ROLE OF THE 5' UNTRANSLATED REGION OF EUKARYOTIC mRNAs IN TRANSLATION INITIATION

by

Jerry Pelletier

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Department of Biochemistry
McGill University
Montreal, Quebec
CANADA

ABSTRACT

Eukaryotic ribosomes are thought to initiate protein synthesis by binding to the 5' end of mRNAs and scanning the noncoding region until the initiation codon is reached. Features of a eukaryotic mRNA which influence the efficiency of initiation include the cap structure and the context sequences of the initiator codon. Artificially increasing the secondary structure within the 5' untranslated region (UTR) of the herpes simplex virus 1 thymidine kinase mRNA was found to reduce the translational efficiency of the mRNA *in vivo* and *in vitro*. Using an ultraviolet light crosslinking assay, the interaction of eukaryotic initiation factor 4B with the cap structure of the thymidine kinase mRNA was found to be inhibited by a hairpin loop structure placed 6 nucleotides from the cap structure, but not when placed 37 nucleotides away. The results show that mRNA secondary structure within the 5' UTR is an important determinant of translational efficiency, and can inhibit translation at different steps of initiation depending on the position of the stem-loop. We also show that the 5' UTR of poliovirus mRNA contains a *cis*-acting element which allows it to translate in a cap-independent fashion. This region functions by allowing internal binding of ribosomes and explains how poliovirus escapes the translational block it imposes on host cellular mRNAs following infection.

RESUME

L'amorce de la traduction chez les eucaryotes comprend l'attachement d'un ribosome au 5' cap de l'ARNm, suivi par la scrutation de la region 5' non codante de l'ARNm jusqu'au codon d'initiation. Les charactéristiques de l'ARNm qui ont une influence sur l'efficacité de la traduction sont la structure 5' cap et les séquences flanquant le codon d'initiation. En augmentant artificiellement la structure secondaire dans la région 5' non codante de l'ARNm du gène de thymidine kinase du virus de l'herpes simplex 1, l'efficacité de la traduction de lARNm est réduite in vivo et in vitro. La structure secondaire lorsque placée à 6 nucleotides de la structure cap, diminue l'intéraction du facteur d'initiation 4B avec la structure cap. Cette protéine, avec la participation d'autres protéines de liaison-cap et de l'ATP, est impliquée dans le "déroulement" de la structure secondaire de l'ARNm. Il est démontré que l'éfficacité traductionelle est inversément proportionnelle au degré de structure secondaire dans la région 5' non codante de l'ARNm. Egalement, il est démontré que la région 5' non codante de l'ARNm du virus de la polio possède un élément qui permet sa traduction indépendamment de la structure cap. Cet élément permet aux ribosomes eucaryotiques de se lier de façon interne à l'ARNm et explique le mécanisme par lequel le virus de la polio échappe au blocage de la traduction qu'il impose aux ARNm cellulaires.

DEDICATION

I dedicate this work to my mother and father for their support, encouragement, and love,

AND

To my wife, Suzanne for her patience, love and understanding.

"When you know a thing, to hold that you know it; and when you do not know a thing, to allow that you do not know it: this is knowledge."

CONFUCIUS, Analects.
Bk.ii, ch. 17.

PREFACE

The work described in Chapters 2-5 of this thesis has been published in the following journals:

Chapter 2. Pelletier, J. and Sonenberg, N. (1985) Cell 40, 515-526.

Chapter 3. Pelletier, J. and Sonenberg, N. (1985) Mol. Cell. Biol. 5, 3222-3230.

Chapter 4. Pelletier, J., Kaplan, G., Racaniello, V.R. and Sonenberg, N. (1987) Mol. Cell. Biol. 8, 1103-1112.

Chapter 5. Pelletier, J. and Sonenberg, N. (1987) Nature, In press.

The work presented in Chapters 2,3 and 5 is entirely my own. Vince R. Racaniello and Geraldo Kaplan provided the infectious poliovirus cDNA clone and some of the deletion mutants for the work described in Chapter 4.

Because of the large scope of the field of translation, I have concentrated mainly with the initiation pathway and the parameters which influence the rate-limiting step of this pathway - binding of the 40S subunit to the mRNA. In areas where there is an overwhelming amount of literature, I limit my references to reviews or to the original articles which forged the path for our understanding of the topic.

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CHAPTER 1

INTRODUCTION

Initiation of Eukaryotic Protein Synthesis

1.1 The Overall Process of Eukaryotic Gene Expression

The synthesis of a protein is one of the most complex cellular processes known. This event can be regulated at many different levels: transcription, mRNA processing, nucleocytoplasmic mRNA transport, translation, mRNA stability, and post-translational protein modification. Disregarding the events leading up to, or immediately after translation, the expression of an mRNA molecule is a formidable task for the cell, involving the synchronized participation of over 150 macromolecules. That the cell would put so much effort and precision towards this process underscores its importance in cellular homeostasis.

Gene expression begins in the nucleus when DNA is transcribed into RNA by one of three cellular RNA polymerases. RNA polymerase I synthesizes the large ribosomal RNAs (45S rRNAs) destined to become, by stepwise processing, the 28S, 18S and 5.8S rRNAs found in ribosomes (1). RNA polymerase III transcribes genes encoding small RNA molecules such as the 5S rRNA, transfer RNAs (tRNAs) and small nuclear RNAs (snRNAs) (2). The formation of a eukaryotic mRNA is carried out by RNA polymerase II (3). The primary transcript, known as heterogeneous nuclear RNA (hnRNA) undergoes several modifications before ending up as a functional unit in the cytoplasm. Shortly after initiation of transcription, a cap structure is added to the 5' end of the hnRNA molecule by guanylyl transferase (4-7). As the RNA chain is made, it becomes associated with a family of highly conserved basic proteins (8,9) and forms a ribonucleoprotein (RNP) complex. During, or shortly after synthesis, ~ 0.1% of the adenosine residues are methylated at the

6 position, usually in the context N_1-(G/A)-m^6A-C-N_2 (where N_1 is usually a purine, N_2 is rarely a guanosine) (10,11). Ninety to 120 seconds following synthesis of hnRNA (which proceeds in the 5' to 3' direction at a rate of about 30 nucleotides per second), the enzyme poly(A) polymerase adds adenylic acid residues to the 3' end of the molecule (12-15). Subsequent splicing of the precursor mRNP is a relatively slow step (16) involving the excision of intervening sequences to form a mature mRNA. There are some mRNAs which do not have all of these modifications, but the majority of eukaryotic cellular mRNAs follow this maturation pathway.

The mature mRNA is then transported out of the nucleus by a still poorly understood, selective mechanism (see ref. 17 for a review). Introns (13,18) and prematurely terminated transcripts (19) are not transported out of the nucleus. Once in the cytoplasm, the mRNA is translated to produce polypeptide chains. These final products are often covalently modified, form active complexes with other peptides, or are proteolytically processed to yield the final gene product. The nascent polypeptides often contain information used to target the molecules to various organelles, such as the mitochondria or nucleus.

1.2 Eukaryotic Protein Synthesis - An Overview

1.2.1 The RNA Components

There are three distinct RNA species involved in protein synthesis: a) mRNA, which contains the nucleotide core specifying the primary structure of the encoded protein; b) tRNA, to which amino acids are attached for subsequent alignment to their translation codon; and c)

rRNA, which migrates along the mRNA and are the site of peptide bond formation.

In the cytoplasm of a eukaryotic cell, there are about 60 different species of tRNAs (20). Consequently, a specific amino acid can be covalently attached to several different tRNAs. A typical tRNA molecule contains from 73 to 93 nucleotides and has a molecular weight of about 25,000 (20). A distinguishing feature of tRNAs is that they contain a number of unusual bases. Over 50 different modified bases have been found in eukaryotic tRNAs, some of which are not only unique in location found on the molecule but also to the tRNA species in which they are located (20). Transfer RNAs possess extensive secondary structure which folds the molecule into a distinct "cloverleaf" structure. Proceeding from the 3' to 5' direction, the tRNA has four major arms: a) the acceptor arm, consisting of the base-paired stem that ends in an unpaired sequence to which amino acids are coupled, b) the TψC arm, c) the anticodon arm, containing the triplet used to decode the mRNA information, and d) the D arm (so named for its content of the modified base-dihydrouridine) (20).

All eukaryotic tRNAs have the common sequence $^{5'}...CCA^{3'}$ at their 3' ends. This sequence is added post-transcriptionally to newly synthesized tRNAs by an enzyme called nucleotidyl transferase (21). Amino acids are coupled to the 3' adenosine residue by a set of enzymes called aminoacyl-tRNA synthetases. There is a different synthetase enzyme for every amino acid and the coupling reaction occurs in two steps as shown below:

1. $E_x + ATP + aa_x \rightarrow aa_xAMP\ E + PP_i$

2. $aa_x \cdot AMP \cdot E + tRNA_x \rightarrow aa_x \cdot tRNA + E + AMP$

where E is the synthetase, aa is the amino acid, PP_i is inorganic pyrophosphate, and the subscript x denotes a particular species. The first step involves the activation of an amino acid by esterification to an adenosine monophosphate (AMP) residue derived from ATP with the concomitant release of pyrophosphate. The AMP-linked carboxyl group of the amino acid is then transferred to the 3' hydroxyl group of the ribose moiety of the 3' terminal adenosine of the tRNA. The aminoacyl-tRNA synthetases form an important link in deciphering the genetic code since, once attached, the amino acid has no effect on codon selection.

The gross anatomy of eukaryotic ribosomes, as revealed by electron microscopy, resembles that of bacterial ribosomes (22), except that eukaryotic ribosomes are bigger. The eukaryotic ribosome consists of two subunits, a large (60S) and a small (40S) subunit, that have independent roles in the initiation of protein synthesis, but work together to catalyze peptide bond formation. The small subunit contains one 18S RNA and approximately 30 polypeptides (23). Changes in ribosome structure by differential expression of the 18S rRNA genes has been postulated to exert a coarse control over protein synthesis by mediating selection of specific mRNA families to be translated (24). The large subunit has one molecule each of 5S, 5.8S, and 25-28S RNA and between 45 to 50 proteins (23). Some of the ribosomal proteins are thought to interact with receptors on the endoplasmic reticulum (25,26), whereas others may be involved in the regulation of translation by mediating the interaction of mRNA with ribosomes (27).

1.2.2. The Site of Protein Synthesis

Eukaryotic cells have several types of protein synthesizing machinery: one in the cytoplasm, one in mitochondria and one in chloroplasts (for those cells harboring chloroplasts). The chloroplast translation system closely resembles the prokaryotic system. Chloroplast and bacterial ribosomes are similar in size, topography, RNA components, protein composition, and the subunits can be functionally interchanged (28). The structure of mitochondrial ribosomes suggests an initiation mechanism different from that in prokaryotes or in the cytoplasm of eukaryotic cells (28). This thesis is concerned with eukaryotic cytoplasmic protein synthesis which will be referred to as eukaryotic protein synthesis.

Polysomes are the site of active protein synthesis and can be found either associated with the outer membrane of the endoplasmic reticulum (ER) or "free" in the cytoplasm. Proteins destined for the ER (secretory and cell surface), like all other peptides, begin their translation on "free" ribosomes. However, as the signal peptide emerges from the translating ribosome, a signal recognition particle (SRP) [composed of six nonidentical polypeptides and a 7S RNA] is postulated to bind to the ribosome and arrest elongation of translation (see ref. 29 for a review). The mechanism by which the SRP interacts with the ribosome and signal sequence to cause recognition and elongation arrest is not known. The SRP-ribosome complex then binds to the membrane of the ER by association with a SRP receptor (29). Once bound, the SRP dissociates from the ribosome, translation resumes, and the protein is vectorially transferred into the lumen of the ER. SRP mediated arrest

of elongation is a potential site for regulation endowing the cell with a rapid switch for controlling translocation-coupled protein synthesis.

Several lines of evidence suggest that actively translating "free" polysomes are actually bound to the cellular cytoskeleton. Using high voltage electron microscopy, Wolosewick and Porter (30) found polysomes associated with the cytoskeleton of whole cell mounts. Sucrose gradient analysis of ribosomes in soluble and cytoskeletal fraction (defined as material remaining after extraction of cells with a mild non-ionic detergent) revealed that most polysomes are in the cytoskeletal fraction while monomeric ribosomes are in the soluble fraction (31,32). Inhibition of cellular mRNA translation by nutrient deprivation (33), virus infection (32,34,35), or treatment with inhibitors of initiation of protein synthesis (31,36) results in the release of cellular polysomes from the cytoskeleton as 80S monomers into the soluble fraction. Maternally inherited mRNAs in sea urchin embryos begin to function only after binding to newly formed cytoskeletal framework (37). These findings suggest that ribosomes are primarily associated with the cytoskeleton when they are translating mRNA and that this association is either much weaker or non-existent when ribosomes are not synthesizing protein. Consistent with this, perturbation of the cytoskeletal framework of HeLa cells with cytochalasin D inhibits protein synthesis (38). However, not all polysomes become dissociated from the cytoskeleton following inhibition of protein synthesis (35,39) suggesting that association is required, but not sufficient for translation. Howe and Hershey (36) showed that ribosomes associate with the cytoskeleton at the initiation step of translation and dissociate

after completion of elongation. Consistent with this, they found that cytoskeletal fractions are enriched in initiation factors relative to the soluble fraction. It is currently not known what anchors the polysome to the cytoskeleton although the cap structure and poly(A) tail do not seem to be involved (35).

1.2.3. The Basic Mechanism of Protein Synthesis

Translation can be thought of as occurring in three mechanistically distinct steps. 1) Peptide chain initiation, where components of the translation machinery are assembled on the initiation signal of mRNA. 2) Peptide chain elongation, where the translation machinery adds one amino acid residue to a growing peptide chain per three nucleotides. 3) Peptide chain termination, where the completed polypeptide chain is released from the translation machinery which subsequently disassembles, releasing the ribosomes for another round of initiation.

(a) Initiation

Initiation involves the positioning of an 80S ribosome and Met-$tRNA_i^{Met}$ on the appropriate initiation codon of an mRNA. This step is catalyzed by initiation factors and sets the reading frame for protein synthesis. The details of this step will be described below.

(b) Elongation

Elongation involves the sequential addition of amino acids to a growing polypeptide chain and can be thought of as occurring in 3 steps. 1) Once a functional 80S complex is formed, an aminoacyl-tRNA binds to the aminoacyl-tRNA binding site (A-site) of the ribosome in such a way that the anticodon nucleotides of the tRNA base pair with the

next codon on the mRNA. This step requires GTP and is catalyzed by a protein known as elongation factor 1 (EF-1). EF-1 consists of three subunits: α, β, γ (40). EF-1α participates to form a ternary complex with GTP and the incoming aa-tRNA. During binding of the aa-tRNA to the A-site of the ribosome, GTP is hydrolyzed to GDP, and EF-1α GDP is ejected from the ribosome. EF-1•β is involved in dissociating the EF-1α• GDP complex and allowing EF-1α to recycle (40). 2) The carboxyl end of the methionine (in the case of the first elongation cycle) is uncoupled from the Met-$tRNA_i^{Met}$ found in the peptidyl-tRNA binding site (P-site) and joined through a peptide bond to the amino group of the aminoacyl-tRNA (40). The exact mechanism of transpeptidation is not understood. The peptidyl transferase center resides on the 60S ribosome and it has recently been speculated that RNA chemistry may be involved in the peptide bond formation (41). The energy for the formation of the peptide bond is derived from the hydrolysis of the ester bond in the peptidyl-tRNA at the P-site. 3) The ribosome then moves three nucleotides along the mRNA, resulting in translocation of the peptidyl-tRNA from the A-site to the P-site, with concomitant ejection of the deacylated tRNA. This process requires GTP hydrolysis and elongation factor 2 (EF-2) (40). Inhibition of eukaryotic protein synthesis by diphtheria toxin involves inactivation of EF-2 by ADP-ribosylation (42).

(c) Termination

The final step of protein synthesis involves release of the completed polypeptide chain from the ribosome-mRNA complex. When one of

three stop codons (UAG,UGA,UAA) is reached, a release factor in the presence of GTP binds to the A-site. Peptidyl-tRNA hydrolysis occurs along with GTP hydrolysis, releasing the polypeptide chain and leaving the deacylated tRNA on the ribosome (43). It is not clear whether the ribosomes dissociate from the mRNA at this point or one or both subunits continue to migrate along the mRNA, eventually falling off the 3' end. The latter possibility seems likely in light of evidence that ribosomes can reinitiate protein synthesis when a second cistron is present on the mRNA template (discussed in detail below).

1.3. Peptide Chain Initiation

The components known to be involved in this process are: initiator tRNAs, initiation factors, the small and large ribosomal subunits, ATP, GTP, and appropriate tonicity.

1.3.1. Eukaryotic Initiation Factors

The assembly of a functional initiation complex between eukaryotic mRNA and 80S ribosomes requires several proteins known as initiation factors. These factors differ from ribosomal structural proteins in that the factors cycle on and off the ribosome. They have been isolated from several sources and assayed for stimulatory activity in reconstituted translation systems or in Met-puromycin synthesis (44-48). The best characterized factors are those from rabbit reticulocyte lysates although many of them have been isolated from a variety of other sources.

Table 1 shows the various initiation factors, their molecular weights and attributed functions. Comparison of protein structure, function, antigenicity (49,50) and cDNA sequences (51) have revealed

TABLE 1
Eukaryotic Initiation Factors +

Factor	Subunit Composition and Molecular Mass (Da)	Function
eIF-1	15,000	Stabilizes mRNA binding to the small ribosomal subunit (57)
eIF-2	36,000(α) 35,000(β) 55,000(γ)	Ternary complex formation; Binding of Met-$tRNA_i^{Met}$ to 40S subunit (45,47)
eIF-2A	65,000	AUG-directed Met-$tRNA_i^{Met}$ binding to 40S subunits (58)
eIF-2B	6 polypeptides ranging from 25,000-85,000	Guanine nucleotide exchange on eIF-2; assists in eIF-2 recycling (59)
eIF-3	9-11 subunits ranging from 25,000-160,000	Maintain dissociation of ribosomal subunits (60,61)
eIF-4A	50,000	RNA-dependent ATPase (62,63)
eIF-4B	80,000	Stimulates in binding of the 43S initiation complex to mRNA (63)
eIF-4C	17,000	Ribosome dissociation; 60S subunit joining (64,65)
eIF-4D	15,000	Stimulates Met-Puromycin Synthesis (47)
eIF-4E	24,000	Binds mRNA cap structures (66)
eIF-4F	24,000 (eIF-4E) 50,000 (eIF-4A) 220,000	Binds mRNA Cap Structures (67) Unwinding of mRNA 5' Secondary Structure (68); Restores translation in poliovirus-infected HeLa cell extracts (48,69)
eIF-5	125,000	Joining of the 60S ribosomal subunit to 48S preinitiation complex (70)
eIF-6	25,000	Anti-associaton; binds to 60S subunit (71,72)

+ This table is a summary of eukaryotic mammalian initiation factors and their probable role in translation initiation. Compilation was based on many sources of data and generally accepted functions of initiation factors (40,73,74). No attempt was made to integrate plant eIFs into this table since they have not been as well characterized as their mammalian counterparts. For a comparison of mammalian and wheat germ factors, see Abramson et al. (75).

that the factors are highly conserved across species. The molecular cloning of several of the factors has been accomplished [eIF-2α (52), eIF-4A (51,53,54) and eIF-4E (55,56)] and should provide valuable insight into the regulation and organization of these genes.

1.3.2. Outline of the Initiation Process

Figure 1 shows a schematic outline of the initiation pathway resulting in the formation of 80S initiation complexes.

(a) Ribosome Dissociation

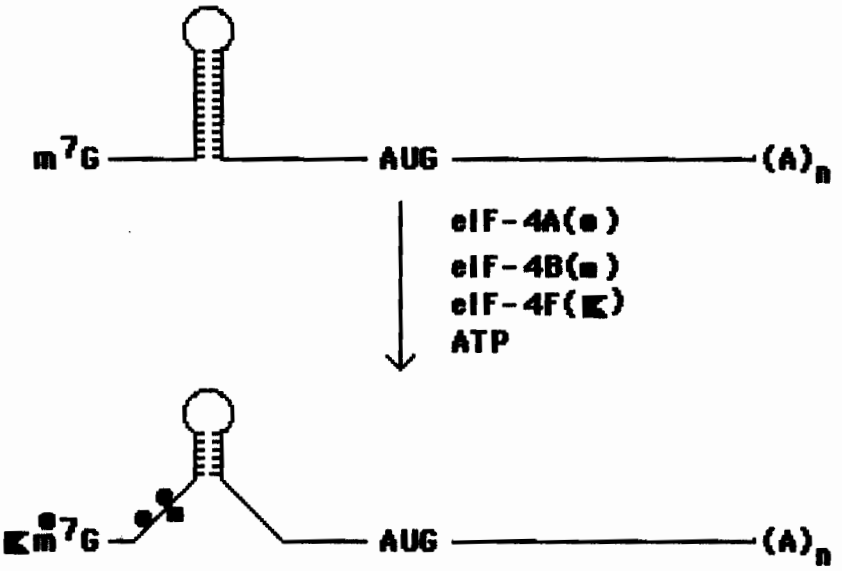
80S ribosomes are unable to bind mRNA directly and are in equilibrium with a pool of 40S and 60S subunits (76). The equilibrium, although favoring 80S formation, can be shifted in the other direction by several initiation factors. eIF-3 has been purified from native 40S subunits (44,60,76) and is capable of binding to purified 40S subunits to form a 43S-46S particle (60). eIF-3 acts as an anti-association factor (44,47,61,76) and the 40S eIF-3 complex which is formed cannot directly associate with 60S ribosomes. One of the subunits of eIF-3 has probably been cloned from Saccharomyces cerevisiae (77-79). Temperature sensitive cells containing a mutant gene, when incubated at the nonpermissive temperature fail to convert 40S subunits to 43S preinitiation compexes (described below) (78). Interestingly, mutants of this gene (termed PRT-1) give rise to cell division control phenotypes (77). Using yeast genetics, the function of the 9-11 polypeptides found in eIF-3 may soon be elucidated and the apparent linkage between protein synthesis and the cell cycle worked out.

eIF-4C has been reported to act as an accessory to eIF-3 in ribosome dissociation (64). This factor can be isolated from 40S

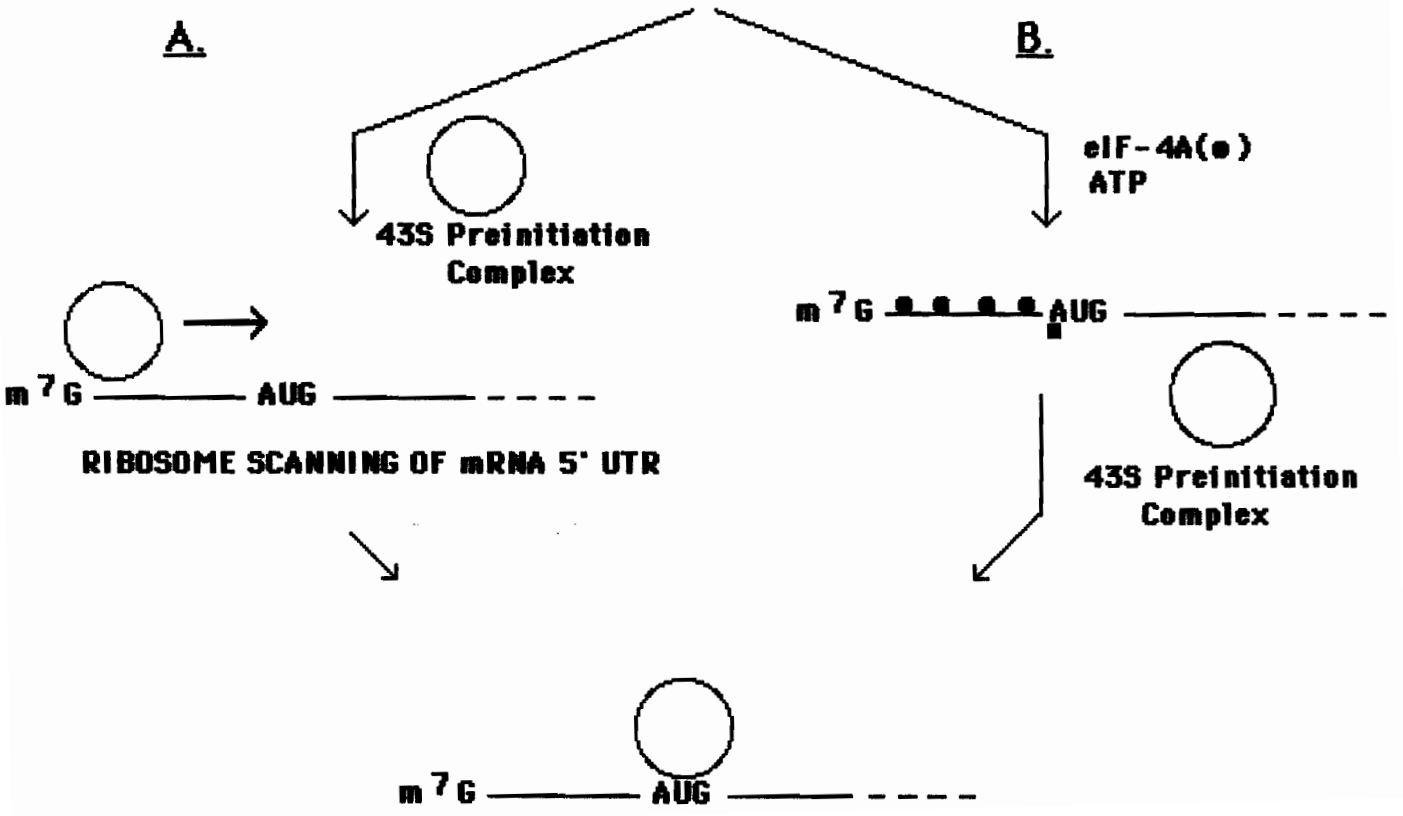
FIGURE 1

The Pathway of Eukaryotic Translation Initiation

MODEL FOR RIBOSOME BINDING



UNWINDING OF mRNA 5' SECONDARY STRUCTURE



subunits and binds in vitro to 40S subunits to prevent association with 60S subunits (66).

In addition, a low-molecular weight dissociation factor from wheat germ and calf liver, called eIF-6, has been postulated to react with 60S subunits and prevent subunit reassociation (71,72). Therefore a model for ribosome dissociation suggests that: eIF-6 binds to 60S subunits and prevents their association with 40S subunits. eIF-3 and eIF-4C bind to 40S subunits to form a 40S•eIF-3•eIF-4C complex incapable of reacting with 60S or 60S•eIF-6 complexes.

(b) Ternary Complex Formation

eIF-2, GTP, and Met-$tRNA_i^{Met}$ form a ternary complex which binds eIF-3•eIF-4C•40S complexes. Ternary complex formation has been well characterized and eIF-2 has been purified from a wide variety of sources. eIF-2 consists of 3 subunits: α, β, and γ found in a 1:1:1 stoichiometry (80). Formation of ternary complexes is specific for Met-$tRNA_i^{Met}$ and even the isoaccepting species Met-$tRNA^{Met}$ cannot be used. Met-$tRNA_i^{Met}$ binds to the γ subunit of eIF-2 (81). Affinity labelling experiments with photoreactive analogues of GTP have demonstrated that GTP binds to the β subunit (82). The formation of a binary complex between eIF-2 and GTP is thought to precede the binding of Met-$tRNA_i^{Met}$. The affinity of eIF-2 for GDP is ~ 100 fold higher than for GTP under physiological Mg^{++} concentrations (83). Following each round of initiation, eIF-2 must be recycled displacing GDP by GTP, in order to allow eIF-2 to participate in a new round of initiation. This event requires eIF-2B. The exact sequence of events involved in eIF-2

recycling are still not known and several models (only one of which is shown in Fig. 1) have been proposed and discussed (40). The requirement for eIF-2B to recycle eIF-2 provides an explanation for the inhibition of initiation obtained with heme deficiency or with double stranded RNA (84-87). The α subunit of eIF-2 becomes phosphorylated on a serine residue (88) under these conditions and reacts with eIF-2B to form a tight complex, sequestering the small amount of eIF-2B available for recycling. The removal of the limiting amounts of eIF-2B from the initiation process explains why translation is almost completely inhibited when only 20-30% of eIF-2 is phosphorylated (89).

(c) 43S Preinitiation Complex Formation

The ternary complex can bind to salt-washed 40S ribosomes to form initiation complexes in vitro. This reaction is greatly stimulated by the presence of eIF-3 (45,47,61,90) and occurs independently of mRNA. The current data suggests that eIF-3 (and eIF-4C) bind first to the 40S subunit, followed by binding of the ternary complex to form the 43S preinitiation complex. eIF-1 seems to stabilize various initiation complexes, including the 43S complex.

(d) Formation of the 48S Preinitiation Complex

The binding of the 43S preinitiation complex to a mRNA to form a 48S preinitiation complex requires eIF-4A, eIF-4B, eIF-4F and the hydrolysis of ATP. This step is the most significant in terms of potential for translational control. It can be thought of as occurring in 2 phases: (i) binding of the 43S preinitiation complex to the mRNA and (ii) recognition of the appropriate initiation codon. This step is rate-limiting for translation (91) and it is here that a mRNA is

selected from a pool of mRNAs to be translated. mRNAs compete for a limiting component of the initiation machinery, eIF-4F, which by virtue of its different affinity for different mRNAs, acts as an mRNA discriminatory factor (92). Selection of the appropriate reading frame is determined at this stage. Several structural features of the mRNA which influence the efficiency of 48S preinitiation complex will be described in detail below.

(e) 60S Subunit Joining

This step requires eIF-5 and involves release of initiation factors from the 48S preinitiation complex. The release of initiation factors requires the hydrolysis of GTP (70,93) originally bound as part of the ternary complex. If a nonhydrolyzable analogue of GTP is bound, release of initiation factors and 60S joining does not take place. The end result of these reactions is the formation of a 80S initiation complex competent for chain elongation.

1.3.3. 48S Preinitition Complex Formation - A Detailed Look

In prokaryotes, base pairing occurs between the pyrimidine-rich 3' end of the 16S rRNA and a purine-rich sequence (Shine-Dalgarno sequence) located approximately 10 nucleotides upstream of the initiator codon and serves to direct the 30S ribosome to the appropriate signal (94). Early experiments with prokaryote mRNAs treated with formaldehyde resulted in activation of otherwise silent, nonfunctional ribosome—binding like regions (95). This lead to the proposal that mRNA secondary structure around and including the Shine-Dalgarno sequence acts to prevent false translation starts (for reviews, see refs. 96,97).

Most eukaryotic mRNAs do not seem to initiate protein synthesis by direct binding of ribosomes to an internal "guide" sequence. The generally accepted hypothesis is that 43S preinitiation complexes or initiation factors bind at or near the 5' end of a mRNA and scan the 5' untranslated region (UTR) until the appropriate initiation codon is reached (98). Comparison of a large number of eukaryotic 5' UTRs revealed no common conserved sequences (98) and experiments in which the 5' UTR of several mRNAs [rabbit β globin (99), polyoma early mRNA (100) and simian virus 40 (SV40) mRNA (101,102)] were deleted with no effect on the fidelity of translation are consistent with a lack of requirement for specific sequences at the 5' end of mRNAs. Insertion of random sequences upstream of the AUG in adenovirus mRNA (103) and Herpes Simplex virus (HSV) thymidine kinase (tk) mRNA (104) similarly did not prevent translation. Ribosome binding studies with <u>in vitro</u> bisulfite-modified reovirus mRNA, converting cytosine residues to uracil residues, and hence resulting in extensive modification of the mRNA primary sequence did not impair ribosome binding (105). The lack of requirement for specific signals within eukaryotic 5'UTRs is consistent with the finding that some closely related mRNAs display considerable evolutionary sequence divergence within the 5' UTR [e.g.-actin mRNAs of slime mold (106) and yeast iso-1- and iso-2-cytochrome c mRNAs (107)].

Two versions of the scanning model are depicted in Fig. 2. One version depicts the 43S preinitiation complex scanning the UTR (Fig. 2A) (108) whereas in the second model, initiation factors scan the UTR (Fig. 2B) (109). At present there is no evidence favoring one model over the other. Although it is clear that once 40S ribosomes are attached to the

mRNA, they are capable of migrating in the 5' to 3' direction (110), these results should not be extrapolated to explain the events occurring in the 5' UTR during initiation (110,111). The ability to trap a 40S subunit upstream of an AUG codon in the absence of ATP on denatured (inosine-substituted) reovirus mRNA was taken as support for the initiation mechanism shown in Fig. 2A (110). However, Tahara et al. (112) subsequently showed that such complexes are not authentic. In any event, the 5' UTRs of most eukaryotic mRNAs are probably scanned prior to 80S initiation complex formation. Signals and factors which influence this efficiency are discussed in the following section.

(a) Cis-Acting Features Which Affect 48S Preinitiation Complex Formation.

(i) The Cap Structure

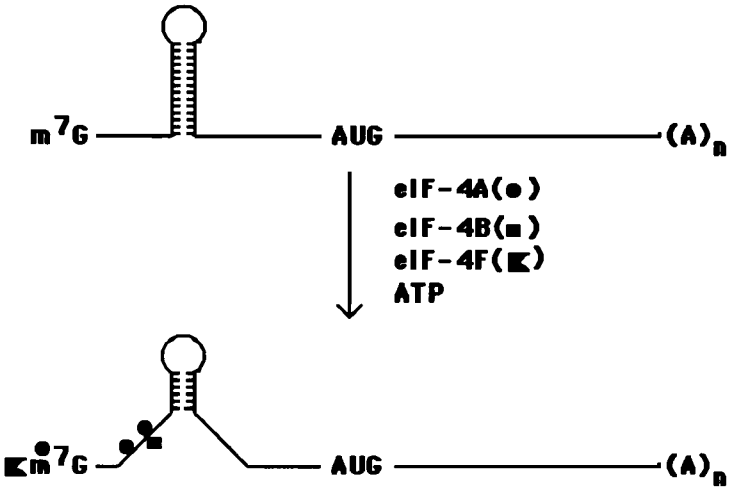
The cap structure, depicted in Figure 3, consists of a 7-methylguanosine linked by an inverted 5' to 5' triphosphate bridge to the penultimate nucleotide of the RNA molecule. It is found at the 5' end of all eukaryotic cytoplasmic and most viral RNAs (113-115) analyzed to date. The only reported exceptions among cellular mRNAs are the poly(A)-containing mRNA of mitochondria which may contain ppA at their 5' ends (116,117). In addition, mRNAs of picornaviruses, calciviruses, and certain plant viruses are not capped. The majority of Semliki forest virus (118) and Sindbis virus (119) mRNA are capped, but a small percentage of them have $m_2^{2,7}G$ and $m_3^{2,2,7}G$ at their 5' terminus.

The cap structure facilitates 48S preinitiation complex formation (113-115) and protects mRNAs from 5' exonucleolytic degradation

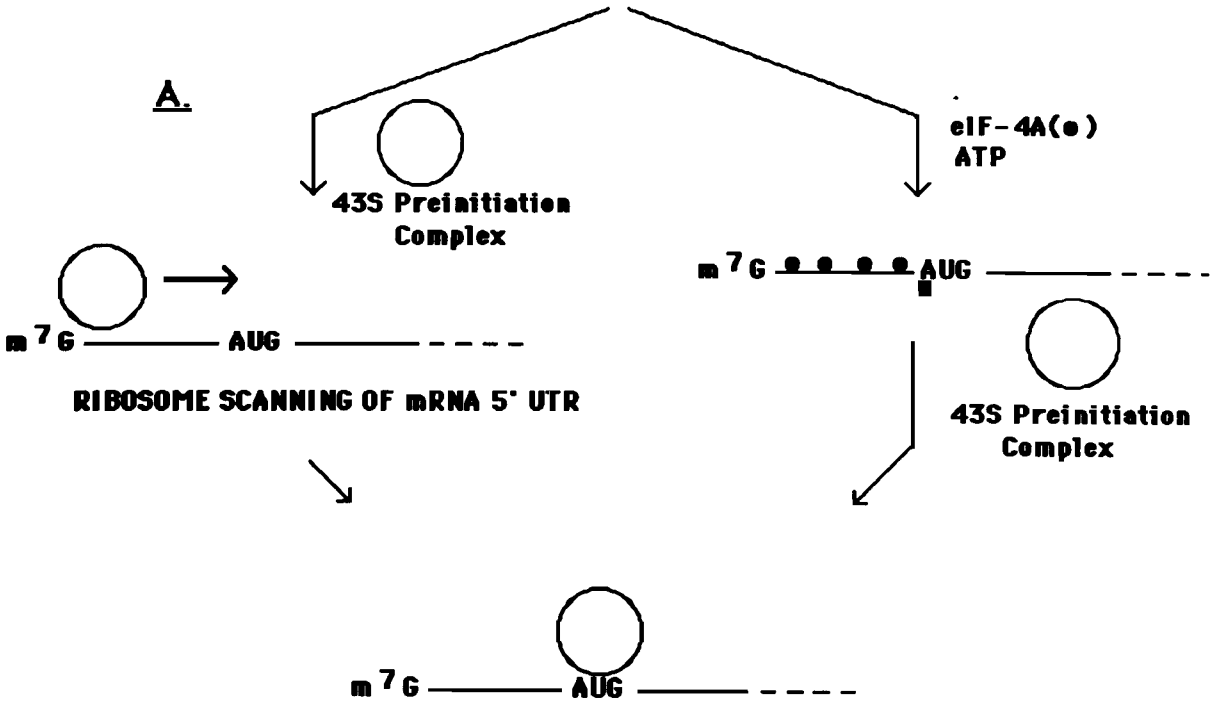
FIGURE 2

The Scanning Model For Translation Intiation of Eukaryotic Protein Synthesis. Two models have been proposed for translation initiation via scanning of eukaryotic mRNA 5' UTRs. The models differ in that one version predicts 43S preinitiation complexes scan the mRNA 5' UTR (Fig. 2A) (108) whereas the other forecasts initiation factors mediating the scanning (Fig. 2B) (109).

MODEL FOR RIBOSOME BINDING



UNWINDING OF mRNA 5' SECONDARY STRUCTURE



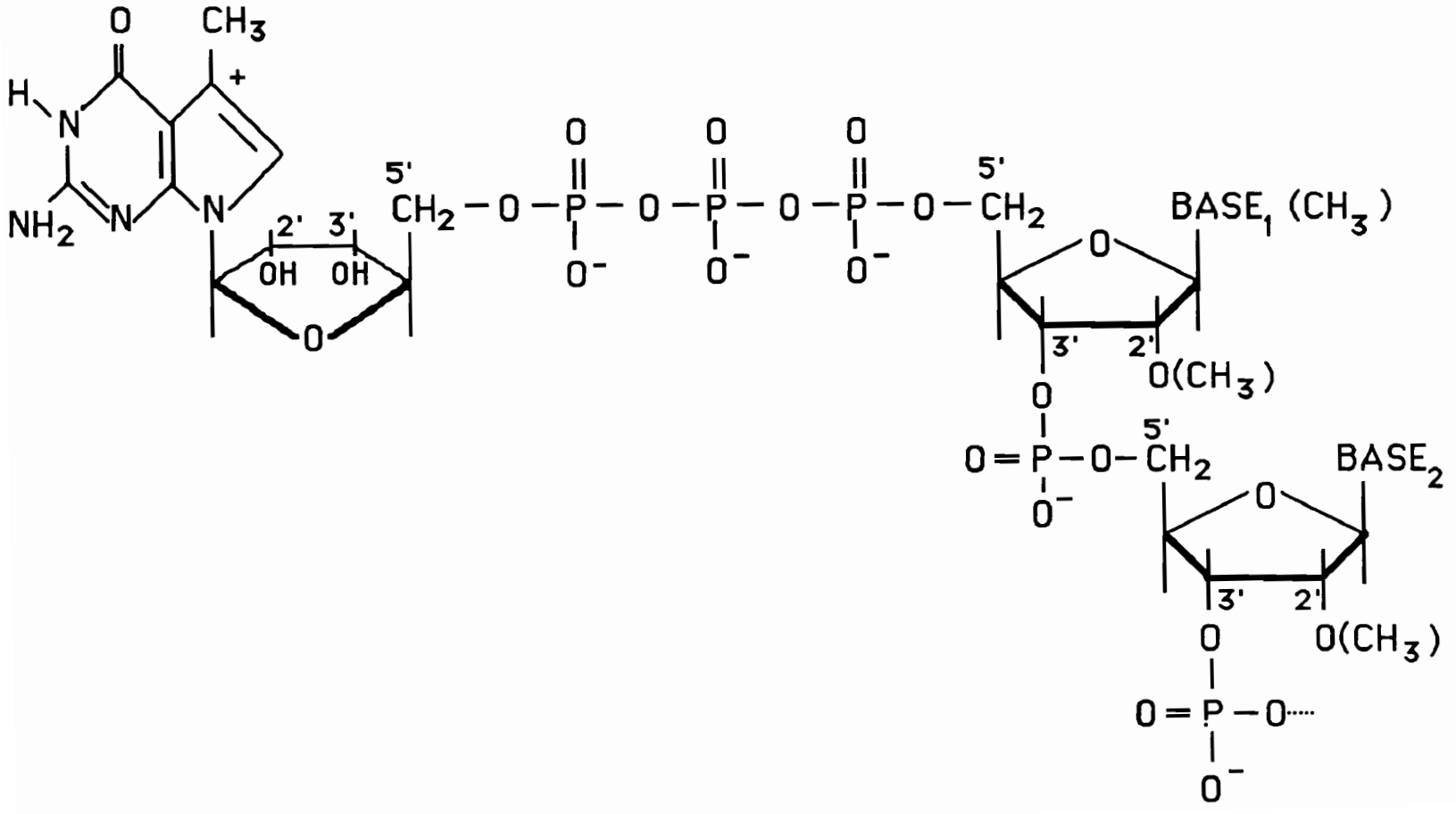
(120,121). The role of the cap structure for initiation was elucidated by comparing the translational efficiencies of capped versus decapped mRNAs, and inhibiting translation systems by the addition of cap analogues (e.g. - m^7GDP). It was originally found that many different capped mRNAs (e.g. - reovirus, vesicular stomatitis virus (VSV) and rabbit globin) are translated more efficiently than their decapped counterparts (122-125). This was shown by *in vitro* translation of *in vitro* synthesized mRNA lacking a cap structure or mRNA in which the cap had been removed by chemical or enzymatic methods. The results indicated that virtually all mRNAs which lacked a cap were poor templates for translation. Consistent with this facilitative nature of the cap, capping a prokaryote mRNA (λ *cro*) greatly increased the efficiency of its translation in wheat germ extracts (126). The translation of the capped *cro* transcript was inhibited by the cap analog, m^7GpppA. These results show that prokaryotic mRNAs contain all of the necessary information for expression in a eukaryotic translation system, except for the cap structure. Ribosome binding studies demonstrated that the cap structure increases the rate and extent by which initiation complexes are formed on several different mRNAs (124, 125-129).

Cap analogues (m^7GDP), but not their unmethylated counterparts (GDP), are specific inhibitors of capped mRNA translation *in vitro* (130-137). By using a variety of chemically modified cap analogues, this assay was used to delineate the important structural features of the cap. At least one phosphate is required since m^7G does not inhibit, while m^7GMP does. m^7GDP is more inhibitory than m^7GMP, whereas m^7GTP,

FIGURE 3

The 5' Cap Structure

THE CAP STRUCTURE



m^7GpppN or m^7GpppN^m behave like m^7GDP (131). Two dissociable hydroxyls on the α-phosphate seem to be important for cap recognition since a methyl ester derivative of m^7GMP was inactive as a cap analogue (135), whereas the β-ester of m^7GDP and γ-ester of m^7GTP are still active (136). The ribose moiety is not involved in cap recognition (136). Consistent with this is the finding that periodate oxidation of capped mRNAs, which causes ribose ring opening to the 2',3'-dialdehyde at the 5' cap, does not inactivate the mRNAs as templates for initiation complex formation (70-80% as active as intact capped mRNAs; ref. 127). C-8 substituted m^7GMP analogues (137) suggest that analogues which preferentially assume the anti conformation (see Fig. 3) are more potent inhibitors than those which are more often in the syn conformation. This data is consistent with NMR spectral data showing that the cap in mRNA adopts a rigid anti conformation (131).

Additional methylations of the 2'-OH ribose moieties of the pentultimate and third nucleotides occurs to varying extents among cellular and viral mRNAs. The additional methylations do not appear to have any significant effects on translation (121,127).

It has been more difficult to assess the function of the cap structure in vivo. This is due to the presence of 5'-exonucleases that degrade uncapped mRNAs (120), methyltransferases that will convert blocked (GpppG...) mRNAs to capped (m^7GpppG...) mRNAs (120), and the existence of a 7-methyl guanosine specific pyrophosphatase activity (138) in the cytoplasm of cells. It has been found that capped mRNAs translate better in Xenopus oocytes than their uncapped (pppG) (121) or unmethylated capped (GpppG...) (120) counterparts although in the latter

case, it was never verified that the uncapped mRNA in translating polysomes was still uncapped. Rose (139) showed that following VSV infection, which generates mRNAs with the 5' structures m^7GpppA..., pppA..., and pppG..., uncapped VSV mRNA is not ribosome associated whereas capped methylated mRNA is. Whether this reflects an absolute requirement for the cap structure or a situation of competitive advantage for capped mRNAs is unclear. Host range mutants of VSV defective in RNA methylation have been isolated which direct the synthesis of full-length unmethylated capped (GpppG...) viral mRNAs has been described (140). The mRNA synthesized by these mutants is not translated *in vivo* (140). These results suggest that possibly *in vivo* the requirement for the cap structure is absolute. Such a dependency for the cap structure has also been shown in sea urchin eggs. Decapped VSV mRNA or naturally uncapped messages such as poliovirus and cowpea mosaic virus mRNAs are not translated in cell-free translation systems derived from sea urchin eggs, whereas they are expressed in reticulocyte lysates (141). The finding that fertilization of sea urchin eggs activates cap methylation of maternal early histone mRNA (142) is consistent with an absolute requirement for the cap structure *in vivo*.

A correlation between fertilization and cap methylation has also been reported by Young (143) who reported m^7GMP appeared in mRNA of mouse one-cell embryo 3 hours after fertilization. Kastern *et al.* (144) has also demonstrated the absence of cap structures in insect oocyte RNA and its presence in embryo mRNA. Presumably, the lack of cap structures in mRNA of oocytes would serve to prevent expression of the mRNA in this system.

The degree of cap dependency for translation varies among different mRNAs and according to many experimental parameters such as temperature, ionic strength and cell-free extract used. The fact that different mRNAs exhibit greater or lesser dependence on the cap suggests a way in which the intrinsic translational efficiency of particular mRNAs might be determined. These peculiarities lead to the suggestion that secondary structure within the 5' noncoding region mediates cap dependency (145).

(ii) Secondary Structure

Many single-stranded ribonucleic acids assume secondary-structure conformations in solution (146,147). Experiments have shown that molecules such as globin mRNA contain considerable secondary structure (148,149). The parameters which dictate the secondary structure(s) a RNA molecule will assume are still poorly understood. One contributing factor is that thermodynamically stable hairpin loops are preferred over less stable ones (150). However, and still unpredictable, protein-RNA interactions may stabilize otherwise unfavorable stem-loop structures. In addition, due to the temporal synthesis of mRNA (from 5' to 3'), short local stem-loop structures may be formed faster (and may hence be selected for) than long-range interactions (151,152).

Initial studies on eukaryotic protein synthesis pointed to an inverse relationship between mRNA secondary structure and translational efficiency. Payvar and Schimke (153) demonstrated that global denaturation of ovalbumin and conalbumin mRNAs with methyl mercury hydroxide enhanced their translation *in vitro*, although the step in protein synthesis at which the effect occurred was not determined. Morgan and Shatkin (154) and Kozak (155), using *in vitro* transcribed

reovirus mRNA in which guanosine was substituted by inosine (thereby reducing the overall secondary structure), showed that these mRNAs bound ribosomes more efficiently (also, binding was less dependent on the cap structure and ATP) than did native reovirus mRNA. Pelletier and Sonenberg (156), by specifically altering the secondary structure within the 5' noncoding region of a eukaryotic mRNA, demonstrated *in vivo* and *in vitro* a decrease in translation efficiency with increasing secondary structure.

