ROLE OF EUCARYOTIC mRNA CAP BINDING PROTEINS IN PROTEIN SYNTHESIS AND REGULATION IN POLIOVIRUS INFECTED HeLa CELLS

by

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CAP BINDING PROTEINS IN POLIOVIRUS INFECTED HELA CELLS

ABSTRACT

The cap structure $m^7GpppX(m)$...at the 5' terminus of eucaryotic mRNAs facilitates ribosomes binding to mRNA via interaction with cap binding proteins (CBP). Polypeptides of 24, 50 and 80 kilodaltons in crude initiation factors can be specifically crosslinked to the cap structure of mRNA. Crosslinking of the 50 and 80 kilodalton polypeptides requires ATP hydrolysis, but shows reduced dependence on ATP if the mRNA has less secondary structure. Purification by m^7GDP affinity chromatography yields the CBP complex, comprising polypeptides of 24, 50 and ~ 220 kilodaltons. The CBP complex and eucaryotic initiation factor-4B (eIF-4B, 80 kilodaltons) are sufficient to allow a cap specific mRNA protein interaction between the 24 and 50 kilodalton polypeptides of the CBP complex, eIF-4B and mRNA. In relation to the cap specific polypeptides in crude initiation factors, the 50 kilodalton polypeptide is eucaryotic initiation factor-4A (eIF-4A) and the 80 kilodalton polypeptide is most probably eIF-4B. This suggests that the cap binding protein complex and possibly eIF-4B, denature mRNA. In poliovirus-infected cells, uncapped poliovirus RNA is translated when cellular (capped) mRNA translation is inhibited. The 220 kilodalton polypeptide of the CBP complex is proteolyzed in poliovirus-infected cells, correlating with a reduction in the crosslinking of the 24, 50 and 80 kilodalton polypeptides, thus probably explaining the inhibition of cellular mRNA translation. mRNAs with reduced secondary structure are less dependent on the fully active CBP complex for ribosome binding, consistent with the suggestion that the CBP complex can denature capped mRNAs. The data indicate an important role for the 220 kilodalton polypeptide in ribosome binding and, that mRNA secondary structure is a significant determinant in translation of capped mRNAs.

RESUME

La structure cap $m^7GpppX(m)....$ au bout 5' des ARNm eucaryotes facilite l'attachement des ribosomes à l'ARNm en interagissant avec des protéines qui se lient au cap (CBP). Des polypeptides de 24, 50 et 80 kilodaltons faisant partie des facteurs d'initiation peuvent spécifiquement se lier à la structure cap de l'ARNm. L'attachement des polypeptides de 50 et 80 kilodaltons nécessite l'hydrolyse d'ATP, mais fait preuve d'une plus faible dépendance à l'ATP lorsque la structure secondaire de l'ARNm est réduite. Le complexe CBP, composé de polypeptides de 24, 50 et ≃ 220 kilodaltons, peut être purifié par chromatographie d'affinité au m^7GDP. Le complexe CBP et le facteur d'initiation 4B (eIF-4B, 80 kilodaltons) suffisent à eux seuls à promouvoir l'interaction ARNm-protéine, spécifique au cap, entre les polypeptides de 24 et 50 kilodaltons du complexe CBP, eIF-4B et l'ARNm. Comparativement aux polypeptides spécifiques au cap faisant partie des facteurs d'initiation, le polypeptide de 50 kilodaltons correspond au facteur d'initiation 4A (eIF-4A) et celui de 80 kilodaltons correspond probablement à eIF-4B. Ceci suppose que le complexe CBP et possiblement eIF-4B dénaturent l'ARNm. Dans des cellules infectées par poliovirus, l'ARN sans cap des poliovirus est traduit alors qu'il y a inhibition de la traduction de l'ARNm cellulaire (avec cap). Dans ces mêmes cellules, le polypeptide de 220 kilodaltons du complexe CBP est protéolysé, en corrélation avec une réduction du niveau de liaison des polypeptides de 24, 50 et 80 kilodaltons à la structure cap, expliquant ainsi probablement l'inhibition de la traduction de l'ARNm cellulaire. Les ARNm ayant une structure secondaire réduite sont moins dépendants au complexe CBP en ce qui a trait à l'attachement des ribosomes, renforçant ainsi l'hypothèse voulant que le complexe CBP puisse dénaturer les ARNm avec cap. Les données recueillies suggèrent que le polypeptide de 220 kilodaltons joue un rôle au niveau de l'attachement des ribosomes et que la structure secondaire de l'ARNm soit un facteur déterminant lors de la traduction des ARNm avec cap.

DEDICATION

I DEDICATE THIS WORK TO MY MOTHER AND FATHER

FOR THEIR LOVE AND CARE

"The lame in the path outstrip the swift
who wander from it."

Francis Bacon

PREFACE

The work described in Chapters 2-7 of this thesis has been published in the following journals:

Chapter 2. Lee, K.A.W. and Sonenberg, N. (1982) Proc. Natl. Acad. Sci. USA 79, 3447-3451.

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The work presented in Chapters 2 and 7 is entirely my own. Denise Guertin assisted with the ribosome binding experiments in Chapters 3 and 4. Isaac Edery performed most of the work in Chapter 5 together with Marcus Humbelin, André Darveau, Susan Milburn and myself. Isaac Edery also collaborated in the work described in Chapter 6 and provided material for some of the work in Chapter 7.

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CHAPTER 1

INTRODUCTION

Initiation of Eucaryotic Translation and Regulation in Poliovirus-Infected HeLa Cells

1.1 The Overall Process of Eucaryotic Protein Synthesis

The expression of eucaryotic structural genes occurs through multiple steps, many of which occur in different cellular compartments and are subject to regulation. In pondering the reasons for this phenomenally complex situation one is led to consider the highly specialized nature of eucaryotic cells, their extensive subcellular structure and their relationship to the whole organism. Such necessities as tissue specific gene expression, the compartmentalization of cellular functions and the ability to communicate with and respond to other cells and physiological conditions, require that the relay of genetic information between its chromosomal location and its site of action is both long and, in bioenergetic terms, arduous. Consequently, although the major steps in gene expression are relatively well understood, the details and the mechanisms of regulation are only now being touched on.

The pathway of expression of eucaryotic structural genes, in out line, is as follows. Transcription of the DNA template by RNA polymerase II (1) produces a primary transcript which contains the mRNA sequences and often intervening sequences or introns. The primary transcript is then modified at both the 5' and 3' termini. The 5' terminal modifica tion involves addition of a cap by guanylyl transferase and methylation and will be described in detail elsewhere in this introduction since it is of major significance to this thesis. The 3' end is modified by cleavage and subsequent addition of adenosine residues to yield a 3' polyA tail (2). There are also internal modifications, notably methylation of some adenine residues (3). With the exception of the 5' terminal modification of the primary transcript, there is currently little idea of the function of these post-transcriptional modifications.

Next, intervening sequences are removed by a cleavage-ligation reaction known as splicing, to produce a mature mRNA sequence (4,5). The reactions described so far occur exclusively in the nucleus and would seem to be a prerequisite for transport of mRNA into the cytoplasm. Once in the cytoplasm the mRNA is translated into the polypeptide sequence by a process to be described in detail in the following section. Finally, polypeptide chains are often covalently modified (for example by phosphorylation), form active complexes with other polypeptides or are proteolytically processed to yield the final gene product. In addition, nascent polypeptide chains sometimes contain information which is required for targeting them to specific subcellular locations, during which process they are processed to yield the mature protein.

Thus, the production of a biologically functional protein is a monumental task for the cell, depending on the efficient and faithful completion of the general pathway described above. Furthermore, for any particular gene, there are likely to be regulatory molecules which interact with the pathway at some point and either increase or decrease its flux. The step of mRNA translation is undoubtedly one of the most complex parts of the pathway and is known to be regulated in many instances. Consequently, a knowledge of the mechanism of translation will provide significant clues in understanding expression of genes encoding proteins.

1.2 Translation of Eucaryotic mRNA

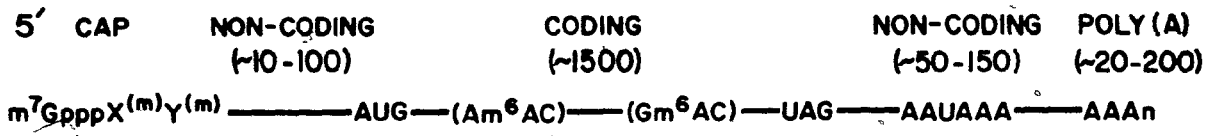
1.2.1. The basic components

Fig. 1 shows the generalized structure of a eucaryotic messenger RNA

FIGURE 1

Primary Structure of Eukaryotic mRNA

EUKARYOTIC mRNA STRUCTURE



(mRNA) with the elementary structure-function relationships indicated. The factors involved in decoding the mRNA, include ribosomes, transfer RNAs (tRNAs), amino acyl-tRNA synthetases, soluble proteins, (initiation factors, elongation factors and termination factors) ATP, GTP, Mg^{++} and appropriate tonicity. Initiation factors are a major concern of this thesis and will be described in detail in a later section.

Transfer RNA (tRNA) is the RNA species which directs amino acids to the site of peptide bond synthesis on the ribosome (6). tRNAs are typically 80 nucleotides in length with extensive secondary and tertiary structure. The 3' terminus of tRNA molecules contains the conserved sequence 3' A-C-C 5' which is often added to the molecule post-transcriptionally in template independent fashion (12). Attachment of amino acids occurs through an acyl linkage to the 2' or 3' hydroxyl group of the terminal adenosine residue, the position depending on the particular amino acid (AA). Proceeding in the 3'→5' direction, the first loop of the tRNA molecule contains the TΨCG conserved sequence, the second loop is variable, the third loop contains the anti-codon triplet and the final loop is referred to as the dihydro-U loop due to the presence of many dihydrouridine residues. One further structural peculiarity of tRNAs is that they contain a high content of the rarer bases such as pseudo uridine and 1-methyl guanosine (6). While decoding the mRNA relies simply on codon-anti-codon base pairing (7) it is clear from thermodynamic considerations that this interaction by itself is not sufficiently stable to account for the observed fidelity of translation. Thus, it is thought that the overall conformation of the tRNA molecule together with constraints imposed by the ribosome are very important in allowing faithful codon-anti-codon interactions (8,9). Besides being

involved in decoding the mRNA there are many novel tRNA species which might play significant regulatory roles. Examples include, nonsense suppressor tRNAs (10) and frameshift tRNAs (11).

Amino acylation of tRNAs is carried out by amino-acyl tRNA synthetases which recognize both the amino acid (AA) and the tRNA. The synthetase first activates the amino acid by esterifying it to an adenosine monophosphate (AMP) residue derived from ATP with the concomitant release of pyrophosphate. The activated amino acid is noncovalently complexed with the synthetase and this complex binds the tRNA molecule, followed by formation of an ester linkage between the carboxy group of the amino acid and the 2' or 3' hydroxyl group of the tRNA and the release of AMP. The process of amino acylation can thus be summarized and the resultant AA-tRNA is a substrate for the elongation step in polypeptide synthesis.

$$(1) \quad AA + ATP \rightleftharpoons AA\sim AMP + PP$$

$$(2) \quad AA\sim AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP$$

The amino acyl tRNA synthetases play a key role in maintaining translational fidelity because, once esterified to tRNA, the amino acid has no effect on codon-anticodon interaction between the mRNA and tRNA. Thus, if a non-cognate aminoacyl-tRNA is synthesized this will result in misincorporation of an amino acid. In view of the fact that errors in translation occur about once every 3000 amino acids (this example is for one particular amino acid (13)) it is clear that amino acylation is a highly specific process.

The characterization of eucaryotic ribosomes is in many respects somewhat rudimentary to date owing to technical limitations. Eucaryotic ribosomes are rather larger than their prokaryotic counterparts while performing in many ways, similar functions. They consist of a large

subunit (60S) and a small subunit (40S). The 60S subunit is comprised of one molecule each of 28S, 7S and 5S RNA and of the order of fifty distinguishable polypeptides. The 40S ribosomal subunit contains one molecule of 18S RNA and about thirty different polypeptides (14). The significance of the larger size of eucaryotic ribosomes in comparison with prokaryotic is not clear at present. It has been suggested that some of the proteins might allow interaction with receptors on the endoplasmic reticulum (15,16). Others might be involved in regulation of translation if they function by mediating interaction of ribosomes with mRNA (17). This seems a distinct possibility, given that the translation mechanism shows clear differences when comparing eucaryotes and prokaryotes, at the step in which ribosomes initially bind to mRNA.

1.2.2 The basic mechanism

In general, eucaryotic mRNAs are functionally monocistronic, i.e. they encode a single primary translation product, due to the fact that initiation of translation is somehow restricted to one site on the mRNA. Translation can be thought of as occurring in three mechanistically distinct phases, namely initiation, elongation and termination.

Initiation

Initiation is defined as the process whereby an 80S ribosome and initiator Met-tRNA (Met-tRNA$_f$) become positioned at the AUG codon of the mRNA and can subsequently function in polypeptide chain elongation. Initiation is a highly complex process which can be readily divided into many sub- reactions, one of which involves mRNA binding to the 40S ribosomal subunit. This event is a major focus of this thesis and, along with other parts of the initiation process will be described in detail in the next section.

