ROLE OF CAP FUNCTION DURING EUKARYOTIC PROTEIN SYNTHESIS AND PRECURSOR MESSENGER RNA SPLICING

by

© Isaac Edery

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Isaac Edery ROLE OF CAP STRUCTURE DURING EUKARYOTIC GENE EXPRESSION Ph.D.

ABSTRACT

The cap structure, m^7GpppX (where x = any nucleotide), is present at the 5' end of all eukaryotic cellular messenger RNAs (except organelles). Previous studies have demonstrated that during protein synthesis the cap structure is recognized by a ~24 kDa cap binding protein (CBP), termed eukaryotic initiation factor 4E (eIF-4E). With the use of a newly developed cap-analog affinity matrix a high molecular weight complex that contains eIF-4E was purified, termed eIF-4F. In addition to eIF-4E this CBP complex consists of eIF-4A and a 220 kDa polypeptide (p220). Using an RNA unwinding assay direct evidence was obtained indicating that eIF-4F, eIF-4A and eIF-4B functionally interelate resulting in helicase activity. Secondary structure in the 5' untranslated region of eukaryotic mRNAs inhibits translation, therefore this unwinding activity is most likely required for efficient 40S ribosomal subunit attachment to mRNA. To facilitate the purification and biophysical characterization of eIF-4E, the yeast homologue was overexpressed in E. coli. mRNA secondary structure can also inhibit translation in trans by another mechanism. This was shown for the unique structure (TAR) at the 5' end of all mRNAs from the human immunodeficiency virus-1 (HIV-1). The mechanism of translation inhibition involves the activation of the double-stranded RNA dependent kinase (dsI), which catalyzes the phosphorylation of eIF-2. This is the first demonstration of a specific naturally occurring mRNA sequence that can activate dsI. A novel translational regulatory mechanism is proposed. Finally, the cap structure is also required for efficient precursor mRNA splicing in HeLa nuclear extracts. These and other studies indicate that the cap structure plays a multifunctional role during regulation of gene expression.

RESUME

La coiffe, m^7GpppX (où X désigne un nucléotide), se retrouve à l'extrémité 5' de tous les ARNs cellulaires (exception faite des ARNs des organites). Des études antérieures ont démontré que lors de la synthèse protéique, la coiffe est reconnue par une protéine de ~24 kDa (CBP, pour protéine de liaison de la coiffe, "cap binding protein") désignée de plus comme facteur d'initiation, eIF-4E (pour "eukaryotic initiation factor"). Le développement et l'utilisation d'une matrice d'affinité pour la coiffe ont permis la purification d'un complexe protéique de haut poids moléculaire désigné eIF-4F. En plus de contenir eIF-4E, le complexe de protéine de liaison de la coiffe est composé d'eIF-4A et d'un polypeptide de 220 kDa (p220). Grâce à l'utilisation d'un test de déroulage de l'ARN, l'intéraction fonctionnelle de l'eIF-4F avec eIF-4A et eIF-4B résultant en une activité hélicasique put être démontrée. La structure secondaire au niveau du 5' non-codant des ARN_{ms} eucaryotes inhibe la traduction. Conséquemment, cette activité hélicasique est fort probablement requise pour assurer un attachement efficace de la sous-unité ribosomale de 40S au niveau de l'ARN_m. De sorte à faciliter la purification et la caractérisation biophysique de l'eIF-4E, son homologue chez la levure fut sur-exprimé chez E. coli. La structure secondaire de l'ARN_m peut inhiber la traduction en trans. Ceci fut démontré pour la structure (TAR) caractéristique des extremités 5' de tous les ARN_{ms} du virus d'immunodéficiencie humain type I (HIV-1). Ce mécanisme inhibiteur de la traduction implique l'activation de la kinase ARN-bicaténaire dépendante (dsI), responsable de la phosphorylation de l'eIF-2. Ceci constitue la première démonstration qu'une séquence se retrouvant naturellement au niveau de l'ARN_m peut

activer dsI. Un nouveau modèle de régulation de la traduction est proposé. Finalement, la coiffe est requise pour permettre un épissage efficace des transcrits primaires dans des extraits nucléaires de cellules HeLa. Cette études et d'autres démontrent donc que la coiffe joue un rôle multifonctionnel dans la régulation de l'expression génique.

DEDICATION

I dedicate this work to my mother and father for their continued love, care and inspiration. And to my brothers and sisters, Lydia, Miguel, Joel, Albert, Luni and Jacky.

"A single feat of daring can alter the whole conception of what is possible".

Graham Greene

PREFACE

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

Chapter 2. Edery, I., Humbelin, M., Darveau, A., Lee, K.A.W., Milburn, S., Hershey, J.W.B., Trachsel, H. and Sonenberg, N. (1983) J. Biol. Chem. 258, 11398-11403.

Chapter 3. Edery, I., Altmann, M. and Sonenberg, N. (1988) Gene, in press.

Chapter 4. Edery, I., Petryshyn, R. and Sonenberg, N. (1988) Submitted.

Chapter 5. Edery, I. and Sonenberg, N. (1985) Proc. Natl. Acad. Sci. USA 82, 7590-7594.

The work presented in Chapters 3 and 5 is entirely my own. For the work presented in Chapter 2, Marcus Humbelin and Hans Trachsel provided antibodies to eIF-4A, Susan Milburn and John Hershey provided antibodies to eIF-4B, Andre Darveau performed the experiment shown in Figure 4, and Kevin Lee performed the experiment shown in Figure 1. For the work presented in Chapter 4 Ray Petryshyn performed the experiment shown in Figure 7.

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ACKNOWLEDGEMENTS

For some reason I find this the hardest part of my thesis to write. There are so many people I want to thank, yet I find it very difficult to faithfully convey my feelings.

First and foremost will always be my parents and family. My endearing love for them has always been a constant source of joy and inspiration. It was my father who first opened up my eyes to the wonders of science. I will never forget those sabbath meals where he considered such diverse topics as the atom and evolution. Although he did not have a formal 'higher education', I was always amazed at his intricate and sophisticated way of thinking. His inspired talks certainly engaged us in learning the art of reasoning (not to mention the occasional art of heated arguing). My mother has sort of become a legend in this department. Everybody knows the care, love and meals, and meals, and meals she gave me. To them both, I will be forever grateful and proud. To my grandmother, mami Luna, I want her to know how much I think about her and love her.

I am deeply grateful to my supervisor Nahum. In the relatively short time it took me to get my Ph.D. (Nahum still is not convinced that 7 years is enough!) I have gotten to respect Nahum not just as a scientist but, I feel more importantly, as a man. I was very fortunate to end up under his guidance. He taught us how to be scientists and instilled good work ethics by example. He made enormous contributions to the field of protein synthesis and is gifted with a rare insight. He allowed me to pursue some pretty wild ideas (sometimes he wasn't too crazy about them) and in so doing, helped me develop a sense of

scientific independence. Although I am leaving the lab soon, I feel I will always be able to call Nahum's lab my other home.

There were many people in "807" and "806" that made my stay immensely pleasant and rewarding. Although I do not like to single out individuals, I must in the case of my two good friends, Kevin Lee and Jerry Pelletier. Kevin was part of what we may now call the early days. I really miss those talks into the late hours without a care in the world. Kevin not only taught me how to do experiments he also taught me the now famous concept of avoiding (at all cost) morning hours. As for Jerry I can only think of the crazy times we shared inside and outside the lab. Jerry is a rare breed. He is a great scientist and yet very modest and simple in his attitude towards life. Always one of the boys. His work here should be an inspiration to the "second generation". I am getting so sentimental that I <u>almost</u> feel like saying I'm sorry for all the times I was late (For further details refer to Jerry Pelletier's acknowledgment section).

I would also like to thank André Darveau for all the help he gave me, he was always at anybody's disposal. Gobinda Sarkar for teaching me the art of acha! acha! I truly enjoyed his company and his easy going manner. To Flo Rozen (one of my disciples) she is no longer a shmoe but a pro (not to mention senior lab member). We finally got a collaboration that worked! Who could ever forget Maria and her endless timer, Rob and his lack (did I say lack) of sarcasm, Anthoula and her embarrassed looks (will she ever use the F word). Okay Phil how did you fertilize those frogs (some of them really did look like you)? To Neilo Porkino whom I never had an argument with, and to Sophie Roy for being such a good listener. Thanks to Denis Guertin (early days), and Chris

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I also want to thank my best friends, Harvey, Lorne and Peter whom I shared most of my life with. To all my basketball buddies at the Y.M.H.A. (my other home) I would have never survived without you. I will really miss not being part of the team, good luck guys. I also want to thank all the people in the department for making my stay a very pleasant one. Thanks to the two Franks, Mohommed and Mina for those friendly talks, and Eileen for all the trouble I put her through. Special thanks to Cathy Teng for putting up with me over the years and her excellent photographic skills. (I never did get those nude pictures you promised me). Finally I thank Margaret for doing such a fast and efficient job of typing my thesis, all while retaining her good sense of humour and wit. By the way I wrote the acknowledgements section after being up all night so if you don't think it sounds like Shakespeare (William that is), try the flip side.

CHAPTER 1

INTRODUCTION

ROLE OF CAP FUNCTION DURING EUKARYOTIC PROTEIN SYNTHESIS AND PRECURSOR MESSENGER RNA SPLICING

1.1 Multifunctional Role of the Cap Structure: Brief Overview

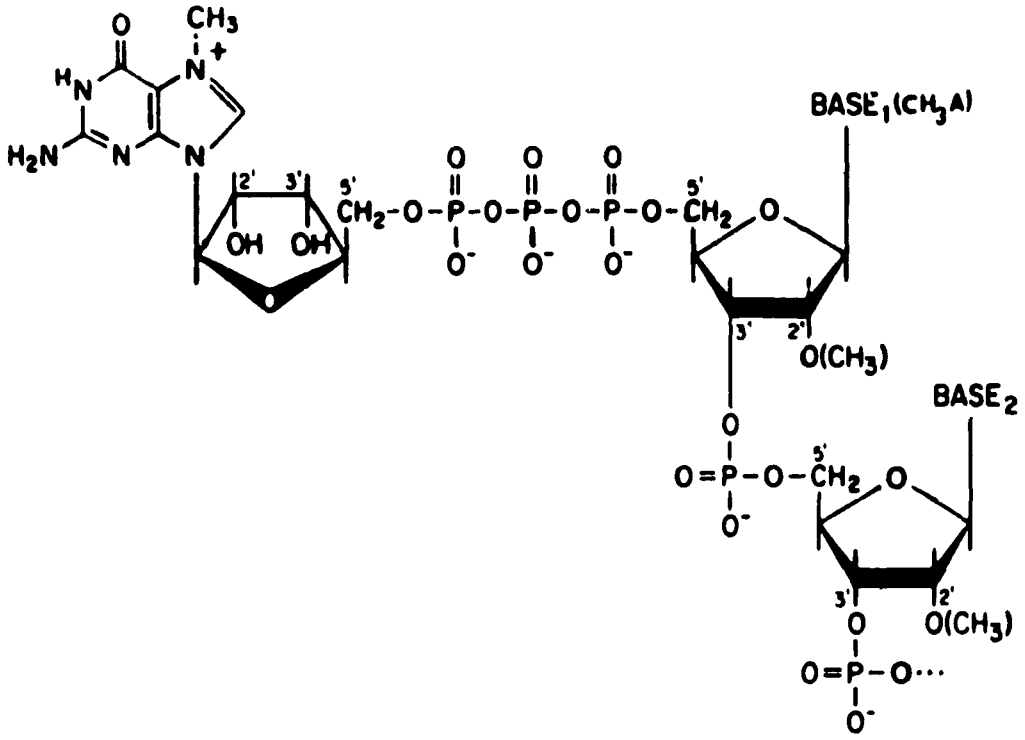
The conversion of nuclear encoded information into proteins that are synthesized in the cytoplasm is arguably the most complex biochemical task undertaken by eukaryotic cells. Of central importance in ensuring faithful completion of this monumental task is the role played by messenger RNA (mRNA). For a eukaryotic mRNA to be functional it must acquire unique structural modifications which are manufactured by a sequence of events collectively known as "RNA processing". This scenario is strikingly different than that experienced by their prokaryotic counterparts. The complexity of eukaryotic mRNA biogenesis and utilization probably reflects the need in higher organisms for more sophisticated control mechanisms. One very important and distinguishing hallmark of eukaryotic mRNAs is the nearly ubiquitous presence of a 5' terminal cap structure (Fig. 1A; for review, see refs. 1,2).

The cap consists of a 7-methyl guanosine residue linked to the 5' position of the penultimate nucleotide through a 5'-5' triphosphate bridge. Lower organisms, such as yeast, contain the least complex cap structure (termed cap 0). In animals and plants, the penultimate base is methylated at the 2'-o-ribose position (cap 1). In addition, the ribose of the second nucleotide also is methylated in vertebrates (cap 2). Thus, there is a general trend towards a more complex cap structure with increase in evolutionary scale. However, this correlation is not absolute. For instance, trypanosome mRNAs have unusual cap 4 structures, the most highly modified 5' termini that has been described on eukaryotic mRNAs (3,4). Albeit the frequent occurrence and proximity to the 5' termini, the significance of 2'-o-ribose methylation is moot

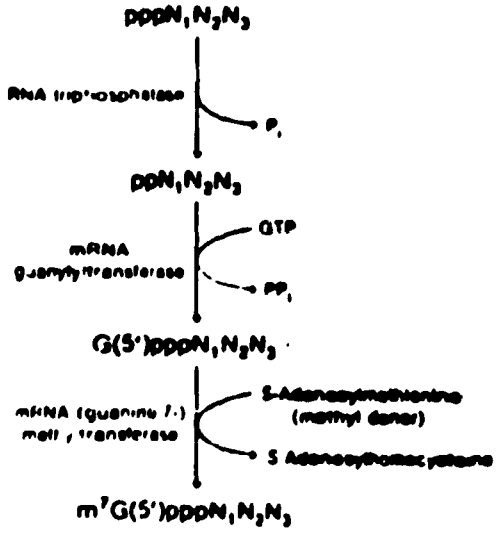
Fig. 1. The Eukaryotic Messenger RNA Cap Structure

a.) Schematic representation of the $m^7G(5')ppp(5')N$ cap structure found at the 5' end of almost all eukaryotic mRNAs. b)Biosynthetic pathway of 5' cap formation. See text for details.

A


BASE1(CH3A)
BASE2
O(CH3)
O(CH3)

B

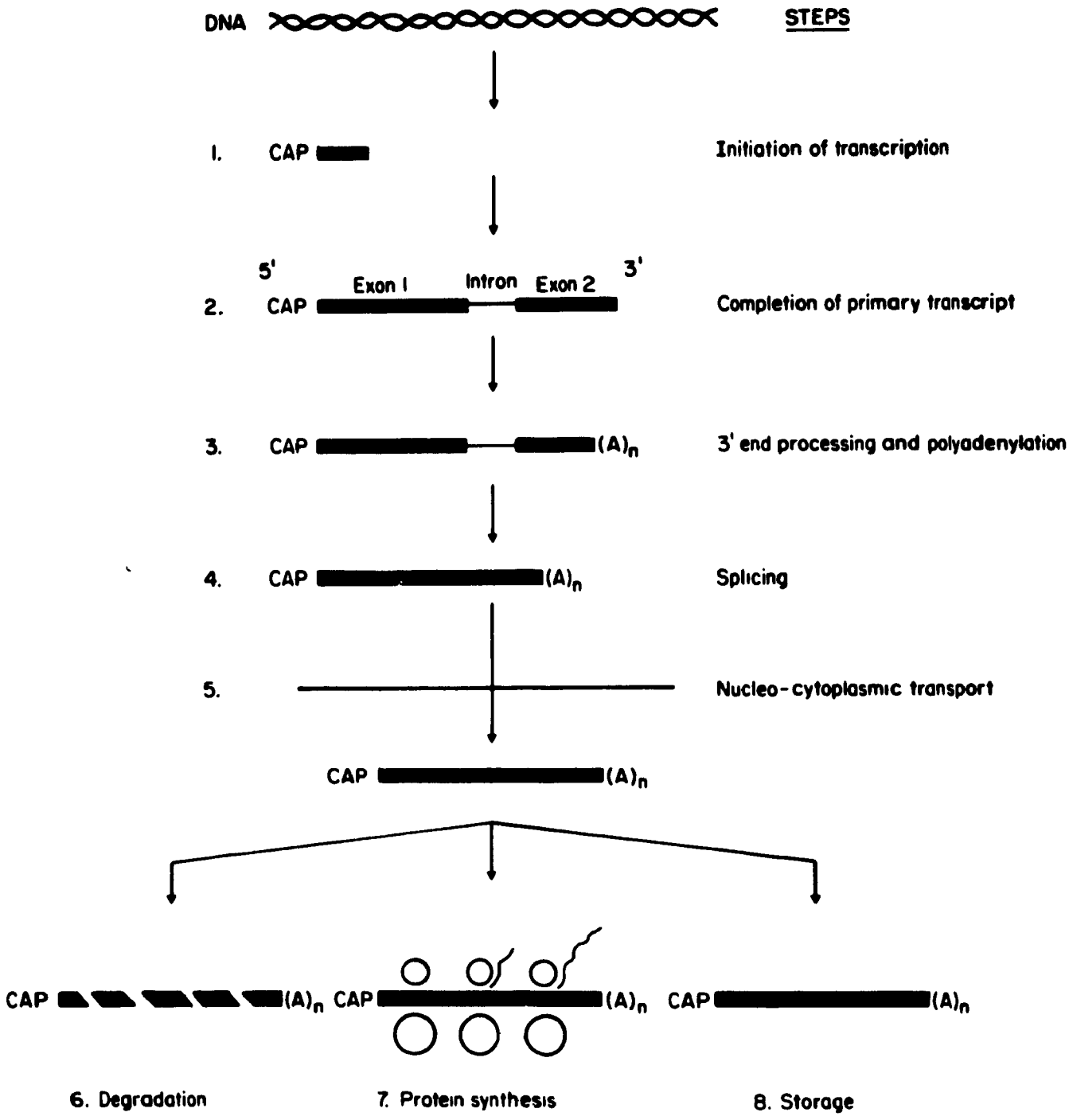

pppN1N2N3
RNA triphosphatase
Pi
ppN1N2N3
GTP
mRNA
guanylyltransferase
PPi
G(5')pppN1N2N3
S-Adenosylmethionine
(methyl donor)
mRNA (guanine-7-)
methyltransferase
S-Adenosylhomocysteine
m7G(5')pppN1N2N3

and does not seem to affect cap function. In addition, the nature of the penultimate base is not important for efficient cap function. Although most viral and cellular mRNAs have a purine as their penultimate base, this preference may simply reflect a peculiarity of the transcription mechanism. The most physiologically significant aspect of the cap structure is the partial positive charge imparted by N-7 methylation of the guanosine residue (section 1.2.6). Therefore, the cap structure can be adequately defined as, $m^7G(5')ppp(5')N$ (where N = any nucleotide). This definition excludes the $m^{2,2,7}G$-terminated small nuclear RNAs (snRNAs) involved in precursor mRNA splicing (section 1.4). However, it is important to note that similar hypermethylated cap structures ($m^{2,7}G$ and $m^{2,2,7}G$) have been identified on mRNAs from Sindbis (5) and Semliki forest (6) viruses. The role, if any, for extra methylation on mRNA caps is not known.

The mechanism of cap formation was initially derived from studies using viruses that replicate in the cytoplasm, and supplied their own capping enzymes (1,2). Subsequently, it was demonstrated that the nucleus of eukaryotic cells contain capping enzymes with similar activities (Fig. 1B: for review, see ref. 7). A phosphohydrolase removes the γ-phosphate from nascent chains priming the ends for capping. Guanosine monophosphate is then added by a guanylyl transferase activity. Finally, methylation of the guanosine is catalyzed by guanine-7-methyl transferase using S-adenosylmethionine (SAM) as the methyl donor. As described above this is usually followed by 2'-o-ribose methylation. The near universal presence of a cap on eukaryotic mRNAs strongly suggest that it has an important function.

Fig. 2. Pathway of Typical Eukaryotic Messenger RNA Life Cycle.

Not shown are the extra methylations of internal adenylate residues and cap proximal base(s) usually occuring in higher organisms. See text for details.


DNA
STEPS
1.
CAP
Initiation of transcription
5'
3'
Exon 1
Intron
Exon 2
2.
CAP
Completion of primary transcript
3.
CAP
(A)n
3' end processing and polyadenylation
4.
CAP
(A)n
Splicing
5.
Nucleo-cytoplasmic transport
CAP
(A)n
CAP
(A)n
CAP
(A)n
CAP
(A)n
6. Degradation
7. Protein synthesis
8. Storage

Indeed, with the advent of efficient cell-free extracts that can faithfully mimic several steps during mRNA biogenesis and its utilization, increasing evidence suggests that the cap structure plays a multifunctional role in regulating gene expression. To better illustrate this point, it is important to consider the steps involved in the "life cycle" of a typical eukaryotic cellular mRNA. The main steps are represented in Fig. 2.

The enzyme RNA polymerase II and auxiliary factors are responsible for transcribing the large heterogenous nuclear RNA (hnRNA) which is the precursor to mature mRNA (for reviews, see refs. 8,9). Several distinct cis-acting recognition elements found in DNA govern the level and start site of transcription (for reviews, see refs. 10,11). Transcription begins at the nucleotide to which the cap is added. The cap structure is synthesized at the initial stage of transcription, followed by elongation of the nascent chain until termination signals are recognized. The 3' termini of mRNAs are generated by RNA processing rather than transcription termination (12). This includes cleavage to form a new 3' terminus and polyadenylation (a notable exception are the histone mRNAs). Most RNA polymerase II transcripts are further processed by a splicing mechanism which removes intervening sequences (introns) followed by exon ligation (section 1.4). Once processed the mature mRNA is transported to the cytoplasm in an ill-defined manner. In the cytoplasm, mRNA is either engaged in protein synthesis and/or degraded (for review, see ref. 13). Furthermore, mRNA can also exist as

cytoplasmic mRNA (cmRNA) which is in a translationally repressed state, presumably accessible to activation (14-16). One classic example of stored mRNAs that are translationally repressed until the proper signals are triggered, occurs during fertilization of oocytes (for review, see ref. 17). Not shown in Fig. 1 are the methylation of adenylate residues and the cap proximal base(s), whose function is only speculative.

Several aspects relating to the 5' cap allow for its potential use as a key target in regulating gene expression at many levels: 1) It is a ubiquitous feature of all nuclear encoded mRNAs; 2) It is added early during transcription and conserved at the 5' termini throughout mRNA biogenesis and protein synthesis (Fig. 2); 3) Eukaryotic mRNAs are synthesized, translated and possibly degraded in a 5' to 3' direction. Therefore, regulatory mechanisms operating via the cap structure can potentially influence many distinct steps during the formation and utilization of mRNA. Moreover, regulation of the different steps would occur at an early stage in the pathway, in accordance with the molecular logic employed by cells. Since its discovery several different roles for the cap have been demonstrated.

For example, the cap structure and/or the association of the capping enzyme system with RNA polymerase II may play an important role in the initiation of transcription of viral (18,19) and cellular (20) mRNAs. This was first noted for purified cytoplasmic polyhedrosis virus (CPV) which requires a capping co-factor, SAM, for mRNA synthesis <u>in vitro</u> (18). This dependency indicates a tight coupling of the two processes. In addition, the presence of a 5' cap on precursor mRNAs enhances 3' end processing (21,22), although in other studies it did not

appear to be required (23,24). Moreover, the cap structure serves to stabilize mRNAs in the cytoplasm (25) and precursor mRNAs in the nucleus (26) against 5' exonucleolytic degradation. A role for the cap during nucleo-cytoplasmic transport has been suggested but has yet to be substantiated by experimentation.

The best documented function of the cap, however, is its role during protein biosynthesis. The main topic of this thesis, concerns a discrete set of proteins that mediate cap function during translation, the cap binding proteins (CBPs). Increasing evidence indicates that a cap binding protein in functional association with other protein factors uses energy derived from ATP hydrolysis to unwind 5'-proximal mRNA secondary structure with subsequent enhancement of 40S ribosomal subunit binding. The existence of a translationally associated helicase activity is not surprising when one considers that secondary structure in the 5' untranslated region (UTR) of mRNAs inhibits translation (27). An intriguing corollary to this is that, in addition to linear information stored in the coding sequence, mRNAs also contain regulatory information in their 5' untranslated regions in the form of secondary (or tertiary) structure. Thus, the ability to generate a variety of 5' UTR "regulatory modules" (for example by alternative splicing) whose effects can be modified by a CBP mediated helicase activity, affords the cell a powerful means of controlling gene expression at the translational level (discussed in Chapter 6).

The main objective of this Introduction section is to review the pertinent literature relating to the role of cap binding proteins and their involvement in the control of translation. In light of an energy

dependent melting activity associated with cap binding proteins, the general features of helicases are also described (section 1.3). The characterization of cap binding proteins and their function during translation is presented (chapters 2 and 3). Initial studies indicating direct evidence for an RNA:RNA unwinding activity is shown (Chapter 6). Furthermore, in the course of analyzing the effects of secondary structure in the 5' untranslated region of mRNAs, evidence was obtained that suggests a novel translational regulatory mechanism (Chapter 4). Finally, and to a lesser extent the process of precursor mRNA splicing (section 1.4) and evidence for the role of the 5' cap in this mechanism (Chapter 5) are presented. Notwithstanding the role of the cap, the mechanisms of protein biosynthesis and precursor mRNA splicing have many other intriguing similarities. In the General Discussion (Chapter 6) an attempt is made to unify the various related topics and their implications.

Due to the vast literature pertaining to protein synthesis and precursor mRNA splicing, reference citation is not exhaustive, key references, especially more recent ones, are generally favoured. The literature search is up to September, 1988.

1.2 Eukaryotic Protein Synthesis

1.2.1 Overview

Protein biosynthesis or translation can be defined (albeit not thoroughly) as the ribosome and template dependent sequential polymerization of amino acids resulting in a polypeptide chain of defined sequence and length. With the deciphering of the genetic code and the realization of its universal application from *E. coli* to man it

was believed that eukaryotes and prokaryotes manufacture proteins in a similar fashion. Indeed, there are several outstanding features in common. The division of labour amongst the three types of RNA molecules (ie. tRNA, rRNA and mRNA) is maintained. Furthermore, small and large ribosomal subunits are required for decoding information stored in mRNA and peptide bond formation. Presently, it is speculated that these gross functional and anatomical similarities reflect the importance and antiquity of an RNA world during the early stages of evolution (28-30). Another notion based on extrapolation from prokaryotes, involves the level at which gene expression is controlled. The main theme can be summarized as, 'to transcribe or not to transcribe, that is the question'.

However, the last 15 years have produced a virtual arsenal of data that strongly challenges the applicability of a prokaryotic vision to the understanding of eukaryotic protein synthesis. The cytoplasmic synthesis of a protein is a dynamic process, requiring the concerted temporal and spatial interplay of literally hundreds of macromolecules that are in a constant state of flux. This elaborate design is consistent with a prevailing dogma, that the evolution of higher organisms neccessitates that complexity be selected at the expense of speed.

Two major differences between prokaryotes and eukaryotes might underlie the need for a more sophisticated network amenable to diverse regulatory pathways: 1) In eukaryotes a nucleus physically separates the acts of transcription and translation thereby allowing independent control; 2) Eukaryotic mRNAs are generally much more stable. Therefore,

a quick response to an environmental stimuli may require the selective translational control of pre-existing mRNAs in the cytoplasm. To date, there is a rapidly increasing list of translational control of gene expression, including during development, neoplastic transformation and viral infection (discussed in detail, section 1.2.7). Moreover, the concept of translational control has recently gained popularity due to the enormous interest in generating highly efficient expression vectors. Elucidation of control mechanisms requires a sound knowledge of the components and sequence of events involved in the pathway.

Before describing the general mechanism, it is important to better qualify the term, eukaryotic protein synthesis. The very distinct protein synthetic pathways employed by organelles is excluded (for review, see ref. 31). Furthermore, it must be emphasized that the majority of the mechanistic details have been based on reconstitution experiments using mammalian (most notably rabbit reticulocytes) cell-free protein synthesis extracts (31-37 and refs. therein). Despite this limitation, it is clear that mammals, plants and fungi are extremely similar in their approach to synthesizing proteins. This is supported by the recent characterization and cloning of initiation factors from widely diverse species. As will be described in greater detail below (section 1.2.4 and 1.2.5) initiation factors isolated from various sources have similar roles, many with the ability to function in heterologous systems. In addition genetic and mutational studies aimed at analyzing the role of the 5' untranslated region on protein synthesis (section 1.2.6) have shown that they have similar properties whether assayed in a yeast or mammalian background. Since all these

aforementioned similarities occur at the initiation level of translation, which is by far the most complex step in the entire process (and most different from prokaryotes), it seems reasonable to assume that all nuclear-encoded protein synthesis occurs by a highly conserved mechanism. One caveat to keep in mind, however, is that different organisms or cells may have evolved distinct regulatory pathways to satisfy their own specialized needs.

1.2.2 The roles of RNA

Three types of RNA molecules perform different but cooperative functions during protein biosynthesis. These are as follows; 1) mRNA, 2) transfer RNA (tRNA), and 3) ribosomal RNA (rRNA). The role of mRNA is mainly to transport the genetic information stored in the nucleus to the cytoplasm where the triplet code can be converted into a defined polypeptide sequence. In addition, the structure of the mRNA (especially the 5' untranslated region) can act to influence the rate at which protein synthesis occurs. The role(s) of mRNA is of central importance to the thesis material and will be considered in greater detail below (sections 1.2.6-1.2.7).

The high fidelity in transfer of nucleotide information into chains of amino acids is imparted by an adapter RNA molecule, transfer RNA (tRNA). Its role is to direct amino acids to the proper sites of peptide bond formation on the ribosome (38). The decoding of mRNA relies on Watson-Crick interactions between the coding triplet on the mRNA and the anticodon (complementary) sequence on the tRNA. This interaction is stabilized by the ribosome.

Attachment of a tRNA molecule with its appropriate amino acid occurs in two steps, both catalyzed by a specific enzyme (for review, see refs. 39) that has dual function. The aminoacyl-tRNA synthetases can recognize both the tRNA molecule and its cognate amino acid. The process of amino acylation can be summarized as follows.

(1) amino acid + ATP $\rightleftharpoons$ aminoacyl-AMP + PPi

(2) aminoacyl-AMP + tRNA $\rightleftharpoons$ aminoacyl-tRNA + AMP

In the first step the amino acid becomes activated by retaining the high energy phosphate bond of the donor ATP. The equilibrium of the reaction is enhanced by cleavage of PPi to inorganic phosphate by a pyrophosphatase. In the second step the activated amino acid is transferred to the tRNA molecule, a form compatible with protein synthesis. Some of the specificity in the reaction catalyzed by the synthetases has recently been described (40). Surprisingly, a single base pair can determine the amino acid specificity of a tRNA molecule. Furthermore, the recognition site in the tRNA does not include the anticodon. There are 20 aminoacyl-tRNA synthetases, each of which can recognize all the tRNAs (up to six) specific for a given amino acid. However, one tRNA can recognize multiple codons due to the wobble effect, whereby nonstandard base-pairing occurs between the third position of the codon and its partner in the anticodon. Fidelity is maintained because although a codon can be recognized by more than one kind of tRNA (degeneracy), each tRNA will bear the same amino acid. In addition to its usual function during protein synthesis there are tRNA species involved in nonsense mutations and frameshifting (for review

see, 41), which may act to increase the coding potential of eukaryotic mRNAs.

The specific interactions between mRNA and tRNA are stabilized by the factory of protein synthesis, the ribosome. Ribosomes have two subunits that have independent roles in the initiation of protein synthesis, but work together to catalyze peptide bond formation. The small subunit (40S) contains one molecule of 18S ribosomal RNA (rRNA) and approximately thirty different polypeptides. The large subunit (60S) is more complex, containing one molecule each of 28S, 5.8S, and 5S rRNA, and 50 polypeptides. The structure of ribosomes is similar but not identical in all cells (for review, see refs. 31). Structurally distinct ribosomes may function to control protein synthesis during development (42). However, due to the large size and complex nature of the ribosome our understanding of this intricate 'enzyme' is somewhat rudimentary. A molecular model for the understanding of its detailed mechanism will have to await better resolution of its three- dimensional structure (43,44). The recent findings by Cech and co-workers that RNA has enzymatic properties (45) has legitimized the notion that rRNA might play a more significant role during protein synthesis than previously anticipated (46).

1.2.3 General Mechanism

Protein biosynthesis is considered to occur in three stages; a) initiation - all the steps required to ensure recognition of the 'proper' initiator codon; b) elongation - stepwise addition of amino acids in a sequence determined by the base sequence of mRNA; c) termination - the final stage, release of ribosome particle and newly

synthesized polypeptide from the mRNA. (For a recent review on protein synthesis, see ref. 47).

a) Initiation - At the end of this stage the Met-tRNA$_i$ (i = initiator) is positioned over the start AUG codon, the interaction stabilized by the 80S ribosome. Since this step is intimately linked to the role of cap binding proteins and translational control (48) it is discussed in the next section (1.2.4).

b) Elongation - The codon adjacent to the initiating AUG (downstream) and all subsequent codons, are recognized during the elongation phase of protein synthesis. During this stage the ribosome catalyzes the sequential addition of amino acids to the growing polypeptide chain in a sequence dictated by the interaction between aminoacyl-tRNAs and the available triplet in the A (acceptor) site in the ribosome. The reaction involves two elongation factors, EF-1 and EF-2, and GTP hydrolysis. Three distinct steps can be discerned, ternary complex formation, peptide bond formation, and translocation.

EF-1 is a multi-subunit factor (α,β,γ) whose role is similar to that of eukaryotic initiation factor 2 (eIF-2; section 1.2.4). It binds GTP to form a binary complex which then associates with aminoacyl-tRNAs, subsequently followed by delivery of aminoacyl-tRNA to the ribosomal particle. EF-1 can recognize all aminoacyl-tRNAs except the initiator methionine-tRNA$_i$ which is specifically recognized by eIF-2. Presumably differences in tRNA structure unique to the initiator species imparts the noted selectivity (31). Once the proper aminoacyl-tRNA molecule has recognized its complementary sequence in the ribosome particle, GTP is

hydrolyzed and an EF-1α•GDP complex is discharged (49). In a similar role as guanine exchange factor (GEF; sections 1.2.4 and 1.2.7) EF-1β catalyzes GDP/GTP exchange on EF-1α in order to recycle the elongation factor (50). Next, peptidyl transferase which is located in the 60S subunit (51) catalyzes peptide bond formation between the two aminoacyl-tRNAs. The deacylated tRNA in the P site is ejected, and the peptidyl-tRNA (one amino acid longer) is attached to the A site. By a mechanism known as translocation, the ribosome 'moves' 5' to 3' by precisely one codon such that the peptidyl-tRNA is now found in the P site and a new A site becomes available. Migration of the ribosomal particle is catalyzed by EF-2 and GTP hydrolysis (52). The entire sequence of events is repeated until a termination codon appears in the A site of the ribosomal particle.

Alternative models for the elongation of protein biosynthesis have been suggested (for review, see ref. 53). Briefly, they involve the simultaneous presence of three tRNA molecules. In addition, studies have shown the presence of a third elongation factor, EF-3, in yeast (54). Although not a frequent occurrence, there are examples of translational control at the level of elongation (55), including a recent study which showed that protein synthesis can be controlled by the selective phosphorylation of EF-2 (56).

c) termination - the final stage in polypeptide synthesis is signalled by the appearance of a stop codon (UAA, UAG, UGA) in the A site for which there, normally, is no meaningful aminoacyl-tRNA. This is followed by the codon-dependent binding of RF (release factor) which catalyzes in a GTP dependent reaction, the release of the polypeptide chain (57). The termination stage is not well understood and it is not

clear if the ribosomal subunits are released upon encountering the stop codon. The demonstrated capability of termination re-initiation (section 1.2.6) argues against release at a stop codon, at least for the 40S subunit. Ribosomes may just simply move along the mRNA until they 'fall-off' the 3' terminus.

1.2.4 Initiation of Protein Synthesis

The importance of this stage is underscored by the observation that in virtually all cell types that have been evaluated, protein synthesis is limited by the rate of initation (for review, see ref. 48). Consequently, it is not surprising that this is a key site for translational control (section 1.2.7). Unlike prokaryotes, where only 3 initiation factors are needed (for review, see 31,58), eukaryotes require numerous initation factors (~8), several auxiliary factors, and the expenditure of energy in the form of ATP hydrolysis (in addition to GTP hydrolysis).

a) Eukaryotic translation initiation factors

The correct positioning of an 80S ribosome particle at the start AUG is dependent upon soluble factors, termed eukaryotic initiation factors (eIFs) (Table I). They are operationally defined by satisfying the following two criteria; 1) stimulation of translation in a reconstituted protein synthesis extract (or partial reactions); 2) transient binding such that they only act during the initiation phase of the cycle and are released prior to 60S subunit joining to mRNA. In the mid 1970s three independent groups extensively purified and characterized at least 7 distinct eIFs required for natural mRNA translation (eIF-1,-2,-3,-4A,-4B,-4C,-5; 31-35,37), and have been the

TABLE I

Eukaryotic (mammalian) initiation factors and their probable function in translation initiation.[a]

Initiation factor	Subunit molecular mass (kDa)	Activity
eIF-1	15	Repositioning of Met-tRNA to facilitate mRNA binding.
eIF-2	35,50,55	Ternary complex formation
eIF-2B, GEF	34,40,55,65,82	eIF-2 recycling
eIF-3	~10 subunits 28-160	Subunit anti-association; 43S pre-initiation complex formation
eIF-4A ATPase	50	mRNA binding to 40S subunit;
eIF-4B	80	mRNA binding to 40S subunit
eIF-4C	17	Subunit anti-association; 60S subunit joining
eIF-4E (24K-CBP,CBP I)	24	5' cap recognition (subunit of eIF-4F)
eIF-4F (CBP II, CBP complex)	24,50,220	mRNA binding to 40S subunit; ATPase; mRNA melting activity
eIF-5	150	Release of initiation factors prior to 60S subunit joining ribosome-dependent GTPase
eIF-6	25.5	Subunit anti-association

a) This table was compiled from many different sources of data, taking into account generally accepted functions (for reviews see refs. 47,48,59,76).

subject of intense review by Hershey (59). Subsequently, a new initiation factor(s) which can bind 5' caps (eIF-4E/eIF-4F) was added to the list (60-63). In addition at least two other factors, co-eIF-2A (64) and GEF (guanine exchange factor, also termed eIF-2B; reviewed in ref. 65) are required for efficient translation. The importance of co-eIF-2A, however, has been a source of unresolved controversy (66,67), and might reflect different degrees of initiation factor purification.