Consistent with the inhibitory effect of mRNA secondary structure is the finding that mouse and human *c-myc* transcripts differing in the length of their 5' UTR (and hence overall secondary structure) exhibit different translational efficiencies (157,158). Increased secondary structure within the 5' UTR, or 3' of the initiation codon, of the *Sacharomyces cerevisiae* iso-1-cytochrome c mRNA was found to be inhibitory to translation (159,160). The 5' UTR of *c-sis* which has the potential to form strong secondary structure has been shown to be inhibitory for translation (161,162). Studies on the 5' and 3' UTRs of the porcine pro-opiomelanocortin mRNA involving secondary structure mapping, ribosome binding, and *in vitro* translations demonstrated that translational efficiency increases when deletions are introduced that specifically eliminate hairpin loop structures (163). Kozak (164) has also found that translational efficiency decreases as secondary structure in the 5' UTR increases.

The effect of secondary structure on cap recognition has also been investigated. It has been noted that the 5' end of alfalfa mosaic virus RNA-4 (AMV-4) is unstructured (165) and that this mRNA is remarkably

efficient in translation. Godefroy-Colburn et al. (166) showed that the degree of cap accessibility of the four AMV mRNAs correlates with their degree of translational efficiency. The message in which the cap was least accessible, RNA-3, showed the lowest translational efficiency. Pelletier and Sonenberg (167), using a photochemical cross-linking assay, demonstrated that thymidine kinase (tk) mRNA with increased secondary structure near the cap site showed a reduced interaction between eIF-4B and the cap, but had no effect on the interaction between the mRNA and eIF-4E.

In some viral mRNAs, sequences at the 3' end show complementarity to those at the 5' end (168,169). The prediction that this may down-modulate translational efficiency has been shown by Spena et al. (170). Zein mRNA containing sequences at the 5' end complementary with those in the 3' UTR translated less efficiently than mRNAs in which the 3' complementary sequences were deleted.

One parameter that directly affects the stability of mRNA secondary structure is ionic strength (148). Lee et al. (171) showed that inhibition of initiation complex formation in a wheat germ extract by high salt concentrations was directly proportional to the degree of secondary structure of the mRNA. Binding of ribosomes to bromouridine-substituted reovirus mRNA (bromouridine has a greater capacity to form hydrogen bonds than uridine) was severely inhibited at high K^+ concentrations, while binding to inosine-substituted mRNA was only slightly inhibited, and binding of native reovirus mRNA was inhibited to an intermediate degree. Edery et al. (172) demonstrated that translation of several capped eukaryotic mRNAs [VSV, reovirus, and

TMV] in extracts from HeLa cells was inhibited when the K^+ concentration was increased from 75 to 215 mM, whereas AMV-4 RNA was resistent to such inhibition. Addition of eIF-4F (involved in melting mRNA higher order structure; described later) reversed the high salt-induced inhibition of translation of VSV, reovirus, and TMV mRNA. Furthermore, it was shown that inhibition of mRNA translation by cap analogues was augmented at high salt concentrations (173). Ribosome binding studies showed that AMV-4 RNA (which has little 5' secondary structure) was insensitive to inhibition by cap analogues at high salt as compared to the binding of reovirus mRNA (165). These results are consistent with the cap dependency of a mRNA being determined by higher order structures within the 5' UTR. The more secondary structure a mRNA will have within its 5' noncoding region, the more that message will be dependent on the cap structure (and hence the proteins which mediate the cap function) for its translation.

(iii) Context Effects of Initiation Codons

The flanking nucleotides of an AUG codon affect the efficiency with which it is recognized by 43S complexes for initiation. An initial compilation of 153 eukaryotic mRNAs (174) followed by a comparison of 699 vertebrate mRNAs (175) revealed that the nucleotide sequence $CC\begin{matrix}A\\G\end{matrix}CCAUGG$ (where AUG is the initiation codon) is significantly conserved. The most highly conserved position in this motif is a purine at position -3 (three nucleotides upstream from the AUG codon): 97% of vertebrate mRNA have a purine in this position. Experimental evidence suggesting that the sequence surrounding the AUG codon, and not simply

its position relative to the 5' end of the mRNA, is important in determining initiation efficiency, was first obtained by Lomedico and McAndrew (176). The contribution to the overall efficiency of the conserved purine at -3 was investigated by mutagenesis of this position in the rat preproinsulin gene and found to affect the level of protein synthesis in transfected COS-1 cells by a factor of 15 (177). The contributions of the other flanking nucleotides was found to be dependent on the nucleotide at the -3 position. A purine in position -3 has a dominant effect; when a pyrimidine replaces this purine, translation becomes sensitive to changes in position -1,-2 and +4 (178). A comparison of consensus sequences from different organisms reveal slight changes in the minor positions [plants: AACAAUGGC (179), Drosophilia: $\begin{matrix}C\\A\end{matrix}AA\begin{matrix}A\\C\end{matrix}$AUG (180), and yeast: $\begin{matrix}A\\C\end{matrix}A\begin{matrix}A\\C\end{matrix}$AAUGU (180)] but the adenine in the -3 position, which has the greatest influence on determining overall efficiency is always conserved. Changes in the -3 position have been found to occur naturally in an α globin mRNA responsible for a form of α+ thalassemia, which carries a two nucleotide deletion at positions -2 and -3 preceding the AUG codon and which affects translational efficiency by 30-50% *in vitro* and *in vivo* (182). Insertion of an artificial ATG triplet upstream of the natural initiation codon of rat preproinsulin or *E. coli* galk genes, has been shown in transfected COS cells to reduce the level of peptide synthesis from the downstream open reading frame (178,183,184). The magnitude of this effect varies and depends on sequences surrounding the inserted ATG triplet, especially in the -3 position.

The mechanism by which the flanking nucleotides exert their effect is not known. It has been proposed by Sargan et al. (185) that a noncontiguous sequence, GG/UGG, brought together by a conserved hairpin near the 3' end of 18S rRNA may base pair with mRNA. Their finding of a CCACC motif in mRNA closely resembles the consensus sequence of eukaryotic mRNAs. Nakashima et al. (186) showed that eukaryotic mRNAs in 40S or 80S initiation complexes can be crosslinked by psoralen to 18S rRNA although the site of crosslinking was not determined. This indicates that mRNAs interact with rRNAs during eukaryotic initiation and that this interact may be stabilized by a consensus sequence.

The extent to which changing the -3 nucleotide affects translational efficiency varies according to the mRNA being studied (182,187) and most likely reflects the contribution of other mRNA structural features (maybe secondary structure) to this process. Increased secondary structure near the AUG codon may slow down ribosomes sufficiently long enough to allow efficient recognition of the initiator codon, making CCACC recognition redundant.

The vast majority of eukaryotic genes invariably initiate translation at AUG. Only a handful of mRNAs initiate at alternate codons. Ribosomes can initiate at UUG (188), but the efficiency is 30-fold lower than at an AUG codon in the same context. The codon ACG is used as initiator codon in the Sendai virus P/C gene (189), when coliphage T7 mRNA is translated in rabbit reticulocyte lysates and wheat germ extracts (190), for the initiation of the adeno-associate virus (AAV) capsid protein B protein (191), and in the mouse DHFR gene when

the natural AUG codon is converted to ACG and expressed in COS cells (192). Recently, Hann et al. (193) have shown that one of the proteins synthesized from the c-myc mRNA derives from alternative initiation at a CUG codon. These non-AUG initiators seem to be less efficient than their AUG counterparts. An ACG codon in favorable context is almost as efficient for translation initiation as an AUG in a less favorable context, but only 10-20% as efficient as an AUG in a more favorable context (189). The ACG codon of the DHFR gene, when transfected into COS cells was found to direct synthesis of this protein at about 5% of the control levels (DHFR having a normal AUG). Changing the purine at -3 and the G at +4 severely reduced the ACG directed DHFR expression (192). It seems that the use of alternate non-AUG initiators may reflect a mechanism for generating protein diversity and quantitative control of the expression of the product.

(iv) Other Features

The average length of a eukaryotic 5' end varies from 20 to 100 nucleotides. The translational efficiency of a mRNA is not simply related to the length of the 5' UTR, based on comparison of a large number of messages (184,194,195). For example, rabbit β globin mRNA, which has a longer 5' UTR (53 nucleotides) than α-globin (36 nucleotides) mRNA, is more efficiently initiated in vitro and in vivo (196). The 5' end of β-globin is, however, less structured than α-globin (149) which probably explains this result. In addition, SV40 late 16S mRNA has a very long 5' end (239 nucleotides), but is more resistant than most host mRNA to hypertonic conditions, which presumably descriminates against less efficient mRNAs (197,198). It may be that

the amount of secondary structure within a 5' UTR generally increases as length of the 5' UTR increases, and that this, rather than length per se, lowers the translational efficiency.

An interesting feature which influences the site of 48S preinitiation complex formation is the distance between the AUG and the cap. In the chicken preproinsulin mRNA (199) and Semliki Forest virus genome RNA (200), nonfunctional AUG codons occur very close to the cap (6 and 1 nucleotides downstream, respectively). This may represent a "blind spot" for the incoming 43S preinitiation complex lying between the cap site and the AUG recognition site. Brome mosaic virus mRNA (210) and VSV N mRNA (202) have functional AUGs that are 10 and 13 nucleotides, respectively, from the cap site. These mRNAs represent the best available measurements of this "blind spot".

(b) Trans-Acting Factors that Influence 48S Preinitiation Complex Formation

Protein factors which play a direct role in mRNA binding are eIF-4A, eIF-4B and eIF-4E/4F.

(i) eIF-4E/F.

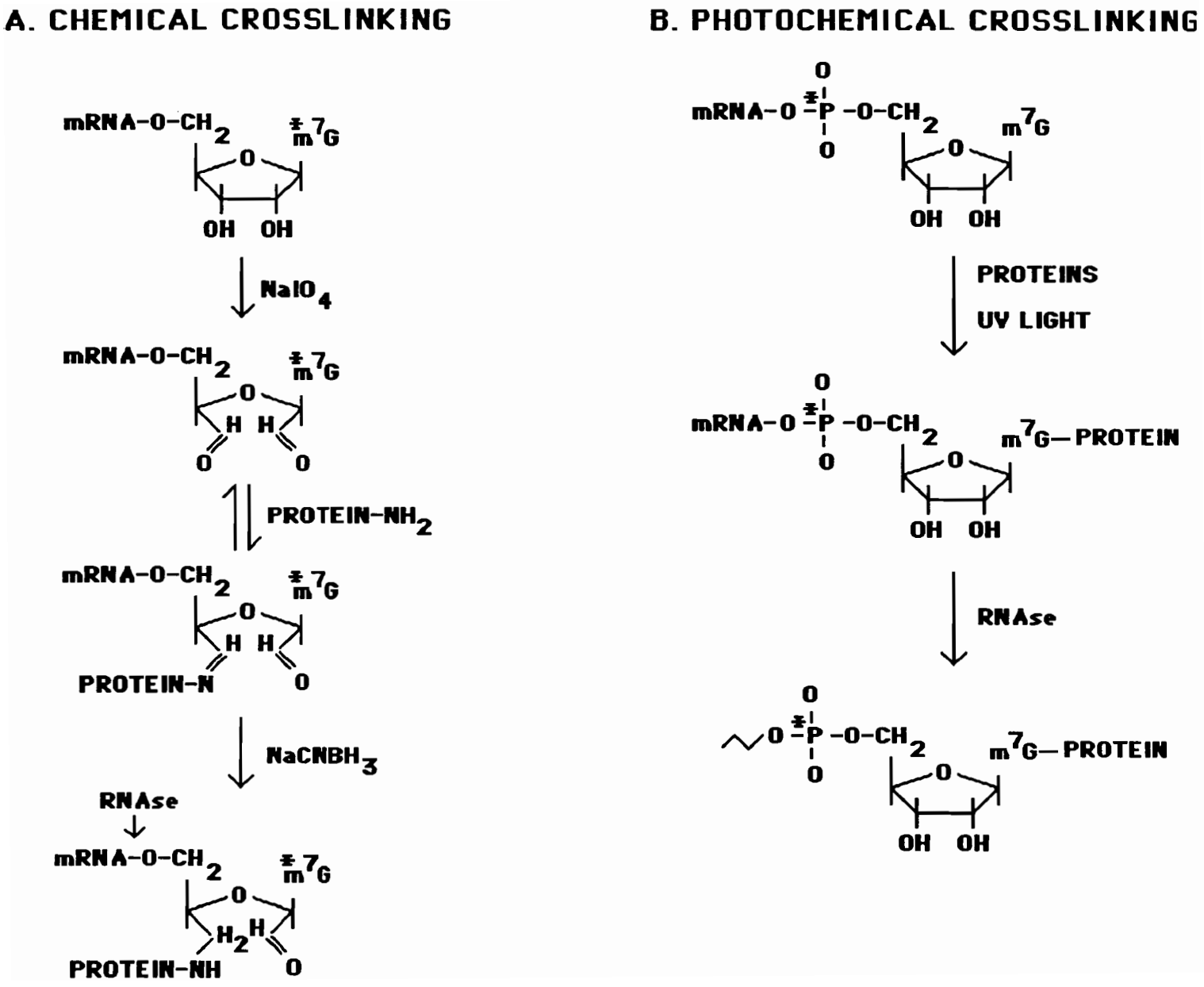
In light of observations that the cap structure facilitates 48S preinitiation complex formation (113-115) and that cap analogues inhibited such interactions (129-137), it was postulated that the cap structure mediates its effect(s) through cap binding protein(s). Sonenberg and Shatkin (203) developed a direct approach to identify polypeptides that bind at or near the cap structure. This assay is depicted in Figure 4. Messenger RNA is synthesized in vitro in the

FIGURE 4

Methods of Crosslinking Proteins to mRNA 5' Ends

A) The Chemical Crosslinking Assay.

B) The Photochemical Crosslinking Assay. In this method the cap structure is labelled with ^{32}P and the proteins are covalently crosslinked to the cap structure with UV light.


A. CHEMICAL CROSSLINKING
mRNA-O-CH2
±m7G
OH OH
NaIO4
PROTEIN-NH2
PROTEIN-N
NaCNBH3
RNAse
PROTEIN-NH
B. PHOTOCHEMICAL CROSSLINKING
mRNA-O
P
O-CH2
m7G
PROTEINS
UV LIGHT
m7G—PROTEIN
RNAse

presence of [methyl-^{3}H]S-adenosylmethionine to specifically label the m^7G ribose moiety which is then oxidized with sodium periodate to yield a reactive dialdehyde. Schiff base formation occurs between the oxidized ribose of the cap structure and primary amino groups (NH_2-terminal or ε-NH_2 groups in lysine) of proteins. These complexes are stabilized by reduction with sodium cyanoborohydride ($NaBH_3CN$). Crosslinked mRNA-protein complexes are then treated with ribonucleases to digest the mRNA, leaving a protein covalently attached to a radiolabeled cap. Complexes are resolved by sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis and visualized by fluorography. Cap specificity is assessed by performing parrallel incubations in the presence of cap analogues.

Using this assay, Sonenberg et al. (204) identified a 24 kDa polypeptide (24K-CBP; eIF-4E) in the ribosomal high salt wash of rabbit reticulocyte ribosomes which specifically interacts with the cap structure. This polypeptide was also found as a contaminant in preparations of eIF-4B and eIF-3, a finding that led to the clarification of several activities previously attributed to these factors. A similar polypeptide has been identified in yeast (205), plants (206,207) and humans (208,209).

The 24K-CBP has been purified to apparent homogeneity by using cap analogues coupled to various affinity resins (210,211). Approaches using conventional protein purification procedures were also employed (212,213). The availability of large amounts of protein lead to the successful cloning of the gene encoding the 24K-CBP from yeast (55) and mammals (56, Jerry Pelletier, Maria Jaramillo, Isaac Edery, Nahum

Sonenberg, In preparation). The gene has been shown to be essential in yeast (55). Northern blot analysis of rat liver poly(A)+ mRNA using the murine cDNA as probe reveals a complex hybridization pattern consisting of 4 mRNA species ranging in size from 1500 nucleotides to 2700 nucleotides (Jerry Pelletier, Maria Jaramillo, Nahum Sonenberg, unpublished observations), suggesting the possible existence of closely related cap-binding proteins. Cap binding proteins have also been found in nuclear extracts from HeLa cells. Two such proteins of molecular mass 20 kDa and 115 kDa were identified by crosslinking proteins to mRNA cap structures using a photochemical crosslinking assay (214). In addition, a crosslinking assay using a photoactive cap analogue identified three polypeptides of molecular masses of 80-, 89- and 120 kDa (215). The potential role of these proteins in mRNA processing events is currently under investigation.

An unusual feature of the 24K CBP is its unusually high tryptophanyl content (216). Eight tryptophans are found in the yeast (55) and mammalian (56) 24K-CBP. It has been shown that tryptophans will stack around the cap structure (217), and therefore, might underlie the mechanism by which the 24K-CBP recognizes the mRNA cap structure. One might also predict the presence of an acidic amino acid at the active site to complex with the positive charge (due to N-7 methylation) of the cap. This ionic interaction would explain in part the two orders of magnitude difference observed with binding of methylated guanosine nucleotides to the 24K-CBP as compared to their unmethylated counterparts (218). The 24K-CBP is a phosposerine-containing protein

with a single phosphorylation site at serine 53 (219). Site-directed mutagenesis of this site in the yeast 24K CBP had no effect on yeast viability or on the crosslinking or cap-binding properties of the protein (M. Altmann and Nahum Sonenberg, unpublished observations).

The 24K-CBP can be isolated as two forms: as a free polypeptide and as part of a multisubunit complex. This high molecular weight complex, called CBP II, was first isolated by Tahara et al. (67) and contained polypeptides of 24-,48-,55- and 225 kDa. Subsequently, a similar complex was purified by two other groups and has been called the CBP complex (comprising of polypeptides of 24-,50- and 220 kDa) (220) and eIF-4F (comprising polypeptides of 24-,46-,73-, and 250 kDa) (221). This complex is functionally different from the free 24K-CBP in that it can restore translation of capped mRNAs in extracts of poliovirus-infected cells where translation of capped mRNAs is inhibited (called restoring activity) (67). In addition, this complex has been shown to stimulate translation in a reconstituted protein synthesis system and is required for maximal binding of mRNA to ribosomes (221).

Several studies were done to characterize the polypeptides of the high molecular weight CBP complex. The CBP complex is deficient in the 55-KDa and 73-KDa polypeptides found in CBP II (67) and eIF-4F (221) respectively, yet still has restoring activity (172). This suggests that these 2 additional polypeptides are contaminants and are not involved in the cap recognition function. The identity of the 24 kDa polypeptide in the CBP complex as eIF-4E was verified by its ability to crosslink to oxidized mRNA in the absence of ATP. The ~48 kDa polypeptides of CBP II, eIF-4F, and the CBP complex are most likely the

same and seem to be very similar, but not identical to eIF-4A. It was shown that the 46 kDa component of eIF-4F comigrated with eIF-4A in a two-dimensional gel system (221). A monoclonal antibody directed against eIF-4A cross-reacts with the 50 kDa polypeptide of the CBP complex (220). In addition, the 50 kDa polypeptide and eIF-4A share nearly identical tryptic peptide maps (220). An intact ~220 kDa polypeptide, which is the third polypeptide of the complex, is necessary for restoring activity in poliovirus-infected HeLa extracts.

The stoichiometry of the polypeptides in the CBP complex (based on estimates by Coomassie blue staining) appears to be 1:1:1 (24 kDa: 50 kDa: 220 kDa), although this awaits confirmation by protein crosslinking studies. The 24K-CBP is the limiting eIF in the cell and has been calculated to be present at a ratio of .02-.05 molecules per ribosome (222,223) whereas all other IFs are present in higher molar amounts (224).

Whereas crosslinking of the 24K-CBP to the cap structure is ATP-independent, when ATP-Mg^{++} are added to IF preparations from rabbit reticulocytes, additional polypeptides of 50- and 80 kDa become specifically cross-linked (145). A similar set of proteins was found in IF preparations from HeLa cells in addition to a 32 kDa polypeptide (209). Non-hydrolyzable analogues of ATP did not substitute for ATP in the cross-linking reaction, leading to the proposal that energy generated from ATP hydrolysis is required for the binding of the 32-,50- and 80 kDa polypeptides to the cap structure. The identity and significance of the 32 kDa polypeptides is unclear. The extent of cross-linking of these polypeptides varies among different HeLa

preparations (K. Lee, I. Edery, N. Sonenberg, unpublished observation). The 32 KDa polypeptide is of similar size to a cap-binding protein identified in HeLa nuclear extracts (F. Rozen and N. Sonenberg, unpublished results) and may leak out of the nucleus during cell fractionation. Its absence in rabbit reticulocytes (which are enucleated) is consistent with this idea.

The 50 kDa polypeptide which becomes crosslinked in response to ATP $-Mg^{++}$ was identified as eIF-4A by the ability of a monoclonal antibody to eIF-4A to specifically immunoprecipitate the crosslinked 50 kDa polypeptide in rabbit reticulocyte IF preparations (220). Using a a photochemical assay (Fig. 4), the cross-linkable 80 kDa polypeptide was identified as eIF-4B (225).

The available data suggests that, of the known cytoplasmic cap binding proteins, the only one with a bona fide cap recognition site is eIF-4E (24K-CBP). It is the only polypeptide which can be purified as a single entity by m^7GDP-affinity chromatography (205,210,211,226). Purified eIF-4A and eIF-4B do not crosslink to mRNA in a cap-dependent fashion individually or in combination, in contrast to eIF-4E (204,220,227).

(ii) eIF-4A and ATP Requirement

Eukaryotic IF-4A exists as a free form ($eIF\text{-}4A_f$) or as a subunit of eIF-4F ($eIf\text{-}4A_c$). The role of eIF-4A in translation initiation is closely linked to the requirement for ATP in this process. eIF-4A possesses an ATPase activity (228). Ribohomopolymers lacking secondary structure are effective as activators of the eIF-4A RNA-dependent ATPase, whereas RNAs with considerable secondary structure or

double-stranded RNAs do not serve as activators (229). Supplementing eIF-4A with eIF-4B and eIF-4F results in large increases in ATP hydrolysis (compared to eIF-4A alone) when globin mRNA is used as activator (228). This activity is sensitive to inhibition by m^7GDP when globin mRNA was the activator, but not when the unstructured homopolymer poly(U) is used (228). Recent experiments have shown that eIF-4A is an ATP-dependent single-stranded RNA binding protein (228). Sarkar et al. (230) have shown that α-^{32}P-ATP can be specifically photochemically crosslinked to eIF-4A, suggesting the existence of an ATP binding site. Inspection of the murine eIF-4A cDNA reveals the presence of such a site (Jerry Pelletier, H. Trachsel and Nahum Sonenberg, unpublished observations). Both forms ($eIF\text{-}4A_f$ and $eIF\text{-}4A_c$) can crosslink ATP, however $eIF\text{-}4A_c$ crosslinks ATP ~60 fold better than $eIF\text{-}4A_f$ on a molar basis. Seal et al. (231) found that the ATP analogue, 5'-fluorosulfonylbenzoyl adenosine, inhibited wheat germ eIF-4A activity, presumably due to irreversible covalant modification of this IF.

Northern blot analysis revealed two discrete eIF-4A mRNA species of ~1600 and ~2000 nucleotides in length (53). Two functional intron-containing eIF-4A genes, eIF-4AI and eIF-4AII, have been isolated from the mouse genome and show variations with respect to tissue-associated expression (54). It is tempting to speculate that the two eIF-4A genes are not functionally equivalent and represent $eIF\text{-}4A_c$ and $eIF\text{-}4A_f$. The different pattern of eIF-4AI and eIF-4AII mRNA expression might thus reflect regulation of eIF-4F abundance in different tissues. It has in fact been found that eIF-4E mRNAs levels parallel those of eIF-4AII in different tissues (P. Nielsen, personal communications).

Two genes in yeast, highly homologous (81%) to eIF-4A have also been cloned (51). Inactivation of either gene by gene disruption has no effect on cell viability whereas inactivation of both genes is lethal to the cell.

(iii) eIF-4B

EIF-4B is an 80 kDa polypeptide. It purifies with the CBP complex through many fractionation steps and interacts functionally with eIF-4F (see ref. 109 for a review). eIF-4B when added to eIF-4A or eIF-4F stimulates the ATPase activity of these latter factors (228). In addition, ATP binding to eIF-$4A_f$ or eIF-$4A_c$ is stimulated when eIF-4B was present (230). Of the factors involved in mRNA binding, the function of eIF-4B is the least understood.

(c) Mechanism of Action of mRNA Binding Factors.

The available evidence suggests a model in which CBPs are involved in the ATP-dependent melting of secondary structure in the mRNA 5' UTR, to facilitate ribosome binding. This model is depicted in Figure 5 and described below:

1. The ATP binding to eIF-$4A_f$ or eIF-$4A_c$, unlike the ATPase activity, is not stimulated by RNA or inhibited by cap analogues. It is thus reasonable to assume that ATP binds to eIF-4A before the mRNA recognition event (230).
2. EIF-4F is the first initiation factor to interact with the cap structure, and does so through the eIF-4E subunit. Binding is ATP independent. Consistent with this is the finding that eIF-4E, either as a purified polypeptide, or as a subunit of intact or

poliovirus-inactivated eIF-4F, can be crosslinked to the cap structure in an ATP-Mg^{++} independent fashion (145,232). The relative availability of the cap structure at this step may be a determinant in the competition of mRNAs for the limiting amounts of eIF-4F (166,233).

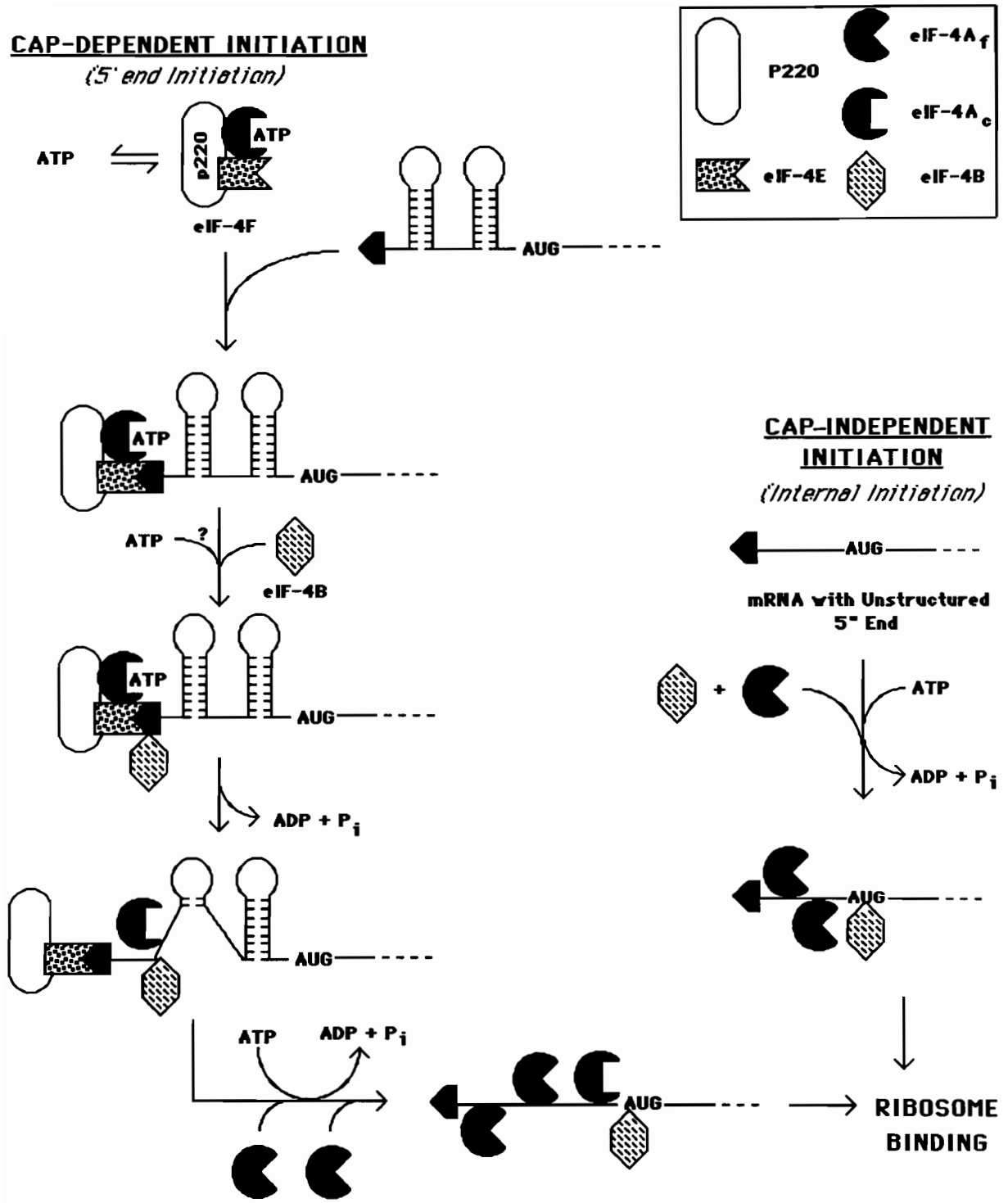
3. EIF-4A and eIF-4B then bind the cap structure, only after initial interaction of eIF-4F with this site, and in an ATP-dependent fashion. Highly purified eIF-4A and eIF-4B are crosslinked to the cap structure only when supplemented with eIF-4F and ATP (220,221). ATP hydrolysis is required for eIF-4A and eIF-4B binding (220,221,229).

4. EIF-4F, eIF-4A and eIF-4B then mediate the ATP-dependent unwinding of mRNA secondary structure. Incubation of reovirus mRNA with eIF-4F in the presence of ATP, but not in its absence, results in local mRNA denaturation as measured by increased sensitivity of the 5' end to single-strand specific nucleases (234). Furthermore, eIF-4F is capable of melting a short (20 base pairs) RNA-DNA heteroduplex in an ATP and cap-dependent manner, and this activity was stimulated by the presence of eIF-4B (234). The eIF-4A component of eIF-4F seems to mediate the unwinding since its removal from eIF-4F by phosphocellulose chromatography abolishes unwinding activity (234). eIF-4A also possesses an ATP-dependent unwinding activity (albeit 20 fold less efficient than eIF-4F), which is stimulated by the presence of eIF-4B and not inhibited by cap analogues (234)

5. The amount of secondary structure in the proximal 5' UTR of an mRNA can hinder the interaction of eIF-4A and eIF-4B. Using a photochemical crosslinking assay, eIF-4B interaction with the cap structure was inhibited by the insertion of a hairpin loop 6 nucleotides from the 5' cap structure, whereas a similar structure 38 nucleotides downstream from the cap structure had no effect (167). Lawson et al. (235) reported that an oligodeoxynucleotide complementary to sequences 15 nucleotides downstream from the cap structure had only a small effect on the cross-linking of eIF-4B and the eIF-4A subunit of eIF-4F, whereas an oligodeoxynucleotide complementary to sequences immediately downstream from the cap strongly inhibited crosslinking of these polypeptides.
6. EIF-4B can mediate the release of eIF-4F and the recycled eIF-4F is capable of rebinding to cap structures (236). The recycled factor is capable of stimulating crosslinking of eIF-4A.
7. Free eIF-4A may maintain the mRNA in a single-stranded configuration by binding to the denatured region of the mRNA in an ATP hydrolysis mediated event, thus shifting the equilibrium between the structured and unstructured conformation of the RNA towards the unstructured form (229). This is suggested by the ability of purified eIF-$4A_f$ to bind to single stranded RNA in an ATP-dependent fashion (228,229). The extremely high concentration of eIF-4A (3 molecules/ribosome), relative to other factors, may reflect the simultaneous involvement of more than one molecule of eIF-4A in the unwinding of the 5' end of each molecule of mRNA.

FIGURE 5

Mechanism of Action of mRNA Binding Factors in Initiation of Translation


CAP-DEPENDENT INITIATION
(5' end Initiation)
P220
eIF-4A f
eIF-4A c
eIF-4E
eIF-4B
ATP
eIF-4F
AUG
?
CAP-INDEPENDENT INITIATION
(Internal Initiation)
mRNA with Unstructured 5" End
ADP + P i
RIBOSOME BINDING

8. EIF-4A and eIF-4B can bind to mRNA lacking secondary structure in the absence of eIF-4F with the same degree of effectiveness and affinity as would normally occur for natural mRNAs in the presence of all three factors (227). There is some experimental evidence suggesting eIF-4B has affinity for AUG triplets (238). This raises the interesting ability that while eIF-4B is important in the cap recognition process, it may also play an important role in AUG recognition.

The mRNA melting model complements the ribosome scanning model. In addition, it opens the interesting possibility that scanning of the mRNA 5' UTR is carried out by eIF-4F, eIF-4A and eIF-4B rather than by the ribosome.

1.4 Modes of mRNA Expression

Eukaryotic mRNAs fall into three classes depending on the strategies used to synthesize their products. These are schematically outlined in Figure 6. The majority of eukaryotic mRNAs have a single open reading frame and a single functional initiation site and belong to the first group of mRNAs. The second class of mRNAs are those in which one AUG codon gives rise to two or more primary protein products. This can occur by (i) elongation beyond the termination codon mediated by a suppressor tRNA or (ii) ribosome frameshifting during translation. The third group of mRNAs comprises 5%-10% of the total mRNA population and consists of those in which 2 or more AUG codons give rise to 2 or more polypeptide products. This can occur by (i) internal in-phase initiation, (ii) initiation from 2 overlapping reading frames or (iii)

mRNAs having 2 or more distinct ORFs. These different modes of expression and the factors which determine their efficiency are addressed below.

1.4.1 Ribosome Scanning versus Internal Ribosome Binding

The scanning model rationalizes the monocistronic nature of most eukaryotic mRNAs. Incorporated into this model is the fact that most ribosomes commence protein synthesis at the first AUG codon encountered in the mRNA 5' UTR.

There is however, a small subset of mRNAs whose translation defies explanation by the scanning model and initiate protein synthesis by internal binding of ribosomes. Using hydrid-arrest translation which selectively inhibits 5'-end mediated initiation, internal binding was reported *in vitro* on the vesicular stomatitis virus (VSV) NS mRNA (239), adenovirus type 2 (Ad2) DNA polymerase mRNA (240), infectious pancreatic necrosis virus (IPNV) segment A (241), and on the encephalomyocarditis virus (EMC) mRNA (242). In addition, the construction of bicistronic mRNAs containing the EMC or poliovirus 5' UTR as intercistronic spacer allowed expression of the second cistron under conditions where 5'-end mediated initiation was blocked (243,244). Although the structural requirements for internal ribosome binding are not known and it may be that very few viral mRNAs exhibit this ability, the possibility that this event occurs on cellular mRNAs deserves more attention.

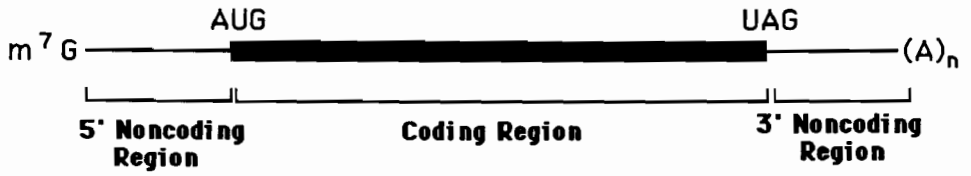
1.4.2 Suppression of Termination

Yeast suppressor tRNAs have been characterized that are capable of suppressing termination of amber and ochre codons (245). The first hint

FIGURE 6

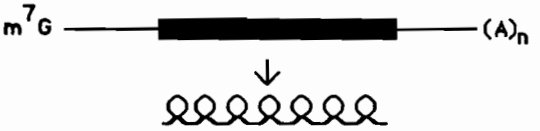
Strategies of mRNA Expression (A) The Primary Structure of a Eukaryotic mRNA. (B) Strategies of mRNA Expression.

A. PRIMARY STRUCTURE OF EUKARYOTIC mRNAs



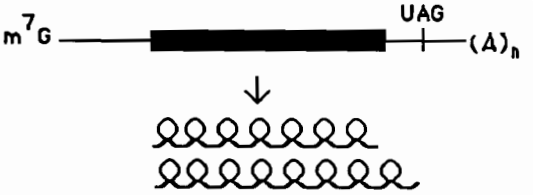
B. STRATEGIES OF TRANSLATION

CLASS 1: 1 AUG ⟶ ≥1 PROTEIN

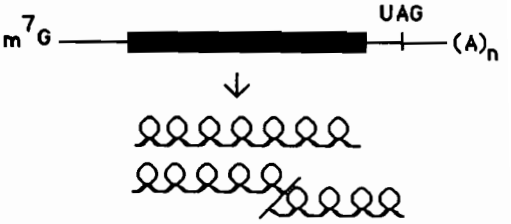


CLASS 2: 1 AUG ⟶ ≥2 PROTEINS

A) Suppression of Termination

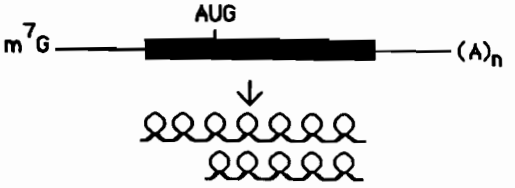


B) Ribosome Frameshifting

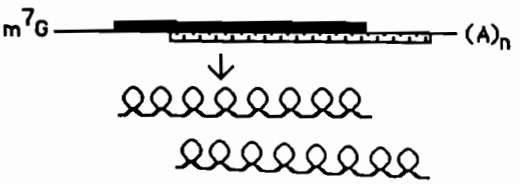


CLASS 3: ≥2 AUGs ⟶ ≥2 PROTEINS

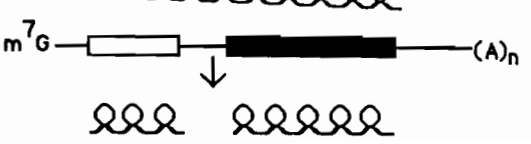
A) Internal In-phase Initiation



B) Overlapping Open Reading Frames



C) Nonoverlapping Open Reading Frames



that suppression of termination occurs in higher eukaryotes came from studies with murine retroviruses. Philipson et al. (246) supplemented a reticulocyte translation extract with yeast suppressor tRNA and found enhanced synthesis of the Moloney murine leukemia virus (MMuLV) gag-pol precursor. Subsequently, Yoshinaka et al. (247,248) showed that the viral protease of MMuLV and feline leukemia virus is a read-through product of the UAG termination codon between the gag and pol genes, with glutamine inserted at the site of the termination codon. The existence of suppressor tRNAs in higher eukaryotes has been demonstrated by the isolation and nucleotide sequence analysis of a glutamine suppressor tRNA from mouse liver (249). This species possesses UmUG (where Um stands for 2'-O-methyluridine) as the anticodon sequence that recognizes the UAG termination codon.

1.4.3 Ribosome Frameshifting

Frameshifting requires that ribosomes correctly switch from one reading frame to another at a discrete position on the mRNA so as to avoid premature termination of protein synthesis (see ref. 250 for a review). The best studied cases of frameshifting involve the expression of the gag-pol polyprotein of Rous Sarcoma Virus (251), mouse mammary tumor virus (MMTV) (252,253), and human immunodeficiency virus-1 (254). Recently, frameshifting has been shown to occur in a non-retroviral eukaryotic virus - the avian infectious bronchitis virus (255).

The events which dictate frameshifting in eukaryotes are not well understood. The primary and secondary structure of the mRNA may be involved. The motifs AAAAAAC and UUUA have been found at several

frameshifting sites (252-254). Noticeable also at such sites are sequences potentially capable of forming stem-loop structures. These may also make important contributions to frameshifting by stalling ribosomes, thereby increasing the chance of a tRNA slippage event at the above mentioned motifs. Alternatively, the existence of frameshift tRNAs (possessing non-triplet anticodons) has been postulated to contribute to the frameshift phenomena in yeast (256).

1.4.4 Leaky Scanning

The flanking nucleotides of an initiation codon in part determines the efficiency with which it is recognized. When the 5' proximal initiation site lies in a suboptimal context, some 40S ribosomes do not recognize it as a start site and continue scanning. This can result in a mRNA molecule that produces two or more unrelated proteins, translated from two different reading frames. It can also result in the production of truncated versions of the same polypeptides, if the reading frames are the same. At present there is no good method of predicting the frequency with which ribosomes initiate at each site. One problem is that in addition to context effects, there may be additional factors which determine the efficiency with which an initiation codon is utilized. There is suggestive evidence (155) that secondary structure around an initiation site may be one such factor. To examplify this point, there is one exceptional bicistronic mRNA which does not behave according to the rules of reinitiation. The first AUG codon of RNA segment b of the influenza B virus is found in the optimal context AxxAUG yet allows a significant number of ribosomes to initiate translation at a downstream AUG (257).

1.4.5 Ribosome Reinitiation

The hypothesis that ribosomes are capable of reinitiation was postulated because of the observation that eukaryotic ribosomes can initiate translation at an internal AUG, when another AUG codon occurs upstream in a highly favorable context (thus ruling out leaky scanning), provided that a termination codon occurs in-frame with the first AUG codon and upstream from the second. This phenomena was reported by Berg and coworkers (258,259) who observed the synthesis of xanthine-guanine phosphoribosyl transferase even though it was preceded by 11 AUG triplets. Consistent with the postulated event of reinitiation, the polypeptides encoded by the upstream minicistrons on several bicistronic mRNAs have been detected *in vivo* (244,260-262) and *in vitro* (243,244,263). Genetic manipulations have implicated reinitiation in the translation of several oncogenes, hormone receptors and growth factors (264). The insertion of upstream minicistrons generally act to decrease the expression from the downstream major ORF (243,244,258,261,265-268). This is probably because not all terminating ribosomes are competent for reinitiation. The efficiency of reinitiation of a preproinsulin gene placed in a bicistronic context has been shown to improve as the intercistronic region was lengthened (269). However, adequate controls for mRNA stability (ie. - it is possible that increased intercistronic length increases the probability of an endonuclease "hit" to this region, thus "liberating" the second cistron for translation) were not performed, and the data should be taken as tentative until it is further verified. The length of intercistronic

region may not be the only factor affecting the efficiency of reinitiation. Perez et al. (270) reported efficient reinitiation with chimeric transcripts of SV40 and Rous sarcoma virus that have an intercistronic gap of only 9 nucleotides. On the other hand, the EBNA 2 protein is expressed inefficiently despite a lengthy intercistronic gap between the leader ORF and the EBNA 2 coding sequence (271,272). Possible signals which may influence the efficiency of reinitiation include: the sequences flanking the downstream AUG codon, the length of the 5' proximal ORF, and the secondary structures of the intercistronic region.

The mechanism by which ribosomes reinitiate is still unknown. One major question which remains to be answered is how a reinitiating 40S (or 80S) ribosome acquires a new Met-$tRNA_i^{Met}$? It is tempting to speculate that an initiation factor, eIF-2A, which was discovered for its ability to bind Met-$tRNA_i^{Met}$ to 40S ribosomal subunits in the presence of AUG ribopolymerers might be involved (see Table 1). This factor, unlike eIF-2, is GTP independent and might be involved in directing Met-$tRNA_i^{Met}$ to reinitiating ribosomes (58).

1.5 Mechanisms of Translational Control

The regulation of a response at the translational level allows for rapid changes in protein levels. With most eukaryotic mRNAs having half-lives greater than several hours, such a swift response could not be achieved at the transcriptional level. The regulation of translation

results in a quantitative or qualitative change of the proteins within a cell. Quantitative control of protein synthesis results in a change in the levels of specific proteins, whereas qualitative control deals with the induced or repressed expression of certain mRNA species. Due to the large number of examples of translational control which have been described in the literature, only the best described and more relevant examples will be dealt with in this section.

1.5.1. mRNA Competition

This form of translational control is most noticeable in virus-infected cells. However, under normal cellular homeostasis the mechanism is of paramount importance. The underlying principle to the concept of mRNA competition (or translational discrimination) is the observation that translation of different mRNAs proceeds at different rates (see ref. 273 for a review). The theoretical models developed to explain translational discrimination (274,275) are based on the notion that mRNAs have different affinities for one or more components of the translational apparatus. Under conditions when this factor becomes limiting, mRNAs with higher affinities or lower requirements will outcompete other mRNAs.

The classic example in which the protein synthetic rate is regulated by competition involves α- and β globin mRNAs which are coordinately regulated in vivo to produce equal amounts of both chains (275), even though there is 1.4 fold more α-globin mRNA (276). This difference in translation was explained as a result of the higher initiation efficiency of β-globin mRNA (273,274). Since the binding of

eukaryotic mRNAs to ribosomes is the overall rate-limiting step in translation (91), the initiation factor eIF-4F, which is found in limiting amounts and participates in the mRNA-ribosome binding step is a prime candidate for modulating this step. Ray et al. (92) found that addition of eIF-4F to a fractionated protein-synthesizing system from Krebs ascites cells relieved the translational competition between reovirus and globin mRNAs. In another study, Sarkar et al. (277) found that of all the IFs tested, only eIF-4F could relieve the translational competition between endogenous α- and β globin in a rabbit reticulocyte lysate. Earlier studies which had attributed discriminatory activity to other IFs have either not been reproduced due to contamination of those IFs with eIF-4F or due to artificial rendition of the protein synthetic system limiting with respect to other factors.

To explain the mechanism by which eIF-4F functions as a discriminatory factor, a kinetic model was developed by Brendler et al. (278). They showed that recognition of the cap structure per se is not required for the discriminatory activity, since a similar hierarchy of translation was observed when relief of translational competition by eIF-4F was achieved when either capped or uncapped reovirus mRNAs were used. Consequently, they proposed that, apart from the cap, mRNAs contain sequence determinants that determine their initiation efficiencies in a competitive situation (278).

Sarkar et al. (277) proposed that the initial binding of the eIF-4E component of eIF-4F to the cap structures of mRNAs occurs with similar efficiencies. During a subsequent step, involving denaturation of mRNA secondary structure (and eIF-$4A_f$ and eIF-4B) the affinity or activity of

eIF-4F will vary inversely with respect to the amount of secondary structure. According to more recent evidence however, it seems that cap accessibility may play a role in the translational discrimination of some mRNAs. Godefroy-Colburn et al. (166,279) found that the rates of initiation efficiency of the alfalfa mosaic virus RNAs correlate with cap accessibility. The interaction of eIF-4F with the cap structure can be greatly impaired by sequestering the cap into a region of secondary structure (235). Thus, a revised model of translational discrimination should include cap recognition as an initial step of discrimination whereby increased cap accessibility results in a better competitive initiator. A subsequent step for discrimination would then include 5' proximal secondary structrue. This model has been described by Lawson et al. (233).

Competition can take two different forms: (1) As mentioned for α- and β globin, the intrinsic translational efficiency of an mRNA will determine its ability to compete for the cellular translational apparatus. (2) Under conditions where the translational machinery of the cell is overloaded by the overproduction of one or several mRNA species, we have competition en masse. For example, this occurs during infection of SC-1 cells by reovirus where, although the intrinsic translational efficiency of reovirus mRNAs is not higher than host cellular mRNAs, reovirus translation dominants because during the infection, the viral mRNAs accumulate and account for up to 45% of the total mRNAs (280).

1.5.2. Poliovirus Infection of HeLa Cells

Upon infection of HeLa cells with poliovirus there is a global shut-off of host protein synthesis. The manner by which the virus induces this block and manages to escape it are described below.

(a) The Virus-Genome and Expression

Poliovirus belongs to the picornavirus family (pico = small; rna = RNA containing). This family is currently divided into four genera: enterovirus, cardiovirus, rhinovirus and aphthovirus (see Table 2).

The poliovirion is a compact icosahedron consisting of a single 7.5 kb RNA molecule and 60 copies of 4 different virally-encoded polypeptides. Following penetration and uncoating (for a review, see ref. 281), all of the crucial events of the virus life cycle take place in the cytoplasm. The poliovirus genome consists of a single-stranded RNA molecule which serves as template for protein synthesis shortly after entry into the cell. The RNA is polyadenylated at the 3' end (282) and covalently attached to a small protein (22 amino acids), VPg [3B in the new nomenclature (283)], at the 5' end (284-286). VPg is removed by an enzyme in the cellular cytoplasm (287). Translated poliovirus mRNA thus is terminated at its 5' end with pU (288,289). The RNA genome of all three poliovirus serotypes has been cloned and sequenced (290-292) and reveals the presence of a large ORF (6616 nucleotides) capable of encoding a polyprotein of ~ 247,000 daltons (293,294) This polyprotein is cleaved by the viral-encoded proteases, 3C (295,296) and 2A (297) into the viral polypeptide products. The 3C protease cleaves between glutamine and glycine residues while the 2A

TABLE 2

The Picarnavirus Family

Genus	Members
Enterovirus	Poliovirus (3 serotypes)
	Coxsackievirus
	Echovirus
	Hepatitis virus A
Cardiovirus	Encephalomyocarditis virus
	Mengovirus
	Maus Elberfeld virus
	Columbia SK
Rhinovirus	More than 115 serotypes
Aphtovirus	Foot-and-Mouth Disease (7 serotypes)

protease cleaves between tyrosine and glycine residues. Not all of these amino acid pairs found in the polyprotein, however, are recognized by the proteases, presumably as a result of steric hindrance. The final cleavage event occurs between an asparagine and a serine residue in 1B (VPO) and is thought to be mediated by the viral RNA (298).