Elongation

Elongation consists of sequential addition of amino acids does not occur in the 5' to 3' direction accompanied by peptide bond formation. When the 80S initiation complex is positioned at the AUG, the second aminoacyl-tRNA is positioned in the form of a ternary complex with elongation factor 1 (EF-1) and GTP (18,19). The triplet codon in the acceptor site on the ribosome (the A site) dictates which amino acyl-tRNA enters the 80S complex. EF-1 consists of three subunits, EF-1$\alpha\beta\gamma$ (20). EF-1α enters in the ternary complex with the aminoacyl-tRNA and GTP. The GTP is then hydrolyzed and an EF-1α-GDP complex is released (21-23). EF-1γ is then involved in dissociating the EF-1α GDP complex and allowing EF-1α to recycle (24). Next, peptidyl transferase which is located on the 60S subunit (25) catalyzes peptide bond formation between the two aminoacyl- tRNAs. The dipeptide remains covalently attached to the tRNA in the A site while the deacylated tRNA in the P site is ejected from the ribosome. Elongation factor 2 (EF-2) (molecular mass ~ 100 kilodaltons (kDa)) then catalyzes translocation of the ribosome, utilizing energy derived from GTP hydrolysis, such that the aminoacyl-tRNA is transferred from the A site to the P site (26-28). This step completes the elongation phase.

Termination

When a termination codon (UAA, UAG or UGA) appears in the A site of the ribosome, a termination factor or release factor (RF) binds and catalyzes release of the completed polypeptide chain. This step requires GTP hydrolysis (29) and both RF and peptidyl transferase. The peptidyl-tRNA in the P site is cleaved, releasing the polypeptide chain and leaving the deacylated tRNA on the ribosome (29). The mechanism of

termination is not well understood although the RF has been purified from rabbit reticulocytes and seems to be a dimer of native molecular weight 115,000 (29,30).

1.2.3 The site of translation

It has been recognized for some time that the site of translation for a particular mRNA will depend on the type of protein it encodes. In general, secreted and membrane proteins are synthesized on polyribosomes associated with the rough endoplasmic reticulum while soluble proteins are synthesized on so called 'free' polyribosomes (31). It is most likely that translation of all mRNAs starts on free ribosomes and then, those encoding membrane or secreted proteins are transferred to the membrane (32). Attachment of polysomes to the membrane must somehow take place by virtue of specific binding sites on the membrane and the properties of the N-terminal nascent peptide, which is usually hydrophobic. The view that the information required to direct ribosomes to membranes resides in the N-terminal peptide being synthesized accords with the fact that the ribosome population in the cell is homogenous and thus plays no role in determining the site of translation.

The studies of Richter and Smith (32) indicate that globin mRNA (normally translated on free polysomes) and zein mRNA (normally tanslated on membrane bound polysomes) do not compete with one another for translation in oocytes. This indicates that the two types of mRNA do have different requirements for translation, given that there is limited initiation capacity in the oocyte. This conclusion is substantiated by the results of a control experiment in which mRNAs of the same type (i.e. either translated on 'free' or membrane found polysomes) were in competition for some limiting component in the oocyte (32). The factor which

appears to be responsible for translocation of appropriate templates to the membrane is the so called signal recognition particle (SRP) which temporarily arrests translation of nascent secretory polypeptides *in vitro* by binding to the amino-terminus of the polypeptide (33). Addition of microsomal fractions alleviates the arrest and so it is thought that SRP plays a role in targeting appropriate mRNAs to the membrane. Thus, SRP is absolutely required for complete translation of mRNAs encoding secretory proteins.

Many observations have suggested that mRNA is translated in certain areas of the cytoplasm only. For example, high voltage electron microscopy of intact cells has shown that polysomes are associated with structures, either membranes or cytoskeleton (34,35). Furthermore, polysomes remain associated with the detergent resistant cytoskeletal framework following extraction and are attached via their mRNA component (36). Finally, a monoclonal antibody which was raised against eucaryotic protein synthesis initiation factor three (eIF-3), and which inhibits translation *in vitro* (37) was found to bind very selectively to the cytoskeleton of baby hamster kidney (BHK) cells (38). These observations prompted a closer look at mRNA-cytoskeleton attachment in relation to translation and an interesting picture is emerging. Penman and coworkers have shown that poliovirus infection of HeLa cells results in release of host mRNAs from the cytoskeleton concomittantly with the inhibition of host mRNA translation (39). Bonneau *et al.*, have repeated and extended these results (40) using various viral systems and shown that mRNA association with cytoskeletal elements is often correlated with translation of the mRNA *in vivo*, although the association by itself is not sufficient for translation. The precise cytoskeletal structures and

soluble factors involved in attachment of mRNAs to the cytoskeleton are not yet known.

1.3 Initiation of Translation

The mechanism and regulation of initiation of translation are major foci of this thesis. Accordingly, I will describe the components and the pathway of the initiation process in some detail.

1.3.1 Eucaryotic translation initiation factors

The assembly of initiation complexes between eucaryotic mRNA and 80S ribosomes requires several soluble proteins referred to as eucaryotic initiation factors (eIF). These factors have been purified and shown to be absolutely required for activity in reconstituted translation systems, by several groups (41-45). An initiation factor is defined by activity in the above assays and by the fact that it is released upon 80S complex formation, as opposed to being an integral ribosomal protein. Most of what is known about initiation factors comes from studies on rabbit reticulocyte factors. This would seem to be a valid model system for several reasons. First, eucaryotic initiation factors appear highly conserved structurally, antigenically and functionally between, for example, rabbits and humans (46). Second, lysates from rabbit reticulocytes are able to efficiently translate a wide variety of eucaryotic mRNAs (47) indicating that the translational machinery does not reflect the highly specialized nature of the reticulocyte (with the exception of the relative abundance of different tRNAs, which correlate with the amino acid composition of globin). Third, there is little convincing evidence for the existence of mRNA specific initiation factors, which might occur in a tissue specific way. For example, it has been shown that globin

TABLE 1

Initiation Factors from Rabbit Reticulocytes

Factor	Molecular weight	Number of polypeptides	pI	Functions
eIF-1	15,000	1	---	mRNA binding
eIF-2	122,000	3	6.4	ternary complex with Met-tRNA$_f$ and GTP
eIF-2A	65,000	1	---	binds Met-tRNA$_f$ to 40S
eIF-3	~400,000	8	6.7	dissociation, promotes Met-tRNA$_f$ and mRNA binding
eIF-4A	49,000	1	5.8	mRNA binding
eIF-4B	80,000	1	6.3	mRNA binding
eIF-4C	17,000	1	5.6	promotes dissociation, Met-tRNA$_f$ binding
eIF-4D	16,000	1	6.1	stimulates Met-puromycin synthesis
eIF-5	160,000	1	6.4	required for 80S complex formation
eIF-4F	~300,000	3/4	---	cap recognition and mRNA binding
Co-eIF-2A	19,000	1	---	stimulates ternary complex formation

mRNA can be efficiently translated in muscle or nerve cells, in which tissue it is normally not expressed (48).

Table 1 (taken from reference 17) shows the various initiation factors, their molecular weights and functions, if known. It remains likely that this list is not exhaustive, either due to the existence of unidentified cofactors or due to the fact that some of the factors described (particularly the multi-subunit eIF-3) probably have separable activities within them. Notwithstanding these caveats however, the listed factors are competent in catalyzing the formation of functional 80S initiation complexes (for a review of the purification of initiation factors see reference 17).

1.3.2 The pathway of translation initiation

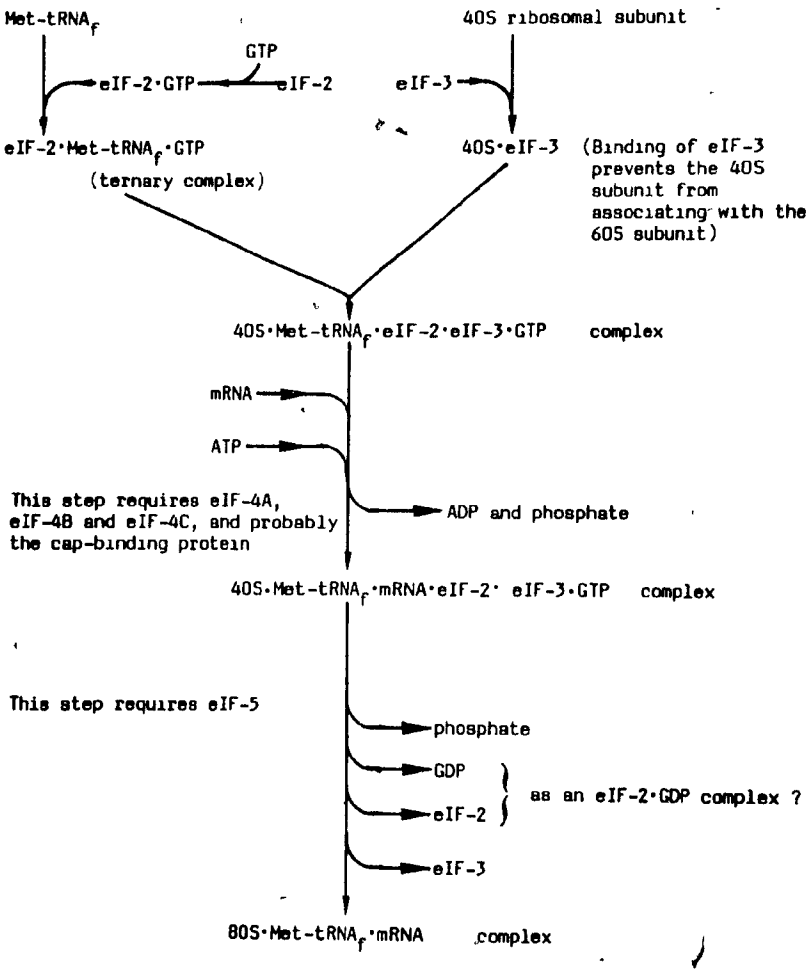
Figure 2 shows a representation of the initiation pathway resulting in the formation of 80S initiation complexes which can function in polypeptide chain elongation (the figure is taken from reference 184). eIF-4F (the cap binding protein complex) has now been shown to function in mRNA binding to 40S ribosomes. Consequently, the 'probable' involvement of cap binding protein (indicated in the figure) is now proven.

(a) Dissociation of 80S ribosomes.

80S ribosomes are unable to bind mRNA directly and are in equilibrium with 40S and 60S subunits (49,50). In vivo, the equilibrium favors formation of 80S complexes but can be shifted in the other direction by eIF-3 (51,52). eIF-3 acts as an anti-association factor since it binds 40S subunits but not 80S subunits (51,52). Binding of eIF-3 to 40S subunits is stoichiometric (43,53) and the eIF-3-40S complex can bind initiator tRNA. It has also been reported that eIF-4C promotes formation of free 40S subunits (54).

FIGURE 2

The Pathway of Translation


Met-tRNA$_f$
GTP
eIF-2·GTP
eIF-2
40S ribosomal subunit
eIF-3
eIF-2·Met-tRNA$_f$·GTP
(ternary complex)
40S·eIF-3
(Binding of eIF-3 prevents the 40S subunit from associating with the 60S subunit)
40S·Met-tRNA$_f$·eIF-2·eIF-3·GTP complex
mRNA
ATP
This step requires eIF-4A, eIF-4B and eIF-4C, and probably the cap-binding protein
ADP and phosphate
40S·Met-tRNA$_f$·mRNA·eIF-2· eIF-3·GTP complex
This step requires eIF-5
phosphate
GDP
eIF-2
as an eIF-2·GDP complex ?
eIF-3
80S·Met-tRNA$_f$·mRNA complex

(b) Formation of the ternary complex and 43S preinitiation complexes.

eIF-2, GTP and Met-tRNA$_f$ form a ternary complex which then binds to eIF-3-40S complexes. The ternary complex is stable, sediments with 40S subunits (53), and has a 1:1:1 stoichiometry. Formation of the ternary complex is specific for the Met-tRNA$_f$ and not even the iso-accepting species Met-tRNA$_m$, which is responsible for decoding internal methionines, can be utilized (55). Preliminary studies have suggested that the α subunit of eIF-2 binds GTP and the β subunit binds the Met-tRNA$_f$ (56). Furthermore, it has been shown that the α and β subunits are substrates for phosphorylation by a variety of protein kinases (57-64) and that phosphorylation of eIF-2 is a means by which translation is regulated.

Binding of the ternary complex to 40S subunits occurs independently of mRNA but is greatly increased by eIF-3 (43,45,53). Stable binding is also enhanced by the AUG codon and influenced by Mg^{++}. Available data suggests that eIF-3 binds first to the 40S subunit, followed by binding of the ternary complex to form the 43S preinitiation complex (43,45,53,65). The 43S preinitiation complex thus contains 40S•Met-tRNA$_f$•eIF-2•eIF-3-GTP.

(c) Formation of the 48S preinitiation complex.

The next step in initiation involves binding of the 43S preinitiation complex to mRNA. It is the least well understood part of the initiation process, while at the same time being the most significant in terms of the whole pathway. The importance of the mRNA binding step is

emphasized for the following reasons:

1. It is apparently the rate limiting step in initiation.
2. It is the step at which mRNAs are recruited for translation.
3. It is influenced by structural features of the mRNA.
4. It requires several soluble initiation factors.
5. It requires ATP hydrolysis (in contrast to the prokaryotic mechanism).
6. It is regulated.

Figure 2 shows the proposed mechanism for 48S preinitiation complex formation and I shall describe the current state of knowledge of this step in some detail.

Cis acting structures of mRNA which influence binding to the 43S preinitiation complex.

In sharp contrast to the prokaryotic system, eucaryotic ribosomes cannot (in general) bind directly to internal sequences of the mRNA. Thus there is a requirement for a free 5' terminus (66) which is consistent with the proposed model in which ribosomes bind initially at or near the 5' terminus of the mRNA (for a review see reference 67). Other evidence also points to the fact that the only absolute structural requirement demanded of the template to enable it to bind a 40S ribosome is that it does have a free 5' end. For example, fragmentation of many different mRNAs (68-73) produces 'cryptic' ribosome binding sites in fragments derived from the interior of the intact mRNA. Furthermore, these cryptic sites are not exposed in the intact mRNA even after extensive denaturation (74).