In a problem alluded to above the rabbit reticulocyte has been the major source for the initial isolation and testing of each initiation factor. It has been suggested that the rabbit reticulocyte may not be a model system for establishing eukaryotic or even mammalian translation initiation due to its highly specialized and differentiated nature. Overwhelming evidence indicates that this concern is not of major significance. Equivalent initiation factors originally characterized from rabbit reticulocytes have been isolated from humans, plants and yeast, and they are remarkably conserved structurally and functionally. An initial study demonstrated that five initiation factors (eIF-2,-3,-4A, -4B, and -5) isolated from human HeLa cells and rabbit reticulocytes are similar by several criteria (68). Furthermore, initiation factors have been shown to function in heterologous systems. In fact, eIF-4F isolated from rabbit reticulocytes was originally assayed on the basis of its activity in a HeLa cell-free extract (62). These lines of evidence are supported by the recent characterization of genes or cDNAs from mammalian and yeast sources that encode initiation factors. These include eIF-4E (69,70), eIF-4A (71,72), and eIF-2β

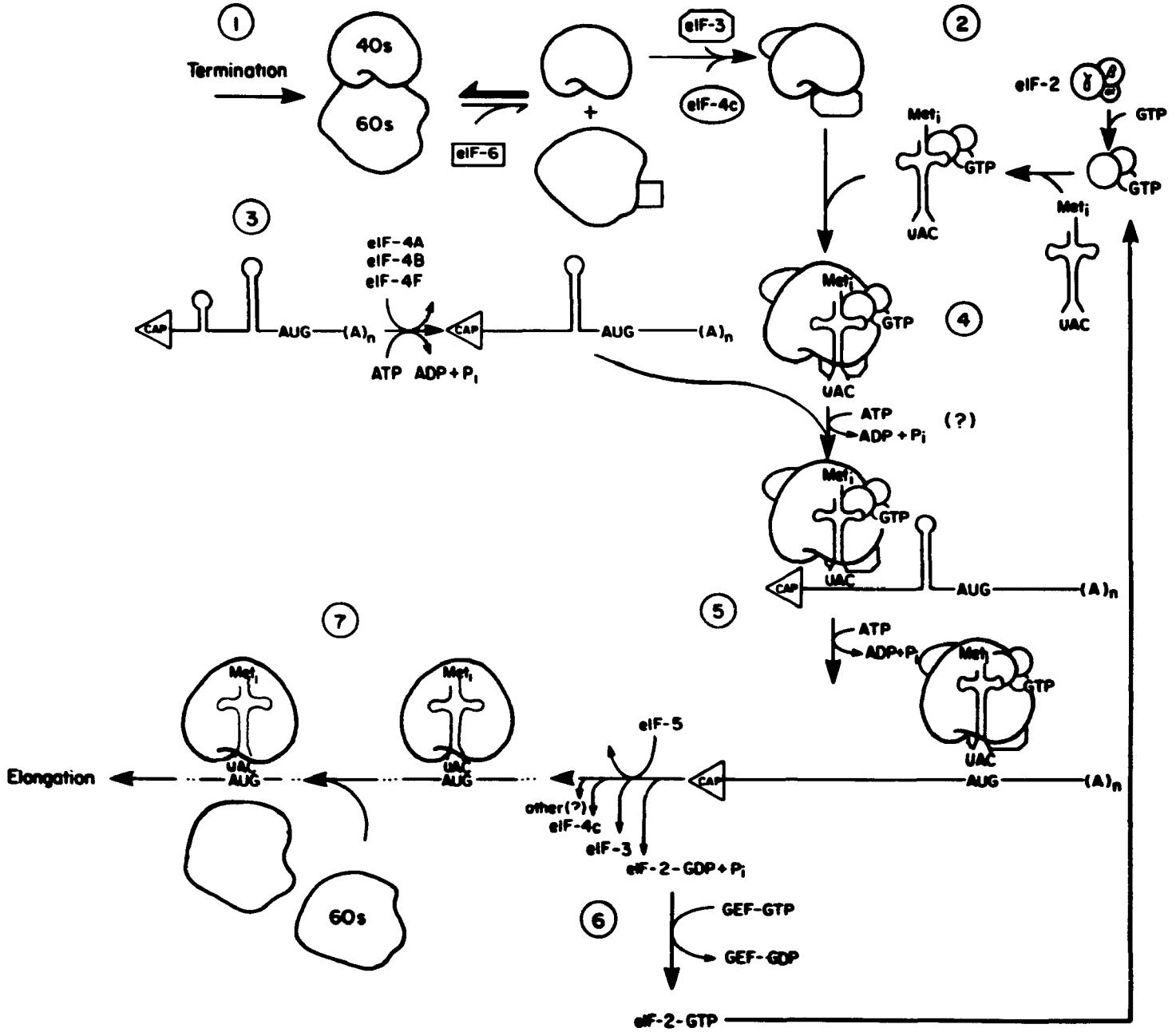
(73,74). Comparative sequence analysis revealed a high preservation in structure and function.

Although there are examples of initiation factor differences (especially when comparing mammalian and wheat germ mRNA binding factors; ref. 75), the overall sequence of events comprising translation initiation as depicted in Fig. 3 is most likely valid for all eukaryotic protein synthesis.

However, two important issues remain unresolved. As noted earlier initiation factors can stimulate protein synthesis in a reconstituted system that is deficient in the added factor(s). A reconstituted system that has the full complement of the initiation factors listed in Table 1 still only has ~5% of the activity obtained from its parental unfractionated system. Why does the fractionated system have such a poor translational efficiency? One possibility is that an initiation factor(s) is lost during fractionation and has yet to be detected. In light of all the effort and various purification schemes employed to isolate initiation factors this seems an unlikely possibility. A more tenable explanation is that initiation factors become impaired during purification either due to instability and/or removal of auxiliary factors required for activity. Consistent with the latter suggestion, Gupta and co-workers claim that auxiliary factors (i.e. co-eIF-2A) are required for maximal eIF-2 activity (66, 75a). Hopefully, the ability to generate from yeast, unfractionated translation extracts harbouring temperature sensitive mutations in selected initiation factors should aid in resolving this issue.

Fig. 3. Sequence of Events during Initiation of Protein Synthesis

The most widely accepted mechanism is depicted. Seven sub-steps are denoted and are as follows. (1) Dissociation of ribosomal subunits. (2) Ternary complex formation and binding to 40S native ribosomal subunit. (3) Unwinding of 5' proximal mRNA secondary structure. (4) Binding of 43S pre-initiation complex to mRNA. (5) Scanning of 43S pre-initiation complex to functional AUG start codon. (6) Release of initiation factors and recycling of inactive eIF-2•GDP binary complex. (7) Joining of 60S ribosomal subunit. See text for details.


1
Termination
40s
60s
eIF-6
eIF-3
eIF-4c
2
eIF-2
GTP
Met$_i$
GTP
GTP
Met$_i$
UAC
3
eIF-4A
eIF-4B
eIF-4F
CAP
AUG
(A)$_n$
ATP ADP + P$_i$
4
Met$_i$
GTP
UAC
ATP
ADP + P$_i$
(?)
5
ATP
ADP + P$_i$
6
GEF-GTP
GEF-GDP
7
Elongation
eIF-5
other (?)
eIF-4c
eIF-3
eIF-2-GDP + P$_i$
eIF-2-GTP

The second unresolved issue involves the possibility of an initiation factor(s) that is specific for one or a limited number of mRNAs. All the factors listed in Table 1 are required for translation of the majority of mRNAs that have been tested. Only eIF-4F, which can bind 5' caps, demonstrates limited mRNA selectivity in that it is not required for the translation of naturally uncapped mRNAs (section 1.2.5-1.2.7). The best evidence for a specific mRNA binding factor that selectively enhances translation is in the case of ferritin mRNA (Chapter 6). Therefore, it is reasonable to assume that all nuclear-encoded mRNAs require (at least to some extent) all the initiation factors shown in Table 1 for efficient translation. As a result all mRNAs should be amenable to translational control mechanisms that operate via modulation of initiation factor activity. Qualitative control is still possible due to the varying requirements or affinities individual mRNAs have for certain initiation factors (section 1.2.7).

Sequence of Events

The sequence of events comprising the initiation of protein synthesis can be reduced to four major steps (Fig. 3). I will concentrate on the 40S subunit binding to mRNA (step 3) due to its relevance to the understanding of cap binding proteins. The initiation pathway has been the subject of several intensive reviews (31,47,48,76).

i) Ribosome Dissociation

Subsequent to the termination phase of translation, 80S ribosomes released from mRNA transiently dissociate into its two subunits. The released 40S and 60S subunits have a high affinity for each other and

rapidly form 74S nonfunctional ribosomes (77). An obligatory step is the generation of free 40S subunits which can then engage in the initiation cycle of protein synthesis. The generation of 'free' 40S subunits is accomplished by the stoichiometric binding of the large multisubunit initiation factor, eIF-3 (78). This activity is reminiscent of the role IF-3 plays during prokaryotic translation initiation (58). Native eIF-3 has a molecular weight of approximately 700,000 (~15S; 7-10 polypeptides, ref.59), and can bind purified 40S subunits to form a faster sedimenting 43S native complex (79).

Evidence indicates that eIF-3 acts as an anti-association factor (78,80) and does not actively dissociate 74S nonfunctional ribosomes. In light of its large size it has been suggested that the anti-association activity of eIF-3 is due to steric hindrance of 60S association, thereby altering the equilibrium of re-association (78). The physiological significance of an eIF-3 mediated anti-association activity is complicated by the presence of two other initiation factors with dissociation activity. The factor eIF-4C is presumed to act as an accessory to eIF-3 by binding 40S subunits (81). In addition eIF-6, reacts with 60S subunits and prevents association (82-84). Thus, the three initiation factors might work in concert to generate 40S subunits that can enter another round of protein synthesis.

ii) Binding of initiator aminoacyl-tRNA to 43S Native Ribosomal Subunit

This step can be further subdivided into two separate events. The delivery of Met-$tRNA_i$ to the 43S native ribosomal subunit (40S ribosome• eIF-3 • eIF-4C discussed above) requires prior formation of a ternary

complex. Formation of the ternary complex has generated much interest because it has been shown to be a controlled event (section 1.2.7). The initiation factor, eIF-2, first binds GTP (non-hydrolyzable analogs also function) to form an intermediate binary complex, followed by the selective binding of the unique Met-tRNA$_i$ to form the ternary complex [eIF-2 • Met-tRNA$_i$•GTP] (32,85). The assay used to detect ternary complex formation is based on the protein-dependent retention of radiolabelled Met-tRNA$_i$ to nitrocellulose filters. The sequence of events is analogous to that catalyzed by elongation factor 1 (EF-1). Importantly, eIF-2 can only interact with the initiator aminoacyl-tRNA, whereas EF-1 has affinity for all the aminoacyl-tRNAs except the initiator molecules. Features distinctive to initiator tRNA molecules are believed to underlie this remarkable selectivity (31,86).

Once the ternary complex has assembled, it is competent to bind 43S native subunits, resulting in a 43S pre-initiation complex. Binding of radiolabelled Met-tRNA$_i$ to 40S subunit (i.e. devoid of eIF-3 and eIF-4C) can be detected in the presence of GTP and eIF-2 (87). The significance of this interaction is not clear. The addition of eIF-3 strongly increases the binding reaction and is not related to its anti-association activity (88). Moreover, a temperature sensitive yeast mutant (ts 187) that can not convert 40S to 43S subunits at the nonpermissive temperature also results in impairment of Met-tRNA$_i$ binding to 40S subunit (89,90). Since the ts187 mutant is most likely eIF-3 (or a subunit) this would suggest that 40S•eIF-3 formation is an obligatory step required for ternary complex binding. The multi-subunit

eIF-3 factor most likely acts to stabilize the interaction between the ternary complex and 43S native subunit.

eIF-2 is usually isolated as a three subunit (α,β,γ; ratio of 1:1:1) polymeric protein (32). The α-subunit ($\sim M_r$ 38,000) binds GTP, but has a 100 fold higher affinity for GDP (91). Binding of GDP by eIF-2α strongly inhibits ternary complex formation. It is noteworthy that upon completion of the initiation cycle eIF-2 is released as an inactive binary complex bound to GDP (92). Mechanistically this presents a severe problem because a major proportion of the eIF-2 in the cell would seem to be bound to a potent inhibitor. This dilemma is circumvented by the catalytic recycling of GDP for GTP by guanine exchange factor (GEF; or also commonly termed eIF-2B to be consistent with initiation factor nomenclature). This results in an eIF-2α•GTP binary complex which can now re-enter another cycle of protein synthesis (Fig. 3). Several models have been proposed to account for this exchange reaction (for review, see ref. 65). Similar scenarios have also been proposed for the recycling of prokaryotic elongation factor EF-Tu. Therefore, the GTP/GDP status of the cell, or energy state, in addition to the GEF catalyzed recycling reaction can potentially regulate ternary complex formation and thus protein synthesis. The role of GEF in translational control is considered in greater detail below (section 1.2.7).

The role of the β- and γ-subunits of eIF-2 are not clear. Early studies suggested that the β subunit binds Met-$tRNA_i$ and mRNA as measured in <u>in vitro</u> partial reactions (91). Consistent with previous

reports showing the binding of eIF-2 to mRNA (93-95). However, the validity of these latter conclusions have been questioned since they were mostly based on nitrocellulose filter binding assays which were shown to be very unreliable for these types of experiments (96). Furthermore, the discovery of cap binding proteins and their role in mRNA binding immediately overshadowed the significance of any mRNA binding activity previously attributed to eIF-2. However, a recent finding indicates that the eIF-2 binding to mRNA saga might still be a viable one. A mutant β-subunit of eIF-2 from yeast allowed initiation to occur at a UUG codon in the absence of the usual AUG start codon (73). Interestingly, the single base change was at a zinc finger motif. These motifs have been implicated in the ability of proteins to recognize nucleic acids (for reviews, see refs. 97,98). Future work aimed at substantiating this elegant mutational approach should settle the long-standing controversy surrounding the specificity and significance of an eIF-2 mRNA binding activity.

iii) Binding of 43S pre-initiation complex to mRNA

This step is considered to be the most crucial during the entire protein synthetic pathway. Several important reasons underscore the significance of this step, and are as follows.

(1) Evidence indicates that this is the rate-limiting step in the overall translation (48,99), and therefore a likely candidate for translational control.

(2) It is at this step that an mRNA is first recruited to enter the cycle of protein synthesis, thus enabling both quantitative and qualitative control of translation (section 1.2.7).

(3) It is a very complex step requiring the participation of at least three initiation factors and the hydrolysis of ATP (prokaryotes do not require ATP, section 1.2.5).

(4) There is no equivalent of a 'Shine-Dalgarno' sequence to guide the 40S subunit to the appropriate site on the mRNA ensuring translation in the proper reading frame. In addition structural features in the 5' untranslated region can influence the efficiency of 43S pre-initiation complex binding to mRNA. Evidently, this step is dramatically different than that employed by prokaryotes (section 1.2.6).

(5) There is increasing evidence that this is a key site for regulating gene expression at the level of translation (section 1.2.7).

I shall briefly summarize the sequence of events most highly regarded as occurring during 43S pre-initiation complex binding to mRNA. Two complementary experimental approaches have resulted in elucidation of this mechanism. They are, 1) Trans-acting factors - isolation and characterization of purified initiation factors required for natural mRNA protein biosynthesis, and ii) Cis-acting factors - a combination of comparative sequence analysis and mutational studies directed at the 5' untranslated region of eukaryotic mRNAs, which ultimately led to the identification of elements that influence translation. In the next two sections (1.2.5 and 1.2.6) each individual approach and their implications are discussed in greater detail.

i) The initiation factors required for 43S subunit binding to mRNA were initially identified by studies of cell-free translation systems

reconstituted from purified rabbit reticulocyte initiation factors and salt-washed ribosomes (i.e. initiation factor free). Early reports (32-34,37) showed the requirement for two initiation factors, eIF-4A and -4B. Subsequently, a new purification scheme (61) led to the discovery of a new initiation factor that can bind the 5' cap, eIF-4F. In addition to these three factors, ATP hydrolysis is also required (34,100,101).

How do the initiation factors in the presence of ATP hydrolysis facilitate 43S subunit binding to mRNA? This question has recently been the subject of several reviews (102-105). Briefly, eIF-4F binds the 5' cap structure and then in conjunction with eIF-4A, -4B and the hydrolysis of ATP unwinds 5' proximal mRNA secondary structure, consequently enhancing small subunit binding. This helicase activity presumably underlies the stimulatory role of the 5' cap structure during protein biosynthesis.

ii) Once the 43S pre-initiation complex binds the 5' end of the mRNA how does it recognize the appropriate AUG initiation codon? To address this question Kozak originally proposed that 43S subunits bind at or near the 5' cap then migrate along the mRNA and stop at the first AUG they encounter whereupon the 60S subunit joins (106). This scanning hypothesis and variations of this theme (107-109) was formulated to account for certain peculiarities of eukaryotic mRNAs. Mainly, their overwhelming monocistronic nature, almost exclusive preference for the functional start AUG to be the 5' most proximal and lack of any equivalent 'Shine-Dalgarno' recognition site on the mRNA. Interestingly, recent evidence indicates that the small ribosomal

subunit can bind a certain class of mRNAs by an alternative mechanism (110).

iv) Initiation Factor Release and 60S Ribosomal Subunit Joining

Prior to large (60S) ribosomal subunit joining, Met-tRNA$_i$, 40S subunit, eIF-2 GTP, eIF-3 and eIF-4C are all present on the mRNA to form a 48S pre-initiation complex. It is important to note that this sedimentation value (i.e. 48S) is calculated under conditions where elongation is inhibited. In the normal situation, initiation events are occurring simultaneously on mRNAs bound to elongating ribosomes (polysomes). With respect to eIF-4A and eIF-4B it is believed that they are not present on the mRNA subsequent to 43S binding to mRNA (34). The fate of eIF-4F is not clear and is complicated by its multi-subunit nature. Using immunological staining techniques two studies reported the subcellular localization of the 24 kDa subunit of eIF-4F. In one study it was shown to be absent on both monosomes and polysomes (111) in stark contrast to another study demonstrating its presence in polysomes (16). Furthermore, the large subunit of eIF-4F (p220) is claimed not to be present on 48S complexes (105). The reasons for this apparent discrepancy are not immediately evident.

In any event, the binding of the 60S subunit is catalyzed by a specific factor, eIF-5 (32-34). The role of eIF-5 is to release the other bound initiation factors (i.e. eIF-2, -3, -4C, at least) in a reaction that is dependent upon the hydrolysis of the GTP molecule originally bound by eIF-2 (114). Release of initiation factors (especially eIF-3) is presumably necessary to remove the steric hindrance imposed by their presence in the vicinity of 60S subunit

binding (113). Non-hydrolyzable analogs of GTP do not allow for 60S subunit binding (101). Once GTP is hydrolyzed and eIF-3, eIF-2•GDP and other factors are released the joining of 60S subunit seems to occur in the absence of any additional factors. With the discovery of eIF-6 and its anti-association activity vis a vis the 40S subunit, this would imply that eIF-6 is released prior to 60S subunit joining. The end of the initiation cycle is achieved when the Met-tRNA$_i$ is positioned over the proper AUG initiation codon and the 80S ribosomal particle is competent to enter the elongation stage of protein synthesis.

1.2.5 Initiation factors that mediate cap function during translation

Despite its importance for translation, the requirement for the cap structure during this process appears to be neither absolute nor universal. Consequently, two pertinent questions arise: (1) What factors influence the degree to which an mRNA is dependent on the cap structure for efficient translation and how is this requirement implicated in regulation of translation? (2) How do naturally uncapped eukaryotic messages (i.e. poliovirus) bypass the requirement for the cap structure for translation? To answer these questions, it is imperative to study the factors that mediate cap recognition and elucidate their function in protein biosynthesis. These studies are important for understanding the control of translation because as noted earlier, binding of 40S ribosomal subunits to mRNA is the overall rate-limiting step in translation and therefore a potential target for regulation.

As detailed below, the factors involved in mediating cap function have been identified, however it is not known precisely what biochemical reactions they perform. Nonetheless, recent findings have clarified

this area immensely and provide a basis to formulate working models. The available data are consistent with the original proposal for the role of the initiation factors that mediate cap function during translation (114,115). Furthermore, this proposed cap binding protein dependent melting of 5' proximal mRNA secondary structure might underlie the discriminatory and regulatory activities attributed to eIF-4F (discussed below).

Two different experimental approaches have been used to identify cap binding proteins (CBPs). One approach involves the formation of covalent complexes between protein factors and capped mRNAs (Fig. 4; ref. 116,117). This method has yielded two sets of 'CBPs' one that does not require ATP to bind to the cap structure and another that is dependent on the hydrolysis of ATP for binding. A second approach has been to affinity purify CBPs by employing cap analogs coupled to a solid support matrix (118-121). The combined results from these approaches established a set of protein factors that interact with each other to mediate cap function. The functional significance of some of these polypeptides for initiation of protein synthesis was studied by making use of poliovirus-infected HeLa cell extracts. As outlined below (section 1.2.7) poliovirus infection inactivates CBP function and, because it does so, proved to be an invaluable tool in studying the role of CBPs in translation initiation.

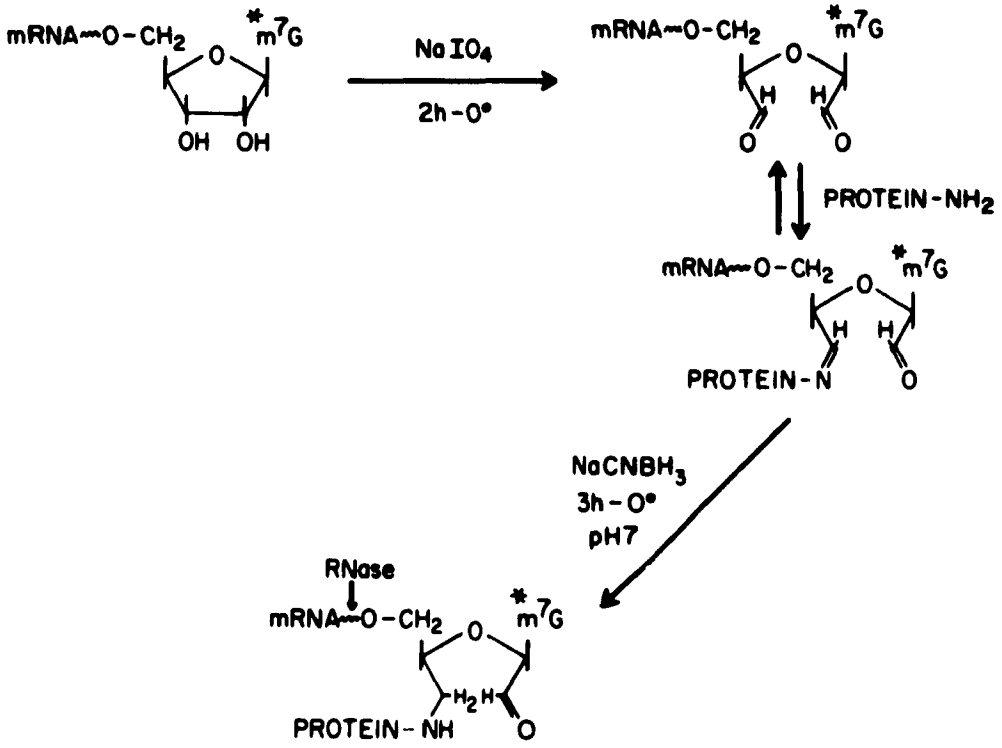
a) Early studies

The role of the cap structure in in vitro translation systems was established by several experiments. First, it was found that capped mRNAs of viral origin [reovirus and vesicular stomatitus virus (VSV)] or

Fig. 4. Chemical Cross-Linking Assay to Detect CAP Binding Proteins

Cap-radiolabeled in vitro transcribed mRNA is oxidized to the reactive dialdehyde. Any putative Schiff base formed between the oxidized cap and amino groups on proteins is stabilized in the presence of $NaBH_3CN$. This is followed by RNAse digestion resulting in a protein covalently attached to a labelled cap. The incubation mixture is resolved by SDS-PAGE and cross-linked proteins visualized by fluorography and autoradiography. Specificity for the cap is determined by performing parallel incubations in the presence of saturating amounts of cap analogs.

SCHEME FOR CROSS-LINKING mRNA 5′-END TO PROTEINS



cellular origin (rabbit globin) are translated more efficiently than their uncapped counterparts (122-124). Second, it was observed that cap analogues, such as m^7GMP or m^7GDP, specifically inhibited translation of capped mRNAs by decreasing 40S ribosomal subunit binding to mRNAs (125-128). It was therefore concluded that cap analogues competitively inhibit the function of one or more proteins required for translation initiation by saturating their cap interacting site(s). Several studies were conducted to identify putative CBPs. The first approach involved the analysis of protein-dependent mRNA retention on nitrocellulose filters (129-131). However, contradictory results were obtained with different purified initiation factors (IF; 130,131). One study found that IF-M3 (eIF-4B) had cap-binding activity (130), whereas another study showed that binding of eIF-2 to capped as well as to uncapped mRNA was inhibited by m^7GMP (131). As noted earlier it was shown that most IFs had a high affinity for RNA, regardless of the species used, and cap analogues inhibited protein-RNA complex formation in a non-specific manner.

In light of these limitations, Sonenberg and Shatkin (116) developed a chemical cross-linking assay to identify polypeptides that can bind at or near the cap structure in a specific manner (Fig. 4). Using this assay, Sonenberg et al. (117) identified a polypeptide of ~24 kDa in IF preparations from rabbit reticulocyte lysates and termed it the 24K cap-binding protein (24K CBP). This polypeptide was present in highly purified preparations of eIF-4B and eIF-3, a finding that ultimately led to the clarification of several activities previously attributed to these factors. Subsequently, similar CBPs were identified in such

diverse species as yeast (132), humans (133, 134) and plants (135,136). Several other cross-linking methods devised to identify m^7G-specific polypeptides invariably detected the 24K CBP in crude IF preparations (137,138).

The 24K CBP was subsequently purified to apparent homogeneity by exploiting its affinity for cap analogues (118). Several different resins were used for the purification of CBPs from rabbit reticulocytes (63,119,120), yeast (132), humans (139), and wheat germ (135,136). Other approaches using conventional protein-purification procedures were also employed (140,141). The 24K CBP purified from rabbit reticulocytes was shown to possess biological activity, since it stimulated the translation of capped mRNAs in a HeLa cell extract (60). In accordance with this activity the 24K CBP was termed eIF-4E (the eIF-4 series is reserved for initiation factors required for 40S subunit binding to mRNA). However, it is not clear whether or not to consider the 24K CBP as an initiation factor (eIF-) in the strict sense. This is because (as outlined below) the 24K CBP does not seem to have any physiologically significant role unless it is complexed with other proteins to form eIF-4F. I will use both 24K CBP and eIF-4E when referring to the small cap binding protein that is translationally associated since both terms are still used in publications.

One of the salient features that distinguish the binding of the small ribosomal subunit to mRNA in eukaryotes from that in prokaryotes is the requirement for ATP hydrolysis in the eukaryotic system (34,100,101). The role of ATP in this step has been a subject of intensive studies and is dealt with in detail in light of the model

proposed for the function of CBPs in translation initiation. Unlike the ATP-independent chemical cross-linking of eIF-4E to mRNA, additional polypeptides of 28-,50-, and 80-kDa in rabbit reticulocyte IF preparations specifically cross-linked to the oxidized cap structure of mRNAs in an ATP-Mg^{2+}-dependent manner (114,115).

A similar set of CBPs was later identified in IF preparations from HeLa cell extracts (134), but a polypeptide of 32 kDa was unique to the HeLa system. Nonhydrolyzable analogues of ATP did not substitute for ATP in the cross-linking reaction. It was suggested that ATP hydrolysis as opposed to protein phosphorylation is required for the cross-linking reaction (114).

The identity of the 50- and 80-kDa CBPs has been established. Grifo et al. (142) reported that based on several criteria the 50- and 80-kDa CBPs were probably eIF-4A (50 kDa) and eIF-4B (80 kDa), respectively. The identity of the ATP-dependent cross-linkable 50 kDa CBP as eIF-4A was confirmed by immunoprecipitating the cross-linked 50 kDa CBP in the presence of a monoclonal antibody to eIF-4A (63). Recently, by the use of a similar approach, it was concluded that the cross-linkable 80 kDa CBP is eIF-4B (143). The identity and significance of the 28- and 32-kDa polypeptides that cross-link to mRNA in a cap-specific manner are unclear. Interestingly, the migration of the 24 kDa CBP is changed from approximately 24 to 28 kDa, as a result of disulfide-bond reduction, probably reflecting a modified protein structure (144). Since the 28-kDa CBP is not always cross-linked, it may represent a different oxidized form of the 24K CBP. With respect to the 32 kDa CBP, a polypeptide in HeLa crude nuclei preparations, of

approximately similar molecular mass (≈37 kDa), cross-linked to the cap by photochemical cross-linking (137,145). Perhaps the 32- to 37-kDa CBP that is specific to the HeLa system leaks out of the nucleus during cell fractionation and cofractionates with IF preparations. Its absence in rabbit reticulocytes (which are enucleated) is consistent with this idea.

Do all the known cytoplasmic CBPs, as identified by the chemical cross-linking assay contain a primary cap-binding site; i.e. could they interact individually and directly with the cap structure. Probably not. Historically, the term CBP was applied to any polypeptide that satisfied the criteria for cap specificity as assayed by chemical cross-linking. The available data suggest that the only "CBP" with a bona fide cap recognition site is the 24K CBP (61-63,116-120,132, 135-138). The most relevant and significant finding is that the only CBP purified as an individual entity by m^7GDP-affinity chromatography is the 24K CBP (118-120,132). Therefore, eIF-4A and eIF-4B are not cap binding proteins per se, but most likely interact with the cap subsequent to 24K CBP binding (as part of eIF-4F, discussed below).

b) Discovery of a new initiation factor

There is a consensus that poliovirus infection of HeLa cells causes the inhibition of host protein synthesis by inactivating a CBP function (for recent reviews, see refs. 146,147). This model is highly attractive, since poliovirus RNA is naturally uncapped (148,149) and therefore must bypass a cap-dependent mechanism for translation.

Several lines of evidence led to the eventual conclusion that the poliovirus-induced lesion to the host cell translational machinery was

at the level of the initiation step of translation resulting from a defect in IF activity (150-155). Two early reports suggested that either eIF-4B (156) or eIF-3 (157) was rendered inactive as a result of poliovirus infection. These different conclusions were soon to be reconciled by the study of Trachsel et al. (140) who showed that the 24K CBP could restore the ability of extracts prepared from infected cells to direct cap-dependent mRNA translation (this ability was defined as restoring activity). The results from the previous two studies (156,157) could therefore be explained as being due to the presence of cofractionating 24K CBP. However, Trachsel et al. (140) claimed that the restoring activity of highly purified 24K CBP was unstable and suggested that other components may be required to stablize the activity.

That the 24K CBP could associate with other proteins had already been suggested by sucrose sedimentation analysis (118,158). Tahara et al. (62) used a modified purification scheme and first described a high molecular weight form of the 24K CBP that can be isolated by m^7GDP-affinity chromatography. Included in this complex (8-10S) were major polypeptides of 48,55, and 210 kDa in addition to the 24K CBP and was termed CBPII to distinguish it from the 24K CBP which they termed CBPI. Importantly, they showed that CBPII, but not CBPI, had stable restoring activity and consequently raised the question as to the actual functional form of the 24K CBP in the cell.

Subsequently, two other reports described similar high molecular weight CBP complexes from rabbit reticulocytes. Grifo et al. (61), showed that their preparation (24K CBP and polypeptides of 46,73 and 220

kDa) stimulated globin mRNA translation in a rabbit reticulocyte reconstituted translation system, and consequently this complex was given bona fide IF status as eIF-4F. Using a different m^7GDP-coupled affinity matrix, Edery et al. (63) described the purification of a similar complex (24K CBP, 50- and 220-kDa polypeptides) and termed it the CBP complex, since it was deficient in some of the polypeptides described by the other two groups.

Several studies were done to characterize the components of the CBP high molecular-weight complex (CBPII, eIF-4F, CBP complex). It is clear that the 24K CBP is present in all the preparations described (61,63,160). The 48-,46-, and 50-kDa polypeptides present in CBP II, eIF-4F and the CBP complex, respectively, are most probably the same polypeptide, that being eIF-4A (61,63,160). This was confirmed by tryptic peptide mapping and immunoblotting with a monoclonal antibody against eIF-4A (63). The 55-kDa and 73-kDa polypeptides present in CBP II and eIF-4F, respectively, are apparently of no significance to the cap-recognition function, since they are absent in the CBP complex. The largest polypeptide described as having an apparent molecular mass between 200 and 220 kDa (termed p220) in the different studies, is required for restoring activity in extracts from poliovirus infected cells and is therefore apparently important for cap function (discussed below).

It is clear that CBP II, eIF-4F, and CBP complex probably reflect a single functional entity as pertains to the cap-recognition event and differ only in their degree of purity. To be consistent with recent reports and IUPAC nomenclature it is referred to as eIF-4F.

c) Structural and functional characterization of individual polypeptides involved in cap function

The following section is concerned with summarizing the important structural and functional attributes of each polypeptide which may allude to their precise physiological role during translation initiation. The major point being that each individual polypeptide has a specialized function that works in concert with the other polypeptides resulting in a multi-subunit helicase activity (discussed in Chapter 6).

i) eIF-4E (cap binding)

The 24K CBP can exist in two molecular forms: free and complexed (with eIF-4A and p220). As mentioned earlier the probable functional entity of the 24K CBP is the complexed form, eIF-4F. Are there structural modifications in 24K CBP which govern its distribution between the free and the complexed form? In this context it is noteworthy that the 24K CBP exists in different isoelectric forms (118,144). Rychlik et al. (144) showed that only one variant present in reticulocytes is significantly labelled in the presence of [^{32}P] orthophosphate. Further, the phosphorylation site of the 24K CBP has recently been identified as ser-53 in the human eIF-4E sequence (161). It was reported that the same four species of 24K CBP, in approximately the same relative proportions, were found in preparations from both the polysomal and postpolysomal fractions (162). This would imply that structural modifications do not govern the distribution of the 24K CBP between the free form and eIF-4F. Notwithstanding, there are examples

of translational control that correlate strongly with the phosphorylated status of eIF-4E (section 1.2.7).

An interesting feature of the 24K CBP is its unusually high tryptophanyl content (144). It was demonstrated that the cap structure has high affinity for tryptophans, especially in a negatively charged environment (163). Since the positive charge (due to N-7 methylation) in the cap is absolutely required for function (164), it is likely that some negatively charged amino acids are present in the cap interacting site to facilitate an ionic interaction. As suggested by Rychlik et al. (144) this might underlie the highly specific interaction between the 24K CBP and the cap.

Strikingly, sequence analysis of cDNA clones of eIF-4E from several species revealed that all tryptophans are conserved evolutionarily in number (8) and position (refs. 69,70,165). Site-directed mutagenesis indicated that tryptophans 1 (amino acid #43) and 8 (amino acid #166) are absolutely required for cap recognition in vitro, whereas other mutations had smaller or no effect on activity (166). In a related study the biophysical properties of native and two mutant forms of eIF-4E were investigated (167). Although the two mutant forms tested, [tryptophan 4 (amino acid #75) and 6 (amino acid #115)] had similar k_d values for m^7GDP, they nevertheless had less specificity for N-7 methylated nucleotides. Therefore, tryptophan residues in eIF-4E are not only required for cap binding but also for specific recognition of N-7 methylated nucleotides. Cap binding might be imparted by base stacking interactions between tryptophans and the nucleotide base moiety. Specificity might require intramolecular tryptophan-tryptophan

TABLE II (CHARACTERISITICS OF mRNA-BINDING INITIATION FACTORS)

initiation factor	origin	Molecular Mass (kDa)	specific cross-linking to mRNA 5' cap		activity[a]
			ATP dependent	ATP independent	
	mammal	50	+	-	stimulates translation; ATPase
$eIF\text{-}4A_f$	plant	51	+	-	stimulates translation; ATPase
	yeast	?	?	?	?
	mammal	80	+	-	stimulates translation; stimulate eIF-4A and eIF-4F ATPase; AUG recognition; recyle eIF-4F
eIF-4B	plant	59-65	+	-	stimulates translation
	yeast	?	?	?	?
	mammal	24		+	binds cap structure
eIF-4E (24K-CBP; CBP I)	plant	i) 26 ii) 28		+	binds cap structure
	yeast	24		+	binds cap structure
	mammal	24, 50, 220	+(50 kDa)	+(24 kDa)	ATPase; mRNA unwinding; restoring activity
eIF-4F (cap binding protein complex; CBP II)	plant	i) 26,220 ii) 28,80 iii) 26,28,75		i)+(26 kDa) ii)+(28 kDa) iii)+(26 or 28 kDa)	i) stimulates translation; ATPase ii) stimulates translation; ATPase ii) stimulates translation
	yeast	24, 150		+ (24 & 150 kDa)	?

a - activity indicated as stimulation of translation refers to stimulation of exogenous mRNA translation in a reconstituted translation system.

interactions to stabilize the protein structure (168). Ultimately, elucidation of the three-dimensional structure of eIF-4E will greatly enhance our understanding of the remarkable specificity it has for the cap structure.

Up till now I have referred to eIF-4E as a singular entity, i.e. a polypeptide of ~24kDa with cap binding activity. This simple relationship may not apply to wheat germ eIF-4E. In wheat germ there are two CBPs, a 26 kDa and 28 kDa polypeptide (169). Slight variations in the eletrophoretic mobility of the mammalian eIF-4E have been attributed to the degree to which the cysteine residues are oxidized (144). However, this observation does not adequately explain the situation for the wheat germ CBPs, since they are antigenically distinct polypeptides (169). Although the two wheat germ CBPs are structurally distinct there is no evidence to suggest that they are functionally distinct (170). As will be addressed below it appears that wheat germ eIF-4B (containing the 28 kDa CBP) is really an isozyme of eIF-4F (containing the 26 kDa CBP) and that the 'real' eIF-4B is another factor recently characterized (135,136). To simplify the properties attributed to the different mRNA binding factors isolated from various sources I have summarized what is known in Table II.

ii) eIF-4A (ATPase)

Elucidating the role(s) of eIF-4A in translation initiation is complicated, because in addition to being a subunit of eIF-4F, the majority (over 90%) of eIF-4A exists as a free form in the postribosomal supernatant (171). On a molar basis, eIF-4A is the most abundant IF at a ratio of about 3 molecules per ribosome (172). The relatively small

amount of 24K CBP (0.2-0.5 molecules per ribosome; ref. 173) in the cell must therefore limit the fraction of eIF-4A molecules found as a subunit of eIF-4F.

The unwinding model predicts that eIF-4F should interact with ATP, and several reports have confirmed this predication. Grifo et al. (174) described an RNA-dependent ATP hydrolysis reaction catalyzed by eIF-4A and eIF-4F. Although eIF-4B had little or no ATPase activity by itself, it stimulated the activity of either eIF-4A or eIF-4F. The authors suggested that ATP is hydrolyzed directly without a phosphoprotein intermediate. In a related study it was shown that the ATPase activity of eIF-4A is only activated by RNA which is lacking in secondary structure (175). Furthermore in the presence of ATP, eIF-4A is capable of binding mRNA. These properties have significant implications for the role of eIF-4A during internal binding of 40S ribosomes to mRNA (Chapter 6). Lax et al. (176) reported on the ATPase activities of wheat germ initiation factors 4A,4B and 4F. In contrast to rabbit reticulocytes, wheat germ eIF-4B alone exhibited RNA-dependent ATPase activity. However as already mentioned, it is not clear whether the eIF-4B preparation used is the equivalent of the rabbit reticulocyte eIF-4B or an isozyme of eIF-4F.

To directly identify the ATP binding protein in eIF-4F, Sarkar et al. (177) performed photoaffinity labelling of purified IFs in the presence of α-[^{32}P] dATP. [Purified eIF-4A was termed eIF-4A$_f$ (f = free), whereas eIF-4A in eIF-4F was termed eIF-4A$_c$, (c = complex)]. Significantly both eIF-4A forms cross-linked to ATP, however eIF-4A$_c$ cross-linking was about 60-fold better on a molar basis. Furthermore, Seal et al. (178) found that the ATP analogue 5'-fluorosulfonylbenzoyl

adenosine inhibited wheat germ eIF-4A activity, presumably as a consequence of irreversible covalent modification of the IF.

Recently, mouse cDNA clones (71) and genes (179) to eIF-4A have been isolated and characterized. The eIF-4A gene family consists of three groups; non-functional retroposons or pseudogenes, possibly functional retroposon and functional genes. The two functional intron-containing eIF-4A genes were termed eIF-$4A_I$ and eIF-$4A_{II}$. Comparative sequence analysis revealed that both have an identical putative ATP binding site, GXGKT (180). The intriguing possibility that the two functional genes (eIF-$4A_I$ and eIF-$4A_{II}$) might encode the two forms of eIF-4A (e.g. eIF-$4A_c$ and eIF-$4A_f$), and that these structural differences can influence activity and/or ability to interact with the other subunits of eIF-4F will be addressed in Chapter 6.

iii) <u>eIF-4B (multiple roles?)</u>

Our understanding of the role(s) eIF-4B plays during translation initiation is far from adequate. Although eIF-4B is absolutely required for natural mRNA translation (34,101) no specific function has yet been uniquely and unequivocally attributed to eIF-4B. The following properties have been ascribed to purified eIF-4B.

(1) In most partial reactions studied the presence of eIF-4B had a stimulatory effect. These include; a) chemical cross-linking of eIF-4A to caps (61,63,160,174); b) ATP binding to eIF-4A (177); c) eIF-4A mediated ATPase activity (174,175); d) eIF-4A or eIF-4F dependent binding to mRNA (175,181); e) eIF-4A or eIF-4F mediated unwinding of mRNA higher order structures (182,183, Chapter 6).