(b) Shut-Off of Host Protein Synthesis

Upon infection of HeLa cells with poliovirus there occurs a rapid shut-off of host protein synthesis (for a review, see ref. 297). Host cellular mRNA is neither degraded (300,301), nor modified with respect to cap structure, methylation, and polyadenylation (302). Most cellular mRNA is not functionally impaired since it can be extracted from infected cells and translated in wheat germ extracts (303). The lesion responsible for the observed inhibition was shown to be at the level of translation initiation (301).

Early experiments mislead investigators into falsely concluding that eIF-4B (304) and eIF-3 (305) were inactivated as a result of poliovirus infection. It was subsequently reported that eIF-4F could restore the translation of capped mRNAs in extracts from poliovirus-infected cells (67) and it seems that the earlier conclusions were due to the fact that eIF-4F cofractionates with eIF-4B and eIF-3 through several purification steps (221). Several lines of evidence are consistent with eIF-4F being inactivated during poliovirus-infection. (1) eIF-4A and -4B are not structurally modified (306) or functionally impaired (305). However, their ability to cross-link to the cap structure, which is dependent on prior interaction of eIF-4F with the

cap, is impaired in IF preparations from poliovirus-infected HeLa cells (167,232,307). Addition of functional CBP complex to crude IF from infected cells restores the cross-linking ability of eIF-4A and eIF-4B (232). (2) A fractionated system has been used to demonstrate that an activity attributable to the CBP complex is inactivated in poliovirus-infected cells (308). (3) Messenger RNAs that are less dependent on the CBP complex for translation (ie. - with reduced 5' end secondary structure) can still function in extracts from poliovirus-infected cells (172, 309).

How does poliovirus inactivate the eIF-4F? No phosphorylation of the complex has been found in poliovirus infected cells (232,310). Immunoblotting experiments using antisera against the 220 kDa component (p220) of eIF-4F revealed that the decline in host protein synthesis is preceded <u>in vivo</u> by the specific cleavage of p220 into two or three polypeptides of 110-130 kDa (311). Purification of this modified complex from poliovirus infected cells directly showed that it contains the cleavage products detected by immunoblotting (232). The cross-linking of the 24K-CBP is drastically reduced in IF preparations from poliovirus-infected cells (232). It is known that the 24K-CBP cross-links ~10 times better when it is part of the CBP complex than when it is in the uncomplexed form (232). It thus seems that the 24K-CBP in the modified complex behaves like the uncomplexed 24 kDa polypeptide in terms of cross-linking efficiency to mRNA. In addition, the presence of eIF-4A in the modified complex is somewhat variable (232) suggesting that eIF-4A dissociates more readily from the modified complex than the native one.

The nature of the viral factor responsible for inducing the shut-off of host protein synthesis is known. Experiments by Bernstein et al. (312) demonstrated that mutating the 2A region of poliovirus resulted in a virus without the ability to mediate the selective inhibition of host protein synthesis. They also demonstrated that p220 was not cleaved following infection with the mutant virus, though it was cleaved by the wild-type virus. The 2A gene product, when synthesized in a rabbit reticulocyte lysate and added back to a HeLa uninfected extract (currently used antibodies only allow detection of HeLa p220, not the reticulocyte p220), induced cleavage of p220 (313). Although 2A seems to be the mediator of p220, several lines of evidence suggest that 2A does not act directly on p220. (1) Antibodies directed against 2A that inhibited cleavage of 3CD to 3C' and 3D' had no effect on the in vitro proteolysis of p220 (314). (2) P220 proteolyzing activity does not copurify with 2A, but instead copurifies with a cellular protein of ~50 kDa (D. Etchison, personal communication). It seems that 2A is not the direct mediator of p220 cleavage, but rather may activate a cellular protein to do this. Consistent with this, the appearance of small amounts of cleavage products has sometimes been observed in preparations of CBP complex from uninfected cells, possibly reflecting a mechanism by which the cell can regulate its own translation (I. Edery, unpublished observations).

Recent experiments have shown that p220 cleavage is necessary, but not sufficient to effect complete translational suppression during poliovirus infection (314b). In cells infected with poliovirus in the presence of guanidine (which inhibits viral replication, such that only

the incoming genomic RNA template is translated) host cell mRNA translation is suppressed by about 70%, yet all of p220 is cleaved (314b). These results imply the existence of a second event to achieve complete translational inhibition. Other models have been proposed for the translational inhibition in poliovirus-infected cells, but none are fully consistent with the available evidence (299).

(c) Poliovirus mRNA Translation

One perplexing question which until recently, remained unanswered, is how does poliovirus, in the face of a complete shut-off of host protein synthesis, manage to translate its own mRNA? The poliovirus 5' UTR is unusually long (~740 nucleotides depending on the serotype) and the Lansing strain of poliovirus type 2 has 7 AUG codons upstream of the major initiator AUG. Three of these AUGs are conserved in position among the poliovirus serotypes, although the ORFs specified by them are not conserved in amino acid content or length (292). Site-directed mutagenesis of the upstream AUGs has revealed that they are not essentially for viral biogenesis (315) although mutagenesis of ATG_{588} resulted in a virus having a small plaque phenotype. This mutation falls within a region which is perfectly conserved among serotypes (316) and which forms part of a conserved stem-loop structure (317). It is not the mere absence of a cap structure which enables poliovirus mRNA to translate in a cap-independant manner since decapping VSV mRNAs did not allow for their translation in poliovirus-infected extracts (318). Deletion mutagenesis of the poliovirus 5' UTR revealed that a sequence between nucleotides 320 to 630 is required for cap-independent translation (319). When this sequence (320-630) was fused to a

heterologous cap-dependent mRNA, it could impart the ability to translate in a cap-independent fashion. Recently, linker scanning mutagenesis has confirmed these results (320). Placement of the poliovirus 5' UTR in a bicistronic mRNA as the intercistronic spacer, allowed expression of the downstream cistron under conditions in which translation of the first cistron was inhibited (eg - in poliovirus-infected cells) (244). These results demonstrate that the poliovirus 5' UTR is able to bind ribosomes internally, thus by-passing the block it imposes on 5'-end mediated initiation of cellular mRNAs.

1.5.3 Translational Control in Heat Shocked Cells

When cultured cells or whole organisms are exposed to elevated temperatures, they respond by synthesizing a small number of highly conserved proteins, the heat shock proteins (hsps) (for a review see ref. 321). Although this response is universal, it is regulated in different ways in different cell types. In yeast, the response is regulated primarily at the level of transcription (321). In Drosophila, regulation is both transcriptional and translational (321). In Xenopus oocytes, the response is at the translational level (322). Thus, in certain cell types there is a rapid change in protein synthesis, resulting in a translational block of pre-existing mRNAs. Within 10 minutes of heat shock of Drosophila cells, normal polysomes disappear (323) and heat shock mRNAs appear and are translated with high efficiencies (324). Pre-existing mRNAs are not degraded in this process and are latter reincorporated into polysomes during recovery from heat shock (325-328).

The mechanism by which cells shut-down their protein synthesis following heat shock is not well understood. The lesion is at the level of translation initiation (329) and although several initiation factors have been reported to become phosphorylated as a result of heat shock, it was never established that phosphorylation was the cause of inhibition (330). Panniers et al. (331) reported that protein synthesis inhibited in cell-free lysates prepared from heat shocked Ehrlich ascites cells could be restored by the addition of eIF-4F. De Benedetti and Baglioni (332) showed that at very early times after heat shock, all protein synthesis was drastically inhibited in HeLa cells, but resumed about 20 minutes later at a rate 50% that of control cells. They attributed this global inhibition of protein synthesis to phosphorylation of eIF-2α. This suggests a two step mechanism by which cells respond to heat shock. Within minutes after heat shock there would be phosphorylation of eIF-2α, resulting in a global shut-down of protein synthesis. This would allow the accumulation of hsp mRNAs in the cytoplasm due to transcriptional activation. Upon release of this translational block, the hsp mRNAs out-compete cellular mRNAs for the limiting amount of eIF-4F in the cytoplasm.

Evidence that hsp mRNAs are efficient mRNAs first came from studies by Hickey and Weber (333). They showed that at low concentrations of cycloheximide (which renders elongation the rate-limiting step of translation) they could stimulate the translation of non-hsp mRNAs, whereas hsp mRNAs were slightly inhibited. Synthesis of heat-shock proteins is much more resistant than that of other polypeptides to inhibition of initiation brought about by hypertonic stress. In

addition, hsp mRNAs have also been shown to be resistant to the inhibitory effect of cap analogues (334).

Recent studies have shown that hsp mRNAs contain cis-acting signals within their 5' UTR which allow for their selective translation during heat shock. Klemenz et al. (335) showed that fusing the gene for alcohol dehydrogenase (Adh) to the Drosophila heat shock transcriptional elements was not sufficient to get Adh expression under heat shock conditions. Rather, only when the Adh gene contained the first 95 nucleotides of the hsp70 5' UTR did they get expression. McGarry and Lindquist (336), by constructing deletion mutantions within the Drosophila hsp70 5' UTR showed that two highly conserved elements within the 5' UTR are not required for translation under heat shock conditions and, an authentic 5' end is required for regulatable expression since the addition of 30 nucleotides to the 5' end of the mRNA abolished translation of elements under heat shock conditions. The translation of a second heat shock gene, hsp22, has also been studied (337). As long as the first 26 nucleotides of the mRNA are left intact, up to 86% of the 5' UTR can be deleted with no effect on the inducible translation of this gene. The functional region was found to coincide with a region of sequence homology between heat shock mRNAs.

What are the structural features of hsp mRNAs that might be used to allow for their preferential translation under heat shock conditions? They have unusually long 5' UTRs (180-250 nucleotides) that are rich in adenosine residues with little potential to form secondary structure. It may be that relaxed secondary structure at the mRNA 5' end allows for their translation at high temperatures and also gives them a

competitive advantage over cellular mRNAs. This hypothesis can easily be tested by analyzing the translation of mRNAs with little or no 5' secondary structure (such as AMV-4) under heat shock conditions. The continued translation of a few non-hsp mRNAs during heat shock (325,333,338) may reflect their status as efficient initiators, possibly due to lack of 5' secondary structure. The lack of secondary structure of hsp mRNAs may also explain why synthesis of hsp70 is more resistant than normal host protein synthesis to inhibition by poliovirus infection (339).

1.5.4 Translational Regulation by Adenovirus Virus-Associated I(VAI)RNA

Following infection of HeLa cells with adenovirus, there is an accumulation of double-stranded (ds) RNA resulting from symmetrical transcription of the adenoviral genome (340). This dsRNA is capable of activating double-stranded RNA-activated protein kinase (DAI) (341) which phosphorylates eIF-2α and inhibits total mRNA translation. In order for adenovirus to express its mRNAs, it prevents this activation by a novel mechanism. Thimmappaya *et al.* (342) found that in cells infected with an adenovirus mutant that produced no virus associated 1 (VAI) RNA, late viral mRNAs accumulated in the cytoplasm, but failed to be translated. In the absence of VAI RNA, translation was blocked at the level of initiation (343) due to activation of DAI with subsequent phosphorylation and inactivation of eIF-2 (344-346). Purified VAI RNA was found to inhibit the activation of DAI *in vitro* (341,347).

The VA RNAs of adenovirus are small RNAs (about 160 nucleotides) synthesized in large amounts late after infection (348,349). These RNA polymerase III products are encoded by two distinct genes termed VAI and

VAII, on the adenovirus genome. VAI RNA is ~40 times more abundant than the VAII species late in infection and both species have extensive secondary structure (350). The VAl RNA has been shown to form a stable complex with DAI in wild-type adenovirus-infected cells (351).

The mechansim by which VAI RNA prevents activation of DAI, thus ensuring translation, has recently been reviewed (352) and rationalizes the perplexing observation that low amounts of dsRNA (ng/ml) activate DAI, whereas higher concentrations (μg/ml) prevent activation. In the absence of VAI, multiple DAI molecules are thought to bind to dsRNA molecules generated from transcription of the adenoviral genome, and phosphorylate each other for activation. Once activated, the kinase phosphorylates eIF-2α, which subsequently forms a tight complex with eIF-2B, thereby inhibiting translation.

The production of high intracellular concentrations of VAI RNA (~100,000 copies/cell) (~100 μg/ml) leads to the formation of monovalent DAI-VAI complexes, thus preventing the kinases from phosphorylating each other. The secondary structure of VAI seems to be important for its function since alteration of the duplex structure results in a mutant VAI not capable of inhibiting DAI activation (353). Preventing activation of DAI may be a general phenomena for viruses, although the mechanism by which this is accomplished does not seem to be conserved (354).

These features of adenovirus mRNA translation have been exploited to produce high efficiency mammalian expression vectors. Kaufman and Murtha (355) have shown that the translational efficiency of mRNA molecules transcribed from plasmid DNA transfected into COS-1 cells can

be increased 10-20 fold by the coexpression of VAI and VAII. This is presumably due to inhibition of DAI activation by dsRNA formed as a result of asymmetric transcription of the transfectd plasmid (355,356). Suprisingly, activated DAI only inhibited translation of mRNA produced from the transfected plasmid and not host cellular mRNA. This argues for a localized, specific depletion of eIF-2, an explanation consistent with results of De Benedetti and Baglioni (357).

An additional feature of adenovirus mRNA translation is that all viral mRNAs synthesized late in infection contain a common 5' UTR, termed the tripartite leader sequence. This sequence is 200 nucleotides long, highly conserved among serotypes, and results from the splicing of three small exons. The presence of a tripartite leader increases the translation of some mRNAs (358-360), but not others (361) in virus-infected cell. Translational enhancement requires that the tripartite leader be placed in close proximity to the AUG start codon of the mRNA (362). At late times in infection, mRNAs containing the tripartite sequence are translated very efficiently at the expense of host cellular mRNAs. The mechanism responsible seems to be one of competition. Messages containing the tripartite leader are not cap-dependent as shown by their ability to be expressed in poliovirus-infected cells, where eIF-4F is inactivated (363,364).

1.5.5 Translation Control via mRNA Structure

(a) Cytomegalovirus mRNA

There are three kinetic classes of genes expressed from the cytomegalovirus (CMV) genome: α (immediate early), β (delayed early), and γ (late). The β gene transcripts contain a cis-dominant regulatory element within their 5' UTR which prevents translation of β transcripts at a time when transcription is fully active (365). Fusing the β 5' UTR to a marker gene under α transcriptional control results in a mRNA species produced early in infection (~6-8 hrs; α kinetics) but, unlike α mRNA, is only translated much later (14 hrs; β kinetics). Deletion of a portion of the β 5' UTR reverts the expression of the marker gene to the α class (365). The nature of the cis-dominant signal(s) and its mode of action remains to be elucidated.

(b) Ferritin mRNA

Ferritin is a multisubunit protein which many, if not all, cells synthesize to provide a reservoir of iron for iron proteins and to detoxify excess iron (for review, see ref. 366). Zahringer et al. (367) showed that the synthesis of ferritin is regulated by iron in the absence of alterations in mRNA levels and proposed that this takes place at the translational level. It was shown that cytoplasmic ferritin mRNA underwent a redistribution from an inactive ribonucleoprotein pool to translationally active polyribosomes after induction (368). The expression of a cloned ferritin H chain gene was shown to be translationally regulated by iron in transiently transfected and stably transformed murine fibroblasts (369,370). The 5' UTR of ferritin was found to be sufficient to achieve regulation and could act in cis when linked to a heterologous mRNA (370). The iron-responsive element (IRE) has been mapped by deletion mutagenesis (371,372) and found to be 26

nucleotides long (372). The IRE is situated 35-38 nucleotides from the cap site and resides within a stem-loop structure (371,372). Synthetic oligonucleotides mimicking this region (372) or differing in primary structure, but conserving certain secondary structure features (373) can impart iron-responsiveness to a normally unresponsive indicator gene. A cytoplasmic protein of ~85-100 kDa has been identified which binds specifically to the IRE of ferritin mRNA (374) and may regulate repression of translation.

In vitro experiments have shown that ferritin mRNA cannot be translated in a wheat germ system unless the mRNA is first treated with phenol (375) consistent with the existence of a translational induction/repression system. Evidence indicates that ferritin mRNA is an unusually strong competitor under derepressed conditions (376).

(c) Translational Regulation by Control of Reinitiation

In the yeast, Saccharomyces cerevisiae, the expression of at least 30 amino acid biosynthetic enzymes in seven different pathways is regulated by a cross-pathway regulatory system known as general amino acid control (see ref. 377 for a review). Starvation of any amino acid out of a group of ten leads to derepression of the biosynthetic enzymes. This derepression is mediated by a DNA binding protein, GCN4, which is the direct transcriptional activator of these genes. The production of GCN4 is under translational control mediated by cis-acting elements within the 5' UTR of the GCN4 mRNA.

The 5' UTR of GCN4 is 591 nucleotides long and contains 4 small ORFs capable of encoding di- or tripeptides (378,379). It has been shown directly that these upstream ORFs are the critical regulatory

sequences in the GCN4 leader: point mutations that eliminate all four upstream AUG codons lead to constitutive expression of GCN4 and have little effect of the levels of steady-state mRNAs (265). Experiments with various permutations of the AUG mutants revealed that the third and fourth ORF are required for repression of GCN4 expression in normal growth conditions, whereas the first and second ORF are required to overcome this repression in amino acid-starved cells (265). The upstream ORFs are efficient translational start sites and can tolerate considerable changes in length, sequence and spacing without drastically affecting regulation (380,381). The translational control mechanism postulated to function here is one of control of reinitiation (381).

Using yeast genetics, a number of negative and positive regulatory trans-acting factors have been identified which are thought to modulate the effects of the upstream ORFs (382-384). One such factor, GCN2, essential for translational derepression of GCN4, has been recently cloned and sequenced (385) and shares strong homology to the catalytic domain of protein kinases. Identification of the factors involved in this regulatory circuit will elucidate the steps involved in the signaling pathway for amino acid starvation.

The manner in which gene expression and regulation has exquisitely evolved is exemplified by the many different forms of translational regulation and intricacy of the translation pathway. The recent identification of cis-acting signals in mRNAs should provide for exciting research in the near future.

CHAPTER 2

INSERTION MUTAGENESIS TO INCREASE SECONDARY STRUCTURE WITHIN THE 5' NONCODING REGION OF A EUKARYOTIC mRNA REDUCES TRANSLATIONAL EFFICIENCY

SUMMARY

The thymidine kinase gene of herpes simplex virus 1 was mutated by inserting oligodeoxynucleotide linkers into the region of the gene corresponding to the 5' untranslated portion of the mRNA. These linkers, when transcribed into mRNA, might be expected to form hairpin loops and hence increase the secondary structure of the 5' end of the mRNA. Thymidine kinase insertion derivatives were examined *in vivo* and *in vitro* to determine translational efficiency. For the *in vivo* studies, thymidine kinase insertion derivatives were transfected into thymidine kinase deficient L cells alone and together with a selectable dominant marker, or were assayed in the COS-1 transient expression system. For *in vitro* studies, thymidine kinase insertion derivatives were subcloned into pSP64. Capped transcripts were analyzed for their ability to bind ribosomes and translate in rabbit reticulocyte lysates and wheat-germ extracts. The results demonstrate that translation efficiency is decreased as the number of linkers is increased and support the view that excessive secondary structure at the 5' end of eukaryotic mRNA impedes translation.

INTRODUCTION

Several studies have demonstrated that both qualitative and quantitative translational control plays an important role in regulating eukaryotic gene expression (15). Qualitative control of translation (that is, differential selection of mRNAs for translation) is exerted at the level of mRNA entry into initiation complexes with ribosomes (20). There is much evidence that the intrinsic translational efficiencies of mRNAs are significant in influencing the level of expression from particular mRNAs in vivo, under competitive conditions (44). The site of competition is thought to involve a step prior to mRNA-ribosome binding, in which mRNAs must compete for a limiting component of the initiation machinery (30), which by virtue of its different affinity for particular mRNAs, acts as an mRNA discriminatory factor. Several features of an mRNA's structure might determine its ability to compete for a discriminatory factor, and hence influence the efficiency of initiation complex formation. Such features include the cap structure (m^7GpppX) and the primary and secondary structure.

It is established that the cap structure enhances the rate of initiation of protein synthesis at the level of mRNA-40S ribosome binding (for review see ref. 3). We, and others, have attempted to elucidate the mechanism by which the cap structure functions in this respect and have identified several polypeptides in crude protein synthesis initiation factors that appear to interact specifically with the 5' cap structure. Two cap binding proteins (CBP), termed 24 CBP (also CBP I or eIF-4E) and the CBP complex (also referred to as CBP II

or eIF-4F), were shown to bind to the cap structure by different assays and to stimulate protein synthesis in several in vitro translation systems (7,33,39). Several years ago it was suggested that a possible function of the cap structure is to mediate the denaturation of 5'-proximal mRNA secondary structure in order to facilitate ribosome binding (17). It was further suggested that this process is mediated by a cap binding protein or proteins, in light of observations that ATP is required for interaction of certain cap binding proteins with the cap structure (34) and that mRNA secondary structure influences this interaction (19). Subsequently, we showed that capped mRNAs with reduced secondary structure are less dependent on the CBP complex for translation initiation (35), suggesting that the putative melting activity resides in (or directly depends on) the CBP complex. Other approaches have shown that the CBP complex exhibits mRNA discriminatory activity and is possibly a limiting factor in translation initiation (7,30). The sum of these results suggests a model in which mRNAs with excessive secondary structure in their initiation region are discriminated against by the translational machinery, and thus inefficiently translated.

Extensive studies with prokaryotic mRNAs have established that mRNA secondary structure plays an important role in the regulation of translation (for review see ref. 37). In eukaryotes, however, there is a paucity of data regarding the effect of mRNA secondary structure on translational efficiency. Payvar and Schimke (27) have demonstrated

that denaturation of ovalbumin and conalbumin mRNA with methyl mercury hydroxide enhanced their translation *in vitro*, although at what step in protein synthesis the effect occurred was not determined. Kozak (17) and Morgan and Shatkin (25), using *in vitro* transcribed reovirus mRNAs with reduced secondary structure (in which inosine was substituted for guanosine), concluded that these mRNAs bound more efficiently to ribosomes than did native reovirus mRNAs. A major drawback of these studies, however, is that the modification is not confined to a discrete region of the mRNA. Other studies attempting to elucidate features of the 5' untranslated region of mRNA that determine translational efficiency compared translational efficiencies of different mRNAs in *in vitro* translation systems (14). The limitation of such analysis is that the various mRNAs compared differ not only in their 5' noncoding sequences but also in their coding and 3' noncoding regions. The latter sequences might be involved in base pairing with sequences in the 5' noncoding region by long-range interactions, thus potentially influencing translation initiation in the manner proposed. Consequently, our approach was to examine the effects on translation of systematically introducing fragments with defined secondary structure exclusively into the 5' noncoding region of a specific gene.

We employed the herpes simplex virus 1 (HSV-1) thymidine kinase (tk) gene as our model system because the expression of this gene can be used as a selectable marker *in vivo* and TK expression can readily be measured by assaying TK enzymatic activity. We found that increasing

secondary structure within the 5' untranslated region of tk mRNA reduces translational efficiency *in vivo* and *in vitro*.

RESULTS

Construction of Thymidine Kinase Insertion Derivatives

The HSV-1 tk gene has been cloned and sequenced (23,43). The region corresponding to the 5' noncoding portion of the mRNA is 109 bp long and contains a unique Bgl II restriction site (see Fig. 1). This restriction site was used to insert from one to several copies of Bam HI linkers, by the procedure shown in Figure 1. The number of Bam HI linkers inserted was determined by size fractionation on 10% polyacrylamide gels after endonuclease restriction and by DNA sequencing (See Experimental Procedures). For the studies described here we selected clones containing inserts of 1, 3, and 11 Bam HI linkers. The clones were designated pXJP13 (1 linker), pXJP15 (3 linkers), and pXJP18 (11 linkers). As a wild-type control we used the original plasmid pX1 throughout.

The introduced Bam HI linkers have dyad symmetry; consequently, when transcribed into mRNA, the prediction is that they will form a stable hairpin loop structure. We predicted the degree of mRNA secondary structure 5' to the first AUG of the wild-type and insertion mutants by computer modeling employing the Zucker program RNA-2 for RNA folding (47). Gehrke et al. (10) have shown, using this program, that the predicted secondary structure of alfalfa mosaic virus-4 RNA is in agreement with that indicated by structure mapping analysis employing structure-specific RNAses. The theoretical standard free energies [determined according to Tinoco et al. (41)] predicted for the various constructs are shown in Fig. 1. In addition, we have indicated the

theoretical free energy values of the hairpin loops generated by folding only the Bam HI linkers. The free energy values of the 5' untranslated regions range from -65 kcal/mole for the wild-type tk mRNA up to -250 kcal/mole for the tk construct containing 11 Bam HI linkers, whereas the standard free energies of the introduced hairpin loops range from -21 kcal/mole to -195 kcal/mole (Fig. 1). Since hairpin loops with estimated free energies of this order are considered stable, it is plausible that the predicted secondary structures generated in our constructs exist in the cell or under our experimental conditions *in vitro* (1).

Expression of tk Insertion Derivatives In Vivo

Three different systems were used to assess the efficiency of TK expression from the different constructs *in vivo*: transfection of TK^- cells with the tk insertion derivatives and determination of efficiency of transformation to the TK^+ phenotype, by selection in HAT media; cotransfection of the tk derivatives with a dominant selectable marker (neomycin resistance) and determination of tk mRNA levels and enzymatic activity in stably transformed neomycin-resistant cells; a transient expression system, in which COS-1 cells were transfected with an expression vector containing the tk derivatives, with subsequent determination of tk mRNA levels and TK enzymatic activity.

Transfection of TK^- Cells with the Insertion Derivatives

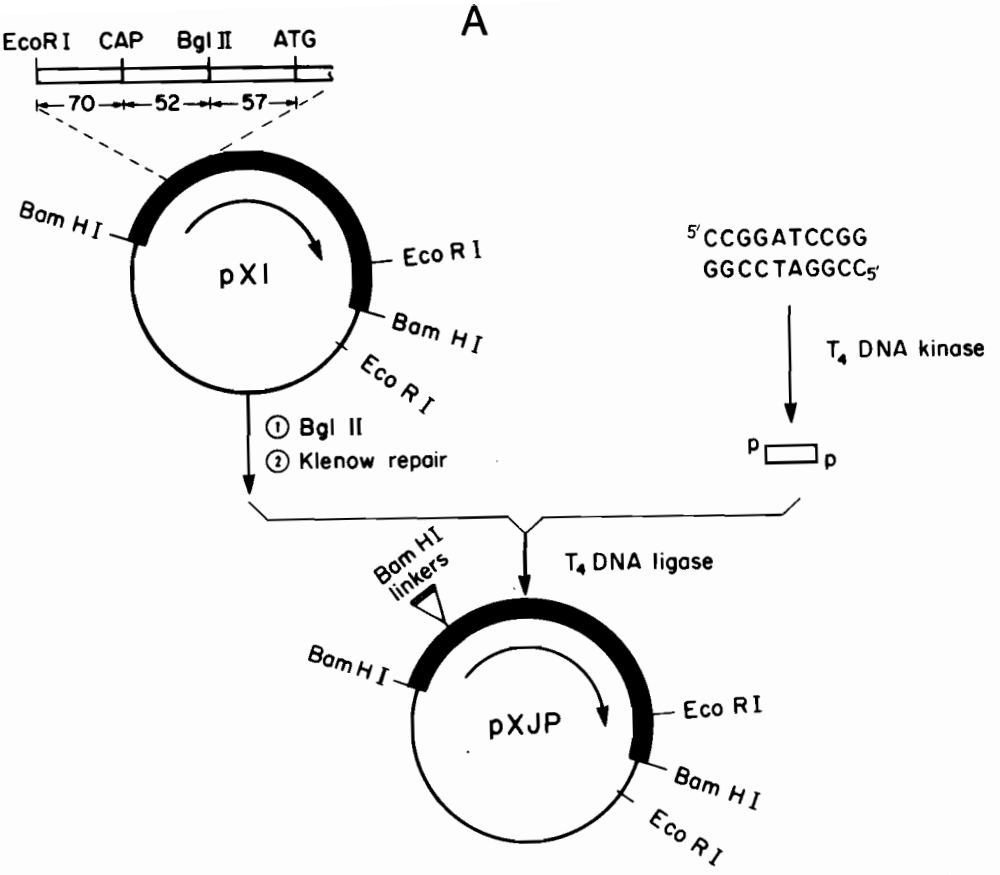
Transfection of TK^- cells with the various tk insertion derivatives was performed by calcium phosphate-DNA precipitation followed by selection of TK^+ transformants in HAT medium. Stably transformed cells were scored after 18 days of selection. Table 1 summarizes the results from two representative experiments obtained by transfecting TK^- cells

FIGURE 1

Construction of Insertion Derivatives of pX1 and Predicted Free Energy Values of the 5' Ends of the Mutant mRNAs

(A) A schematic diagram showing the general strategy used to construct the insertion derivatives of pX1. The position and structure of the 5' untranslated region of the tk mRNA are indicated. Linker derivatives were constructed from pX1 as described in Experimental Procedures. The filled box denotes the tk mRNA coding region, the hatched box denotes the tk promoter, and the empty boxes denote flanking sequences. The black arrow denotes the direction of transcription of the tk gene.
(B) The predicted free energy values are given for the 5' untranslated region and inserted hairpin loops of the tk variants (1 cal = 4.184 J).

A



B

Construct	ΔG° of inserted hairpin loop (kcal/mole)	ΔG° of mRNA 5' Non-coding region (kcal/mole)
pX1	-	- 64.6
pXJP13	- 20.7	- 73.1
pXJP15	- 61.1	-113.5
pXJP18	-195.3	-247.7

with the tk insertion derivatives. Generally, the insertion derivatives showed a decrease in transformation frequency in direct proportion to the number of Bam HI linkers introduced. The effect of one Bam Hl linker inserted into pXl (clone pXJP13) was variable (between 3 to 6 fold decrease), but the insertion of three linkers (clone pXJP15) abolished the transformation capacity. However, rare transformants (two to eight colonies in some plates) were observed. Unexpectedly, the number of transformants obtained upon transfection with pXJP18 (11 Bam HI linkers), albeit low, was higher than that obtained with pXJP15. Although we have no clear explanation for this, one possibility is that the multiple repeats of the Bam HI linkers enhance the frequency of their deletion via rearrangements or recombinational events when transfected into cells. This could also explain the low level of transformants observed with pXJP15. However, we have not analyzed the nature of the integrated DNA in these transformed colonies. We conclude that the reduced transformation capacity of the tk insertion derivatives probably results from reduced expression in the cells.

Cotransfection of the Insertion Derivatives with Neomycin Resistance Marker

It was important to measure TK activity in transfected cells grown in the absence of selective pressure, since under these conditions it is possible to correlate the level of TK expression with the level of tk mRNA. In addition, it was necessary to verify that the lack of TK expression observed with pXJP15 and pXJP18 results from a translational block, as opposed to an effect on transcription or mRNA processing. To investigate this, we cotransfected the tk derivatives with a dominant

Table 1

Transformation Frequencies of tk Insertion Derivatives

Construct	No of Colonies/Plate	Average No. of Colonies/ng DNA	Relative Frequency of Transformation[a]
Experiment 1:			
None (Control)[b]	0; 0; 0; 0	0	0
pX1	314; 210; 258; 218	1.25	1
pXJP13	34; 22; 16	0.2	0.16
pXJP15	0; 0; 2; 0	0.01	0.01
pXJP18	10; 16; 10; 8	0.05	0.04
Experiment 2:			
None (Control)[b]	0; 0; 0; 0	0	0
pX1	588; 752; 576; 640	1.65	1
pXJP13	176; 248; 270	0.64	0.39
pXJP15	0; 0; 4; 8	0.01	0.01
pXJP18	20; 8; 0; 28	0.07	0.04

Plasmids were transfected into mouse LTA cells as supercoiled DNA and transformation frequences to TK^+ phenotype determined as described in Experimental Procedures.

[a] Frequency of transformation obtained with pX1 was normalized to 1.

[b] As control for spontaneous transformation, only carrier DNA was used for transfection.

selectable marker, and subsequently analyzed the selected clones for the levels of tk mRNA, TK enzymatic activity, and protein levels. It has been demonstrated that when nonlinked genes are cotransfected with a selectable marker more than 80% of the selected transformants contain the nonselected gene stably integrated in the genome (46). TK constructs were cotransfected with pSVtkneoβ (described by Nicolas and Berg (26); this plasmid contains, among other elements, the gene conferring neomycin resistance and will be referred to as p^{neo}) into TK^- cells followed by selection for neomycin resistance in media containing the antibiotic G418. Following 9 days of selection the number of surviving colonies was determined, grown to mass culture, and analyzed for TK enzymatic activity and tk mRNA content.

We obtained similar numbers of neomycin-resistant transformants by cotransfection with the different constructs (1000-2000 colonies per 100 mm dish). We measured the levels of total cellular poly(A^+) tk mRNA in the transformed cells by dot blot analysis and found that the yield of mRNA from the different constructs varied between 0.5 to 1.7 relative to the levels of wild-type mRNA (Table 2). Analysis of TK enzymatic activity in the transformants shows that activity was significantly reduced when 3 and 11 Bam HI linkers (clones pXJP15 and pXJP18 respectively) were inserted into the 5' noncoding region of the tk mRNA; insertion of one Bam HI linker (clone pXJP13) did not have any significant effect, while insertion of 11 Bam HI linkers resulted in almost background levels of TK activity (Table 2). It seems clear that the insertion derivatives are faithfully transcribed and processed and that the lack of TK activity is explained either by the fact that the

mRNAs produced are translated poorly (if at all) or that they direct the synthesis of an inactive TK protein. The latter alternative has to be considered because of the possibility that alterations of the tk coding region occurred during the *in vitro* manipulations.

We have immunoprecipitated TK protein from ^{35}S-methionine-labeled transfected cells with an anti-HSV-1 TK rabbit polyclonal antibody (generously provided by J. Smiley, McMaster University, Hamilton) and have found that no aberrant proteins are synthesized, as determined by SDS/polyacrylamide gel analysis (data not shown). Quantitation of the TK product by densitometry demonstrates that the reduction in TK protein levels correlates well with the level of TK enzymatic activity in extracts from transfected cells (Table 2). We conclude from these observations that the lack of TK expression from pXJP15 and pXJP18 is due to the failure of the mRNAs produced to engage in protein synthesis.

Expression of the Derivatives in COS-1 Cells

To substantiate the conclusions drawn thus far, we studied the expression of the tk derivatives in a transient expression system employing COS-1 cells. In contrast to the stable transformation described above, in which multiple copies of the cloned gene are sometimes rearranged or joined to various segments of host or carrier DNA, in the COS system all the cloned genes in the pSVOd vector are identical with respect to flanking sequences. To express the tk constructs in COS-1 cells we subcloned the tk derivative genes into pSVOd (24), which contains the SV40 origin of replication and therefore

Table 2.

TK Expression in Long-Term and Short-Term (Transient) Expression Systems

Construct	Relative tk mRNA Level[a]	TK Protein Level[b]	TK Activity[c] cpm of [^{125}I]IdC/Dish (%of control)
Experiment A: p^{neo}	ND	0	1,415; 1,445 (0)
p^{neo}+ pX1	1	1	134,769; 102,721 (100)
p^{neo}+ pXJP13	1.7	1.78	149,520; 133,741 (119)
p^{neo}+ pXJP15	0.8	0.10	43,520; 51,577 (39)
p^{neo}+ pXJP18	0.5	0.02	2,102; 2,339 (1)
Experiment B: pSVOd	0		15,797; 17,769 (0)
pX1/SVOd	1		55,174; 57,640 (105)
pXJP13/SVOd	1.9	ND	38,333 (58)
pXJP15/SVOd	0.8		26,296; 21,074 (17)
pXJP18/SVOd	1.0		19,809; 18,818 (7)

Experiment A: Plasmids were transfected into mouse LTA cells as supercoiled DNA as described in Experimental Procedures. Experiment B: Plasmids were transfected into COS-1 cells as supercoiled DNA.

[a]Levels of tk mRNA were determined after selection of total poly A^+ mRNA, followed by dot blot analysis and quantitation of autoradiographs as described in Experimental Procedures. The values obtained for transfection with wild-type (pX1) tk construct were normalized to 1.

[b]TK protein levels were determined by densitometric tracing of immunoprecipitated material from ^{35}S-methionine-labeled cells using an anti-HSV TK antibody. The value obtained for p^{neo} + pX^1 transfected cells was normalized to 1.

[c]TK enzymatic activity was determined as described in Experimental Procedures. Values obtained when transfection was carried out without tk plasmids (p^{neo} in experiment A and pSVOd in experiment B) where substracted from all the other measurements.

can use endogenous T antigen expressed in COS-1 cells to enhance plasmid replication. Mellon et al. (24) have shown that human α-globin gene inserted into pSVOd is faithfully transcribed and accurately processed to produce authentic α-globin mRNA. The structure of the recombinant tk/pSVOd plasmids is shown in Figure 2A. COS-1 cells were transfected with the recombinant pSVOd plasmids by the calcium phosphate-DNA precipitation method, and after 48 hr., tk mRNA levels and TK enzymatic activity were measured. Again, measurement of tk mRNA levels as determined from dot blot analysis and quantitation by densitometry (Fig. 2B and Table 2) revealed that transcription was not impaired in clones pXJP15 and pXJP18 (Fig. 2B). The measurements of TK activity, summarized in Table 2, show an inverse relationship between the degree of introduced secondary structure into the mRNA and expression of TK protein. With clone pXJP13 expression is reduced to 55% of wild-type levels, whereas with clones pXJP15 and pXJP18 expression is reduced to 18% and 7%, respectively, of wild-type levels.

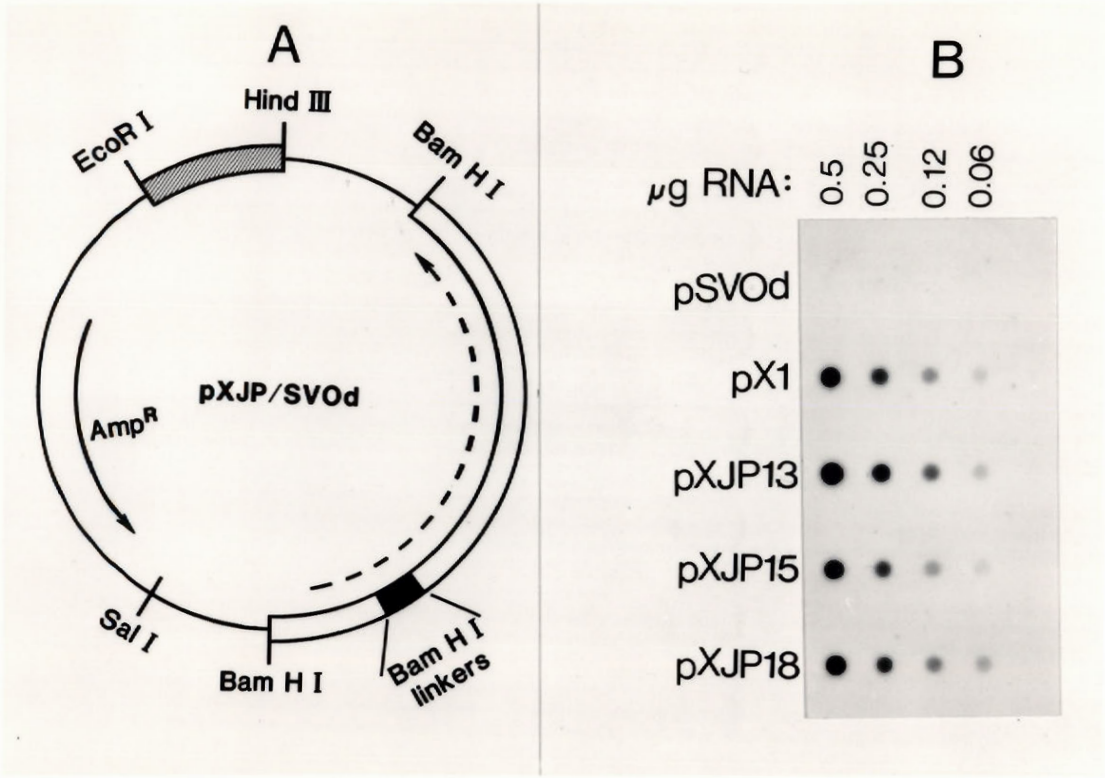
Expression of TK Gene Constructs In Vitro

To study the effects of the insertions on in vitro translation, we placed the tk variants under the control of the SP6 promoter in the pSP64 plasmid as shown in Figures 3A and 3B. The new recombinant plasmids were termed pX1/SP, pXJP13/SP, etc. These plasmids were subsequently linearized with Eco RI, which cleaves 28 nucleotides downstream from the original Pvu II restriction site (see Fig. 3B). In addition, we generated a series of other recombinant tk/SP molecules to use as controls. We introduced one and two Bam HI linkers into the Hind III site of pX1/SP (called pXJPA/SP and pXJPB/SP respectively) to serve

FIGURE 2

Schematic Diagram of pXJP/SVOd Recombinants and Analysis of tk mRNA Expression in COS-1 Cells

(A) tk insertion derivatives were subcloned into pSVOd as described in Experimental Procedures. The hatched box indicates the SV40 origin of replication. (B) For determination of mRNA levels, $1x10^6$ COS-1 cells were transfected with 20 µg of pXJP/SVOd recombinants and 20 µg of DNA as carrier. mRNA was isolated at 48 hr after transfection and prepared for dot blotting experiments. Poly (A^+) mRNA from cells that have been transfected with the indicated plasmids has been quantitated by hybridization to [^{3}H]polyuridilic acid as described in Experimental Procedures, and the amounts indicated were spotted on nitrocellulose paper (S&S) and hybridized to ^{32}P-labeled nick-translated pX1 DNA ($3x10^6$ cpm; $5x10^8$ cpm/µg) prepared according to Rigby et al. (31), essentially as described by Thomas (40). Blots were exposed against Fuji film and the intensities of the dots were quantified by soft laser densitometry. The results are tabulated in Table 2.


A
Hind III
EcoR I
Bam H I
pXJP/SVOd
Amp^R
Sal I
Bam H I
Bam H I linkers
B
μg RNA:
0.5
0.25
0.12
0.06
pSVOd
pX1
pXJP13
pXJP15
pXJP18

as controls for site-specific effects. We have also introduced seven Eco RI linkers (^{5}'GGAATTCC^{3}') into the Bgl II site of pX1/SP (called pXJP/Eco/SP) to control for sequence-specific effects of the linker DNA. To account for any effect on translation due to changes in length of the 5' untranslated region of the mRNA molecule, we created a deletion mutant by removing the Hind III/Hinc II fragment of pX1/SP to generate pX1/SP1 (see Fig. 3B). In addition, we increased the length of the 5' untranslated region by subcloning the Hinc II/Pvu II tk fragment into the Sac I site of pSP64, to generate pX1/SP2 (Fig. 3B).

Green et al. (13) have shown that a eukaryotic gene inserted next to the SP6 promoter in pSP64 can be transcribed with purified SP6 RNA polymerase. The polymerase is highly specific for the SP6 promoter and hence does not initiate transcription from other E. coli promoters on the plasmid. Transcription of linearized plasmid from the SP6 promoter yields run-off transcripts that can be used for translation in vitro. However, mRNAs transcribed in this system lack a cap structure; consequently these mRNAs would translate poorly in eukaryotic translation systems in vitro (3). Green et al. (13) have previously used the capping enzyme from vaccinia virus to posttranscriptionally cap and methylate globin mRNA transcripts made by SP6 polymerase. However, we were unable to achieve consistent capping of the majority of the transcripts (efficiency of capping varied between 20% and 60%), and because it was imperative to have a homogenous population of capped mRNAs, we attempted an alternative approach suggested by Contreras et al. (6). In this method, a cap analogue, $m^{7}GpppX$, (in which the nucleotide denoted by X corresponds to the nucleotide at which trans-

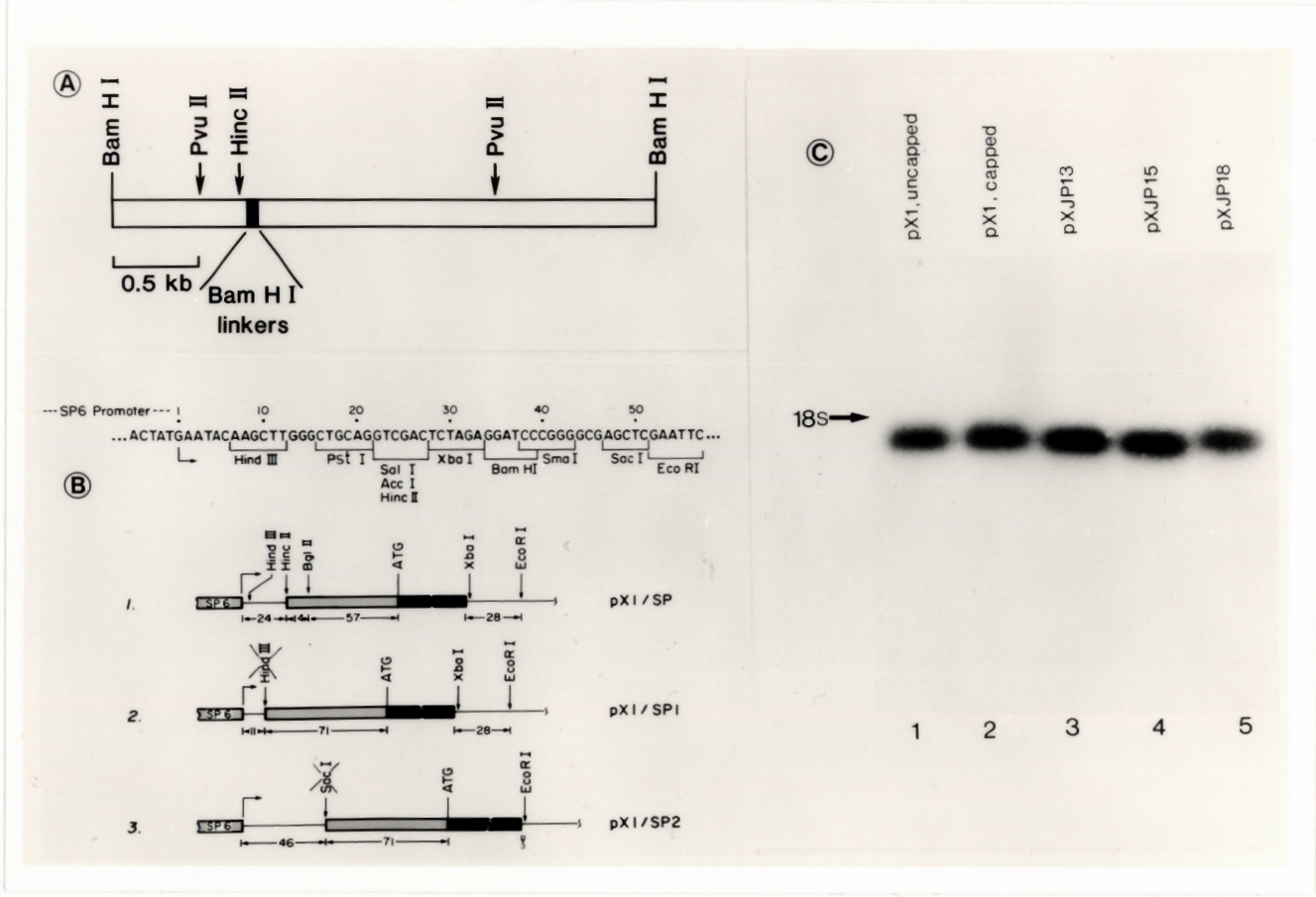
cription initiates), is added in the transcription reaction at a relatively high concentration (500 μM) while the concentration of the nucleoside triphosphate that normally initiates transcription is reduced (20 μM). Consequently, the cap analogue is utilized by the RNA polymerase with sufficient efficiency to serve as a primer for transcription.