Binding of ribosomes to prokaryotic initiation sites requires base pairing of the 3' end of the 16S ribosomal RNA (rRNA) with a purine rich

sequence in the mRNA (the Shine and Dalgarno sequence, 75-77). The lack of a conserved sequence in the 5' untranslated region of eucaryotic mRNAs, which is complementary to 18S rRNA, strongly suggests that such a mechanism is not of general significance to the eucaryotic ribosome binding process (78). It is not excluded however, that base pairing between 18S rRNA and mRNA plays a role in the case of some mRNAs which do have substantial complementarity with the 3' end of 18S rRNA (79-81). There is evidence that the 3' end of 18S rRNA is juxtaposed to mRNA in 80S initiation complexes (82) and so it seems a distinct possibility that stable base pairing might significantly increase the stability of 80S complexes. This however, remains to be elucidated.

Many other studies also support the view that specific sequences near the 5' end of mRNAs are not required for binding of ribosomes. For example, translation of simian virus 40 (SV40) mRNA (83) polyoma early mRNA (84) and rabbit β globin mRNA (85) is not affected by deletions of 5'-noncoding sequences. Similarly, simple insertion of nucleotides upstream of the AUG in adenovirus mRNA (86) and Herpes simplex virus (HSV) thymidine kinase (tk) mRNA (87) has no effect on ribosome binding. In addition, 5' noncoding regions of eucaryotic mRNAs show considerable variation in both length and composition and there appears to be no simple correlation between the length of 5' noncoding region and translational efficiency (88). Finally, the 5' untranslated regions of closely related mRNAs show considerable sequence divergence. For example, the actin mRNAs of slime molds (89) and the yeast iso-I- and iso-2-cytochrome c (90). The sum of these observations strongly suggest (without proving) that primary sequence elements residing in the 5' untranslated regions of eucaryotic mRNAs are not significant determinants in translation.

In contrast, mRNA secondary structure appears to influence binding of ribosomes to eucaryotic mRNAs. In general, mRNA secondary structure is postulated to have a negative effect on translational efficiency in accord with the following pieces of evidence. It has been shown that denaturation of mRNA with methylmercury hydroxide enhances translation (91). Other preliminary studies have shown that irreversibly denatured (inosine substituted) reovirus mRNAs bind more efficiently to ribosomes compared to native reovirus mRNAs (74). More recently, attempts have been made to map regions in the mRNA where secondary structure might exert its negative effect, although there remains a paucity of data available. Herson et al. have compared the efficiency of translation of different mRNAs in vitro, but the drawback with these studies was that the mRNAs differed in their 3' ends as well as their 5' ends (88). In a more systematic study, Pelletier and Sonenberg have shown that introduction of secondary structure into the 5' noncoding region of the HSV-1 tk mRNA sequences severely restricts tk expression in vivo (and translational efficiency in vitro) at the translational level (87). As expected from previous studies, 5' mRNA secondary structure blocks translation by preventing ribosome attachment to the mRNA (87). Chapters 3 and 4 of this thesis pertain to the effects of mRNA secondary structure on translation and a more detailed evaluation of the current evidence is included in the general discussion in Chapter 8.

Finally, the poly A tail and specific sequences towards the 3' end of the mRNA are thought, in general, not to influence ribosome binding to mRNA. In the case of the poly A tail it is not required for translation in vitro (92) but seems to enhance long term expression (928,93). How-

ever, whether the latter effect is due to a positive influence on reinitiation, or simply due to stabilization of mRNA is not established. Deletion of 3' sequences or comparison of translation of mRNAs differing only in their 3' terminal sequences have indicated that 3' sequences are most probably not of general significance to translation initiation (94,95). On the other hand, mRNAs from two α globin loci in humans which differ only in their 3' untranslated regions are translated at different rates (96). Thus, in specific cases, the 3' terminal sequences of mRNA might influence translational efficiency.

The function of the cap structure $m^7GpppX(m)pY(m)$ at the 5' terminus of eucaryotic mRNAs.

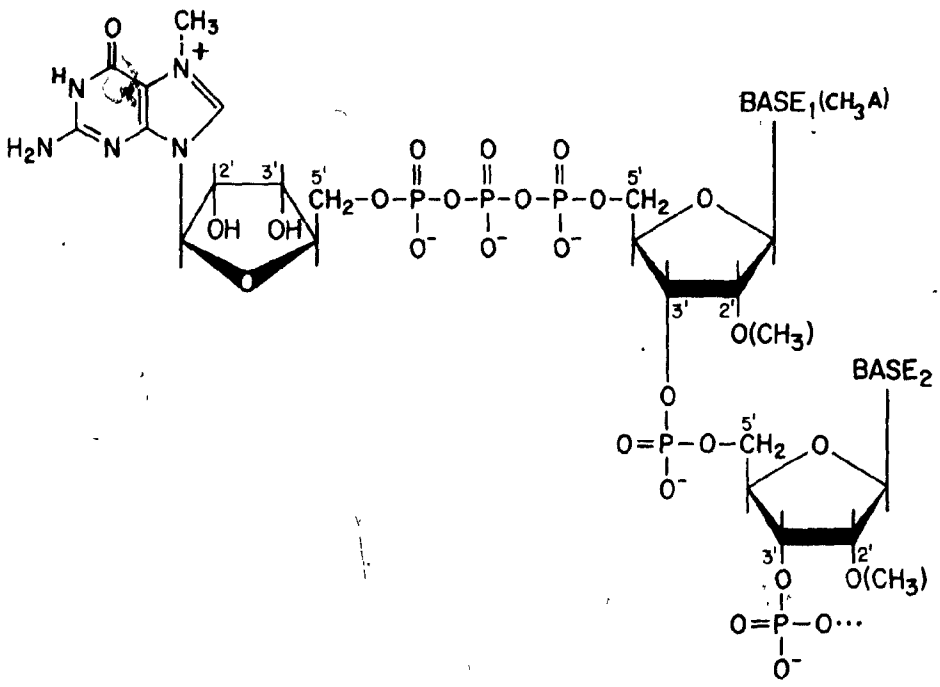
The cap structure, depicted in figure 3, is found at the 5' terminus of all eucaryotic cellular and most viral RNAs (97). The distinguishing structural features of the cap are (1) 7-methylation of the guanine moiety and (2) an inverted 5'→ 5' triphosphate linkage to the penultimate nucleotide of the mRNA. These two features give the so called 'cap zero' structure. Further 2'-O-methylations of the ribose moieties of the second and third nucleotides of the RNA chain occur to varying extents among cellular and viral mRNAs and give 'cap one' and 'cap two' structures, respectively. The significance of these 2-O-methylations is not clear at present although it appears that they have no significant effects on translation of natural mRNAs (111). Consequently, I will not distinguish between the different types of cap structure during the rest of this thesis.

Caps are added to nascent mRNA transcripts early during mRNA biogenesis, but after initiation of transcription and are conserved throughout the lifetime of the mRNA (98-100). (For a detailed descrip-

FIGURE 3

The mRNA 5' Cap Structure

STRUCTURE OF 5′-CAP



tion of the mechanism of the capping reactions, see references 97,101,-102.)

The requirement for the cap structure in translation, in vitro, was established using two approaches. First, it was found that many different capped mRNAs (reovirus, vesicular stomatitis virus (VSV) and rabbit globin mRNAs) are translated much more efficiently than their decapped counterparts (103,104,112). Second, cap analogues (such as 7-methylguanosine monophosphate m^7GMP and m^7GDP) but not their unmethylated counterparts are specific inhibitors of capped mRNA translation in vitro (105-110). Subsequently, ribosome binding studies indicated that the cap structure increases the rate and extent of ribosome binding to several different capped mRNAs (112,121-124). By examining the effects of chemically modified cap analogues as inhibitors in the in vitro assays described above, the significant structural features of the cap have been determined. The m^7G nucleoside is not active while m^7GMP is active, indicating the requirement for at least one 5' phosphate group (105). m^7GDP is more inhibitory than m^7GMP, while m^7GTP, m^7GpppN or m^7GpppN^m behave the same as m^7GDP (106,107). 7-ethyl- and 7-benzyl GDP also behave the same as m^7GDP. Similarly, reovirus mRNAs synthesized in vitro with a 7-ethylguanosine 5' terminus, bind ribosomes as efficiently as native reovirus mRNAs and direct synthesis of authentic reovirus polypeptides (125). Thus, it appears that the positive charge resulting from the 7-methylation is the significant factor, rather than methylation per se (126). Finally, the 2-amino group of m^7GDP is also important as evidenced by the fact that 7-methylinosine phosphates are less inhibitory than the corresponding m^7G phosphates (106,126).

It has been difficult to assess the function of the cap structure *in vivo*. Attempts to block methylation have generally been unsuccessful due to difficulties in blocking the 7-methylation of the guanine (113,114). However, some drugs which inhibit methylation have produced results which are consistent with the view that 5' terminal methylation of mRNA is required for translation (115,116). More direct evidence comes from two sources. In one case, uncapped mRNA with the 5' terminus GpppN, was stable when injected into Xenopus oocytes but was not translated (117), while the corresponding methylated template was translated. In another case, it has been demonstrated that polysomal VSV mRNAs are capped, while the small population of VSV mRNAs not associated with ribosomes are not capped (118). Finally, perhaps one of the most persuasive pieces of evidence is provided by the observation that in poliovirus-infected HeLa cells capped mRNAs are unable to enter initiation complexes, and a factor which mediates cap function is inactivated (119,120). Thus, while direct experimental evidence to demonstrate the requirement for the cap structure for translation *in vivo* is hard to obtain, there seems little reason to question its *in vivo* role.

The degree of dependence on the cap structure for translation varies among different mRNAs and according to many experimental parameters such as temperature, ionic strength and the source of the translation system. The fact that different mRNAs exhibit greater or lesser dependence on the cap is interesting, since it suggests a way in which the intrinsic translational efficiency of particular mRNAs might, in part, be determined. This in turn might implicate the cap in regulation of translation and this possibility will be further addressed in chapter 8. The effect of different conditions on cap function *in vitro* is a subject of particular

relevance to chapters 3 and 4 of this thesis and is addressed in detail in these chapters.

Initiation factors involved in mRNA binding to ribosomes.

By following 48S preinitiation complex formation using radiolabeled globin mRNA it is evident that in addition to eIF-2, eIF-3 and eIF-4C which enter with the 40S ribosome, eIF-4A, eIF-4B and eIF-1 are also required for maximal mRNA binding (43,127). More recently, the eIF-4B activity has been shown to have two separable activities, one associated with the 80 kDa polypeptide which is now thought to represent homogenous eIF-4B and another which represents eIF-4F (128) (otherwise referred to as CBPII (119) or the cap binding protein (CBP) complex (129).

Of the above mentioned factors, eIF-1, eIF-4A, eIF-4B and eIF-4F are directly required for mRNA binding to ribosomes (128,130,131). However, it remains to be determined whether eIF-2, eIF-3 and eIF-4C are directly involved in this step. This is a difficult question to address because all of these factors are involved in previous steps in the initiation pathway and consequently their involvement in mRNA binding is not clear. The presence of eIF-4A, eIF-4B or eIF-1 on the 40S subunit has yet to be demonstrated. Perhaps they are not bound with sufficiently high affinity to withstand sedimentation, or maybe they never bind (43). This point thus remains contentious.

eIF-2

eIF-2 is required for formation of the 48S preinitiation complex and can be found on the surface of the ribosome (53,54). The nature of the interaction between eIF-2 and mRNA is however, obscure. Two observations show that eIF-2 does have affinity for RNA. Originally, it was claimed that eIF-2 specifically interacts with the cap structure of mRNA (132).

This was based on the finding that eIF-2 could specifically retain labeled capped mRNA on a nitrocellulose filter. This assay however did not unequivocally identify the cap binding component of eIF-2. Furthermore, there are nonspecific effects of cap analogues in the filter binding assay as evidenced by the fact that m^7GMP also blocks the binding of E. coli RNA polymerase to 18S ribosomal RNA (133). In subsequent experiments it has been shown that eIF-2 can be cross-linked to oxidized capped mRNA but that the interaction is not cap specific (134). This, along with the fact that eIF-2 binds to uncapped mRNA (135) indicates that eIF-2 is not a cap binding protein. It has been reported that eIF-2 has a high affinity for mRNA, as opposed to tRNA, rRNA and negative strand RNA (136-138) which points to a direct function in mRNA recognition. It is also pertinent that eIF-2 can apparently relieve translational competition between α and β globin mRNAs (139) and thus acts as a mRNA discriminatory factor. In summary, eIF-2 is most likely directly involved in mRNA binding to ribosomes although the way in which it acts is not clear. (For a review see reference 135)

eIF-3

eIF-3 is a very large entity indeed, being comprised of about ten polypeptides with a sedimentation coefficient of ~ 16S and a corresponding molecular mass of 500-700 kDa (44,51,140). Preparations of eIF-3 are heterogeneous with respect to polypeptide composition and consequently the native structure of eIF-3 is not precisely known. Furthermore, some eIF-3 subunits may be related to one another as indicated by comparative peptide mapping (51). This might result from proteolysis of larger subunits during purification and may also account for the heterogeneity of eIF-3 particles.