(2) The recycling of the eIF-4E component of eIF-4F (and possibly other components of eIF-4F) in mRNA cap binding has been attributed to eIF-4B (184).

(3) Recognition of the AUG translation initiator codon (185,186).

From these combined studies it would seem that eIF-4B plays a catalytic role during cap function, yet might have alternative or additional functions. However, it is important to note that eIF-4B is one of the most difficult initiation factors to isolate in a pure state (most are 60-70% pure). Thus, one has to entertain the serious possibility that some of the activities 'shown' for eIF-4B might in actuality reflect those of minor contaminants. In addition there has been some confusion as to the wheat germ eIF-4B. There is a growing consensus that wheat germ eIF-4B (135,169) identified by having a similar size than mammalian eIF-4B is really an isozyme of eIF-4F and that the real eIF-4B is what was claimed to be a new wheat germ specific factor eIF-4G (75.170,176,187,188). eIF-4G is most likely the ~60 kDa eIF-4B described by Seal et al. (136,189). The cloning of eIF-4B will significantly enhance our understanding of its role(s) during translation initiation.

iv) p220 (Functional alignment of components?)

The p220 subunit is the least characterized component of eIF-4F. There are three or four polypeptides that are collectively termed p220. They are present in approximately equimolar amounts in eIF-4F. Several lines of evidence indicate that the different forms are structurally similar: (1) Monoclonal antibodies directed against p220 recognize all the forms with equal intensity in HeLa lysates (190). (2) Tryptic

peptide mapping indicates that they are almost identical (I. Edery, unpublished observations). (3) They are all substrates for poliovirus-induced protease activity (190-192), as well as rhinovirus-14 (193). The fact that cleavage of the p220 subunit of eIF-4F by poliovirus infection causes inactivation of eIF-4F (section 1.2.7) implies that its structural integrity is critical. This has led to the notion that the p220 component interacts with eIF-4E, eIF-4A and possibly eIF-4B to align them in a proper configuration resulting in enhanced activity.

It is noteworthy that p220 associates with eIF-3 under physiological conditions (191,192). The multisubunit eIF-3 has been shown to bind 40S ribosomal subunits and is also required for maximal binding of the 43S pre-initiation complex to mRNA. Thus, the interaction between eIF-3 and p220 may direct small subunit binding to the 5' end of mRNA.

1.2.6 The role(s) of mRNA sequences and structures in translation

There are several features present at the 5' ends of eukaryotic mRNAs that affect translational efficiency. These include: (1) cap structure, (2) secondary (or tertiary) structure, (3) sequences flanking the AUG codon, and (4) presence of upstream AUG codons. The role(s) of the 5' untranslated region (5' UTR) during protein synthesis has been studied by comparing natural mRNA sequences and by *in vitro* manipulations. From these studies some interesting 'rules' have emerged which are encompassed in the now familiar, modified scanning mechanism. However, recent evidence indicates that ribosomes can bend or even ignore these rules. What follows is a summary of all the rules and the

two most established models proposed for the binding of 43S pre-initiation complexes (commonly referred to as simply 40S subunits) to mRNA.

a) Relationship between cap structure, ATP and secondary structure

The dependency for a cap structure for efficient 40S subunit binding to mRNA has been correlated with the degree of mRNA secondary structure and the need for ATP hydrolysis. Sonenberg et al. (115) showed that a monoclonal antibody with anti-cap binding protein activity preferentially inhibits initiation complex formation with native reovirus but not inosine-substituted reovirus (resulting in less stable secondary structure). Furthermore, in a related study it was shown that ribosomes in extracts from poliovirus-infected cells could form initiation complexes with denatured reovirus mRNA, in contrast to their inability to bind native reovirus mRNA (194). That the defect in infected cells is in eIF-4F is consistent with it having an activity that is required to a lesser extent for the translation of mRNAs with reduced secondary structure. Two other studies demonstrated that binding of ribosomes to inosine-substituted reovirus mRNA is less dependent on ATP than binding of native reovirus mRNA (195,196).

These studies are consistent with the observation that naturally uncapped RNAs such as cowpea mosaic virus RNA (197) and STNV RNA (discussed in ref. 198) do not require ATP for initiation complex formation. Several earlier studies were also compatible with the suggestion that cap function is related to mRNA secondary structure. For example, Weber et al. (199,200) reported that inhibition of translation of several mRNAs by cap analogs was augmented when potassium concentrations were increased and temperatures lowered. Moreover, the

translation of several capped eukaryotic mRNAs [VSV, reovirus, and tobacco mosaic virus (TMV)] in extracts from HeLa cells were inhibited at elevated K^+ concentrations, whereas alfalfa mosaic virus 4 (AMV-4 RNA), which is devoid of stable secondary structure at its 5' end (201), was resistant to such an inhibition (202). Low potassium concentrations and higher temperatures favor a more relaxed structure of the mRNA (203) and thus partially obviate the requirement for the cap and consequently eIF-4F.

b) Stable stem and loop structure inhibits translation

All the aforedescribed studies (section 1.2.6a), although suggestive in nature, are flawed to certain degrees in experimental design. There are several drawbacks to using inosine-substituted mRNAs. (1) they result in global denaturation and thus do not provide precise insight into mechanistic details. (2) they have an AUI (instead of AUG) as their initiation codon thereby not permitting translation. (3) It was suggested that they form artefactual initiation complexes in the absence of ATP (204). Finally, the contribution of increased salt concentration to other events outside of increased mRNA secondary structure are difficult to establish.

A more direct study by Pelletier and Sonenberg (27) showed that increasing secondary structure within the 5' noncoding region of a eukaryotic mRNA inhibited translation both *in vitro* and *in vivo*. Bam HI linkers having dyad symmetry were inserted into the DNA region corresponding to the 5' UTR of the herpes simplex thymidine kinase mRNA (tk mRNA). Kozak (205) introduced hairpin structures into the 5' UTR

of a chimeric SV40-rat proinsulin II gene and demonstrated the same qualitative results. Moreover, a recent study (206) analyzing the effects of hairpin structures in CYC1 mRNA translated *in vivo* (in yeast) reached a similar conclusion. One notable difference was in the degree of secondary structure (measured in predicted ΔG) required to manifest itself as refractory to translation. It was suggested that translation in yeast (S. cerevisiae) is more sensitive to secondary structures than is translation for higher eukaryotes (206).

c) AUG codon requirement and AUG context effects

In contrast to prokaryotes, AUG is almost exclusively the only initiation codon used in wild-type genes of eukaryotes (for review, see ref. 31). There are limited examples of non AUG codon usage to initiate translation. One of the adeno-associated virus (AAV) capsid mRNAs (207,208) and the Sendai virus P/C mRNA (209,210) both initiate at an ACG. Recently, a non-viral mRNA was shown to initiate translation at a CUG codon (211). Interestingly, the CUG start codon present in exon 1 of the oncogene c-myc is disrupted by a translocation event in Burkitt's lymphomas. It is still not known if the very low translational efficiency observed with non AUG start codons (212) is used *in vivo* to regulate gene expression.

Notwithstanding the importance of an AUG, sequences in the immediate vicinity of the start codon can also influence translational efficiency (31). The sequence (GCC) $GCC^{A}_{G}CCAUGG$ (where the AUG start codon is underlined) emerged as the consensus sequence for initiation of translation in vertebrates (213). This motif was substantiated by a systematic experimental study demonstrating that alterations at the -3

position produced the most dramatic effects on translation, consistent with the fact that this position is the most conserved nucleotide in vertebrates (214). Similar studies have analyzed the importance of a consensus sequence flanking translational start sites in Drosophila (215), plants (216) and yeast (217,218,219). The following consensus sequences were derived: Drosophila - $^{C}_{A}AA^{A}_{C}\underline{AUG}$, plants - AACA$\underline{AUG}$GC, and yeast - $A^{A}_{U}\underline{AAAUG}UCU$. These differences probably do not represent mechanistic differences in translation initiation inasmuch as they all exhibit identical preferences and restrictions at the crucial -3 position (e.g. A usually favored). The only exception might be yeast (S. cerevisiae) mRNAs which are A-rich throughout the entire 5' untranslated region. Therefore an A at the -3 position might just reflect the need for unstructured mRNA for efficient translation initiation in yeast. Two lines of evidence support this contention. (1) Stem and loop structures inhibit translation in yeast more than in mammalian cells (27,205,217). (2) whereas changes in the -3 position can alter translational efficiency over a 20-fold range in mammalian cells (214), no more than a twofold change in expression was observed in yeast (217). One of the major drawbacks in studies analyzing AUG context effects has been the almost exclusive use of pre-proinsulin (in mammals) and iso-1-cytochrome C (in yeast) mRNAs. Future studies using other mRNAs will be useful in establishing the physiological importance of this motif in governing translational efficiency.

d) AUG selection

The majority of eukaryotic mRNAs (~90%) analyzed do not have AUG triplets upstream of the 'functional' start codon, and furthermore initiate translation from the AUG closet to the 5' terminus (108,213). This would imply that there is selective pressure against the presence of upstream AUG triplets. This supposition has been confirmed experimentally by showing the inhibitory effects upsteam AUG triplets have on translation from the normal initiation site (214,220). In addition Kozak (214) showed that an AUG codon with an improved context becomes a more effective barrier to translation from the downstream AUG.

e) Modified scanning model

The monocistronic character of most eukaryotic mRNAs can be rationalized by a scanning mechanism in which both position (ie., proximity to the 5'-terminus) and flanking sequences dictate which AUG codon(s) will initiate translation. Kozak (31) proposed that 40S ribosomal subunits bind at or near the 5' cap and migrate linearly until they reach the first AUG codon. Deviations from this model as incorporated in the modified scanning model can also be explained. If the first AUG codon lies in an optimal context the 40S subunit stops followed by 60S joining and the elongation cycle of translation. If, however, the first AUG codon is in a suboptimal context (position -3 being most critical) a percentage of 40S ribosomes will bypass the first AUG codon and initiate at the next AUG codon downstream (termed "leaky scanning"). In some cases a downstream AUG codon is still utilized despite the presence of a good context surrounding an upstream AUG. To

account for this variation it was noted that in most cases the upstream AUG is followed by an in frame stop codon that is upstream of the downstream AUG. In this scenario termed termination re-initiation 40S ribosomes initiate translation at the upstream AUG, reach the in-frame stop codon, continue scanning and re-initiate at a downstream AUG (for recent review, see ref. 221).

f) Internal binding of 40S subunits to mRNA

The idea that 40S ribosomes can bind to internal regions of mRNA thus bypassing upstream sequences and the requirement for a cap structure has been around for several years (222). It has been suspected that poliovirus (in fact all picornaviruses) does not initiate translation by the proposed scanning model. Unlike most eukaryotic mRNAs poliovirus does not have a 5' cap structure (148,149), has a long 5' untranslated region (~750 nucleotides) containing 7-8 AUG codons some in the optimal context.

Recent studies have indicated that poliovirus has a sequence of about 300 nucleotides (RLP = ribosome landing pad) that is sufficient for direct binding of 40S ribosomal subunits (223,224). Pelletier and Sonenberg (224) constructed bicistronic mRNAs whereby the intercistronic spacer was the polio RLP sequence. In poliovirus infected cells translation from the first cistron was inhibited yet expression of the downstream cistron was not affected. Leaky scanning, termination re-initiation or mRNA cleavage to liberate a 5' end in the intercistronic spacer were dismissed as likely possibilities. Similar conclusions were reached by Jang et al. (225) for encephalomyocarditis

virus (EMC). Internal initiation is consistent with the result that *in vitro* mutagenesis of upstream AUG codons in the polio RNA did not lead to a concomitant increase in translational efficiency as might be predicted from the scanning hypothesis (226). Other examples of internal initiation have been suggested (227,228). It will be of interest to demonstrate internal initiation on cellular mRNAs and how this might relate to translational control. The possible role of initiation factors in mediating internal initiation will be discussed in Chapter 6.

1.2.7 Examples of translational control

Translational control can generally be defined as a change in the efficiency of mRNA translation, i.e., in the number of amino acids polymerized per unit time per mRNA molecule. This may be reflected as either a quantitative change in the overall amount of proteins synthesized, or a qualitative change in the species of mRNAs translated, or both. In a steady state situation the overall rate of protein synthesis is governed by the number of active ribosomes per cell, whereas qualitative control is achieved by the availability of cytoplasmic mRNAs (for review, see refs. 48,229). However, during changes from one steady state level to another a rapid response is usually achieved by changes in the rate of initiation of translation. Although difficult to measure directly most translational control mechanisms operate at this step.

At the level of translational control certain features of the initiation process are important to consider. Regulation of eIF-2 recycling may affect protein synthesis in a global manner since all mRNAs require this initiation factor. Yet there are examples of

selective inhibition of translation via modulation of eIF-2. The factors that bind mRNA and mediate cap-function (eIF-4A, -4B and -4F) are expected to control translation in a qualitative manner and indeed there are several examples of this. Finally, cis-acting features particular to mRNAs such as secondary structure or cap accessibility have been shown to differentially influence translational efficiency. In this section I give selected examples to illustrate each case, placing more significance on those which direct evidence for the involvement of eIF-4F has been obtained.

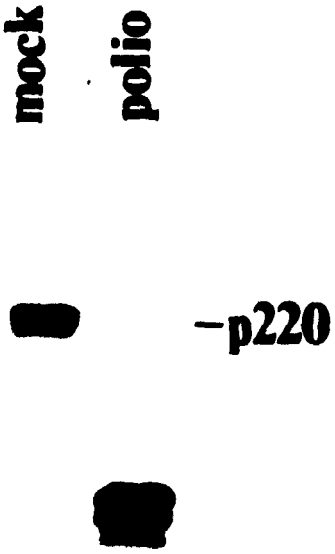
a) Poliovirus infection of HeLa cells

The best documented case in which eIF-4F is involved in translational control occurs during poliovirus infection of HeLa cells. Poliovirus infection causes a selective translational switch whereby host mRNA translation is inhibited, followed by exclusive synthesis of viral-specific products (for review, see refs. 146,147). The currently accepted model to explain this phenomenon proposes that the shutoff is a direct consequence of a viral-induced degradation of the p220 component of eIF-4F (Fig. 5), which results in the inactivation of eIF-4F function (139,191,192). This is an attractive hypothesis since poliovirus RNA is naturally uncapped and therefore must translate by a cap-independent mechanism. While there are sufficient data to illustrate the deleterious effects that inactivation of eIF-4F has on the translation of cellular mRNAs, there are several unanswered questions. It is not clear that p220 degradation by itself is sufficient for effecting the shutoff. In addition, it is known that a viral protease is required for p220 cleavage, however, it is not known if it acts directly or activates a cellular protease.

Fig. 5. Immunoblot analysis of the p220 Component of eIF-4F in extracts from poliovirus-infected cells.

Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 100 PFU per cell. Cells were extracted with PLB solution (0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 1mM PMSF) 3.5 h after infection. Samples representing equivalent number of cells were resolved by SDS-PAGE and analyzed by immunoblotting with a rabbit anti-p220 polyclonal antibody. Lanes: 1, post mitochondrial extract (S-10) from mock-infected cells; 2, S-10 from poliovirus-infected cells.

IMMUNOBLOT ANALYSIS OF p220 AFTER POLIO INFECTION



A key question concerns the mechanism by which proteolysis of eIF-4F results in loss of function. The extent of cross-linking of eIF-4E to mRNA in IF preparations from poliovirus-infected cells is severely impaired (134). Identical results were obtained when the photochemical cross-linking technique was used (138). Two groups examined whether modifications of eIF-4E contribute to its reduced cross-linking ability. This question was of interest in light of a report showing that several polypeptides, including a 24-kDa polypeptide, became phosphorylated in poliovirus-infected CV-1 cells (230). However, no differences in the isoelectric variants of eIF-4E were detected after poliovirus infection (139,161). In addition, two other initiation factors required for mediating cap function, eIF-4A and eIF-4B, are not impaired as a consequence of poliovirus infection (231). Therefore, if eIF-4E, -4A and -4B are not altered by poliovirus infection how does p220 cleavage result in inactivation of cap function? It is noteworthy that the extent of cross-linking of eIF-4E to capped mRNA is at least 20-fold greater on a molar basis when eIF-4E exists as part of eIF-4F than when it is in the free form (139). Consequently, it is tempting to speculate that eIF-4E in the modified eIF-4F complex from poliovirus-infected cells behaves like free eIF-4E in terms of affinity for the cap. As mentioned earlier, the structural integrity of p220 may be critical in juxtaposing the individual polypeptides in a functional complex.

Since poliovirus codes for two proteinases (232,233), it was investigated whether one of these proteinases mediates the cleavage of p220. Studies with polypeptide 3C (234,235) and more recently 2A (236) showed that neither enzyme copurified with p220 cleavage activity, and

antibodies to neither proteinase inhibited cleavage of p220 in vitro. However, Bernstein et al. (237) obtained a poliovirus mutant that contains a single amino acid insertion in 2A. Strikingly, the mutant fails to cleave p220 and inhibit protein synthesis, thus providing evidence for a causal relationship between the cleavage of p220 and the selective suppression of host protein synthesis after poliovirus infection. In a recent study Krausslich et al. (238) showed that a 2A coding transcript generated from a hybrid plasmid could mediate p220 cleavage in cell-free extracts. In accord with previous data (236) they suggested that 2A does not directly cleave p220 but presumably induces a cellular protease. This mechanism is probably not unique to poliovirus since Human rhinovirus 14 infection also leads to p220 cleavage (190). In addition, rhinovirus 14 and poliovirus have closely related 2A polypeptides (236). The occasional observation of p220 cleavage products (similar in size to those generated by poliovirus infection) in uninfected cell-free extracts suggests that p220 cleavage may be a general mechanism employed by cells to regulate translation.

Although the shutoff phenomena has been correlated directly with p220 cleavage, recent evidence indicates that cleavage of p220 is necessary but may not be sufficient to induce complete shutoff in poliovirus-infected cells (239-242). Following poliovirus infection an increase in the activity of a kinase (dsI) that specifically phosphorylates eIF-2α (thus inhibiting recycling of eIF-2; discussed below) might account for the second event required for the selective translational shutoff (241,242). More studies aimed at elucidating the

contributions of p220 cleavage and dsI activation on this phenomenon are necessary. In addition, the intriguing possibility that the 5' untranslated region of poliovirus might itself exert an effect by having enzymatic properties (ribozyme) is still an open question.

b) eIF-4E phosphorylation/dephosphorylation

Numerous studies have established that translational control can be achieved through the reversible phosphorylation of initiation factors. The best demonstrated situation involves eIF-2 (discussed below). However, there is a strong correlation between the ability to translate and the phosphorylated status of eIF-4E. The best studied case occurs during heat shock.

Cells exposed to elevated temperatures respond by a rapid change in the pattern of gene expression (reviewed in refs. 243-245). A selective translational switch allows for the translation of heat-shock proteins (HSPs) mRNAs at the expense of normal cellular mRNAs present in the cytoplasm. The characterization of the factor(s) responsible for the observed translational discrimination was attempted in studies using HeLa and Ehrlich cells. Duncan et al. (173) demonstrated that the extent of phosphorylation of eIF-4E decreased on heat shock. However, eIF-4F containing either phosphorylated or non-phosphorylated eIF-4E possessed similar activities in in vitro translation assays. They suggested that limitations in the in vitro system may have accounted for the failure to detect phosphorylation-dependent activity differences. Panniers et al. (246) reported that protein synthesis in cell-free lysates prepared from heat-shocked Ehrlich ascites cells was inhibited

and that synthesis was restored by the addition of eIF-4F. Although dephosphorylation of eIF-4E may contribute to the translational switch it is noteworthy that other initiation factors are also modified upon heat-shock, including eIF-2α (173,247).

The selective translation of HSP mRNA in Drosophila depends on sequence information in the 5' untranslated region (248,249). It is not clear what the distinctive structural features in the 5' leader are that account for this phenomenon. However, the 5' leader sequences are unusually long (180-250 nucleotides) contain a high proportion of adenylate residues, and have little potential to form stable secondary structure. It is possible that relaxed secondary structure in the mRNA 5' UTR may be the crucial feature by allowing the preferential translation of HSP mRNAs at elevated temperatures which induce inactivation of eIF-4F. The lack of secondary structure in these mRNAs may also explain why hsp70 (an HSP) protein synthesis is more resistant to inhibition after poliovirus infection than is normal host protein synthesis (250).

Changes in the extent of phosphorylation of eIF-4E also occurs during other major biological events. The rate of protein synthesis in metaphase-arrested cells is reduced as compared to interphase cells. In addition, eIF-4E from mitotic cells is metabolically labeled with ^{32}P to a lesser extent than the protein purified from interphase cells (251). The increased rate of protein synthesis observed during fertilization of sea urchin eggs has also been attributed (in part) to modulation of eIF-4F activity (252) presumably resulting from a change in eIF-4E

phosphorylation. Very recently it has been shown that protein kinase C appears to be directly involved in the modulation of eIF-4F in response to mitogens (253).

c) Competition (discriminatory factor)

Numerous studies have established that translation of different mRNAs proceeds at different rates (for review, see ref. 254). To explain these differences, several theoretical models have been developed (254,255). They are based on the notion that mRNAs have different affinities for one or more components of the translational apparatus that determine overall translational efficiency. Under competitive conditions when this component(s) becomes limiting, mRNAs with either a higher affinity or a lower requirement for the component(s) will out compete other mRNAs. Initiation is the overall rate-limiting step in translation, and eIF-4F is the least abundant initiation factor (173), thus eIF-4F is an ideal candidate for such a discriminatory activity.

Several cases in which protein synthesis rate is regulated by competition were studied in detail, including the shutoff of cellular protein synthesis on encephalomyocarditis (EMC) virus infection of mouse plasmacytoma cells and regulation of the ratio of α/β-globin chain synthesis in reticulocytes. In the former case, it was reported that EMC virus infection induces the shutoff of host protein synthesis due to the ability of the viral RNA to outcompete cellular mRNAs by virtue of its high affinity for a certain IF (256,257). Translation of α- and β-globin mRNAs in coordinately regulated *in vivo* to produce equal

amounts of both chains (258), despite there being approximately 1.4-fold more α-globin mRNA (259). This equality of translation was explained as the result of the higher translational efficiency of β-globin mRNA (254,258).

Thach and co-workers (257) developed a kinetic model to explain how EMC virus RNA outcompetes cellular mRNAs and showed that this model could be applied to explain the relief of translational competition between reovirus and globin mRNAs by eIF-4A and eIF-4F, in a fractionated in vitro protein-synthesizing system from Krebs ascites cells (260). They also concluded that recognition of the cap structure is not essential for the discriminatory activity, since both factors relieved translational competition when either capped or uncapped reovirus mRNAs were used, according to similar hierarchies. In light of this observation, they suggested that each mRNA contains a unique feature or set of features, apart from the cap, that determines its initiation efficiency in a competitive situation.

In another study, Sarkar et al (261) studied the translation of endogenous globin mRNA in a rabbit reticulocyte lysate to analyze IF discriminatory acitivity and found that only eIF-4F and no other factor, including eIF-4A, relieved the translational competition between α- and β-globin mRNAs. The advantage of this system is that it mimics more accurately the in vivo translational control of the relative synthesis of α-vs. β-globin chains. Other studies may have rendered a nondiscriminatory factor limiting by nuclease treatment or by fractionation to achieve a reconstituted system. In contrast to Ray et al. (260), who postulated that the translational discriminatory factor recognizes a specific structure of the mRNA, Sarkar et al. (261)

suggested that the discriminatory factor (namely, eIF-4F) binds initially to all mRNAs with similar affinity via the interaction with the cap structure. However, during a subsequent step involving denaturation of the mRNA, the affinity of the discriminatory factor for the mRNA will vary in inverse proportion to the amount of mRNA secondary structure. Consistent with this proposal, cross-linking studies have shown that eIF-4E binds mRNA caps with equal efficiency. Pelletier and Sonenberg (138) showed that two sets of constructed tk mRNAs with secondary structure inserted at either 6 or 37 nucleotides from the cap site were equally bound by eIF-4E. A similar conclusion was reached by Lawson et al. (183) using short cDNAs to generate secondary structure in the mRNA.

However, one recent study showed that eIF-4E cross-links to the different reovirus mRNAs with different degrees of efficiency under competitive conditions (262). The steric accessibility of the cap to eIF-4E may thus be a major determinant of the mRNA translation rate. This is consistent with the fact that AMV-4 mRNA has a very accessible cap structure (263), is a highly efficient mRNA under competitive conditions (264-266) and requires little eIF-4F to translate (115,159,187). Therefore it is important to consider that secondary structure (or tertiary) in the 5' untranslated region may not just act to limit eIF-4F mediated unwinding but also the mRNAs ability to bind eIF-4F in the first place.

d) Involvement of the cap structure in control of gene expression in other systems

Most of the maternal mRNA present in oocytes is translationally inactive until fertilization (267,268). Activation of maternal mRNAs

after fertilization occurs at the level of translation initiation (e.g. ref. 269). Both et al. (270) suggested that methylation of the 5' cap structure of maternal mRNA might be involved in its translational induction. This hypothesis is consistent with two reports: Young (271) found that m^7GMP appeared in the RNA of the mouse 1-cell embryo 3 hr after fertilization. In addition, Kastern et al. (272) reported the absence of cap structures in insect oocyte RNA. It is important, however, to confirm this finding before it can be concluded that some cytoplasmic mRNAs are uncapped. More recently, Caldwell and Emerson (273) showed that fertilization of sea urchin embryos activated RNA cap methylation of early histone genes H1,H4 and H6. The authors proposed that cap-methylating enzymes are present in the unfertilized eggs, but for some unknown reason cannot function until fertilization.

Another system in which cap methylation was implicated in translational control was described by Cordell et al. (274). The authors found that the expression of two allelic rat insulin genes in an insulinoma is different by a factor of 10. The levels of the two mRNAs were similar, but protein levels differed. When the mRNAs were capped in vitro, the ratio of protein synthesized was 1:1. It was concluded, therefore, that one of the mRNAs was not capped and therefore translated inefficiently. Again, more direct evidence has to be given to substantiate the claim that some cytoplasmic mRNAs are uncapped.

The mechanism for the shutoff of host protein synthesis after reovirus and Semliki forest virus (SFV) infection was also suggested to involve the inactivation of CBPs. Zarbl et al. (275) reported that reovirus mRNAs that are found on polysomes late in infection had pGp 5' termini, instead of the m^7GpppG cap structure present on reovirus mRNAs

early in infection. These findings are consistent with the reports that reovirus progeny subviral particles contained masked guanylyl-and methyltransferases (276). Furthermore, Skup and Millward (277) showed that extracts obtained from reovirus-infected cells at late times after infection supported the translation of uncapped but not capped reovirus mRNAs. Consequently, it was suggested that reovirus exerts its shutoff of host protein synthesis by redirecting the protein synthesis machinery from a cap-dependent to a cap-independent mechanism of translation initiation. This model is also consistent with the finding that the natural uncapped RNA of EMC virus translated with high efficiency in an extract prepared from reovirus-infected cells late after infection (278). The conclusions from the latter studies were questioned by Morgan-Detjen et al. (279) who showed that extracts prepared from reovirus-infected mouse SC-1 cells translated capped or uncapped mRNAs with similar efficiencies. The reasons for the apparent discrepancies are not immediately clear .

SFV shuts off host protein synthesis after infection. IFs from SFV-infected neuroblastoma cells did not support the translation of cellular mRNAs or early SFV RNA in a reconstituted protein-synthesizing system (280). However, translation of late SFV mRNA was stimulated by the same IF preparations. Subsequently, it was found that eIF-4E and eIF-4B restored the ability of IF preparations from SFV-infected cells to stimulate translation of early SFV and cellular mRNAs (280). Later experiments showed, however, that eIF-4E and eIF-4B in infected cells are not inactivated, but rather a trans-acting factor inhibits their activity (281). In any event, these results show that translation of late SFV mRNA is less dependent on eIF-4E activity for translation as

compared to early SFV or cellular mRNAs. This reduced dependency is like that of AMV-4 RNA (115) and might point to a general characteristic of late viral mRNAs that generally code for coat or capsid proteins that have to be synthesized in abundant amounts.

e) Phosphorylation of the α-subunit of eIF-2

Numerous studies initially performed using rabbit reticulocyte lysates demonstrated that phosphorylation of the α-subunit of eIF-2 correlates strongly with inhibition of translation. This was shown for reticulocyte translation extracts incubated in the absence of hemin (282) or presence of low amounts of double-stranded RNA (dsRNA)(283-286). The phosphorylation of eIF-2α is in itself not inhibitory (287) but impairs the ability of guanine exchange factor (GEF or eIF-2B) to catalyze the conversion of inactive eIF-2•GDP to active eIF-2•GTP (65,288). Inhibition of protein synthesis presumably occurs due to the stoichiometric binding of the limiting amounts of GEF in an inactive complex that is not amenable to recycling (288). Phosphorylation of eIF-2α also occurs in nonerythroid cells under such diverse conditions as heat shock, serum deprivation and cell growth (173,247). In all these cases a concomitant decrease in protein synthesis is observed. Translational control can also be manifested by changes in the activity of GEF (289).

Although it is not entirely clear what the normal physiological function of dsI is, it plays an important role during viral infection. Most viruses that infect cells activate dsI, presumably by dsRNA generated from asymetrical transcription of the viral genome (290). It

is becoming increasing clear that different viruses have developed distinct defense mechanisms to escape dsI activation (290-293). The classic example involves adenovirus which produce a small virus-associated RNA (VA_1) that antagonizes dsI activation (290,291).

As part of the cellular response to block viral gene expression, cells synthesize the anti-viral agent interferon (for review, see ref. 294). Interferons elicit their anti-viral activity, in part, by increasing the level of dsI. Recently, it was speculated that active dsI may itself induce transcription of β-interferon thereby potentially creating a positive feedback mechanism for its own synthesis (295). In addition to dsI activation, interferon synthesis can also lead to inhibition of protein synthesis by activating the (2'-5')-oligoadenylate synthetase-nuclease system which causes the degradation of rRNA, mRNA and polysomes (294). Moreover, interferon production can trigger numerous changes in the cell such as differentiation. Consequently the presence of dsRNA in a cell can potentially induce a myriad array or responses.

Usually phosphorylation of eIF-2α is considered in the context of global protein synthesis. This notion is based on the fact that eIF-2 is required for a step previous to mRNA recognition. However, it is possible that selective inhibition of mRNA binding to ribosomes can be achieved by localized activation of dsI (296). This contention is further supported by the specific translational increase in mRNAs derived from transfected DNA under conditions that antagonize dsI activation (297,298). The intriguing interplay between dsRNA, eIF-4F and eIF-2 is considered in Chapter 4.

1.3 General Features of Helicases

Helix destabilizing proteins (HDPs) are involved in shaping the conformation of DNA or RNA and can be divided into those using ATP and those independent of the supply of chemical energy (299). The most extensive studies have been performed with proteins that bind single-stranded DNA (or RNA) with much higher affinity than to double-stranded DNA. A typical example is the calf thymus UP1 protein which is non-enzymatic and lacks nucleotide specificity (300). In stark contrast, a helicase uses the energy derived from ATP hydrolysis to actively induce RNA or DNA strand separation. In recent years, helicases of various sorts have been demonstrated in a variety of systems involving the dynamics of nucleic acid interactions. Their existence is not surprising considering the many pathways involved in RNA biogenesis that require the constant need to destabilize Watson-Crick interactions.

From the several well studied examples of helicases (301-305) several remarkable conserved functional and structural properties have emerged. (1) They can bind single-stranded RNA (or DNA) in the presence or absence of ATP. (2) They contain an ATPase activity that is stimulated by single-stranded RNA. (3) Unwinding is unidirectional, either 5' to 3' or 3' to 5' but not both. (4) They can unwind DNA:RNA hybrids but if, for example, it is an RNA helicase it will not unwind dsDNA (or dsRNA if DNA helicase). (5) As assayed *in vitro* there is high ATP turnover relative to the number of base-pairs opened. (6) They have a conserved nucleoside triphosphate binding motif (306,307). Examples of helicases include, transcription termination factor Rho (303), Simian virus 40 large T antigen (302,305), and eIF-4A (182).

During protein synthesis it has been shown that elongating ribosomes have a translationally associated helicase activity (308,309). This activity is presumably required for the local unwinding of mRNA coding sequences to allow codon-anticodon interactions. A developmentally regulated RNA duplex unwinding activity has been demonstrated in fertilized Xenopus oocytes (310,311). The presence of this melting activity has had grave implications for the general feasibility of anti-sense technology, whereby gene expression is blocked by hybridizing the targeted mRNA with its cognate antisense RNA (for review see ref. 312). An efficient RNA duplex unwinding activity has also been demonstrated in nuclear extracts capable of precursor mRNA splicing (313). Recently, a nuclear protein with sequence homology to eIF-4A was cloned and postulated to be a helicase (314). Direct evidence for eIF-4F/-4A mediated RNA duplex helicase activity and its implications are presented in Chapter 6.

1.4 Splicing of Messenger RNA Precursors

Most eukaryotic nuclear mRNAs are produced from precursors (pre-mRNAs) that contain multiple introns (for reviews, see refs. 315,316). The development of efficient *in vitro* splicing extracts have led to the formulation of a two-stage process for splicing in higher eukaryotes (317,318). First, the pre-mRNA is cleaved at the 5' splice site (donor) to yield a linear upstream moiety and a downstream characteristic lariat structure in which the 5' end of the intron is covalently joined by a 5'-2' linkage to an A residue (317-319) some 30 nucleotides upstream of the 3' splice site (donor) (318). Cleavage of

the 5' splice site is coupled with lariat formation, whereas cleavage at the 3' splice site is concomitant with exon ligation. The phosphate moieties at both the 5' and 3' splice sites are conserved in the products (319). Thus, both steps may be considered trans-esterification reactions, where a hydroxyl group reacts with a phosphodiester bond displacing a hydroxyl group while forming a new phosphodiester bond.

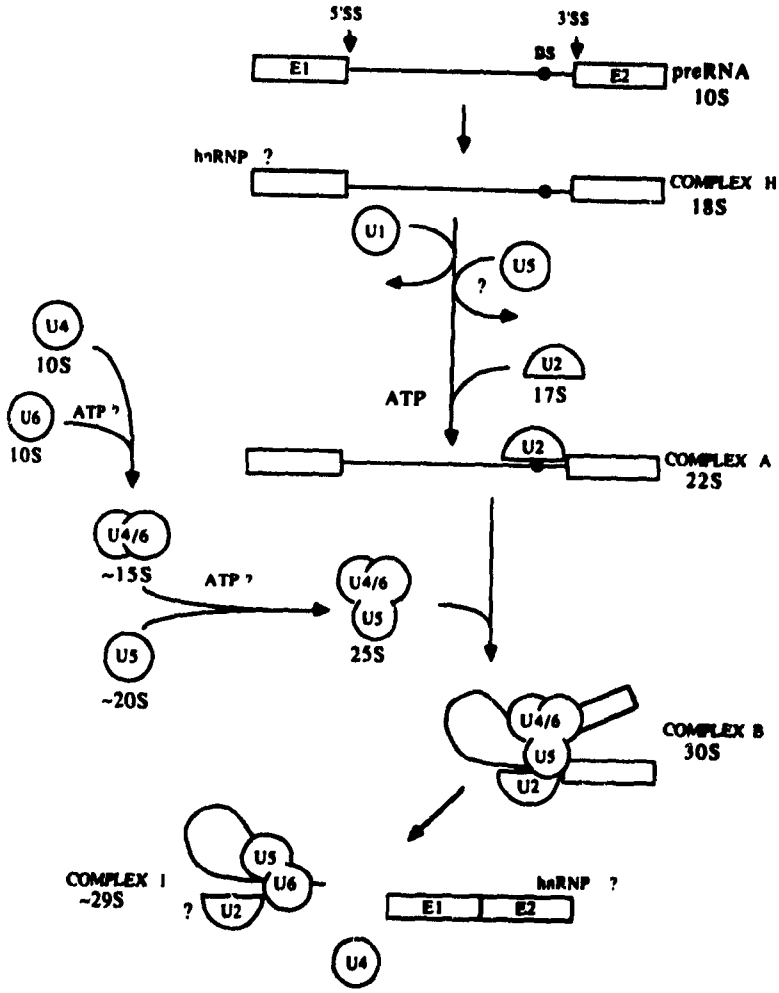
Of particular importance in understanding the mechanism of splicing is elucidating how the precise excision of introns is achieved. Numerous studies have shown that only limited stretches of primary sequence information on the mRNA are neccessary (320). At the 5' splice junction is a conserved GU and at the 3' splice junction is a conserved AG, both maintained in the intron (321). Using in vitro transcribed mRNAs that were mutated in these regions it was shown that the 5' GU need not be conserved for 5' cleavage and that it plays an important role in cleavage and exon joining at the 3' splice site (322,323). In contrast, 3' AG mutations drastically reduce 5' cleavage and abolish splicing (322,323). The proximaty of the 5' and 3' splice sites is also an important determinant in the selection of splice sites (324). Yeast introns contain an additional region required for accurate and efficient splicing in vivo. The TACTAAC box is absolutely conserved and is located near (20-60 nucleotides upstream of) the 3' splice junction, the downstream most adenosine in this sequence being the site at which the yeast lariat is formed (325). A weakly conserved functional analog of this sequence has been described in mammalian introns (318).

The precise recognition and exision of intervening sequences is most likely imparted by the role of the small nuclear RNAs (snRNA). Small nuclear RNAs are a group of short RNAs found in the nuclei of all

Fig. 6. Schematic Pathway of the Assembly of Splicing Complexes.

Top line shows a structure of a preRNA substrate that contains two exons (E1 and E2) separated by an IVS. 5'SS, 3'SS, and BS indicate 5' and 3' splice sites and branch site, respectively. Complex A is formed by binding of U2 snRNP to sequences upstream of the 3' splice site of preRNA. Complex B represents a probable structure of the splicesome. Splicing products - the excised lariat IVS associated with snRNPs (complex 1) and the spliced exons E1-E2, probably released in an hnRNP complex - are shown on the bottom line. The relative positions of snRNPs with respect to themselves and to preRNA sequences in complexes U4/5/6,B, and Z are arbitrary.

note: The above Figure was taken from Konarska et al. (331).


5'SS
3'SS
BS
E1
E2
preRNA
10S
hnRNP ?
COMPLEX H
18S
U1
U5
?
U4
10S
U6
10S
ATP ?
ATP
U2
17S
U2
COMPLEX A
22S
U4/6
~15S
ATP ?
U4/6
U5
25S
U5
~20S
U4/6
U5
U2
COMPLEX B
30S
U5
U6
COMPLEX I
~29S
?
U2
hnRNP ?
E1
E2
U4

eukaryotic cells, where they are complexed with specific proteins to form small nuclear ribonucleoprotein particles (snRNPs). The U-snRNAs (so named because they are U-rich) have at their 5' ends a unique trimethyl cap structure (except U-6), 2,2,7-trimethylguanosine (for review see ref. 326). The notion that snRNPs are involved in splicing of pre-mRNAs was initially based on the observation that sequences at the 5' end of U1-snRNA are complementary to the consensus 5' splice site (327,328). Evidence for this has been obtained by showing that a compensatory base change in U1 snRNA can suppress a 5' splice site mutation (329). The most recent studies have employed electrophoretic separation of ribonucleoprotein particles in nondenaturing gels to analyze the splicing intermediates and role of snRNPs (330,331). Early in the reaction, a complex formed consisting of the U2 snRNP bound to sequences upstream of the 3' splice site is probably the committing step to splice and requires ATP hydrolysis. Subsequently a single multi snRNP particle containing U4/6 and U5 snRNPs are required for lariat formation and exon ligation. U1 snRNP is not detected in any of the splicing complexes consistent with its absence in affinity purified complexes (332), indicative of a weak association (the splicing pathway is shown in Fig. 6).