It was important to determine whether the inclusion of the cap analogue in the transcription mixture affects the fidelity of transcription and whether there was any effect of Bam HI linker insertion on the amount and size of the corresponding tk transcripts made in vitro. To this end, radiolabeled transcription products were analyzed on denaturing agarose gels followed by autoradiography. The results (Fig. 3C) indicate that the sizes of tk mRNAs produced are very similar in the presence and absence of the cap analogue, m^7GpppG (compare lanes 1 and 2). More than 90% of the transcripts migrated as a single band with the expected size of 1800 nucleotides. The capped transcripts made from the tk insertion constructs appear to have the same size as wild-type tk mRNA, with the exception of pXJP18/SP mRNA, which migrates somewhat slower than the other mRNAs, consistent with the expected extra 110 nucleotides as compared to wild-type tk mRNA (Fig. 3C, lanes 3-5). Furthermore, the amount of mRNA made from the insertion constructs was generally equal to that made from pX1/SP (1-2 μg mRNA/3 μg DNA template, as determined from the specific activity of the mRNA produced). The possibility that the SP6 RNA polymerase prematurely terminates at the Bam HI linker region in the tk variants, thus producing short capped transcipts of ~ 50 nucleotides, which could compete with the mRNA in translation, was ruled out by analyzing the

FIGURE 3

Structure of tk Derivatives Inserted into pSP64 and Size Analysis of In Vitro Transcribed tk Derived mRNAs

(A) Diagram of the Bam HI fragment containing the tk gene in the pXJP constructs and restriction sites used for subcloning into pSP64. (B) tk insertion derivatives were subcloned into pSP64 as described in Experimental Procedures. Shown above is the polylinker region of pSP64. The right-angled arrows denote the start and direction of transcription, and the restriction sites that are X-ed out were destroyed during the manipulations. The numbers represent distances in base pairs. The diagrams are schematic representation of: (1.) the tk gene in the Hinc II site of pSP64 (termed pX1/SP); (2.) a deletion mutant of pX1/SP in which the Hind III/Hinc II fragment has been excised (termed pX1/SP1); (3.) the Hinc II/Pvu II fragment of the tk gene in the Sac I site of pSP64 (called pX1/SP2). (C) Transcription of tk derivatives inserted into pSP64 was carried out in the presence of α-^{32}P-ATP and in the absence or presence of m^7GpppG, as described in Experimental Procedures. An aliquot of the products was analyzed on 1.4% agarose gel containing 5 mM methylmercury hydroxide. The gel was treated with 10% methanol, 10% acetic acid, 20 mM β-mercaptoethanol for 3 hr, then dried and exposed against Cronex x-ray film for 1 hr. Lane 1, 30,000 cpm of uncapped wild-type (pX1/SP) tk mRNA; lane 2, 30,000 cpm of capped wild-type (pX1/SP) tk mRNA; lane 3, 30,000 cpm of capped mRNA from pXJP13/SP; lane 4, 30,000 cpm of capped mRNA from pXJP15/SP; lane 5, 15,000 cpm of capped mRNA from pXJP18/SP. HeLa rRNA was run in adjacent lanes and visualized by staining with ethidium bromide.


A
Bam H I
Pvu II
Hinc II
Pvu II
Bam H I
0.5 kb
Bam H I
linkers
B
---SP6 Promoter---
...ACTATGAATACAAGCTTGGGCTGCAGGTCGACTCTAGAGGATCCCGGGGCGAGCTCGAATTC...
Hind III
PSt I
Sal I
Acc I
Hinc II
Xba I
Bam HI
Sma I
Sac I
Eco RI
pX1/SP
pX1/SP1
pX1/SP2
SP6
ATG
Xba I
Eco R I
Bgl II
C
pX1, uncapped
pX1, capped
pXJP13
pXJP15
pXJP18
18S
1
2
3
4
5

transcription products on a 20% polyacrylamide/7M urea gel. No radioactive bands corresponding to low molecular weight RNAs were detected by autoradiography after overexposure of X-ray film (data not shown).

Analysis of 5'-Terminal Structures of pSP64/tk Transcripts

We determined the proportion of transcripts from wild-type and tk insertion constructs containing cap structures. Efficiency of priming by m^7GpppG was determined in several ways. Transcription was performed with α-^{32}P-ATP, which is the second nucleotide from the initiation site of transcription, in the absence or presence of m^7GpppG. Transcripts were digested with RNAse T2, and digestion products were analyzed by DEAE-cellulose column chromatography. With uncapped transcripts, a major peak of radioactivity eluted from the column with a negative charge of approximately -5 (Fig. 4A). (The radioactive peak at position -2 is nucleoside monophosphates while the material eluting at approximately position -3 is remaining ^{32}P-ATP.) With m^7GpppG in the transcription mixture, a new peak with a net negative charge of approximately -4.5, consistent with the structure $m^7GpppGp$, was found (Fig. 4B). The radioactive material migrating at approximate positions of -4.5 and -5 was desalted and further analyzed on TLC plates developed with 2M pyridinium-formate (pH 3.4). Most of the material eluting at position -5 from the DEAE-cellulose column comigrated with the pppGp marker compound (Fig. 4C, lane 1), whereas most of the material possessing a negative charge of -4.5 migrated faster and is most probably m^7GpppG^*p (lane 2). Further analysis of the putative m^7GpppG^*p product by nucleotide pyrophosphatase and calf intestinal alkaline phosphatase resulted in release of pG^*p and ^{32}P-free phos-

phate, respectively, as determined by rechromatography on PEI plates in 1.2 M LiCl, consistent with the postulated structure (data not shown).

In a different analysis, transcripts were made with α-^{32}P-GTP in the absence or presence of m^7GpppG. Digestion of transcripts, produced in the absence of cap analogue, with RNAse T2 followed by chromatography on PEI cellulose plates developed with 2M pyridinium-formate (pH 3.4), reveals a distinct spot comigrating with pppGp near the origin of application, in addition to material that is retained at the origin of application (Fig. 4C, lane 3). However, the radioactive spot corresponding to pppGp was absent when transcription was performed in the presence of m^7GpppG, indicating that few uncapped transcripts were synthesized under these conditions (Fig. 4C, lane 4). Thus, the results of two different analyses lead us to conclude that the majority of transcripts (>95%) made in our system contain a m^7GpppG cap structure. Similarly, analysis of cap structures of transcripts made from the three tk insertion derivatives revealed that the transcripts were capped to the same extent (data not shown).

In Vitro Translation of pSP64/tk Transcripts

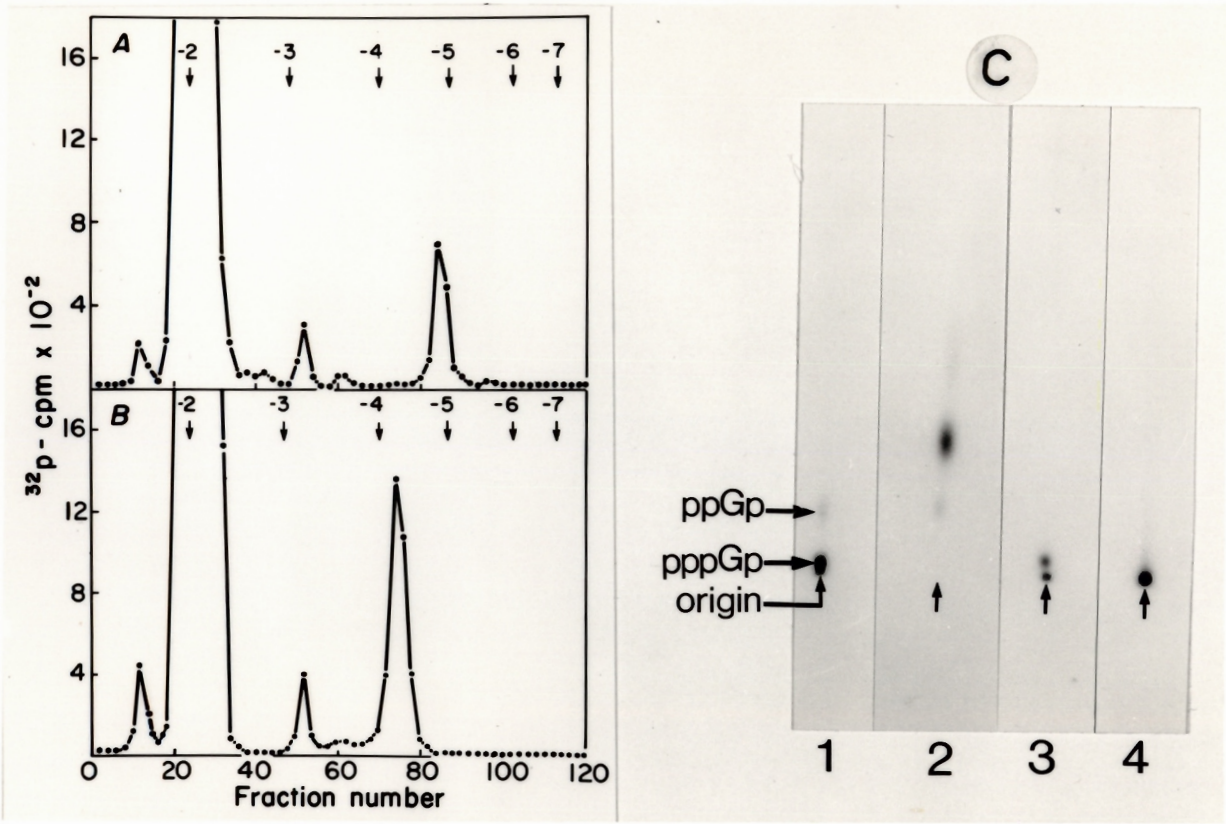
To examine the effect of linker insertions on translation we translated the mRNAs in a reticulocyte lysate. The results in Figure 5A show that the major translation product from pX1/SP mRNA (wild-type tk mRNA) is a polypeptide with an apparent molecular weight of ~ 44,000 (lane 2), consistent with the published molecular weight of the HSV-1 TK protein (29). This polypeptide is not synthesized in the absence of added mRNA (lane 1) or translated at a much lower efficiency when

FIGURE 4

Analysis of 5' Terminal Structures of tk mRNA Transcribed from pX1/SP in the Absence or Presence of m^7GpppG

Transcription of tk mRNA was carried out as described in Experimental Procedures in a total volume of 100 µl with 150 µCi of α-^{32}P-ATP and a final concentration of 100 µM ATP. Transcription products were phenol extracted and ethanol precipitated two times with 2 M NH_4OAc. Transcripts synthesized in the absence ($5x10^6$ cpm) or presence ($1x10^7$ cpm) of m^7GpppG were treated with 5 units of RNase T2 for 4 hr at 37°C in a total volume of 200 µl in 50 mM NaOAc (pH 4.5) and 2 mM EDTA. A fraction containing 180 µl of the reaction mixture was mixed with an RNAse A digest of calf liver tRNA as charge marker and analyzed by chromatography on a DEAE-cellulose (DE-11, Whatman) column equilibrated with 50 mM Tris (pH 7.5) containing 7 M urea and 25 mM NaCl. Oligonucleotides were eluted with a linear gradient of NaCl (100 ml each of 25 mM NaCl and 0.3 M NaCl) in the same buffer. Fractions of 1.3 ml were collected and aliquots of 400 µl were counted in 10 ml of ACS (Amersham). Approximately 95% of radioactive material was recovered. (A) Transcription in the absence of m^7GpppG. (B) Transcription in the presence of 500 µM m^7GpppG. (C) For further analysis of radioactive material eluting from the DEAE-cellulose column, fractions corresponding to material eluting at approximately position -5 in A and approximately -4.5 in B were pooled, diluted 3-fold in H_2O, and applied on a (0.5 cm x 4 cm) DEAE-cellulose (DE-11) column pre-equilibrated in 5 mM triethylammonium bicarbonate (TEAB) (pH 7.5), washed extensively with 5 mM TEAB, and eluted with 1 M TEAB (pH 7.5). Samples were lyophilized to dryness, applied on polyethyleneimine plates, and chromatographed in 2M pyridinium formate (pH 3.4). The nucleotides pppGp and ppGp (PL

Biochemicals) used as markers were visualized by UV light. Lane 1, material from approximately position -5 in A. Lane 2, material from approximately position -4.5 in B. For lanes 3 and 4, tk mRNA was transcribed as above, except that α-^{32}P-GTP substituted for α-^{32}P-ATP in the reaction mixture. Transcripts synthesized in the absence (1.6×10^5 cpm) or presence (1.1×10^5 cpm) of m^7GpppG were treated with RNAse T2 as above and analyzed directly on polyethylenimine plates. ^{32}P-labeled nucleoside monophosphates released by RNAse T2 digestion migrated with the solvent front and are not shown. Lane 3, transcription in the absence of m^7GpppG. Lane 4, transcription in the presence of m^7GpppG. The upward-pointing arrows indicate the origin of sample application.


A
B
C
-2
-3
-4
-5
-6
-7
16
12
8
4
^{32}p - cpm x 10^{-2}
0
20
40
60
80
100
120
Fraction number
ppGp
pppGp
origin
1
2
3
4

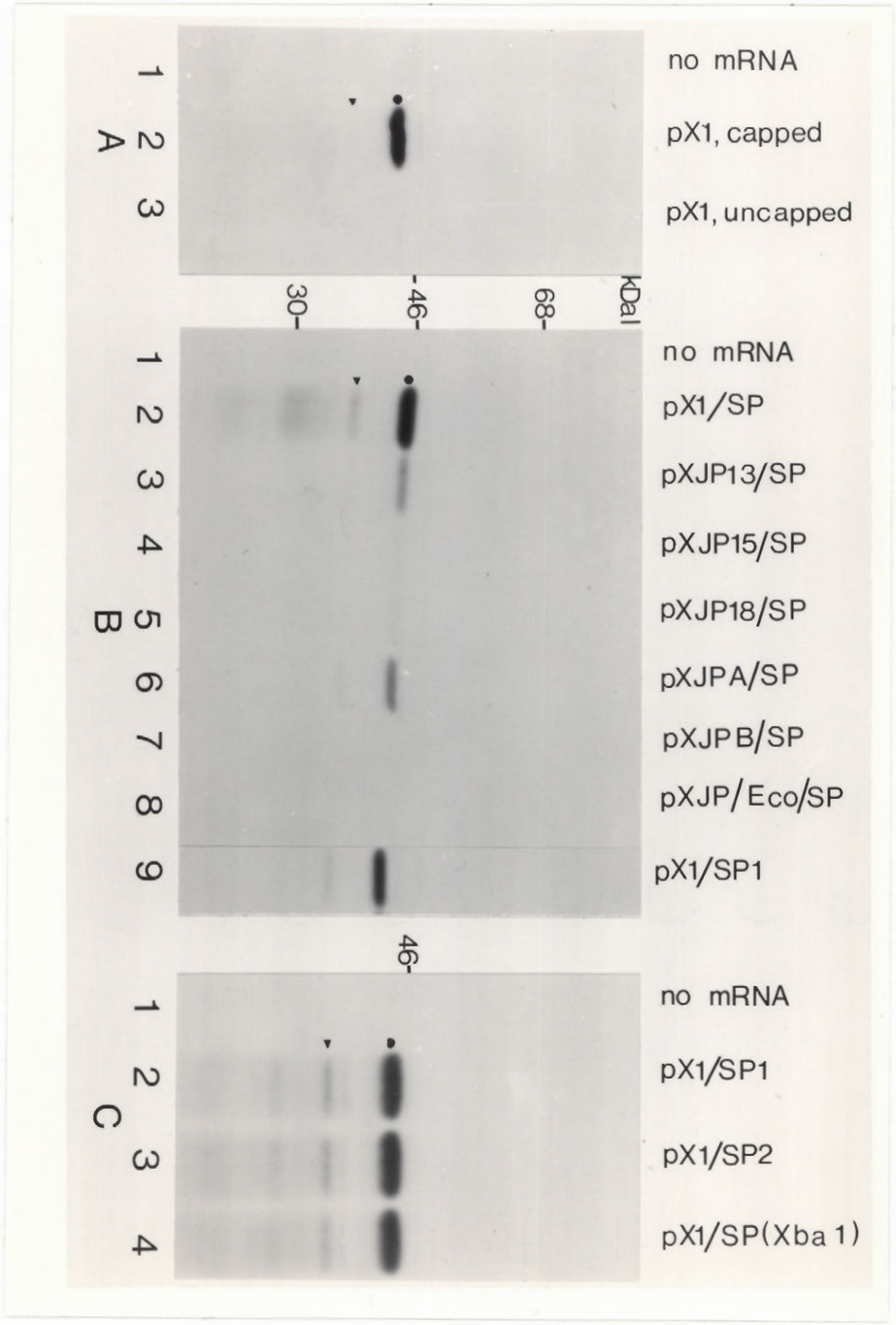
uncapped tk mRNA is used (compare lane 3 to lane 2). In addition to the major 44,000 dalton polypeptide, a minor polypeptide of ~ 39,000 daltons is synthesized in response to the mRNA (lane 2; the synthesis of this polypeptide is more pronounced in Fig. 5B). This polypeptide probably corresponds to a second translation product, which initiates from the second in-phase AUG downstream from the first initiation codon (29). Examination of the translation products from the modified mRNAs reveals that mRNA from pXJP13/SP translates less efficiently than mRNA derived from pX1/SP (~10 fold less, Figure 5B, lane 3). However, translation of mRNA from clones pXJP15/SP and pXJ18/SP is dramatically reduced (Fig. 5B, lanes 4 and 5).

We have prepared mRNAs from several constructs described in Figure 3 for control experiments. Introduction of Bam HI linkers into the Hind III site of pX1/SP, instead of the Bgl II site (see Fig. 3), has the same effect on translation. mRNA prepared from pXJPA/SP is translated ~ 10-fold less efficiently than mRNA from pX1/SP (Fig. 5B, lane 6), while the translation product of mRNA from clone pXJPB/SP (two Bam HI linkers) is detectable only after overexposure of the autoradiogram (lane 7). In addition, mRNA prepared from pXJP/Eco/SP (seven Eco RI linkers in the Bgl II site of pX1/SP) also translated much less efficiently than mRNA from pX1/SP (20-fold, lane 8). In order to synthesize mRNA from pXJP/Eco/SP we linearized the plasmid with Xba I, since Eco RI could not be used. Consequently, the 3' untranslated region is different than in pX1/SP; it was thus necessary to determine if sequences 3' to the tk mRNA had any influence on translation. We therefore linearized pX1/SP with Xba I and found that the translational

FIGURE 5

Polyacrylamide Gel Electrophoresis of Proteins Synthesized in Rabbit Reticulocyte Lysate Programmed with In Vitro Transcribed tk mRNA

Translation was done in 12 µl reaction mixtures containing the amounts indicated below of [^{3}H]CTP-labeled mRNAs made from the various tk constructs as described in Experimental Procedures. Incubation was for 60 min, at which time 4 µl of incubation mixture was withdrawn, mixed with electrophoresis sample buffer, and run on 10% SDS/polyacrylamide gels that were treated with EN^{3}HANCE, dried, and autoradiographed. Gels were exposed against Cronex film (Fuji) at -70°C for 3 hr. Radioactive bands corresponding to the TK protein were quantified by soft laser densitometry (LKB). Values obtained relative to the amount of TK protein synthesized from wild-type pX1/SP transcript, which is set as 1, are indicated in brackets. (A) Translations were performed with 0.5 µg of mRNA. Lane 1, no mRNA added; lane 2, capped mRNA from pX1/SP (1); lane 3, uncapped mRNA from pX1/SP (0.02); (B) Translations were carried out with 0.2 µg of capped mRNAs obtained from the following plasmids. Lane 1, no mRNA added; lane 2, pX1/SP (1); lane 3, pXJP13/SP (0.1); lane 4, pXJP15/SP (0.01); lane 5, pXJP18/SP (0.01). lane 6, pXJPA/SP (0.33); lane 7, pXJPB/SP (0.01); lane 8, pXJP/Eco/SP (0.01); lane 9, pX1/SP cleaved with Xba I (0.45). (C) Translations were carried out with 0.2 µg of capped mRNAs from the following plasmids. Lane 1, no mRNA added; lane 2, pX1/SP1 (1); lane 3, pX1/SP2 (1); lane 4, pX1/SP linearized with Xba I (1). The position of the ~ 44,000 dalton TK protein is indicated by a dot, and that of the ~ 39,000 dalton TK protein is indicated by a triangle.


A
1
2
3
no mRNA
pX1, capped
pX1, uncapped
30-
46-
68-
kDa
B
1
2
3
4
5
6
7
8
9
no mRNA
pX1/SP
pXJP13/SP
pXJP15/SP
pXJP18/SP
pXJPA/SP
pXJPB/SP
pXJP/Eco/SP
pX1/SP1
46-
C
1
2
3
4
no mRNA
pX1/SP1
pX1/SP2
pX1/SP(Xba 1)

efficiency of the mRNA made from this plasmid is the same as mRNA from pX1/SP1 (Fig. 5C, compare lanes 4 and 2). To control for any effects of increasing the length of the 5' leader region on translation, we prepared mRNA from the deletion mutant pX1/SP1 (see Fig. 3B) and found that it translates with approximately the same efficiency as mRNA from pX1/SP (2-fold difference; compare lanes 9 and 2, Figure 5B). Furthermore, we have used the pX1/SP2 construct (Fig. 3) which contains an extra 22 bp at the 5' noncoding region as compared to pX1/SP. The former plasmid was linearized with Eco RI, and the translational efficiency of mRNA produced from this vector was compared to mRNA from Xba I linearized pX1/SP. The two mRNAs are translated to the same extent (Fig. 5C, compare lanes 3 and 4). Taken together, these results strongly support the hypothesis that excessive secondary structure in the 5' noncoding region of the mRNA impedes protein synthesis.

Ribosome Binding of pSP64/tk Transcripts

It was expected that the level at which the block in translation occurs is that in which the mRNA forms initiation complexes with ribosomes. Consequently, we carried out ribosome binding studies on the various modified mRNAs by incubating ^{32}P-labeled tk transcripts in a reticulocyte lysate or wheat-germ extract and analyzing initiation complex formation by glycerol gradient centrifugation. Capped tk mRNA could bind to rabbit reticulocyte (see Fig. 6) and wheat-germ ribosomes (Fig. 7A). Control experiments showed that this binding was authentic and cap-specific and was inhibited by m^7GDP (data not shown). It is of interest that in addition to 80S initiation complexes, a significant

amount of disomes were formed (Fig. 6A). This is not unexpected, inasmuch as the distance between the cap and the first AUG, 95 nucleotides, is long enough to accomodate two 80S ribosomes (9).

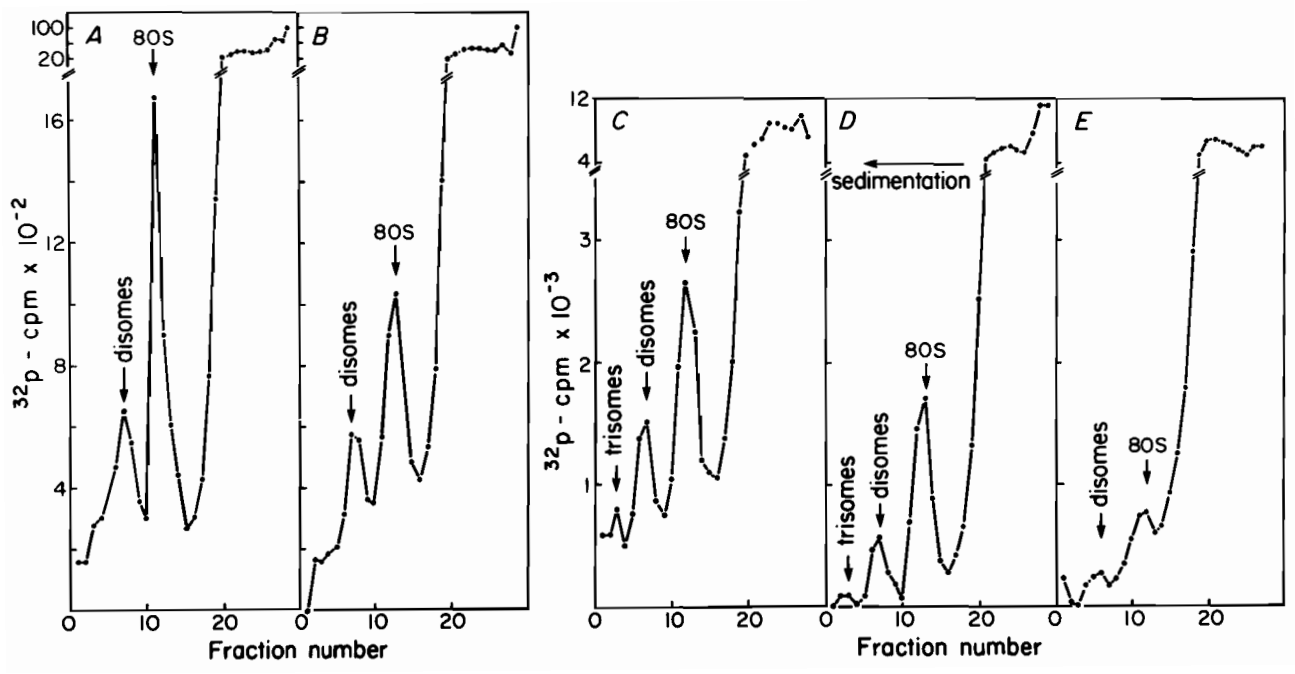
Initiation complex formation analysis with the different modified mRNAs shows that both in wheat-germ and in reticulocyte lysate, insertion of base-paired regions reduced the efficiency of mRNA binding to ribosomes. In a reticulocyte lysate (Fig. 6), 13% of wild-type tk mRNA bound ribosomes while 12-13%, 8% and 2% of added mRNAs of clones pXJP13/SP, pXJP15/SP, and pXJP18/SP respectively, bound to ribosomes. The low levels of trisomes (Fig. 6C and 6D) are probably due to ribosome migration past the first AUG initiation codon and binding to the second in-phase AUG, which allows the synthesis of the 39,000 dalton TK protein as explained above. These results indicate that the lower efficiency of translation of tk mRNA with excessive 5' secondary structure is due to impaired 80S initiation complex formation. To support this conclusion, we prepared mRNA <u>in vitro</u> from pXJP15/SP and pX1/SP, in which inosine was substituted for guanosine, thus producing an mRNA of the same length as control mRNA but with less stable secondary structure (17). If our conclusion is correct, then it is predicted that ITP-substituted mRNA from pXJ15/SP will bind to ribosomes to a similar extent as mRNA from pX1/SP. The ITP-substituted tk mRNA prepared from pX1/SP bound to wheat-germ ribosomes with 32% efficiency, whereas control tk mRNA from pX1/SP bound with 20% efficiency (see Figs. 7A and 7B). The increase in the binding efficiency (1.6 fold) of the ITP-substituted tk mRNA probably reflects decreased secondary structure and hence greater

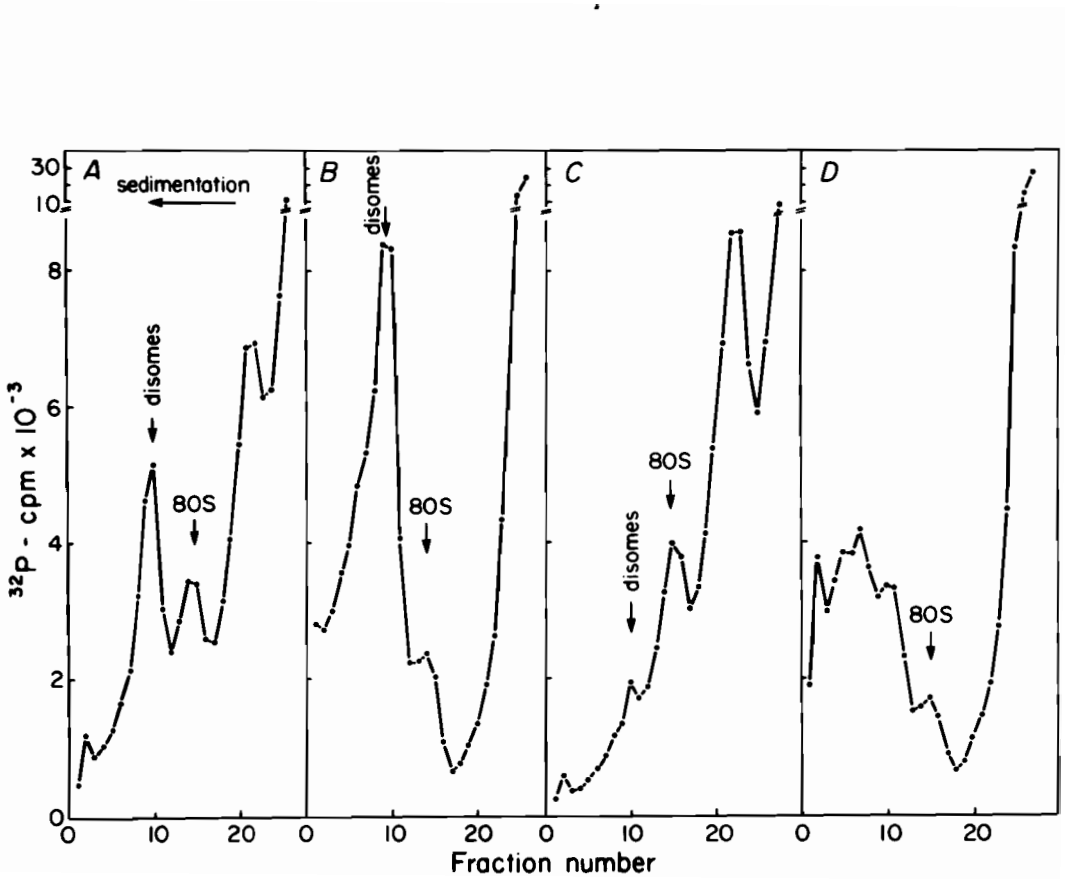
ability to form initiation complexes (17). Messenger RNA from pXJP15/SP bound with only 8% efficiency to wheat-germ ribosomes. However, binding of ITP-substituted mRNA from pXJP15/SP to wheat-germ ribosomes was significantly stimulated (from 8% to 23%; approximately 3-fold stimulation). Thus, reducing (but not fully eliminating) the secondary structure of mRNA from pXJP15/SP results in an increase of mRNA binding to ribosomes to a level comparable to wild type mRNA.

FIGURE 6

Ribosome Binding of In Vitro Transcribed tk mRNAs From Wild-Type and Insertion Derivative Constructs in Rabbit Reticulocyte Lysate

Two separate experiments were performed in which α-^{32}P-ATP-labeled tk mRNA ($6x10^4$ cpm, $3x10^5$ cpm/µg for the experiment shown in A and B; and $1x10^5$ cpm, $3x10^5$ cpm/µg for the experiment shown in C through E) was incubated in 50 µl of rabbit reticulocyte lysate at 30°C for 10 min. Initiation complexes formed were analyzed on glycerol gradients as described in Experimental Procedures. Fractions of 21 drops were collected, and radioactivity was determined by scintillation counting. The tk constructs from which mRNA was synthesized and percentages of input mRNA bound were as follows: (A) pX1, 13%; (B) pXJP13, 12%; (C) pXJP13, 13%; (D) pXJP15, 8%; (E) pXJP18, 2%.


A
B
C
D
E
80S
disomes
trisomes
sedimentation
^{32}p - cpm x 10^{-2}
^{32}p - cpm x 10^{-3}
Fraction number
Fraction number


A
sedimentation
disomes
80S
B
disomes
80S
C
80S
disomes
D
80S
30
10
8
6
4
2
0
^{32}P - cpm x 10^{-3}
0
10
20
Fraction number

DISCUSSION

There is much evidence to support the view that a significant degree of translational control of gene expression occurs in eukaryotes. Features such as posttranscriptional modification and the relatively long half-life of eukaryotic mRNAs, together with the complexity of the translation initiation machinery, might readily form the basis of regulatory functions. One factor that might be significant in regulation of translation is the intrinsic translational efficiency of particular mRNAs, especially since it appears to vary widely among different mRNAs. It is thought that the step at which mRNAs are differentially selected for translation is initiation complex formation. Consequently, an important question concerns the nature of structural features of mRNA that influence the efficiency with which they bind to ribosomes. In view of the proposed mechanism for the 48S preinitiation complex formation (33), it is thought that the relevant information resides in the 5' noncoding region of the mRNA, although there is little clue as to exactly what this information might be.

Our aim was to study the effect of modifying mRNA 5' secondary structure on translation. The results demonstrate that the introduction of a series of restriction enzyme oligonucleotide linkers, which should increase secondary structure, into the 5' noncoding region of the tk mRNA has a negative effect on the efficiency of translation. We carried out several control experiments to rule out alternative mechanisms by which the Bam HI linkers may be acting to impair translation. It is possible that introduction of Bam Hl linkers into the Bgl II site

destroyed some sequence (primary or secondary) essential to translation, resulting in decreased translation. By introducing Bam HI linkers upstream of the Bgl II site and obtaining the same effect on translation as found with pXJP13 and pXJP15, we excluded this possibility. Introduction of Eco RI linkers into the Bgl II site of pX1/SP with the resultant observed decrease in translation (Fig. 5B) argues against the possibility that the reduction in translation of the pXJP derivatives is due to a sequence-specific effect of the introduced linker DNA. That the effect observed with the pXJP derivatives results from the increased length of the 5' noncoding region, rather than from increased secondary structure is not likely either, because translation of mRNA prepared from pX1/SP2, which possesses a 5' noncoding length of 117 nucleotides, is as efficient as pX1/SP1 mRNA (having a 5' noncoding length of 82 nucleotides). In addition, the fact that ITP-substituted mRNA from pXJP15/SP can enter initiation complexes with efficiency comparable to that of mRNA from pX1/SP argues that the length of the inserted fragment in pXJP15/SP mRNA is not responsible for its inability to bind ribosomes (Fig. 7).

In summary, our data suggests that <u>in vivo</u> and <u>in vitro</u>, increased 5' mRNA secondary structure decreases translational efficiency in eukaryotes. However, we cannot conclude whether the effect is due to failure of 40S ribosomes to attach to mRNA or to inhibition of 40S migration between the 5' cap and the AUG codon [according to the scanning mechanism (18)]. Given the extensive, although variable, amount of secondary structure within eukaryotic mRNAs, it seems likely

in view of our data, that mRNA secondary structure contributes significantly to the intrinsic translational efficiencies of different mRNA.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. Calf intestine alkaline phosphatase and Klenow fragment of DNA polymerase I were obtained from Boehringer Mannheim. T4 DNA polymerase was from New England Biolabs. RNAse-free DNAse I and RNAse T2 were from BRL. Oligodeoxythymidylate-cellulose and m^7GpppG were from P-L Biochemicals. 5-^{3}H-cytidine triphosphate (21.8 Ci/mmol), 5-^{125}I-2'- deoxycytidine (2200 Ci/mmol), ^{35}S-methionine (1000 Ci/mmol), SP6 RNA polymerase, and T4 DNA ligase were purchased from NEN. α-^{32}P-deoxynucleoside triphosphates (3000 Ci/mmol), α-^{32}P-nucleoside triphosphates (400 Ci/mmol), and γ-^{32}P-adenosine triphosphate were obtained from Amersham or NEN. Synthetic Bam HI (^{5}'CCGGATCCGG^3') and Eco RI (^{5}'GGAATTCC^3') linkers were from Collaborative Research. Genticin (G418 sulfate) was purchased from Gibco Laboratories. Human placenta RNAse inhibitor was from Promega Biotec.

General Methods

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, nick translation of DNA, DNA end-labeling, DNA ligation, and bacterial transformation were carried out using standard methods (ref. 21, and references therein). DNA sequencing was accomplished by the Maxam and Gilbert method (22).

Plasmid Constructions

Plasmid pX1 (43) was provided by Y. Nishioka (Montreal) and contains a 3.4 kbp Bam HI fragment from HSV-1, which includes the tk

gene, promoter, and flanking sequences, inserted into the Bam HI site of pBR322 (see Figure 1). Bam HI linker derivatives were prepared from pX1, by inserting Bam Hl linkers into the unique Bgl II site, which is 57 bp upstream from the first ATG (see Figure 1), as follows. Plasmid pXl was cut at the Bgl II site, filled in with DNA polymerase 1 (Klenow fragment) in the presence of deoxyribonucleoside triphosphates, and blunt-end ligated, using T4 DNA ligase, with Bam HI linkers that had been previously kinased. After transformation of E. coli strain HB101, analysis of plasmid DNA from individual colonies was carried out by testing their susceptibility to Bam HI restriction endonuclease digestion. The Bam HI linkers containing plasmid were termed pXJP and are shown in Figure 1. Those likely to contain linkers were analyzed for length by endonuclease restriction and analysis on 10% polyacrylamide gels. The integrity of the Bam HI linkers was confirmed by DNA sequencing (22) for clones pXJP13 and pXJP15.

The tk gene and its linker derivatives were subcloned into pSVOd (a generous gift from Y. Gluzman, Cold Spring Harbor) by first isolating the Hind III/Sal I restriction fragments of each tk construct (see restriction map in Fig. 1) from agarose gels, using the method of Vogelstein and Gillespie (42). Hind III and Sal I are unique restriction sites in pXl; thus no internal cleavage of the 3.4 kbp Bam HI tk fragment occurs. pSVOd was digested with Hind III and Sal I, treated with calf intestinal alkaline phosphatase, and ligated to the Hind III/Sal I fragment of the tk insertion derivatives. The recombinant plasmids termed pXJP/SVOd are shown in Fig. 2A. After

transformation of E. coli HB101, individual colonies were screened and mapped with restriction endonuclease digestion to ensure that the tk gene was in the expected orientation.

For in vitro studies we have subcloned the tk insertion derivatives into pSP64, obtained from D. Melton and M. Green (Cambridge, Mass.). This plasmid contains the SP6 promoter, a polylinker region, and a selectable ampicillin gene. The coding region, a portion of the 5' noncoding region, and 3' noncoding sequences of the tk gene were subcloned into this vector as follows. The Hinc II/Pvu II fragment of the tk gene (see Fig. 3A) was separated from pBR322 sequences by isolating the fragment from an agarose gel. The fragments were blunt-end ligated into the Hinc II site of pSP64, which is 24 bp downstream of the transcription start site. The structure of the recombinant plasmids was termed pXJP/SP as shown in Fig. 3B. E. coli strain HB101 was transformed with the ligated mixture, and colonies were selected with the tk gene in the sense orientation. To insert the tk gene into the Sac I site of pSP64 the vector was cleaved with Sac I, rendered blunt ended using T4 DNA polymerase and blunt-end ligated to the Hinc II/Pvu II tk fragment mentioned above.

The plasmid pSVtkneoβ, which confers resistance to the neomycin analog G418 (36), was a gift from D. Skup (Montreal). This plasmid, along with pXl and pXJP derivatives, was used directly for cotransformation of LTA cells.

Cell Lines and Transfection Procedures

Mouse LTA cells, an adenine-phosphoribosyl-transferase-deficient

derivative of cell line LM (TK^-) (16), were obtained from C. Stanners (Montreal) and maintained in α-minimal essential medium (α-MEM) without nucleosides, supplemented with 10% fetal calf serum (FCS). For transfection experiments, all plasmid preparations were spun through a second cesium chloride gradient. Cells were incubated with calcium phosphate-DNA coprecipitates, prepared as described by Wigler et al. (45) with the modifications of Graham et al. (12). Briefly, $8x10^5$ cells in 100 mm plates with 10 ml of media (plated 1 day before transfection) were incubated with 1 ml of precipitate containing 200 ng of plasmid DNA and 10 μg of calf thymus carrier DNA. After incubation at 37°C for 24 hr, the medium was removed and replaced with fresh nonselective medium. After an additional 24 hr at 37°C, the medium was replaced with fresh HAT-containing medium (HAT is 100 μM hypoxanthine, 1 μM aminopterin, 40 μM thymidine). Colonies were scored 18 days after selective pressure had been applied.

Cotransfection experiments of LTA cells were carried out using the same technique except that 1 ml calcium phosphate-DNA coprecipitate contained 15 μg of pSVtkneoβ and 15 μg of recombinant tk plasmid, without carrier DNA. Transfectants were selected with medium containing 400 μg/ml of G418 and scored 9 days after selective pressure had been applied. Transient expression assays were carried out using COS-1 cells (11), obtained from Y. Gluzman, through J. Hassell (Montreal). Cells were grown in Dulbecco's modified Eagle's medium (DME) with 10% calf serum, and conditions used for transfection were as previously described by Mellon et al. (24).

Analysis of TK Activity and tk mRNA Levels

TK enzymatic activity was assayed by the method of Summers and Summers (38). Cytoplasmic RNA was prepared from cells as described by Favaloro et al. (8). RNA used in blotting experiments was treated with RNAse-free DNAse in 100 µl of 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, and 100 units RNAsin, at 37°C for 15 min, extracted with phenol, and ethanol precipitated. Poly(A)$^+$ mRNA was selected according to Aviv and Leder (2) and quantitated by [^{3}H]poly(U) hybridization according to Bishop et al. (4). RNA dot blots were prepared using a BRL manifold apparatus according to the manufacturer's instructions and hybridization conditions with nick-translated ^{32}P-labeled pX1 DNA were according to Thomas (40).

In Vitro Transcriptions

For in vitro transcription of pSP64/tk constructs, plasmid DNA was linearized with the appropriate restriction enzymes, phenol extracted, passed over a G-50 spun-dialysis column, and ethanol precipitated. Transcription reactions using pSP64 derivatives as template were carried out according to Green et al. (13) except for the following modifications: when capped transcripts were required, 500 µM m^7GpppG was included in the reaction mixture and the GTP concentration was lowered to 20 µM. Incubation times were for 2 hr at 37°C. Following incubation, 2 µg RNAse-free DNAse 1 and 1 µg carrier tRNA was added and incubation continued for 10 min at 37°C. The mixture was phenol extracted, passed over a G-50 spun-dialysis column, ethanol precipitated twice with 2 M NH_4OAc and once with 0.15 M KOAc, and washed with 70%

ethanol. To prepare ITP-substituted mRNA, the GTP was replaced with ITP at a concentration of 500 μM.

In Vitro Translation and Ribosome Binding Studies

In vitro translations in micrococcal-nuclease treated rabbit reticulocyte lysates were carried out as previously described (28). Conditions for ribosome binding and analysis of initiation complex formation in 10%-30% glycerol gradients were as previously described (5,33) except that K^+ concentrations were raised to 130 mM by the addition of 60 mM KOAc. Centrifugation was for 3½ hr at 39,000 rpm and 4°C in an SW40 rotor. Fractions were collected and counted in a Beckman scintillation counter in 10 ml of Aquasol II (NEN).

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CHAPTER 3

PHOTOCHEMICAL CROSSLINKING OF CAP BINDING PROTEINS TO EUKARYOTIC mRNAs: EFFECT OF mRNA 5' SECONDARY STRUCTURE

SUMMARY

We used ultraviolet light-induced crosslinking to study the interactions of cap binding proteins with the 5' cap structure of eukaryotic mRNAs. Thymidine kinase gene (herpes simplex virus type I) transcripts prepared *in vitro* using the SP6 RNA polymerase transcription system were capped and methylated post-transcriptionally with [α-^{32}P]GTP and S-adenosyl-L-methionine to yield cap-labeled transcripts. Irradiation of capped transcripts in the presence of crude rabbit reticulocyte initiation factors and ATP-Mg^{2+}, resulted in the cap-specific crosslinking of two polypeptides with molecular masses of 24- and 80 kilodaltons. The crosslinking characteristics of these polypeptides resemble those of the cap binding proteins previously detected by a chemical crosslinking assay (N. Sonenberg, D., Guertin, D. Cleveland, and H. Trachsel, Cell 27:563-572, 1981). However, the relative efficiency of crosslinking of these two polypeptides to the cap structure was different from that in previous studies, and there was no detectable crosslinking of the previously described 50 kDa polypeptide. In addition, we present data indicating that insertion of secondary structure into the 5' noncoding region of tk mRNA, 6 nucleotides from the cap structure, decreases the cap-specific crosslinking of the 80 kDa, but not the 24 kDa polypeptide. In contrast, insertion of secondary structure 37 nucleotides from the cap structure had no significant effect on the crosslinking of either the 24- or the 80 kDa cap-specific polypeptides. These results demonstrate that the position of mRNA 5'-proximal secondary structure relative to the cap structure

can influence the cap-specific interaction between the mRNA and a translation initiation factor.

INTRODUCTION

All cellular eukaryotic mRNAs analyzed to date are blocked at their 5' end by the cap structure m^7GpppN (1,6,27). Numerous studies have indicated that the cap structure facilitates translation initiation complex formation (see ref. 1 for a review), protects the mRNA against 5' exonucleolytic degradation (7), and more recently, might be involved in pre-mRNA processing (13). To identify protein factors which recognize the cap structure and may be involved in translation, Sonenberg and Shatkin (33) developed an assay which involves covalent crosslinking of mRNA with an oxidized cap structure to polypeptides that bind at or near the cap structure. Using this assay, Sonenberg et al. (32) initially identified a 24-kilodalton (kDa) polypeptide (termed 24K-CBP [cap binding protein]) in the high-salt wash of rabbit reticulocyte ribosomes which could be specifically crosslinked to the oxidized cap structure of reovirus mRNA. A polypeptide of similar mobility on sodium dodecyl sulfate (SDS)/polyacrylamide gels and with identical crosslinking characteristics has been detected in initiation factors (IFs) from mouse Ehrlich ascites cells (32) and human HeLa cells (12,17). It was subsequently demonstrated that additional polypeptides of 28-, 50- and 80 kDa can be specifically crosslinked to the oxidized cap structure in an ATP-Mg^{2+} -dependent manner (29,31). Using purified initiation factors, Grifo et al. (10) suggested that the 50- and 80 kDa polypeptides correspond to eIF-4A (eukaryotic IF-4A) and eIF-4B, respectively. This has been confirmed for eIF-4A by Edery et al. (4) and is probably true for eIF-4B (based on several indirect observations;

4,11). Hence, we will refer to the 50-and 80 kDa polypeptides as eIF-4A and eIF-4B, respectively.

The molecular mechanism by which cap recognition factors act in facilitating mRNA-ribosome interactions is not well characterized. Several studies have implicated eIF-4A, eIF-4B, and more recently, eIF-4F (also referred to as CBP II [35] or CBP complex [4,5]) in the ATP-dependent binding of mRNA to 43S preinitiation complexes (2,36). Sonenberg (29) postulated that cap recognition factors facilitate ribosome binding by melting mRNA secondary structure in an ATP-Mg^{2+} dependent process. Subsequently, Lee et al. (16) showed that cap-specific crosslinking of eIF-4A (50 kDa polypeptide) and eIF-4B (80 kDa polypeptide) in crude IF preparations has a reduced dependence on ATP, if the mRNA has less stable secondary structure. This suggested that eIF-4A and eIF-4B can be crosslinked to the cap structure only after secondary structure has been melted. Edery et al. (4) reported that the eIF-4A in the CBP complex (comprising three major polypeptides of ~ 24-[24K-CBP], 50-[eIF-4A], and 220 kDa) cannot be crosslinked to mRNA unless eIF-4B is present. This implies that any putative melting activity is not present solely in the CBP complex but is dependent on eIF-4B or, alternatively, that eIF-4B mediates cap recognition by eIF-4A after denaturation of the mRNA. These data suggest a model by which the CBP complex binds to the 5' cap structure and mediates the melting of 5' secondary structure of the mRNA, possibly in conjunction with eIF-4B, followed by 40S ribosome binding (4,5,29-31). This model is consistent with our recent studies showing that introduction of secondary structure into the 5' noncoding region of herpes simplex virus type 1 tk mRNA acts

to decrease translational efficiency *in vivo* and *in vitro* (22). We concluded from these studies that the 5' mRNA secondary structure blocks translation by preventing 80S initiation complex formation.

In an attempt to analyze the step at which the block in initiation complex formation occurred, we studied here the interaction of the cap-specific polypeptides with the cap structure of mRNAs differing in secondary structure within the 5' noncoding region. We adopted the UV light-induced crosslinking technique used by Greenberg (9) and others (26,37) and noticed differences in the crosslinked protein profiles obtained using this technique and the chemical crosslinking method introduced by Sonenberg and Shatkin (33). In addition, we found differences in the degree of crosslinking of eIF-4B when we compared tk mRNAs differing in the position of 5' noncoding secondary structure relative to the cap structure. The results demonstrate that secondary structure near the mRNA 5' terminus can influence the interaction of eIF-4B with the cap structure, whereas the presence of secondary structure farther downstream in the 5' noncoding region does not affect this interaction.