Again, because eIF-3 is involved in steps prior to mRNA binding it is difficult to assess its role here. eIF-3 is found in stoichiometric amounts on the 43S preinitiation complex (43,54) and several of its subunits can be chemically cross-linked to oxidized capped mRNAs (141). In the latter experiments the mRNA was 5' end labeled and three of the labeled eIF-3 bands comigrated with polypeptides labeled in the same way in 40S initiation complexes. These data suggest that eIF-3 and the 5' end of mRNA are closely opposed in initiation complexes (141). Several pieces of evidence indicate that eIF-3 is also associated with a cap recognition factor under physiological conditions (100 mM salt) as will be described fully in a later section (134,142). Finally, the formation of 48S preinitiation complexes shows a very strong dependence on eIF-3 (128) although again this probably reflects the involvement of eIF-3 in steps other than mRNA binding to the 43S preinitiation complex. The sum of these observations indicate the importance of eIF-3 in formation of 48S preinitiaton complexes and, most probably, in the partial reaction whereby the 43S preinitiation complex binds mRNA. The mechanism of action and the assignment of functions to eIF-3 subunits remain to be worked out.

eIF-4B

eIF-4B was purified by several groups to varying degrees of homogeneity, the major constituent being an 80K polypeptides (41,43,143). Among the activities attributed to these partially purified eIF-4B preparations are (1) cap recognition (144), (2) mRNA discrimination (145), (3) restoration of capped mRNA function in poliovirus-infected HeLa cells (146) and, (4) ATP-dependent binding of mRNA to ribosomes (147). This factor was very difficult to obtain pure, mainly due to

some form of contaminating cap binding protein (134), which made it difficult to evaluate which activities were due to the 80K polypeptide and which were due to cap binding protein. This problem was recently solved by Grifo et al. (128) who successfully resolved the 80 kDa polypeptide and the cap binding protein and demonstrated that the 80 kDa polypeptide is required for maximal binding of mRNA to 43S preinitiation complexes (128). Thus, the role of eIF-4B in mRNA binding is established while the other activities previously attributed to eIF-4B need to be re-examined in order to assess the contribution of the 80 kDa polypeptide. The role of eIF-4B in the cap recognition process and in the poliovirus induced shut-off of cellular protein synthesis are discussed throughout this thesis.

Cap binding proteins (eIF-4E and eIF-4F).

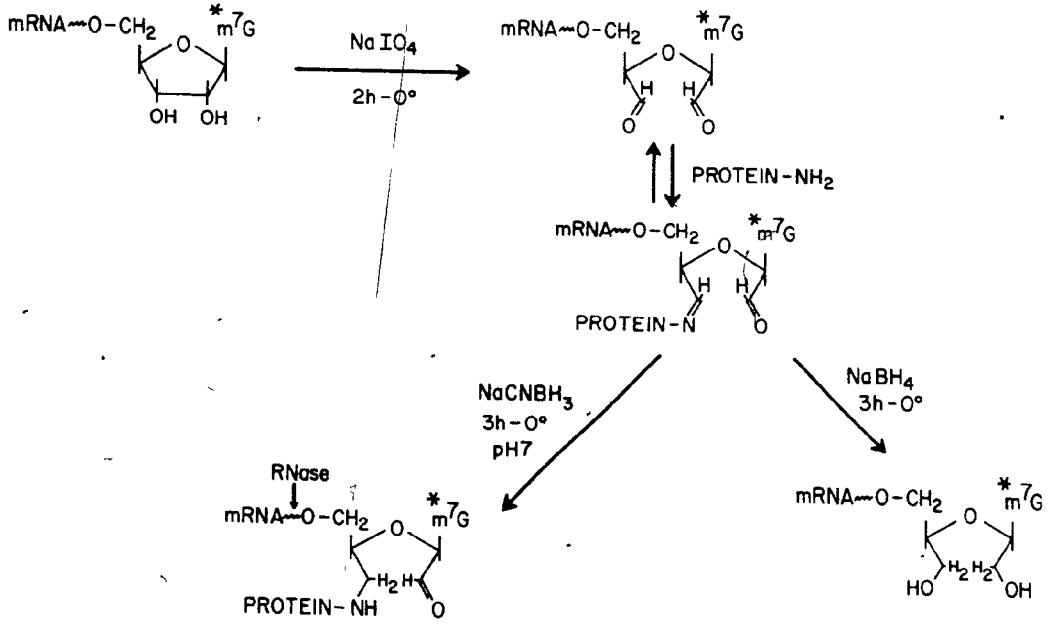
Following the discovery that the cap structure plays a significant role in the translation of eucaryotic mRNAs, it was reasoned that cap function would be mediated via recognition by cap binding proteins. Originally, filter binding assays were employed to ask whether individual initiation factors could retain radiolabeled mRNAs in a manner which was sensitive to inhibition by cap analogues. Using this approach, it was claimed that eIF-2 (132) and eIF-4B (144) specifically interacted with the cap structure. However, in the case of eIF-2, the limitations of this assay are alluded to earlier (see page 19) and the same cautions apply to eIF-4B (133).

A direct approach to identify proteins that bind at or near the cap was developed by Sonenberg and Shatkin (148) and is depicted in Figure 4. Periodate oxidized [^{3}H]methyl-labeled reovirus mRNA can be chemically cross-linked to proteins. Schiff base formation occurs between the

FIGURE 4

The Chemical Crosslinking Assay For
Detecting Cap Binding Proteins

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



reactive dialdehyde (in the 2 and 3 positions of the ribose moiety of the cap structure) and primary amino groups (N-terminal or ε-NH_2 in lysine) of proteins. These relatively unstable complexes can be stabilized by reduction with sodium cyanoborohydride ($NaBH_3CN$). Cross-linked mRNA protein complexes can then be treated exhaustively with ribonuclease (RNase) to degrade all but the cap portion of the mRNA and cap labeled polypeptides can be resolved and visualized by sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis and fluorography. Under these conditions, many polypeptides present in crude initiation factors are labeled. In order to assess cap specific labeling, incubations are performed in the presence or absence of cap analogues (usually m^7GDP) and cap specific cross-linking is defined by inhibition in the presence of competing cap analogue.

Using this assay, Sonenberg et al. (134) identified a 24 kDa poly peptide (24K-CBP) in the ribosomal high salt wash of rabbit reticulocyte ribosomes which specifically interacts with the cap structure. A polypeptide which comigrates in SDS/polyacrylamide gels and with identical cross-linking characteristics has subsequently been detected in initiation factors from a variety of mammalian sources (134,120,149). Cross-linking experiments with purified initiation factors indicated that the 24K-CBP associates with eIF-3 and cosediments with eIF-3 in sucrose gradients, under low salt conditions (134,155). Similarly, the 24K-CBP is generally detected in eIF-4B preparations and, as mentioned earlier, is somewhat difficult to remove. The functional significance of these associations, if any, remains unclear. Addition of ATP/Mg^{++} to the cross-linking incubation using crude initiation factors (IF), results in cap specific labeling of polypeptides of 28, 50 and 80 kDa (150). The

identity of these polypeptides in relation to eucaryotic initiation factors and the ATP/Mg^{++} requirement for cross-linking, are questions addressed in chapters 4 and 5 of this thesis.

The 24K-CBP polypeptide has been purified to near homogeneity by using m^7GDP coupled to various affinity resins (151-153). This polypeptide was called the 24K-cap binding protein (24K-CBP), CBPI (119) or eIF-4E. Purified 24K-CBP has been shown to stimulate translation of capped mRNAs in extracts from HeLa cells, but has no effect on translation of naturally uncapped mRNAs (154). Because cap recognition is important in the formation of 48S preinitiation complexes, it is inferred that the 24K-CBP functions during this step. However, it has not been possible to demonstrate this in a reconstituted system to date, most probably due to the fact that other factors (eIF-3 and eIF-4B) contributed saturating amounts of the 24K-CBP as a contaminant.

The 24K-CBP is also associated with a high molecular weight protein complex (156,119,128,155,157). This complex was first isolated by Tahara et al. (119) using the m^7GDP affinity chromatography technique and comprised the 24K-CBP and major polypeptides of 48, 55 and 225 kDa. A similar protein complex was subsequently purified by two other groups (128, 157). This complex is functionally different from the free 24K-CBP in that it can restore translation of capped mRNAs in extracts from poliovirus-infected cells (119). Furthermore, in contrast to the 24K-CBP, it has been shown to be required for translation in a reconstituted protein synthesis system and for maximal binding of mRNA to ribosomes (128). The complex has been called CBPII (119), the CBP complex (157) or eIF-4F (128). A major goal of this thesis was to characterize the CBP complex purified in our laboratory (157), in terms of its poly

peptide composition, its interaction with mRNA and its role in the poliovirus-induced shut-off of cellular protein synthesis.

eIF-4A

The role of eIF-4A in initiation was something of an enigma for many years. It purifies as a single polypeptide of molecular weight ~ 45 kDa (147) and is required for maximal binding of mRNA to ribosomes (43,130, 147). eIF-4A is an acidic protein (pI = 6.1 (147)) and is reported to be heterogeneously glycosylated (157a). It does not appear to bind mRNA directly, and attempts to show that eIF-4A is part of the 48S preinitiation complex have thus far been unsuccessful (43). However, there is evidence that eIF-4A is involved in mRNA recognition. Originally eIF-4A was named the 'EMC factor' because it appeared to preferentially stimulate translation of encephalomyocarditis virus (EMCV) RNA (158). In addition, eIF-4A was reported to have mRNA discriminatory activity, although the effects observed were not that large (159,145,160). Recently, it has been demonstrated that eIF-4A is a component of the CBP complex (128, 157) and has a role in mRNA cap recognition. Only a small proportion of total eIF-4A is present in the CBP complex however, and there are conflicting data relating to whether the free eIF-4A (which represents the bulk of eIF-4A) is required for initiation. The results presented in this thesis along with others (referred to in the general discussion in chapter 8), serve to further characterize the role of eIF-4A in mRNA binding to ribosomes. The situation, however, remains far from clear.

Other protein factors which are involved in mRNA binding.

eIF-1 consists of a single polypeptide of 15 kDa and is required for maximal binding of mRNA to the 43S preinitiation complex, although the

dependence is not strong (130,131,41). Its role is unknown, although it has been suggested that it might stabilize the interaction between Met-tRNA$_i$ and the AUG codon (161). eIF-2A is also found on the 40S ribosome although its function is unknown (162).

(d) Repositioning of the 40S ribosome on the mRNA.

The model for the binding of ribosomes to eucaryotic mRNAs which best fits the available evidence, is the 'scanning mechanism' proposed by Kozak (for a review see 67). The general postulates of the scanning mechanism are as follows (163-166):

1. A 40S subunit binds at the 5' terminus of the mRNA
2. The 40 S subunit then repositions at the AUG, probably by migrating along the template
3. A 60S subunit joins to form the 80S initiation complex.

The evidence which supports a 5' terminal 'entry site' for the 40S ribosome was mentioned in an earlier section (see page 12). Furthermore, since in some cases the initiator AUG codon is much further from the 5' end of the mRNA than the distance occupied by one 40S ribosome, it follows that the AUG is not part of the initial recognition site for the ribosome. Consequently, 40S subunits should be able to bind to RNA molecules lacking an AUG. This prediction has been experimentally verified (167).

The scanning model offers a good explanation as to how initiation of translation is (generally) restricted to the 5' proximal AUG, thus rendering eucaryotic mRNAs functionally monocistronic. There is now some very elegant genetic evidence that the functional AUG in a mRNA is defined by its position relative to the 5' end of the mRNA. Kozak constructed mutants of the preproinsulin gene which contained copies of

the initiation site (AUG plus flanking nucleotides) inserted upstream of the AUG of the wild type gene. In each case, initiation of translation started at the AUG nearest the 5' end of the mRNA, in vivo (168). Similar results have been obtained by another group (169). Exceptions to the scanning mechanism are addressed in the general discussion of this thesis, in light of more recent data.

The scanning mechanism clearly requires that 40S ribosomes can migrate along the mRNA. In order to demonstrate this convincingly it was necessary to use conditions under which the migration of 40S subunits is not arrested at the AUG. This can be achieved (among other ways (74,170)) by using the drug edeine (171) which prevents 60S subunits binding and somehow impairs recognition of the AUG by the 40S subunit. In the presence of edeine and ATP as energy source, 40S ribosomes can protect the ^{32}P-labeled 3' PolyA tail from digestion by nuclease. In the absence of ATP there is no protection of the 3' polyA tail, and so it seems that ATP allows migration of 40S subunits along the mRNA. Further evidence to support this view comes from the fact that in the absence of ATP, a single 40S subunit is found near the 5' end of denatured mRNA (164).

(e) Formation of 80S initiation complexes.

The formation of 80S initiation complexes occurs rapidly after 48S preinitiation complexes, indicated by the fact that the 48S complexes cannot be detected under conditions in which 60S subunits can bind (172). The point at which 60S subunits bind is not entirely clear. The scanning mechanism suggests that 60S subunits do not bind until the 40S subunit arrives at the AUG. However, if 80S complexes are arrested at the AUG by using an inhibitor of elongation, then there is evidence that a second

80S ribosome can bind to mRNA (173-175). The pertinent question which arises is whether the second 80S ribosome is a potentially functional initiation complex or whether it represents an abortive complex produced under the prevailing assay conditions. This remains in doubt at present.

Formation of 80S complexes is readily followed by a model assay involving methionyl-puromycin synthesis. Using this assay, it has been shown that eIF-5 is required for junction of 60S subunits with the 48S preinitiation complex, and that eIF-2, eIF-3 and eIF-4C are released for recycling (176). This step requires GTP hydrolysis and it is the GTP which entered in the ternary complex which is utilized (177). eIF-2 is released, most probably, as an inactive eIF-2-GDP complex which requires a recycling factor to remove the GDP. This requirement was anticipated in light of the fact that GDP has a 100-fold higher affinity for eIF-2 than GTP (178). The recycling factor has now been identified and plays a central role in regulation of eIF-2 activity (179,180).

(f) Requirement for ATP.