The two-step splicing mechanism results in a bimolecular kinetic intermediate that must be juxtaposed correctly to ensure correct exon ligation. This is probably achieved by the fact that splicing occurs in a large ribonucleoprotein complex termed the spliceosome (333-335). It is still not understood how the splicing of pre-mRNAs containing multiple introns ensures that only neighboring exons are joined. One

model proposes that the splicing system first recognizes the donor splice site and then moves along the intron until it encounters a suitable acceptor splice site (336). However evidence against a simplistic scanning model of RNA splicing has been presented (337). This is supported by the ability to form two spliceosomes that can form simultaneously and independently on double-intron pre-mRNAs (338). Moreover, a simple scanning model is not easily compatible with alternative splicing.

In vivo polyadenylation occurs prior to pre-mRNA splicing (8). However, a polyA tail is not required for efficient pre-mRNA splicing. In contrast splicing in HeLa extracts (but not yeast extracts) is more efficient with 5' capped pre-mRNAs (339,340). A recent study has shown the preferential excision of the 5' proximal intron from pre-mRNAs with two introns as mediated by the cap structure (341). When a pre-mRNA was capped, the upstream intron was spliced out more efficiently than the downstream intron.

In addition to intramolecular splicing, pre-mRNAs can also be spliced in trans (342,343). The trypanosome mRNAs are generated in this fashion (344,345). Finally, secondary structure in the pre-mRNA can cause alternative splicing (346). The similarities between pre-mRNA splicing and protein synthesis will be considered in Chapter 6.

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CHAPTER 2

INVOLVEMENT OF EUKARYOTIC INITIATION FACTOR 4A IN THE CAP RECOGNITION PROCESS

FOOTNOTES

[1]The abbreviations used are: CBP, cap binding protein; eIF, eukaryotic initiation factor; kDa, kilodalton; A.S., ammonium sulfate; Hepes, 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TBS, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl; BSA, bovine serum albumin; 2-D, 2-dimensional; SDS, sodium dodecyl sulfate; ELISA, enzyme-lined immunosorbent assay.

SUMMARY

Antibodies against eukaryotic initiation factor 4A (eIF-4A) were used to study the involvement of this factor in recognizing the 5' cap structure of eukaryotic mRNA. We demonstrate that an ~50 kilodalton polypeptide present in rabbit reticulocyte ribosomal high salt wash which can be specifically cross-linked to the 5' oxidized cap structure of reovirus mRNA [Sonenberg, N. (1981) Nucleic Acids Res. 9, 1643] reacts with an anti-eIF-4A monoclonal antibody. We also show that antibodies against eIF-4A react with a 50 kilodalton polypeptide present in a cap binding protein complex obtained by elution from a m^7GTP-agarose affinity column. Comparative peptide analysis of eIF-4A and the 50 kilodalton component of the cap binding protein complex indicates a very strong similarity between the two polypeptides.

INTRODUCTION

Cap binding proteins[1] (CBPs) from rabbit reticulocyte ribosomal high salt wash that interact directly or indirectly with the cap structure of eukaryotic mRNAs have been identified by specific chemical cross-linking to the 5' oxidized cap structure of reovirus mRNA (1-3). Polypeptides of M_r = 24,000, 28,000, 50,000 and 80,000 have been detected using this assay and except for the M_r = 24,000 species [24K-CBP (1)] the cross-linking is absolutely dependent on the presence of ATP-Mg^{++} (2,3). These CBPs are most likely involved in the process of ribosome binding during translation initiation and consequently their relationship to previously characterized initiation factors is of interest. Recent findings have indicated that mRNA binding of two initiation factors, eIF-4A and eIF-4B, can be stimulated by ATP-Mg^{++}, is partially sensitive to cap analogues and requires the presence of both factors (4). In addition, it has been reported that eIF-4A and eIF-4B, when present together, can be specifically cross-linked to the oxidized cap structure of reovirus mRNA in the presence of ATP-Mg^{++} (4). These observations suggested that the 50- and 80-kDa cap-specific polypeptides previously detected by the cross-linking assay in crude initiation factor preparations correspond to eIF-4A and eIF-4B, respectively.

In poliovirus-infected HeLa cells, the mechanism of cap recognition is impaired (5). Earlier studies suggested that the 24K-CBP was inactivated by poliovirus since apparently homogenous preparations of this polypeptide could restore translation of capped, vesicular stomatitis virus mRNA in extracts from poliovirus-infected cells (6). However, this restoring activity was labile and subsequently Tahara et

al. (7) have isolated a stable form of restoring activity using a m^7GDP-Sepharose affinity column (8) consisting of 24K-CBP (termed CBP I) and polypeptides of M_r = 48,000, 55,000 and 225,000 (9) which was termed CBP II. Furthermore, Etchison et al. have shown that a 220 kDa polypeptide is cleaved during poliovirus infection and that this polypeptide is antigenically related to the largest polypeptide in the CBP complex (10). The identification and functional significance of the polypeptides in the CBP complex is, consequently, an interesting question both with respect to regulation of translation during poliovirus infection and the cap recognition process in general.

Here, we have purified a high molecular weight protein complex consisting of the 24K-CBP and other major polypeptides of M_r = 50,000 and 220,000 by means of affinity chromatography on a m^7GTP-coupled agarose column. Based on its composition and its ability to restore translation of capped mRNAs in extracts from poliovirus-infected cells, this complex appears functionally analogous to the CBP II previously described (7,9) and will be referred to as the CBP complex. In addition, we have determined the relationship between the 50 kDa polypeptide in crude initiation factor preparations that can be cross-linked to the oxidized cap structure, the 50 kDa polypeptide in the CBP complex and eIF-4A.

MATERIALS AND METHODS

Cell and Virus: Growth of L cells and infection with reovirus (Dearing 3 strain) were as described (11). [^{3}H]methyl-labeled reovirus mRNA was synthesized to a specific activity of 2 x 10^4 cpm/µg and periodate oxidized as previously described (12).

Protein Synthesis Factors: Preparation of rabbit reticulocyte lysate, high salt wash of ribosomes (as the source of IFs) and subfractionation of the latter fraction to a 0-40% A.S. fraction were as described by Schreier and Staehelin (13). Rabbit reticulocyte eIF-4A and eIF-4B were purified through steps 15 and 7 respectively, according to Benne et al. (14).

Preparation of CBP Complex: Purification was essentially as described by Etchison et al. (10). A 0-40% A.S. fraction of rabbit reticulocyte ribosomal high salt wash (14 A_{280}) was layered on a 12 ml 10-35% linear sucrose gradient in buffer A (20 mM Hepes, pH 7.5, 0.2 mM EDTA, 0.5 mM PMSF and 7 mM 2-mercaptoethanol) containing 0.5 M KCl. Centrifugation was for 24 hr at 38,000 rpm in an SW 40 rotor at 4°C. The top half of the gradient, excluding fast sedimenting eIF-3 (>10S), was pooled and dialyzed against buffer A containing 0.1 M KCl and 10% glycerol. The dialyzed material (3-7 A_{280}) was then loaded directly onto a 1 x 0.7 cm m^7GTP-agarose affinity column (preparation of this column will be described in Chapter 3) equilibrated in buffer A containing 0.1 M KCl and 10% glycerol. Proteins which bound to the column non-specifically were eluted by washing with 50 ml of buffer A containing 0.1 M KCl and 10% glycerol followed by 4 ml of 100 µM GTP in the same buffer. Cap-specific proteins were eluted with 4 ml of 75 µM m^7GTP. A final

wash with buffer A containing 1 M KCl and 10% glycerol was used to elute the remaining adsorbed material.

Cross-linking of mRNA to Protein Synthesis Factors: [^{3}H]Methyl-labeled, oxidized reovirus mRNA was incubated with IF preparations for 10 min at 30° essentially as described before (2), followed by the addition of $NaBH_3CN$ (Aldrich, freshly prepared) and RNase A to degrade the mRNA. The samples were resolved on SDS/polyacrylamide gels, and labeled bands were detected by fluorography as described in the Legends to Figures. Quantitation of protein labeling was performed by scanning the radioautograph with a soft laser scanning densitometer (LKB).

Preparation of anti-eIF-4A Monoclonal Antibody: Immunization was achieved by injection of BALB/cJ female mice with rabbit reticulocyte eIF-4A that had been purified through steps 1-4 (15). Mice were injected intraperitoneally (i.p.) with 200 µl of eIF-4A (30 µg) in TBS/complete Freund's adjuvant (1:1). The injection was repeated after two weeks, with incomplete Freund's adjuvant. Four weeks later a final injection was given (400 µl TBS/incomplete Fruend's adjuvant, containing 125µg eIF-4A). Spleen cells from this mouse were fused with FO myeloma cells as described (16). Culture supernatants were tested by using a solid phase enzyme-linked antibody assay (ELISA) and positive cultures were recloned by limiting dilution as described (16). The ELISA was performed by applying eIF-4A (5-10 µg/ml in TBS) to microtiter plates (Dynatech) and allowing it to adsorb for 1 hr followed by addition of 200µl per well of 2.5% BSA in TBS for 1 hr to saturate free protein binding sites. The plates were washed briefly with TBS and hybridoma supernatants were added (50 µl/well). Following incubation for 2-16 hr, plates were washed 4-5 times with TBS and incubated for 3 hr with 1

µg/ml of peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako) in TBS containing 0.5% BSA. The plates were washed again and stained with 0.4 mg/ml 5-amino-2-hydroxybenzoic acid, 0.003% H_2O_2 in TBS (50 µl/well). For preparation of purified antibody, cells (1-2 x 10^7) of the clone were injected i.p. in BALB/cJ mice. About two weeks later the mice were sacrificed and the ascites fluid collected. Antibodies were purified from the ascites fluid by $(NH_4)_2SO_4$ precipitation (1.75 M final concentration) and DEAE-cellulose chromatography (17).

Preparation of anti-eIF-4A and -4B Antibodies: Polyclonal antisera against HeLa cell eIF-4A and -4B were raised in goats as described (18). The two sets of antibodies which react with rabbit reticulocyte eIF-4A and eIF-4B, respectively, were affinity purified before use (18).

Immunoblot Analysis: All incubations were carried out at room temperature. Polypeptides were resolved on 10-18% linear gradient SDS/polyacrylamide gels and transferred to nitrocellulose paper essentially as described by Towbin et al. (19) for 1 hr at 25 volts and 1 Amp. The nitrocellulose paper was incubated for 1 hr with 2.5% BSA and 5% horse serum in saline and then washed with TBS. The washed paper was incubated with anti-eIF-4A antibody (ascites fluid 1:20,000 diluted in TBS and 1% BSA/0.5% horse serum) overnight followed by washing with TBS. Bound antibody was detected by incubating the blot with peroxidase conjugated mouse IgG (Sigma; 1:500 dilution in TBS) for 3 hr, washing in TBS and development by a color reaction with diaminobenzidine (19). For the experiments described in Fig. 3 (lanes 2 and 3) the procedure of Meyer et al. (18) for immunoblotting was followed.

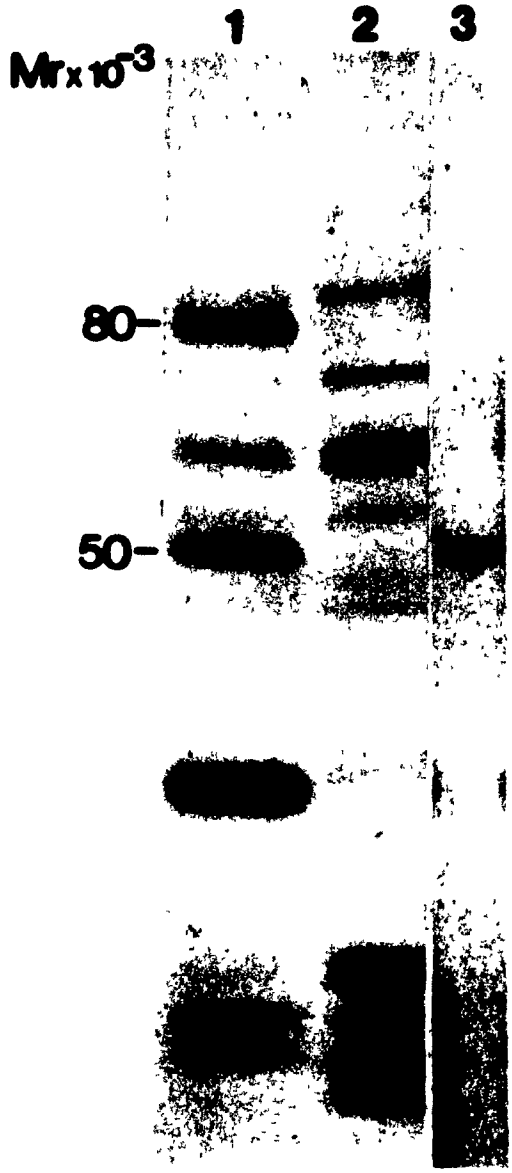
RESULTS

We attempted to determine whether the 50 kDa polypeptide present in rabbit reticulocyte crude IF preparations that can be cross-linked to the oxidized cap structure of reovirus mRNA in an ATP dependent m^7GDP sensitive manner, corresponds to eIF-4A as previously suggested (4). To this end, we used a monoclonal antibody directed against eIF-4A to immunoprecipitate total IF that were cross-linked to oxidized reovirus mRNA. Fig. 1 shows that cross-linking of the major polypeptides of 24,50 and 80 kDa was inhibited by the addition of m^7GDP (compare lane 2 to 1) as established previously (2,3). The immunoprecipitation of cross-linked IF with anti-eIF-4A monoclonal antibody is shown in Fig. 1, lane 3, and it is seen that only the cross-linked 50 kDa polypeptide precipitated.

Sonenberg *et al.* (8) have previously purified the 24K-CBP by centrifugation of a 0-40% A.S. fraction of rabbit reticulocyte ribosomal high salt wash on sucrose gradients in 0.1 M KCl and application of the slow sedimenting fractions on a m^7GDP-Sepharose affinity chromatography column followed by elution with m^7GDP. This procedure yielded an apparently homogeneous preparation of the 24K-CBP. However, Tahara *et al.* (7,9) have shown that other polypeptides can be retained and eluted specifically from a m^7GDP-Sepharose affinity column if rabbit reticulocyte ribosomal high salt wash is fractionated on sucrose gradients in 0.5 M KCl and fractions excluding the fast sedimenting eIF-3 are applied to the column. In an attempt to characterize these other polypeptides we have modified the procedure of Tahara *et al.* (7) and in addition, used a m^7GTP-agarose column for affinity chromatography

Fig. 1. Identification of the cross-linked 50 kDa polypeptide in total reticulocyte initiation factors as eIF-4A.

A high salt ribosomal wash fraction from rabbit reticulocytes (~10 A_{280}, 100 µl) was incubated under cross-linking conditions (as described in Materials and Methods and in Ref. 3) in a final volume of 300 µl for 10 min at 30°, in absence (lanes 1 and 3) or presence of 0.7 mM m^7GDP (lane 2). Following the addition of 30µl of 0.2 M $NaBH_3CN$ and incubation overnight at 4°, RNase A (20 µl, 10 mg/ml) was added and incubation was continued for 30 min at 37°. SDS-sample buffer (150 µl) was added and the samples were boiled for 5 min. For lanes 1 and 2, 100 µl were applied on a 10% SDS/polyacrylamide/gel for electrophoresis (20). For lane 3 the following procedure was followed: A 100 µl fraction of cross-linked proteins was precipitated with 900 µl of cold acetone at -20°, pelleted and dissolved in 20 µl of TBS containing 0.5% NP-40, 0.25% sodium deoxycholate and 0.25% SDS. Purified anti-eIF-4A monoclonal antibody (5 µg) was added followed by incubation at 4°C for 2 hr and addition of 20 µl of rabbit anti-mouse immunoglobulin (Dako) at equivalence. After incubation overnight at 4°, the precipitate formed was pelleted in an Eppendorf centrifuge and washed 3 times in TBS containing 0.5% NP-40, dissolved in SDS-sample buffer and applied to the gel. Following electrophoresis, gels were treated with 1 M sodium salicylate and exposed to XAR-5 Kodak film.

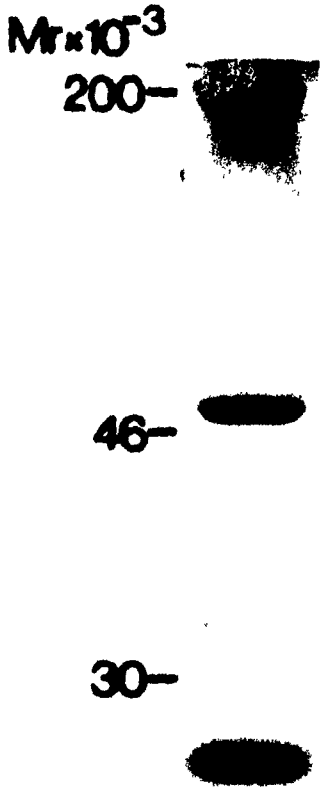

1
2
3
Mr x 10^-3
80-
50-

in the final step of purification. Fig. 2 shows a typical Coomassie blue staining pattern of the polypeptides eluted from the m^7GTP-affinity column, the major components being polypeptides of M_r = 24,000, 50,000 and 220,000. The 50 kDa component comigrates with eIF-4A, consistent with observations of Tahara et al. (9) and the 24 kDa polypeptide comigrates with purified 24K-CBP isolated from rabbit reticulocyte S-100 fraction (data not shown). In addition to these major components the CBP complex preparation contained minor polypeptides of variable intensity depending on the preparation (e.g. polypeptides of ~160 kDa). We believe that some of these polypeptides are degradation products of the 220 kDa polypeptide, since polyclonal antibodies affinity purified with the 220 kDa polypeptide also react with the lower molecular weight polypeptides (10). The different polypeptides eluted from the m^7GTP-agarose column are most probably in the form of a complex, since they cosediment in a sucrose gradient in 0.1 M KCl and 0.5 M KCl (data not shown).

Since eIF-4A can be cross-linked specifically to the cap structure (4), it seemed a likely possibility that the 50 kDa polypeptide component of the CBP complex which comigrates with eIF-4A, is identical with eIF-4A. Consequently, we analyzed the ability of the anti-eIF-4A monoclonal antibody to react with the 50 kDa polypeptide of the CBP complex following transfer of the complex polypeptides to nitrocellulose. Fig. 3 (lane 1) shows the immunostaining profile of the CBP complex polypeptides when probed with anti-eIF-4A monoclonal antibody, indicating that only the 50 kDa polypeptide reacts with the antibody. In agreement with this is the observation that

Fig. 2. Analysis of the CBP complex polypeptides by SDS/polyacrylamide gel electrophoresis.

CBP complex (~2 μg), purified as described in Materials and Methods, was resolved on a 12.5% SDS/polyacrylamide gel followed by Coomassie blue staining.

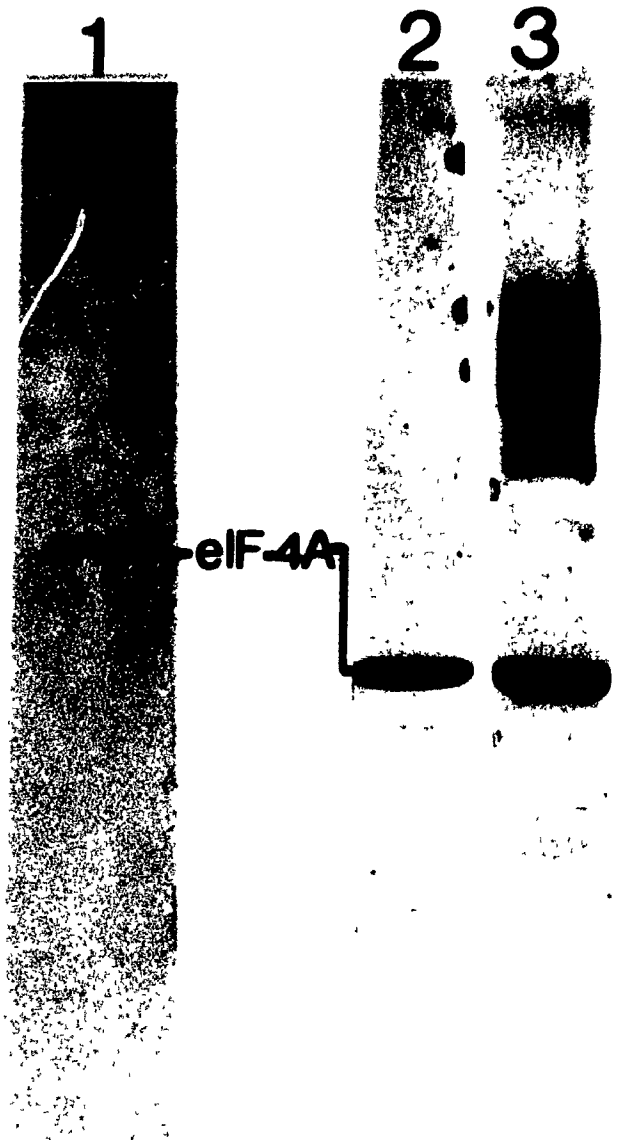

Mr×10-3
200
46
30

affinity-purified polyclonal antibodies against eIF-4A also react specifically with the 50 kDa component of the CBP complex (lane 2). In the latter experiment we used, affinity-purified polyclonal antibodies against eIF-4B, in addition to anti-eIF-4A, to test for the presence of both eIF-4A and eIF-4B in the CBP complex but no reaction with a polypeptide corresponding to eIF-4B was detected (lane 2). In a control experiment, it can be seen that the mixture of antibodies against eIF-4A and eIF-4B reacted strongly with their cognate antigens (lane 3; streaking of eIF-4B has been observed on several occasions but its cause is unknown). These results indicate that eIF-4A but not eIF-4B is present in the CBP complex preparation. This observation is pertinent in light of the report that eIF-4B is required for the ATP-Mg^{++}-dependent cross-linking of eIF-4A (4) and might indicate that eIF-4B can associate or interact with the CBP complex but is not an integral part of it (7,9).

To support the immunological data indicating structural similarity between the 50 kDa polypeptide present in the CBP complex preparation and eIF-4A, peptide analysis of the two polypeptides was performed. Fig. 4 shows the tryptic maps of eIF-4A (panel A), 50 kDa polypeptide (panel C) and a mixture of eIF-4A and the 50 kDa polypeptide (panel B). It is clear that the majority of peptides are common to eIF-4A and the 50 kDa polypeptide (these peptides are indicated by small arrowheads). However, one consistent and possibly significant difference in the peptide maps of the two polypeptides is noted by the heavy and thin arrows. Whereas the peptide indicated with the heavy arrow appears to

Fig. 3. Immunoblot analysis of the CBP complex using anti-eIF-4A and anti-eIF-4B antibodies.

Samples were resolved either on a 10-18% gradient (lane 1) or a 10% (lanes 2 and 3) SDS/polyacrylamide gel. For the experiment in lane 1, proteins in the gel were transferred onto nitrocellulose paper and probed for with anti-eIF-4A monoclonal antibody as described in Materials and Methods. For the experiment in lanes 2 and 3, the proteins in the gel were transferred electrophoretically to nitrocellulose paper and processed as previously described (19). After transfer the paper was incubated in TBS containing 3% BSA followed by incubation with a mixture of affinity-purified polyclonal anti-eIF-4A and anti-eIF-4B antibodies. Excess antibody was washed away followed by incubation with ^{125}I-labeled rabbit anti-goat IgG. The paper was then washed and exposed to Kodak X-Omat SB5 film. Lanes 1 and 2 contained ~4 µg of CBP complex. Lanes 3 contained 1 µg each of eIF-4A and eIF-4B.


1
2
3
eIF-4A

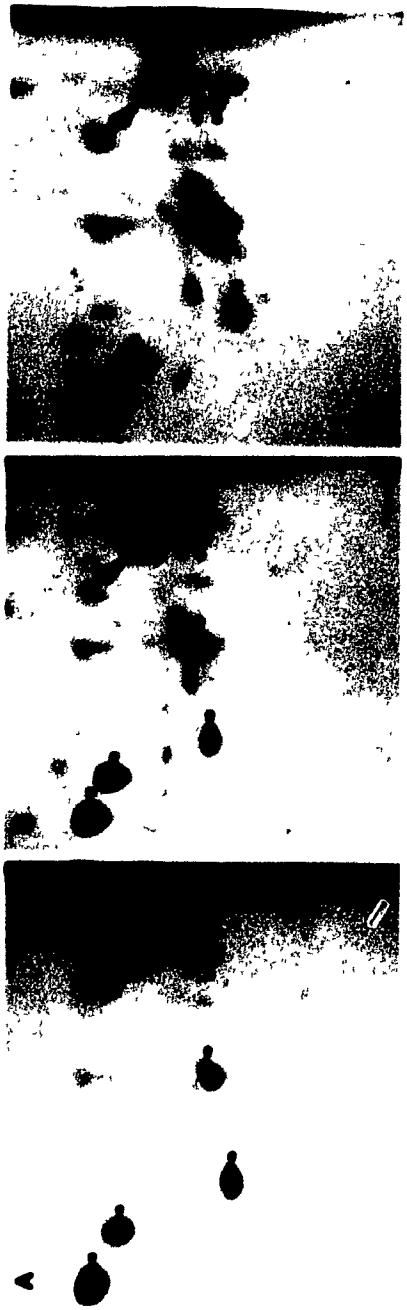
be prominent in the eIF-4A preparation (panel A), the peptide indicated with the thin arrow is prominent in the 50 kDa polypeptide of the CBP complex (panel C). This difference may reflect a modification of eIF-4A that could contribute to the observed distribution of eIF-4A between its free form and the CBP complex.

An important question raised by these findings concerns the functional significance of eIF-4A in relation to its distribution between the free form and the CBP complex. In order to gain insight into this question we analyzed the cross-linking characteristics of the different species containing eIF-4A, and since eIF-4B has been implicated in the cap recognition process (4), we examined its involvement here.

In the absence of ATP, incubation of oxidized reovirus mRNA with the CBP complex alone results in cross-linking of the 24K-CBP (Fig. 5, lane 1) which is m^7GDP sensitive (lane 2), while no cross-linking of the eIF-4A component of the complex is seen. Addition of eIF-4B to the CBP complex in the absence of ATP-Mg^{++} resulted in cross-linking of eIF-4B in addition to the 24K-CBP (lane 3). However, cross-linking of eIF-4B under these conditions is apparently not due to specific interaction with the cap since it was enhanced about 4 fold in the presence of m^7GDP (lane 4). This non-specific cross-linking which has been observed before (1) is enhanced in the presence of m^7GDP probably because under these conditions the 24K-CBP cannot compete for mRNA binding. These results are consistent with previous findings using crude initiation factor preparations, which showed no cap specific cross-linking of

Fig. 4. Peptide map analysis of eIF-4A and the 50 kDa component of CBP complex.

eIF-4A (1 μg) and CBP complex (~3 μg) were resolved on a 10-18% SDS/polyacrylamide gel which was stained with Coomassie blue. The gel pieces containing eIF-4A and the 50 kDa component of CBP complex (see Fig. 2) were excised and labeled with ^{125}I (0.4 mCi/slice) by the chloramine-T method according to Elder *et al.* (21). The gel pieces were washed to remove free ^{125}I and the proteins digested in the gel with 25 μg of trypsin (Worthington). The resulting peptides were eluted from the gel and lyophilized. Peptides ($1.5x10^5$ to $2x10^5$ cpm) were analyzed by electrophoresis in the first dimension and chromatography on cellulose coated thin layer plates (Brinkman) in the second dimension (22). Electrophoresis was in pyridine/acetic acid/acetone/water (1:2:8:40 v/v) at pH 4.4 for 75 min at 800 volts. Chromatography was in n-butanol/acetic acid/water/pyridine (15:3:12:10 v/v) for 5-6 hr. Plates were exposed to Cronex-4 X-ray film and Cronex Hi-plus intensifying screens for 16-24 hr. A. eIF-4A; B. eIF-4A + 50 kDa polypeptide of CBP complex; C. 50 kDa polypeptide of the CBP complex.

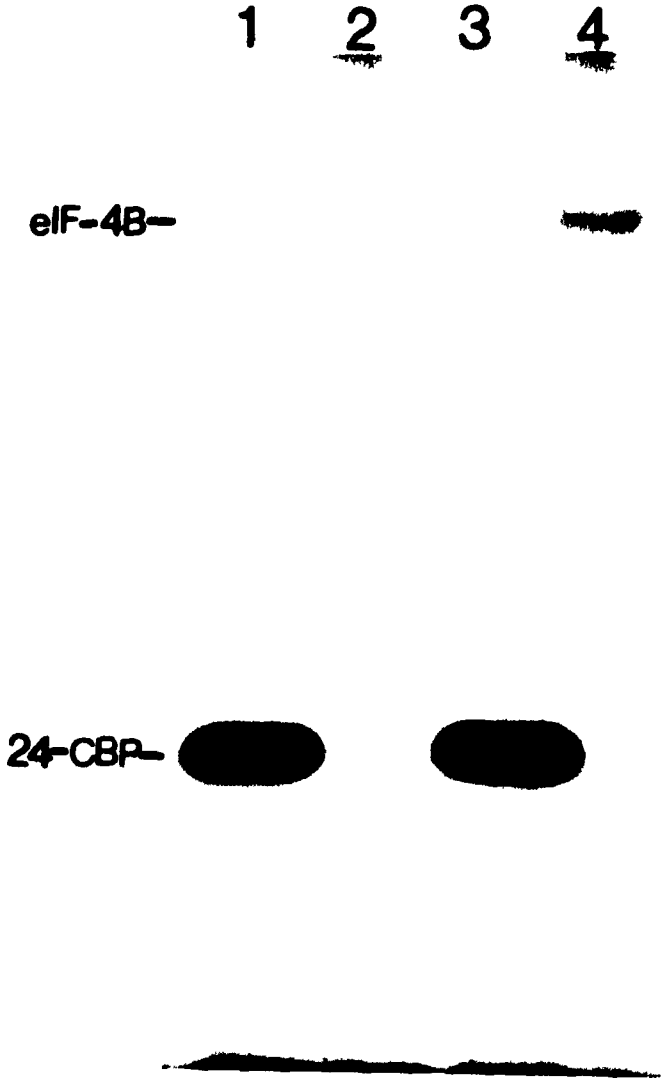


eIF-4A or the 80 kDa polypeptide (probably eIF-4B) in the absence of ATP (1,23).

Recently, Grifo et al. (4) have demonstrated cap specific, ATP dependent cross-linking of eIF-4A and eIF-4B when both of these factors are present together. The results from these studies also indicated the presence of some form of cap binding protein in the factor preparations used, since there was also detectable cross-linking of the 24K-CBP. It is therefore possible that the cross-linking of eIF-4A and eIF-4B is also dependent on the 24K-CBP, as pointed out by the above authors, and consequently, we were led to examine the cross-linking characteristics of combinations of eIF-4A, eIF-4B and the CBP complex in the presence of ATP. Using only eIF-4A together with eIF-4B there was no detectable cross-linking of any nature (Fig. 6, lane 1). This indicates that our preparations of eIF-4A and eIF-4B alone are not significantly contaminated by 24K-CBP and that eIF-4A and eIF-4B alone are not sufficient for a cap specific interaction with mRNA. The cross-linking profile obtained using the CBP complex by itself is identical to that in the absence of ATP, showing cap specific cross-linking of the 24K-CBP only (lane 3). However, addition of eIF-4B to the CBP complex results in cross-linking of 24K-CBP, the eIF-4A component of the CBP complex and eIF-4B (lane 5). This cross-linking is due to a cap specific mRNA-protein interaction as indicated by the substantial inhibition on addition of m^7GDP (74% and 60% inhibition of eIF-4A and eIF-4B cross-linking, respectively) on addition of m^7GDP (lane 6) and shows that cross-linking of eIF-4A in the CBP complex is dependent on eIF-4B. The cross-linking of both eIF-4A and eIF-4B is likewise dependent on an

Fig. 5. Cross-linking profile of CBP complex to [^{3}H]-methyl-labeled oxidized reovirus mRNA in the absence of ATP-Mg^{++}.

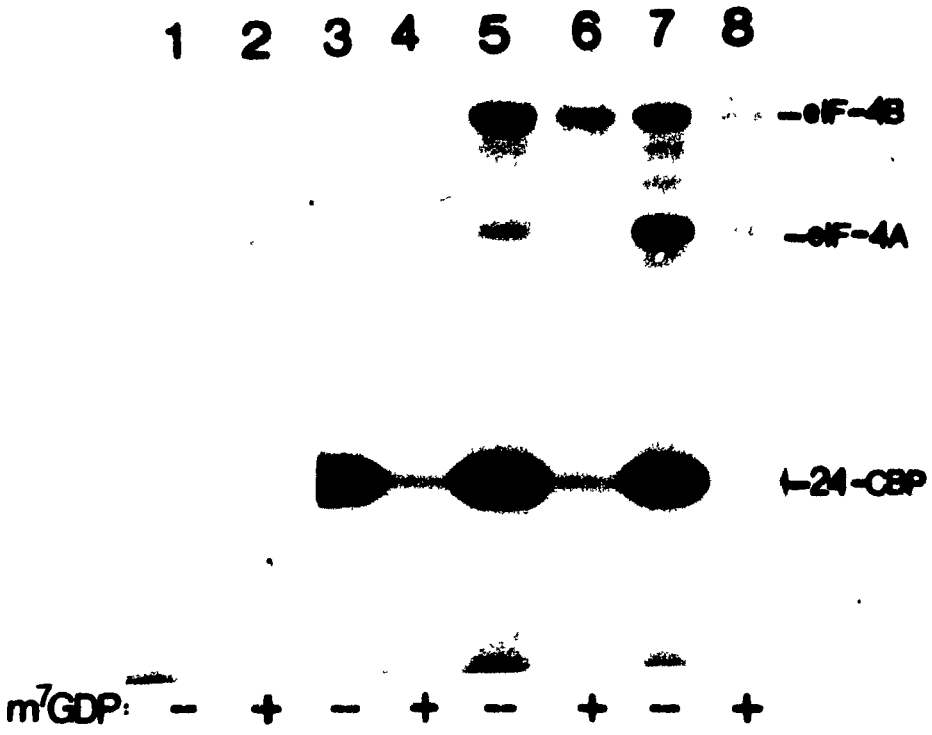
Cross-linking was performed as described in Materials and Methods and in the legend to Fig. 1, in a final volume of 40 µl containing 2 µg of CBP complex and 6 µg of BSA in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 0.7 mM m^7GDP. Lanes 3 and 4 also contained 1 µg of eIF-4B.


1
2
3
4
eIF-4B
24-CBP

activity present in the CBP complex, since we have found that a combination of 24K-CBP, eIF-4A and eIF-4B is not sufficient to enable specific cross-linking of eIF-4A and eIF-4B (data not shown). It is of interest, however, that addition of eIF-4A to the CBP complex in the presence of eIF-4B results in significant stimulation (about 5-fold) of eIF-4A cross-linking (compare lane 7 to 5). It is not clear, however, from these data whether the cross-linked eIF-4A is the eIF-4A component of the CBP complex or the exogenously added eIF-4A. In summary, these data indicate that the CBP complex contains an activity that is required for the cap specific cross-linking of both eIF-4A and eIF-4B, and that eIF-4B mediates cap recognition by eIF-4A in the CBP complex.

Fig. 6. Effect of CBP complex on cross-linking of eIF-4A and eIF-4B to 5' [3H]-methyl-labeled oxidized mRNA in the presence of ATP-Mg^{++}.

[^{3}H]-Methyl-labeled oxidized reovirus mRNA was incubated with initiation factors and samples processed for SDS/polyacrylamide gel electrophoresis and fluorography as described in Materials and Methods and in Fig. 5. Cross-linking was performed in the presence of 1 mM ATP and 0.5 mM Mg^{++} and in the presence or absence of 0.7 mM $m^{7}GDP$ as indicated in the figure. The following amounts of factors were used: eIF-4A, 0.6 µg; eIF-4B, 0.5 µg; CBP complex, 0.8 µg. Lanes 1 and 2, eIF-4A + eIF-4B. Lanes 3 and 4, CBP complex. Lanes 5 and 6, eIF-4B + CBP complex. Lanes 7 and 8, eIF-4A + eIF-4B + CBP complex.


1 2 3 4 5 6 7 8
eIF-4B
eIF-4A
4-24-CBP
m7GDP: – + – + – + – +

DISCUSSION

The observation that a monoclonal antibody with anti-CBP activity reacted with polypeptides (predominantly of M_r = 50,000 and 210,000) sharing common peptides with the 24K-CBP isolated by affinity chromatography on m^7GDP-Sepharose 4B columns, led to the suggestion that higher molecular weight cap specific polypeptides detected by the cross-linking assay might be precursors of the 24K-CBP (2,3,24). Since the anti-CBP antibody also inhibits cross-linking of all the cap specific polypeptides detected in crude initiation factor preparations (24,28,50 and 80 kDa polypeptides) it seemed a likely possibility that the cross-linked 50 kDa polypeptide was identical to the 50 kDa polypeptide recognized by the anti-CBP antibody. Consequently, in light of the suggestion by Grifo et al. (4) that the 50 kDa polypeptide which can be cross-linked is eIF-4A and the data presented here, it is of importance to clarify the relationship between the 50 kDa polypeptide recognized by the anti-CBP antibody and eIF-4A. The results presented here show that the cross-linkable 50 kDa polypeptide is indeed eIF-4A, as suggested by Grifo et al. (4), since an anti-eIF-4A monoclonal antibody precipitates the cross-linked 50 kDa polypeptide (Fig. 1). However, peptide analysis shows that eIF-4A and the 50 kDa polypeptide which reacts with the anti-CBP antibody are distinct polypeptides (H.T., unpublished results) implying that the 50 kDa polypeptide recognized by the anti-CBP antibody is not cross-linked to oxidized cap structures when using crude initiation factors.

An interesting question raised by the data presented here concerns the functional significance of eIF-4A. Most of the eIF-4A found in

association with ribosomes fractionates in the 40-70% A.S. fraction of IF (14,15), while cross-linkable eIF-4A is found in the 0-40% A.S. fraction. This suggests that cross-linking of eIF-4A is dependent on factors present in the 0-40% A.S. fraction which could be complexed with eIF-4A. Fractionation of the complexed portion of eIF-4A in the 0-40% A.S. fraction might then be due to its association with the CBP complex, as recently suggested by Tahara et al. (9). These observations raise the question as to how eIF-4A might be partitioned between its free and complexed forms. It is possible that a modification of eIF-4A permits its association with another component of the CBP complex since in tryptic peptide maps of the two forms we have consistently observed a difference in the relative amounts of two peptides that migrate close to each other (Fig. 4). However, it remains to be determined whether this is due to two forms of the same peptide and whether this putative modification has any functional significance. In this respect it is of interest that the molar ratio of eIF-4A to ribosomes in HeLa cells is 3, whereas that for other initiation factors to ribosomes is about 0.5 to 0.8 (25).

Several observation led to the hypothesis that a cap binding protein(s) facilitates ribosome binding by melting mRNA secondary structure (2,3). However, such an activity is as yet uncharacterized except for the fact that it appears to require ATP-Mg^{++} and is inhibited by an antibody with anti-CBP activity. Lee et al. have recently shown that cap specific cross-linking of the 50 kDa polypeptide in crude preparations of rabbit reticulocyte ribosomal high salt wash (shown here

to correspond to eIF-4A) can take place in the absence of ATP if the mRNA is devoid of stable secondary structure (23). This suggested that eIF-4A can interact with the cap structure only after the energy dependent melting of mRNA secondary structure. The observation that the eIF-4A in the CBP complex cannot be cross-linked to the cap structure unless eIF-4B is present (Fig. 6) could imply that any putative melting activity is not solely present in the CBP complex but is dependent on eIF-4B or alternatively that eIF-4B directly mediates cap recognition by eIF-4A. The latter possibility is consistent with results from Grifo et al. who have demonstrated cap specific ATP-Mg^{++} dependent cross-linking of purified eIF-4A and eIF-4B when both are present together (4), although these results are at variance with the data presented here which demonstrate that CBP complex is required for cap specific interaction between eIF-4A, eIF-4B and mRNA (Fig. 6). A likely explanation is that Grifo et al. (4) had CBP II (the CBP complex) as a contaminant in their eIF-4B preparations, since these investigators obtained significant cross-linking of the 24K-CBP in their reactions, while no such cross-linking is evident in our experiment (Fig. 6, lane 1). The possibility that Grifo et al. had the 24K-CBP (CBP I) as the only contaminant of their eIF-4B preparations seems unlikely, in light of the fact that we have found no stimulation of the m^7GDP sensitive cross-linking of eIF-4A and eIF-4B by the addition of purified 24K-CBP (K.A.W.L., I.E. and N.S., unpublished observations).