MATERIALS AND METHODS

Materials and General Methods

Restriction endonucleases and RNAse A were purchased from Boehringer Mannheim. RNAse-free DNAse I and vaccinia virus guanylyltransferase were from Bethesda Research Laboratories, Inc. Human placenta RNAse inhibitor was from Promega Biotec. m^7GpppG, GpppG, GDP and m^7GDP were purchased from P-L Biochemicals. [5-^{3}H]CTP (23.7 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), and SP6 RNA polymerase were obtained from New England Nuclear Corp. Preparation of plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis of DNA were performed by standard methods (18). Preparation of high-salt wash of ribosomes from rabbit reticulocytes and HeLa cells as the source of IF was by the method of Schreier and Staehelin (25) and Lee and Sonenberg (17), respectively. Poliovirus type 1 (Mahoney strain) infection of HeLa cells was as described by Lee and Sonenberg (17), and IF preparations were made 3 hrs after poliovirus infection.

In Vitro Transcriptions and Capping Reactions

The construction of pX1/SP, pXJP15/SP and pXJPB/SP has been described in detail elsewhere (22; the important features of these constructs are shown in Fig. 3.) Before transcription with SP6 RNA polymerase, plasmids were linearized with Eco RI, phenol extracted, washed six times with ether, and passed over NACS prepac columns (Bethesda Research Laboratories) to remove trace amounts of contaminating bacterial RNA. Uncapped pSP64/tk mRNA transcripts were synthesized from Eco RI-linearized DNA templates essentially as

described by Green et al. (8), except that [5-^{3}H]CTP was used to monitor the level of RNA synthesis. RNA transcripts were capped and methylated as described by Monroy et al. (20) using vaccinia virus guanylyltransferase in the presence of α-^{32}P-GTP and S-adenosyl-L-methionine. For the preparation of capped but nonmethylated transcripts, S-adenosyl-L-methionine was omitted from the reaction mixture. Methyl-^{3}H-labeled reovirus mRNA was synthesized with viral cores in the presence of S-[methyl-^{3}H]adenosyl-L-methionine (New England Nuclear), and oxidation of reovirus mRNA was performed as described by Muthukrishnan et al. (21) and Sonenberg and Shatkin (33).

Crosslinking of mRNA to Initiation Factors

Chemical crosslinking of oxidized reovirus mRNA to IFs was performed as described by Lee et al. (16). Photochemical crosslinking of reovirus mRNA and pSP64/tk transcripts was performed essentially as described by Ulmanen et al. (37). Briefly, 2 x 10^4 to 4 x 10^4 cpm of ^{32}P-mRNA (cap labeled) (~6 x 10^4 cpm/μg) was incubated in a total volume of 30 μl in 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5)-0.5 mM magnesium acetate [$Mg(OAc)_2$]-2 mM dithiothreitol-3% glycerol-1 mM GTP (no changes were observed when this compound was omitted)-100 mM potassium acetate (KOAc) (unless otherwise indicated) - IF preparations (high salt wash of ribosomes) at 30°C for 10 min. Reaction mixtures were irradiated at 254 nm at 4°C at a distance of 4 cm with a 15-W General Electric G15T8 germicidal lamp for 1 hour. The samples were then digested for 30 min at 37°C with 20 μg RNAse A, boiled for 5 min in electrophoresis sample buffer, and subjected to electrophoresis on 10 to 15% gradient SDS-polyacrylamide gels (15),

followed by autoradiography. Quantitation of labeled bands was performed by scanning autoradiograms with a model 1650 transmittance-reflectance scanning densitometer (Bio-Rad Laboratories).

RESULTS

Crosslinking of oxidized reovirus mRNA to crude IF preparations from rabbit reticulocytes and other sources has been used to identify proteins that interact with the cap structure of eukaryotic mRNAs (12,29,31,32). We wished to extend this methodology to study the effect of the location (relative to the 5' cap structure) of 5'-proximal mRNA secondary structure on the efficiency of mRNA crosslinking to the different cap-specific polypeptides.

In previous studies, reovirus mRNAs were synthesized by reovirus cores in the presence of S-[methyl-^{3}H]adenosyl-L-methionine as the methyl donor. As a consequence, the cap structure of reovirus mRNAs became exclusively labeled with a methyl-^{3}H group. The ribose moiety of the cap structure was then oxidized to yield a dialdehyde which is capable of Schiff base formation with primary amino groups of proteins. Polypeptides that interact with mRNA in a cap-specific manner were detected by incubating oxidized methyl-^{3}H-labeled reovirus mRNA with IF preparations followed by reductive stabilization of RNA-protein complexes with sodium cyanoborohydride. The reaction mixtures were treated with RNAses A and T1 to degrade the mRNA portion of the complex, and the polypeptides that were covalently linked to the radioactive cap structure were resolved on SDS-polyacrylamide gels and detected by fluorography. Cap specificity was assessed by performing parallel experiments in the presence of the cap analogue, m^{7}GDP. It was impractical to use reovirus mRNA to study the effect of mRNA secondary structure on the interaction of CBPs with the cap structure, because it is not possible to generate reovirus mRNAs in the standard *in vitro*

transcription system with localized modifications in the nucleotide sequence. Consequently, we chose to use the SP6 expression system to synthesize mRNAs in vitro (8). In this system, the desired gene is inserted downstream from the SP6 promoter in pSP64 (described in ref. 19), and the construct is linearized using a restriction site downstream from the inserted gene followed by transcription with SP6 polymerase. Messenger RNAs synthesized in this system are then capped with [α-^{32}P]GTP and methylated with S-adenosyl-L-methionine by using the enzyme guanylyltransferase from vaccinia virus. In this system, the gene in question can be mutated by different techniques, thus giving rise to mRNAs with altered primary or secondary structures. In addition, the mRNA is labeled exclusively in the cap structure with ^{32}P instead of ^{3}H, resulting in a much reduced exposure time for autoradiography. The amounts of RNA transcribed in the SP6 transcription system, although relatively large (up to 50 µg of uncapped RNA for 3 µg of DNA in a total reaction mixture of 100 µl), are smaller than the milligram amounts produced by reovirus cores. Consequently, to minimize losses of mRNA that are likely to occur during the oxidation and subsequent purification of small amounts of mRNA, we circumvented these steps and adopted the photochemical technique for crosslinking of the mRNA to cap-specific polypeptides. This method of identifying cap-specific polypeptides has several other important advantages, as will be addressed in the discussion.

Initially, we wished to compare the efficiency and pattern of crosslinking obtained by the photolabeling technique with that obtained by the chemical crosslinking technique. To this end, we performed the

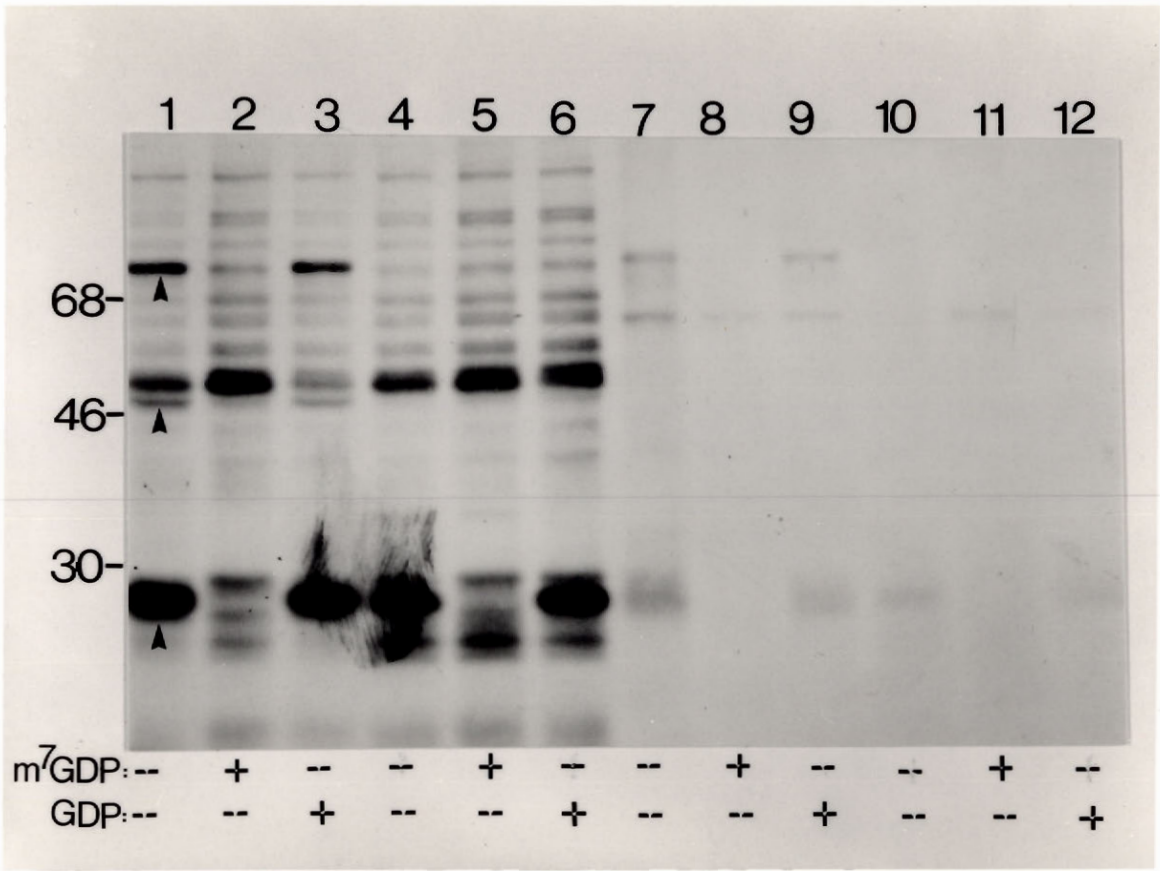
chemical crosslinking assay using methyl-^{3}H-labeled reovirus mRNA that had been oxidized to convert the 5'-terminal m^7G to the reactive dialdehyde. The polypeptides that became labeled by the chemical crosslinking assay in the presence of ATP-Mg^{2+} are shown in Fig. 1 (lane 1). These include the 24 kDa CBP, 50 kDa protein (eIF-4A), and the 80 kDa protein (eIF-4B), which are indicated by arrowheads, in addition to several other polypeptides. Crosslinking of the former polypeptides is cap specific, because 0.6 mM m^7GDP completely inhibited (>90%, as determined by scanning densitometry) their crosslinking (lane 2), whereas the same concentration of GDP had no effect on the crosslinking pattern (lane 3), in accordance with previous reports (29,31). In the absence of ATP-Mg^{2+}, IFs 4A and 4B did not crosslink to mRNA, in agreement with previous results (4,10,29), whereas crosslinking of the 24K-CBP was reduced to some extent (~ 50%, lane 4). This crosslinking was inhibited by m^7GDP (>90%, lane 5) but not GDP (lane 6). The crosslinking pattern obtained by using the photochemical crosslinking method in the presence and absence of ATP-Mg^{2+} is shown in lanes 7 to 12. In the presence of ATP-Mg^{2+}, three major polypeptides of ~ 24-,65- and 80 kDa became crosslinked to the cap structure (lane 7). Crosslinking of the 24- and 80 kDa polypeptides was inhibited by 0.6 mM m^7GDP (lane 8) but not by the same concentration of GDP (lane 9), whereas crosslinking of the 65 kDa polypeptide was not inhibited by either nucleotide. In contrast to the pattern obtained by the chemical crosslinking method, eIF-4A (50-kDa polypeptide) did not become crosslinked to a significant extent by UV light irradiation.

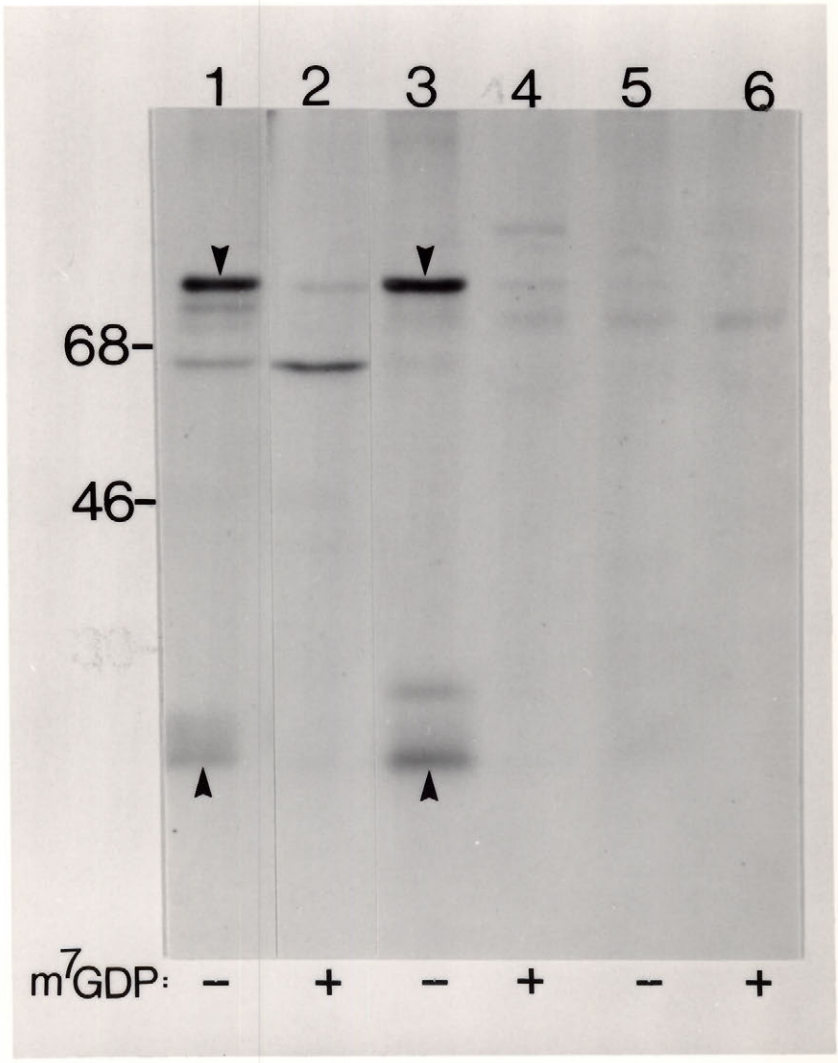
Photochemical crosslinking of the 24-kDa polypeptide occurs in the absence of ATP-Mg^{2+}, whereas that of the 80 kDa polypeptide requires ATP-Mg^{2+} (cf. lane 10 with 7). The effect of ATP-Mg^{++} cannot be due to the effect of ATP on UV absorption, since a non-hydrolyzable analogue, AMP-$P(CH_2)P$, could not substitute for ATP in the crosslinking reaction (data not shown). The ATP-Mg^{2+}-independent photochemical crosslinking of the 24 kDa polypeptide to mRNA is inhibited by m^7GDP (lane 11) but not by GDP (lane 12). Based on the photochemical crosslinking characteristics of the 24- and 80 kDa polypeptides with respect to inhibition by m^7GDP and dependence on ATP-Mg^{2+}, in addition to their comigration on SDS-polyacrylamide gels with the chemically crosslinked 24- and 80 kDa polypeptides, it is very likely that these polypeptides correspond to the 24K-CBP and eIF-4B. Moreover, we have recently obtained evidence, using polyclonal antibodies directed against eIF-4B, that the photoinduced crosslinked 80 kDa polypeptide is eIF-4B, thus reinforcing the belief that the 80-kDa polypeptide identified by the chemical crosslinking is indeed eIF-4B (S. Milburn, J. P, J.W.B. Hershey, and N.S., In press). It is clear, however, that the relative crosslinking efficiencies of the 24K-CBP and eIF-4B are different using the two crosslinking techniques. Crosslinking of the 24K-CBP is approximately sevenfold more efficient than that of eIF-4B (as determined by scanning densitometry) when the chemical crosslinking method was used, whereas the photolabeling method resulted in an equal intensity of crosslinking of the 24K-CBP and eIF-4B in this experiment. As will be shown below, in other experiments crosslinking of eIF-4B was significantly greater than that of the 24K-CBP using the photochemical

FIGURE 1

Comparison of CBP Crosslinking Patterns Obtained by the Chemical and Photochemical Crosslinking Techniques

Rabbit reticulocyte IF preparations (180 µg) were incubated with ~0.5 µg (40,000 cpm) of methyl-^{3}H-labeled oxidized reovirus mRNA (lanes 1 to 6) or methyl-^{3}H-labeled unoxidized reovirus mRNA (lanes 7 to 12) for 10 min at 30°C in a total volume of 30 µl under the conditions described in Materials and Methods. Chemical (lanes 1 to 6) or photochemical (lanes 7 to 12) crosslinking was performed, and samples were analyzed on a 10 to 15% SDS-polyacrylamide gel followed by autoradiography as described in Materials and Methods. Reaction mixtures included 1 mM ATP in lanes 1 to 3 and lanes 7 to 9, and m^7GDP or GDP at a concentration of 0.6 mM was added where indicated in the figure. The molecular masses of standard proteins are indicated in kilodaltons in the left margin, and the chemically crosslinked cap-specific polypeptides are indicated by arrowheads in lane 1.


1 2 3 4 5 6 7 8 9 10 11 12
68-
46-
30-
m7GDP: -- + -- -- + -- -- + -- -- + --
GDP: -- -- + -- -- + -- -- + -- -- +


1
2
3
4
5
6
68-
46-
m7GDP: – + – + – +

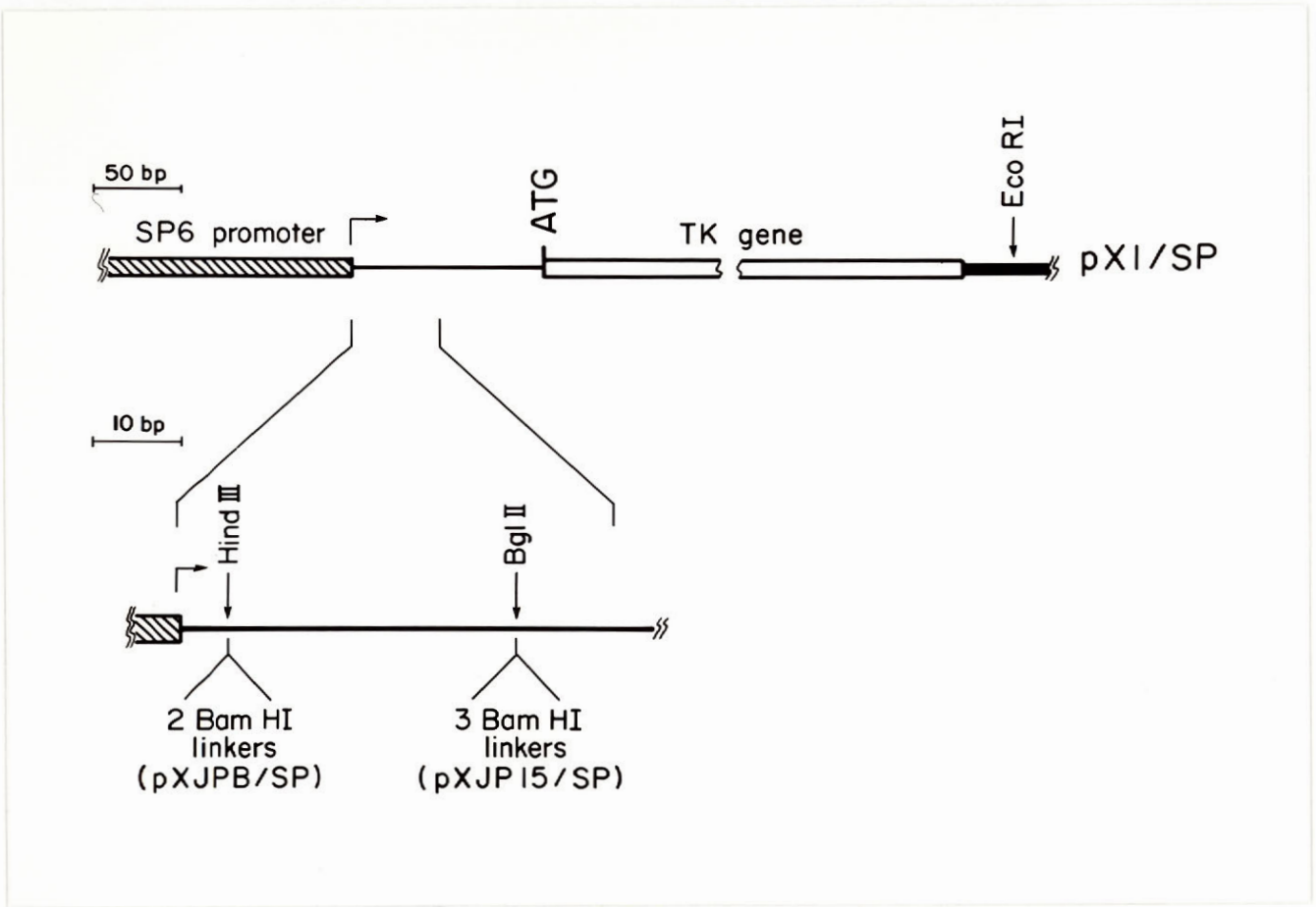
eIF-4A. The ability of the cap specific polypeptides to interact with the cap structure is, however, impaired in poliovirus-infected IF preparations (cf. lanes 5 and 3). Thus, with the exception of the 50-kDa polypeptide (eIF-4A) and the 28-kDa polypeptide, the UV crosslinking assay gave similar results (qualitatively) to those of the chemical crosslinking assay in terms of cap specificity, ATP-Mg^{2+} requirement, and effects of poliovirus infection. However, whereas it is clear that in the case of the chemical crosslinking technique, crosslinking of 24K-CBP and eIF-4B occurs via the oxidized ribose of the m^7G group, it is not clear that the photochemical crosslinking of these polypeptides also occurs to this residue. To establish that the actual site of the photochemical crosslinking is indeed to the m^7G group, as opposed to bases downstream from the cap structure, we performed following experiment. Photochemical crosslinked mixtures were digested with the enzyme tobacco acid pyrophosphatase (Bethesda Research Laboratories) or with RNase A and analyzed on SDS-polyacrylamide gels. The extent of cap-specific crosslinking of eIF-4B was similar with both enzymes (data not shown). Because tobacco acid pyrophosphatase should cleave only the pyrophosphate bonds in the cap structure, we conclude that the photoaffinity crosslinking of eIF-4B occurs via the m^7G group of the cap structure.

To study the interaction of cap-specific polypeptides with mRNAs differing in 5' secondary structure, we used the SP6 system to prepare mRNA from tk gene constructs differing in the number of Bam HI linkers inserted into the region of the gene corresponding to the 5' noncoding portion of the mRNA. The various constructs chosen for this study are

FIGURE 3

Schematic Representation of the Structure of tk/SP64 Derivatives

Constructions were performed as described in reference 22. The Hinc II-Pvu II fragment of the tk gene was inserted into the Hinc II restriction site of pSP64. The thick line at the right end of the pX1/SP construct represents sequences originating from pSP64. The right-angled arrow denotes the start and direction of transcription. In enlarged form are shown the restriction sites used to insert Bam HI linkers as described in Materials and Methods.


50 bp
SP6 promoter
ATG
TK gene
Eco RI
pX1/SP
10 bp
Hind III
Bgl II
2 Bam HI
linkers
(pXJPB/SP)
3 Bam HI
linkers
(pXJP15/SP)

shown in Fig. 3. Recombinant plasmid pX1/SP contains a Hinc II-Pvu II fragment of the herpes simplex virus type I tk gene placed under the control of the SP6 promotor (see ref. 22 for details). Plasmid pXJP15/SP is a derivative of pX1/SP that contains three Bam HI linkers ($^{5'}$CCGGATCCGG$^{3'}$) 42 nucleotides (nt) downstream from the cap site, whereas pXJPB/SP contains two Bam HI linkers 11 nt downstream from the cap site. In the cloning procedure, the staggered ends generated by Hind III and Bgl II were filled in with the Klenow fragment of DNA polymerase I. This resulted in the addition of four extra nucleotides on each side of the inserted linker fragment. The introduced linkers and five additional nucleotides flanking the insert on each side have dyad symmetry; hence, when transcribed into mRNA in the SP6 system, they should form a stable stem-loop structure. We experimentally verified the presence of these structures by demonstrating that the region of the mRNAs (synthesized from pXJPB/SP and pXJP15/SP) containing the Bam HI linkers was resistant to cleavage by the single-stranded specific S1 and T1 RNAses and sensitive to the double-stranded-specific V1 nuclease (data not shown). We have previously shown that the translational efficiency of pXJP15/SP- and pSJPB/SP-derived mRNAs is greatly reduced as compared to pX1/SP-derived mRNA in both <u>in vitro</u> and <u>in vivo</u> assays (22). Furthermore, our studies indicated that the cause of the decreased translational efficiency of the pXJP15/SP transcript is reduced ribosome binding to this mRNA (22), and we have recently found that ribosome binding to the pXJPB/SP transcript is also inefficient as compared to pX1/SP transcript binding (data not shown). Ribosome binding is most probably dependent on prior interaction of CBPs with the

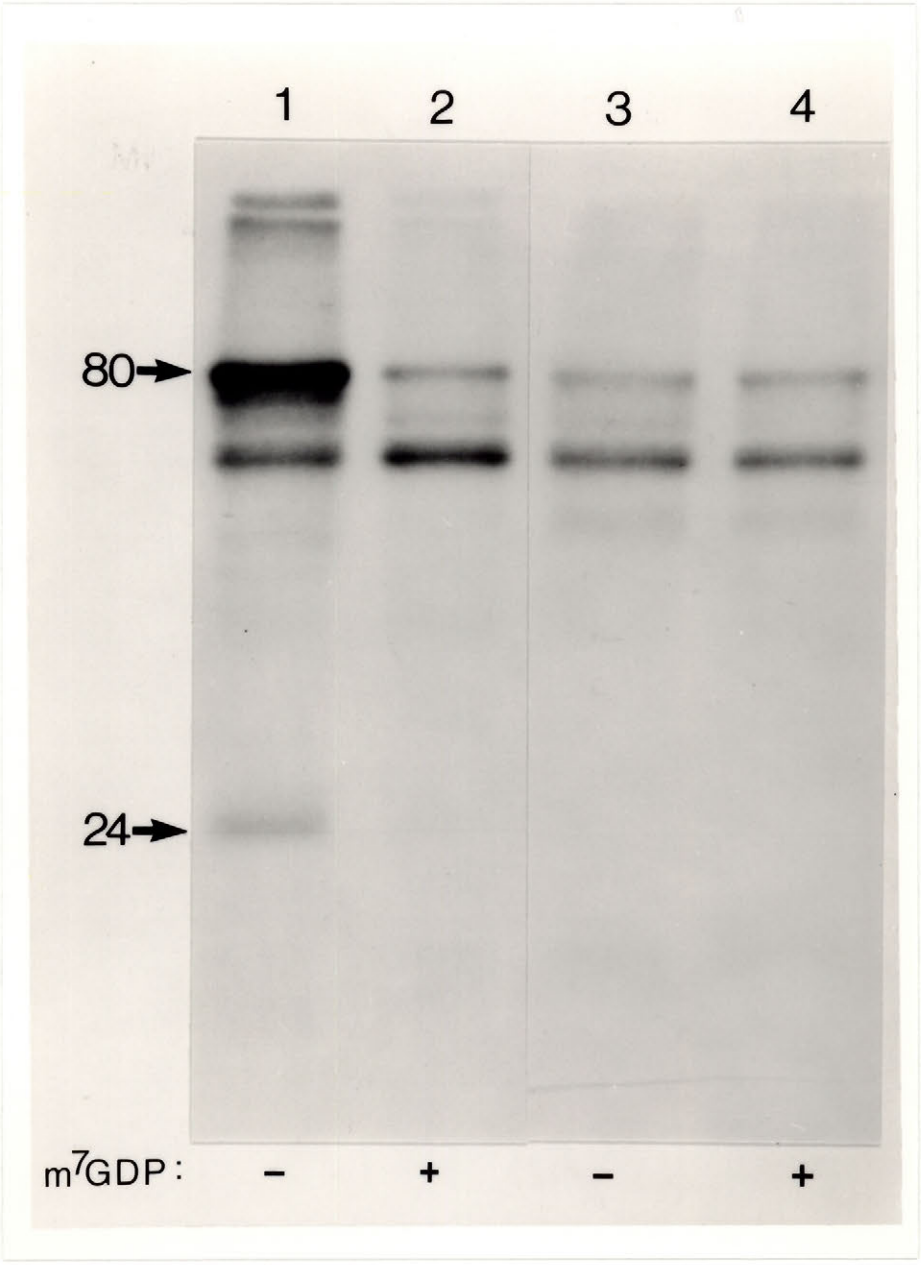
mRNA cap structure (4,5,11,27). Thus, it is plausible that the secondary structure of pXJP15/SP- and pXJPB/SP-derived mRNAs prevents interaction of cap recognition factors with the cap structure and consequently impedes ribosome binding. To test this possibility, we examined the interaction of cap-specific proteins with the various tk mRNA derivatives using the UV crosslinking technique.

Transcription of Eco RI-linearized pX1/SP and derived plasmids yields runoff transcripts that have a 5'-terminal pppG structure (22). For studies of UV-induced crosslinking, mRNAs were capped by using vaccinia virus guanylyltransferase and labeled exclusively at their 5' terminus by using [α-^{32}P]GTP in the reaction mixture (8,20). We have found that although only 10-15% of the mRNAs are capped, more than 95% of these capped mRNAs are methylated (data not shown). This results in the production of mRNAs with a ^{32}P label in the α phosphate of the cap structure ($m^7G\overset{*}{p}pppG...$; in which * denotes the ^{32}P label). We investigated the specificity of the photochemical crosslinking reaction of the tk transcripts by using $m^7G\overset{*}{p}pppG...$- and $\overset{*}{G}pppG...$-terminated pX1/SP mRNAs in the presence and absence of the cap analogue, m^7GDP. UV irradiation of $m^7G\overset{*}{p}pppG...$-terminated pX1/SP mRNA with rabbit reticulocyte IF resulted in the cap-specific crosslinking of the 24K-CBP and eIF-4B (Fig. 4; cf. lane 1 with lane 2 which includes m^7GDP), consistent with the results obtained with methyl-^{3}H-labeled reovirus mRNA. However, no proteins were found to specifically crosslink to $\overset{*}{G}pppG...$-terminated pX1/SP mRNA, as m^7GDP had no effect on the crosslinking of any of the polypeptides (cf. lanes 3 and 4, a

FIGURE 4

UV-Light Induced Crosslinking of ^{32}P-labeled G$\overset{*}{p}$pppG...- and m^{7}G$\overset{*}{p}$pppG...-terminated pX1/SP mRNA to Rabbit Reticulocyte CBPs.

IF (~ 100 µg) was incubated with [^{32}P]m^{7}G$\overset{*}{p}$pppG...-terminated mRNA (4 x 10^4 cpm, ~0.7 µg; lanes 1 and 2) or [^{32}P]G$\overset{*}{p}$pppG... -terminated mRNA (4 x 10^4 cpm, ~0.7 µg; lanes 3 and 4) in the presence of 1 mM ATP, followed by UV light irradiation as described in Materials and Methods. Samples were resolved on a 10 to 15% gradient SDS-polyacrylamide gel that was dried and exposed against Fuji X-ray film for 12 hr at -70°C. m^{7}GDP (0.6 mM) was added as indicated in the figure. Positions of the 24- and 80 kDa crosslinked polypeptides are indicated in the left margin.


1
2
3
4
80
24
m7GDP:
–
+
–
+

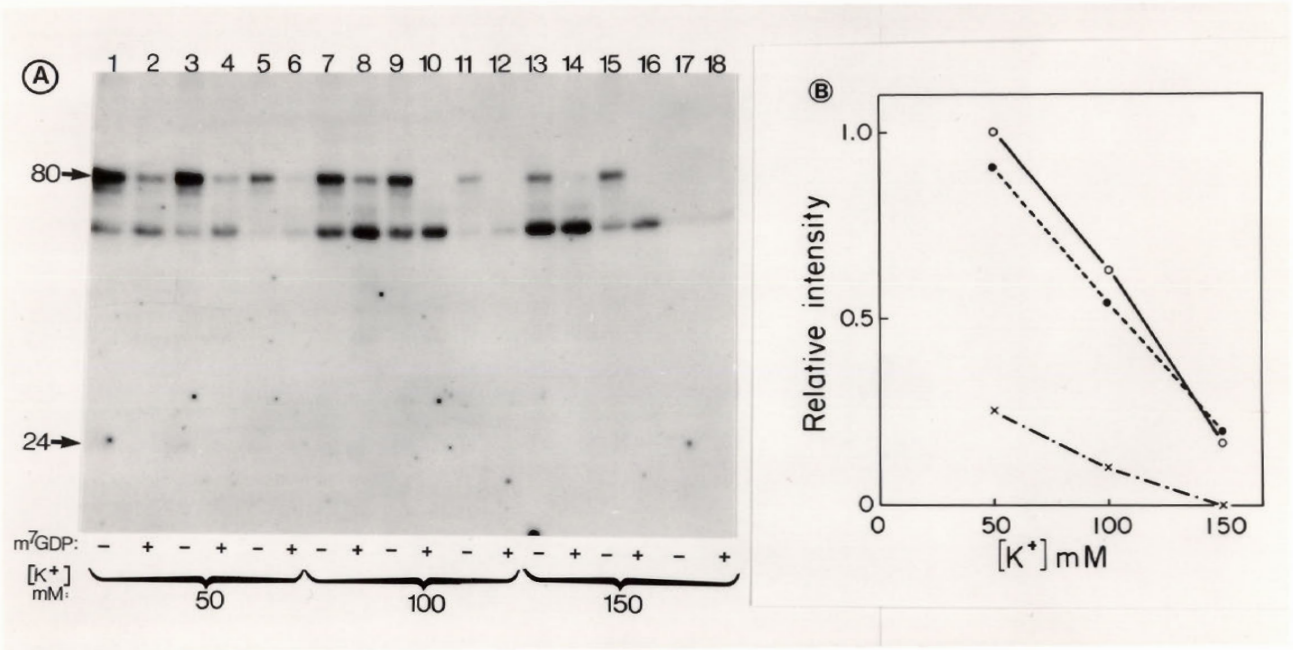
polypeptide that comigrates with eIF-4B crosslinks to this mRNA, but this crosslinking is not affected by the addition of m^7GDP: the identity of this polypeptide is not known, but it is possible that it is eIF-4B, because it can crosslink to some extent in a cap-independent manner; 32).

In light of the observations showing that salt concentration influences the degree of cap dependency exhibited by capped mRNAs for translation (3,5,38), and for interaction with cap-specific polypeptides (18), we examined the cap specific crosslinking pattern obtained with various tk constructs at different salt concentrations. In these experiments, decreased amounts of reticulocyte IF were used with the intention of limiting the components that might be involved in melting mRNA 5' secondary structure (29-31). The crosslinking pattern obtained with mRNA prepared from pX1/SP, pXJP15/SP and pXJPB/SP constructs after irradiation in the presence of rabbit reticulocyte IF at different salt concentrations (50, 100, and 150 mM K^+) is shown in Fig. 5A. Cap-specific crosslinking in this experiment has occurred primarily to eIF-4B (80-kDa polypeptide), crosslinking of the 24K-CBP being less pronounced than in previous experiments (see, for example Fig. 4). The crosslinking of eIF-4B decreased with increasing concentrations of K^+ from 50 to 150 mM with all three mRNAs (cf., for example, lane 13 with lane 1 or lane 17 with lane 5), as might be expected if secondary structure is increased at higher salt concentrations. However, the significant result in this experiment is that the extent of crosslinking of pX1/SP and pXJP15/SP mRNAs to eIF-4B was similar under all salt concentrations (Fig. 5A; cf. lanes 3,9, and 15 with lanes 1,7 and 13,

FIGURE 5

Effect of K^+ Concentration on UV Light-Induced Crosslinking of Rabbit Reticulocyte CBPs to mRNA Prepared from pX1/SP, pXJP15/SP and pXJPB/SP.

[^{32}P]-mRNA (cap labeled) (2 x 10^4 cpm, ~0.3 µg) was incubated with 24 µg of rabbit reticulocyte IFs in the presence of 1 mM ATP for 10 min at 30°C, UV irradiated, and processed for autoradiography as described in Materials and Methods. The autoradiograph is shown in panel A. KOAc was added to give the final concentrations indicated in the figure. The mRNAs used were as follows. Lanes 1,2,7,8,13 and 14,pX1/SP; lanes 3,4,9,10,15 and 16,pXJP15/SP; lanes 5,6,11,12,17 and 18,pXJPB/SP. m^7GDP (0.6 mM) was added as indicated in the figure, and the positions of the 24- and 80 kDa crosslinked polypeptides are indicated in the left margin. (B) Quantitative analysis of the labeled 80 kDa polypeptide band from panel A. The value obtained for lane 1 was set as a relative intensity of 1: symbols: (o—o), pX1/SP; (•----•), pXJP15/SP; (x—•—x), pXJPB/SP.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
80
24
m7GDP: – + – + – + – + – + – + – + – + – +
[K+] mM:
50
100
150
B
Relative intensity
1.0
0.5
0
0
50
100
150
[K+] mM

respectively; see Fig. 5B for quantitative analysis). This similarity is in sharp contrast to the low efficiency of pXJPB/SP mRNA crosslinking to eIF-4B at all salt concentrations as compared to pX1/SP mRNA (cf. lanes 5, 11 and 17 with lanes 1,7 and 13, respectively; see Fig. 5B for quantitative analysis). The results also indicate that the deleterious effect of the increase of salt concentration on eIF-4B crosslinking is more pronounced with pXJPB/SP mRNA as compared to the two other mRNAs. Increasing the K^+ concentration from 50 to 100 mM resulted in a 40% inhibition of eIF-4B crosslinking to pX1/SP and pXJP15/SP mRNA, whereas the inhibition was 70% for pXJPB/SP mRNA crosslinking. This is consistent with the idea that the increase of secondary structure at higher salt concentration is responsible for the reduced extent of crosslinking. In contrast to the differential pattern of eIF-4B crosslinking with regard to the mRNA used, there was no difference in the degree of 24K-CBP crosslinking to the different mRNAs at any given salt concentration (this could be clearly seen on a longer exposure of the X-ray film than that used in the experiment shown in Fig. 5A [data not shown]). This reinforced previous findings that the crosslinking of the 24K-CBP is independent of ATP and mRNA secondary structure, whereas the crosslinking of eIF-4B is dependent on ATP and the degree of mRNA secondary structure (16,29). On the basis of these results, we conclude that the ability of eIF-4B to crosslink to mRNA is influenced by secondary structure near the 5' terminus (6 nt from the cap structure) but is not affected by secondary structure farther downstream (37 nt from the cap structure).

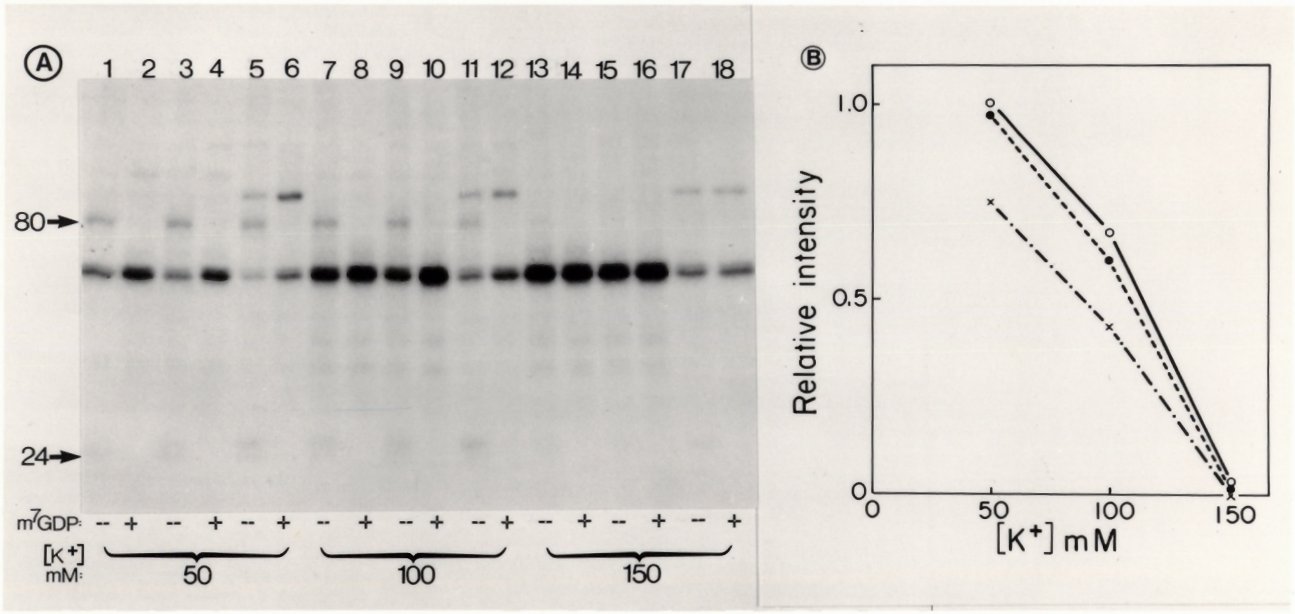
We performed experiments to examine the idea that the secondary structure, and not the mere extra nucleotides introduced downstream from

the cap structure prevents the interaction of eIF-4B with the cap structure. We prepared transcripts from pX1/SP, pXJP15/SP and pXJPB/SP in which inosine was substituted for guanosine, resulting in a less structured mRNA (14). If our interpretation of the results is correct, then inosine-substituted mRNA derived from pXJPB/SP might, in the absence of ATP-Mg^{2+}, crosslink to eIF-4B with an efficiency similar to that of pX1/SP mRNA. We have previously shown that inosine-substituted reovirus mRNA is capable of crosslinking to eIF-4B (identified as an 80-kDa polypeptide) in the absence of ATP-Mg^{2+}, in contrast to the inability of native reovirus mRNA to do so (16). We interpreted our results to indicate that the requirement of ATP-Mg^{2+} for the crosslinking of eIF-4B to native reovirus mRNA reflects the requirement for melting the 5'-proximal secondary structure of the mRNA. The crosslinking profiles obtained with pX1/SP, pXJPB/SP, and pXJP15/SP inosine-substituted mRNAs under three different salt concentrations, in the absence of ATP-Mg^{2+} are shown in Fig. 6A. Cap-specific crosslinking occurred to eIF-4B, consistent with previous results (16) and to a doublet in the region region of the 24K-CBP (cf. lane 1 to lane 2 which contains m^7GDP). There is an ~96-kDa polypeptide that crosslinks only to pXJPB/SP in a cap-independent manner (for example, see lane 5 and 6). We have no explanation for this, although it may be due to the base content of the mRNA, because a similar polypeptide is not seen with guanosine-containing mRNA (see Fig. 5, lanes 5 and 6). Significantly, the extent of crosslinking of the cap-specific polypeptides to the three mRNAs was very similar at all salt concentrations. (At 50 and 100 mM KCl, crosslinking was equal, but then it droped sharply at 150 mM KCl as

FIGURE 6

Photochemical Crosslinking Pattern of CBPs to Inosine-substituted mRNA Prepared from pX1/SP, pXJP15/SP and pXJPB/SP as a Function of K^+ Concentration.

[^{32}P]-mRNA (cap labeled) (2 x 10^4 cpm, ~0.3 µg) was incubated with 24 µg of rabbit reticulocyte IF preparation for 10 min at 30°C, UV irradiated, and processed for autoradiography as described in Materials and Methods. The autoradiograph is shown in panel A. KOAc was added to give the final concentrations indicated in the figure. The mRNAs used were as follows. Lanes 1,2,7,8,13 and 14, pX1/SP; lanes 3,4,9,10,15 and 16, pXJP15/SP; lanes 5,6,11,12,17 and 18, pXJPB/SP. m^7GDP (0.6 mM) was added as indicated in the figure, and the positions of the 24- and 80 kDa crosslinked polypeptides are indicated in the left margin.
(B) Quantitative analysis of the labeled 80 kDa polypeptide band from panel A. The value obtained for lane 1 was set as a relative intensity of 1. Symbols: (o—o), pX1/SP; (•---•), pXJP15/SP; (x—•—x), pXJPB/SP.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
80
24
m7GDP: - + - + - + - + - + - + - + - + - + - +
[K+] mM: 50 100 150
B
Relative intensity
1.0
0.5
0
0 50 100 150
[K+] mM

is evident from the quantitation of the eIF-4B bands on the autoradiograph [Fig. 6B]). This behaviour is in sharp contrast to the lower efficiency of native pXJPB/SP mRNA crosslinking to eIF-4B in the presence of ATP-Mg^{2+} (cf. Fig. 6 with Fig. 5). Similar results were obtained when the experiments shown in Fig. 6 were performed in the presence of ATP-Mg^{2+} (data not shown). Thus, the reduction of secondary structure of pXJPB/SP mRNA abolished the differential crosslinking of this mRNA to eIF-4B relative to pX1/SP mRNA.

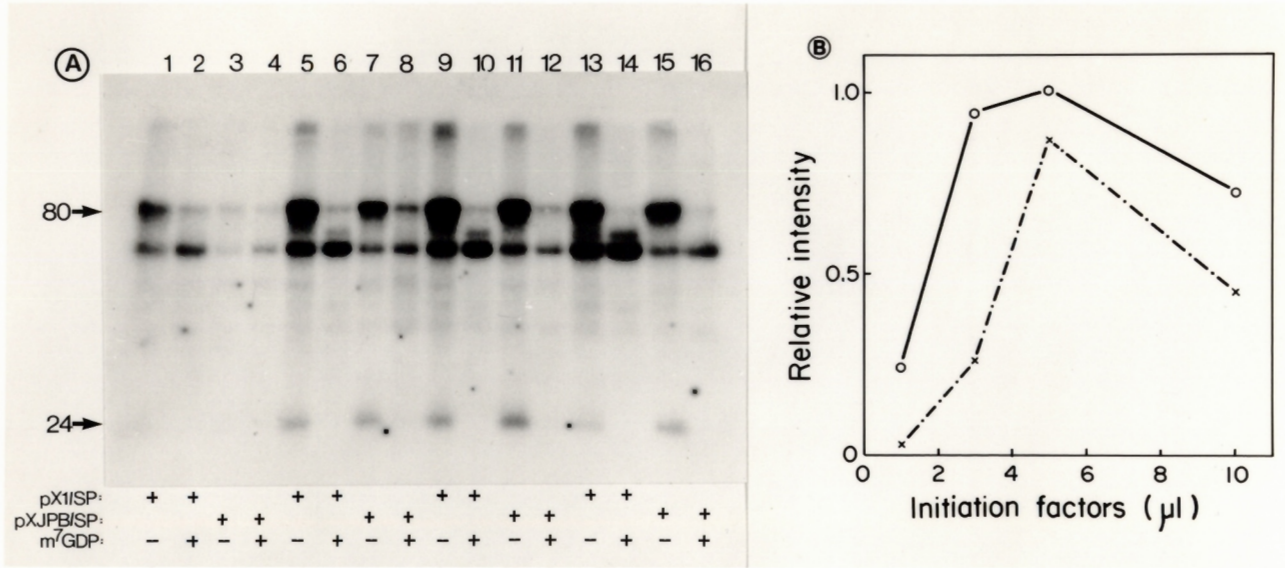
In light of the hypothesis that the CBP complex, possibly in conjunction with eIF-4B, is involved in the melting of 5' mRNA secondary structure (5,30) and that this complex is limiting in the cell (23,24), it is conceivable that the addition of this factor to the crosslinking reactions containing pX1/SP or pXJPB/SP mRNAs will abolish the differential crosslinking of these mRNAs to eIF-4B (i.e., the CBP complex will preferentially stimulate crosslinking of eIF-4B to pXJPB/SP mRNA). Unfortunately, we could not test this prediction directly, because our latest preparations of the CBP complex contained an ~80 kDa polypeptide (possibly a contaminant; 11) that crosslinks nonspecifically to the cap structure and consequently interfered with the interpretation of the data. We alternatively used total IFs from rabbit reticulocytes. Albeit less satisfactory than that obtained for the purified CBP complex, the data obtained should indicate whether IF preparations contain a factor which is required more for mRNAs having increased secondary structure. We crosslinked increasing amounts of total IF preparations to pX1/SP and pXJPB/SP mRNAs, and the analysis of the crosslinked polypeptides on an SDS-polyacrylamide gel is shown in

Fig. 7A. When 12 µg of IFs was used, the major cap-specific crosslinked protein is eIF-4B, whereas crosslinking of the 24K-CBP is not evident at this exposure (lane 1). In addition, crosslinking of pX1/SP mRNA was more efficient than of pXJPB/SP mRNA (cf. lane 3 with 1), as shown before (Fig. 5). The addition of increasing amounts of IF increased the extent of crosslinking of both mRNAs to eIF-4B and the 24K-CBP, but the efficiency of crosslinking of pX1/SP mRNA to eIF-4B reached a plateau after the addition of 36µg of IF (lane 5), whereas the efficiency of crosslinking of pXJPB/SP mRNA to eIF-4B reached a similar level only when 60µg of IF was added (lane 11; the quantitation of crosslinking is shown in Fig. 7B). The results also show that there was no significant differential crosslinking of the 24K-CBP to the two mRNAs under any of the conditions (cf. for example, lanes 7 and 5 or lanes 11 and 9), consistent with the contention that crosslinking of the 24K-CBP to the 5' end of the mRNA is independent of the degree of mRNA 5' secondary structure, whereas crosslinking of eIF-4B is dependent on this structure.