The need for ATP as an energy source is a salient feature of the mechanism of translation initiation in eucaryotes, compared to prokaryotes (181,130,43). ATP hydrolysis is required for binding of the 43S preinitiation complex to mRNA and appears to be required for repositioning of the 40S ribosome between the 5' terminus and the initiator AUG codon (163, 164). The reason for the ATP requirement is not yet clear although it appears not to lie in the need to phosphorylate initiation factors but is strictly an energy source (182).

Many observations point to the possibility that ATP hydrolysis and the function of the cap structure are related. This possibility is explored during the course of this thesis (chapters 4 and 5). First, it

was found that denatured mRNAs are less dependent on both the cap structure and ATP for initiation complex formation (183,74,164). Second, Jackson has reported that naturally uncapped mRNAs (cowpea mosaic virus (CMV) RNA and EMCV RNA) are much less dependent on ATP than capped mRNAs, for initiation complex formation (184). Lastly, cap specific mRNA recognition by polypeptides of 50 and 80 kDa present in crude initiation factors was shown to be dependent on ATP/Mg^{++} (150). These results prompted several of the experiments described in this thesis and, along with other recent developments are discussed in chapter 8.

1.4 Regulation of Gene Expression at the Translational Level

1.4.1 General

It is currently believed that expression of eucaryotic structural genes is largely regulated at the transcriptional level. This is consistent with the economy in the molecular logic of cells, which aims at regulating anabolic pathways near their beginning and thus conserving energy and metabolites. In eucaryotes however, transcription is well separated both temporally and spatially from translation, and cytoplasmic mRNAs are in general, very stable (184a). Thus, there are many steps which might be amenable to regulation and moreover, economical use of pre-existing mRNAs is a likely end of translational control. In accord with this situation, there is increasing evidence that post-transcriptional events are significantly regulated. For example, differential splicing of primary transcripts or use of alternative transcriptional promoters, has been documented in many cases, thus allowing expression of different cytoplasmic mRNA sequences from the same gene (185-188,357,370). In some cases, differential splicing will give rise to different

protein coding sequences, whereas in others, only the non-coding region of the mRNA is affected (187). While there is no precedent at present, it remains entirely likely that production of different mRNAs by differential splicing would allow translational control of the gene, either by altering the intrinsic translational efficiency of the mRNA or by deleting (including) sequences required for response to a trans-acting regulatory molecule.

Translational control has now been demonstrated in many cases. Prime examples are: (1) shut-off of cellular protein synthesis during infection by many eucaryotic viruses (222); (2) during heat shock (206); (3) during the cell cycle (223,223a); (4) following fertilization in oocytes (212) and (5); following administration of hormones or changes in nutritional state of animals or tissue culture cells (224,224a). Translational control might also be of particular importance in allowing the cell a rapid response to changes in its environment. Such responses can either be of the type in which protein synthesis is quantitatively inhibited or, can involve preferential translation of certain mRNAs. The classic example of quantitative translational control occurs in rabbit reticulocytes in which case, phosphorylation of eIF-2 brings about a very rapid shut-off of all protein synthesis (189,190,225,226). Although protein kinases which phosphorylate eIF-2 are present in many cell types other than reticulocytes, their regulatory significance in these cell types remains to be demonstrated. Thus, the general significance of eIF-2 phosphorylation in nucleated cells is not yet established. The second kind of response which involves discrimination between different mRNAs has been reported in many instances, although the mechanisms are only just becoming apparent. Two factors though which can clearly

contribute to mRNA discrimination are: (i) the intrinsic translational efficiencies of particular mRNAs and (ii) the activities which are responsible for mRNA binding to ribosomes. Because these topics are major concerns of this thesis, I will describe in some detail the current evidence relating to mRNA selection phenomena. The description is by no means exhaustive but hopefully will include examples which appear to be of general significance.

1.4.2 Mechanisms of mRNA selection

The fact that different mRNAs are translated at different rates in eucaryotic cells is established (191-193). In general, initiation of translation is rate limiting and binding of the 40S ribosomal subunit to mRNA is the most likely rate limiting step (191-193, 172). Thus, the rate at which a particular mRNA is translated is dictated largely by its affinity for some factor(s) which is required for mRNA binding to ribosomes. This factor must have two properties in order that it might affect translation of particular mRNAs to different degrees. First, it must be subsaturating relative to the total mRNA such that mRNAs must compete for it. Second, it must bind to different mRNAs with different affinities (i.e. exhibit mRNA discrimination). Under these conditions, mRNAs with high affinity for the discrimination factor will be translated at the expense of those with lower affinity. This has become known as the competition model. Kinetic studies have established that such a discriminatory factor exists (194,196) and a possible mechanism for its action is discussed in chapter 8 of this thesis.

The competition model is able to explain some examples of translational regulation, mainly during viral infection and cellular growth control (194-197). However, the significance of this competition

mechanism to more routine cellular events remains to be determined. Indeed, it seems unlikely that mRNA competition will account for all of the qualitative changes in translation. In prokaryotes there are many examples of specific repression of translation by diffusable factors. Such examples include the T4 gene 32 and reg A proteins (198,199) and several ribosomal proteins of E. coli (200,201). These proteins turn off their own synthesis by acting as translational repressors. Examples of this kind of mechanism are less well documented in eucaryotes but are reported to occur for the yeast ribosomal proteins (202) collagen (203,204) and apoferritin (205). Another interesting possibility for which there is some preliminary evidence, is that small RNA species are important in regulating translation in either a non-specific or a highly sequence specific and hence mRNA discriminatory manner (227-231). For example, an inhibitory RNP particle containing a small RNA species appears to be involved in translational control during muscle differentiation (232). In addition, it has been reported that vaccinia virus RNA transcripts inhibit cellular (globin, HeLa) mRNA translation in vitro but have no effect on translation of vaccinia virus mRNA (233). In summary while the existence of specific translational repressors is a very attractive mechanism for regulating expression, their general significance in allowing regulation of translation by mRNA selection in eucaryotes remains to be determined.

Other examples of qualitative translational control occur in which mRNA competition seems not to play a role, and mRNA specific factors might be important. For example, during the heat shock response, the bulk of cellular protein synthesis is inhibited while a small number of heat shock mRNAs are preferentially translated (206,207). This kind of

response is observed in many systems, notably Drosophila (206,207), Xenopus oocytes (208) and HeLa cells (209). When Xenopus oocytes are allowed to recover from heat shock, the translation of heat shock mRNAs persists while the overall protein synthesis rate increases back to normal (208). This clearly indicates that the translation of other cellular mRNAs does not competitively inhibit translation of heat shock mRNAs, thus raising the possibility that mRNA specific factors are responsible for allowing translation of heat shock mRNAs. Consistent with this idea, cell extracts from heat-shocked cells will translate heat shock mRNA but not 'normal' (non heat shock) mRNA (207) and addition of ribosomes from normal cells can restore translation of normal mRNAs These results suggest that heat shock inactivates a factor which is not required for translation of heat shock mRNAs.

Finally, on the topic of mRNA selection, it is important to consider that a proportion of mRNA in all cells exists as untranslated cytoplasmic mRNP particles (210-212). For example, sea urchin oocytes contain a store of untranslated maternal mRNA, which becomes translationally active following fertilization (or after piercing the oocyte with a needle!) and functions during early development (213,214). This phenomenon appears to be quite general during early development, being found in starfish (215) through mammals (216) on the evolutionary scale. However, the mechanism underlying mobilization of mRNA into polysomes are unknown. mRNA extracted from cytoplasmic mRNP particles is active in cell free translation systems (217) which has led to the supposition that the proteins present in cytoplasmic mRNP particles act as 'masking agents', either specifically or non-specifically (218,219). The fact that the types of mRNA in cytoplasmic mRNAs (untranslated) and polysomal mRNPs (translated)

are different is also taken as support for this view. It should be noted however, that the intrinsic translational efficiency of particular mRNAs will result in an equilibrium distribution, which will influence their relative abundance in untranslated mRNPs versus polysomal mRNPs. Notwithstanding this caution though, the stage-specific translation of mRNAs in developing embryos (220) and the fact that some mRNAs are clearly not in equilibrium between untranslated mRNPs and polysomal mRNPs (22) strongly suggest that repression of translation by factors present in untranslated mRNP is an important regulatory mechanism. Recent evidence to support this contention comes from in vitro reconstitution experiments in which oocyte specific proteins from Xenopus have been shown to reversibly inhibit tranlation (235).

1.5 Poliovirus-induced Inhibition of HeLa Cell Protein Synthesis

1.5.1 The significance of the system

The replication strategies of many eucaryotic viruses have thrown considerable light on the mechanism of expression of eucaryotic structural genes. In general, this most probably stems from the requirement of viruses to effectively compete for the host cells capacity to synthesize macromolecules (DNA, RNA and proteins) and hence to redirect this capacity toward production of new virus. In many cases, there is a negative pleiotropic response of the host cell to virus infection, which is viewed as an attempt by the cell to inhibit viral replication by shutting down its own normal functions (383-385). However, there is abundant evidence that eucaryotic viruses have tended to acquire genes which are expressed with very high efficiency and in many cases, by a route which is uncommon and hence difficult to detect in the uninfected

cell. For example, some of the most efficient promoters of transcription are found in retroviruses (236), a transcriptional enhancer sequence was first identified in SV40 (237), differential splicing of pre-mRNA was first observed in adenovirus and SV40 (238,239) and last, viral mRNAs are among the most efficiently translated mRNAs (240). Thus, while the expression of viral genes generally occurs via the same mechanism as cellular genes, there is an efficiency and economy in the expression of viral genomes which has proven invaluable in identifying structural elements of genes involved in directing expression. Moreover, many seemingly novel modes of expression were first discovered during expression of viral genes. Indeed, when disparagingly referring to viruses as 'parasites', we might ponder the likely extent of our knowledge of eucaryotic gene expression without them.

In many cases, infection by eucaryotic viruses drastically inhibits cellular protein synthesis, while translation of viral mRNA occurs with high efficiency. There appear to be many different routes by which this phenomenon (known as "shut-off") occurs and it was a major goal of this thesis to elucidate the mechanism in the case of poliovirus.

While the shut-off of host protein synthesis may well be of utmost significance to the 'survival instincts' of poliovirus, this author's bias lies towards the translational control which occurs in infected cells and how this phenomenon can inform on the fascinating topic of translation initiation. Translational control in poliovirus-infected HeLa cells exhibits some notable characteristics. First, the site of inhibition of translation, is the step in which the 43S preinitiation complex binds cellular mRNAs. Thus a mRNA selection process is imposed, in which only viral mRNA is efficiently translated. Second, the mRNA binding step is

the least well understood partial reaction in the initiation pathway. Poliovirus inactivates one of the factors involved in this step, and thus provides a relatively simple assay for the factor. Third, there is a very discreet structural basis for the selection of viral mRNA (naturally uncapped) over cellular mRNA (capped) for translation. Fourth, the translational specificity seen *in vivo*, is faithfully mimicked *in vitro*. That is, extracts from poliovirus-infected cells can translate viral mRNA but cannot translate cellular (capped) mRNA. Consequently, the *in vitro* system can be employed to study questions related to the mechanism of shut-off and also to the mechanism of translation for cellular mRNAs. In a sense, extracts from infected cells might be considered to come from cells carrying a mutation in the translation initiation machinery. In view of the lack of eucaryotic mutants in protein synthesis (perhaps for obvious reasons!) this highlights the significance of poliovirus-infection, in studying translation initiation.

1.5.2 Structure of poliovirus

(a) Classification

Poliovirus belongs to the group of mammalian picornaviruses (*pico* = small, *rna* containing) which can be classified according to the scheme shown in Table 2 (copied from reference 241).

(b) Structure of the virion

The genome

The poliovirus genome consists of a single stranded RNA molecule which serves as mRNA in the infected cell and has a molecular weight of approximately 2.6×10^6 daltons (7,433 nucleotides). The RNA contains approximately equal numbers of the different ribonucleotides and has recently been sequenced (242-243). It is polyadenylated at the 3' end

TABLE 2

Vertebrate Picornaviruses

Genus Enterovirus:	Polio (3 serotypes) Coxsackie A (23) Coxsackie B (6) Echo (31) Enteroviruses of mice, swine, cattle Enterovirus 70	Sedimentation coefficient ~155S Buoyant density ($CsCl$) ~1.34g/ml Virions stable at pH 3-10 Empty capsids produced *in vivo*
Genus cardiovirus:	EMC ME Mengo Columbia-SK MM	Sedimentation coefficient ~155S Buoyant density ~1.34g/ml Virions labile pH 5-7 in the presence of 0.1M Cl^- or Br^- No empty capsids *in vivo*
Genus Rhinovirus:	More than 120 serotypes	Sedimentation coefficient ~155S Buoyant density ~1.40g/ml Virions labile pH 5 Empty capsids produced *in vivo*
Genus Aphtoviurs:	Foot-and-Mouth Disease Virus, 7 serotypes	Sedimentation coefficient ~145S Buoyant density ~1.43g/ml Virions labile pH 6.5 Empty capsids produced *in vivo*

(244,245) and terminated with pUp at the 5'end (246,247). In virions, a small protein known as VPg is covalently attached to the 5' terminus (248). In contrast to some other picornavirus RNAs (249,250) there is not a poly C tract near the 5' end of the molecule.

The capsid

The poliovirus capsid consists of four capsid proteins, designated VP1-4, of molecular mass 35, 28, 24 and 5.5 kDa, respectively. They are arranged in 60 groups to form the capsid, each group comprising an equimolar amount of the four polypeptides (251,252). The overall shape of the capsid is spherical but actually possesses icosahedral symmetry. In addition to the four capsid polypeptides, the virion might also contain an uncleaved precursor in which the VP2 and VP4 amino acid sequences are still covalently linked (241). The arrangement of individual capsid proteins in the virion is not entirely clear. VP1 appears to be the major antigenic determinant but there is also evidence that VP2 and VP3 are able to elicit production of neutralizing antibody (253). This would suggest that these polypeptides are all surface components of the virion.