A cap binding protein complex (CBP II) was originally purified by Tahara et al. (7,9) and functionally characterized in that it could restore translation of a capped mRNA in extracts from

poliovirus-infected HeLa cells. Since the polypeptide composition of the CBP complex described here is to some extent deficient (missing a 55 kDa polypeptide) compared to that obtained by Tahara et al. it was of importance to determine the biological activity of our CBP complex. To assess this, we assayed for the ability to restore translation of a capped mRNA (tobacco mosaic virus RNA) in extracts from poliovirus-infected HeLa cells. The results obtained indicated that the components present in the CBP complex are sufficient for activity in the above assay (I.E. and N.S manuscript in preparation). Further work will be aimed at determining the mechanism of action of the CBP complex in eukaryotic translation initiation and its mode of inactivation during poliovirus infection.

We have recently learned that Grifo et al. (26) have purified a cap binding protein complex analogous to CBP II that has been termed eIF-4F. This complex consists of four major polypeptides of ~24,46,73 and 200 kDa. However, it is claimed that the 73 kDa polypeptide is not an integral component of the CBP complex in agreement with our data.

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CHAPTER 3

HIGH LEVEL EXPRESSION IN *E. COLI* OF FUNCTIONAL CAP BINDING EUKARYOTIC INITIATION FACTOR eIF-4E AND AFFINITY PURIFICATION USING A SIMPLIFIED CAP ANALOG AFFINITY MATRIX

SUMMARY

Numerous studies have established the important role that eukaryotic initiation factor-4E (eIF-4E) plays during protein biosynthesis. However, biochemical characterization of eIF-4E has proved difficult, mainly because of its low abundance in cells. To facilitate studies on eIF-4E, we have overexpressed Saccharomyces cerevisiae eIF-4E in E. coli. The isolation of eIF-4E was simplified by using a cap-analog affinity matrix that is considerably less demanding to prepare than those previously reported. We describe a simple and rapid purification scheme that can yield 2-5 milligrams of a homogenous and active preparation of eIF-4E from 1 liter of E. coli. E. coli-expressed eIF-4E is active as determined by its ability to bind the cap structure. The results demonstrate that the cap binding activity of eIF-4E is not dependent on the presence of other proteins that are present at low levels in eIF-4E preparations isolated from eukaryotic cells.

INTRODUCTION

The cap structure present at the 5' end of all eukaryotic cellular (except organelles) mRNAs, plays a multifunctional role during gene expression. Capped RNA substrates are more efficient during protein synthesis (reviewed in refs. 1,2), splicing (3,4), and 3' end processing (5). Consistent with the diverse roles of the cap during gene expression, a number of different cap binding proteins (CBPs) have been identified in the cytoplasm (for recent reviews see, refs. 6,7) and nucleus (8,9). It is imperative to purify the CBPs in order to gain an understanding of their functions. Rapid and efficient purification has been accomplished by using affinity chromatography based on cap-analogs covalently attached to a solid support matrix (10-12). To date the first described and best characterized cap binding protein is eukaryotic initiation factor 4E (eIF-4E; 10,13).

Initially, eIF-4E was isolated from rabbit reticulocyte ribosomal high salt wash using an m GDP-Sepharose affinity matrix. The protein was purified to apparent homogeneity and shown to consist of a single polypeptide of approximately 24 kDa (10). Subsequently, a similar homolog has been found in different eukaryotic cells, including yeast (14) plants (15,16), and human (17,18). eIF-4E can also be isolated in association with other polypeptides as a distinct high molecular weight form, termed eIF-4F (19), previously also termed CBP II (20) or the CBP complex (21). Numerous studies indicate that eIF-4F (in association with eIF-4A and eIF-4B) can facilitate 40S ribosomal subunit binding to mRNA by its ability to act as a helicase and denature 5' proximal mRNA secondary structure (e.g. 22; reviewed in refs. 7,23). The interaction

between eIF-4E (as a component of eIF-4F) and the cap structure is of particular importance because it is probably the first event in committing an mRNA to enter the cycle of protein synthesis. In addition, it participates in mRNA binding which is the rate-limiting step in translation (24), and therefore is a likely target for translational regulation. It is also noteworthy that eIF-4E is the least abundant initiation factor (25,26) and as a result it may modulate the amount of functional eIF-4F present in the cell.

To study structure-function relationships in eIF-4E, we have used the cloned Saccharomyces cerevisiae eIF-4E gene (14) to attain high levels of eIF-4E expression in E. coli. A simplified purification scheme is described that can easily yield 2-5 milligrams of pure eIF-4E from 1 L of culture. To facilitate the isolation of CBPs we improved the procedure using a cap-analog affinity matrix that is much less demanding to prepare than those previously reported (10-12).

MATERIALS AND METHODS

(a) m^7GDP-agarose resin.

The synthesis of a resin consisting of 7-methyl guanosine diphosphate (m^7GDP) coupled to agarose was performed by a modification of the procedure initially described by Lamed et al. (27). The synthesis is schematically represented in figure 1. All steps, unless otherwise indicated, were performed at 4°C. The numbers given below are based on using 1 ml of the reactive solid support matrix.

(1) Oxidation of m^7GDP. To a 0.5 ml solution of 10 mM m^7GDP (P-L biochemicals) dissolved in water, 0.1 ml of 0.1 M NaOAc, pH 6.0, containing 5 μmoles (1 equivalence) of sodium metaperiodate (Fisher) was added. The mixture was briefly vortexed and allowed to stand for 1.5 h in the dark, and then used in step 2.

(2) Coupling reaction. Adipic dihydrazide agarose (1 ml; P-L biochemicals) was washed in a sintered glass funnel with 20 ml of water followed by 20 ml of 0.1 M NaOAc, pH 6.0, under a slight vacuum. The equilibrated adipic dihydrazide agarose was transferred, by using a total of 2 ml of 0.1 M NaOAc, pH 6.0, directly to the tube containing the oxidized m^7GDP (step 1). The slurry was incubated end over end for 1.5 h. The hydrazone bond formed was stabilized by the addition of 5 mg of solid sodium cyanoborohydride (Aldrich) and incubation for approximately 15 h. To remove non-covalently bound m^7GDP the resin was washed with succesive 5 ml aliquots of 1M NaCl for 15 min each. The 1 M NaCl washes were continued till the $O.D._{260}$ readings were close to zero. Finally, the resin was equilibrated in buffer A (0.1 M KCl, 20 mM Hepes pH 7.5, 0.2 mM EDTA) and stored at 4°C.

Coupling efficiency was determined by comparing the $O.D._{260}$ of the starting amount of oxidized m^7GDP and the 1M NaCl washes.

(b) Overexpression of yeast eIF-4E in E. coli.

(1) Vector construction. All recombinant DNA manipulations were performed by standard procedures essentially as described in Maniatis et al. (28). The methodology employed is displayed in figure 2. A plasmid vector containing a Hind III genomic fragment of the S. cerevisiae eIF-4E gene (for details see ref. 14) was restricted with Avr II and Hpa I. The eIF-4E fragment containing the entire reading frame and portions from the 5' and 3' noncoding regions was isolated and directly subcloned into the plasmid pGEM•2 (Promega) between the Xba I and Sma I sites of the polylinker region. The resulting hybrid plasmid, pT74E, places eIF-4E expression in the sense orientation under the control of the T7 RNA promoter. Propagation of pT74E was done in either E. coli HB101 or Y1090.

(2) Overexpression. High-level expression of eIF-4E was essentially performed by the method of Studier and Moffatt (29). Briefly, an overnight culture of E. coli cells transformed with pT74E is diluted (1:75) into 1 L of LB media (containing 3 mM $MgCl_2$, 0.2% (w/v) maltose and 75 µg/ml ampicillin). The culture was incubated at 37°C till an $O.D._{600}$ of approximately 0.8 is achieved. To supply T7 RNA polymerase, cells were infected with a constructed T7 RNA polymerase containing phage, CE6, at a ratio of 3:1 (CE6/cell; CE6 was constructed to yield high-level expression of target genes under the control of a T7 promoter, and was a kind gift from W. Studier, Brookhaven National

Laboratory, U.S.A.). Cells were harvested 2 h post-infection and placed on ice.

(c) Purification of eIF-4E.

All steps were performed at 4°C. E. coli cells expressing eIF-4E (step b) were suspended in sonication buffer (0.5 M KCl, 20 mM Hepes, pH 7.5, 0.2 mM EDTA, 0.5% NP-40) using 1/40th the volume that was used for the initial LB culture media. Resuspended cells were sonicated 5 times for 30 seconds each, taking care to cool the probe in between cycles of sonication. The homogenate was clarified by centrifugation for 10 min at 10,000 g. The resulting supernatant was carefully removed and re-centrifugated (see text for details) in a Ti60 rotor for 2 h at 40K rpm. The supernatant from the high speed spin was diluted with 20 mM Hepes, pH 7.5, to achieve a final concentration of 125 mM KCl. At this stage eIF-4E can be affinity purified using a cap-analog affinity matrix. Aliquots of 50 ml were incubated with 0.3 ml of the m^7GDP-agarose resin for 30 min. The beads were sedimented by a slow speed spin and the supernatant carefully removed. Each aliquot was washed with 40 ml of buffer A for 15 min, followed by 20 ml of buffer A containing 0.1 mM GDP. The m^7GDP-agarose resin was pooled and specific elution of eIF-4E was achieved by 3 successive incubations of the resin with 6 ml aliquots of buffer A containing 0.1 mM m^7GDP. A final wash step with 6 ml of buffer A containing an additional 0.9 M KCl, was performed to remove all adsorbed proteins. The resin was re-equilibrated in buffer A prior to reusing. Typical results are shown in figure 3B.

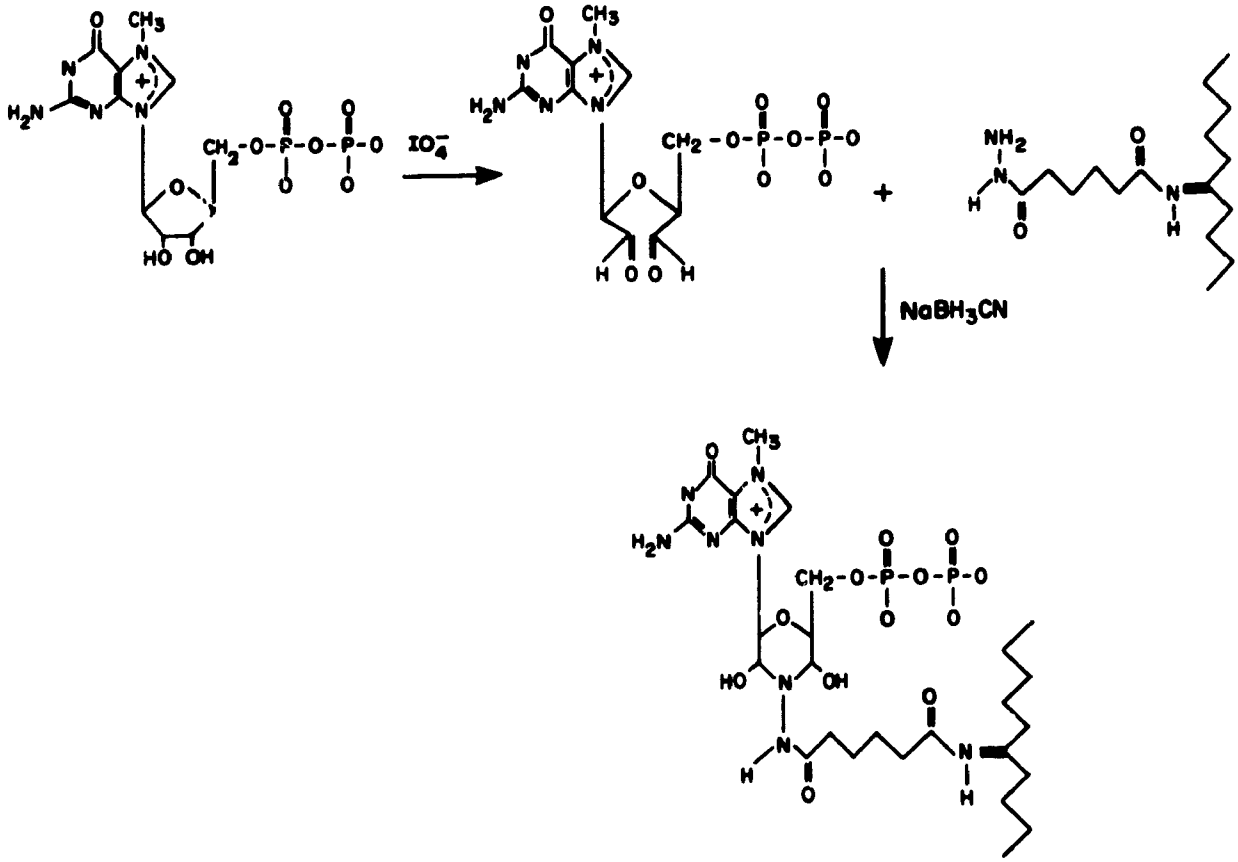
(d) *In vivo* radiolabeling of eIF-4E.

To obtain radiolabeled eIF-4E of high specific activity, we modified the above procedure. Typically, 5 ml of *E. coli* cells transformed with pT74E were grown until the $O.D._{600}$ has reached approximately 1. A 3:1 ratio (CE6/cell) was added and incubated with shaking for 10-12 min at 37°C to allow phage adsorption. Cells were immediately harvested at room temperature by a low speed spin, followed by resuspension in an equal volume of labeling media (M9 salts, 0.001% (w/v) cassamino acids, 75 µg/ml ampicillin). Once resuspended, 250-1000 µCi of a mixture of ^{3}H labeled amino acids (I.C.N.) was added. Labeling was performed for 2 h at 37°C and eIF-4E purified as described above.

(e) Western blotting.

This was done as previously described (30) using a rat polyclonal anti-yeast eIF-4E antibody. The blot was incubated with a rabbit anti-rat antibody (DAKO) followed by alkaline phosphatase conjugated anti-rabbit antibody (Promega). Color development was according to manufacturer's (Promega) recommended procedures.

Fig. 1. Affinity resin for CBPs. The key steps in the synthesis are illustrated and described in detail in Materials and Methods (section a).


IO_4^-
$NaBH_3CN$

RESULTS

(a) Cap-analog affinity matrix.

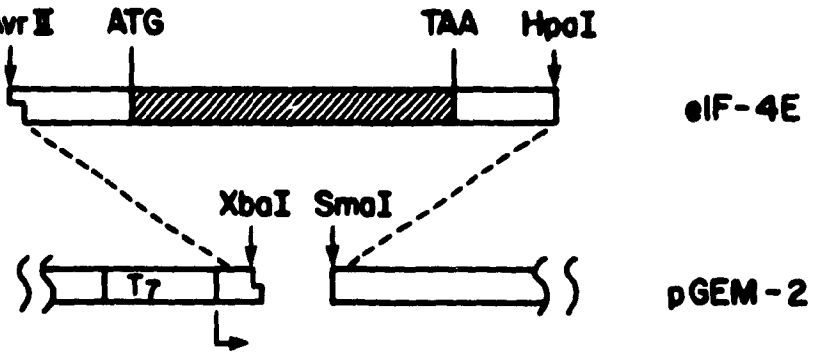
The use of affinity resins consisting of cap-analogs covalently attached to a solid support matrix has proven the method of choice for the purification of cap binding proteins (10-12). However, all the previously described resins require rather demanding chemical synthesis steps. To simplify the procedure we have synthesized an m^7GDP-agarose resin (Fig. 1) based on a combination of the chemical cross-linking assay used to detect CBPs (31) and methods previously described by Lamed et al. (27). The cap-analog, m^7GDP, was oxidized to the reactive dialdehyde in the presence of periodate. The oxidized ligand was then incubated with a solid support matrix containing hydrazide functional groups. A hydrazone bond intermediate was formed and stabilized by sodium cyanoborohydride. Typically, we have achieved greater than 90% ligand binding efficiency and the resin is stable for months with no apparent loss in the capacity to bind CBPs when stored at 4°C.

(b) High-level expression of eIF-4E.

The recent cloning of eIF-4E from yeast (14), human (32) and mouse (Pelletier et al., in preparation) has, in principle, made it possible to obtain large quantities of the protein. We chose to express eIF-4E in E. coli because prokaryotes do not contain endogenous CBPs and therefore a homogeneously pure preparation of eIF-4E could be obtained. Part of the yeast eIF-4E gene (containing the full coding sequence for the 24 kDa protein) was subcloned such that its expression in the sense orientation is under the control of T7 RNA polymerase (Fig. 2A). A fortuitous Shine-Dalgarno (SD) sequence (Fig. 2B) present in the eIF-4E

Fig. 2. eIF-4E expression vector. A) A fragment containing the entire reading frame of yeast eIF-4E was subcloned directly into the pGEM.2 vector as described in Materials and Methods (section b). Expression in the sense orientation is under the control of the T7 RNA promoter. The site of transcription initiation and direction are shown by the arrow. B) The putative Shine-Dalgarno (SD) sequence present in the yeast eIF-4E gene (14). The start ATG codon is typed in large letters and the SD sequence is underlined.

a)



b) ...TAGTGAAAGGAAGAAAAATG...

gene obviated the need to construct a SD sequence into the expression vector (14).

Our main objective was to design a simplified overexpression procedure that could easily yield milligram amounts of pure protein. Several methods were attempted, and the best results were obtained by using the procedure described by Studier and Moffatt (29). In this system high levels of protein were synthesized from cloned sequences that were placed under the control of the T7 RNA promoter on an appropriate plasmid. E. coli HB101 produced approximately twice as much protein as Y1090 cells. (10-20 mg of eIF-4E was obtained for 1 liter of culture).

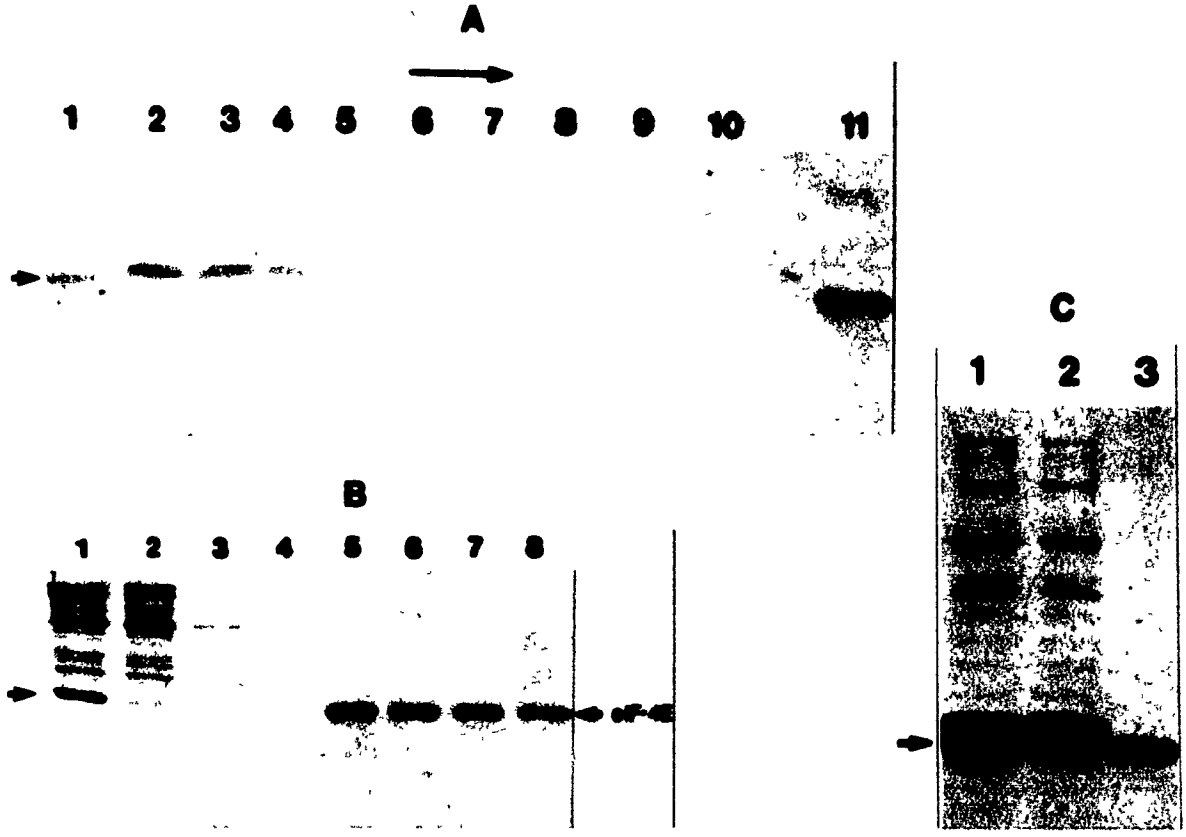
(c) Affinity purification of eIF-4E.

It was difficult to solubilize a significant portion of eIF-4E under conditions whereby it still retained its activity. We found that a combination of vigorous sonication in the presence of 0.5 M KCl and detergent significantly increased the yield of solubilized protein. However, the clarified homogenate obtained after a low speed centrifugation to remove cellular debris, bound poorly to the m^7GDP-agarose resin. Only 5% of the total solubilized eIF-4E (~50% of total eIF-4E produced) could bind and be specifically eluted from the resin. We reasoned that this could be due to the possible presence of inactive complexed eIF-4E which was inhibiting functional eIF-4E from binding. The presence of fast sedimenting eIF-4E was confirmed by the experiment depicted in Fig. 3. When the supernatant fraction resulting from the low speed spin is subjected to centrifugation through a sucrose

gradient two distinct sedimentation forms of eIF-4E could be readily detected (Fig. 3A, compare lanes 1-4 with 11). Removal of the fast sedimenting eIF-4E (about 50% of total eIF-4E present, lane 11) allows for the high efficiency purification of the remaining functional eIF-4E (Fig. 3B, compare lanes 1 and 2). Consequently, approximately 90% of the eIF-4E present in the supernatant subsequent to a ultracentrifugation step can bind the m^7GDP-agarose resin. Thus, about 25% of total eIF-4E produced can be isolated in a pure and active form. The purity of eIF-4E is shown in figure 3B (lanes 5-8).

To study certain biochemical aspects of eIF-4E (e.g. stability, association with other factors, etc.), radiolabeled eIF-4E is required. Radiolabeled eIF-4E of high specific activity (200,000 cpm/μg) was prepared. Only two prominent bands are apparent after labeling (Fig. 3C, lane 1), one of which (≈24 kDa) was specifically eluted from the column with m^7GDP and is eIF-4E (compare lane 1 to 3). The other polypeptide (≈ 28 kDa) which did not bind to the column is most likely β lactamase (29). We could not label the yeast eIF-4E with ^{35}S-methionine, because there is only one methionine present at the amino terminus (14) and it is presumably enzymatically removed in E. coli. The labeling media can be re-used several times without loss of labeled amino acid incorporation into eIF-4E since only 5-10% of the input radioactivity is taken up by the cells.

Fig. 3. Analysis of E. coli-expressed yeast eIF-4E. A) Sedimentation analysis.eIF-4E was expressed and a supernatant obtained after a low speed centrifugation as described in Materials and Methods (section c). The supernatant was layered onto a 12 ml linear 10-30% sucrose gradient containing 0.5 M KCl, 20 mM Hepes, pH 7.5, 0.2 mM EDTA and 0.5% (V/V) NP-40. Centrifugation was performed at 4°C in a SW-40 rotor for 20 hr at 40 K rpm. The gradient was fractionated into 1.2 ml fractions and the pelleted material resuspended (lane 11). Equal proportions from each fraction (lanes 1-10) and the pellet (lane 11) were resolved and analyzed by Western blotting (Materials and Methods, section e). The arrow above indicates the direction of sedimentation. B) Affinity purification of eIF-4E. E. coli expressed eIF-4E was purified as described in Materials and Methods (section c). The purification results shown above were from 1L of culture and represent typical yields and purity. Fractions were analyzed by Coomassie blue staining following SDS/polyacylamide gel electrophoresis. The fractions are as follows: starting material prior to incubation with cap analog resin (lane 1), non-bound material (lane 2), buffer A wash (lane 3), GDP wash (lane 4), m^7GDP elutions (lanes 5-7), buffer A containing 0.9 M KCl wash (lane 8). 60 µl representing 0.1% of the collected volume (lanes 3-8) was analyzed. C) Purification of in vivo radiolabeled eIF-4E. E. coli expressed eIF-4E was radiolabeled and purified as described in Materials and Methods (section d). In this experiment, 1 mCi of a mixture of ^{3}H-labeled amino acids (I.C.N.) was concentrated to 0.2 ml and then diluted into 5 ml of labeling media. Following affinity purification equivalent portions of different fractions were resolved by SDS/PAGE and visualized by fluorography (EN^{3}HANCE, NEN) and autoradiography. The following fractions were analyzed: material loaded onto the affinity matrix (lane 1), flow thru material (lane 2), first m^7GDP elution (lane 3). An XAR-5 (Fuji) film was exposed for 10 hr. In each lane 0.5% of the total material was analyzed. The position of eIF-4E is indicated to the left.


A
1 2 3 4 5 6 7 8 9 10 11
B
1 2 3 4 5 6 7 8
C
1 2 3

DISCUSSION

Our main objective in this report was to devise a simple and efficient system to produce large quantities of pure eIF-4E. We accomplished this by developing two complementary procedures. (a) The synthesis of a simplified cap-analog affinity matrix and (b) a high expression system for the production of eIF-4E.

The purification and characterization of cap binding proteins is vital to understanding the diverse functions of the 5' cap structure during gene expression. Although several different CBPs have been identified, to date the only bona fide cap binding protein isolated in a relatively pure state is eIF-4E (10,12,33). Affinity purification based on cap-analogs covalently attached to a variety of solid support matrices (10-12) has proven to be an extremely efficient method for the selective isolation of CBPs. Although a cap-analog resin described by Rhoads and coworkers (12), is commercially available (Pharmacia), the design of our resin has certain advantages. It is less expensive to prepare, and importantly can easily be adapted for the general binding of compounds that have a free cis diol. One noteworthy application would be the synthesis of a resin that contains derivatives of the trimethyl cap ($m_3^{2,2,7}G$) found at the 5' end of most of the U-series small nuclear RNAs involved in precursor mRNA splicing (for a review see ref. 34).

To obtain large amounts of eIF-4E, we expressed the protein in E. coli. As evidenced by the purification shown in Fig. 3B, high levels of

highly pure protein can be attained. Significantly, most of the eIF-4E could be purified only after being subjected to an ultracentrifugation step to remove heavy sedimenting material (Fig. 3A). It is evident that eIF-4E aggregates strongly when overexpressed. Indeed, a major problem in dealing with the intracellular overexpression of foreign proteins in bacteria is the relative difficulty of protein solubilization under non-denaturing conditions. Removal of fast sedimenting protein may be of general use for the isolation, in a functional state, of other overexpressed proteins.

One major advantage of using bacteria to overexpress eIF-4E, is that there are no endogenous CPBs in E. coli, resulting in a very pure preparation of eIF-4E (Fig. 3B). The ability of E. coli-expressed eIF-4E to bind the cap structure demonstrates unequivocally two important properties. First, post-translational modifications are not required for eIF-4E cap binding capacity. Mammalian eIF-4E can exist in a phosphorylated form (26,35,36) and it has been suggested that this modification may influence the affinity of eIF-4E for the mRNA cap structure (35). However, non-phosphorylated eIF-4E (as a component of eIF-4F) can still bind a cap-analog resin (26,35,36). The results shown here support this conclusion. Secondly, the ability of eIF-4E to bind caps is not dependent on the presence of any other eukaryotic protein factors. For example, the previously reported ability of eIF-4A and eIF-4B to specifically bind the cap structure was probably due to low levels of contaminating eIF-4F in the eIF-4B preparation (37). Likewise, cap binding activity could presumably be dependent on low

levels of proteins associated with eIF-4E. However, the current findings demonstrate that an E. coli-synthesized eIF-4E possesses cap-binding activity. However, the ability of eIF-4E to recognize the cap structure might be enhanced by other components of the translation machinery (38).

One of the important issues concerning the mechanism of function of eIF-4E is to elucidate the structure-function relationship responsible for its cap binding activity. These studies are best addressed by a combination of in vitro mutagenesis coupled with direct physical measurements. Recently, using the methods described herein we purified yeast eIF-4E which had been mutated in tryptophan residues, and determined the important residues for cap binding activity (M. Altmann., I. Edery., Hans Trachsel and N. Sonenberg., submitted). Understanding structure-function relationship in eIF-4E should lead to the development of a more efficient eIF-4E that when introduced into cells will enhance the rate of protein synthesis.

In summary, the procedures described in this report should significantly enhance our understanding of the crucial role(s) eIF-4E plays in protein synthesis, and facilitate the isolation and characterization of nuclear cap binding proteins of which little is presently known.

ACKNOWLEDGMENTS

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CHAPTER 4

ACTIVATION OF DOUBLE-STRANDED RNA DEPENDENT KINASE (dsI) BY THE TAR REGION OF HIV-1 mRNA: A NOVEL TRANSLATIONAL CONTROL MECHANISM

SUMMARY

All mRNAs of human immunodeficiency virus-1 (HIV-1) contain in their 5' untranslated region a sequence termed TAR that responds to trans-activation by the tat (trans-activating) protein. This RNA sequence assumes a stable secondary structure and its cap structure is relatively inaccessible. Here we report that these structural properties of the TAR sequence underlie the ability of TAR to inhibit in trans the translation of other mRNAs. This mechanism of translation inhibition involves the activation of the double-stranded RNA dependent kinase (dsI), which in turn phosphorylates the protein synthesis initiation factor-2 (eIF-2). Mutations in the TAR region which diminish the stability of the secondary structure cause a significant reduction in the trans-inhibition. A similar reduction in dsI activation occurs when TAR is placed further downstream of the cap structure. This is the first demonstration of a specific naturally occurring mRNA sequence that can activate dsI. We suggest a novel translational regulatory mechanism which interdigitates the activities of eIF-2 and eIF-4F.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of the acquired immune deficiency syndrome (AIDS; 1,2). Replication of HIV-1 is an extremely complex process which involves several *trans*-acting genes and responding *cis*-acting sequences (3-8). One of the *trans*-activators which plays a pivotal role in virus replication is the *tat* protein. The mechanism by which *tat* trans-activates HIV-1 gene expression is not clear, although evidence suggests both transcriptional and post-transcriptional mechanisms (7-12). The *cis*-acting sequences responding to *tat trans*-activation (termed TAR) are located downstream of the transcription initiation site, and map to nucleotides +1 to +80 (5). The TAR sequence is present in all HIV-1 mRNAs and assumes a stable stem and loop structure *in vitro* as determined by RNA nuclease mapping (10).

Excessive secondary structure in the 5' untranslated region (UTR) of eukaryotic mRNAs interdicts translation initiation (13). Recently, Parkin *et al.*, (14) demonstrated that the TAR region, when fused to a heterologous mRNA, exhibited a strong inhibitory effect on translation in cell-free extracts and *Xenopus* oocytes. Two factors determined the degree of inhibition by the TAR region: secondary structure and accessibility of the cap structure (14).

The secondary structure at the 5' proximal end of a eukaryotic mRNA is believed to be melted during translation initiation by the eukaryotic initiation factor 4F (eIF-4F) in conjunction with several other

initiation factors (for a recent review see ref. 15). Consequently, the accessibility of the cap structure to eIF-4F is an important determinant in controlling translational efficiency (16,17). Consistent with this, increasing the accessibility of the cap structure of HIV-1 mRNA using site-directed mutagenesis mitigated the translational inhibitory effect of the TAR sequence (14).

The presence of double stranded RNA can potentially inhibit protein synthesis in trans by a different mechanism. Double-stranded RNA sequences can mediate the autophosphorylation and activation of the double-stranded RNA-dependent kinase (dsI)(17a). The phosphorylated and active dsI specifically phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2) rendering it incapable of recycling (for reviews see refs. 17b,18). Although it is not entirely clear what the normal physiological function of dsI is, it plays an important role during viral infection. As part of the cellular response to block viral gene expression, cells synthesize the anti-viral agent interferon (reviewed in ref. 19). Interferons elicit their anti-viral activity, in part, by increasing the level of dsI.

The results in this paper demonstrate that the TAR region can inhibit mRNA translation in trans and that this inhibition is mediated by the presence of the unique TAR stem and loop structure. Translational inhibition is correlated with phosphorylation of dsI and the α subunit of eIF-2. Activation of dsI mediated by the TAR region might have important implications for the control of viral and host gene expression after HIV-1 infection. From these observations we suggest a novel translational regulatory mechanism.

Materials and Methods

Plasmid Constructs

All recombinant DNA techniques were performed by standard methods (20). The plasmids CAT and TAR/CAT (Fig. 2A) and the TAR mutants, TAR3/CAT, TAR3R/CAT and TAR3R3/CAT (Fig. 4), were previously described (14). The plasmid polio/CAT (Fig. 2A) was described by Pelletier *et al.*, (21). The plasmid PLTAR/CAT (Fig. 2A) contains 23 nt between the SP6 promoter and the +1 nucleotide of the TAR element (mostly polylinker sequences) and was a kind gift from Dr. C. Rosen (Roche Institute, Nutley, N.J.). To generate the hybrid plasmids TAR/polio/CAT and PLTAR/polio/CAT (Fig. 2A), plasmid polio/CAT (designated Δ5'-320/CAT in ref. 21) was digested with Hind III and BamHI. The released insert containing the poliovirus 5' UTR and the CAT coding sequence was gel purified, and inserted into the HindIII-BamHI site of the TAR/CAT plasmid resulting in TAR/polio/CAT. The plasmid PLTAR/polio/CAT was created by first performing a partial HindIII digestion on the plasmid PLTAR/CAT. The partially digested plasmids were gel-purified and incubated with BamHI. The desired HindIII-BamHI backbone was purified and ligated to the HindIII-BamHI insert from polio/CAT. All poliovirus clones contain nucleotides 320 to 733 of the poliovirus 5' UTR.

In Vitro Transcriptions

Plasmids were linearized at the unique BamHI site downstream of the CAT coding sequence followed by phenol extraction and ethanol precipitation. Linearized plasmids were used as templates for *in vitro* synthesis of m^7GpppG-primed mRNA as previously described (13), except

that the concentration of GTP and m^7GpppG were raised to 0.2 and 1mM, respectively. Yields of transcripts were calculated from the incorporation of [^{3}H]-CTP into RNA. All mRNAs were analyzed for integrity on 1% agarose-formaldehyde gels and visualized by fluorography and autoradiography. Autoradiography showed the presence of a single RNA species migrating at the appropriate size. For each experiment where different mRNAs were directly compared, they were all synthesized simultaneously. For the experiments involving gel-purified mRNAs (Fig. 7B) transcription was performed with [α-^{32}P]GTP. The resulting mRNAs were purified by preparative gel electrophoresis using a 40 cm long sequencing gel (7 M urea, 4% acrylamide, 0.2% bis-acrylamide, 1 X TBE). The corresponding radiolabeled mRNAs were identified by autoradiography and purified as described in Grabowski et al. (22). The quality of the gel-purified mRNAs was determined by analysis on a 1% agarose-formaldehyde gels and autoradiography. All mRNAs were resuspended in sterile water and stored at -70°C.

In Vitro Translations

In vitro translations were performed in a nuclease treated rabbit reticulocyte lysate (Promega) according to the manufacturer's recommended procedure. trans-Inhibition experiments were done as follows: dried mRNA was resuspended in 4.4µl of nuclease treated rabbit reticulocyte lysate and pre-incubated for 10 min at 30°C, except where indicated otherwise, and immediately placed in an ice water-bath. Subsequently, the reaction mixture was supplemented with ^{35}S-Methionine, amino acid mixture (minus methionine) (Promega) and the indicator CAT mRNA (or polio/CAT mRNA in Fig. 6), and translation was

done at 30°C for 60 min. Radiolabeled CAT protein was analyzed by resolution on a 12.5% polyacrylamide-SDS gel followed by fluorography and autoradiography. The autoradiograms were quantitated using an LKB scanning densitometer.

Cell Culture and Extract Preparation.

3T3-F442A cells were grown to confluence in Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with 10% fetal or calf serum and S10 extracts were prepared as previously described (23). Mouse inteferon-beta (IFNβ)(Lee Bio Molecular Research Laboratories, San Diego, Ca) was added to cultures (50 IU/ml) 18 hr prior to preparation of extracts (24). These S10 extracts were used as a source of crude dsI in the protein kinase assays. Highly purified latent dsI (25) and eIF-2 (26) were prepared from rabbit reticulocyte lysates as described. Guanine nucleotide exchange factor (GEF) was a kind gift of Dr. Robert L. Matts (Oklahoma State University) and prepared as described (27).

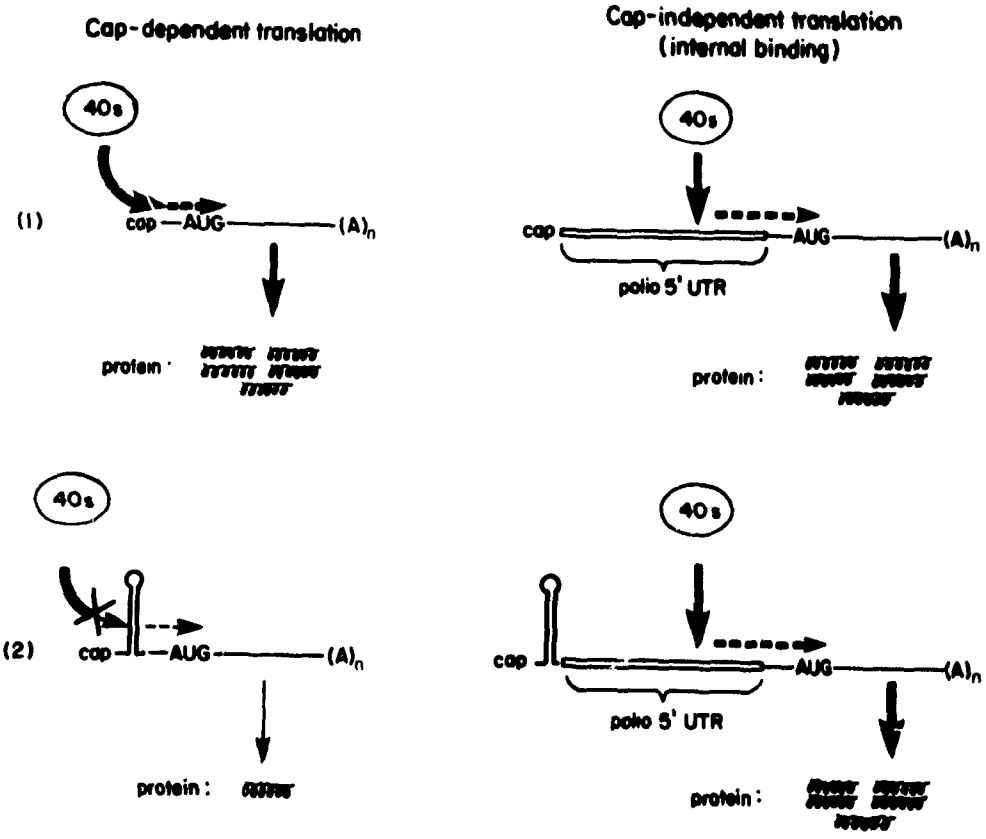
Protein Kinase Assays.

Protein phosphorylation assays (20 µl) using 3T3-S10 extracts (30 µg) and purified latent dsI were carried out under conditions previously described (23,25). All incubations were for 10 min at 30°. Other additions or changes are as indicated in the figure. The reactions were terminated by the addition of SDS-denaturing buffer (28) and heated at 95° for 2 min. Proteins were separated on a 7.5% polyacrylamide-SDS gel and analyzed by autoradiography (23).

Figure 1. Models for the two mechanisms of 40S ribosome binding to eukaryotic mRNAs.

(1) Left: Cap-dependent translation. The 40S ribosomal subunit binds at or near the cap structure and scans the 5' untranslated region (UTR) of the mRNA until an appropriate AUG initiation codon is encountered (reviewed by Kozak, (29). The thickness of the arrow and the number of protein products represent the relative translational efficiency. Right: Direct binding of the 40S ribosome to an internal region of the poliovirus mRNA 5' UTR. See text for details.