FIGURE 7

Effect of IF Concentration on UV Light-Induced Crosslinking of CBP to [^{32}P]-mRNA from pX1/SP and pXJPB/SP Constructs

[^{32}P]-mRNA (cap labeled) (2 x 10^4 cpm, ~0.3 µg) was incubated with increasing amounts of rabbit reticulocyte IFs (12 mg/ml) in the presence of 1 mM ATP, followed by UV irradiation and SDS-polyacrylamide gel analysis of the crosslinked products as described in Materials and Methods. The autoradiograph is shown in panel A. The amounts of IF added were as follows. Lanes 1 to 4, 12 µg; lanes 5 to 8, 36 µg; lanes 9 to 12, 60 µg; lanes 13 to 16, 120 µg. mRNAs used are indicated in the figure. m^7GDP (0.6 mM) was added where indicated, and the positions of the 24- and 80 kDa crosslinked polypeptides are indicated in the left margin. (B) Quantitative analysis of the labeled 80 kDa polypeptide band from panel A. The value obtained for lane 9 in panel A was set as a relative intensity of 1. Symbols: (o—o), pX1/SP; (x-•-x), pXJPB/SP.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
80
24
pX1ISP:
pXJPBISP:
m7GDP:
B
Relative intensity
1.0
0.5
0
0 2 4 6 8 10
Initiation factors (μl)

DISCUSSION

The chemical crosslinking assay has been very useful in identifying polypeptides that bind at or near the cap structure of eucaryotic mRNAs and elucidating their possible roles in facilitating initiation complex formation (4,10,32). This assay, however, has several limitations which preclude its application to a range of experiments as detailed in the Results section. One of the disadvantages of the chemical crosslinking technique is that the interaction between the aldehyde groups of the oxidized ribose moiety of the cap structure occurs via ε-amino or α-terminal amino groups of proteins. These groups might not be present in the cap binding site of the different polypeptides that interact with the cap structure. In contrast, it has been shown that as many as eleven different amino acids can form covalent bonds with uracil upon UV irradiation (28). Thus, the probability of detecting CBPs by the photolabeling technique is a priori greater than with the chemical crosslinking technique. Furthermore, the application of the photochemical technique is not restricted to those few viral mRNAs which can be purified to large amounts, this feature being particularly attractive with the advent of the SP6 transcription system. Finally, because [α-^{32}P]GTP is used to prepare the labeled cap structure, results are obtained with much shorter exposure of the autoradiograms to X-ray film as compared with the chemical crosslinking method in which tritium is used.

The high-energy intermediates that are formed during irradiation of mRNA have a relatively short half-life, and as a consequence, the site of covalent linkage in the protein must be very close to the cap

structure. This decreases the probability of non-specific crosslinking, and, indeed, the data in Fig. 1 demonstrate that there is less nonspecific crosslinking with the photolabeling technique as compared to the chemical technique.

We identified two polypeptides of 24- and 80 kDa that can be photochemically crosslinked to the cap structure of reovirus and tk mRNAs. These polypeptides are most probably the same as the 24-kDa (24K-CBP) and 80-kDa (eIF-4B) polypeptides observed in the chemical crosslinking assay, based on their similar behaviour in terms of cap specificity, ATP-Mg^{2+} requirement for crosslinking, and their inability to interact with the cap structure after poliovirus infection of HeLa cells. It is significant, however, that there are qualitative and quantitative differences between the crosslinking patterns obtained by the two techniques. Unlike with the chemical crosslinking assay, we did not observe cap specific crosslinking of eIF-4A in the photochemical assay. A simple explanation for this is that the position of eIF-4A relative to the mRNA is not within the reactive range of the high-energy and short-lived intermediate species produced by irradiation of mRNA. However, because it is crosslinked in the chemical assay, eIF-4A must be in proximity to the cap structure. In the chemical crosslinking assay, the 24K-CBP is crosslinked with the highest efficiency (Fig. 1), whereas in the photochemical assay eIF-4B crosslinks with greater efficiency than the 24K-CBP. It is possible that this difference is due to the fact that the chemical crosslinking reaction proceeds through the ribose group of the m^7G moiety of the cap structure (33), whereas the covalent link between the cap structure and proteins, induced by UV

light, is probably via the ring structure of the m^7G group. This is based on the studies of Steinmaus et al. (34) indicating that the 8-position of the guanine ring is the most reactive group and that a free radical is generated upon the photolysis of this compound. Thus, these results suggest that eIF-4B is positioned in closer proximity to the ring of the m^7G than to its ribose moiety, whereas the opposite applies for the 24K-CBP.

Photochemical crosslinking has been previously used to identify proteins that interact with eukaryotic mRNAs, but no attempt was made in these studies to determine the proteins that crosslink specifically to the cap structure (9,26). It is of interest, however, that a polypeptide termed p78X was found to crosslink to mRNA by UV light irradiation and that this polypeptide was different from the protein that binds to the poly(A) tail of eukaryotic mRNAs (termed p78A; ref. 26).

We found an inverse relationship between the efficiency of mRNA crosslinking to eIF-4B and K^+ concentration (Fig. 5). This is expected according to our model, because the degree of secondary structure is increased at higher salt concentrations. That the high salt concentration per se is affecting the activity of a factor(s) involved in the melting of the mRNA secondary structure is an unlikely possibility, because it cannot explain the differential crosslinking patterns obtained with mRNA from pX1/SP and pXJPB/SP constructs at the different salt concentrations used. These results are, however, different from those obtained by the chemical crosslinking technique (16). In the latter study, it was reported that the crosslinking efficiency of oxidized reovirus mRNA to the 80 kDa polypeptide (eIF-4B)

increased with increasing K^+ concentrations (16). A possible explanation for these differences is that the mRNA crosslinking site in eIF-4B is different in the two techniques. This would seem a reasonable argument, considering the differences in the mechanism of chemical and photochemical crosslinking.

The results presented here indicate that eIF-4B is unable to interact with the cap structure if excessive secondary structure exists close to the cap structure (6 nt in pXJPB/SP) yet is able to do so if the stem-loop structure is farther downstream (37 nt in pXJP15/SP). The crosslinking of the 24K-CBP, however, does not seem to be affected by the insertion of Bam HI linkers at either position. This observation is consistent with a mechanism by which the 24K-CBP as part of the CBP complex binds to the mRNA in an ATP-independent fashion, followed by ATP-dependent melting of mRNA secondary structure and subsequent interaction of eIF-4B (4,30).

In a recent report from this laboratory (22), we showed that increasing tk mRNA secondary structure within the 5' noncoding region results in a decreased translational efficiency in <u>in vivo</u> and <u>in vitro</u> systems. mRNAs from pXJP15/SP and pXJPB/SP showed a drastic reduction in translational efficiency when compared with mRNA obtained from pX1/SP. Because the position of the hairpin loops is different in each of the constructs, the data presented here suggest that the block in translation of pXJPB/SP (but not pXJP15/SP) is at least partially the result of the inability of eIF-4B to interact with the 5'-terminal cap structure, with subsequent impediment of ribosome binding and protein

synthesis. The impaired translation of pXJP15/SP is probably due to a step subsequent to cap recognition and eIF-4B binding, involving further melting of the mRNA 5' noncoding region perhaps concomitantly with 40S ribosome binding or migration along the mRNA 5' leader region.

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CHAPTER 4

CAP-INDEPENDENT TRANSLATION OF POLIOVIRUS mRNA IS CONFERRED BY SEQUENCE ELEMENTS WITHIN THE 5' NONCODING REGION

SUMMARY

Poliovirus polysomal RNA is naturally uncapped, and as such, its translation must bypass any 5' cap-dependent ribosome recognition event. To elucidate the manner by which poliovirus mRNA is translated, we have determined the translational efficiencies of a series of deletion mutants within the 5' noncoding region of the mRNA. We found striking differences in translatability among the altered mRNAs when assayed in mock- and poliovirus-infected HeLa cell extracts. The results identify a functional *cis*-acting element within the 5' noncoding region of the poliovirus mRNA which enables it to translate in a cap-independent fashion. The major determinant of this element maps between nucleotides 320 and 631 of the 5' end of the poliovirus mRNA. We also show that this region (320 to 631), when fused to a heterologous mRNA, can function in *cis* to render the mRNA cap-independent in translation.

INTRODUCTION

Poliovirus polysomal RNA, unlike most eukaryotic mRNAs, is not capped at its 5' end, but rather terminates in pUp (15,27). The mRNA contains a major translation initiation site approximately 750 nucleotides from the 5' end (4). The long 5' noncoding region of poliovirus mRNA (compared to an average of 100 nucleotides for eukaryotic mRNAs) is scattered with multiple upstream AUGs, of which only three are conserved in position among all three serotypes (17,31,39). These AUGs are not removed by splicing of the viral mRNA (4), and their function, if any, remains unclear. The first 650 nucleotides of the 5' end are highly conserved (greater than 80% homology) among the three serotypes and are thought to be involved in some essential functions (39). Alterations within the 5' noncoding region have been correlated with changes in neurovirulence, cytopathic effects, and translational efficiency (9,19,21,28,32,38).

The mechanism by which poliovirus mRNA translates is enigmatic in several major respects. First, poliovirus RNA must bypass the requirement for a cap structure for its translation. This translation is efficient in extracts prepared from poliovirus-infected HeLa cells, in sharp contrast to the low translational levels exhibited by cellular mRNAs in these extracts. Second, it has been shown that poliovirus-mediated inactivation of the cap-binding protein complex (a translation initiation factor required for translation of most mRNAs; for a recent review, see reference 6), in conjunction with an as yet unidentified second event (2), allows poliovirus to usurp the cellular

translational apparatus without the need to compete with cellular mRNAs (for reviews, see references 7 and 36).

A scanning model has been suggested to explain the manner by which eukaryotic messages bind ribosomes (18). As postulated for other mRNAs, this model predicts that ribosomes bind at or near the 5' end of poliovirus mRNA and proceed to scan the long leader region until the major translation initiation codon is reached. An alternative possibility is that ribosomes can bind internally near the major initiation site. In a recent report, Hassin et al. (13) presented evidence that such a mechanism occurs on the adenovirus type 2 mRNA of DNA polymerase. In a previous report, Herman (14) demonstrated internal initiation of translation on the vesicular stomatitis virus nonstructural mRNA, thus supporting the contention that internal initiation is possible on some eukaryotic mRNAs.

To gain a better understanding of the mechanism of translation initiation of poliovirus mRNA, we have analyzed the effects of deletions within the 5' leader region of poliovirus mRNA on its translation in mock- and poliovirus-infected HeLa cell extracts. We show that poliovirus mRNA contains a cis-acting sequence within the 5' noncoding region which mediates cap-independent translation. In addition, when fused to a heterologous mRNA species, this region confers upon the mRNA the ability to translate in a cap-independent fashion.

MATERIALS AND METHODS

Construction of Deleted Templates

Recombinant DNA techniques were performed by standard methods (24). The plasmid used to construct the deletion mutants was pP2-5' (Fig. 1). This plasmid was constructed by inserting a Hind III-Sma I fragment of P2/Lansing cDNA (21) representing bases 1 to 1872 of the viral genome into pSP64. The 5' 68 nucleotides of this cDNA fragment were derived from a P1/Mahoney cDNA (32) in which the first nucleotide of the viral RNA is preceded by 18 nonviral nucleotides, including a Hind III linker. Linearization of pP2-5' with Sma I followed by *in vitro* transcription using SP6 RNA polymerase produces an RNA containing 24 nonviral nucleotides preceding the first poliovirus nucleotide.

The deleted templates Δ3'-631, Δ3'-381, Δ5'-733, and Δ5'-632 were constructed by cleaving pP2-5' with combinations of restriction endonucleases Hind III, Bal I, Eco RV and Hae III, removing the appropriate DNA fragments, and religating the ends. The deleted templates Δ5'-33, Δ5'-80, Δ5'-96, Δ5'-140 and Δ5'-320 were constructed by cleaving pP2-5' with Hind III followed by treatment with nuclease Bal 31 for various periods. The truncated inserts were released from the vector by cleavage with Sst I and ligated into M13mp18 for sequencing by the chain termination technique (34). Deleted DNA templates were cleaved from the M13 vector by digestion with Hind III and Sst I and cloned into pT7SP6, so that transcription with SP6 polymerase yields positive-strand RNA transcripts.

Poliovirus/CAT fusion constructs were made as follows: Δ3'-70/CAT, Δ3'-461/CAT, Δ3'-631/CAT, and pP2/CAT were constructed by cleaving the

parent poliovirus plasmid pP2-5' with Asp 718, Bsm I, Bal I, and Eco RV, respectively. The resulting vectors were subjected to digestion with Bam HI (or Eco RI for Δ3'-461/CAT) and purified from a low-melting agrose gel as described previously (22). The chloramphenical acetyltransferase (CAT) gene was isolated from pCAT (Hind III-Bam HI CAT fragment inserted between the Hind III-Bam HI sites of pSP64) by restricting with Hind III, blunt ending with Klenow, and excision with Bam HI, or by partial Eco RI restriction; it was then gel purified and directionally inserted into the various pP2-5' deletion vectors using T4 DNA ligase.

The Δ5'-33/CAT, Δ5'-140/CAT, and Δ5'-320/CAT constructs were created by using the above mentioned strategy. Briefly, the corresponding parental vectors (Δ5'-33, Δ5'-140, and Δ5'-320, respectively) were restricted with Eco RV and Bam HI. The Hind III(blunt-ended)-Bam HI CAT gene was directionally inserted into the parental vectors with T4 DNA ligase. The Bsm I-Eco RV polio fragment was inserted into pCAT. The fragment was rendered blunt end with T4 DNA polymerase, ligated to synthetic Hind III linkers, restricted with Hind III, agarose gel purified, and ligated to Hind III-restricted pCAT.

Δ5'-320/Δ3'-461/CAT and Δ3'-461/CAT were constructed in a similar manner using Δ5'-320 and pP2-5' as starting parental vectors. Δ5'-463/CAT was constructed from partial Bsm I restriction of pP2/CAT, blunt ending with T4 DNA polymerase, restricting with Hind III, and then filling in with Klenow. The resulting vector was religated with T4 DNA ligase. Dideoxy Sequencing (34) was performed on Δ5'-463/CAT, Δ3'-461/CAT and Δ5'-320/Δ3'-461/CAT to precisely map the nucleotide at

which T4 DNA polymerase digestion terminated. Δ5'-465/Δ3'-631/CAT was constructed from Δ5'-465/CAT by digestion with Bal I and Bam HI. The CAT gene (Hind III-blunted Bam HI fragment) was then inserted into this vector with T4 DNA ligase. Δ5'-320/Δ3'-631/CAT was constructed in a similar fashion, except that Δ5'-320/CAT was used as the starting template.

In Vitro Transcriptions

Plasmids were linearized with Sma I (for pP2-5' deletion constructs) or Bam HI (for pP2/CAT fusion constructs), and transcription reactions were carried out by the method of Pelletier and Sonenberg (29), except that the GTP concentration was 50μM. Yields of transcripts were calculated from the incorporation of [^{3}H]CTP into RNA. A portion of each transcription reaction was analyzed on 1.4% formaldehyde gels to ascertain that equal amounts of mRNA were used for translation. Autoradiography of the gels showed the presence of a single RNA species migrating at the appropriate size.

In Vitro Translations

Poliovirus type 1 (Mahoney strain) infection of HeLa cells was done as described by Lee and Sonenberg (23). Extracts from mock- or poliovirus-infected HeLa cells were prepared at 3 hr after infection, essentially as described previously (33), but with minor modifications (23). Extracts were judged to be infected by assaying for p220 proteolysis (a component of the cap-binding protein complex [8], by the inability of oxidized reovirus mRNA to crosslink to cap-binding proteins (23), and by the detection of poliovirus proteins following *in vitro* translation of endogenous mRNA. Translation in HeLa extracts was

carried out by the method of Rose et al. (33). Following translation of pP2-5' deletion mRNAs, RNAse T1 (200 U) and RNAse A (1 ug) in 3 M urea-25 mM EDTA-0.5 mM phenylmethylsulfonyl fluoride was added and the incubation was continued at 30°C for 1 hr. For gel analysis of translation products, portions from translation mixtures were mixed with sodium dodecyl sulfate sample buffer (20), boiled for 5 min, and applied on sodium dodecyl sulfate-10 to 15% polyacrylamide gels. Gels were fixed in 40% methanol-7.5% acetic acid, treated with EN^{3}HANCE (New England Nuclear Corp.), and exposed against X-ray film at -70°C. Translations for each experiment were performed at least twice with two different mRNA preparations.

RESULTS

To study the sequence requirements for the cap-independent translation of poliovirus mRNA, we used RNA transcribed from a type 2 poliovirus cDNA clone (30) using SP6 RNA polymerase. To avoid the analysis of several translation products generated by processing of the poliovirus precursor polyprotein (NCVPOO), we used a truncated cDNA clone termed pP2-5', extending to nucleotide 1872 in the VP3 region (Fig. 1). This cDNA template gave rise to the expected 1.8-kilobase RNA product following transcription in the SP6 system and analysis on formaldehyde gels (data not shown). Various deletion mutants in the 5' noncoding region were constructed and are shown in Fig. 1. Following linearization of the plasmids, transcription by SP6 polymerase was performed in the presence of m^7GpppG or GpppG to generate methylated and unmethylated capped transcripts, respectively. It has been previously shown that for cap-dependent mRNAs, the capped methylated (m^7GpppG...-terminated) form translates more efficiently than its capped unmethylated (GpppG...-terminated) counterpart (3). In addition, cap analogues (such as m^7GDP), but not their unmethylated counterparts, are specific inhibitors of methylated capped mRNA translation in vitro (16). We reasoned that deletions extending into the region responsible for cap-independent translation of the mRNA would render the mRNA cap stimulated. We attempted to identify the region responsible for cap-independent expression of poliovirus mRNA by comparing the translational efficiencies of the m^7GpppG...- and GpppG...-terminated mRNA species transcribed from the poliovirus deletion constructs. We have also translated uncapped poliovirus transcripts [terminating with a

GMP-P(CH_2)P group] and found that they behave similarly to capped unmethylated transcripts (data not shown). All of these transcripts are more stable under translation conditions than pppG...-terminated mRNAs, which are rapidly degraded in several cell-free extracts (10).

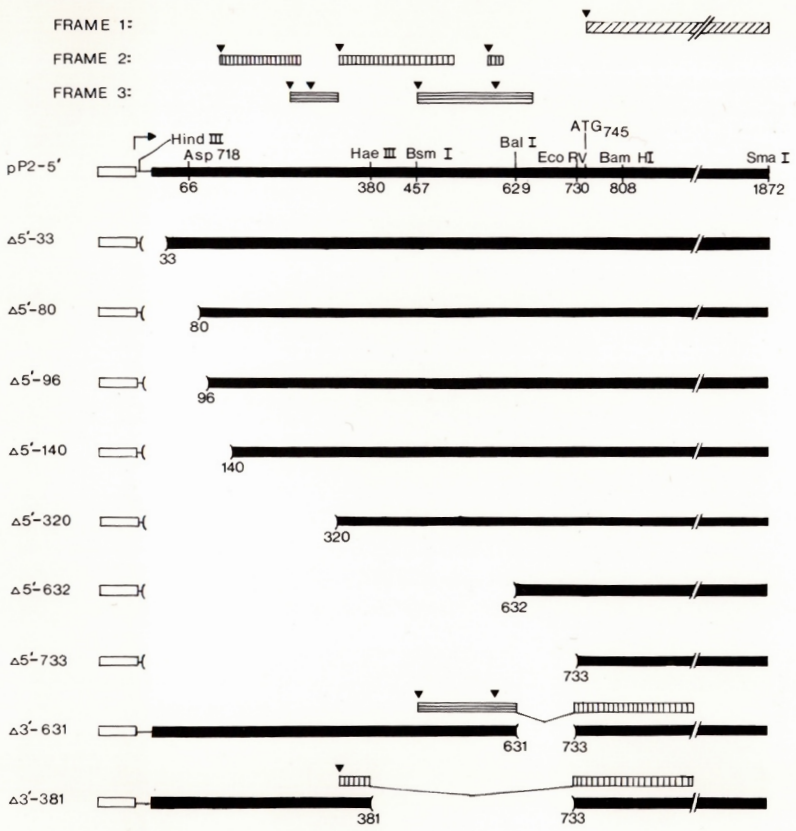
Translation of pP2-5' Deletion mRNAs in Extracts from Mock-Infected HeLa Cells.

We wanted to define features of the 5' noncoding region of poliovirus mRNA that enable it to translate in extracts from poliovirus-infected cells, in contrast to most capped mRNAs, which do not translate in these extracts. The translation of mRNAs containing deletions within the 5' noncoding region was first assessed by using extracts from mock-infected HeLa cells. The choice of this cell line was dictated by its almost universal use in studies of poliovirus. In this, and all subsequent in vitro translation experiments, S-adenosyl-L-homocysteine was included in the translation incubations to prevent methylation of unmethylated capped transcripts by endogenous methyltransferases (3). The results are shown in Fig. 2 and are summarized in Fig. 1. Translation of pP2-5' mRNA in a HeLa mock-infected extract yields a 46-kilodalton (kDa) polypeptide (Fig. 2, lane 2). The predicted molecular mass of the VP4-VP2-VP3 polypeptide encoded by pP2-5' mRNA is 42 kDa. Thus, the translation product migrates slightly slower than anticipated, but can be immunoprecipitated by anti-VP4 antibodies (data not shown). (The slower migration may be due to residual tRNA nucleotides remaining attached to protein following RNAse treatment, which was performed after translation. The tRNA molecule could not be naturally released when ribosomes reached the end of the mRNA because of

FIGURE 1

Structure of Deletion Constructs of Poliovirus RNA

The open box represents the SP6 polymerase promoter, and the right-angled arrow indicates the transcription start site. The thin line represents 24 extra, nonviral nucleotides between the SP6 promoter and the first nucleotide of the viral genome. The black box represents P2/Lansing viral sequences. RNAs transcribed from these templates contain the initiator ATG (shown at nucleotide 745) of the viral polyprotein encoding an ORF for a truncated poliovirus polyprotein consisting of VP4 (69 amino acids), VP2 (271 amino acids) and 37 amino acids of VP3 (predicted total molecular weight, 42,000 [21]). The ORFs defined by the AUGs in the 5' noncoding region are represented at the top of the diagram. The triangles denote AUG positions. When a deletion causes an alteration within an upstream ORF resulting in termination within the major poliovirus polyprotein ORF, it is diagramatically represented above the respective construct. Deleted sequences are bracketed. The cleavage site of restriction enzymes used to produce the deletion mutants are shown on the pP2-5' template. The numbers shown represent distances, in nucleotides, from the first nucleotide of the 5' end of the viral template. The relative translational efficiencies of the deletion constructs obtained in mock- and poliovirus-infected HeLa extracts are indicated to the right. Radioactive bands corresponding to poliovirus protein product were quantified by soft laser densitometry (LKB Instruments, Inc.), and the value obtained for capped unmethylated pP2-5' mRNA was set as 1.0. NT; not tested; -, not detected.



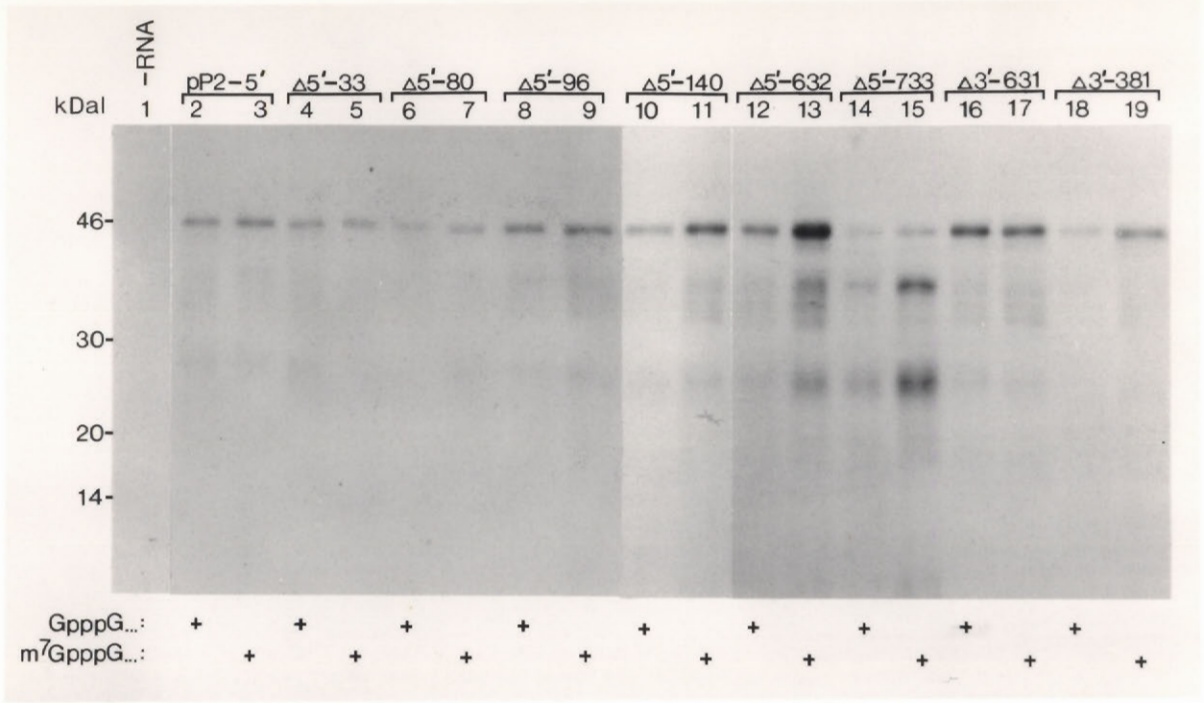
	UNINF. HELA		INF. HELA	
	GpppG	m^7GpppG	GpppG	m^7GpppG
pP2-5'	1.0	1.1	1.0	1.0
Δ5'-33	1.0	1.0	NT	NT
Δ5'-80	0.9	0.9	0.7	0.7
Δ5'-96	1.2	1.3	1.0	1.0
Δ5'-140	1.1	2.7	0.7	0.7
Δ5'-320	NT[a]	NT	0.2	0.2
Δ5'-632	2.3	12	—[b]	—
Δ5'-733	2.2	6.0	—	—
Δ3'-631	3.2	2.3	1.0	1.0
Δ3'-381	0.5	1.8	—	—

the lack of a termination codon). Since VP4 contains only one methionine (derived from ATG_{745}), these results prove that translation on pP2-5' commences with ATG_{745}. As anticipated, the translation of pP2-5' mRNA was not stimulated by the presence of a methylated cap structure on the mRNA (Fig. 2, compare lane 3 to 2). The translational efficiency of deletion mutants Δ5'-33, Δ5'-80, and Δ5'-96 was similar to that of pP2-5' mRNA and likewise not cap stimulated (lanes 4 to 9). A further deletion extending to nucleotide 140 resulted in a 2.4-fold difference in the translational efficiency between unmethylated (lane 10) and methylated (lane 11) capped mRNA. A significantly larger effect was obtained with deletion mutants Δ5'-632 and Δ5'-733 (lanes 12 to 15). The translation of capped Δ5'-632 mRNA is severalfold more efficient than that of pP2-5' mRNA (compare lane 13 with lane 3); this is most probably due to the differences in the efficiencies between cap-dependent (Δ5'-632) and cap-independent (pP2-5') mechanisms of initiation. It is of interest that synthesis of the major 46 kDa polypeptide from Δ5'-733 mRNA was greatly diminished, while ~27- and ~40 kDa protein species became more prominent (lanes 14 and 15). Polypeptides of these sizes are consistent with initiation at AUG codons that are present downstream of AUG_{745}. The short 5' end of the Δ5'-733 transcript most probably does not allow for efficient recognition of the major AUG codon by 40S ribosomes. Translation of mRNA containing deletions upstream of nucleotide 733 shows that nucleotides 631 to 733 are not important for cap-independent translation (compare lane 16 to lane 17). In contrast, translation of methylated capped Δ3'-381 mRNA was 3.5-fold more efficient than its unmethylated counterpart (compare

FIGURE 2

Translation of pP2-5' Deletion mRNAs in Mock-infected HeLa Cell Extracts

Translations were performed by using 12.5µl as described in Materials and Methods, with 0.4 µg of pP2-5' deletion mRNAs. Incubations were carried out for 60 min. at 30°C followed by RNAse treatment, and samples were processed for electrophoresis as described in Materials and Methods. Values obtained after scanning of the autoradiograph relative to the amount of protein synthesized from unmethylated capped pP2-5' mRNA are indicated in Fig. 1. Kinetic analysis and dose-response curves indicated that the rates of translation were in the linear range. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.


kDal
-RNA
pP2-5'
Δ5'-33
Δ5'-80
Δ5'-96
Δ5'-140
Δ5'-632
Δ5'-733
Δ3'-631
Δ3'-381
1
2 3
4 5
6 7
8 9
10 11
12 13
14 15
16 17
18 19
46-
30-
20-
14-
GpppG...: + + + + + + + + + +
m7GpppG...: + + + + + + + + + +

lane 19 with lane 18). The upstream overlapping open reading frames (ORFs) in Δ3'-631 and Δ3'-381 (shown diagramatically in Fig. 1) are not efficiently recognized by ribosomes, since the predicted polypeptides (~17 kDa for Δ3'-631 and ~12 kDa for Δ3'-381) were not detected. On the basis of the scanning model (18), one would expect the upstream ORFs to decrease translational efficiency, which definitely is not the case with Δ3'-631, in which even a slight stimulation is observed compared to pP2-5' mRNA.

The absence of a translation termination codon on the pP2-5' deletion mRNAs does not render termination rate-limiting, since if this was the case, one would expect to see equal translational efficiency for all constructs. In addition, the ability to detect an effect of methylation of the cap structure on translation of several of the constructs (e.g., Δ5'-632; compare lanes 13 with lane 12) argues that an event at the level of initiation is occurring. From the results shown in Fig. 2, we conclude that the major element responsible for cap-independent expression of pP2-5' mRNA lies between nucleotides 140 and 631.

Translation of pP2-5' Deletion mRNAs in Extracts from Poliovirus-Infected HeLa Cells.

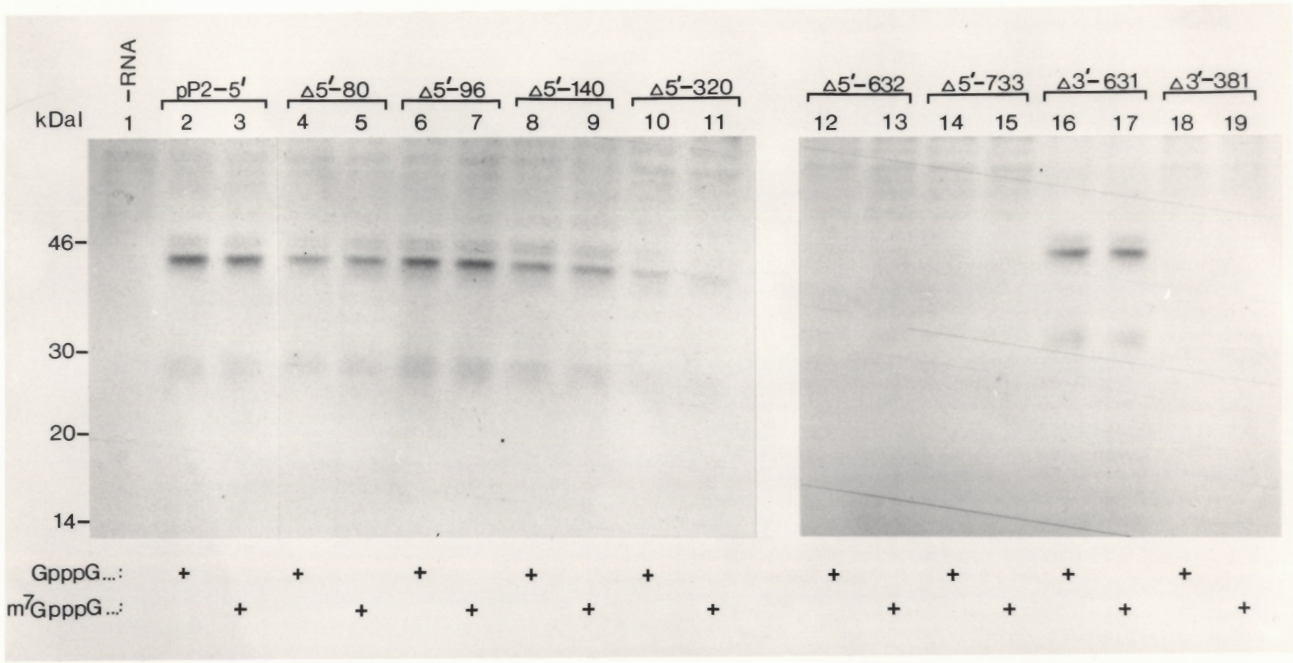
The translation of mRNAs in poliovirus-infected HeLa cell extracts can occur only via a cap-independent mechanism (7,36). pP2-5' mRNA containing the sequence element(s) conferring cap-independent translation would therefore be expected to translate in extracts prepared from poliovirus-infected cells. Poliovirus pP2-5' mRNA translated efficently in an extract from poliovirus-infected HeLa cells,

and translation was not dependent on a methylated cap structure (Fig. 3, lanes 2 and 3). Thus, the pP2-5' mRNA contains all the information necessary for cap-independent translation. The major polypeptide synthesized had an apparent molecular mass of 42 kDa as compared with the 46 kDa polypeptide synthesized in extracts from mock-infected HeLa cells (compare Fig. 3 with Fig. 2). In addition, there is a minor product migrating above the major 42 kDa polypeptide product. It is likely that the translation product of pP2-5' mRNA is processed by endogenous $3C^{pro}$ to yield VP4-VP2 (predicted molecular mass, 38 kDa) and a truncated VP3 product (4 kDa), which is not seen. The residual 46 kDa protein is then unprocessed precursor. The translational efficiencies of mutants Δ5'-80, Δ5'-96, and Δ5'-140 were within 1.5-fold of that of pP2-5' and were not cap stimulated (lanes 4 to 9). The translation of Δ5'-320 was reduced five fold (lanes 10 and 11) compared with that of pP2-5' (lanes 2 and 3), whereas expression of capped unmethylated and methylated mRNA from mutants Δ5'-632 and Δ5'-733 was undetectable (lanes 12 to 15). These results argue that sequences downstream of nucleotide 140 are important for cap-independent translation. The removal of 102 nucleotides just upstream of the major initiator AUG in Δ3'-631 did not affect cap-independent expression (compare lanes 16 and 17 with lanes 2 and 3), whereas removal of 354 nucleotides (Δ3'-381) resulted in an mRNA species incapable of translating in a poliovirus-infected extract (lanes 18 and 19). Taken together, the results shown in Fig. 2 and 3 position the major body of the element responsible for cap-independent translation between nucleotides 320 and 631, with contributions from sequences between 140 and 320. The fact that Δ5'-320 translates at a

FIGURE 3

Translation of pP2-5' Deletion mRNAs in Poliovirus-infected HeLa Extracts

Translations were performed as described in the legend to Fig. 2, and the products were resolved by polyacrylamide gel electrophoresis and visualized by fluorography. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.


-RNA
pP2-5′
Δ5′-80
Δ5′-96
Δ5′-140
Δ5′-320
Δ5′-632
Δ5′-733
Δ3′-631
Δ3′-381
kDal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
46–
30–
20–
14–
GpppG...: + + + + + + + + + +
m7GpppG...: + + + + + + + + + +

lower efficiency than pP2-5' in poliovirus-infected extracts denotes a graded response to 5'-end deletion mutagenesis.

Translation of pP2/CAT Deletion mRNAs in Extracts from Mock-Infected HeLa Cells

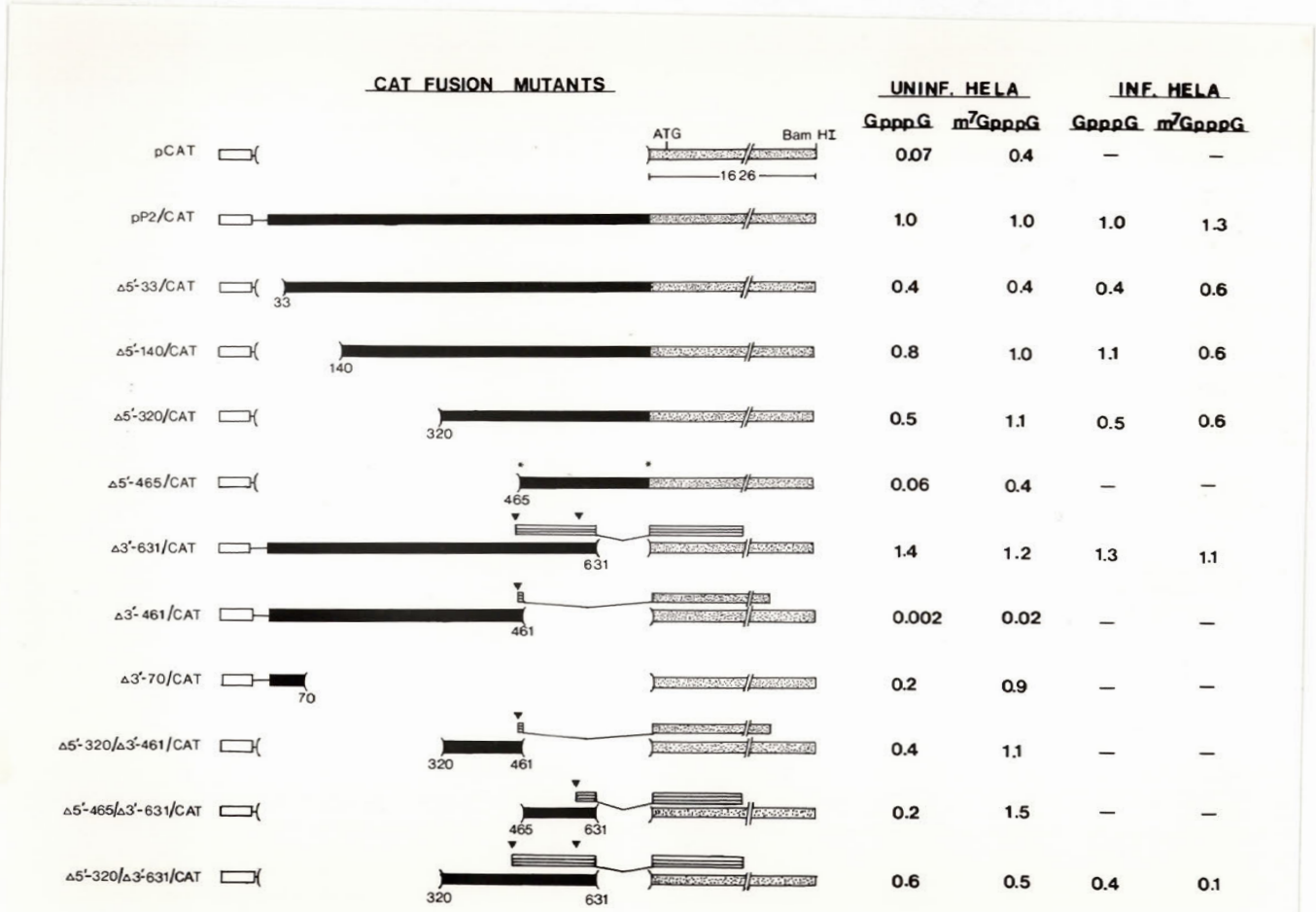
It is possible that the effects observed with the poliovirus mRNA 5' noncoding sequences on translation are due to interactions between the 5' noncoding and the downstream coding region. In addition, since none of our deletion constructs contained termination codons, the possibility (although very unlikely) exists that the rate of termination was somehow influencing (directly or indirectly) the expression from the various mutants. To experimentally address these potential pitfalls and determine whether the poliovirus mRNA 5' noncoding region could function independently of the coding region, we fused the 5' noncoding region of the deletion mutants to the bacterial CAT coding region. To better delineate the region responsible for cap-independent expression, additional constructs were produced and are shown in Fig. 4.

Translation of methylated and unmethylated SP6-derived CAT mRNAs was performed by using extracts from mock-infected HeLa cells in the presence and absence of m^7GDP to assess mRNA cap dependency. This was important to demonstrate, since CAT is a bacterial mRNA and its expression in an eukaryotic translation system may not *a priori* be cap dependent. Translation of unmethylated capped CAT mRNA yielded the expected size polypeptide of 25 kDa (Fig. 5, lane 2). To demonstrate that the 25 kDa polypeptide corresponds to CAT, we immunoprecipitated the protein from the translation extract using a monoclonal antibody directed against CAT (data not shown). The second polypeptide at ~16

FIGURE 4

Structure of Fusion Constructs of pP2-5' and the CAT Gene

The dotted boxes denotes the CAT gene and the black boxes refer to sequences derived from pP2-5'. Alterations in ORFs caused by deletion mutagenesis are drawn above the appropriate construct. The asterisks above Δ5'-465/CAT denote the position of synthetic Hind III linkers. The relative translational efficiencies obtained with the pP2/CAT fusion constructs are indicated to the right. The value obtained for capped unmethylated pP2/CAT was set as 1.0. -, Not detected.



	UNINF. HELA		INF. HELA	
	GpppG	m^7GpppG	GpppG	m^7GpppG
pCAT	0.07	0.4	—	—
pP2/CAT	1.0	1.0	1.0	1.3
Δ5'-33/CAT	0.4	0.4	0.4	0.6
Δ5'-140/CAT	0.8	1.0	1.1	0.6
Δ5'-320/CAT	0.5	1.1	0.5	0.6
Δ5'-465/CAT	0.06	0.4	—	—
Δ3'-631/CAT	1.4	1.2	1.3	1.1
Δ3'-461/CAT	0.002	0.02	—	—
Δ3'-70/CAT	0.2	0.9	—	—
Δ5'-320/Δ3'-461/CAT	0.4	1.1	—	—
Δ5'-465/Δ3'-631/CAT	0.2	1.5	—	—
Δ5'-320/Δ3'-631/CAT	0.6	0.5	0.4	0.1

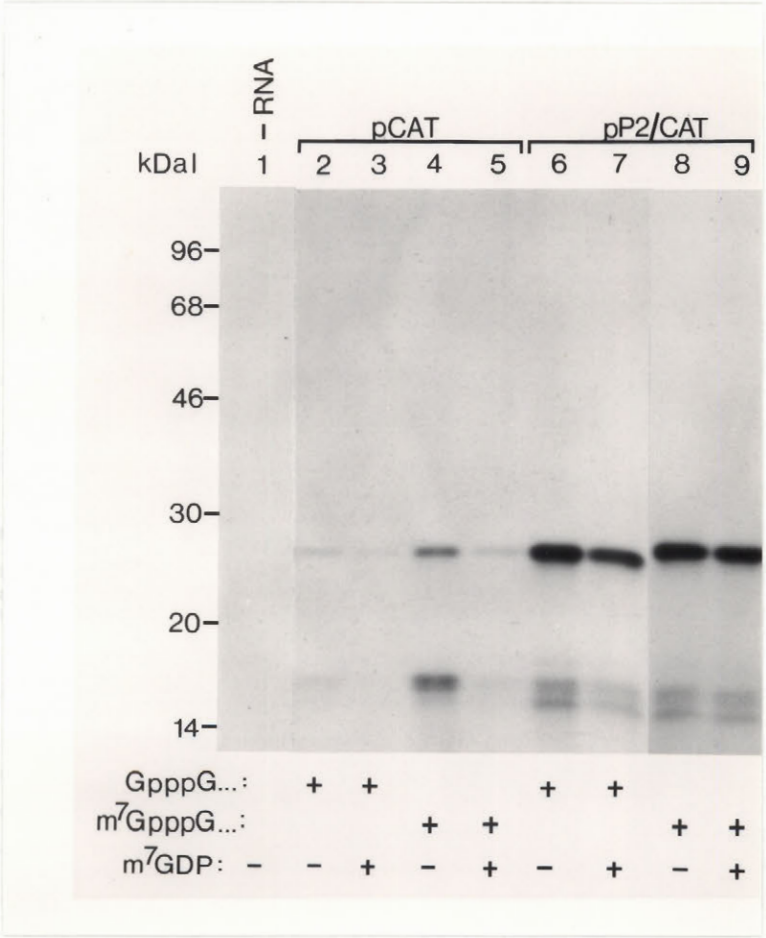
kDa most probably arises from initiation at a downstream in-frame AUG codon that is expected to yield a polypeptide of this size (12). Translation of unmethylated CAT mRNA was not affected by the addition of m^7GDP (compare lane 3 with lane 2), whereas translation of methylated CAT mRNA was five fold better than that of its unmethylated counterpart (compare lane 4 with lane 2). Addition of m^7GDP to methylated CAT mRNA reduced its translation to the level observed with the unmethylated CAT mRNA (compare lane 5 with lane 2). These results demonstrate that translation of CAT mRNA in mock-infected HeLa cell extracts is cap stimulated. When the poliovirus mRNA 5' untranslated region was fused to the CAT mRNA, translation of pP2/CAT transcripts was efficient and better than CAT mRNA, but not cap-stimulated (compare lanes 8 and 6 with lanes 4 and 2, respectively). Translation of both methylated and unmethylated pP2/CAT mRNAs was not affected by the addition of m^7GDP (compare lanes 9 and 7 with lanes 8 and 6, respectively).

Figure 6A shows the effects of the deletions in the 5' noncoding region of pP2/CAT on translational efficiency in mock-infected HeLa cell extracts. The translation of Δ5'-33/CAT (lanes 6 and 7) and Δ5'-140/CAT (lanes 8 and 9) was not stimulated by the presence of a cap structure, whereas that of CAT mRNA was (compare lane 3 with lane 2). These results indicate that nucleotides 1 to 140 are not essential for cap-independent translation of pP2/CAT. Deleting to nucleotide 320 and beyond, however, resulted in increasing cap-stimulated expression, as observed with Δ5'-320/CAT (2-fold, compare lane 11 with lane 10) and Δ5'-465/CAT (6.5-fold, compare lane 13 with 12). Fusing the CAT gene to the 5'-proximal 631 nucleotides of poliovirus mRNA (Δ3'-631/CAT)

FIGURE 5

Effect of Cap Analogue on Translation of mRNA Derived from pCAT and pP2/CAT in Mock-Infected Extract

Translations were performed with mRNA concentrations of 15 µg/ml. m^7GDP (0.1 mM) was added where indicated. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.


- RNA
pCAT
pP2/CAT
kDal
1
2
3
4
5
6
7
8
9
96
68
46
30
20
14
GpppG...:
m7GpppG...:
m7GDP:

generated an mRNA which translated in a cap-independent fashion (lanes 14 and 15). When the 5' proximal 461 nucleotides of the poliovirus genome were fused to the CAT gene (lane 16), there was a significant inhibition of CAT mRNA translation (0.2% as compared to pP2/CAT; lane 4) and translation was cap stimulated (10-fold, compare lane 17 with lane 16). The cap dependency of Δ3'-461/CAT was further confirmed by m^7GDP inhibition studies (data not shown). The reasons for the low translational level obtained with this deletion construct are addressed in the Discussion. When the deletion mutant Δ3'-70/CAT was tested, translation was efficient and cap stimulated (lanes 18 and 19). Taken together, these results place the major cap-independent region between nucleotides 320 and 631, with some effect of nucleotides 140 to 320. To substantiate this conclusion, we created three subclones containing fragments spanning the majority of this region (see Fig. 4 for constructs). Translation of Δ5'-320/Δ3'-461/CAT (Fig. 6A, lanes 20 and 21) or Δ5'-465/Δ3'-631/CAT (Fig. 6B, lanes 3 and 4) was cap-stimulated, although the degree of dependency was different between the two constructs. Only Δ5'-320/Δ3'-631/CAT (Fig. 6B, lanes 5 and 6) translated in a cap-independent fashion. Therefore, nucleotides 320 to 631 can function in this assay, albeit at a lower efficiency, consistent with this region containing the major determinant(s) for cap-independent translation.

