylinker of pSP65A plasmids (and their rabbit β-globin (RBG) derivatives) were characterized by restriction digests (EcoRI-HindIII) which bracketed the A/T region. Digestion products were fractionated on a 5% polyacrylamide gel (in TBE) and stained with ethidium bromide. Lane 1, pRBG+0A's; lane 2, pRBG+23A's; lane 3, pRBG+32A's; lane 4, pRBG+68A's; lane 5, HinfI cut pBR322; lane 6, an example of a pRBG plasmid preparation with a heterogeneous A/T region arising from deletions accumulated during replication in E. coli HB101.

(C) Poly(A) tail lengths of in vitro transcripts (labeled with α-AT32p) were measured by digestion with either RNases A and T1 or RNases A, T1, and T2. Digestion products were purified on oligo(dT)-cellulose and analyzed on a 5% polyacrylamide gel (in 8M urea and TBE). Odd numbered lanes were digested with RNases A, T1, and T2; even numbered lanes were digested with RNases A and T1. Lanes 1 and 2, RBG mRNA with 0A's; lanes 3 and 4, RBG mRNA with 23A's; lanes 5 and 6, RBG mRNA with 32A's; and lanes 7 and 8, RBG mRNA with 68A's.

(D) Synthetic rabbit β-globin mRNAs (labeled with α-AT32p) containing 68 (A+) or 0 (A-) A's were fractionated on oligo(dT)-cellulose. The resulting bound and unbound fractions were analyzed on a 5% polyacrylamide gel (in 8M urea and TBE). Lane 1, unbound A+; lane 2, bound A+; lane 3, unbound A-; and lane 4, bound A-.

(E) To assess the extent of in vitro capping, a short (10 nucleotide) RNA was transcribed from pSP65 digested (within the polylinker) with EcoRI.
Transcription reactions included $\alpha$-AT$^{32}$P and 7MeGpppG. Transcripts were digested with RNases A, T$_1$, and T$_2$ and digestion products were separated by two-dimensional thin layer chromatography.
Figure 2A.

- C-A+
- C-A-
- UC-A+
- UC-A-
Figure 2B.

mRNA concentration (ug/ml)

0.4 2.0 4.0 6.0 8.0

C-A+  

C-A-  

UC-A+  

UC-A-  

Figure 2C.

RBG decay

VSV.N decay

% of time 0

time (min)
Figure 2. Relative translational efficiency and stability of synthetic mRNAs differing in poly(A) tail length and/or 5'-cap structure.

(A), (B) VSV.N in vitro transcripts, of indicated structure and concentration, were incubated for 30 min at 37°C in an mRNA-dependent, cell-free, rabbit reticulocyte translation extract supplemented with 35S-methionine. Synthesis of VSV N polypeptide in equal volumes of each translation reaction was analyzed by SDS-polyacrylamide gel electrophoresis and quantitative fluorography. (A) Average of the densitometric quantitation of N polypeptide in two independent experiments. (B) Autoradiograph of one of the two experiments depicted in (A).

(C) Capped, 32P-labeled RBG or VSV.N in vitro transcripts, containing either 68 (A+) or 0 (A-) 3'-adenylate residues, were incubated at 37°C in a reticulocyte translation extract. Aliquots were withdrawn at the indicated times and RNA levels were quantitated by autoradiography and densitometry as described in Experimental Procedures.
Figure 3A.

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

% of total

Fraction Number
Figure 3B.

C–RBG–68A vs C–RBG–0A

% of total

Fraction Number
Figure 3C.

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

% of total

Fraction Number
Figure 3D.

C–RBG–68A vs UC–RBG–68A

% of total

Fraction #

Fraction Number
Figure 3E. C-RBG-68A vs UC-RBG-OA

% of total

Fraction Number
Figure 3F.
Figure 3. Polysomal distribution of synthetic mRNAs differing in poly(A) tail lengths and/or 5'-cap structure.

(A)-(E) Equal amounts of a $^{32}$P-labeled, capped, RBG or VSV.N in vitro transcript containing 68 3'-adenylate residues (□) and a $^{3}$H-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 reactions were fractionated on 15-50% sucrose gradients. Fractions were collected from the bottom of the gradients directly into ice cold 5% trichloroacetic acid and filtered. Insets present the ratio of $^{32}$P/$^{3}$H cpm across the polysomal regions of the gradients. Arrows indicate the position of the 80S peak.

(F) In experiments analogous to those of (A)-(E), the percentage of $^{32}$P-labeled, capped, RBG mRNA with 68A's found on polysomes was compared to that of $^{3}$H-labeled, capped, RBG mRNA of indicated poly(A) tail length. All fractions sedimenting faster than 80S were considered to be polysomal. Data in the figure are normalized, such that the percentage of $^{32}$P-labeled RBG mRNA with 68 A's present on polysomes is considered to be 100%.
Figure 4A.
Figure 4B.
Figure 4C.
Figure 4D.
Figure 4E.
Figure 4. Formation of mRNPs, 48S pre-initiation complexes, and 80S initiation complexes by poly(A)$^+$ and poly(A)$^-$ mRNAs.

Formation of mRNPs and initiation and pre-initiation complexes was monitored by incubating equal amounts of a $^{32}$P-labeled, capped poly(A)$^+$ in vitro transcript (□) with a $^3$H-labeled capped, poly(A)$^-$ in vitro transcript (+) in reticulocyte extracts supplemented, incubated, and fractionated as indicated. Experiments A-D included poly(A)$^+$ (68A's) and poly(A)$^-$ (0A's) RBG mRNAs and experiment E included poly(A)$^+$ (61A's) and poly(A)$^-$ (0A's) VSV.N mRNAs.