(2) Predicted effect of introducing stable secondary structure at the 5' end of eukaryotic mRNAs. Left: The presence of a stable stem and loop structure in the 5' UTR of a cap-dependent mRNA inhibits the ability of the 40S ribosome to either bind and/or scan the mRNA resulting in a reduced translational efficiency. Right: The presence of a stable stem and loop structure upstream from the polio 5' UTR should not impede internal binding by 40S ribosomes.


Cap-dependent translation
Cap-independent translation
(internal binding)
40s
(1)
cap
AUG
(A)n
protein
polio 5' UTR
(2)

RESULTS

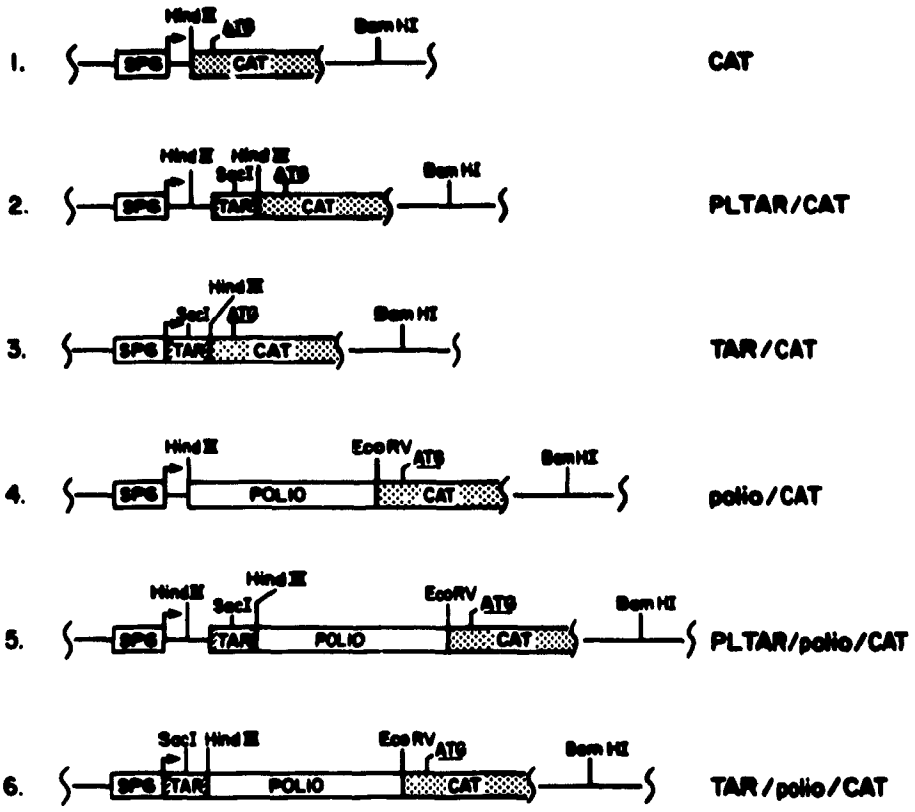
The HIV-I TAR Sequence Upstream of the Poliovirus 5' UTR Inhibits Translation

The *trans*-inhibitory properties of the TAR region were investigated as a consequence of experiments designed to analyze the translational effects of secondary structure upstream of the poliovirus 5' untranslated region (UTR). There are two possible mechanisms by which 40S ribosomal subunits can initiate translation on eukaryotic mRNAs (illustrated in Fig. 1). It is thought that the majority of mRNAs initiate translation in a cap-dependent manner, whereby the 40S subunit binds at or near the 5' cap structure and scans the 5' UTR until an appropriate AUG initiation codon is recognized (Fig. 1, top left; see Kozak, (29)). Recently, it has been shown that poliovirus and EMC virus mRNAs, which are naturally uncapped, initiate translation by a different mechanism. The 40S subunit can bind directly to an internal region of the picornavirus' 5' UTRs bypassing upstream sequences and the requirement for the cap structure (Fig. 1, top right; 30,31). The presence of secondary structure in the 5' UTR of cap-dependent mRNAs has been shown to inhibit translation (13). This inhibition is presumably caused by the reduced ability of the 40S subunit to bind and/or scan the 5' UTR (Fig. 1, bottom left). However, the introduction of secondary structure upstream of the poliovirus mRNA 5' UTR does not significantly impede protein synthesis since translation can initiate internally (ref. 30; Fig. 1, bottom right).

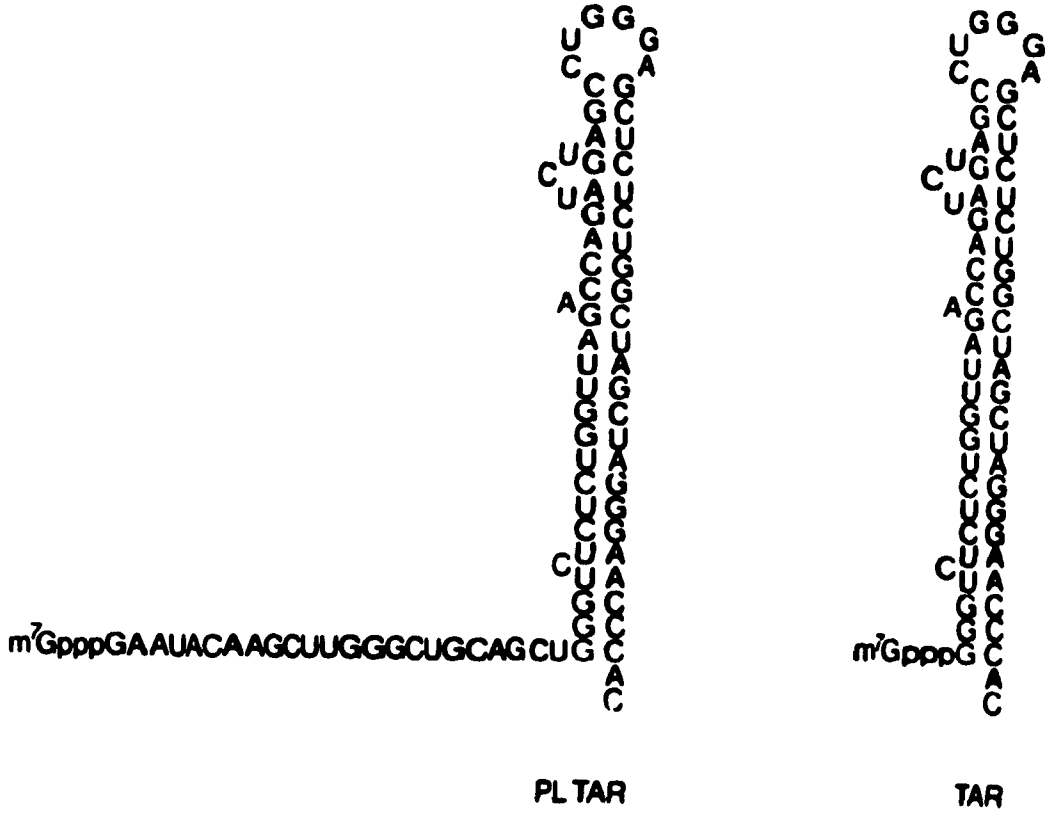
We generated hybrid mRNAs consisting of 5' untranslated sequences derived from HIV-1 and poliovirus mRNA fused 5' to the chloramphenical

Figure 2.(A) Structure of DNA templates used for *in vitro* transcriptions: ⌐→, site for transcription initiation; ATG, initiation codon for CAT protein synthesis; TAR, nucleotides +1 to +80 of the HIV-1 mRNAs; POLIO, nucleotides +320 to +733 of poliovirus (Lansing strain) mRNA 5' UTR; CAT, chloramphenicol acetyl transferase coding sequences; BamHI, site used to linearize templates. For details concerning plasmids see Experimental Procedures and text.
(B) Predicted secondary structure folding of PLTAR and TAR RNA. Only the first 61 nucleotides of the TAR region are shown.

A


1.
SPS
CAT
CAT
2.
TAR
PLTAR/CAT
3.
TAR/CAT
4.
POLIO
polio/CAT
5.
PLTAR/polio/CAT
6.
TAR/polio/CAT

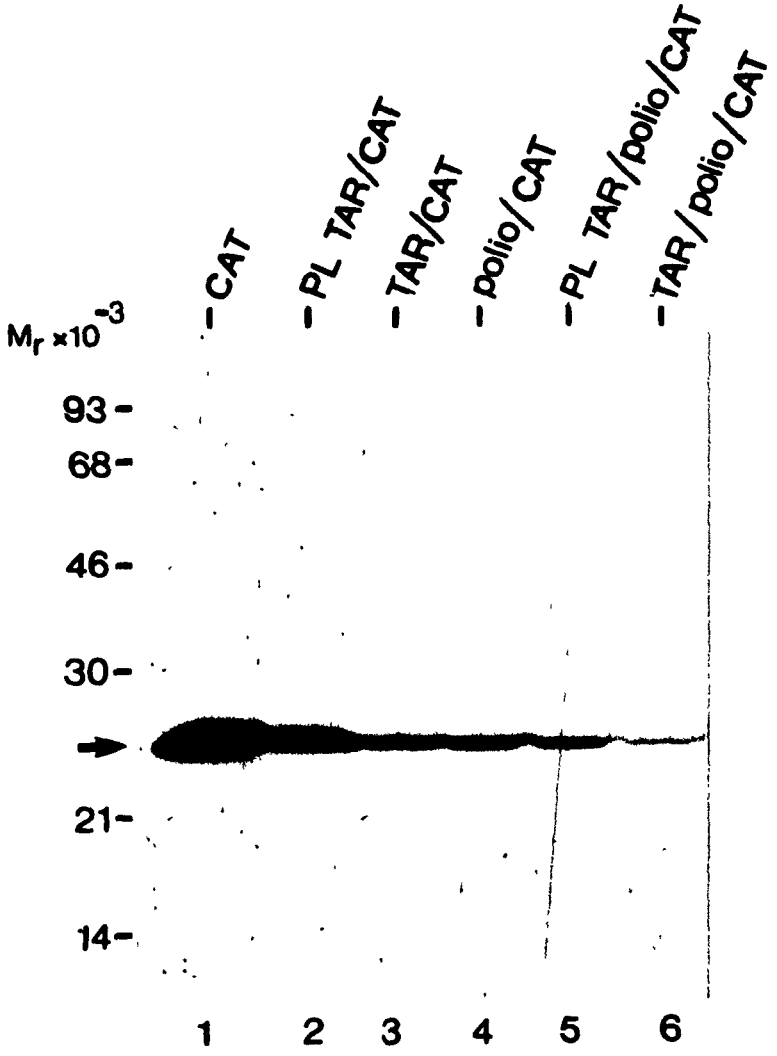
B


m7GpppGAAUACAAGCUUGGGCUGCAGCUG
PL TAR
m7GpppG
TAR

acetyltransferase (CAT) coding sequence (Fig. 2A). The stem and loop structure of PLTAR and TAR (constructs 2 and 3, Fig. 2A) was derived from the 5' proximal 59 nucleotides of HIV-1 mRNAs (14). PLTAR is identical to TAR except that it contains an extra 23 nucleotides upstream of the TAR sequence originating from the polylinker region in the pSP64 plasmid and a synthetic PstI linker (compare constructs 2 and 3 in Fig. 2A). When transcribed into mRNA, both PLTAR and TAR sequences are predicted to fold into the same stem and loop structure (Fig. 2B). However, it is of importance that the cap structure is more accessible to the translational machinery in PLTAR than in TAR RNA. The parental vector (construct 4) that was used to synthesize the hybrid mRNAs (constructs 5 and 6), contains poliovirus sequences sufficient for promoting internal initiation (30).

In vitro transcription of the linearized templates (Fig. 2A) yielded capped mRNAs that were translated in a rabbit reticulocyte lysate (Fig. 3). The presence of PLTAR 5' to the CAT coding sequence significantly inhibited translation (3 fold; compare lanes 2 and 1). In agreement with an earlier study (14), TAR/CAT mRNA translated less efficiently (10 fold) than CAT mRNA (compare lanes 3 and 1). A different translational hierarchy was obtained using mRNA that had the poliovirus 5' UTR inserted between the secondary structure sequence of TAR and the indicator CAT coding sequence (Fig. 2A, constructs 4-6). PLTAR/polio/CAT mRNA was translated nearly as efficiently (1.3 fold less) as polio/CAT mRNA (Fig. 3, compare lane 4 to 5), consistent with the scenerio depicted in Fig. 1. However, surprisingly, and at variance

Figure 3. Translation of hybrid mRNAs in rabbit reticulocyte lysate. Translations were performed at a final mRNA concentration of 4 µg/ml and analyzed as described in Experimental Procedures. mRNAs added are indicated at the top of the figure. The position of the CAT protein synthesized is indicated by the arrowhead to the left. Dose-response curves confirmed that the mRNA concentration used was in the linear range. Molecular weight standards are indicated to the left.

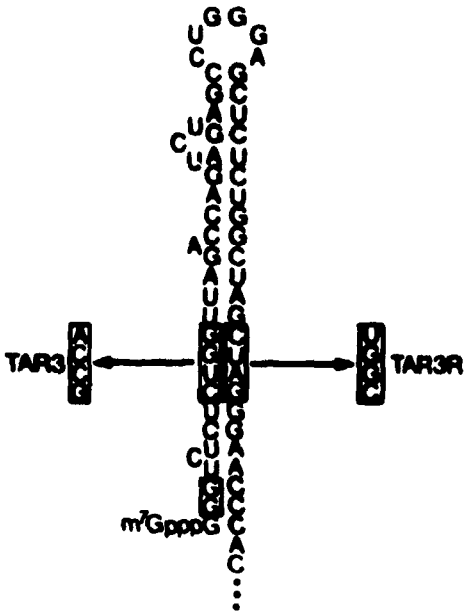

CAT
PL TAR/CAT
TAR/CAT
polio/CAT
PL TAR/polio/CAT
TAR/polio/CAT
Mr x10-3
93
68
46
30
21
14
1
2
3
4
5
6

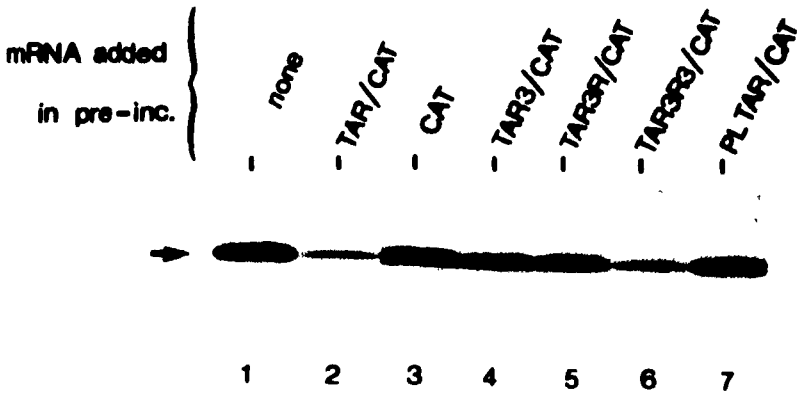
with the expected scenario (Fig. 1), TAR/polio/CAT mRNA was poorly translated relative to polio/CAT (compare lanes 4 and 6). All mRNAs had similar stabilities (data not shown), and therefore the effect must have occurred at the translational level. These results led us to investigate a possible trans-inhibitory effect of the TAR region on translation.

trans-Inhibition: TAR Structural Requirements.

To investigate the possibility of a TAR mediated trans-inhibition of translation we performed the following experiment: a reticulocyte translation lysate was pre-incubated with small amounts of TAR/CAT mRNA (350 ng/ml) in the absence of protein synthesis, followed by translation of the indicator CAT mRNA. The presence of TAR/CAT mRNA during the pre-incubation step caused a marked inhibition (10 fold) in the translation of added CAT mRNA (Fig. 4; compare lanes 1 and 2). Pre-incubation in the absence of added mRNA (lane 1) or in the presence of CAT mRNA (lane 3) did not result in any detectable reduction in CAT yield. The low levels of CAT mRNA present during the pre-incubation step (lane 3) did not cause a detectable increase in the total synthesis of CAT protein. To examine the possibility that the inhibitory effect mediated by TAR/CAT mRNA is due to its unique stem and loop structure (Fig. 2B), we performed similar experiments in the presence of mRNA containing mutations in the TAR region (Fig. 4; lanes 4-7). These mutants were created by site-directed mutagenesis (Fig. 4, top), and their translational efficiencies were previously reported (14). Two mutants (TAR3 and TAR3R) exhibiting reduced secondary structure, as compared to wild type TAR, were considerably less potent

Figure 4. *trans*-inhibition of CAT mRNA translation by mRNAs containing TAR and mutant TAR sequences. Top. The stem and loop structure of TAR RNA is shown with the sequences changed in the indicated mutant forms. TAR3R3/CAT contains both indicated mutations. Bottom. *trans*-inhibition of CAT mRNA translation by prior pre-incubation of the extract in the presence of mutant TAR/CAT mRNAs. A pre-incubation step (described in Experimental Procedures) in the presence of the indicated mRNAs (final concentration of 0.35 µg/ml) was performed for 10 min at 30°C. CAT mRNA (final concentration of 8 µg/ml) and ^{35}S-Methionine were added and incubation continued for 1 hr at 30°C. Polyacrylamide-SDS analysis was as described in Experimental Procedures. The position of the CAT protein is indicated by the arrowhead at the left.


TAR3
TAR3R
m7GpppG


mRNA added
in pre-inc.
none
TAR/CAT
CAT
TAR3/CAT
TAR3R/CAT
TAR3R3/CAT
PL TAR/CAT
1
2
3
4
5
6
7

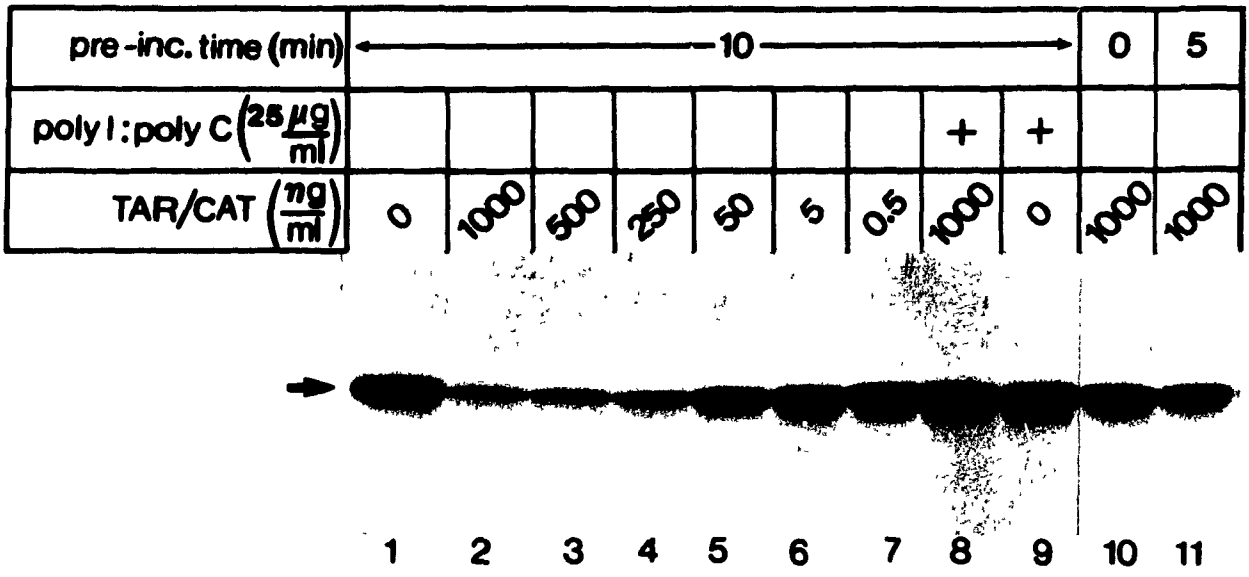
in eliciting the trans-inhibitory effect (Fig. 4; compare lanes 4 and 5 to 2). However, the mRNA generated from the double mutant TAR3R3/CAT, which restores the wild-type stem structure, was as effective as TAR/CAT mRNA in trans-inhibition (compare lanes 6 and 2). These results demonstrate that secondary structure rather than the nucleotide sequence of TAR is important for the inhibitory activity.

The importance of a stable stem and loop structure notwithstanding, it is not the only criterion necessary for effective trans-inhibition by the TAR region. PLTAR/CAT mRNA which has a similar secondary structure as TAR/CAT mRNA, but its cap structure is accessible (Figure 2B), exhibited low but detectable trans-inhibitory activity (Figure 4, compare lanes 7 and 2). This result suggests that cap accessibility is also a strong determinant in effecting trans-inhibition.

Reversal of trans-Inhibition by polyI:polyC and Guanine Exchange Factor (GEF).

TAR/CAT mRNA begins to lose its effect as a trans-inhibitor of protein synthesis at a concentration below approximately 50 ng/ml (Figure 5, lanes 1-7). For example, at a concentration of 1 μg/ml TAR /CAT mRNA (lane 2) there is a 10 fold reduction in CAT synthesis compared to only a 2.2 fold inhibition in the presence of 50 ng/ml TAR/CAT mRNA (lane 5). Considering that the length of the TAR/CAT mRNA is ~ 1600 nucleotides and that of the TAR stem and loop structure is 59 nucleotides (Fig. 2B) this would imply an effective concentration of TAR in the range of approximately 2 ng/ml. It has been observed that low levels of double-stranded RNA strongly inhibit protein synthesis (32). This inhibition is due to activation of a double-stranded RNA-

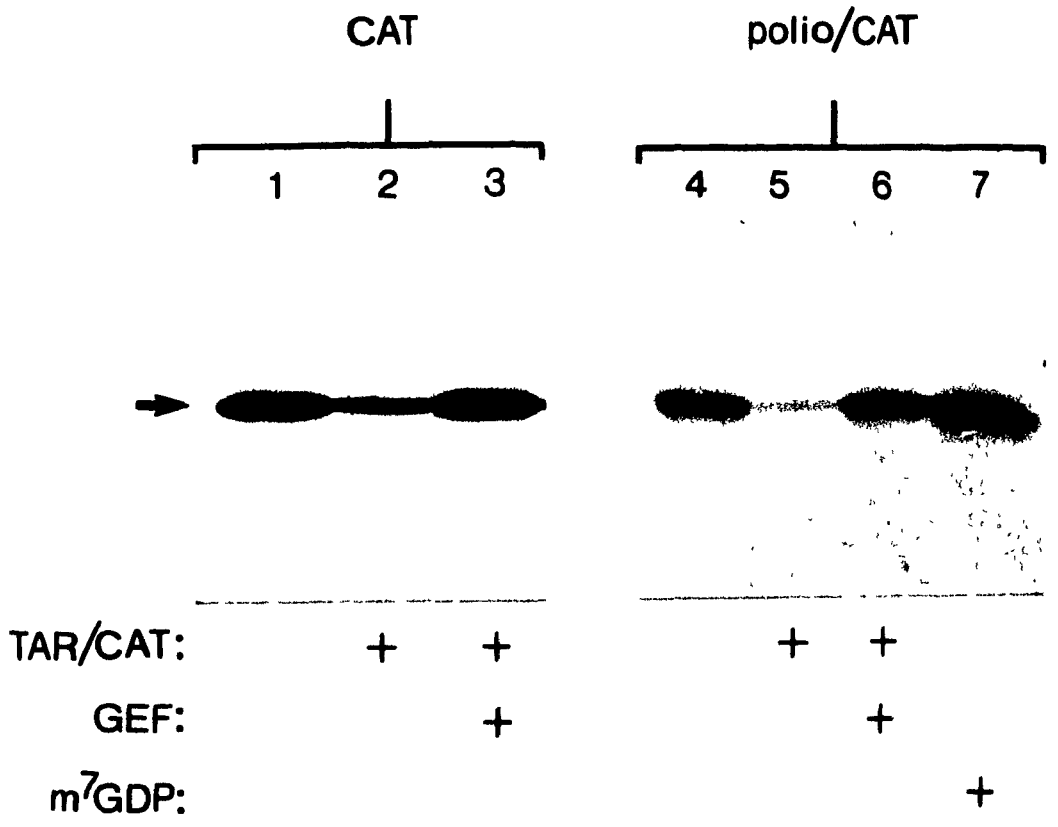
Figure 5. Dose-response curve and pre-incubation requirements for *trans*-inhibition of CAT mRNA translation by TAR/CAT mRNA. TAR/CAT mRNA was added at the indicated final concentrations and pre-incubated for the indicated times. CAT mRNA (final concentration of 12 µg/ml) was subsequently added and analyzed as described in the legend to Fig. 4. The polyI:polyC was added together with the indicated amount of TAR/CAT mRNA prior to the pre-incubation step such that a final concentration of 25 µg/ml polyI:polyC was achieved during the pre-incubation step.


pre-inc. time (min)
10
0
5
poly I : poly C (25 μg/ml)
+
+
TAR/CAT (ng/ml)
0
1000
500
250
50
5
0.5
1000
0
1000
1000
1
2
3
4
5
6
7
8
9
10
11

dependent protein kinase (dsI), which phosphorylates the α subunit of eIF-2 (17a,33,34). One approach to determine that dsI is involved in the inhibition of protein synthesis is the reversal of inhibition by high levels of polyI:polyC. In a seemingly paradoxical fashion, high levels (≈25 μg/ml) of double-stranded RNA prevent the activation of dsI by low levels of double-stranded RNA (35). When TAR/CAT mRNA was pre-incubated in the presence of 25 μg/ml polyI:polyC *trans*-inhibition was no longer detected (Fig. 5, compare lanes 8 and 2). The addition of polyI:polyC alone during the pre-incubation step had no effect on CAT mRNA translation (compare lanes 9 and 1). The *trans*-inhibition by TAR/CAT mRNA increased with longer pre-incubation time [1.5 fold (lane 10), 2.5 fold (lane 11), and 10 fold (lane 2)]. This observation is also consistent with the observed lag period required to attain a sufficient level of dsI activation and eIF-2α phosphorylation which precedes inhibition of protein synthesis (35).

To further support the contention that dsI is activated by the TAR region, we examined the ability of guanine exchange factor (GEF) to reverse the translational inhibition (27). The mechanisms by which dsI mediates protein synthesis inhibition are partly known (reviewed in London *et al.*, (18)). eIF-2 enters the initiation cycle as a complex with GTP and once the cycle is completed, eIF-2 is released as an inactive binary complex bound to GDP, which is exchanged for GTP by GEF for another round of initiation. Phosphorylation of the α-subunit of eIF-2 by dsI diminishes the ability of GEF to catalyze the exchange reaction, and leads to inhibition of protein synthesis. The

Figure 6. Reversal of TAR/CAT mRNA mediated *trans*-inhibition of translation by guanine exchange factor (GEF). TAR/CAT mRNA (final concentration of 125 ng/ml) was added at the start of the pre-incubation step (10 min, 30°C) as indicated, followed by the addition of either CAT mRNA (final concentration of 4µg/ml) or polio/CAT mRNA (final concentration of 4µg/ml) and further processed as described in Experimental Procedures. A highly purified preparation of GEF (0.5µl, 1.5 mg/ml) was added immediately after the pre-incubation step in the indicated lanes. The cap-analog, m^7GDP (P-L biochemicals), was added (lane 7) immediately after the pre-incubation step at a final concentration of 0.8mM.

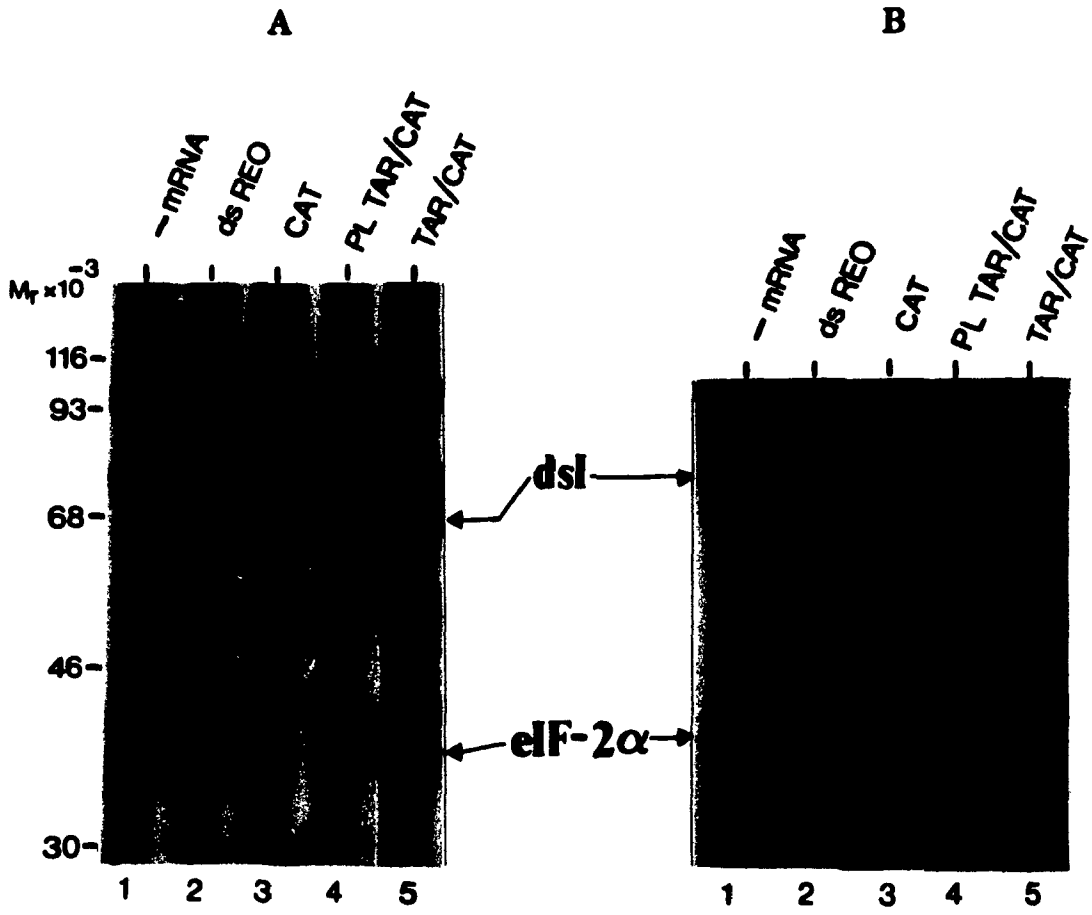

CAT
polio/CAT
1
2
3
4
5
6
7
TAR/CAT:
GEF:
m7GDP:

inhibition can be overcome by the addition of exogenous GEF (27). The addition of GEF at the end of the pre-incubation step completely reversed the inhibitory effect observed in the presence of TAR/CAT mRNA (Fig. 6; lanes 1-3). The addition of GEF to CAT mRNA in a reaction mixture that was not pre-incubated with TAR/CAT mRNA resulted in only a 10% increase in translational efficiency (data not shown). These results indicate that phosphorylation of the α subunit of eIF-2, which prevents the recycling reaction, is the cause of trans-inhibition. TAR/CAT mRNA can also inhibit in trans the translation of polio/CAT mRNA (Fig. 6, compare lane 5 to 4), and this inhibition was also completely alleviated by GEF (lane 6). These results indicate that both cap-dependent and cap-independent (lane 7; See also Pelletier et al., 21) translations (see Fig. 1) are amenable to trans-inhibition by TAR. This is an expected result, in light of eIF-2 requirement for the translation of all mRNAs. The ability of GEF to reverse the TAR mediated inhibition was specific inasmuch as all the other purified initiation factors, except for eIF-2, could not relieve this inhibition (data not shown).

Activation of dsI and Phosphorylation of eIF-2α.

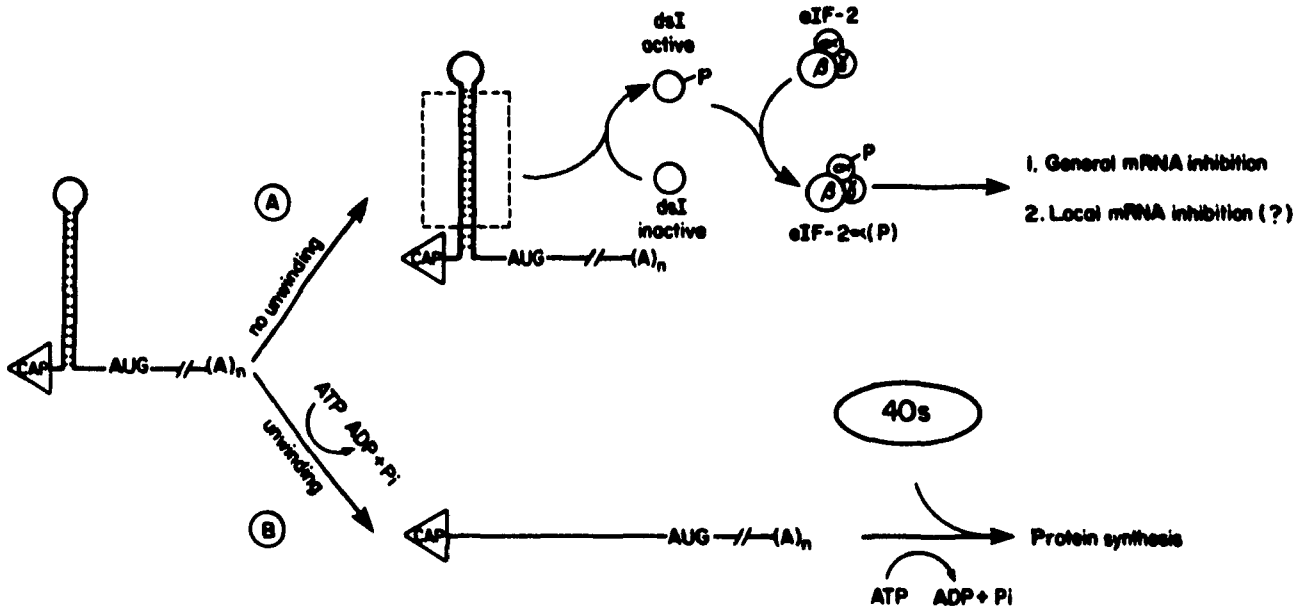
Direct evidence for the TAR mediated activation of dsI and phosphorylation of eIF-2α was obtained (Fig. 7). Protein kinase assays containing crude (Fig. 7A; (23)) or highly purified (Fig. 7B; (25)) preparations of dsI were carried out in the presence and absence of the various RNAs and γ-^{32}P ATP. All assays were supplemented with eIF-2 (23). Phosphoproteins were resolved by SDS-polyacrylamide gel

Figure 7. Effect of RNAs on the Phosphorylation of dsI and eIF-2α. (A) Protein kinase assays containing 30 μg of S10 protein from 3T3-F442A cells were performed and analyzed as described in Experimental Procedures. Assays contained a final concentration 20 ng/ml reovirus dsRNA and 0.3 μg/ml of CAT, PLTAR/CAT or TAR/CAT mRNAs as indicated. Lane 1 received no RNA. (B) protein kinase assays containing purified latent reticulocyte dsI (≈ 10 ng protein) were performed as described in Experimental Procedures. Additions were as described above except that gel purified mRNAs were added to a final concentration of 0.5 μg/ml. All assays were supplemented with highly purified eIF-2 (80% pure, 0.1 μg. The positions of phosphorylated dsI and eIF-2α are indicated. Molecular weight standards are indicated to the left.


A
B
– mRNA
ds REO
CAT
PL TAR/CAT
TAR/CAT
$M_r \times 10^{-3}$
116–
93–
68–
46–
30–
dsI
eIF-2α
1 2 3 4 5
1 2 3 4 5

electrophoresis and visualized by autoradiography. In control assays the addition of low levels of double-stranded reovirus RNA (dsReo) resulted in the phosphorylation of dsI and eIF-2α which were not observed in the absence of added dsReo (Fig. 7 A and B, compare lanes 1 and 2). A similar phosphorylation of dsI and eIF-2α was observed in the presence of TAR/CAT mRNA (Fig. 7 A and B, lane 5), but no or little phosphorylation was observed with CAT or PLTAR/CAT mRNAs (Fig. 7 A and B, lanes 3 and 4, respectively). A small but reproducible increase in the level of dsI phosphorylation occurs in the presence of PLTAR/CAT relative to CAT mRNA when using highly purified dsI (Fig. 7B). The phosphorylation of dsI and eIF-2α by TAR/CAT mRNA was prevented when high concentrations of poly I:poly C (25 μg/ml) were included in the assays (data not shown). The specific activation of dsI by the TAR/CAT mRNA and the subsequent phosphorylation of eIF-2α observed in these experiments most likely explains the TAR/CAT mRNA mediated trans-inhibition of translation (Figs. 4 and 5). Similar results were also obtained using RNAs which were subjected to gel-purification (Fig. 7B). This minimizes the possibility that the trans-inhibition and phosphorylation of dsI and eIF-2α were due to contaminants in the TAR/CAT mRNA preparation. In other experiments the addition of native polio, TMV or globin mRNAs and tRNA at similar concentrations resulted in no phosphorylation of dsI and eIF-2α (data not shown).

Figure 8. Model of a translational regulatory mechanism which correlates the state of phosphorylation of eIF-2 with the ability of eIF-4F and associated factors to mediate denaturation of mRNA 5' proximal secondary structure. See Discussion for details.


dsI
active
P
eIF-2
dsI
inactive
P
eIF-2∝(P)
1. General mRNA inhibition
2. Local mRNA inhibition (?)
A
no unwinding
CAP
AUG—//—(A)n
ATP ADP + Pi
unwinding
B
40s
CAP
AUG—//—(A)n
Protein synthesis
ATP
ADP + Pi

DISCUSSION

The results in this paper demonstrate that a unique stem and loop structure present at the 5' proximal end of HIV-1 mRNAs can activate the cellular dsRNA-dependent kinase resulting in *trans*-inhibition of translation. The presence of secondary structure in the 5' untranslated region of eukaryotic mRNAs can also inhibit translation in *cis* by a different mechanism (13). We suggest a novel translational regulatory phenomenon which can link these separate inhibitory mechanisms. Thus, an intriguing aspect of this work is the interdigitation between the translational control pathways involving two initiation factors, eIF-4F and eIF-2.

Control of eIF-2 Activity by eIF-4F.

The role of eIF-4F is to bind the 5' cap structure, then in conjunction with two other initiation factors (eIF-4A and eIF-4B) mediate the ATP-dependent unwinding of 5' proximal mRNA secondary structure (15,36,37). This helicase activity underlies the stimulatory effect of the cap on 40S subunit binding to mRNA. It is significant that eIF-4F is present in limiting amounts in the cell (38) and acts at the rate-limiting step in translation (39). As a result, the activity of eIF-4F can regulate translational efficiency. Therefore, it is conceivable that under conditions whereby eIF-4F activity is impaired, an increase in the effective concentration of double-stranded RNA will occur. This increase in RNA secondary structure could lead to the activation of the latent dsI followed by inactivation of eIF-2 recycling. This model is shown in Fig. 8.

There are several examples which suggest that this can occur *in vivo*. In cells exposed to elevated temperatures, the activities of eIF-2 and eIF-4F are impaired. Inhibition of eIF-2 activity is caused by the phosphorylation of its α-subunit (40,41), whereas the inhibition of eIF-4F activity is correlated with dephosphorylation of the 24 kDa subunit of eIF-4F (38). Significantly, the addition of eIF-4F to extracts prepared from heat-shocked Ehrlich cells not only stimulated translation to control levels, but surprisingly also enhanced the activity of eIF-2 (42). This observation can be explained by our proposed model. Other examples of conditions leading to translational repression where eIF-4F and eIF-2 activities are both inhibited are serum deprivation (43) and poliovirus infection: poliovirus infection of HeLa cells, which causes eIF-4F inactivation (44,45) results in phosphorylation of dsI and eIF-2α (Black *et al.*, submitted). These examples may point towards the generality of such a translational control phenomenon.