(c) Genetic map of poliovirus

During the course of the work described in this thesis, there was a change in picornavirus protein nomenclature. The old nomenclature is used throughout this thesis. Figure 5 shows a map of the poliovirus genome (copied from reference 292). Table 3 shows both the old and new nomenclatures together with the known functions and molecular weights of poliovirus proteins.

FIGURE 5

Processing map of the polioviral polyprotein. The polyprotein (heavy line) is divided into three regions (P1, P2, and P3) for convenience in classiying cleavage products. Amino acid pairs (sites) known to be cleaved are indicated by filled symbols; apparently uncleaved sites are indicated by open symbols; (▽, ▽) glutamine-glycine (QG); (o,•) tyrosine-glycine (YG); (◇ ◆) asparagine-serine (NS). The glutamine-glycine sites are all believed to be cleaved by P3-7C, a virus-encoded coded protease. The agents responsible for cleavage of sites NS-2, YG-6, and YG-8 have not yet been identified . Assignments for proteins 7a and 1c are tentative. (■) VPg.

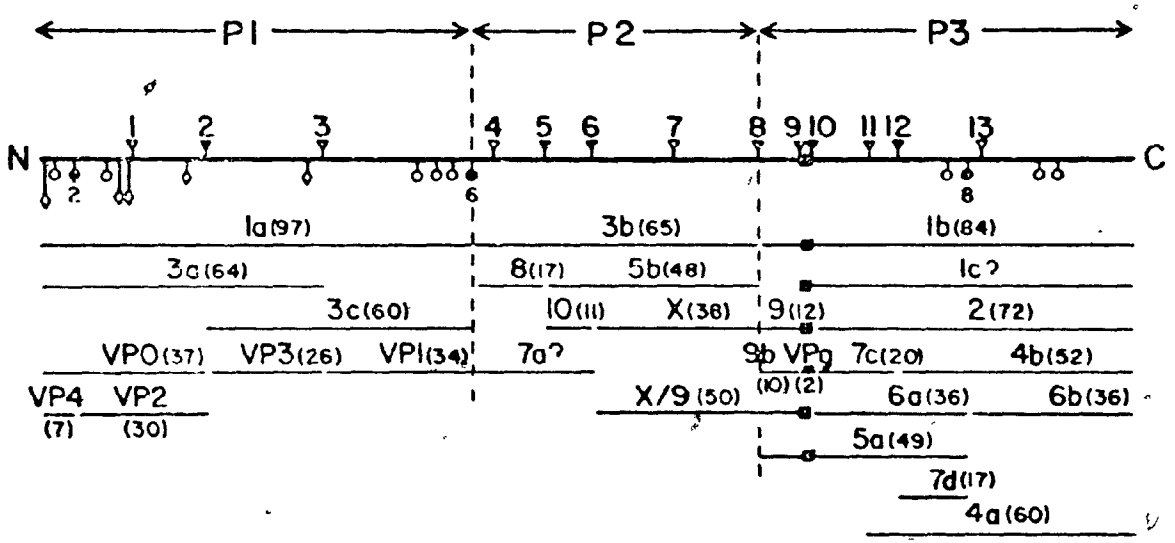

P1
P2
P3
N
C
1
2
3
4
5
6
7
8
9
10
11
12
13
1a(97)
3b(65)
1b(84)
3a(64)
8(17)
5b(48)
1c?
3c(60)
10(11)
X(38)
9(12)
2(72)
VP0(37)
VP3(26)
VP1(34)
7a?
9b
VPg
(10)
(2)
7c(20)
4b(52)
VP4
(7)
VP2
(30)
X/9(50)
6a(36)
6b(36)
5a(49)
7d(17)
4a(60)

TABLE 3

Protein Function and Nomenclature

PROTEIN			
Old System	New System	Mol. Wt.	FUNCTION
P1-1a	1	97,247	Capsid Precursor
P3-1b	3	84,234	
3b/9	2-3AB	77,000	
P3-1c	3BCD	76,000	
P3-2	3CD	72,132	
P1-3a	1ABC	63,786	
P2-3b	2	64,953	
P1-3c	1CD	59,930	
P3-4a		60,000	
P3-4b	3D	52,481	Replicase
X/9	2C-3AB	50,000	
P3-5a		48,550	
P2-5b	2BC	48,273	Replication Complex Formation
VP0	1AB	37,352	Capsid Precursor
P3-6a	3C'	36,450	
P2-X	2C	37,555	
VP1	1D	33,521	Capsid Protein
P3-6b	3D'	35,700	
VP2	1B	29,985	Capsid Protein
VP3	1C	26,410	Capsid Protein
P2-7a	2AB	25,500	
P3-7c	3C	19,669	Proteolytic Processing
P3-7d		16,780	
P2-8	2A	16,680	
P3-9	3AB	12,100	
P3-9b	3B	9,750	
P2-10	2B	10,720	
VP4	10	7,385	Capsid Protein
VPg	3B	2,354	Replication

1.5.3 The replicative cycle

(a) Adsorption and penetration

The initial virus cell interaction is mediated by viral capsid proteins and specific receptors which probably determine the host range and tissue tropism of poliovirus (254,255). The involvement of viral capsid proteins is elegantly indicated by the fact that infection of cells susceptible to Coxsackie virus but not to poliovirus with a virus comprising poliovirus RNA and Coxsackie capsid components, yields high levels of wild type poliovirus (256). Interaction between the cell and viral capsid appears to modify the virus, because the infectivity of previously adsorbed virus is markedly reduced (257). The modified virus particle has altered antigenic properties and is partially uncoated as indicated by increased sensitivity of the RNA to RNAses (257). In vitro experiments indicate that plasma membrane but not microsomal fractions from the host cell have the ability to effect this modification to virions, thus suggesting a role for membranes in the uncoating process. The natural target cells for poliovirus are in the gut and the central nervous system and yet for practical purposes the life cycle of poliovirus has generally been described in HeLa cells. In view of the fact that replication of poliovirus is not greatly dependent on specialized host cell functions (to be described later in this section) beyond the requirement for receptors, one might expect replication of the virus to be similar in natural target cells. It should be borne in mind however, that this has not been directly examined.

(b) Expression of the viral genome

The available evidence suggests that translation of viral RNA is the first viral function occurring in the infected cell and that production

of viral proteins (even if only in small amounts) is absolutely required to initiate the infectious cycle. Thus, while the most obvious effect on the cells is the abrupt shut-off of protein synthesis long before the detectable appearance of viral proteins, synthesis of the latter actually precedes, and is required for the shut-off.

The input virion harbours an RNA template which serves directly as mRNA in the infected cell (258-260). The mRNA does not have the covalently attached VPg at the 5' end, since polysomal RNA from infected cells is lacking VPg (246,247). Moreover, while the presence of VPg does not prevent translation of poliovirus RNA in vitro (260), it is clearly not required for translation. This rules out the possibility that VPg somehow substitutes for the cap structure in poliovirus RNA, as had been speculated. The mechanism by which poliovirus RNA is translated is not clear and is addressed in chapter 8. Of particular significance is the fact that the major translation initiation site is 743 nucleotides from the 5' end of the RNA (an extremely long distance for a eucaryotic mRNA) and, there are no less than 8 other potential initiator AUGs in this region. The AUG at position 743 from the 5' end is followed by a long open reading frame which encodes most (if not all) of the poliovirus proteins. The polyprotein encoded by this region is ~ 240 kDa, although this product never appears in vivo because it is proteolytically cleaved co-translationally. It can be observed in infected cells if processing is inhibited by using amino acid analogues (262,263). Recently, RNA and protein sequence data have confirmed the initiation site for synthesis of the polyprotein and it is established that all of the poliovirus proteins are derived from the polyprotein by proteolysis (242,264,265). A major unanswered question is whether or not there are other cistrons in polio-

virus RNA. Under certain conditions, in vitro, there appears to be more than one initiation site for translation. By varying the Mg^{2+} ion concentration it was shown that a small polypeptide (5-10 kDa) is synthesized from a unique initiation site (266-267). This site has not been mapped, but it might be one of AUGs 5' to nucleotide 743. Of the 8 AUGs present in this region, 6 are very closely followed by in-phase translation termination signals but the other two are followed by sufficient coding capacity for a small polypeptide. In other experiments, it has been claimed that there is another translation initiation site in vitro which maps in the P3 region (288). Initiation from this site is apparently abolished in the presence of viral components which suggests that it may have some significance in vivo (288). In summary, while the major translation unit of the poliovirus genome is well defined, the mechanism of initiation of translation and the possible existence of additional cistrons are still in question.

(c) Processing of poliovirus proteins

All of the known viral proteins are produced by proteolytic cleavage of the same polyprotein precursor. This process is most probably dependent on both cellular and viral proteases and most of the cleavages occur between glutamine-glycine amino acid pairs (289,265,242,290-292). All of the glutamine-glycine cleavages are carried out by viral protein P3-7C, as indicated by the inhibitory effect of anti-P3-7C anti-sera (293). Other cleavages occur at tyrosine-glycine pairs (2) (291 and 293) and one at an asparagine-serine pair. The protease(s) involved in these latter cleavages are not yet identified and they might be carried out by a viral or cellular protein (294,295,243,293).

Cleavage of the polyprotein to yield the primary cleavage products occurs cotranslationally, which raises many interesting questions. Firstly this led to the suggestion that a cellular protease might be involved although there is no conclusive evidence to support this possibility directly at present. It is suggestive however, that extracts from uninfected HeLa cells are able to cleave the poliovirus polyprotein into polypeptides with the size and antigenicity of the authentic primary cleavage products (294-296). Secondly, because P3-7C is involved in primary cleavage events but is not part of the input virion, it must be able to act in cis, i.e. autocatalytically. There is now good evidence that this is the case. P3-7C sequences have been cloned in an E. coli expression system which produces a P3-7C/E. coli fusion protein. This fusion protein is efficiently cleaved to produce authentic P3-7C and mutations in the P3-7C gene abolish this cleavage event (297).

Major unanswered questions concern (i) the identity of the protease responsible for cleavages not involving glutamine-glycine amino acid pairs and (ii) the additional signals required for cleavage at glutamine-glycine pairs, because the dipeptide by itself is not sufficient to direct cleavage by P3-7C (297).

(d) Effects on host cell functions

Expression of the viral genome, including proteolytic processing of viral precursor proteins, allows poliovirus to inhibit host cell macromolecular synthesis. In the case of poliovirus, the shut-off of cellular protein synthesis is extraordinarily rapid compared to other picornaviruses and is reviewed in the latter part of this introduction.

Inhibition of cellular RNA synthesis approximately parallels inhibition of protein synthesis (300,301) but there appears to be

different effects on polymerase I (ribosomal RNA), polymerase II (mRNA) and polymerase III (5S ribosomal RNA and tRNA). Studies using isolated nuclei (298) or whole cell transcription extracts (299) have indicated that transcription by RNA polymerase II is impaired following poliovirus infection and polymerase I is inhibited to a lesser degree. RNA polymerase II itself appears to be unaltered in poliovirus-infected cells (302,303), and polymerase II transcription can be restored in extracts from poliovirus-infected cells by addition of a component(s) present in the soluble fraction of uninfected cells. This component might be one of the transcription factors identified by Matsui et al. (304) but this has not been established yet. The mechanism by which RNA polymerase II transcription is inhibited is therefore not particularly clear, although it does require a functional viral genome (306) and is presumably mediated by a poliovirus protein in the nucleus of infected cells.

At later times during infection, cellular DNA synthesis is inhibited and this is thought to occur as a secondary effect of the inhibition of cellular protein synthesis. This is indicated by the fact that DNA synthesis in uninfected cells treated with protein synthesis inhibitors is affected in the same way as in infected cells (307).

(e) Replication

This topic has been one of intensive investigation and is reviewed by R. Perez-Bercoff (308). Consequently, I shall describe the main features and some recent developments.

The template for the initial event in replication is the viral mRNA. From this, a minus strand is synthesized in the 5'→ 3' direction by a virally encoded polymerase (309). This process most probably requires VPg as a primer for transcription (310,311), ATP hydrolysis (312) and a

host factor (313). Following synthesis of the minus strand, which is fully complementary to the mRNA, the minus strand is transcribed by the same mechanism to produce new mRNA, which can either enter polysomes or be assembled into virions. The whole process appears to occur in so called 'replication complexes' which can be isolated from the cytoplasm of infected cells and are membrane bound (314,315). The vast majority of viral RNA in the cell at any one time is mRNA which means that the replication process is asymmetric. The mechanism by which this occurs is not clear although it is postulated that the presence of minus strand hybridized to the 3' end of mRNA somehow prevents any further initiation by the polymerase. In contrast, the 3' end of the minus strand is not prevented from interacting with the polymerase (308).

(f) Virion assembly and cell lysis

If guanidine is used to inhibit replication, then 80S structures comprising the whole capsid component of the virion accumulate in infected cells (251). Moreover, when the guanidine block is removed, these 80S structures are incorporated into virions by association with viral RNA, which strongly suggests that the 80S structures are authentic intermediates in the assembly process. However, there is also evidence that 14S particles of which the 80S particles are made, can form virions directly with viral RNA (252) and so the assembly pathway is not yet clear. Association of either the 14S particles or 80S procapsids with viral RNA is followed or accompanied by cleavage of VP0 (present in the procapsid) to VP2 and VP4 which gives rise to the mature virion. Virion assembly seems not to be regulated but merely depends on the production of sufficient quantities of capsid proteins and viral RNA.

Cell lysis most probably results from a combination of the inhibi-

tion of cellular processes and direct effects of viral proteins, although may not require viral replication (316).