(A) mRNP formation in a reticulocyte lysate containing 20mM EDTA. Following incubation for 5 min at 37°C, the reaction was fractionated on a 5-20% sucrose gradient.

(B) 48S pre-initiation complex formation in a reticulocyte extract containing 10μM edeine. After 5 min at 37°C, the translation reaction was fractionated on a 10-30% sucrose gradient.

(C) 80S initiation complex formation in a reticulocyte extract supplemented with 75μg/ml cycloheximide.

(D) 80S initiation complex formation in a reticulocyte extract supplemented with 1μg/ml anisomycin.

(E) 80S initiation complex formation in a reticulocyte extract supplemented with 75μg/ml cycloheximide.
Figure 5A.

RNase: A T1 T2 A
RNA: A+ A- A+ A-
Figure 5B.
Figure 5C.
Figure 5D.
Figure 5E.

![Diagram showing blot results with labels for mGMP and their corresponding RNA. The diagram includes molecular weight markers at 200, 92.5, and 69.]
Figure 5. mRNP proteins associated with poly(A)$^+$ and poly(A)$^-$ mRNAs.

Individual mRNP proteins were identified by a label transfer procedure in which $^{32}$P-labeled RBG mRNA was incubated in a reticulocyte lysate at 37°C to reconstitute mRNPs, irradiated at ambient temperature to cross-link bound mRNP proteins to the mRNA, and digested with RNase to transfer $^{32}$P-label to the proteins. Labeled proteins were visualized by SDS polyacrylamide gel electrophoresis.

(A) $\alpha$-AT$^{32}$P-labeled, capped, RBG mRNA with 68 (A$^+$) or 0 (A$^-$) 3'-adenylate residues was UV-irradiated in an EDTA-treated reticulocyte lysate and then digested with the indicated RNase(s).

(B) $\alpha$-AT$^{32}$P, $\alpha$-CT$^{32}$P, or $\alpha$-UT$^{32}$P labeled, capped, RBG mRNA with 68 (A$^+$) or 0 (A$^-$) 3'-adenylate residues was UV-irradiated in a reticulocyte translation extract and then digested with RNase A.

(C) $\alpha$-AT$^{32}$P-labeled, capped, RBG mRNA with 68, 32, 23, 5, or 0 3'-adenylate residues was UV-irradiated as described in Figure 5B.

(D) $\alpha$-AT$^{32}$P-labeled, capped, RBG mRNA with 68 3'-adenylate residues was UV-irradiated as described in Figure 5B in the presence of the indicated concentrations of competitor poly(A) or 0.4mM $m^7$GMP ($m^7$GMP). Other samples were irradiated without competitor and were digested with proteinase K at 2mg/ml for 30 min at 37°C (proteinase K) or were irradiated without labeled RNA (no RNA).

(E) Capped RBG mRNA (labeled only in the 5'-cap) with 68 (A$^+$) or 0 (A$^-$) 3'-adenylate residues was UV-irradiated as described in Figure 5B in the presence (+) or absence (-) of 0.4mM $m^7$GMP.

The arrow in (A)-(D) indicates the position of the p78 monomer of PABP.
Figure 6.
Figure 6. 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 mRNA was then added to a final concentration of 0.25μg/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).
A) Isolation of HB101A.

Transformation of conventional bacterial hosts (such as HB101) with pSP65A plasmids usually resulted in a poor efficiency of transformation. The few resulting transformants were small, yellowish in color, and slow growing relative to normal HB101. Plasmids isolated from such colonies invariably contained deletions within the dA:dT region. Occasionally, larger white transformants were found. Although these transformants also grew slowly, they almost always resulted in plasmids with small or no deletion within the dA:dT region. One such transformant was "cured" of its plasmid in the following manner: 1) the colony was transferred to non-selective liquid media and grown for several generations; 2) a sample of this stock was streaked for isolation on non-selective media; and 3) individual colonies were "picked" onto grids of selective (ampicillin) and non-selective media. Individual isolates which grew on the non-selective media but not on the selective media were judged to be "cured" of plasmid. One such isolate was used in subsequent transformations and found to support replication of pSP65 plasmids without deletion. This isolate was designated HB101A.

B) Translational elongation rates.

The rates of translational elongation on synthetic VSV.N mRNAs were measured by a modification of the method described by Palmiter (1973). The transit time along poly(A)+ and poly(A)-VSV.N mRNAs was determined by monitoring the incorporation of 35S-methionine into total and released VSV N polypeptide in rabbit reticulocyte lysates (figure 7). The lag time between the appearance of total and released VSV N polypeptide equals one-half of the VSV.N mRNA transit time (Palmiter, 1973). The apparent transit time along
both poly(A)$^+$ and poly(A)$^-$ VSV.N mRNA is 144 seconds (figure 7). VSV.N mRNA encodes a 422 amino acid polypeptide (Sprague et al., 1983). Therefore, the translational elongation rate on either VSV.N mRNA would be 422 amino acids/144 seconds or 2.9 amino acids/second.
Figure 7.
Figure 7. Rates of translational elongation on poly(A)$^+$ and poly(A)$^-$ mRNAs.

Capped VSV.N in vitro transcripts were incubated for 0, 0.5, 1, 2, 3, and 6 minutes at 37°C in an mRNA-dependent, cell-free, rabbit reticulocyte translation extract supplemented with $^{35}$S-methionine. Each sample was analyzed for synthesis of total and released VSV N polypeptide by SDS-polyacrylamide gel electrophoresis and quantitative fluorography as described in Experimental Procedures.

(A) Translational elongation on capped, poly(A)$^+$ VSV.N mRNA
(B) Translational elongation on capped, poly(A)$^-$ VSV.N mRNA
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