The control of eIF-2α phosphorylation by eIF-4F does not exclusively implicate a general inhibition of protein synthesis. De Benedetti and Baglioni (46) invoked local activation of dsI leading to selective inhibition of mRNA translation. This contention is further supported by the specific translational increase in mRNAs derived from transfected DNA under conditions that antagonize dsI activation (47,48). Since dsI requires dsRNA for its activation (33), local activation would presumably occur when dsI interacts with the secondary structure elements present on that mRNA. Local effects on translation might be

maintained in the cell by compartmentalization or anchoring of certain factors to the cytoskeletal framework (49). It is possible that dsI binds the TAR region and locally catalyzes eIF-2α phosphorylation causing a selective down regulation of HIV-1 expression. Since all HIV-I mRNAs contain the TAR region they could be coordinately regulated. An important implication of this hypothesis is that this sequence of events might be required to maintain the latency period observed during the HIV-1 replicative life cycle .

mRNA Structural Requirements for dsI Activation.

It is presumed that most mRNAs have base-paired regions. However, it is unlikely that the majority of mRNAs can activate dsI. The results here (Fig. 4) indicate that the presence of a stable stem and loop structure is necessary but not sufficient to activate dsI. This is best illustrated by comparing TAR/CAT and PLTAR/CAT mRNAs. The latter mRNA has a similar stem and loop structure to TAR/CAT except that its cap structure is significantly more accessible (Fig. 2B). There are two likely explanations for the inability of PLTAR/CAT mRNA to significantly activate dsI which are not mutually exclusive. First, the increased accessibility of the cap structure in PLTAR/CAT may increase the efficiency of eIF-4F mediated unwinding, thus causing a reduction in dsRNA structure. In agreement with this, crosslinking experiments showed that the cap structure of PLTAR/CAT mRNA is more accessible to eIF-4F than that of TAR/CAT mRNA (N. Parkin, personal communication). A second possibility is that the increased accessibility of the cap

results in a structure that cannot be recognized by dsI. This is consistent with the fact that although tRNA, rRNA and VAI RNA (50) have extensive regions of secondary structure they do not activate dsI (51). HIV-1 may have evolved a highly specialized stem and loop structure in its mRNAs to activate dsI. The difficulty in unwinding the TAR region by the initiation factors may significantly contribute to its effectiveness as an activator of dsI.

Double-stranded mRNA regions that activate dsI do not have to be contained exclusively in the 5' UTR, as is the case for the TAR element of HIV-1. Baum and Ernst (52) reported that a crude preparation of cytoplasmic poly A^+ mRNA could phosphorylate eIF-2 in vitro. Pratt et al., (53) extended these findings by showing that polysomal mRNA could cause eIF-2α phosphorylation. The element(s) that mediates the phosphorylation of eIF-2α was not investigated in these reports. It is possible that sequences in the coding or 3' untranslated region could also activate dsI.

Possible Physiological Significance of TAR Induced dsI Activation.

One important aspect of our findings lies in the possible implications for the regulation of the life cycle of HIV-1. It was postulated that active dsI can specifically induce the synthesis of β-interferon (54). Thus, phosphorylation of dsI mediated by the TAR secondary structure can have important consequences in HIV-1 infected cells. If HIV-1 mRNAs have the capacity to induce interferon production in vivo it could serve in an attempt to establish an anti-viral state in individuals infected with the AIDS virus. Moreover, interferon can

suppress the production of growth factors required for cell proliferation (reviewed in ref. 19). The establishment of an anti-viral state and/or the inability of cells to proliferate could contribute to the repression of virus replication.

In summary, we show that HIV-1 mRNAs have a unique structure that can activate dsI which catalyzes eIF-2α phosphorylation. This is the first report of a specific mRNA sequence that has this capacity. This may have profound implications for translational control in general and HIV-1 replication in particular.

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CHAPTER 5

CAP-DEPENDENT RNA SPLICING IN A HeLa NUCLEAR EXTRACT

SUMMARY

We have studied the involvement of the 5' cap structure in the splicing of precursor mRNAs in a HeLa nuclear extract. We show that precursor mRNAs are efficiently spliced only when they possess a cap structure and that pre-incubation of a HeLa nuclear extract rendered the splicing reaction highly sensitive to inhibition by cap analogues. This sensitization was dependent on exogenous Mg^{++} but not exogenous ATP. These results demonstrate that splicing in a nuclear extract is highly dependent on the cap structure as was demonstrated for the splicing process in a HeLa whole cell extract [Konarska, M.M., Padget, R.A. and Sharp, P.A. 1984, Cell, 38, 731-736], thus supporting the contention that cap recognition is an important feature of eukaryotic mRNA biogenesis.

INTRODUCTION

All eucaryotic cellular mRNAs analyzed to date are blocked at their 5' terminus by the cap structure, $m^7G(5')ppp(5')N$ (1). There is now considerable evidence demonstrating that the cap structure enhances translational efficiency by facilitating ribosome binding to mRNA (1). This interaction is mediated by a distinct group of proteins defined as cap binding proteins (2,3).

With the advent of efficient *in vitro* splicing systems (4-10), two recent studies have suggested that the cap structure may play a significant role in the splicing process as well (6,7). Using a HeLa nuclear extract, Krainer *et al.* (7) were able to show efficient splicing of *in vitro* synthesized truncated human β-globin transcripts. They showed that although uncapped transcripts could still be spliced, the efficiency was two to three fold lower than with their enzymatically capped counterparts. In addition splicing of uncapped precursor mRNAs (pre-mRNAs) resulted in the production of aberrant splice products, not observed with capped transcripts. A more striking result was obtained by Konarska *et al.* (6) who used a HeLa whole cell extract and showed that the efficiency of splicing was greatly enhanced when a capped as opposed to an uncapped precursor RNA was used. In addition, cap analogues inhibited splicing by up to 90%. The specific inhibition of splicing by cap analogues suggests that cap recognition factors are required for precursor mRNA processing. Thus, the cap structure may play a key role in directing and regulating the assembly of mRNA processing complexes.

Here, we show that when a HeLa nuclear extract is pre-incubated prior to addition of pre-mRNA, the splicing is rendered highly sensitive to cap analogue inhibition. In addition, Mg^{++} is required to elicit this response. We also show that precursor mRNA terminated with a 5' non-hydrolyzable GTP analogue is a poor substrate for splicing. Thus, our data demonstrate that splicing in a HeLa nuclear extract, can be tightly cap dependent, as with the HeLa whole cell extract, and point to an important function of the cap structure in mRNA biogenesis.

MATERIALS AND METHODS

Materials. Restriction enzymes and nucleoside triphosphates were from Boehringer Mannheim. SP6 RNA polymerase and [α-^{32}P]ATP were purchased from New England Nuclear. RNasin was from Promega biotec. Cap analogues were purchased from P-L Biochemicals and Polyvinyl alcohol (type II) and creatine phosphate were from Sigma.

Plasmids, SP6 Transcription. Preparation of plasmid DNA and restriction enzyme digestion were carried out using standard methods (11). The plasmid pSP64-HβΔ6 (see bottom of Fig. 1; a generous gift from M. Green, Harvard) was linearized at the Bam HI site. In vitro transcriptions with SP6 RNA polymerase primed with cap analogues, in the presence of [α-^{32}P]ATP, to yield SP6/β-globin [^{32}P]-RNA were done under conditions previously shown to generate greater than 95% capped transcripts (12). The transcription was either primed with 350 μM of the cap dinucleotide, m^7GpppG or GpppG, or with the non-hydrolyzable analogue of GTP, GMP-PCP in the presence of 40 μM GTP to yield substrate pre-mRNA with the appropriate 5' termini. The transcripts were stored at -70°C in water and used directly in the splicing reaction.

In Vitro Splicing Reaction. HeLa nuclear extracts were prepared as described (13). All experiments described throughout were performed using the same extract preparation. Splicing reactions were carried out in a volume of 25 μl containing 15 μl of nuclear extract at 30°C for 3.5 hr under optimized splicing conditions, exactly as described by Krainer et al. (7), unless otherwise indicated in the text. Typically, 1-5 ngs (approximately 30,000 cpm) of substrate RNA was added per incubation: varying the amounts of substrate RNA in this concentration range was

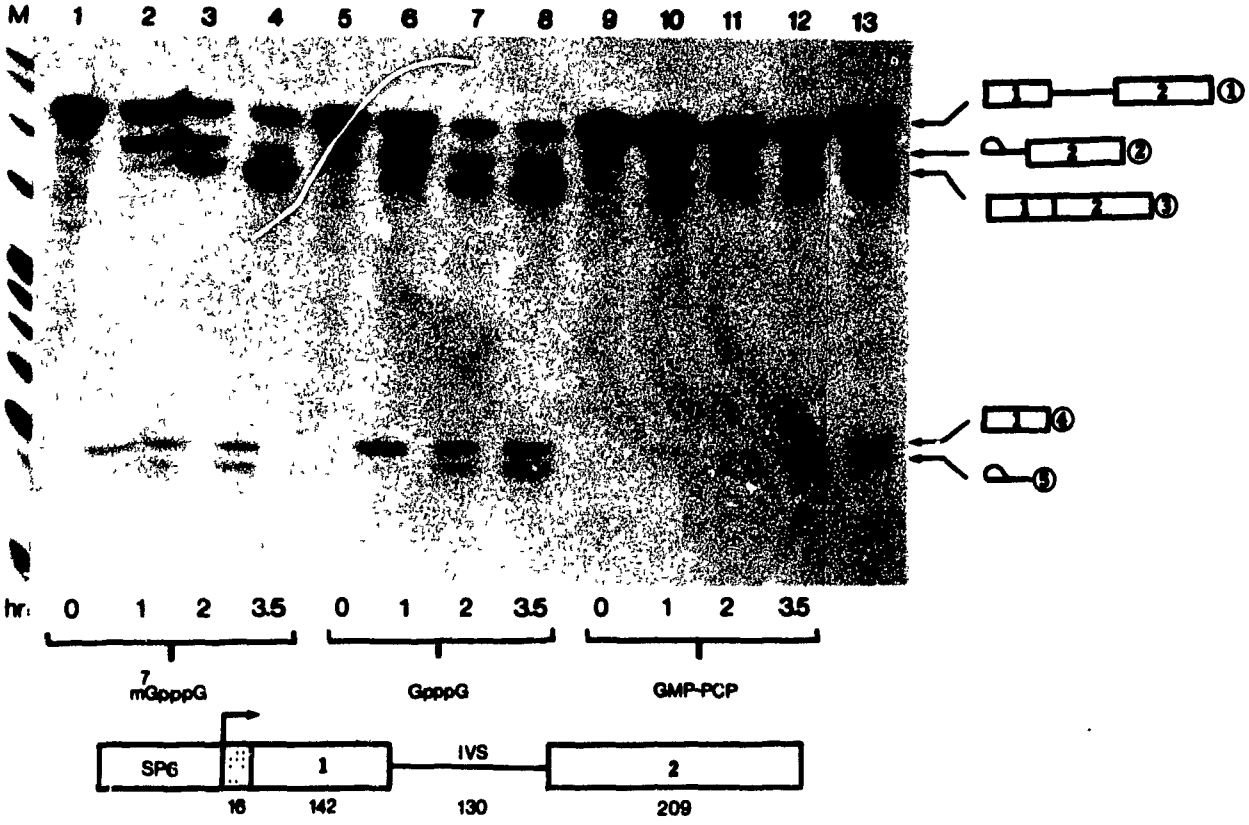
shown not to affect splicing efficiency (7). Pre-mRNA processing products were analyzed directly on 5% polyacrylamide - 7 M urea gels followed by exposure against Fuji X-ray film as described (8). Pre-incubation of nuclear extract was performed under the same conditions as for splicing reactions, for 15 min at 30°C, in the absence of pre-mRNA, except where otherwise indicated, followed by the addition of m^7GpppG terminated pre-mRNA for the splicing reaction. Components omitted during the pre-incubation were added with pre-mRNA to achieve a final optimal concentration for splicing in this system (7).

Quantitation of Splicing Efficiency. Splicing efficiency used throughout the text is defined as a percentage based on the ratio of final spliced product relative to the sum of spliced product and input RNA still remaining at the end of the incubation. Quantitation was performed by scanning X-ray films after short exposures with an LKB soft laser densitometer. Numbers obtained were normalized to molar amounts.

RESULTS

To characterize the splicing activity of our HeLa nuclear extracts we used the plasmid pSP64-HβΔ6. This plasmid and its use in the nuclear splicing system were described by Krainer et al. (7) and Ruskin et al. (8). For preparation of substrate RNA for splicing we linearized the plasmid with the restriction enzyme Bam HI (as shown in Fig. 1) and transcribed the globin DNA with SP6 RNA polymerase in the presence of the methylated cap dinucleotide analogue, m^7GpppG, as a primer for transcription. Fig. 1 (lanes 1 through 4) shows the kinetics of splicing of these transcripts under optimized conditions over a period of 3.5 hr. The different RNA species obtained during the reaction were identified according to their migration relative to DNA size markers and to their order of appearance during the splicing reaction as shown by Ruskin et al. (8). Furthermore, we have analyzed on gels the RNA species produced in our reaction mixture, side by side with splicing reaction products obtained from B. Ruskin and M. Green (that have previously been characterized; ref. 8) and found them to comigrate. The different species were assigned numbers that are shown in the right margin of Fig. 1, as follows: 1, input precursor RNA; 2, lariat form containing the intron and second exon; 3, spliced product: 4, first exon; 5, branched intron. The kinetics of splicing is consistent with the report of Ruskin et al. (8) with regard to the order of appearance of the intermediates and final product (#3) of the splicing reaction. Lanes 5-8 represent a time course of the splicing reaction using a GpppG terminated SP6 globin transcript as substrate. The kinetics and efficiency of splicing in this case were the same as with the m^7GpppG

FIGURE 1. Time course of <u>in vitro</u> splicing of SP6/HβΔ6-globin pre-mRNAs differing in 5' termini. Pre-mRNA labeled with [α-^{32}P]ATP was incubated in a HeLa nuclear extract for the time periods indicated in the figure and the RNA products were analyzed on a 5% polyacrylamide denaturing gel followed by autoradiography as described in Materials and Methods. Substrate RNAs used in the splicing reactions were primed with either m^7GpppG, GpppG, or GMP-PCP as indicated in the figure. Lane 13 shows the products made in a standard splicing reaction containing equal amounts of m^7GpppG and GMP-PCP terminated pre-mRNAs. M;[^{32}P]-labelled markers of Hpa II digested pBR322 DNA. Structures in the right margin represent the RNAs produced in the reaction (8). These products (numbered from 1 to 5) are described in the text. The structure of the DNA transcription template is shown at the bottom (see ref. 8). The length of the different regions is shown in numbers of base pairs. Boxes numbered 1 and 2 represent the first and second globin exons; IVS represents the intervening sequence; the box indicated by SP6 represents the SP6 promoter; dotted box represents the transcribed SP6 sequence and the arrow denotes the initiation site and direction of transcription.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hr: 0 1 2 3.5 0 1 2 3.5 0 1 2 3.5
7 mGpppG
GpppG
GMP-PCP
SP6
1
IVS
2
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142
130
209

terminated transcript. This is consistent with the results of Konarska et al. (6) in the whole cell extract splicing system; these authors also demonstrated that N^7 methylation of the GpppG structure at the 5' end of the SP6 transcript takes place very quickly and with high efficiency in their extract. Consequently, it is very likely that methylation also takes place in our system. To determine the importance of the cap structure for splicing in our system, we have used a GMP-PCP terminated SP6 globin transcript. When this pre-mRNA was incubated in the nuclear extract and the kinetics of splicing was determined we found that although the order of appearance of products was similar, the efficiency of splicing was markedly reduced (lane 12, 13% splicing efficiency as compared to 83% with m^7GpppG terminated pre-mRNA, lane 4). To exclude the possibility that an inhibitor of splicing was present in the GMP-PCP terminated substrate pre-mRNA, we mixed this RNA with m^7GpppG terminated RNA and found no inhibition of splicing (lane 13). These results clearly demonstrate that the cap structure is required for efficient processing of pre-mRNA.

To further establish the requirement of cap recognition for pre-mRNA processing we examined the effects of cap analogues on pre-mRNA processing in the nuclear splicing system. In contrast to the remarkable inhibitory effect of cap analogues on <u>in vitro</u> splicing in a HeLa whole cell extract (6), addition of the cap analogue, m^7GDP to the nuclear splicing system resulted only in a partial inhibition (2-3 fold inhibition in the presence of 20 μm m^7GDP, but inhibition was not increased even when m^7GDP concentration was increased to 1 mM; (data not shown). Similar results were obtained by other groups (M. Konarska and P. Sharp, A. Krainer and T. Maniatis; personal communications). Thus,

it was worrisome that a significant proportion of pre-mRNA processing is not affected by addition of the cap analogue m^7GDP and that these findings were different from those obtained in the HeLa whole cell extract. The results suggested however, that a significant fraction of the splicing machinery is inaccessible to the inhibitory action of cap analogues. Consequently, we reasoned that pre-incubation of the nuclear extract in the presence of cap analogues might facilitate cap analogue recognition by the splicing machinery. As will be reported below, this was indeed the case. However, pre-incubation of the nuclear extract by itself (in the absence of cap analogues) was also sufficient to sensitize the nuclear splicing machinery to cap analogues (see below).

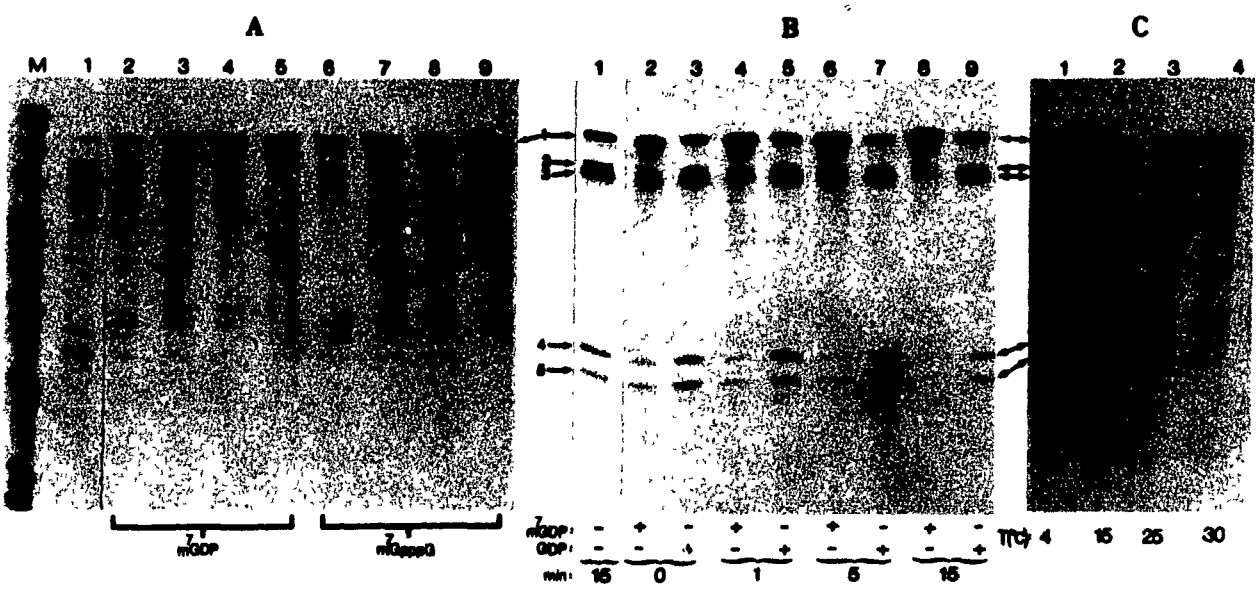
To characterize the requirements for cap analogue inhibition of splicing as a function of pre-incubation, we titrated the reaction against m^7GDP concentration. Pre-incubation (15 min at 30°C) of the nuclear extract in the absence of cap analogue had no significant effect (~ 10% as compared to a non pre-incubated extract) on splicing efficiency (data not shown). Lane 1 Fig. 2A, shows a control splicing reaction, in the absence of cap analogue (in this experiment some degradation of pre-mRNA, that occurred prior to its use in the splicing reaction, is observed, and also in fig. 3C) . The cap analogue, m^7GDP was present during pre-incubation of the nuclear extract at a final concentration of 1-40 μM (lanes 2 through 5). The results show that inhibition of splicing was 50% when 1 μM m^7GDP was used (lane 2) and greater than 95% when 5 μM m^7GDP was used (lane 3). A similar pattern was obtained in the presence of m^7GpppG (lanes 6 through 9). The magnitude of inhibition obtained in this experiment is dramatically higher than without pre-incubation (20 fold as compared to 2-3 fold,

FIGURE 2. Conditions influencing cap-analogue inhibition of *in vitro* splicing reaction. Nuclear extract was pre-incubated under different conditions followed by the addition of pre-mRNA for splicing reactions and the resulting RNA products analyzed as described in Materials and Methods.

(A) Effect of pre-incubation in the presence of cap analogue on splicing. Lane 1, control reaction pre-incubated in the absence of cap-analogue. For pre-incubation, m^7GDP or m^7GpppG as indicated in the figure was present during pre-incubation at the following concentrations and the percent of splicing efficiency were as follows: Lanes 2 and 6, 1 μM 50%; 3 and 7, 5 μM < 5%; 4 and 8, 20 μM, < 5%; 5 and 9, 40 μM, < 5%. M; markers. Note that recovery of radioactivity in lanes 2 and 6 is only ~ 60%, as determined by densitometry of the X-ray film, as compared to the other lanes.

(B) Effect of pre-incubation time in the presence of m^7GDP on splicing. Nuclear extract was pre-incubated in the presence or absence of 40 μM m^7GDP or GDP for the time periods indicated in the figure. The percent splicing efficiency was as follows: Lane 1, 71%; 2, 22%; 3, 80%; 4, 13%; 5, 64%; 6, 8%; 7, 64%, 8, < 2%; 9, 55%.

(C) Effect of pre-incubation temperature on splicing inhibition by m^7GDP. Pre-incubation of nuclear extract was performed in the presence of 5 μM m^7GDP at the temperatures indicated in the figure. The efficiency of splicing was as follows: Lane 1, 29%; 2, 29%; 3, 21%; 4, 11%.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B
C
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1 2 3 4 5 6 7 8 9
1 2 3 4
m7GDP
m7GppppG
m7GDP: - + - + - + - + -
GDP: - - + - + - + - +
min: 15 0 1 5 15
T(°C) 4 15 25 30

respectively) and is very similar to the effects of cap analogues observed in the whole cell extract splicing system (6).

The importance of the pre-incubation step was further ascertained by a time course experiment in which m^7GDP and GDP at a concentration of 40 μM were added to the nuclear extract and pre-incubated for different time periods. Lane 1 (Fig. 2B) is the control experiment in the absence of cap analogue, and lanes 2 and 3 show the effect of m^7GDP and GDP on pre-mRNA processing without pre-incubation, respectively (~ 65% inhibition obtained with m^7GDP, whereas no inhibition was obtained with GDP). Inhibition of the splicing reaction due to pre-incubation of the nuclear extract in the presence of m^7GDP was time dependent: 82% inhibition after 1 min pre-incubation (Fig. 2B; lane 4), 89% after 5 min (lane 6) and 97% inhibition after 15 min of pre-incubation (lane 8). In contrast, pre-incubation of the nuclear extract in the presence of GDP did not significantly inhibit the splicing even after 15 min of pre-incubation (~ 20% inhibition; lane 9).

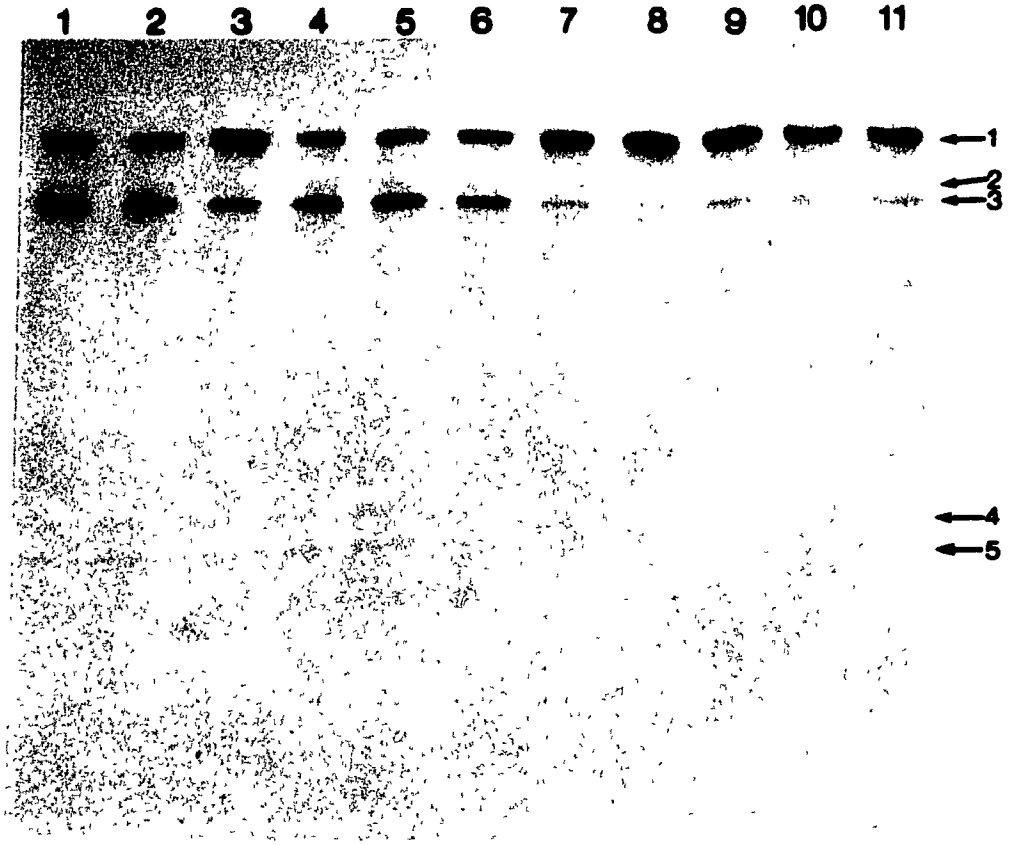
We also examined the effect of pre-incubation temperature on the inhibitory action of m^7GDP. Fig. 2C shows that increasing the temperature of pre-incubation results in a greater inhibition of splicing by m^7GDP (4°C and 15°C, lane 1 and 2, both ~ 60% inhibition; 25°C lane 3, 70% inhibition and at 30°C, lane 4, 85% inhibition; the control lane for this experiment is lane 1 of Fig. 2A that was exposed for a shorter time period). Pre-incubation at 37°C in the absence of cap analogue completely abolished splicing activity (data not shown).

We also examined the importance of the cap analogue phosphate groups for the inhibitory activity. In a control experiment in the

absence of any cap analogues, splicing efficiency of 63% was obtained (Fig. 3, lane 1). Five μM of either GDP (lane 2), GMP-PCP (lane 4), or m^7G(lane 5) did not inhibit pre-mRNA processing. As is the case for in vitro translation (16) inhibition by m^7G containing cap analogues increased with the increase of the number of phosphate groups (18% inhibition for m^7GMP, lane 6, in contrast to 86% inhibition for m^7GDP, lane 7). Pre-incubations were also performed in the presence of cap dinucleotides. The cap analogue, GpppG, inhibited the splicing reaction by 51% (lane 3). The nature of the N nucleotide present in the cap analogue $m^7Gppp(p)N$ did not significantly affect the degree of inhibition (~90% inhibition, lanes 8 through 11).

We investigated the potential role of several components required for in vitro splicing in influencing cap analogue inhibition. In control experiments (Fig. 4A, lanes 1 and 2) the nuclear extract was pre-incubated in the absence of m^7GDP resulting in a 70% splicing efficiency (lane 1). When the nuclear extract was pre-incubated in the presence of polyvinyl alcohol only, followed by addition of ATP, $MgCl_2$ and creatine phosphate, no loss in splicing activity was observed (Fig. 4A, lane 2, 75% splicing efficiency). This result indicates that omission of the latter components from the pre-incubation mixture does not affect splicing activity. Surprisingly, no difference in the extent of inhibition of splicing activity (~ 90%) occurred when m^7GDP was added prior to or after completion of the pre-incubation in the presence of all components (compare lanes 4 and 3, respectively). This finding is of great significance to the interpretation of the results as will be addressed in the Discussion. In the remaining experiments described in Fig. 4A, m^7GDP was present during pre-incubation. When pre-incubation

FIGURE 3. Effect of pre-incubation with different cap analogues on splicing. Nuclear extract was pre-incubated in the presence of 5 μM of the nucleotides and cap analogues indicated below, followed by splicing reaction and analysis of splicing products as described in Materials and Methods. The percent of splicing efficiency was as follows: Lane 1, control 63%; 2, GDP, 63%; 3, GpppG, 31%; 4, GMP-PCP, 63%; 5, m^7G, 62%; 6, m^7GMP, 52%; 7, m^7GDP, 9%; 8, m^7GpppG, 4%; 9, $m^7GppppG^7m$, 10%; 10, m^7GpppA, 10%; 11, m^7GpppU, 12%.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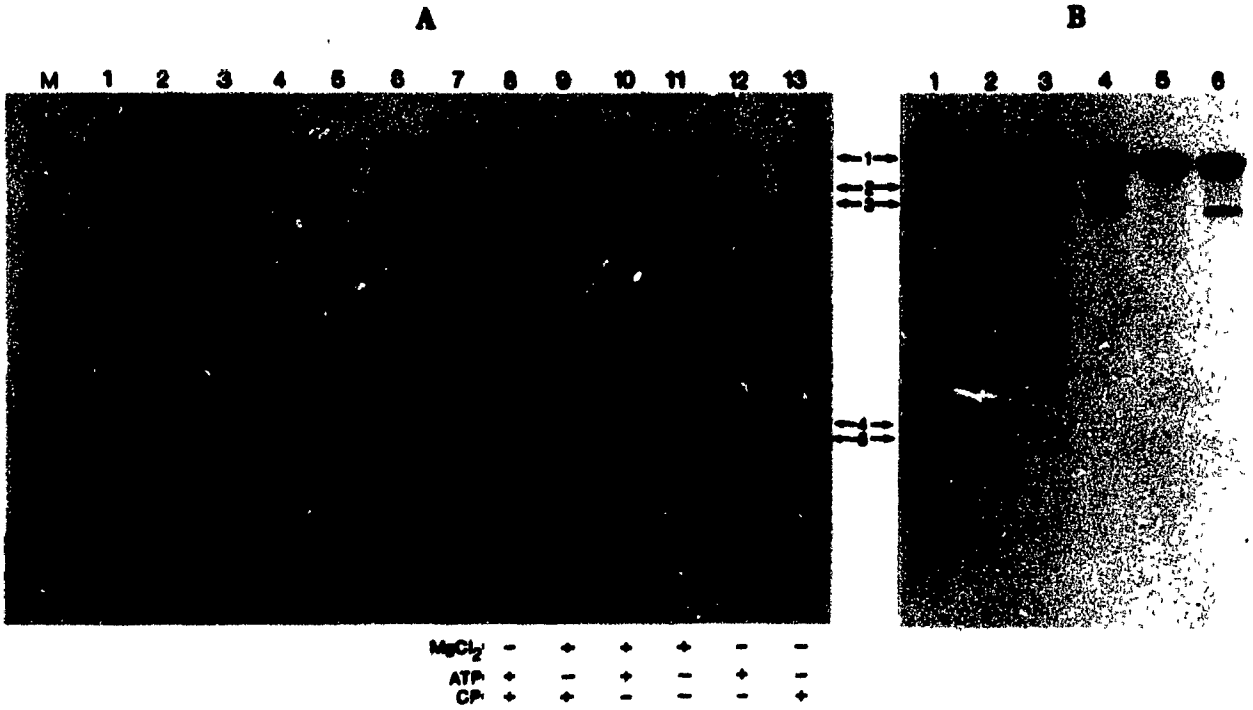
was performed in the absence of ATP, $MgCl_2$ and creatine phosphate a significant reduction in inhibition by the cap analogue as compared to the experiment described in lane 4, was observed (51% inhibition, lane 5). The extent of inhibition in this case was comparable to the inhibition obtained when the pre-incubation was performed at 4°C in presence of all components (51% inhibition, lane 6). When polyvinyl alcohol was omitted during the pre-incubation step (lane 7) only 40% inhibition by m^7GDP was obtained. Thus, the increased inhibitory action is observed only in the presence of polyvinyl alcohol, which is used in the splicing reaction to stimulate splicing efficiency, because of its property to concentrate macromolecules by an excluded volume effect (7). This compound might function during pre-incubation in a similar manner. To determine the other component(s) necessary for the increased inhibition, ingredients were omitted either individually (lanes 8 through 10) or in pairs (lanes 11 through 13). The results show that the presence of exogenous $MgCl_2$ during pre-incubation resulted in a cap analogue inhibition of approximately 90% (lanes 9 through 11). Conversely, when $MgCl_2$ was omitted a significant decrease (a reduction from ~ 90 to 45%) in the inhibitory activity of m^7GDP was observed (lanes 8, 12, and 13). Thus, exogenous $MgCl_2$ but not exogenous ATP, is required during pre-incubation to produce the increased inhibitory effect by m^7GDP.

The effect of $MgCl_2$ concentration during pre-incubation on cap analogue inhibition is shown in Fig. 4B (lanes 1 through 6). After pre-incubation at different Mg^{++} concentrations (in the absence of ATP

FIGURE 4. Effect of the presence of splicing reaction components during pre-incubation on cap analogue inhibition of splicing.

(A) Pre-incubation of nuclear extract was performed in presence or absence of splicing reaction mixture components (ATP, $MgCl_2$ and CP) and in the presence of 5 μM m^7GDP. Lanes 1 through 3 were pre-incubated in the absence of m^7GDP except that m^7GDP was added after pre-incubation to lane 3. Pre-mRNA and the missing reaction mixture components were then added for splicing reaction as described in Materials and Methods. Pre-incubation was as follows: Lanes 1,3,4,6 and 7 in the presence of all reaction mixture components except that lane 6 was carried out at 4°C and lane 7 was pre-incubated in the absence of polyvinyl alcohol; Lanes 2 and 5, in the absence of reaction mixture components; the reaction mixture ingredients present or absent in lanes 8 through 13 are indicated in the figure. The percent of splicing efficiency was as follows: Lane 1, 70%; 2, 75%; 3, 8%; 4, 10%; 5, 34%; 6, 34%; 7, 43%; 8, 43%; 9, 10%; 10, 9%; 11, 8%; 12, 37%; 13, 37%. M; markers.

(B) Effect of $MgCl_2$ concentration during pre-incubation on cap analogue inhibition of splicing. Pre-incubation was done in the presence of 5 μM m^7GDP and $MgCl_2$ at the concentrations indicated below. For the splicing reaction, $MgCl_2$ was supplemented to give a final concentration of 3.2 mM. $MgCl_2$ concentration and percent of splicing efficiency were as follows: Lane 1, 1 μM, 23%; 2, 10 μM, 30%; 3, 100 μM, 26%; 4, 500 μM, 7%; 5, 1 mM, < 2%; 6, 3.2 mM, 9%.


A
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3 4 5 6
MgCl2 - + + + - -
ATP + - + - + -
CP + + - - - +

and creatine phosphate) the reaction mixture was supplemented with $MgCl_2$ to attain a final concentration of 3.2 mM, the optimal concentration for in vitro splicing in this system (7). In a control experiment 75% splicing efficiency was obtained (data not shown). Increasing the concentration of exogenous $MgCl_2$ in the pre-incubation reaction resulted in an increased inhibitory effect by m^7GDP. The addition of $MgCl_2$ at a concentration range of between 1 μM and 100 μM caused an inhibition of ~ 60% (lanes 1 through 3) as compared to only 45% when completely omitted (see lane 8 in Fig. 4A). A significant reduction in pre-mRNA processing was observed at a concentration of 500 μM $MgCl_2$ (Fig. 4B, lane 4, 91% inhibition) which reached a maximum at a concentration of 1 mM (lane 5, > 98% inhibition) and then dropped at a concentration of 3.2 mM (lane 6, 88% inhibition).

The result obtained in Fig. 4A, lane 3 prompted us to reexamine whether the presence of m^7GDP during pre-incubation is required to elicit the inhibitory effect on the splicing reaction. We have repeated the experiments described in Fig. 4 with the exception that m^7GDP was added only after the pre-incubation step. The results showed that the requirements for the increased cap analogue inhibition, as described in Figs. 2 and 4, were identical in all respects (data not shown). Thus, these results demonstrate that the presence of m^7GDP is not required during pre-incubation of the nuclear extract to elicit the strong cap analogue inhibition of in vitro splicing.

DISCUSSION

Our results provide strong evidence for the generality of the requirement for the cap structure for efficient splicing of eukaryotic mRNAs. Splicing in a nuclear splicing system is shown here to be cap-dependent under certain conditions, as is in vitro splicing in a HeLa whole cell extract (6). Cap recognition for mRNA processing is most probably an early event since no splicing intermediates were generated in the presence of cap analogues. Thus, it is possible that recognition of the pre-mRNA cap structure by a specific nuclear cap binding protein(s) may serve as a signal for the assembly of mRNA processing complexes that have been recently described (14,15). The absence of a cap structure from polymerase I and III transcription products might be responsible in part for the exclusion of these RNAs from mRNA-processing complexes.

We believe the strongest evidence for the important role of the cap structure in processing of eukaryotic precursor mRNA is the complete and specific inhibition of this process which we have obtained in this study by micromolar concentrations of cap analogues. The degree of inhibition is proportional to the number of phosphate groups in the methylated cap analogues, which is characteristic also of the inhibitory effect of cap analogues on translation (16). It is, however, striking as pointed out before (6), that the concentration of cap analogue required to inhibit splicing is two orders of magnitude smaller than that required to inhibit mRNA translation to the same extent. This raises the possibility that the concentration of the putative nuclear cap binding proteins required for splicing is low.

The most noteworthy finding in this report is that cap analogue inhibition of splicing could be achieved to a significant extent only when the HeLa nuclear extract was pre-incubated (even in the absence of cap analogue) before the splicing reaction. When cap analogue was added to a splicing reaction without pre-incubation, inhibition was only partial (~ 60%) and was not increased when higher concentrations of cap analogue were used. As a possible explanation for this differences, we suggest that in a non pre-incubated extract a high proportion of added pre-mRNA associates with pre-formed splicing complexes and this association is not dependent on cap recognition. However, upon pre-incubation, pre-formed splicing complexes are dissociated and the subsequent assembly of de-novo complexes is dependent on cap recognition. It might also be of significance that the presence of exogenous Mg^{++} during the pre-incubation was required to elicit the increased inhibition by cap analogues, lending support to the idea that a conformational change of the splicing machinery took place. Pre-incubation of the nuclear extract to produce the observed increased inhibitory action by cap analogues is time and temperature dependent (Figs. 2B and C), but not dependent on exogenous ATP or GTP (Fig. 4A), implying that the putative conformational change that leads to the sensitization of the splicing machinery towards cap analogue inhibition is not dependent on energy derived from hydrolysis of a high energy phosphate bond.

In summary, the results presented here demonstrate that <u>in vitro</u> splicing in a HeLa nuclear extract can be dependent on the 5' cap structure and that a remarkable inhibition by cap analogues can be

demonstrated if the extract is pre-incubated before the splicing reaction. The development of a cap dependent nuclear splicing system should facilitate the isolation and characterization of putative nuclear proteins involved in cap recognition during the early steps of eukaryotic mRNA processing.

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CHAPTER 6

GENERAL DISCUSSION

6.1 Model for eIF-4F Mediated Unwinding and Implications for Translational Control

One of the major concerns of this thesis is the role of eIF-4F during eukaryotic protein synthesis. Increasing evidence supports the original proposal for the role of the cap binding protein (and associated factors) in mediating the denaturation of 5' proximal mRNA secondary structure thus facilitating 43S pre-initiation complex binding to mRNA (1,2).

Many lines of indirect evidence have been obtained in the last 7 years that are consistent with this proposal. Briefly they are: (a) Cross-linking of eIF-4A and eIF-4B to the cap structure does not require ATP hydrolysis in the presence of inosine-substituted mRNAs in contrast to native mRNAs (3). In addition, binding of 43S pre-initiaton complex to inosine-substituted mRNA occurs in the absence of ATP (4,5) and in extracts prepared from poliovirus-infected cells (6). These results indicate that mRNAs with less stable secondary structures are less dependent on functional eIF-4F. (b) A monoclonal antibody possessing anti-CBP activity preferentially inhibits initiation complex formation with mRNAs having more significant secondary structure (2). (c) Translation of naturally capped AMV-4 RNA, which has negligible secondary structure at its 5' end (7), is highly efficient in extracts from poliovirus-infected HeLa cells (8) and requires low amounts of eIF-4F to translate at optimum levels in a reconstituted system (9). (d) The high-salt induced inhibition of capped mRNA translation in HeLa extracts can be reversed by eIF-4F (8).