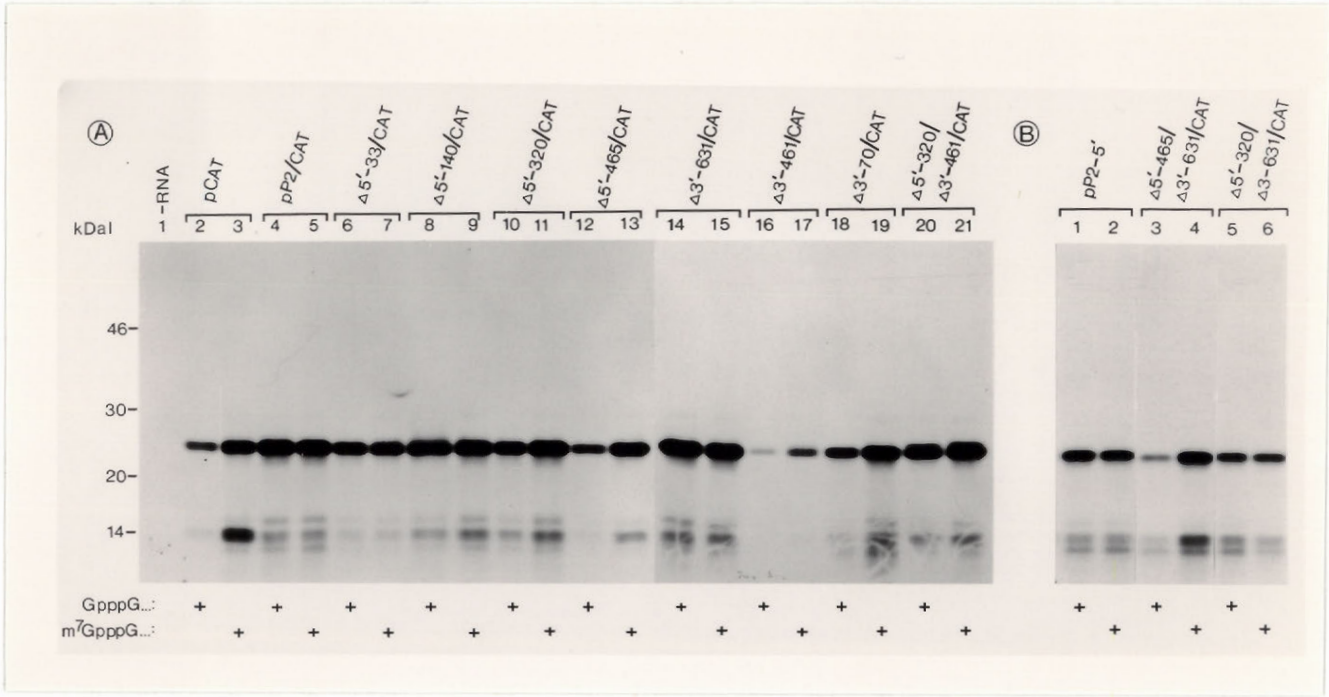
Translation of pP2/CAT Deletion mRNAs in Extracts from Poliovirus-Infected HeLa Cells

The conclusions drawn from the studies of translation of pP2/CAT mRNAs in extracts from mock-infected cells were further supported by

FIGURE 6

Translation of pP2/CAT Fusion mRNAs in Extracts from Mock-infected HeLa Cells

Translations were performed with mRNA concentrations of 30 µg/ml and processed as described in Materials and Methods. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNAs are indicated.

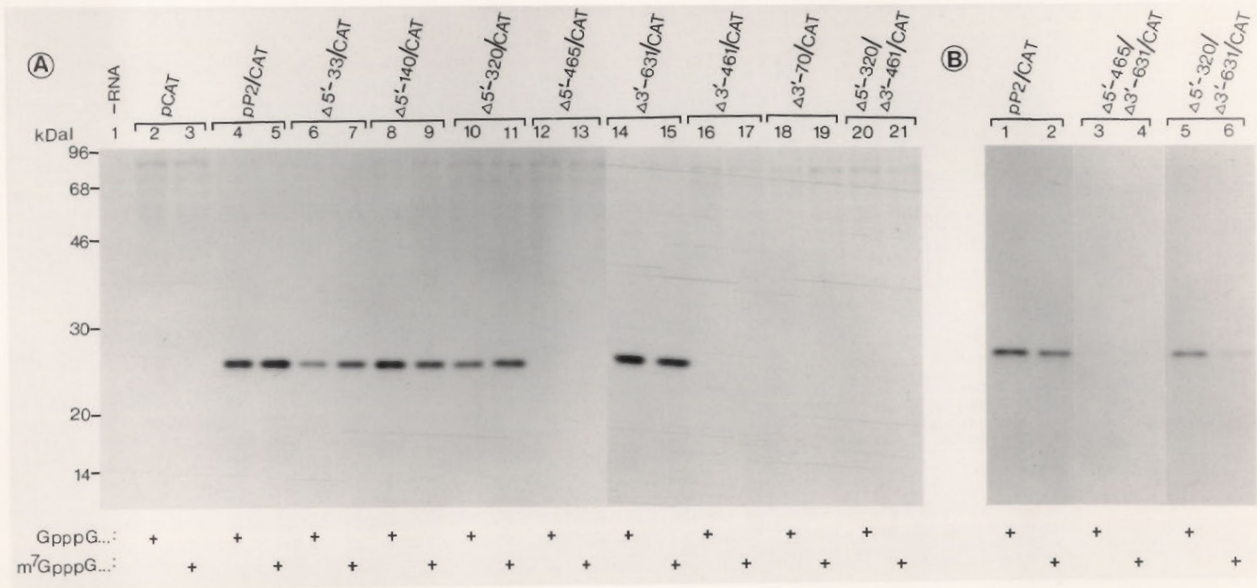

Ⓐ
-RNA
pCAT
pP2/CAT
Δ5'-33/CAT
Δ5'-140/CAT
Δ5'-320/CAT
Δ5'-465/CAT
Δ3'-631/CAT
Δ3'-461/CAT
Δ3'-70/CAT
Δ5'-320/ Δ3'-461/CAT
kDal
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
46–
30–
20–
14–
GpppG...: + + + + + + + + + + + +
m7GpppG...: + + + + + + + + + + + +
Ⓑ
pP2-5'
Δ5'-465/ Δ3'-631/CAT
Δ5'-320/ Δ3-631/CAT
1 2 3 4 5 6
+ + + + + +
+ + + + + +

results obtained from translation in extracts from poliovirus-infected cells (Fig. 7). Translation of unmethylated or methylated CAT mRNA in extracts from poliovirus-infected cells was undetectable, as anticipated (Fig. 7A, lanes 2 and 3). Fusion of the poliovirus 5' noncoding sequences to CAT mRNA resulted in synthesis of the CAT product (lanes 4 and 5). Deletion mutant Δ5'-33/CAT mRNA translation was reduced twofold as compared with that of pP2/CAT mRNA (lanes 6 and 7) and Δ5'-140/CAT (lanes 8 and 9) translated with similar efficiency to pP2/CAT. The expression of Δ5'-320/CAT (lanes 10 and 11) was reduced twofold compared with that of pP2/CAT. Removal of nucleotides beyond 320, however, yielded an mRNA species (Δ5'-465/CAT) not capable of translating in a poliovirus-infected HeLa cell extract (lanes 12 and 13). The 5'-proximal 631 nucleotides of poliovirus conferred the ability to express CAT in a poliovirus-infected HeLa cell extract as assessed by translating Δ3'-631/CAT (lanes 14 and 15), but further 3' deletions, Δ3'-461/CAT (lanes 16 and 17) and Δ3'-70/CAT (lanes 18 and 19), abolished translation. The fusion mutants containing internal 5' poliovirus sequences, Δ5'-320/Δ3'-461/CAT (lanes 20 and 21) and Δ5'-465/Δ3'-631/CAT (Fig. 7B, lanes 3 and 4), also did not translate in the poliovirus-infected extract. Only Δ5'-320/Δ3'-631/CAT, containing nucleotides 320 to 631 from the poliovirus genome, translated in a cap-independent fashion (Fig. 7B, lane 5 and lane 6). (The reason for the lower translational efficiencies of methylated capped Δ5'-320/Δ3'-631/CAT [Fig. 7B, lane 6] compared with its unmethylated counterpart [lane 5] is not clear). These results are consistent with

FIGURE 7

Translation of pP2/CAT Deletion mRNAs in Extracts from Poliovirus-infected HeLa Cells

Translations were performed with mRNA concentrations of 30 µg/ml and processed as described in Materials and Methods. Lanes containing unmethylated (GpppG...) and methylated (m^7GpppG...) capped mRNA are indicated.


Ⓐ
Ⓑ
-RNA
pCAT
pP2/CAT
Δ5'-33/CAT
Δ5'-140/CAT
Δ5'-320/CAT
Δ5'-465/CAT
Δ3'-631/CAT
Δ3'-461/CAT
Δ3'-70/CAT
Δ5'-320/ Δ3'-461/CAT
pP2/CAT
Δ5'-465/ Δ3'-631/CAT
Δ5'-320/ Δ3'-631/CAT
kDal
96
68
46
30
20
14
GpppG...:
m7GpppG...:

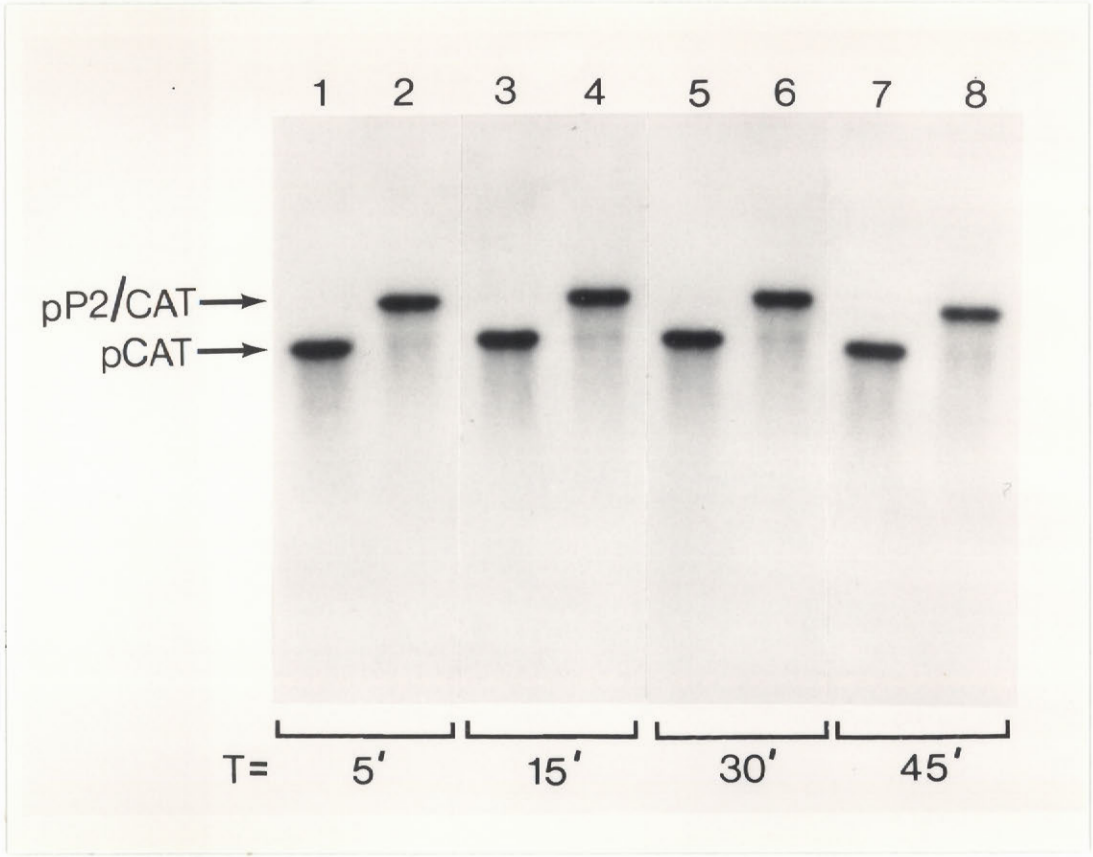
those obtained with the pP2-5' mutants (Figs. 2 and 3), with some differences involving nucleotides 140 to 320; the possible reasons for this are addressed in the Discussion.

We confirmed in all of the experiments described above that the stability of the GpppG...-terminated mRNAs was identical to their m^7GpppG...-terminated counterparts. The stabilities of CAT and pP2/CAT mRNAs at different periods of translation in a poliovirus-infected extract were assayed (Fig. 8). When analyzed on a 1.0% formaldehyde agarose gel, the two transcripts were found to have similar stabilities (Fig. 8). Thus, the observed translational results are not due to differential mRNA turnover rates.

FIGURE 8

Stability of pCAT and pP2/CAT mRNAs in Poliovirus-infected HeLa Cell Extracts

[^{32}P]-labeled mRNA (30ng; specific activity = 1.5 X 10^7 cpm/µg) was incubated in a translation system prepared from a poliovirus-infected HeLa extract as described in Materials and Methods. At the various times indicated, a portion was removed, diluted 10-fold in proteinase K buffer (0.1M Tris-hydrochloride [pH 7.5], 12mM EDTA, 0.15M NaCl, 1% sodium dodecyl sulfate) and incubated with Proteinase K (400µg/ml) at 30°C for 30 min. Samples were phenol extracted, ethanol precipitated, and analyzed on a 1% formaldehyde-agarose gel. The gel was treated with EN^{3}HANCE, dried, and exposed against XAR-5 film (Eastman Kodak Co.) The mRNA species and times of incubation are indicated.


1 2 3 4 5 6 7 8
pP2/CAT
pCAT
T= 5′ 15′ 30′ 45′

DISCUSSION

The results in this paper demonstrate the existence of a *cis*-acting element(s) within the poliovirus mRNA 5' noncoding region necessary for its translation under conditions that prevent cap-dependent translation. This element also confers cap-independent translation to a heterologous RNA (CAT) when fused to that RNA (Figs. 5 to 7). The effects of the mRNA 5' noncoding deletions on translation could not be explained by differential mRNA stability, because all of the deleted mRNAs studied exhibited similar stabilities in the different translation systems (Fig. 8; unpublished results). These findings are not peculiar for the CAT mRNA, since similar results were obtained with the herpes simplex virus thymidine kinase gene (unpublished results). We have mapped the major body of the element that confers cap-independent translation to poliovirus mRNA between nucleotides 320 and 631. This conclusion is based on translation data obtained with deletion mutants of the 5' noncoding region, and particularly Δ5'-320/Δ3'-631/CAT mRNA that translates in a cap-independent fashion in extracts from mock- and poliovirus-infected cells (Fig. 6B and 7B). The efficiency of the latter mRNA is reduced only two fold compared to constructs containing the full-length poliovirus 5' noncoding region. There may also be contributions to the cap-independent translation from nucleotides 96 to 320. For example, deletion of nucleotides 1 to 140 (Δ5'-140) produced an mRNA that is slightly (2.4-fold) cap stimulated in uninfected extracts (Fig. 2) and translated slightly less efficiently in infected extracts (Fig. 3). However, when the same deletion was introduced into pP2/CAT, there was little if any cap stimulation and no loss of

translation efficiency in infected extracts (Fig. 7). Deletion of nucleotides 1 to 320 in pP2-5' (Δ5'-320) produced an mRNA with fivefold-reduced expression in a poliovirus-infected extract (Fig. 3, lanes 10 and 11). However, when the same deletion was introduced into pP2/CAT, only a two fold reduction in translation efficiency in infected extracts was observed (Fig. 3). The reasons for these differences in translation are not clear, although it is possible that translation is influenced, to some degree, by the coding region of the pP2-5' mRNA. In any case, it is apparent that the element(s) specifying cap-independent translation in mock-infected extracts allows translation of these mRNAs in poliovirus-infected HeLa extracts.

What might be the mechanism which enables poliovirus mRNA to initiate translation via a cap-independent fashion? Our data show that a large area is encompassed by the cap-independent region (nucleotides 320-631) and that a graded translational response (as opposed to an all-or-none effect) is obtained in poliovirus-infected extracts upon deletion of nucleotides 140 to 320 (Fig. 3, compare lanes 8 and 9 with lanes 10 and 11). One possibility is that an internal guide sequence in the 5' noncoding region of poliovirus mRNA acts to direct ribosomes to the mRNA, bypassing the requirement for a cap structure. Another possibility is that a region lacking secondary structure is required for internal binding. mRNAs with little potential to form stable secondary structure at their 5' ends were shown to function in extracts from poliovirus-infected HeLa cells (37). For example, the naturally capped viral alfalfa mosaic virus type 4 mRNA, which contains little secondary structure at its 5' end (11), is able to translate in poliovirus-

infected HeLa cells (37). Thus, it is possible that poliovirus mRNA contains a single-stranded region enabling 40S ribosomes to bind internally. We and others have presented data to support a model by which a cap-binding protein complex (eIF-4F) binds to the 5' cap structure of mRNA, followed by the binding of eIF-4A and eIF-4B, with subsequent unwinding of mRNA secondary structure to facilitate ribosome binding (36). It is conceivable that eIF-4A and eIF-4B bind directly to the cis-acting element(s) within the 5' noncoding sequence of poliovirus mRNA, followed by internal binding of ribosomes. This is an attractive possibility in light of results showing that eIF-4A is able to bind single-stranded RNA in an ATP-dependent fashion (1).

It is of interest that the CAT fusion mutant containing poliovirus nucleotides 1 to 461 (Δ3'-461/CAT) translates very poorly compared with mRNA containing the full length 5' noncoding region in HeLa mock-infected extracts (Fig. 5 and 6). This effect is not seen with Δ3'-70/CAT, implying the existence of a translational restrictive element between nucleotides 70 to 461 which is normally bypassed on the P2/CAT mRNA. This element may play a role in preventing 5'-end-mediated initiation that could interfere with internal initiation between nucleotides 320 and 631.

Experiments by Dorner et al. (5) have suggested that ribosomes can bind internally on poliovirus mRNA. These authors showed that in reticulocyte lysate a significant proportion of translation initiation originates in the P3 region located in the 3' one third of the RNA. Evidence from electron microscopy studies showing ribosome binding to the mRNA P3 region supports this conclusion (25). Recent experiments by

Shih et al. (35) using cDNA hybridization to arrest translation of encephalomyocarditis virus mRNA demonstrated that the region near nucleotide 450 but not the 5'-proximal 338 nucleotides is required for translation. This raises the possibility that internal initiation is also possible on encephalomyocarditis virus mRNA, although direct proof is still lacking.

A single base change (C to U at base 472) in the 5' noncoding region of the poliovirus genome was found to be a major determinant of the attenuation of the Sabin type 3 polio vaccine (9,40). A single base change is thought to contribute to the attenuation of the Sabin type 1 strain as well (26). The molecular mechanism by which this mutation alters poliovirus neurovirulence is not understood. However, it has been found that mRNAs from attenuated strains of poliovirus types 1 and 3 exhibit diminished translational activity in extracts from Krebs-2 ascites cells compared to mRNAs from their virulent counterparts (38). Consequently, it was suggested that nucleotide sequences encompassing position 472 might interact with translation factors and that this interaction might be affected by mutations (38). It is also of interest that the C-to-U mutation at position 472 has a significant effect on the predicted secondary structure of the viral RNA (9). In light of these results and our data, nucleotide 472 (and flanking nucleotides) may play an important role in the translation of poliovirus RNA.

Recently, Kuge and Nomoto (19) created deletion and insertion mutants of the Sabin strain of type 1 poliovirus. They found that viruses lacking the genome region at positions 600 to 726 appeared fully

viable, whereas 3' deletions extending to nucleotide 580 failed to produce cytopathic effects or detectable plaques when assayed in cell culture. These observations are consistent with our data that nucleotides 631 to 733 do not play an essential role in translation of poliovirus mRNA. We are currently performing experiments *in vivo* to demonstrate the relevance of our *in vitro* studies to the *in vivo* translation of poliovirus. In this regard, isolation of viable mutants which have lost the ability to translate in a cap-independent manner would be helpful.

In summary, our results identify a *cis*-acting element within the 5' noncoding region of poliovirus RNA. The major element maps between nucleotides 320 and 631 and enables poliovirus mRNA to translate in a cap-independent fashion. The identification of *trans*-acting factors which mediate this effect will be of importance in elucidating the mechanism by which poliovirus mRNA is translated.

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CHAPTER 5

INTERNAL INITIATION OF TRANSLATION OF EUKARYOTIC mRNA DIRECTED BY A SEQUENCE DERIVED FROM POLIOVIRUS mRNA

SUMMARY

Poliovirus RNA is naturally uncapped, therefore its translation must proceed via a cap-independent mechanism. Translation initiation on poliovirus RNA occurs by binding of ribosomes to an internal sequence within the 5' noncoding region. This novel mechanism of initiation may explain the disparate translation of several other eukaryotic mRNAs.

INTRODUCTION AND RESULTS

The mechanism of initiation of translation on poliovirus RNA is enigmatic. Poliovirus RNA, unlike most eukaryotic mRNAs is not blocked at its 5' end by the cap structure, m^7GpppX (where X is any nucleotide). Instead, a small polypeptide termed VPg is covalently linked to the 5' end of genomic RNA (8,25) and is removed in the cytoplasm (2). The 5' end of polysomal RNA terminates in pUp (12,31). Poliovirus RNA possesses an unusually long 5' untranslated region (UTR) (~ 750 nucleotides) which is highly conserved among the three poliovirus serotypes (18,38,47). This region contains 7-8 AUGs, of which only 3 are conserved in position, although the open reading frames (ORFs) specified by them are not conserved in length or amino acid content (47). The translation products originating from these ORFs have not been observed in vivo or in vitro and are not necessary for viral biogenesis(J.P., M.E. Flynn, G. Kaplan, V.R. Racaniello and N.S., submitted).

A scanning model has been proposed to explain the mechanism by which eukaryotic mRNAs initiate translation (21). According to this model, ribosomes and associated factors bind at or near the 5' end of the mRNA in a process that is facilitated by the presence of a cap structure, and scan the mRNA until the appropriate initiator codon is reached (21). Two modifications were introduced to the model to explain translation on polycistronic eukaryotic mRNAs. It was postulated that ribosomes can terminate translation at an upstream ORF, then resume scanning and initiate translation at the downstream ORF (14,22,26) and that ribosomes could skip an upstream AUG and initiate at a downstream

AUG (the latter process was termed leaky scanning, ref. 22). In either case, the scanning model excludes the possibility of independent internal ribosome binding on eukaryotic mRNAs (20).

The translation of poliovirus RNA is not satisfactorily accommodated by the scanning model. Because poliovirus RNA is naturally uncapped, it must translate by a cap-independent mechanism. We used deletion mutagenesis to show that an internal sequence in the 5' UTR of poliovirus RNA is required for cap-independent translation and this sequence can also confer cap-independent translation to heterologous mRNAs (36). This raises the interesting possibility that ribosomes can bind internally to this region of the poliovirus 5' UTR. But, in these experiments the possibility that ribosomes bind to the 5' end of poliovirus mRNA and then migrate towards the region important for cap-independent translation was not rigorously excluded.

We designed experiments to test the hypothesis that an internal sequence of the 5' UTR of poliovirus mRNA can mediate internal initiation. An assay was devised whereby the poliovirus 5' UTR could direct translation initiation independently of 5'-end-mediated initiation. The poliovirus 5' UTR was inserted as the intercistronic spacer in an artificially created bicistronic mRNA (Fig. 1A). The bicistronic plasmid contains as the first cistron the herpes simplex virus-1 thymidine kinase (TK) gene and as the second cistron, the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1A). A control plasmid contained the 5' UTR of CAT as the intercistronic spacer. The rationale for the experiment is that if the poliovirus 5' UTR mediates internal initiation, then under conditions which abolish

initiation at the first cistron (for example, in poliovirus-infected cells), translation of the second cistron (CAT) in TK/P2CAT should be unaffected (Fig. 1A).

Translation In Poliovirus-Infected Cells

To determine whether internal initiation could occur in vivo, the bicistronic constructs, TK/CAT and TK/P2CAT, were subcloned into the pSV2 expression vector. The pSV2 constructs were transfected into COS-1 cells and TK and CAT protein levels were determined by pulse-labelling with ^{35}S-methionine, followed by immunoprecipitation analysis. Parallel dishes of transfected COS-1 cells were infected with poliovirus and labelled with ^{35}S-methionine 4 hours post-infection; at this time cellular mRNA translation is abrogated because of inactivation of eIF-4F (a translation initiation factor required for cap dependent mRNA translation) (7). Antisera directed against TK immunoprecipitated a polypeptide of ~46 kDa which corresponds to HSV-1 TK in extracts from cells which had been transfected with pSV2/TK/CAT and pSV2/TK/P2CAT (Fig. 1B, lanes 2 and 3 respectively), but not from extracts of mock-transfected COS-1 cells (lane 1). The TK product was not detected in pSV/TK/CAT and pSV/TK/P2CAT transfected COS-1 cells which were subsequently infected with poliovirus (Fig. 1B, lanes 4 and 5, respectively). Anti-CAT antibodies immunoprecipitated a polypeptide of ~25 kDa from extracts of cells transfected with pSV/TK/CAT (lane 7) and pSV/TK/P2CAT (lane 8) but not from extracts of mock-transfected cells (lane 6). Following poliovirus infection, CAT synthesis was detected in pSV2/TK/P2CAT (lane 10), but not in pSV2/TK/CAT (lane 9) transfected COS-1 cells. Because poliovirus infection blocks 5'-end-mediated

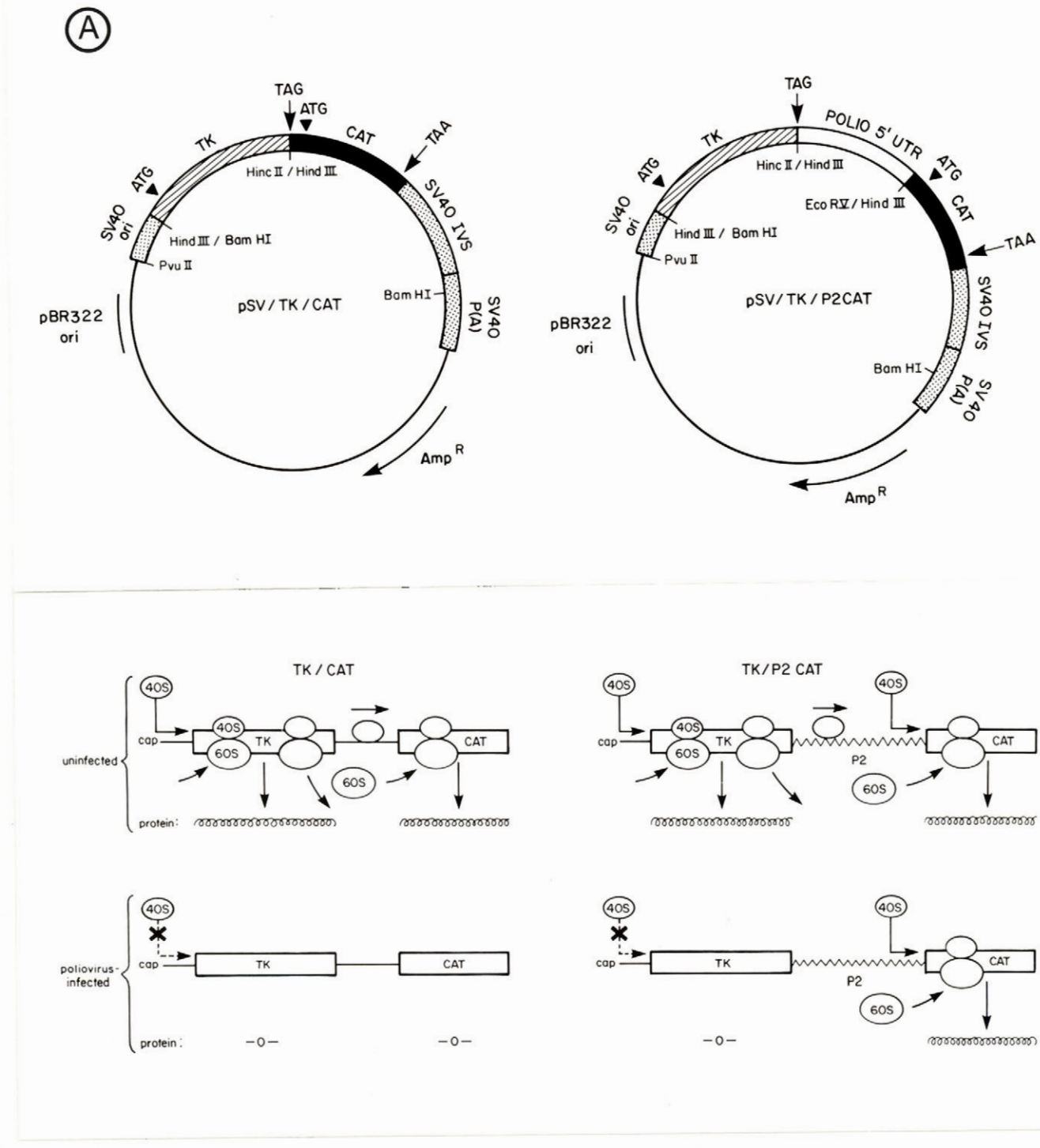
FIGURE 1

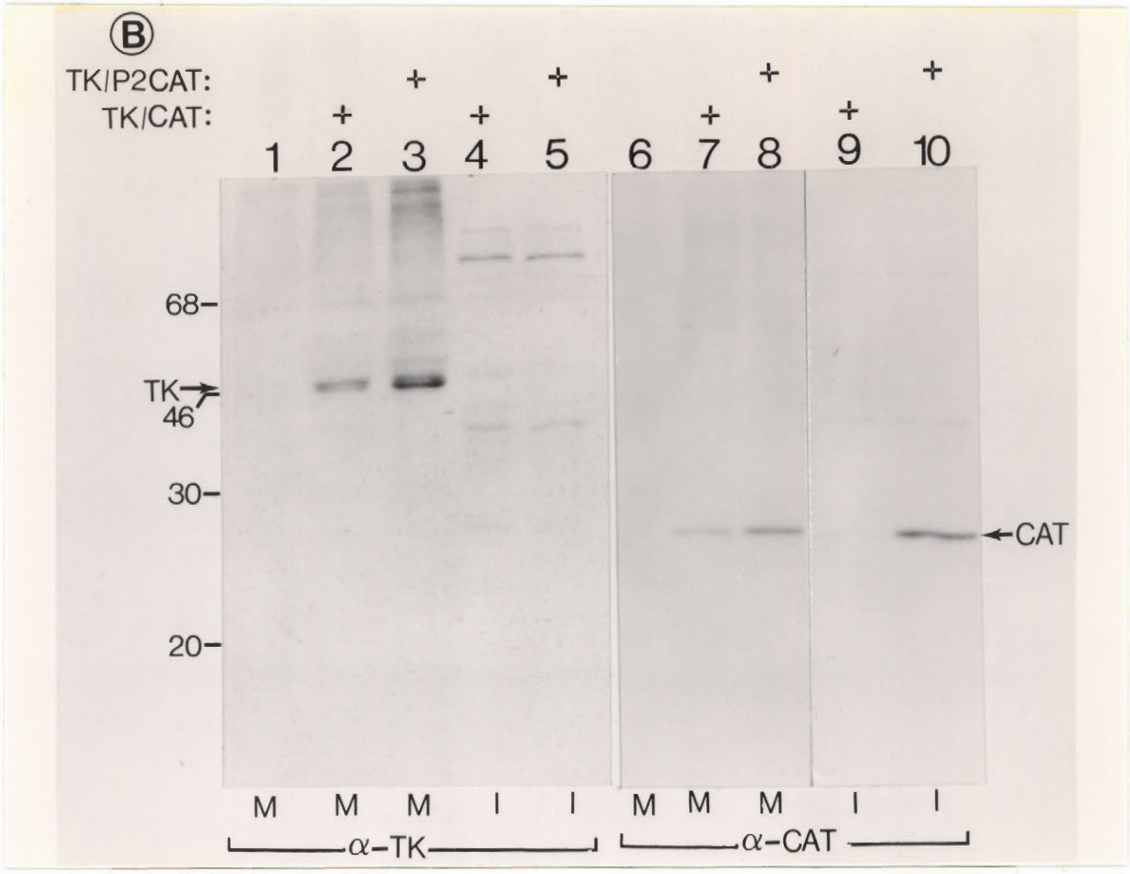
(A) Schematic Diagram of Bicistronic Genes, Showing the Eukaryotic expression Vector used for DNA Transfections and Experimental Rationale.

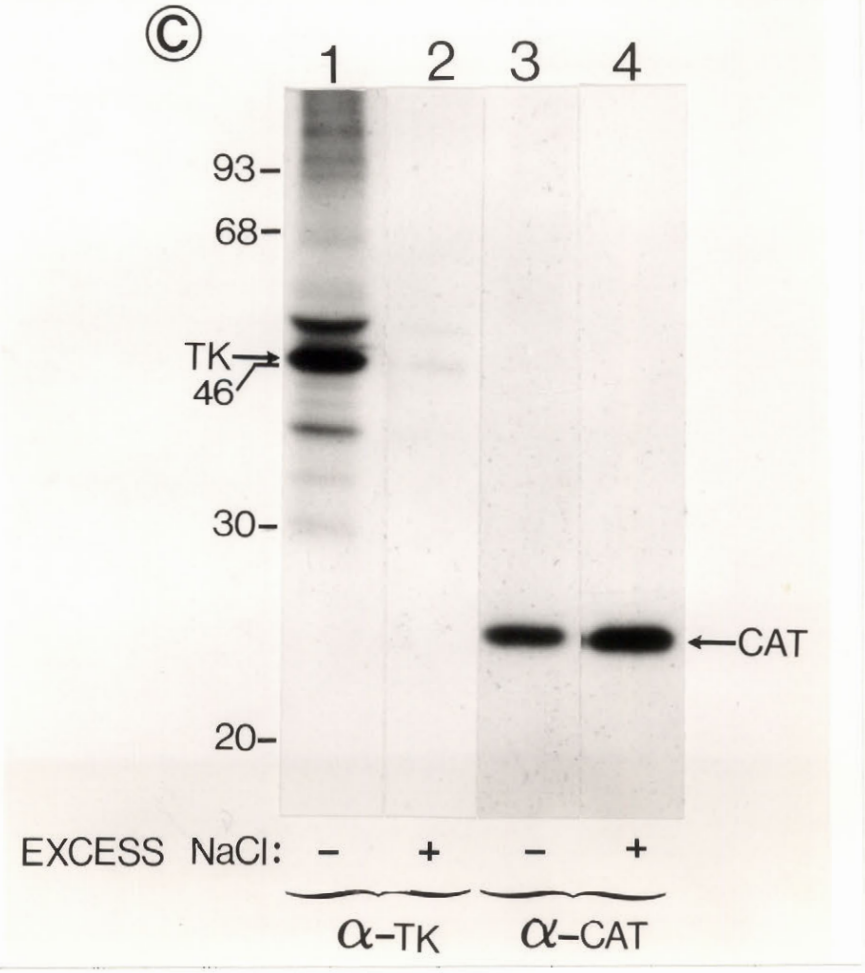
(B) Immunoprecipitation of ^{35}S-Labelled Cell Extracts Prepared from Mock (M) or Poliovirus-Infected (I) COS-1 Cells. Cells were transfected with no DNA (lanes 1 and 6), pSV/TK/CAT (lanes 2,4,7,9), or pSV/TK/P2CAT (lanes 3,5,8,10). After 48 hours, cells were infected with poliovirus type 1 (Mahoney strain, 50 pfu per cell) according to Rose et al. (41). Four hours post-infection, cells were labelled with ^{35}S-methionine (100 µCi) in 1 ml α-minimal essential media (-Met) +10% dialyzed fetal calf serum at 37°C for 1 hr. Lanes immunoprecipitated with anti-TK or anti-CAT antibodies are indicated at the bottom of the figure.

(C) Immunoprecipitation of ^{35}S-Labelled Cell Extracts from Mock-or Hypertonic Treated COS-1 Cells. Cells were transfected with pSV/TK/P2CAT. After 48 hours, the media was replaced with fresh media containing excess 190 mM NaCl and incubation was for 15 min. followed by ^{35}S-methionine labelling as described for 1B.

Derivatives of pSV2 containing TK/CAT or TK/P2CAT were constructed from JP15/CAT and JP15/P2CAT (see Fig. 2A and ref. 37) which contain 3 Bam HI linkers within the TK 5' UTR. TK/CAT and TK/P2CAT were excised with Bam HI and the fragments were inserted into the Hind III/Bam HI sites of pSV2/CAT. COS-1 cells (seeded one day before transfection at 7.0×10^5 cells/100 mm petri) were transfected with DNA (10 µg) using the DEAE-dextran method (10). Cell extracts were prepared (10), divided into two portions and immunoprecipitated with anti-CAT or anti-TK antibodies. Immunoprecipitates were analyzed on 12% SDS/polyacrylamide gels that were treated with EN^{3}HANCE, dried, and autoradiographed. Following immunoprecipitations, all cell extracts were put through a second round of immunoprecipitation to ensure there was no TK or CAT protein remaining in the supernatant.


A
TAG
ATG
CAT
TAA
TK
ATG
SV40 ori
Hinc II / Hind III
Hind III / Bam HI
Pvu II
SV40 IVS
Bam HI
SV40 P(A)
pSV / TK / CAT
pBR322 ori
Amp R
TAG
POLIO 5' UTR
TK
ATG
CAT
Hinc II / Hind III
Eco RV / Hind III
SV40 ori
Hind III / Bam HI
Pvu II
TAA
SV40 IVS
pSV/ TK / P2CAT
pBR322 ori
Bam HI
SV40 P(A)
Amp R
TK / CAT
40S
cap
60S
TK
CAT
uninfected
protein:
TK/P2 CAT
P2
poliovirus-infected
—O—


B
TK/P2CAT:
TK/CAT:
1 2 3 4 5 6 7 8 9 10
68–
TK
46
30–
20–
CAT
M M M I I M M M I I
α-TK
α-CAT


C
1 2 3 4
93–
68–
TK
46
30–
20–
CAT
EXCESS NaCl: – + – +
α-TK
α-CAT

initiation on the TK gene, CAT synthesis observed in poliovirus-infected COS-1 cells transfected with pSV/TK/P2CAT (lane 10) is not the result of termination/reinitiation events or leaky scanning, but is probably due to internal binding of ribosomes.

We used another strategy to substantiate the finding that translation initiation at the two cistrons occurs by two independent mechanisms. Translation of most cellular mRNAs is reduced when cells are incubated in hypertonic conditions. It has been previously shown that poliovirus translation is more resistant to hypertonic inhibition than cellular mRNAs (4,33). We reasoned that under hypertonic conditions, translation of the TK cistron should be diminished to a greater extent than the downstream CAT cistron which is preceded by the poliovirus 5' UTR. Figure 1C shows the synthesis of TK and CAT proteins directed by pSV/TK/P2CAT (as determined by immunoprecipitation) under physiological and hypertonic (excess 190 mM NaCl) conditions. Translation of TK is inhibited (~ 50 fold) when cells are grown under hypertonic conditions (compare lane 2 to 1). In contrast, CAT translation is even slightly stimulated under high salt conditions (compare lane 4 to 3). These results agree well with a model predicting independent and separate access points for ribosomes translating the TK and CAT cistrons.

An argument against internal ribosome binding could be that in poliovirus-infected cells, ribosomes do bind to the 5' end of the bicistronic mRNA, scan through all upstream AUGs, then for some unknown reason become "activated" for AUG recognition by a signal in the poliovirus 5' UTR to initiate translation at the downstream CAT ORF.

This possibility is extremely unlikely because of the overwhelming data showing that the CBP complex (cap binding protein) (eIF-4F) cannot bind to the cap structure of mRNAs following poliovirus-infection (23,24), thus excluding ribosome binding to the mRNA 5' end. Also, the results shown below (Fig. 2) argue strongly against this interpretation.

Translation In Vitro

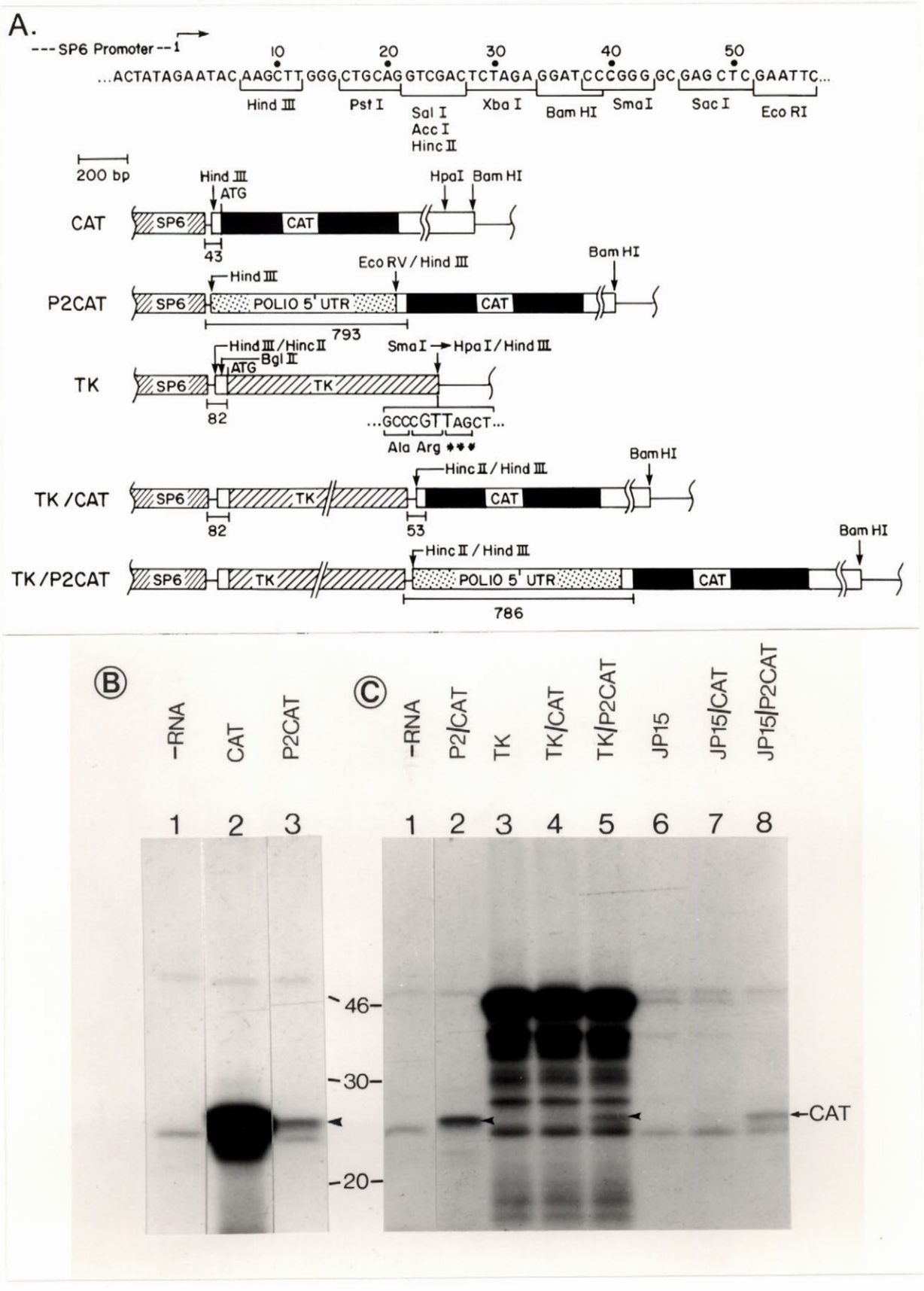
Because the in vivo experiments established internal initiation on poliovirus mRNA, we wished to reproduce these results in in vitro translation systems. If internal initiation could be observed in vitro, it would justify the use of translation extracts for the identification of putative trans-acting factors involved in internal initiation of translation and ultimately, elucidation of the molecular mechanism of this process. Translation studies were performed in a reticulocyte lysate using mRNAs synthesized in vitro in the SP6 transcription system (Fig. 2A). Translation of poliovirus mRNA is inefficient in this system (43) and we defined an inhibitory sequence for translation in reticulocyte lysates within the proximal 5' UTR (nucleotides 70-381; ref 36). When the 5' UTR is juxtaposed 5' to the CAT coding sequence, translation of CAT is reduced ~ 200 fold (Fig. 2B, compare lane 3 to 2). Translation of TK mRNA gave a major polypeptide of 46 kDa (Fig. 2C, lane 3), which corresponds to HSV-1 TK. The origin of the minor faster migrating polypeptides is not clear, but they are detectable only because the autoradiograph was greatly overexposed to detect the CAT protein. Translation of TK/CAT gave an identical pattern of proteins to that of TK mRNA, indicating that the downstream CAT cistron was not

FIGURE 2

(A) Schematic Representation of Constructs for In Vitro Translation Studies. The polylinker region of pSP64 is shown at the top of the diagram as a reference to the restriction sites used to create the various bicistronic genes described below. The right-angled arrow denotes the start and direction of transcription of SP6 polymerase. The numbers below the constructs represent distances in base pairs. The result of modifying the TK gene (pX1; see ref. 37) is diagramed below TK. The new artifically created stop codon in TK allowed us to easily manipulate intercistronic distance. The large letters represent bases derived from the HpaI linker. The blank boxes denote 5' and 3' UTRs, and the thin line represents sequences from pSP64.

(B) SDS-Polyacrylamide Gel Electrophoresis of Proteins Synthesized in Rabbit Reticulocyte Lysate Programmed with In Vitro Transcribed mRNA. Transcription of Sma I linearized plasmids using SP6 polymerase was performed as described (37). Translations were done in 12 µl reaction mixtures containing 0.2 µg mRNA at 30°C for 1 hr (34). Four microliters of mixture was withdrawn after incubation, analyzed on 12% SDS-polyacrylamide gels that were treated with EN^{3}HANCE, dried, and autoradiographed.

CAT and P2CAT have been previously described (35). TK was derived from pX1 (37) by replacing the SmaI site, which resides within the seventh codon before the termination codon, with a HpaI linker (^{5}'GTTAAC^3'). This derivative was subcloned into pSP64 by restricting with HincII (which cleaves within the TK 5' UTR) and Hpa I and inserting into the blunted Hind III site of pSP64. This generates an in-frame amber termination codon. TK/CAT and TK/P2CAT were created by first digesting TK with HincII and BamHI. The CAT and P2CAT fragments isolated from CAT and P2CAT by restricting with Hind III were blunted with Klenow, followed by Bam HI digestion and ligated to TK. JP15 derivatives containing 3 Bam HI linkers in the non-coding region of TK were similarly constructed by using pXJP15 from ref. 37.


A.
---SP6 Promoter--1
10
20
30
40
50
...ACTATAGAATAC AAGCTT GGG CTGCAG GTCGAC TCTAGA GGATCC CCGGG GC GAG CTC GAATTC...
Hind III
Pst I
Sal I
Acc I
Hinc II
Xba I
Bam HI
Sma I
Sac I
Eco RI
200 bp
CAT
SP6
CAT
Hind III
ATG
HpaI
Bam HI
43
P2CAT
SP6
POLIO 5' UTR
CAT
Hind III
Eco RV / Hind III
Bam HI
793
TK
SP6
TK
Hind III / Hinc II
Bgl II
ATG
Sma I → Hpa I / Hind III
82
...GCCCGTTAGCT...
Ala Arg ***
TK /CAT
SP6
TK
CAT
Hinc II / Hind III
Bam HI
82
53
TK /P2CAT
SP6
TK
POLIO 5' UTR
CAT
Hinc II / Hind III
Bam HI
786
B
-RNA
CAT
P2CAT
1
2
3
C
-RNA
P2/CAT
TK
TK/CAT
TK/P2CAT
JP15
JP15/CAT
JP15/P2CAT
1
2
3
4
5
6
7
8
46
30
20
CAT

used in this construct (compare lane 4 to 3). But, when TK/P2CAT was translated, CAT was synthesized (indicated by arrowhead; lane 5), most probably by internal initiation, as was shown in vivo (Fig. 1). This contention is further supported by translation of insertion derivatives of TK and TK/CAT (termed JP15 and JP15/CAT) that contain 3 tandem repeats of Bam HI linkers ($^{5'}$CCGGATCCGG$^{3'}$) having the potential to form stable secondary structure within the TK 5' UTR (37). The translation of mRNAs from these constructs is strongly inhibited in a reticulocyte lysate (37). Translation of JP15 and JP15/CAT mRNAs was very inefficient as anticipated (lanes 6 and 7) (37). Remarkably, translation of the downstream cistron of CAT in JP15/P2CAT was not affected by the secondary structure upstream of the TK cistron (compare lane 8 to 5). These in vitro results mimic those obtained in vivo (Fig. 1) and provide evidence for internal initiation in vitro.