1.5.4 The mechanism of poliovirus induced shut-off of host protein synthesis

(a) General

It has been known for twenty years that poliovirus infection of HeLa cells results in a very rapid and extensive inhibition of cellular protein synthesis (317,318). Depending on the multiplicity of infection (318), cellular polysomes disassemble after approximately one hour, followed by assembly of viral specific polysomes and a peak of viral protein synthesis at about four hours post infection (319). The rate of protein synthesis recovers to approximately forty percent of the rate in uninfected cells.

Elongation rates in residual host specific polysomes appears unaffected during polysome disaggregation, which points to a block in the initiation phase of protein synthesis (320-322). This was demonstrated directly *in vivo* by the inability of cellular mRNAs to form 80S initiation complexes (323) and subsequently an *in vitro* system provided evidence that binding of 40S ribosomes to mRNA was impaired following infection (324). Several investigators were led to examine the structural and functional integrity of host mRNAs following infection. They were found to be unchanged in size (321,322,325), polyadenylation (326) or 5' terminal methylation (325). Moreover, cellular mRNA extracted from infected cells is active in translation, *in vitro* (327). The possibility that cellular mRNA becomes sequestered in an inactive state *in vivo* seems to be ruled out by experiments with vesicular stomatitis virus (VSV) infected-poliovirus superinfected HeLa cells. Poliovirus-superinfection

of VSV-infected cells results in inhibition of VSV mRNA translation by an apparently identical mechanism to that by which cellular translation is inhibited (328) and thus VSV mRNAs can be considered a model for cellular mRNAs. VSV mRNA extracted from superinfected cells is functional *in vitro* (328) consistent with earlier studies of cellular mRNAs (327). The VSV mRNA which accumulates in superinfected cells enters mRNP complexes with cellular proteins and these mRNPs are also active in *in vitro* translation (329). This suggests that untranslated cellular mRNAs are active templates *in vivo*, and that they are not translated because of a defect in the cellular initiation machinery.

(b) *Shut-off requires viral gene expression*

Studies with guanidine, a drug which somehow inhibits viral replication, have indicated that viral replciation is not required for shut-off to occur (330-332). This suggests that the input virion RNA is competent in shut-off, although it should be noted that guanidine does not completely suppress viral replciation (333). In contrast, if inhibitors of protein synthesis are present at the time of infection, then there is a lag period between removal of the inhibitors and the onset of shut-off (332,334), suggesting that expression of the viral genome is required for shut-off. This conclusion was vindicated by the observation that treatment of virions with UV light (332,335,336) proflavine (33) or hydroxylamine (334) abolish the shut-off function, under conditions in which viral RNA but not capsid proteins are damaged. Moreover, the inactivation follows 'single hit kinetics' and the target size appears to be the whole genome (334, 336). This might seem surprising, but may result from the fact that poliovirus proteins are all derived from a single precursor polypeptide (337). Because of this, single hits in the genome could

prevent proper cleavage of the precursor or, disrupt reading frames 3' to the hit, besides directly inactivating single genes. The sum of these results indicate that a viral gene product is responsible for (directly or indirectly) the shut-off of cellular protein synthesis.

(c) Proposed mechanisms for shut-off

This thesis sought to test what appeared at the time to be the most likely hypothesis to explain the poliovirus-induced shut-off, namely that a cellular protein synthesis initiation factor (which for some reason is not required for poliovirus RNA translation) is inactivated. The rationale for this hypothesis and the evidence supporting it are described and expanded in chapters 2,3 and 6. There are however, other models and I shall briefly review these, for the main purpose of indicating that, while they are valid models for other viruses (even closely related picornarviruses) they are almost certainly not applicable to poliovirus.

1) Effects of double-stranded RNA.

The original observation which suggested a role for double stranded (ds)RNA in the shut-off came from experiments in which extracts from poliovirus-infected HeLa cells were found to inhibit translation in reticulocyte lysate, while extracts from uninfected cells had no effect (338). The inhibitor was identified as dsRNA (an intermediate in the poliovirus replicative cycle) (339) but it was subsequently found that translation of viral RNA was also sensitive to inhibition (340). Thus it was difficult to envision how this could explain the preferential selection of viral mRNA for translation in vivo. Later work on the mechanism of inhibition by dsRNA demonstrated that there is activation of a protein kinase which phosphorylates the α-subunit of eIF-2 and consequently prevents its catalytic utilization (341). While eIF-2 is

most probably involved in mRNA binding to ribosomes, phosphorylation of the α-subunit affects formation of ternary complexes prior to mRNA binding and thus affects all mRNAs equally. This presumably explains why dsRNA does not have mRNA specific effects and is therefore not involved in selective inhibition of host protein synthesis.

2) mRNA competition.

The competition model for shut-off was described earlier (page 30). Because translation of poliovirus RNA in vivo is more resistant to hypertonic conditions than cellular mRNA it was suggested that poliovirus RNA might be an intrinsically efficient mRNA and thus compete favourably for translation (342,343). However, two kinds of evidence indicate that this is not the case. First, poliovirus RNA is uncharacteristic of viral RNAs (particularly picornavirus RNAs), in that the available data suggest it to be a 'poor' messenger. It is outcompeted by VSV mRNA in vitro (344) and yet inhibits VSV mRNA translation in vivo (146), and is generally translated at low efficiency in in vitro systems (344). Second, the shut-off occurs in the absence of accumulating viral RNA (330-332). Thus, mRNA competition plays no role in shut-off, in the case of poliovirus.

3) Alterations in membrane permeability.

Carrasco and co-workers are the main proponents of a model in which viral infection alters membrane permeability and creates intracellular ionic conditions which favor the translation of viral mRNA (345,346, and reviewed by Carrasco and Lacal ref. 347). In this proposed mechanism, the entry of Na^+ ions into the cell is considered particularly important for two reasons. First, it was found that translation of cellular mRNAs was inhibited in vitro by 30 mM Na^+ ions whereas trans-

lation of EMCV RNA was slightly stimulated (345). Second, the membrane associated Na^+/K^+ ATPase activity declined during the course of infection, resulting in elevated levels of intracellular Na^+ ions (345) concomittantly with the peak of translation of EMCV RNA. Although membrane leakiness does occur during poliovirus infection of HeLa cells, this event occurs well after the shut-off of cellular translation (348-350) and additionally appears to require replication of the viral genome, which shut-off does not. It seems clear therefore that changes in ionic environment are not responsible for the early shut-off observed in poliovirus-infected HeLa cells.

4) Involvement of initiation factors.

The available evidence suggests very strongly that poliovirus inhibits cellular protein synthesis by inactivating a factor which is required for translation of cellular mRNAs, but not for poliovirus RNA. This thesis addresses the identity of the factor and the way in which it is inactivated in poliovirus-infected HeLa cells. A description of the preliminary evidence is given in the introductory sections of chapters 2 and 6 of this thesis and recent data are discussed in chapter 8. In addition the initiation factors which are implicated in the shut-off and their roles in translation initiation are described in the earlier part of this introduction.

Footnote: To avoid duplication, the literature cited in this Introduction is included along with references for the general discussion following Chapter 8..

CHAPTER 2

Inactivation of Cap-binding Proteins Accompanies the Shut-off of Host Protein Synthesis by Poliovirus

SUMMARY

Infection of HeLa cells with poliovirus results in a rapid shut-off of host protein synthesis. It has been suggested that inactivation of a protein that binds to the cap structure of cellular mRNAs would explain the selective inhibition of host protein synthesis because the naturally uncapped poliovirus RNA can be translated by a cap-independent mechanism. To test directly for the presence of cap-binding proteins in poliovirus-infected and mock-infected cells, we analyzed initiation factor preparations for their ability to specifically cross-link to the 5' cap structure of oxidized reovirus mRNA. The data presented here show that the crosslinking ability of the different cap-binding proteins (24-, 28-, 32-, 50-, and 80-kilodalton polypeptides) is reduced in preparations from poliovirus-infected compared to mock-infected cells. This reduction correlates with the inability of initiation factor preparations from infected cells to restore translation of capped mRNAs in extracts of poliovirus-infected cells. In addition, initiation factor preparations from poliovirus-infected cells have the ability to rapidly inactivate cap-binding proteins and can also impair the restoring activity of initiation factors from mock-infected cells.

INTRODUCTION

Viral infection of mammalian cells often results in the shut-off of host protein synthesis (1), and this effect has been extensively studied in poliovirus-infected HeLa cells (2,3). Poliovirus infection neither induces the degradation of host mRNA (4,5) nor causes detectable changes in the patterns of host mRNA capping, methylation, and polyadenylylation (6). Furthermore, Ehrenfeld and Lund have demonstrated that host mRNA extracted from infected cells remains functional in a wheat-germ cell-free translation system (7).

It was established that the inhibition of host protein synthesis occurs at the initiation step (5), and subsequently it was shown that ribosomal high-salt-wash fractions from infected cells stimulated the translation of poliovirus mRNA, but not of endogenous mRNA, in HeLa cell extracts (8). Rose et al. (9), using vesicular stomatitis virus (VSV) mRNA as a model for host mRNAs (8,10), and Helentjaris et al. (11) have presented evidence that suggested inactivation of eukaryotic initiation factors (IF) eIF-4B and eIF-3. However, a more recent study indicated that a 24-kilodalton (kDal) protein isolated by a multistep procedure (12) copurified with the ability to restore the capacity of poliovirus-infected HeLa cell extracts to efficiently translate VSV mRNA (this activity will be referred to as restoring activity) (12). This polypeptide was found to be identical to the 24-kDal cap-binding protein (24K-CBP) isolated from rabbit reticulocytes by affinity chromatography on a column of 7-methylguanosine diphosphate (m^7GDP) coupled to Sepharose 4B(13). The demonstrated copurification of the 24K-CBP with eIF-3 and eIF-4B (12,14,15) suggests that the effects ascribed to these factors were due to the presence of the 24K-CBP and that the shut-off phenomenon might actually be a result of inactivation of the 24K-CBP.

Recently, Tahara et al. (16) described an 8-10S protein complex purified by m^7GDP affinity chromatography from rabbit reticulocyte ribosomal high-salt wash. This complex consisted of several higher molecular weight proteins in addition to the 24K-CBP and possessed stable restoring activity. This finding is consistent with recent results demonstrating the existence of several higher molecular weight polypeptides that are structurally related to the 24K-CBP of rabbit reticulocytes (17,18). Moreover, some polypeptides with molecular weights that are strikingly similar to those of the latter polypeptides can specifically recognize the cap structure, as determined by cross-linking to oxidized mRNA (19).

In contrast to the almost ubiquitous nature of the cap structure at the 5' terminus of eukaryotic cellular and viral mRNAs, poliovirus RNA lacks a capped 5' end (20,21) and its translation must therefore bypass any 5'-cap-dependent ribosome recognition mechanism. Inactivation of one or more of the CBPs would most likely result in a reduction of host mRNA translation and favor poliovirus RNA translation.

In an attempt to determine the fate of CBPs after poliovirus infection, we analyzed the ability of polypeptides in crude initiation factor preparations from poliovirus-infected and mock-infected cells to specifically cross-link to the 5' terminus of oxidized reovirus mRNA. In this report, we show that IF from poliovirus-infected cells contain significantly lower levels of CBPs as determined by the cross-linking assay. In addition, we demonstrate that IF preparations from infected cells have the ability to effect this reduction and can also impair the restoring activity of IF from mock-infected cells.

MATERIALS AND METHODS

Cells and Viruses. Mouse L-929 cells and HeLa S3 cells were grown in suspension in 10% calf serum. Infection of L cells with reovirus Dearing 3 strain (10 plaque-forming units/cell) and virus purification were carried out as described (22). Infection of HeLa cells with polio virus Mahoney 1 strain (10-20 plaque-forming units/cell) and virus isolation were according to Rose et al. (9). Sindbis virus infection of chicken embryo fibroblasts (23) and encephalomyocarditis virus (EMC) infection of L cells (24) were as described.

Preparation of RNAs. Synthesis of [^{3}H]methyl-labeled reovirus mRNA to a specific activity of ≈ 80,000 cpm/μg with viral cores in the presence of S-adenosylmethionine (specific activity ≈ 70 Ci/mmol, New England Nuclear; 1 Ci = 3.7 X 10^{10} becquerels) and periodate oxidation were according to Muthukrishnan et al. (25). EMC virus RNA was isolated as described (24). Total Sindbis RNA was obtained from infected chicken embryo fibroblasts and consisted of approximately 85% 26S RNA, the remainder being the 42S RNA species (23).

Preparation of Cell Extracts and IF. Cell-free extracts from poliovirus-infected or mock-infected HeLa cells were prepared at 3 hr after infection, essentially as described before (9), except that the extracts were not preincubated under translation conditions, but were dialyzed for 2 hr against buffer containing 90 mM KOAc, 10 mM Hepes buffer at pH 7.6, 1.5 mM $Mg(OAc)_2$, and 1 mM dithiothreitol before freezing. Initiation factors were prepared essentially as described (26). Briefly, S10 extracts were centrifuged at 48,000 rpm for 2 hr in a Beckman SW 50.1 rotor. The ribosomal pellet was resuspended in buffer containing 0.1 M KCl, 20 mM Hepes at pH 7.5, 1 mM dithiothreitol, 0.2 mM EDTA, and 0.2 mM

phenylmethylsulfonyl fluoride ($PhMeSO_2F$), and 3 M KCl was added to a final concentration of 0.6 M. The mixture was stirred for 30 min and centrifuged as above, and the supernatant was dialyzed for 4 hr against 100 mM KOAc/20 mM Hepes, pH 7.5/1 mM dithiothreitol/0.2 mM EDTA/0.2 mM $PhMeSO_2F$.