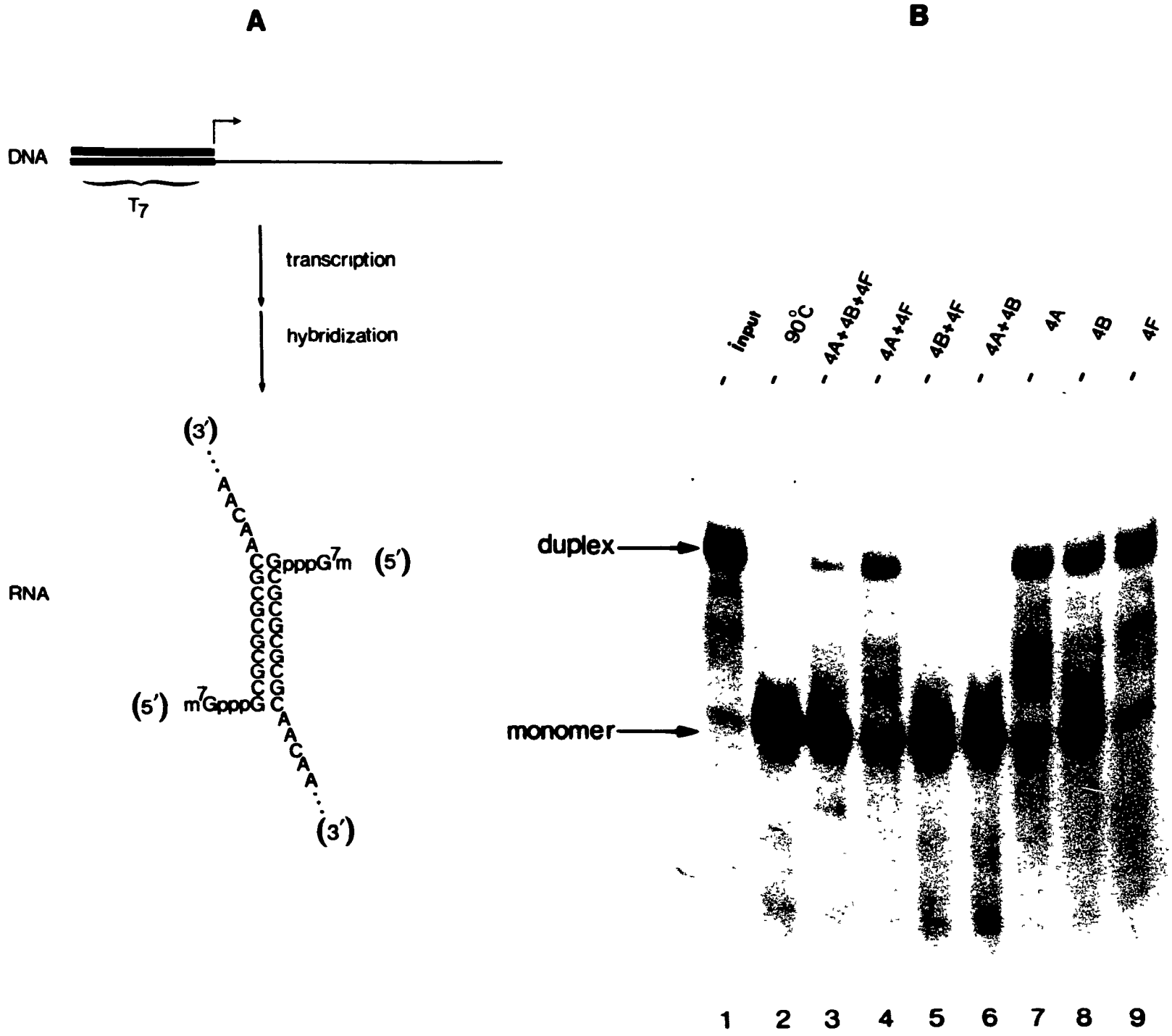
The most direct experimental evidence indicating that eIF-4F has an unwinding activity was reported by Ray et al. (10). They used a nuclease sensitivity assay whereby structural changes in mRNA were monitored by increased susceptibility of the mRNA towards single-strand specific nucleases. Using this assay it was demonstrated that eIF-4A has an ATP-dependent mRNA unwinding activity. Importantly, stoichiometric comparisons indicated that eIF-4F is approximately 20-fold more efficient than eIF-4A in catalyzing this reaction. The melting activity detected in eIF-4F was attributed to the eIF-4A subunit. Moreover, the unwinding activity of eIF-4F was inhibited by the cap analog m^7GDP, while that of eIF-4A was not.

Although all the aforedescribed studies strongly suggest that eIF-4F (and eIF-4A) has a helicase activity, direct evidence for an RNA duplex unwinding property has not been described. For example, the nuclease sensitivity assay described by Ray et al. (10) is not suitable to differentiate between secondary or tertiary structural changes in mRNA conformation. In addition it is difficult to investigate more subtle mechanistic details using this assay.

In light of these limitations and in an attempt to provide direct evidence for an RNA duplex helicase activity we (I. Edery, F. Rozen, N. Sonenberg, manuscript in preparation) recently developed a new RNA:RNA unwinding assay (Fig. 1A). The critical feature is the ability to generate virtually an unlimited number of short RNAs of defined length and sequence that can base-pair to form hybrids. Oligoribonucleotides are synthesized using the method of Milligan et al. (11). RNAs are transcribed by using T_7 RNA polymerase and templates of synthetic DNA

Fig. 1 Direct Evidence for Initiation Factor Mediated RNA Duplex Unwinding Activity

a) RNA duplex unwinding assay. T_7 RNA polymerase directs the *in vitro* synthesis of m^7GpppG primed ^{32}P-radiolabeled transcripts from short DNA oligonucleotides. The RNA transcripts have complementary sequences that can base pair to form an RNA duplex. The RNA duplex is purified and incubated with the indicated amounts of initiation factors for 10 min at 37°C. RNA is resolved by low-ionic strength non-denaturing gel electrophoresis and visualized by autoradiography. b) The following amounts of eIF-4A (3.0 µg), eIF-4B (3.0 µg) and eIF-4F (1 µg) were added as indicated (lanes 3-9). Lane 1, input RNA; lane 2, duplex RNA heated to 90° C for 3 min. The migration of the duplex and monomer forms of the RNA are indicated by the arrows to the left.


A
DNA
T7
transcription
hybridization
RNA
(3′)
AACAACGpppG7m (5′)
(5′) m7GpppGCAACAA
(3′)
B
input
90°C
4A+4B+4F
4A+4F
4B+4F
4A+4B
4A
4B
4F
duplex
monomer
1 2 3 4 5 6 7 8 9

which contain the T_7 promoter (Fig. 1A). During the transcription reaction only the promoter portion of the template has to be double-stranded, this results in the additional advantage of requiring the synthesis of only a single DNA fragment for each different RNA oligonucleotide desired. Transcription is primed in the presence of the cap analog m^7GpppG, resulting in over 50% capping efficiency. The inclusion of complementary sequences in the RNA molecule allows for quantitative inter-molecular annealing producing an RNA dimer.

Using this assay we addressed the ability of purified initiation factors eIF-4A,-4B,-4F and combinations thereof to melt the RNA duplex. A preliminary experiment (performed by F. Rozen) provides direct evidence for an RNA duplex helicase activity (Fig. 1B). The combination of all three IFs in the presence of ATP results in highly efficient unwinding (lane 3). In the absence of eIF-4B no unwinding was observed. This result is in contrast to Ray et al. (10) who demonstrated nuclease sensitivity in the presence of either eIF-4A or eIF-4F alone. In most studies (described in Chapter 1) the role of eIF-4B has been suggested to be facilitative in nature, enhancing already existing (but weak) activities. However, the obligatory requirement for eIF-4B in our RNA duplex unwinding assay is consistent with its absolute requirement to mediate the cross-linking of eIF-4A (as a subunit of eIF-4F) to the cap structure (12). Taken together these results indicate a more significant role for eIF-4B during the melting process than previously suggested. It would seem that in contrast to previous indications eIF-4B alone has limited capacity to unwind RNA duplexes (lane 8). Inasmuch as eIF-4B is notoriously "contaminated" with low levels of

eIF-4F, it is not clear what the significance of this result is. The cloning of eIF-4B will no doubt be a significant step towards understanding its role(s) during cap function.

With the available data what model can we propose for the eIF-4F mediated unwinding of 5' proximal mRNA secondary structure? At present any model should be considered tentative. Notwithstanding that disclaimer the following is probably the most likely sequence of events, a schematic of which is shown in Fig. 2.

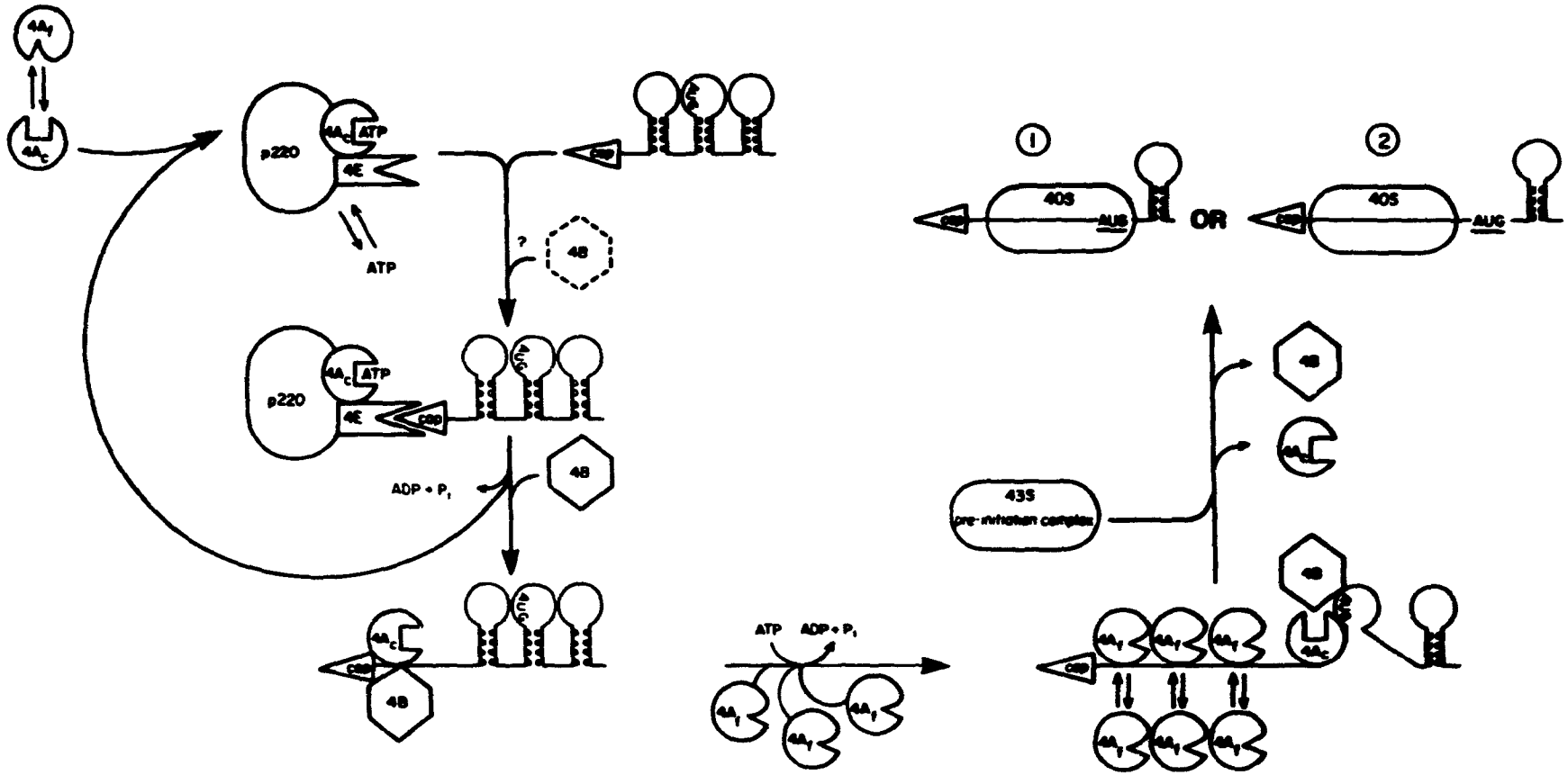
(1) First, eIF-4F must be assembled from its individual components, eIF-4E, eIF-$4A_c$ and the p220 component (the stoichiometry has not been determined, however it is approximately 1:1:1). The assembly of eIF-4F will be considered below.

(2) The multi-subunit eIF-4F binds ATP via the eIF-$4A_c$ subunit. Evidence for this is suggested by the fact that the binding of ATP by eIF-$4A_c$ is approximately 60-fold more efficient than in the case of eIF-$4A_f$ (13). Furthermore, binding of ATP by eIF-$4A_c$ is not inhibited by cap analogs (13) indicating that binding occurs in a step prior to eIF-4F interaction with the 5' cap structure.

(3) The cap structure is recognized by the eIF-4E component of eIF-4F in an ATP-hydrolysis independent binding. This is supported by the following evidence: a) The eIF-4E component of eIF-4F can bind the 5' cap structure in the absence of ATP and is not enhanced in the presence of ATP (14,15). b) The eIF-4E component of eIF-4F has 20-fold higher affinity for the cap structure (determined by chemical cross-linking assay) as compared to uncomplexed eIF-4E (16), indicating that <u>in vivo</u> mRNA caps are most likely recognized

Fig. 2 Proposed Model for the eIF-4F Mediated Denaturation of 5' Proximal mRNA Secondary Structure.

See text for details.


p220
4E
ATP
?
4B
ADP • Pi
OR
40S
AUG
43S
pre-initiation complex

by eIF-4F than free eIF-4E. This step is considered to be a weak interaction (17), although this remains to be proven. It is noteworthy that at this step the binding efficiency of eIF-4F to the cap can be influenced by the degree of cap accessibility (18). In fact under normal physiological conditions this interaction may be the most important determinant in translational efficiency (17-19).

(4) One unresolved issue is the stage at which eIF-4B interacts with eIF-4F to mediate cap function. It is not clear whether eIF-4B interacts with eIF-4F prior to or subsequent to binding of eIF-4F to the cap. The ability of eIF-4B to stimulate the cap independent binding of ATP by eIF-$4A_c$ (13), implies that, in principle, eIF-4B can interact with eIF-4F prior to cap recognition.

(5) The next step requires the hydroly is of ATP, provided by the ATPase activity of eIF-$4A_c$ (15,20). Upon hydrolysis of ATP a change in the spatial arrangement of eIF-$4A_c$ and eIF-4B relative to the 5' cap most likely occurs. Evidence for this is provided by the fact that although eIF-$4A_c$ is present near the cap structure (as a subunit of eIF-4F) it can only cross-link to the cap subsequent to ATP hydrolysis (12,14,15). The putative movement of eIF-$4A_c$ and eIF-4B along the mRNA is inhibited by secondary structure since its presence at the 5' proximal end of mRNAs diminishes the ability of eIF-4A (17,18) and eIF-4B (17,18,21) to interact with the cap structure.

(6) The movement of eIF-$4A_c$ (and eIF-4B) results in the eIF-4E and

p220 components of eIF-4F residing at the cap. It has been suggested by Ray et al. (22) that eIF-4B mediates the release of eIF-4E (at least) from the cap structure. This should enable another round of eIF-4F binding to the cap structure.

(7) In one of the least understood steps eIF-$4A_c$ actively melts secondary structure. Since eIF-$4A_c$ has characteristics unique to helicases (described in Chapter 1 section 1.3) it might move along the mRNA in a processive 5' to 3' direction destabilising RNA secondary structure. As shown (Fig. 2) eIF-$4A_f$ has strong affinity for RNA sequences that are highly single-stranded in nature (15,23). It is plausible that once eIF-$4A_c$ initiates the 'melting' process, eIF-$4A_f$ binds to the newly formed (or previously existing) single-stranded regions thus preventing re-folding of the RNA. If, as suggested, the role of eIF-$4A_f$ is to bind unstructured RNA this might help explain why eIF-$4A_f$ is the most abundant eIF at 3 molecules per ribosome (24).

(8) Melting might occur until a functional AUG is recognized, a likely candidate for this function being eIF-4B (25,26), however there is also evidence for invoking eIF-2β in this capacity, (refs. 27,28). In this scenario it is postulated that the initiation factors in actuality "scan" the 5' UTR and then the 43S pre-initiation complex binds. Conversely, IF mediated unwinding is restricted to the 5' end of the mRNA. Once the 5' end is accessible, the small ribosomal subunit binds and moves along the mRNA unwinding secondary structure with its own associated helicase activity (29,30).

It will be of interest to synthesize RNA duplexes whereby the 5' most species is a natural mRNA and the 3' most species forms a stable stem structure (similar to that described in Fig. 1) in an attempt to determine any distance constraints. In addition, the presence of AUGs upstream to and downstream from the site of annealing might provide evidence for an eIF-4B mediated recognition of the initiator triplet as previously proposed (25,26).

The recent cloning of initiation factors should aid in our understanding of structure-function relationships. Of particular importance in this context is eIF-4A. As previously noted (Chapter 1) the role of eIF-4A during translation initiation is complicated by the fact that the majority of eIF-4A in the cell exists in a free form (eIF-$4A_f$) and not as part of eIF-4F (eIF-$4A_c$). Peptide map analysis revealed that although both eIF-$4A_f$ and eIF-$4A_c$ are very similar they are nevertheless not identical (12). Recently, it was shown that there are two functional eIF-4A genes in mouse (32) and yeast (33). The two eIF-4A genes in mouse (termed eIF-$4A_I$ and eIF-$4A_{II}$) are differentially expressed in a tissue specific manner. It is tempting to speculate that the two eIF-4A genes encode eIF-$4A_c$ and eIF-$4A_f$. If this is the case, it follows that eIF-$4A_f$ is derived from the eIF-$4A_I$ gene since the amino acid sequence of eIF-$4A_f$ corresponds exactly to eIF-$4A_I$ (discussed in ref. 32). Consistently, it is noteworthy that the pattern of eIF-4E mRNA abundance is similar to that of eIF-$4A_{II}$ (P. Nielson, personal communication). Thus it is conceivable that the protein products of eIF-$4A_I$ and eIF-$4A_{II}$ are functionally not interchangeable and as suggested by Nielson and Trachsel (32), the presence of eIF-$4A_{II}$ in

certain tissues may provide a translational advantage. However if the two mouse eIF-4A genes are functionally equivalent to the two yeast eIF-4A genes it is unlikely that eIF-$4A_I$ and eIF-$4A_{II}$ encode eIF-4A proteins that perform distinct functions. This prediction is based on the fact that inactivation of either eIF-4A yeast gene had no effect on cell viability yet inactivation of both genes was lethal to the cell (33).

With respect to eIF-4A the important question to address is a three part problem: 1) Does eIF-$4A_c$ have an intrinsic functional difference as compared to eIF-$4A_f$? 2) Do the structural differences in eIF-$4A_c$ impart the ability to associate with the other subunits in forming eIF-4F? 3) Is the increased activity of eIF-$4A_c$ soley a direct consequence of association with eIF-4F? Not enough information is available to consider the different possibilities in a totally adequate manner. Nevertheless, I believe that the structural differences particular to eIF-$4A_c$ do not provide it directly with increased activity, such as ATPase. In support of this contention is the study of Ray *et al.* (10). They removed the eIF-$4A_c$ component of eIF-4F and assayed their activities by cross-linking to cap-labelled mRNA. As previously demonstrated eIF-$4A_f$ present alone can not cross-link the cap (12,14), however the addition of eIF-$4A_f$ to the eIF-4F devoid of eIF-$4A_c$ enabled eIF-$4A_f$ to bind the cap (similar to that observed for eIF-$4A_c$ when part of eIF-4F). This would imply that eIF-4A assumes enhanced activity as a consequence of associating with eIF-4F.

How could eIF-4F impart upon eIF-4A increased activity? It is noteworthy that the interaction of eIF-4A with single-stranded regions

of mRNA activates its ATPase, and that this activity is greatly stimulated by eIF-4B (15,20,23). Bearing this property in mind and the striking similarities amongst helicases, it was shown that in the case of transcription termination factor rho (a helicase, discussed in chapter 1) a conformational alteration in the protein was induced by the activator RNA (34). In addition, Dombroski and Platt (35) postulated that with respect to rho, the domains for RNA binding and ATP binding comprise separate regions of the protein but that interaction between them is necessary for ATP hydrolysis. The hydrolysis of ATP is believed to be required for generating the energy to actively melt secondary structure and/or the energy required for the processive translocation of the helicase as it destablizes secondary structure.

Taken together these data may suggest that the enhanced helicase activity of the eIF-4A component in eIF-4F is a direct result of the increased ability complexed eIF-4A acquires in binding mRNA. In this fashion eIF-4A is in close proximity to the "proper" RNA activator which in turn stimulates ATP hydrolysis and subsequent unwinding. Therefore, the role of eIF-4F would be to enhance the rate with which eIF-4A binds RNA, a seemingly necessary prerequisite for helicase activity. Since unwinding by eIF-4A is most likely unidirectional (5' to 3') the 5' cap structure automatically aligns the helicase activity at the correct start site. Furthermore, eIF-4A has a high preference for single-stranded RNA. It is probable that stretches of mRNA completely devoid of secondary structure (at least 12-18 bases, ref. 15) are not frequently encountered <u>in vivo</u> (not to mention the additional problem that proteins already bound to mRNA may cause). The cap structure may

thus provide the necessary attachment site irrespective of the nature of the mRNA. This mechanism may explain why mRNAs with regions devoid of secondary structure in the immediate vicinity of the cap are such efficient translators. They provide the necessary single-stranded RNA activator in the right place, next to the eIF-$4A_c$ entry site. In this context it is noteworthy that cleavage of the p220 component of eIF-4F induced by poliovirus infection results in loss of the eIF-4A component from the structurally modified eIF-4F complex (16). Although poliovirus-infection does not modify the activity of eIF-4A directly, inhibition of host protein synthesis could presumably occur due to eIF-4A now having significantly reduced capacity to interact with mRNA at the appropriate place.

Are any of the initiation factors required for mediating cap function also involved in internal binding of ribosomes? It has been known for several years that eIF-4A and eIF-4B but not eIF-4F are required for initiation complex formation on poliovirus and other naturally uncapped RNAs (Chapter 1). The finding that eIF-4A and eIF-4B can bind to a mRNA (lacking in secondary structure), with essentially the same degree of effectiveness and affinity as would occur for natural mRNAs in the presence of eIF-4A, -4B and -4F, suggests a possible role for eIF-4A and eIF-4B in internal initiation (23). The model proposes that eIF-4A in conjunction with eIF-4B, binds to an internal sequence of the mRNA (presumably devoid of secondary structure). This would then signal the binding of ribosomes. This is consistent with the abnormally high concentration of eIF-4A required by poliovirus (and EMC) to ensure optimal translation (36). If eIF-$4A_f$ is involved in internal binding it

might follow that the mere abundance of eIF-$4A_f$ in the cell (3 molecules per ribosome) would cause unwanted spurious cap-independent translation events on cytoplasmic mRNAs. However, as suggested above, binding of eIF-$4A_f$ to mRNA independent of eIF-4F may be a difficult task *in vivo*. This assumption further implies that the polio 5' UTR has a specialized structure that can mediate the binding of eIF-$4A_f$. Unlike polio however, any putative cytoplasmic mRNAs that can undergo genuine internal initiation are still faced with the problem of having a cap at their 5' ends. Therefore, ribosomes should still be able to bind by 5' mediated cap-dependent events which may occlude the internal initiation pathway. This may explain why capping poliovirus mRNA *in vitro* actually leads to a reduction in translation (36a).

It is important to also consider that there are at least two types of cap-independent mRNAs. One case typified by AMV-4, is devoid of 5' proximal mRNA secondary structure and thereby demonstrates independence of an eIF-4F mediated unwinding activity. The second case typified by poliovirus, can circumvent the need for eIF-4F by having a relatively unique RNA sequence that can signal small ribosomal subunits to bind directly. Pelletier and Sonenberg (37) showed that poliovirus has a sequence of several hundred nucleotides in its 5' UTR that is sufficient to transfer internal binding to heterologous mRNAs. The size of the region required for internal binding suggests that secondary and tertiary structure are required for efficient internal ribosomal binding. It is probable that the 5' leader of poliovirus (and EMC, ref. 38) has evolved a specialized structure that can either supply an eIF-4F like function or can bind additional factors that can mediate internal

initiation. It should be feasible to identify the two different types of cap-independent mRNAs by using the bicistronic system described by Pelletier and Sonenberg (37).

The fact that poliovirus has such a long 5' UTR with the potential to form extensive secondary structures raises another important issue. Does the presence of secondary structure in the 5' UTR of mRNAs a priori imply that they will be more dependent on eIF-4F? Studies using the adenovirus tripartite leader suggest that the answer is no. Adenovirus late protein synthesis is resistant to the inhibition of translation induced by poliovirus (39,40). The tripartite segment is required for preferential translation of viral mRNAs at late times during infection (41). The secondary structure of the tripartite leader predicted by computer folding programs indicated a surprising degree of secondary structure (40). If adenovirus late protein synthesis does not occur by internal binding it is not immediately clear how the lack for functional eIF-4F is achieved (40). Lawson et al. (18) suggested that cap accessibility is the prime determinant in translational efficiency under normal conditions. One speculation requires that secondary structure in the tripartite leader be present to ensure that the 5' proximal sequences to the cap are not engaged in short range interactions with downstream elements. In this situation a stable stem-loop structure downstream from the 5' end would maintain a relatively unstructured region in the immediate vicinity of the cap, fascilitating the binding and initial interaction of eIF-4F with mRNA (the rate-limiting step, ref. 18). If indeed this is the case, it is worrisome that many studies analyzing the role of particular 5' UTRs in determining the

translational efficiencies of mRNAs produced by in vitro transcription are not devoid of 5' vector nucleotides (42; a good example provided by PLTARCAT and TAR CAT, Chapter 4). Moreover, secondary structure in the 5' UTR has even been found to stimulate translation. A stable stem-loop iron-responsive element in the 5' UTR of the ferritin mRNA enhances translation in an iron-dependent manner (42-44). Therefore the presence of secondary structure in the 5' UTR can affect the rate of protein synthesis in different ways.

The available evidence strongly indicates, however, that secondary structure in the 5' UTR of mRNAs mainly inhibits translation (Chapter 1). There are several examples of translational control by the existence of multiple 5'-untranslated regions upstream of a common coding sequence. One intriguing example is the c-sis/platelet-derived growth factor 2 (PDGF-2) which has transforming potential. The c-sis/PDGF-2 transcript contains a long 5' UTR that is highly G.C rich, which was demonstrated to be a potent translational inhibitor (45,46). Thus, their findings raise the possibility that changes in regulation at the level of c-sis/PDGF-2 translation may play a role in development of the neoplastic phenotype. This situation is analogous to that proposed for c-myc (47,48). Alternative RNA splicing could generate a variety of different 5' UTRs that influence the translational efficiency. For example, the expression of the gene for the β subunit of mouse thyrotropin results in multiple mRNAs differing in their 5' UTRs, derived by alternative splicing (49), as are the mRNAs for the Ia antigen-associated invariant chain (50). The combination of generating 5' UTR 'regulatory units', whose effects can be further modulated by the

eIF-4F helicase activity might prove to be a powerful method of regulating gene expression.

A very important question is how eIF-4F, -4A and -4B work *in vivo* when the entire ribosomal particle is present. These initiation factors are certainly capable of melting RNA duplexes *in vitro* using the described helicase assay (Fig. 1), without any other components of the translation machinery. However, the presence of other translational components may be necessary for maximum helicase efficiency and cap function. Some evidence implicates an interaction with eIF-4F and eIF-3 bound to the 43S pre-initiation complex. eIF-4E (presumably as part of eIF-4F) co-sediments with eIF-3 under physiological salt conditions (51). It has been speculated that eIF-4F may form a "bridge" between the 5' proximal end of mRNA and the incoming 43S pre-initiation complex via association with ribosomal bound eIF-3. Furthermore, it is not known if the helix-destabilizing activity associated with the elongating 80S ribosome (29,30) is related to the eIF-4F mediated unwinding. The ability of elongating 80S ribosomes to destablize secondary structures in the coding and 3' non-translated region indicate that the associated helicase activity is independent of eIF-4A,-4B and -4F since they only participate during the initiation phase of translation.

Other related aspects are also important to consider when dealing with the mechanism and regulation of protein synthesis. The 'hypothesis' that the cytoskeleton is involved in the regulation of translation requires further examination (for review, see ref.52). The association of translational initiation factors and ribosomes with the cytoskeletal framework has been shown (example ref. 53). In addition,

the association of mRNA with the cytoskeleton is postulated to be necessary for mRNA translation in vivo (54-56). This may explain why in vitro extracts have such low translational efficiencies. The progressive reorganization of the host cell cytoskeleton during viral infection may also contribute to the selective translation of viral mRNAs (57). Furthermore, mRNA injected into Xenopus oocytes exhibits a differential capacity for translation. mRNAs translated in the 'free' cytoplasm are translated efficiently whereas mRNAs translated on the rough endoplasmic reticulum membrane are translated inefficiently (58,59). In summary, the translation of mRNA in vivo does not occur as a soluble reaction in the typical sense of an in vitro reaction.

mRNA in the cell also exists bound to specific proteins giving rise to messenger ribonucleoprotein complexes. It is not well understood what the role of these RNA binding proteins is, and how they might influence the structure of the RNA and the initiation phase of translation. Another consideration as pertains to the in vivo situation concerns the fashion in which ribosomes attach to polysomes. Is the binding of the first 40S subunit an mRNA encounters in the cytoplasm similar to all subsequent 40S subunit bindings? It is possible that the movement of a 40S subunit along the mRNA will alter the structure of the 5' UTR behind it. In addition, the binding of eIF-$4A_f$ to the 5' UTR (Fig. 2) is speculated to retain the RNA in an unstructured conformation. If this situation is maintained long enough an incoming 40S subunit should encounter less difficulty in binding and scanning the 5' leader. This mechanism would negate the inhibitory effects of 5' proximal mRNA secondary structure, a situation not consistent with the

known effect of stable stem-loop structures on mRNA translatability *in vivo* (60-62). Therefore, it is likely that eIF-4A$_f$ is removed from the mRNA once the 40S ribosome binds, with concomitant restructuring of the mRNA secondary structure preceding its attachment site. In such a way secondary structure in the 5' UTR and the activity of eIF-4F can be used as effective modulators of mRNA translatability.

In summary, a challenging problem concerns relating activities determined *in vitro* with the overall situation occurring *in vivo*. Furthermore, better means of determining mRNA higher order structure (especially *in vivo*) will greatly contribute to our understanding of the role the 5' UTR plays during protein synthesis.

6.2 Control of eIF-2 Activity by eIF-4F

An intriguing aspect of the work presented in this thesis is the interdigitation between the translational control pathways involving two initiation factors, eIF-4F and eIF-2 (Chapter 4). We proposed that under conditions whereby eIF-4F activity is impaired, an increase in the effective concentration of double-stranded RNA will occur. This increase in RNA secondary structure could lead to the activation of the latent double-stranded RNA activated kinase (dsI), followed by phosphorylation of the α-subunit of eIF-2. Conceivably, even small increases in eIF-2α phosphorylation could have large inhibitory effects on translation due to the low amounts of guanine exchange factor. The local activation of dsI and eIF-2α phosphorylation could result in the selective inhibition of mRNA translation. This model was based on the finding that HIV-1 mRNAs have a unique stem-loop structure at their 5' ends that can activate dsI *in vitro*.

What potential role could the activation of dsI by mRNA (or other RNA species) secondary structure play under normal cellular conditions? It was shown that total polysomal mRNA can activate dsI in _in vitro_ extracts (63,64). The activation of dsI has been suggested to induce the synthesis of interferon (65). Interferon production is usually associated with induction of an anti-viral state, however recent evidence indicates that they possess a much broader range of activities (for review, see ref. 66). These activities include modulation of many components of the immune response, inhibition of growth of some cell types, and modification of cellular phenotypes such as alterations in cell differentiation. For example, Petryshyn _et al._ (67) reported that mouse 3T3-F442A cells spontaneously produce and secrete interferon and exhibit a pattern of dsI phosphorylation that is related to specific stages of growth and differentiation. Their findings support a role for dsI during growth and differentiation of 3T3-F442A cells. It is possible that dsI is activated in these cells by the regulated synthesis of specific double-stranded RNA molecules.

6.3 Similarities between Protein Synthesis and Precursor mRNA Splicing

Several reports have shown that the presence of a cap structure at the 5' termini of precursor mRNAs (pre-mRNAs) increases splicing efficiency _in vitro_ (68-72). Konarska _et al._ (69) demonstrated that cap analogs inhibit splicing when added at the start of the reaction but not at later times of incubation. This suggests that cap recognition might be an important step in the formation of a specific ribonucleoprotein complex required for splicing. Presumably, the de novo assembly of these complexes is dependent on cap recognition since pre-incubation of

nuclear splicing extracts is required to render the reaction sensitive to inhibition by cap analogs (70; Chapter 5). Recently, Patzelt et al. (71) showed that the assembly of pre-mRNA splicing complex (spliceosome) is markedly increased in the presence of capped substrates. The cap recognition step may direct the ATP-dependent formation of the spliceosome. This is consistent with the observed ATP-dependent cap-analog sensitive lag period required in vitro prior to the first appearance of pre-mRNA splicing intermediates (69).

Interestingly, Ohno et al. (72) used a double intron pre-mRNA and showed that the cap structure enhances the splicing reaction leading to the preferential excision of the upstream intron but not the downstream intron. When the relative order of the two introns was reversed, the upstream intron was still spliced out more efficiently than the downstream intron if the pre-mRNA was capped. It appears that a spliceosome is formed, albeit rather inefficiently, at the splice junctions of the downstream intron independently from the cap-mediated formation of the complex at the upstream intron. This is consistent with the simultaneous and independent formation of two spliceosomes on synthetic double-intron pre-mRNAs (73). Furthermore, each of the two 50S spliceosomes formed simultaneously on a double-intron pre-mRNA is assembled in a stepwise fashion via a 22S-35S-50S pathway as is the case with a 50S splicing complex assembled on a single-intron precursor. Unfortunately, this study did not address the involvement of the 5' cap during double intron spliceosome assembly. In any event these (73) and other results (74,75) exclude the possibility of a cap mediated processive scanning mechanism for splicing. However, it is noteworthy

that *in vivo* spliceosome formation occurs while transcripton is still in progress (76) thus increasing the possibility of a cap mediated 5'-3' polarity in the formation of spliceosomes on the multiple introns. This need not imply an obligatory order of intron removal inasmuch as the rate of splicing might be determined by other factors independent of time of spliceosome assembly.

In contrast to the stimulatory role played by the 5' cap during pre-mRNA splicing in mammalian extracts, the yeast splicing system was shown not to be inhibited by cap analogs (77). Differences in the *in vitro* splicing conditions used in the yeast system may account for this apparent discrepancy. Extracts prepared from yeast have extensive nuclease activity which imposes several constraints on the optimization of conditions. The splicing of pre-mRNAs in yeast extracts has no significant lag period, is over in 15 min., and is performed at 25°C (77). Konarska *et al.* (69) showed that the cap-analog only inhibits pre-mRNA splicing during the lag period (~30 min) characteristic of mammalian splicing extracts. The absence of a significant lag period during pre-mRNA splicing in yeast may explain the apparent lack of a 5' cap requirement. Moreover, the mRNA-decapping enzyme from yeast has a unique specificity for long RNA chains (78). It was demonstrated that a synthetic capped RNA (540 nucleotides) was not reduced in size, while as much as 80% was decapped (unlike the presumptive mammalian homolog that has a preference for oligonucleotides of less than 10 nucleotides in length, ref. 79). As a result it is probable that the input capped pre-mRNA substrates are significantly decapped in the whole-cell yeast

splicing system. This would lead to an underestimation of the enhancing effects of the 5' cap structure.

This is reminiscent of the controversy surrounding initial studies analyzing the relative importance of the cap on translation of mRNAs in different systems (80). For example, the cap structure is not important for the translation of AMV-4 RNA. Many studies were required before it became apparent why this is likely to be the case. In a similar fashion, there is insufficient data pertaining to the role of the cap structure during pre-mRNA splicing. Future studies aimed at analyzing a variety of pre-mRNA substrates and isolation of the cap-binding trans-acting factor(s) should reveal the importance of the 5' cap structure during mRNA biogenesis.

Notwithstanding the role of the 5' cap structure the acts of protein synthesis and pre-mRNA splicing have other striking similarities. The specificity in excision of an intron from pre-mRNA appears to be determined by the interactions of trans-acting splicing components with the specific RNA sequences which govern the formation of a multicomponent splicing complex, the spliceosome. A similar scenario is invoked for the assembly of ribosomes for translation. Formation of both spliceosomes and polysomes requires ATP hydrolysis and is enhanced by recognition of the cap structure. In addition there is limited sequence information on the pre-mRNA or the mRNA that is required for efficient utilization. In the case of pre-mRNA a 5' splice junction GU and a 3' splice junction AG are recognized, whereas in translation an AUG triplet serves. The functional interplay of specialized RNA molecules organized in a large ribonucleoprotein complex

ensures faithful recognition and execution. snRNAs function, in part, by complementary base pairing with pre-mRNA consensus regions in an analogous fashion to the recognition of the triplet codon by tRNA. The different proteins particular to each complex most likely stabilize the neccesary RNA:RNA interactions. The presence of secondary structure in pre-mRNA can inhibit splicing (or induce an alternative splice) (81-83) indicating that RNA structure is also an important parameter as is the case for translation. Spliceosome assembly, however, does not seem to be processive from 5'-3' (with the possible exception of the first intron), a situation more similar with internal binding of ribosomes than that proposed in the scanning hypothesis. The similarities between pre-mRNA splicing and translation might reflect the antiquity and importance of an RNA world.

6.4 Summary

The role of the cap structure during translation initiation is to fascilitate the binding of a multi-subunit complex termed eIF-4F that functionally interacts with two other initiation factors eIF-4A and eIF-4B resulting in a helicase activity. The individual polypeptides have specialized functions that work in concert to maximize the reaction. Unwinding of 5' proximal mRNA secondary structure enhances the efficiency of 40S ribosomal subunit binding to mRNA. Since this step is the overall rate limiting step in translation, regulation can be achieved by modulating the activity of the trans-acting factors (especially eIF-4F) and/or the cis-acting features present in the 5' UTR of mRNAs. The ability of RNA to form complex higher order structures may provide intricate regulatory information in the 5' UTR of mRNAs.

The cap structure and the activity of helicases are also required for other events involving nucleic acid interactions. Regulation of gene expression by inducing controlled changes in RNA conformation is likely to be a widespread phenomena. These studies provide evidence for the multifunctional role of the cap structure during gene expression and its control.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. The synthesis of a new cap-analog affinity matrix that is less demanding to prepare than those previously described. Using this resin we purified and characterized a high molecular weight cap binding protein complex (termed eIF-4F). Studies showed that the 50 kDa component of eIF-4F is almost identical to eIF-4A. Furthermore eIF-4F is required for the ATP-dependent interaction of eIF-4A and eIF-4B with the cap structure of mRNA.
2. The development of a novel RNA duplex unwinding assay provided direct evidence for initiation factor mediated helicase activity. We show that the combination of eIF-4A,-4B and -4F results in highly efficient melting activity.
3. The unique stem-loop structure present at the 5' proximal end of the human immunodeficiency virus (HIV-1) can activate the double-stranded RNA dependent kinase (dsI) resulting in *trans*-inhibition of translation. This is the first demonstration of a specific naturally occurring mRNA sequence that can activate dsI.
4. The cap structure at the 5' termini of precursor mRNAs enhances *in vitro* splicing. These studies provide evidence for the multi-functional role of the cap structure during mRNA biogenesis and utilization.