Mapping of Ribosome Binding Site.

A series of deletion mutants within the poliovirus 5' UTR intercistronic region were constructed to map the region required for internal initiation of translation (Fig. 3A). Translations were performed in extracts from poliovirus-infected HeLa cells (Fig. 3B), which do not permit the translation of cap-dependent mRNAs (24,44). Translation of P2CAT mRNA was efficient in this extract (lane 2) and translation of the downstream CAT cistron in TK/P2CAT was as efficient (compare lane 5 to 2). As was shown in vivo under conditions of poliovirus infection, CAT was not expressed from the bicistronic construct TK/CAT (lane 4). As expected, there was no expression from the upstream TK cistron in these extracts for any of the constructs.

Deletion of 33 nucleotides from the 5' UTR had no effect (lane 6), whereas deletion of 140 nucleotides reduced CAT translation 3 fold (lane 7). A further deletion of 465 nucleotides from the 5' poliovirus UTR completely abolished internal initiation (lane 8). A deletion removing 100 nucleotides from the 3' proximal UTR [TK/(Δ3'-631)/CAT] slightly stimulated (2-fold; lane 9) translation, showing that the 3' proximal nucleotides of the poliovirus 5' UTR are not required for internal initiation. But a further deletion from the 3' end extending to nucleotide 461 completely abolished CAT translation (lane 10).

These results indicate that sequences between nucleotides 140 and 630 of the 5' UTR contain the internal ribosome-binding site. Similar results were obtained when the constructs were translated in uninfected extracts, except that the appearance of TK products was observed. Similar results were also observed when JP15 was used as the first cistron for the constructs shown in Fig. 3A and translations were performed in poliovirus-infected or uninfected HeLa extracts, except that TK production was reduced in uninfected HeLa extracts (data not shown). This finding is consistent with our previous result that an internal sequence of the poliovirus UTR confers cap-independent translation when fused 5' to a heterologous mRNA (35).

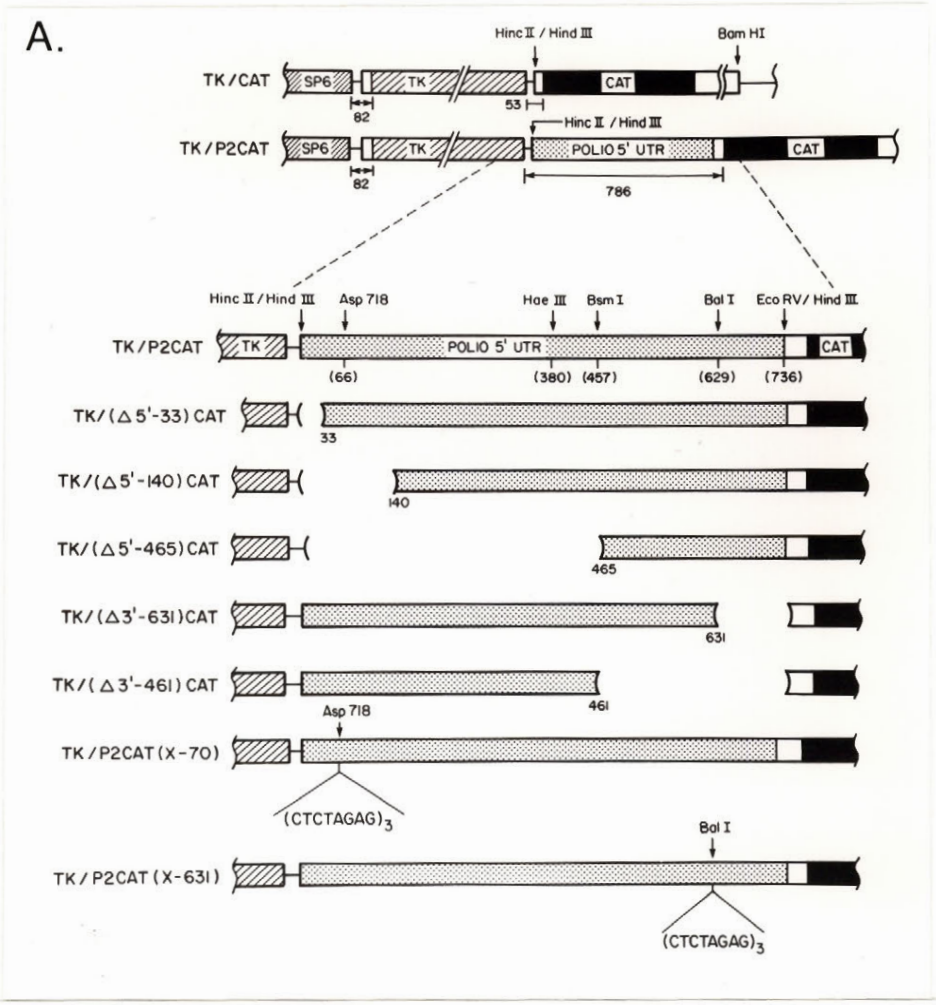
The results suggest that ribosomes bind first to an internal sequence of the poliovirus 5' UTR and are then translocated, presumably by scanning, to the initiator AUG of the CAT ORF. This interpretation is supported by the experiment shown in Fig. 3C. Three Xba I linkers ($^{5'}$CTCTAGAG$^{3'}$), having the potential to form a hairpin structure in the mRNA (ΔG°= -30 kCal/mole) and thus inhibit translation initiation (37),

FIGURE 3

(A) Structure of Poliovirus 5' UTR Deletion Mutants for Mapping the Region Responsible for Internal Initiation. The starting P2CAT mutants used to construct the bicistronic genes have been previously described (35). Deletion derivatives of P2CAT were subcloned into pSP64 as described for TK/P2CAT in the legend to Fig. 2. The cleavage sites of restriction enzymes used to produce the deletion mutants are shown on the TK/P2CAT template. The numbers represent distances, in nucleotides, from the first nucleotide of the poliovirus 5' UTR.

(B) Translation of TK/P2CAT Deletion mRNAs in Extracts from Poliovirus-Infected HeLa Cells. Translations were performed and processed as described by Pelletier et al. (37) with mRNA concentrations of 30 µg/ml.

(C) Translation of Xba I ($^{5'}$CTCTAGAG$^{3'}$) Linker Derivatives of P2CAT in Poliovirus-Infected HeLa Cell Extracts. Linker derivatives were created and characterized as described (37). Transcriptions and translations were performed and processed as described (35).


A.
TK/CAT
SP6
TK
CAT
Hinc II / Hind III
Bam HI
82
53
TK/P2CAT
POLIO 5' UTR
786
Asp 718
Hae III
Bsm I
Bal I
Eco RV/ Hind III
(66)
(380)
(457)
(629)
(736)
TK/(Δ5'-33)CAT
33
TK/(Δ5'-140)CAT
140
TK/(Δ5'-465)CAT
465
TK/(Δ3'-631)CAT
631
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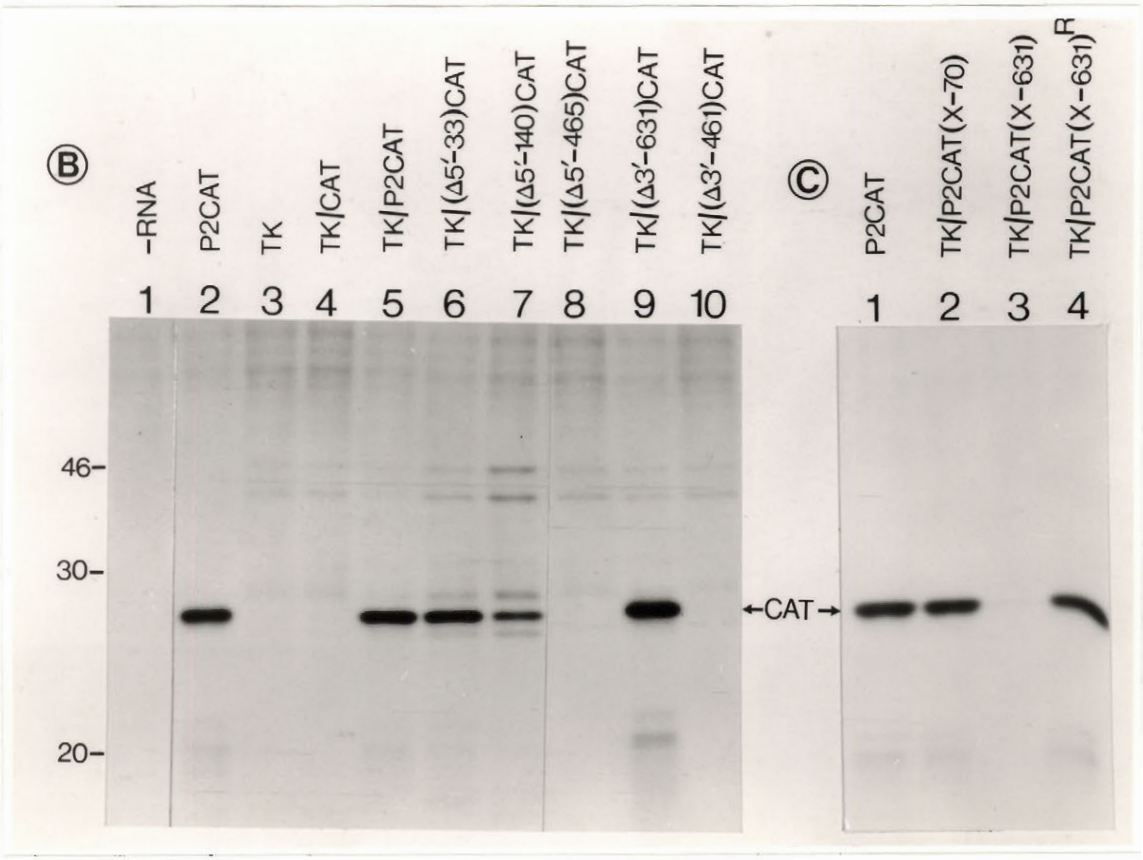
(CTCTAGAG)3
TK/P2CAT(X-631)


B
-RNA
P2CAT
TK
TK/CAT
TK/P2CAT
TK/(Δ5'-33)CAT
TK/(Δ5'-140)CAT
TK/(Δ5'-465)CAT
TK/(Δ3'-631)CAT
TK/(Δ3'-461)CAT
1 2 3 4 5 6 7 8 9 10
46-
30-
20-
C
P2CAT
TK/P2CAT(X-70)
TK/P2CAT(X-631)
TK/P2CAT(X-631)R
1 2 3 4
←CAT→

were introduced into the 5' proximal region (at position 70) or at the 3' distal region (at position 631) of the poliovirus 5' UTR. Insertion of the Xba I linkers at position 70 had no effect on translation of CAT (compare lane 2 to 1), whereas insertion at position 631 dramatically inhibited P2CAT translation (compare lane 3 to 1). The observed inhibition is not due to a second site mutation because removal of the Xba I linkers [TK/P2CAT(X-631)^{R}] restored translation to levels observed with P2CAT mRNA (compare lane 4 to 1). Thus, ribosomes must bind to the poliovirus 5' UTR upstream of position 631, but downstream of position 70, and subsequently reach the AUG initiator at position 745 to initiate translation.

Translated mRNA Is Intact

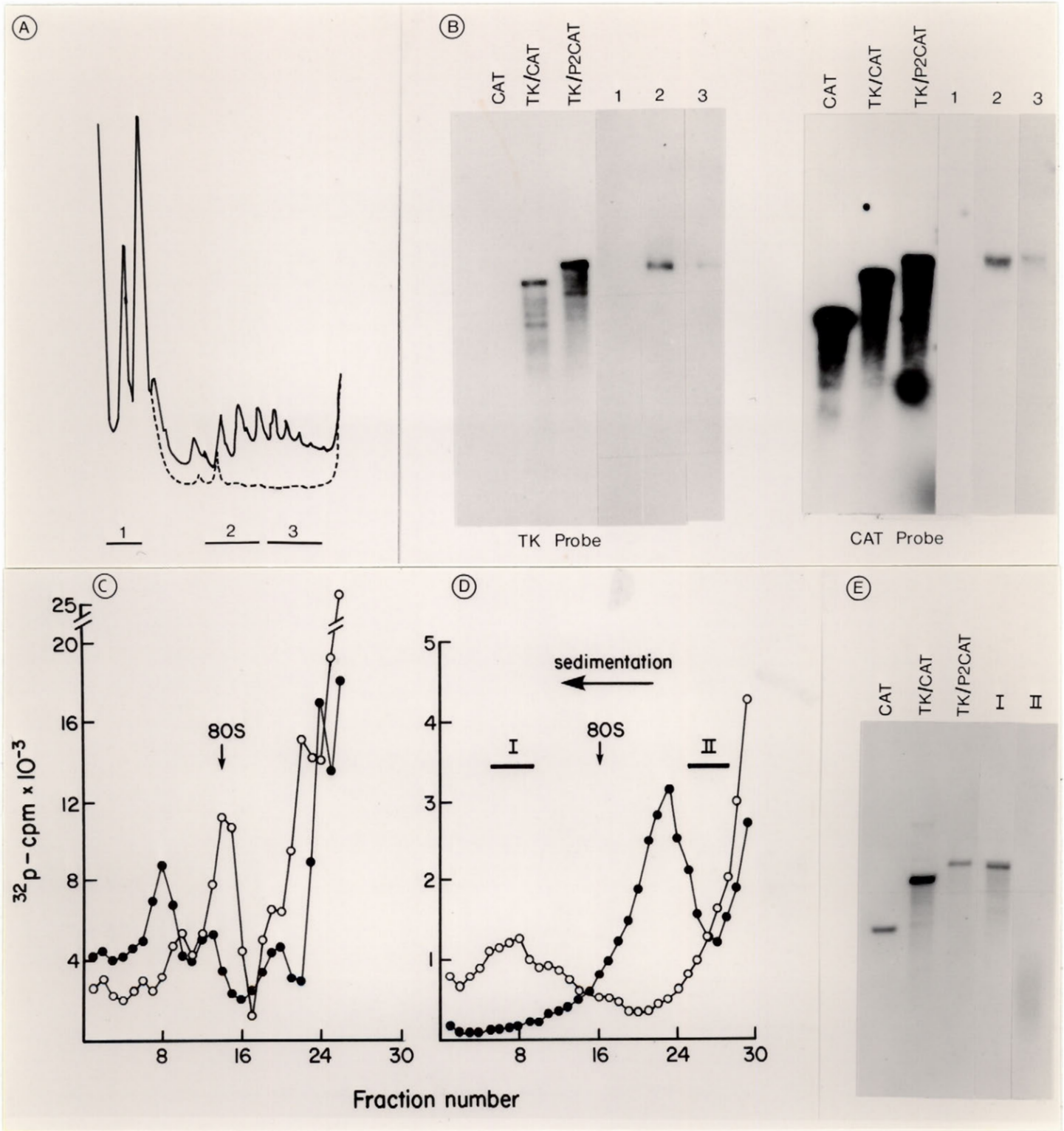
Our results can best be explained by ribosomes binding internally to the poliovirus 5' UTR. We wished however, to exclude the unlikely possibility that nucleolytic cleavage of the bicistronic TK/P2CAT mRNA generated fragmented mRNAs capable of translation in poliovirus-infected cells. To do this, we analyzed the integrity of TK/P2CAT mRNA obtained from polysomes isolated from poliovirus-infected cells. The polysome profile from uninfected and poliovirus-infected COS-1 cells is shown in Fig. 4A. Poliovirus-infection caused a sharp decrease in the size and amount of polysomes (44). A northern blot of mRNA isolated from different fractions of the polysome gradient (Fig. 4A), probed with a ^{32}P-TK or ^{32}P-CAT DNA fragment, shows that the mRNA is intact in poliovirus-infected, TK/P2CAT transfected COS-1 cells (Fig. 4B). Most of the mRNA is present on small polysomes, consistent with only the CAT portion of the mRNA being translated. The translation of the CAT

cistron in TK/P2CAT mRNA could not have been achieved by translation of a fragmented mRNA.

We also examined the integrity of TK/P2CAT mRNA in initiation complexes formed in extracts from poliovirus-infected cells. Figure 4C shows a glycerol gradient sedimentation analysis of initiation complexes formed with CAT and P2CAT mRNA in extracts from uninfected HeLa cells. CAT mRNA formed mainly an 80S initiation complex as expected. But P2CAT mRNA was engaged mainly in disomes, indicating that most P2CAT molecules bound two ribosomes, one presumably at the CAT AUG initiator and a second ribosome upstream in the poliovirus mRNA 5' noncoding region. TK/P2CAT mRNA formed a similar size complex in extracts from poliovirus-infected HeLa cells (Fig. 4D). Initiation complexes were not formed in the presence of EDTA (Fig. 4D) or at 4°C (data not shown), consistent with them being translation specific complexes. Also, TK/CAT mRNA which does not contain any poliovirus RNA sequence, did not form initiation complexes in poliovirus-infected HeLa cell extracts (data not shown). Messenger RNA from initiation complexes (region I in Fig. 4D) and from the top of the gradient (region II in Fig. 4D) was extracted and analyzed on a formaldehyde agarose gel (Fig. 4E). The RNA present in the initiation complex is intact, whereas the RNA from the top of the gradient is mostly degraded. We conclude that TK/P2CAT mRNA entering initiation complexes is intact, thus excluding the possibility that fragmentation of the mRNA is a prerequsite for translation of this mRNA.

FIGURE 4

Integrity of TK/P2CAT In Vivo and In Vitro. (A) Polysome profiles from uninfectected COS-1 cells (black line) and poliovirus-infected, TK/P2CAT transfected COS-1 cells (dashed lines). (B) Northern blot analysis of mRNA fractions purified from portions of the polysome gradient denoted in A. The probes used consisted of the ^{32}P-labelled HincII-SmaI TK fragment from pX1 (37) and the Hind III-BamHI CAT fragment from pSV2/CAT (10). The lanes indicated by CAT, TK/CAT, and TK/P2CAT contained SP6 generated transcripts used for size markers. Polysomes were prepared, fractionated, and analyzed as described by Katze et al. (17). (C-E) Ribosome binding and integrity of mRNAs containing the poliovirus 5' UTR. (C and D) α-^{32}P-ATP labelled CAT, P2CAT and TK/P2CAT (2 X 10^5 cpm;1µg for the experiment shown in C, and 6X 10^5 cpm; 3µg for the experiment in D) were incubated in 32 µl of uninfected (C) or poliovirus-infected (D) HeLa extract at 30°C for 10 min as described by Weber et al (49). Initiation complexes were formed and analyzed on glycerol gradients (49). Fractions were collected and radioactivity was determined by scintillation counting (C) or by Cherenkov counting (D). The horizontal bars designated I and II indicate the fractions pooled for mRNA analysis. (E) The pooled fractions from experiment B were treated with proteinase K (400 µg/ml) at 37°C for 30 min., phenol extracted and ethanol precipitated. The transcripts were analyzed on a 1.2% formaldehyde agarose gel.


A
1
2
3
B
CAT
TK/CAT
TK/P2CAT
1
2
3
TK Probe
CAT Probe
C
25
20
16
12
8
4
80S
^{32}p - cpm x 10^{-3}
8
16
24
30
Fraction number
D
5
4
3
2
1
sedimentation
I
80S
II
E
CAT
TK/CAT
TK/P2CAT
I
II

DISCUSSION

In this paper we present evidence that eukaryotic ribosomes can bind internally to the 5' noncoding region of poliovirus RNA. This finding is consistent with several peculiarities of poliovirus translation including the absence of a cap structure (8,12,25,31), expression under conditions where the cap binding protein complex (eIF-4F) is inactivated (7,23,44) and the presence of an internal sequence in the 5' noncoding region which confers cap-independent translation to the mRNA (35). It is not the mere difference in length of the intercistronic region which allows TK/P2CAT to be expressed in infected extracts because insertion of 445 nucleotides from the mouse c-myc 5' UTR as the intercistronic region did not allow translation of CAT in mock-infected HeLa extracts (unpublished observations). Also, TK/(Δ3'-461)CAT, which had an intercistronic spacer of 511 nucleotides did not translate in poliovirus-infected extracts (Fig. 3B, lane 10). These results support the contention that eukaryotic ribosomes are capable of binding internally on certain mRNAs. The strongest evidence against this possibility is the finding that eukaryotic ribosomes are unable to bind to circular RNA, whereas prokaryotic ribosomes can (19,20). Unfortunately, these studies suffer from several disadvantages. The RNA fragments were small (less than 80 nucleotides) and most probably lacked important signals for internal ribosome binding (19) or their circularization could have sterically prevented ribosome binding (19,20).

It is possible that poliovirus RNA constitutes an extreme exception for internal ribosome binding and that the majority of other eukaryotic

mRNAs do not have the necessary signal sequences. Several *in vitro* results with other mRNAs have been interpreted as indication that translation initiation at internal sequences can occur without scanning from the 5' end. The most relevant study is of another member of the picornavirus family, encephalomyocarditis (EMC) virus: complementary DNA fragments to the 5' proximal 450 nucleotides of EMC virus RNA had no effect on the synthesis of the EMC polyprotein (42). Moreover, a recent study showed directly that an internal sequence in the 5' noncoding region of EMC virus RNA directs internal binding of ribosomes *in vitro* (16). Because all picornavirus RNAs have similar translational characteristics in that they are cap independent, it is most probable that all of them are initiated by direct internal binding of ribosomes. Results from *in vitro* hybrid-arrest translation experiments were also interpreted as evidence for internal initiation on other viral mRNAs including the NS (P) mRNA of vesicular stomatitis virus (VSV) (11); adenovirus 2 polymerase mRNA (9) and infectious pancreatic necrosis virus segment A (30).

Internal Initiation on Cellular mRNAs?

There are several potential and interesting candidates for internal initiation among cellular mRNAs - for example, the yeast GCN4 mRNA which contains 4 minicistrons (2-3 amino acids) upstream of the GCN4 long ORF (13,46). These minicistrons regulate translation from the downstream GCN4 ORF. The 5' proximal upstream ORF acts as a positive regulator under starvation conditions (28,48). It is possible that under these conditions translation of the first minicistron facilitates internal initiation at the GCN4 AUG. Several other candidates for internal

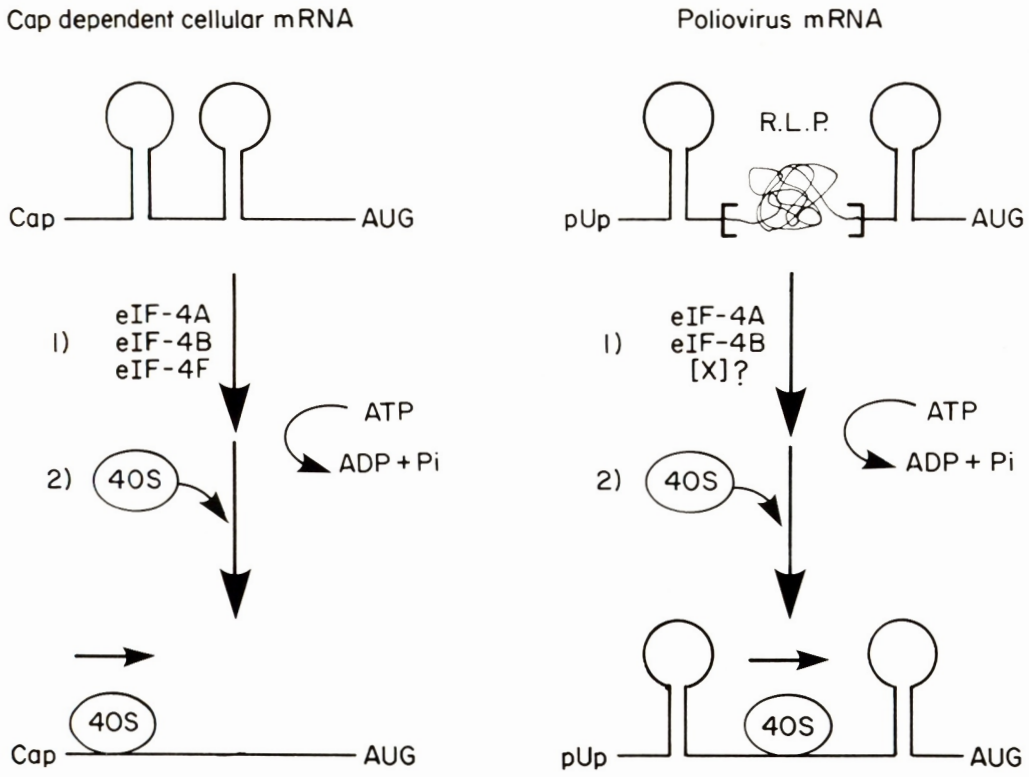
initiation are mRNAs that possess unusually long 5' noncoding regions containing multiple AUGs. For example, one of the transcripts of the Antennapedia gene of Drosophila melanogaster contains a 5' noncoding region of approximately 1100 nucleotides with 15 AUG codons (45). One of the human c-abl transcripts (type 1b) has a 5' noncoding region of 1200 nucleotides containing 13 AUG codons (3) and the hamster HMG CoA reductase gene produces a family of mRNAs with variable 5' UTR length, the longest containing 670 nucleotides and 8 AUG codons upstream of the initiation site for translation (40). Heat-shock mRNAs constitute another set of mRNAs that might initiate translation by internal ribosome binding. Internal sequences of Drosophila heat-shock mRNAs 5' UTR are required for translation under heat-shock conditions (15,27). Moreover, there is some evidence that the CBP complex (eIF-4F) may be rendered inactive at elevated temperatures (33), suggesting that translation of heat shock mRNAs is less dependent on the cap structure than the bulk of cellular mRNAs. This is consistent with the finding that hsp mRNAs are more resistant than the bulk of cellular mRNAs to the shut-off of host protein synthesis after poliovirus infection (29).

Model for Ribosome Internal Binding

What is the molecular mechanism that explains internal binding of ribosomes to eukaryotic mRNAs? There is considerable evidence that the cap structure of eukaryotic mRNAs mediates the melting of regions of secondary structure in the 5' UTR through the action of the cap binding protein complex (eIF-4F) (5,39), thus facilitating 40S ribosomal subunit binding. This melting activity is dependent on two other initiation

FIGURE 5

Models for Cap-Dependent and Internal Binding of Ribosomes to mRNA. The mechanism for cap-dependent initiation is based on many lines of evidence as reviewed by Edery et al. (6). The internal binding mechanism might be facilitated by additional factors (denoted by X) and mRNA signals (depicted by RLP [Ribosome Landing Pad]). The requirement for the hydrolysis of ATP in internal binding is as yet ill-defined.


Cap dependent cellular mRNA
Cap
AUG
1)
eIF-4A
eIF-4B
eIF-4F
ATP
ADP + Pi
2)
40S
40S
Cap
AUG
Poliovirus mRNA
R.L.P.
pUp
AUG
1)
eIF-4A
eIF-4B
[X]?
ATP
ADP + Pi
2)
40S
40S
pUp
AUG

factors, eIF-4A and eIF-4B (1,39). Poliovirus RNA translation proceeds by a cap-independent mechanism and most probably does not require eIF-4F, but does require eIF-4A and eIF-4B. The model we propose for poliovirus translation is schematically shown in Fig. 5. According to our proposed model, eIF-4A in conjunction with eIF-4B, binds to an internal sequence of the mRNA. The nature of the sequence, recognized by eIF-4A and eIF-4B ,which we have termed the ribosome landing pad (RLP) is not known. But an intriguing possibility is that this sequence is naturally devoid of secondary structure or is melted by an unknown factor(s) (X in Fig. 5). ATP-dependent binding of eIF-4A to unstructured RNA has previously been documented (1). This would then signal the binding of ribosomes. The importance of single-stranded regions does not exclude, however, the possibility that secondary and tertiary structure of the RLP region serve as recognition signals for factors involved in ribosome binding. The size of the region required for internal binding (several hundred nucleotides; Fig. 3B) suggests that secondary and tertiary structure or additional factors (denoted as X in Fig. 5) are required for efficient internal ribosome binding. The system we have described should allow the testing of putative 5' UTRs of cellular mRNAs which may be involved in internal ribosome binding.

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CHAPTER 6

GENERAL DISCUSSION

6.1 mRNA Secondary Structure and Translation

Our inability to predict the translational efficiency of a mRNA from knowledge of its primary structure stems in part, from our poor understanding of the initiation process. A lack of direct evidence of how the large number of protein factors involved in eukaryotic translation interact with each other and with mRNAs or how 40S ribosomes select the appropriate AUGs are a main contributing factor to this ignorance. Three parameters of mRNA structure are known to affect translational efficiency: (1) the 5' cap structure, (2) the degree of 5' secondary structure and (3) the nature of the nucleotides flanking an initiator codon. Before the work described in this thesis was undertaken, there was suggestive evidence indicating that the function of the Cap Binding Protein Complex (eIF-4F), in conjunction with eIF-4A and eIF-4B, was to mediate unwinding of mRNA 5' secondary structure through the hydrolysis of ATP. Morgan and Shatkin (154) and Kozak (155) have shown that reovirus mRNAs with reduced secondary structure are less dependent on both the cap structure and ATP for initiation complex formation than native reovirus mRNA. Jackson (386) has reported that naturally uncapped mRNAs such as those of cowpea mosaic virus and EMC virus are less dependent on ATP for initiation complex formation than capped mRNAs. Lee et al. (171) demonstrated a direct relationship between the degree of secondary structure and the extent to which ribosome binding is inhibited by high salt concentrations. We were able to show a negative effect of mRNA 5' secondary structure on translation (156).

It is *a priori* difficult to predict the amount of secondary structure one can introduce within the 5' UTR of a mRNA before drastically affecting translation. This is due to the variable amount of secondary structure within eukaryotic mRNA 5' UTRs and the positional effects such secondary structure might have. The introduction of a hairpin loop having a ΔG° = -20.7 kcal/mole within the tk 5' UTR (thus increasing the overall ΔG° of the 5' UTR from -64.6 to -73.1 kcal/mole) resulted in appreciable inhibition of translation *in vivo* and *in vitro*. Kozak (164) however, found that the introduction of a moderately stable hairpin (ΔG° = -30 kcal/mole) into the preproinsulin 5' UTR had no effect on translational efficiency *in vivo*, whereas the introduction of a hairpin loop having a ΔG° of -50 kcal/mole strongly inhibited translation. However, analysis of the preproinsulin mRNA 5' UTR employing the Zuker program RNA-2 for RNA folding (386) reveals that it is not as structured as that of the tk mRNA and contributes only -30 kcal/mole to the overall 5' secondary structure (J. Pelletier, unpublished results). The inhibition of translation observed with tk mRNA (but not preproinsulin mRNA) with the insertion of a stem-loop structure having a ΔG° of -20.7 kcal/mole may be due to an additive effect from neighboring hairpin loops (of which preproinsulin has less).

An interesting outcome of these studies is that excessive secondary structure can exert its effect at different levels of the initiation step (167). When introduced close to the cap structure (6 nucleotides), it hampers the interaction of eIF-4B with the cap structure, presumably

preventing ribosome binding and thus inhibiting translation. However, when excessive secondary structure was introduced 37 nucleotides from the cap structure, it had no effect on the interactions of the CBPs with the mRNA (167), but did prevent 80S ribosome formation in a ribosome binding assay (156). These latter constructs presumably inhibit translation by preventing ribosome scanning. A similar conclusion was reached by Lawson et al. (235) who showed that hybridization of an oligodeoxynucleotide complementary to sequences adjacent of the cap structure (hence forming a stable secondary structure) inhibited crosslinking to the cap structure of eIF-4B and the eIF-4A component of eIF-4F. A second oligodeoxynucleotide targeted to a region 15 nucleotides from the cap site had only a small effect on the crosslinking of these factors. An interesting follow up to these studies would be to determine the effects of altering the secondary structure around the AUG codon, in the coding region, and within the 3' noncoding region. Sherman and coworkers (159) have found that point mutations in the yeast CYC1 gene near the AUG codon (13 nucleotides into the coding region) having the potential to severely alter the local secondary structure drastically inhibited translation. This suggests that excessive secondary structure within the coding region may render elongation rate-limiting and decrease translational efficiency. Kozak (155) found that 40S ribosomes inefficiently recognized the initiation codon of a less structured mRNA (where inosine was substituted for guanosine) than in the native mRNA, suggesting that possibly secondary structure around an initiator codon may function to slow down scanning ribosomes, giving them more time to efficiently recognize the start

signal. Secondary structure within the coding region of an mRNA has also been suggested to induce pausing of 80S ribosomes (388). Whether or not introduced secondary structure in the coding or 3' noncoding region would affect translational efficiency (or fidelity) awaits experimental investigation.

The current belief that mRNAs with less structured 5' UTRs are, in general, better translators and more efficient competitors seems to be true (389). There are however notable exceptions. In the case of ferritin mRNA (having a predicted stem-loop structure of ΔG° = ~-45 kcal/mole at the 5' end, ref. 371) or mRNAs containing the adenovirus tripartite leader (having a predicted free energy of -55.3 kcal/mole, ref. 364), these mRNAs are efficiently translated under appropriate conditions and the hairpin-loop structure in these cases may serve as binding sites for trans-acting proteins that facilitate initiation. Alternatively, until proven otherwise, there also remains the formal possibility that these mRNAs initiate by internal binding of ribosomes thus bypassing the inhibitory stem-loop structure.

Another manner by which mRNA secondary structure has been found to inhibit translation is via phosphorylation of eIF-2 (390). Poly $(A)^+$ mRNA from several mammalian sources is capable of phosphorylating eIF-2 and subsequently inhibiting translation in vitro. De Berredetti and Baglioni (357) found that hybridization of the poly $(A)^+$ tail of VSV mRNAs to poly(U) to form a dsRNA duplex resulted in phosphorylation of eIF-2 and inhibited initiation complex formation in vitro. Although the physiological relevance of these studies remains to be established, it has been found that an mRNA containing a stable stem-loop structure at

the cap site, thus sequestering the cap structure, and rendering it inaccessible to eIF-4F, also results in phosphorylation of eIF-2 by DAI, and inhibits global mRNA translation (I. Edery, personal communication). This mRNA is a poor translator *in vitro* and *in vivo* (N. Parkin, personal communication).

6.1.1 Translational Associated Helix-Destabilizing Activities

A RNA helix destabilizing protein (HDP) causes the denaturation of helices in RNA. An unwinding activity specific for eukaryotic mRNA was first described by Ilan and Ilan (391) and attributed to eIF-3. This factor promoted the synthesis of polyphenylalanine when added to a cell free system containing a hybrid duplex in which AUG(U)n was hybridized to poly(A) containing 10% U. Moreover, eIF-3 was found to decrease the melting temperatures of globin mRNA and double-stranded RNA. Subsequently, Sonenberg (145) attributed a mRNA melting activity to CBPs in light of the ability of cap-labelled mRNA to crosslink to several of these polypeptides only in the presence of ATP. It is interesting that eIF-3 preparations are known to be contaminated with CBPs (66), thus the properties previously attributed to eIF-3 might have been due to the presence of contaminating CBPs.

Ray *et al.* (68) have recently described a nuclease sensitivity assay to determine the effect of purified initiation factors on mRNA higher order structure. Although not a "melting" assay *per se*, they reported that eIF-4A (at greater than 100-fold molar excess of protein to mRNA) caused a structural change in mRNA configuration, in an ATP-dependent reaction, resulting in increased sensitivity of the cap structure to single-strand specific nuclease digestion. eIF-4F was also found to sensitize the mRNA cap structure to nuclease digestion and it

was 20-fold more efficient than eIF-4A in catalyzing this reaction.

The possibility of creating cell lines which have increased HDP activity may help in the characterization of this factor. The ability to select several colonies in HAT media with tk constructs containing large amounts of 5' secondary structure suggests that this may be easier than anticipated (see Table 1, Chapter 2). Since these revertants were not characterized, it is possible that they contain rearranged tk genes that have lost the inhibitory feature. This is likely since it is well documented that transfection of cultured cells gives rise to mutations and alterations of the introduced gene (392,393). The potential of pXJP18 to form large cruciform structures at the DNA level may induce recombinational events. This clone was difficult to propagate in E. coli and constantly had to be checked for rearrangements. On the other hand, the interesting possibility exists that some of these cell colonies are actual mutants that overexpress the helix destabilizing protein (possibly eIF-4F) required to overcome the translational block. It would be interesting to determine if these colonies are more resistant to infection by poliovirus (which inactivates eIF-4F) or if cell extracts made from these cells would have a greater translational capacity.

Techniques have recently been developed for the direct visualization of mRNA-protein complexes (394) and to directly measure mRNA unwinding (395). Although used to study mRNA splicing, adapting these methods to the study of translation should enable us to directly measure the kinetics and molecular mechanism involved in mRNA unwinding.

Translationally associated helix-destabilizing activity has also

been associated with the 80S ribosome (396,397). This was assessed by determining the ability of ribosomes to read-through mRNA/cDNA duplexes. Hybridization of globin mRNA to cDNA fragments which extend into the 5' UTR fully blocked the translation of the hybridized mRNA, whereas hybridization to cDNA fragments which covered regions 3' to the initiation codon had no adverse effect on globin translation. Although the ribosomes read through the mRNA/cDNA duplexes 3' of the AUG, it did not remove the cDNA from its homologous mRNA. This suggests that the translating ribosome destablizes secondary structures in a local fashion. Experiments with RNA/RNA duplexes between part of the coding region and the 3' UTR of lysozyme mRNA yielded similar conclusions (398).

The use of RNA or synthetic oligodeoxyribonucleotides complementary to mRNA to inhibit gene expression has become increasingly popular. The mechanism of inhibition however, is dependent on the technique and cell used. It is not known how antisense DNA constructs, introduced into the nucleus of cells (most often by transfection) inhibit gene expression. Possibilities include: (a) impairment of processing and/or transport of the mRNA or (b) affecting the stability and/or translation of the mRNA in the cytoplasm. In the case where antisense oligodeoxyribonucleotides and their derivatives are used to inhibit gene expression in wheat germ extracts and Xenopus oocytes, the mRNA-DNA hybrids activate RNAse H, which subsequently cleavages and inactivates the mRNA (399-402). The use of antisense RNA, however, in Xenopus oocytes (403,404) or antisense oligodeoxyribonucleotides in reticulocyte lysates (396,397,399) does not lead to degradation of the mRNA template. In these cases, inhibition is

only observed if the 5' UTR of the mRNA is targeted. This is consistent with the finding that 5' secondary structure within the 5' UTR inhibits translation (104). It remains to be seen whether the cell utilizes its own form of antisense "technology" to control gene expression *in vivo*.

6.1.2 Developmentally Associated Helix-Destabilizing Activities

It was recently reported that an RNA melting activity increases dramatically when *Xenopus* oocytes are induced to mature (405,406). This activity exists at high levels in eggs and early embryos, is barely detectable in oocytes, and is not present in Drosophila (407) and mouse embryos (408). A similar unwinding activity has also been found in various mammalian cells (409) and exhibits a transient increase in expression during the cell cycle. The unwindase activity is very low in 3T3 cells when they are arrested into quiescence, but increases when cells are released into renewed growth by serum. The unwindase activity does not require ATP (406) nor is it inhibited by cap analogues (406,409). The identity of this activity is presently unknown.

6.3 Poliovirus mRNA Translation

Upon infection of HeLa cells by poliovirus there is a drastic decrease in host protein synthesis (reviewed in Chapter 1). In addition to cleavage of a component of the CBP complex (which inactivates the protein), there seems to be a second ill-defined event required for the shut-off phenomenon (314b). A long neglected aspect of poliovirus biogenesis concerns the manner by which the mRNA is translated in a cap independent fashion. Indeed, the virion mRNA is a strange template for

ribosomes to encounter. It is uncapped, has 7-8 upstream ORFs 5' to the major translation initiation site (depending on the serotype), and has an unusually long 5' end (~745 nucleotides compared to an average of ~100 nucleotides for eukaryotic mRNAs).

It is not the presence of a cap structure *per se* which does not allow translation of host mRNAs under poliovirus-infection conditions. Conversely, it is not the absence of a 5' cap structure which permits poliovirus to translate by a cap independent mechanism. It was demonstrated that capped mRNAs with reduced 5' secondary structure can function in poliovirus-infected extracts (306). Secondly, decapping VSV mRNA (which is normally cap-dependent) does *not* enable it to translate in extracts from poliovirus-infected cells (318). Thirdly, the addition of a cap structure to the 5' end of *in vitro* synthesized poliovirus chimeric mRNAs (see Chapter 4) did not change the cap-independency of the mRNA (319). The observation that a viral mRNA lacking in secondary structure, like AMV-4, can translate in poliovirus extracts raises the interesting possibility that some cellular mRNAs might be efficiently translated in the absence of functional cap binding proteins. If the CBP complex is a target for regulation in situations other than in poliovirus-infection, as has been suggested when cells enter mitosis (410), then this may have important consequences. This would be a mechanism by which the cell would allow the translation of a few choice mRNAs under the imposed translational block.

How would such a mechanism function? A modified scanning model predicts that ribosomes should reach the downstream initiation site of

the poliovirus 5' UTR due to (i) leaky scanning of the 5' UTR, somehow missing 8 AUG codons, two of which are in an optimal context in the Lansing strain and/or (ii) termination/reinitiation reactions until the initiator codon is reached. There are several points however that argue against these possibilities. Firstly, abolishment of the upstream ORFs individually, or in combination, did not increase the translation of a chimeric mRNA containing the poliovirus 5' end (315). In fact, mutating AUG_{588} within the Lansing strain 5' UTR decreased translational efficiency, a result contrary to that predicted by the scanning model (315). Secondly, alteration of the upstream ORFs by linker scanning mutations (411), which resulted in alterations of reading frames of the 5' ORFs did not affect virus viability or their in vitro translation. Thirdly, deletion mutagenesis of the poliovirus 5' UTR resulted in the mapping of an internal region (encompassing nucleotides 140 to 630) which is responsible for the cap independent translation of the mRNA (319). In several of the constructs used in this study, deletions within the 5' UTR resulted in the creation of new ORFs having the potential to generate 17 kDa and 12 kDa polypeptides, none of which were observed in in vitro translation systems. This suggests that the upstream ORFs are not recognized by ribosomes.

The identification of an internal sequence required for cap-independent translation suggested to us, the possibility that ribosomes were binding internally to the 5' UTR, thus bypassing the uORFs. This mechanism had been previously speculated for initiation on poliovirus mRNA (412) due to the supportive evidence that under conditions where elongation of protein synthesis is blocked, ribosomes

did not accumulate on the long leader region of poliovirus mRNA. It was suggested that an adenine-uridine rich region just preceding the denatured region in the mRNA may be responsible for internal binding (413). We have found however that deletion of this adenine-uridine rich region had no deleterious effect on the cap-independent expression of poliovirus (319). We found that we could insert the poliovirus 5' UTR as an intercistronic spacer in a bicistronic mRNA, and under conditions of poliovirus-infection where 5'-end-mediated initiation is inhibited (and hence leaky scanning and reinitiation are prevented), we could obtain expression from the second cistron (244). The majority of polysome-associated mRNA (and hence actively translating mRNA) was found to be intact (244). It thus seems that poliovirus mRNA initiates translation by allowing internal binding of ribosomes. This makes good sense because it allows poliovirus mRNA to overcome the general block on translation which the virus imposes after infecting a cell. Although we attempted to circularize the poliovirus 5' UTR using T4 RNA ligase to directly demonstrate internal binding, we were unable to achieve circularization the mRNA, due to the fact that RNA molecules longer than 80 nucleotides are inefficient templates for the ligase. The actual site to which the 43S preinitiation complex binds awaits ribosome binding and nuclease protection analysis.

These results are consistent with data obtained by Shih et al. (242) and Jang et al. (243) showing that EMC (also a member of the picornaviruses) mRNA can initiate translation by internal binding. It is likely all picornaviruses initiate translation by internal ribosome binding. Studies using mengovirus RNA, another member of this family,

in which ribosome binding sites have been identified by nuclease protection experiments have also indicated that ribosomes can bind internally to mengovirus RNA (412).

6.3.1 The Mechanism of Internal Initiation

The mechanism by which internal ribosome binding occurs remains speculative. A recent model for ribosome binding (Fig. 2, Chapter 1) can accommodate ribosome binding near the 5' end of the mRNA as well as internally on the mRNA (109). The internal binding pathway can be explained by the direct binding of eIF-4A and eIF-4B to internal regions of the mRNA that are devoid of secondary structure (237). This however, can only be part of the explanation since a denatured region is insufficient by itself, to allow internal binding of ribosomes (155). The participation of ancillary proteins and/or specific primary mRNA sequences in this event is a likely possibility. This pathway is expected to function in a cap-independent manner. The large region required for cap-independent expression, as defined by deletion mutagenesis suggests that perhaps some higher-order structure (e.g.: secondary or tertiary) also participates in this reaction. It would be useful to develop more efficient fractionated translation systems so that individual factor requirements for picornavirus RNAs could be determined.

6.3.2 Tissue-Specific Expression

The tissue-specific expression of proteins at the translational level still remains speculative. For many viral systems, the basis for host-range or tissue-specific restriction is a lack of cell-surface receptors that mediate attachment and penetration of the virus. A

host-range restriction at the translational level has recently been shown to be operative in the infection of mouse L cells by avian reovirus S1133 (414). There is growing evidence that the translation of poliovirus mRNA is restricted in a tissue-specific fashion. The translation of poliovirus mRNA is very poor in reticulocyte lysates, wheat-germ extracts or Xenopus oocytes compared to extracts from HeLa cells and the element responsible for this inhibitory effect lies within the 5' UTR (415). These results are consistent with recent results showing that translation of poliovirus RNA in U-937 (a human monocytic cell line) is impaired (416). Poliovirus RNA extracted from infected U-937 cells was shown to be functional by its ability to translate in a cell-free extract. In addition, poliovirus infection of several established human blood cell lines showed that viral replication differed depending on the differentiation stage and cell lineage (417). It was not determined in the latter study at what stage in the viral replication cycle the restriction occurred but our results (415) would suggest that a block at the translational level is a clear possibility. The interesting possibility exists that cells in which poliovirus translation is restricted lack a factor required for internal ribosome binding (and hence cannot initiate translation of poliovirus mRNA) and that attenuated vaccine strains which contain nucleotide changes within the 5' UTR (418,419) can no longer bind this factor.

What does the future hold for the field of translation? The study of RNA-protein interactions using gel retardation assays of the kind used to elucidate the splicing pathway (394) will prove an invaluable tool for elucidating the molecular mechanism of initiation, in

particular, 48S preinitiation complex formation. Cloning of the genes coding for different initiation factors and the ability to mutate them for functional analysis will undoubtably increase our understanding of their function. The power of yeast genetics, which has been overlooked by many in this field, should serve as a unique window into finding novel and unusual forms of translational control. The mRNA itself may be more dynamic than anticipated. The understanding of the translational process is an important step in unraveling the implementation and control of gene expression in eukaryotic cells.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Studies with the HSV-1 tk gene directly showed that secondary structure within the mRNA 5' UTR can inhibit translation *in vivo* and *in vitro*. The mRNA secondary structure was shown to prevent 80S initiation complex formation.
2. A new technique was developed to co-transcriptionally cap mRNAs made *in vitro* from the SP6 system. This procedure turned out to be much more efficient than the conventional post-transcriptional capping procedure using vaccinia guanylyltransferase and is used by many labs when generating *in vitro* synthesized capped mRNA.
3. The inhibitory effect on translation of mRNA having secondary structure near the cap site was shown to correlate with the inability of eIF-4B to interact with the cap structure. This was shown using a novel photochemical crosslinking assay.
4. Using deletion mutagenesis, a *cis*-acting element within the 5' UTR of the poliovirus mRNA was identified which enables it to translate in a cap-independent fashion. This element can confer cap-independent translation to a heterologous mRNA when fused to the 5' end of that RNA.
5. Initiation of translation by poliovirus mRNA was shown to occur by internal binding of ribosomes within the poliovirus 5' UTR.