Binding and Crosslinking of mRNA to IF. [^{3}H]Methyl-labeled oxidized reovirus mRNA was incubated with IF preparations under the conditions described in the figure legends for 10 min at 30°C in a final volume of 30 µl. After incubation, 3 µl of 0.2 M $NaBH_3CN$ was added and the mixture was left on ice for 3 hr, followed by the addition fo 3 µl of RNase A (5 mg/ml) and incubation for 30 min at 37°C to degrade the mRNA. The latter step was carried out according to the modification of Hansen and Ehrenfeld (27). Crosslinked proteins were resolved in $NaDodSO_4$/12.5% polyacrylamide gels, followed by treatment with 2,5-diphenyloxazole/dimethyl sulfoxide or EN^{3}HANCE (New England Nuclear) and exposure of Kodak X-Omat XR-1 film at -70°C as described (14).

In Vitro Protein Synthesis. Translation in HeLa cell extracts was carried out essentially according to Rose et al. (9). Incubation mixtures at a final volume of 25 µl contained 13 µl of micrococcal nuclease-treated S10 extract in 20 mM Hepes buffer, pH 7.6/130 mM KOAc/0.8 mM $Mg(OAc)_2$/1 mM ATP/54 µM GTP/9 mM creatine phosphate/0.6 µg of creatine kinase/2 mM dithiothreitol/0.2 mM spermidine/11 µM of each of 19 amino acids (minus methionine)/20 µCi of [^{35}S]methionine (>1,000 Ci/mmol, New England Nuclear). After incubation for 1 hr at 37°C, 20-µl samples were analyzed by electrophoresis on $NaDodSO_4$/12.5% polyacrylamide gels, which were processed as described above.

FIGURE 1

Cell-free translation in poliovirus-infected and mock-infected HeLa cell extracts and the effect of IF. Translation was performed as described in the text and translation products were resolved on $NaDodSO_4$/polyacrylamide gels and visualized by fluorography (14). Lanes 1-5, translation products in extracts from mock-infected HeLa cells. Lanes 6-10, translation products in extracts from poliovirus-infected HeLa cells. The following amounts of mRNA and IF were added: Lanes 1 and 6, no RNA; lanes 2 and 7, 0.5 μg of EMC RNA; lanes 3 and 8, ≈ 1 μg of Sindbis mRNA; lanes 4 and 9, ≈ 1 μg of Sindbis mRNA plus 90 μg of IF from mock-infected cells; lanes 5 and 10, ≈ 1 μg of Sindbis mRNA plus 90 μg of IF from poliovirus-infected cells.

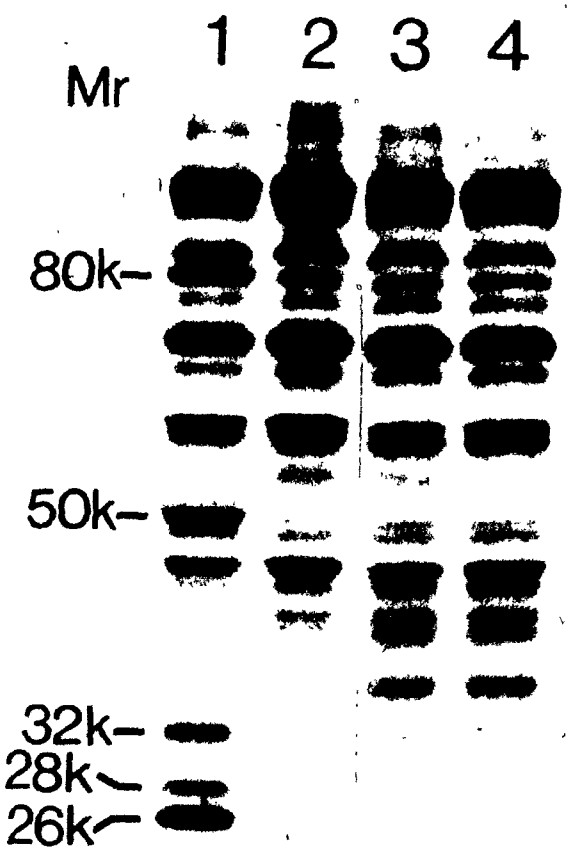

1 2 3 4 5 6 7 8 9 10
Mr
93k–
68k–
46k–
30k–

RESULTS

Cell-free extracts prepared from poliovirus-infected HeLa cells have a reduced ability to translate capped mRNAs, whereas translation of naturally uncapped mRNAs is not impaired (9,11-13). Fig. 1 shows that our extracts had these characteristics. EMC RNA, which does not contain a cap structure at its 5' terminus (28), was translated with similar efficiencies in extracts from mock-infected and infected cells (Fig. 1, lanes 2 and 7, respectively), a finding that is consistent with earlier studies with in vivo and in vitro translation systems (29-31). In contrast, Sindbis mRNA (consisting of 26S and 42S RNA species - both capped mRNAs) was translated efficiently in extracts from mock-infected cells to yield mainly the coat protein (≈ 33 kDal, lane 3), whereas translation was restricted in extracts from poliovirus-infected cells (compare lane 8 to lane 3). Crude IF preparations from mock-infected cells had very little effect on the translation of Sindbis mRNA in extracts from mock-infected cells (Fig. 1, compare lane 4 to lane 3), indicating that the IF do not contain any active component missing in the cell extracts. However, IF preparations from mock-infected cells stimulated the translation of Sindbis RNA in extracts from poliovirus-infected cells by approximately 4-fold (compare lane 9 to lane 8). In contrast, preparations from infected cells showed no such restoring activity: they had no effect on Sindbis mRNA translation in extracts of poliovirus-infected (Fig. 1, compare lane 10 to lane 8) or mock-infected cells (compare lane 5 to lane 3). These results confirm previous reports that a factor, crucial for translation of capped mRNAs and residing in high-salt wash of ribosomes, is inactivated in IF preparations from poliovirus-infected cells (8,9,11).

FIGURE 2

Crosslinking pattern of CBPs from poliovirus-infected and mock-infected HeLa cells in the presence of Mg^{2+}/ATP. IF preparations from mock-infected (92 μg) or poliovirus-infected cells (98 μg) were incubated with 0.7 μg (57,000 cpm) of [^{3}H]methyl-labeled oxidized reovirus mRNA in 25 mM Hepes buffer (pH 7.6) containing 45 mM KOAc, 0.5 mM $Mg(OAc)_2$, 10 μM of each of 19 amino acids (minus methionine), 2.5 mM dithiothreitol, 50 μM GTP, 9 mM creatine phosphate, 0.7 μg of creatine kinase, 0.1 mM $PhMeSo_2F$, 0.1 mM EDTA, 0.2 mM spermidine, and 1 mM ATP. After incubation samples were processed for electrophoresis and fluorography. Lanes 1 and 2, IF from mock-infected cells; lanes 3 and 4, IF from poliovirus-infected cells; lanes 2 and 4 contained 0.67 mM m^7GDP.


1
2
3
4
Mr
80k–
50k–
32k–
28k–
26k–

To test the hypothesis that the activity of a CBP is impaired in poliovirus-infected cells (9,12,13) we analyzed IF preparations by crosslinking to [^{3}H]methyl-labeled oxidized reovirus mRNA. Crosslinking was performed in the presence of Mg^{2+}/ATP, which had previously been shown to be an absolute requirement for the cap-specific crosslinking of several polypeptides, other than the 24K-CBP (19). The polypeptides from rabbit reticulocyte IF that required Mg^{2+}/ATP to crosslink had molecular masses of 28, 50, and 80 kDal and are referred to as CBPs throughout the text (17-19). Fig. 2 shows that oxidized reovirus mRNA could be crosslinked to several polypeptides in crude IF preparations from mock-infected HeLa cells (lane 1). Addition of m^7GDP prevented the crosslinking of the 24K-CBP* in addition to the 28-, 50-, and 80-kDal polypeptides (Fig. 2, lane 2). Crosslinking of an ≈ 32-kDal polypeptide was also inhibited by the addition of m^7GDP, although crosslinking of this polypeptide has not been observed in rabbit reticulocyte IF. Incubation of crude IF from poliovirus-infected cells with oxidized reovirus mRNA resulted in a markedly reduced levels of crosslinking of the 24, 28, 32, 50, and 80-kDal polypeptides (Fig. 2, lane 3). Crosslinking of the 28-, 32-, 50-, and 80-kDal polypeptides was reduced to the level observed with IF from mock-infected cells in the presence of m^7GDP (compare lane 3 to lane

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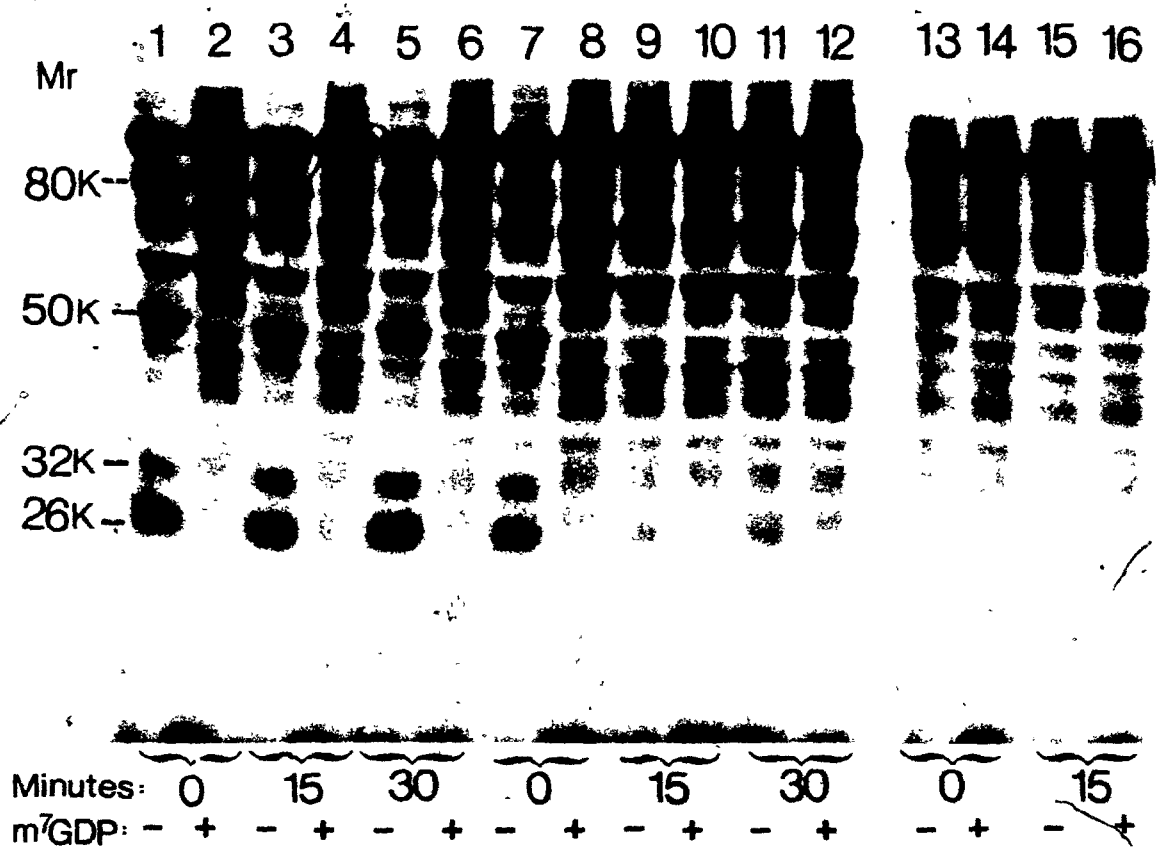
*Note that the CBP with the fastest mobility has been assigned a molecular mass of 26 kDal. This was also observed by Hansen and Ehrenfeld (27) and with IF from other species (unpublished results). However, because this protein was originally termed the 24K-CBP (13,14) we refer to it throughout the text as the 24K-CBP or 24-kDal polypeptide in order to avoid confusion.

FIGURE 3

Effect of mixing IF from mock-infected and poliovirus-infected cells on crosslinking of CBPs to mRNA. IF from mock-infected cells (92 µg) were preincubated with IF from poliovirus-infected cells (36 µg) at 37°C for the times indicated in the figure, prior to the addition of [^{3}H]methyl-labeled oxidized reovirus mRNA (0.5 µg, ≈ 42,000 cpm) under crosslinking conditions in the presence of 1 mM ATP as described in the legend to Fig. 2. As control experiments, IE from mock-infected and poliovirus-infected cells were preincubated separately. Each incubation was perfomred in the absence or presence of 0.67 mM m^7GDP. After incubation samples were processed for electrophoresis, Lanes 1-6, prein cubation of IF from mock-infected cells; lanes 7-12, preincubation of IF from mock-infected cells with IF from poliovirus-infected cells; lanes 13-16, preincubation of IF from poliovirus-infected cells.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Mr
80K
50K
32K
26K
Minutes: 0 15 30 0 15 30 0 15
m7GDP: - + - + - + - + - + - + - + - +

2). The low residual level of crosslinking of these proteins observed in IF from infected cells is also resistant to the addition of m^7GDP (compare lane 4 to lane 3), indicating that this is not cap specific. In contrast, it is noteworthy that the residual crosslinking of the 24K-CBP observed in IF from poliovirus-infected cells is totally prevented by the addition of m^7GDP (lane 4).

Crude IF preparations from poliovirus-infected cells contain additional proteins, absent from mock-infected preparations, which can crosslink nonspecifically to oxidized reovirus mRNA. The most notable of these migrates slightly faster than the 24K-CBP (lanes 3 and 4) and is probably the poliovirus capsid protein VP3, which is known to be a "sticky" protein and has been found in association with ribosomes from infected cells (32). We have observed this protein very consistently and believe that it serves as a useful and reliable marker of infection.

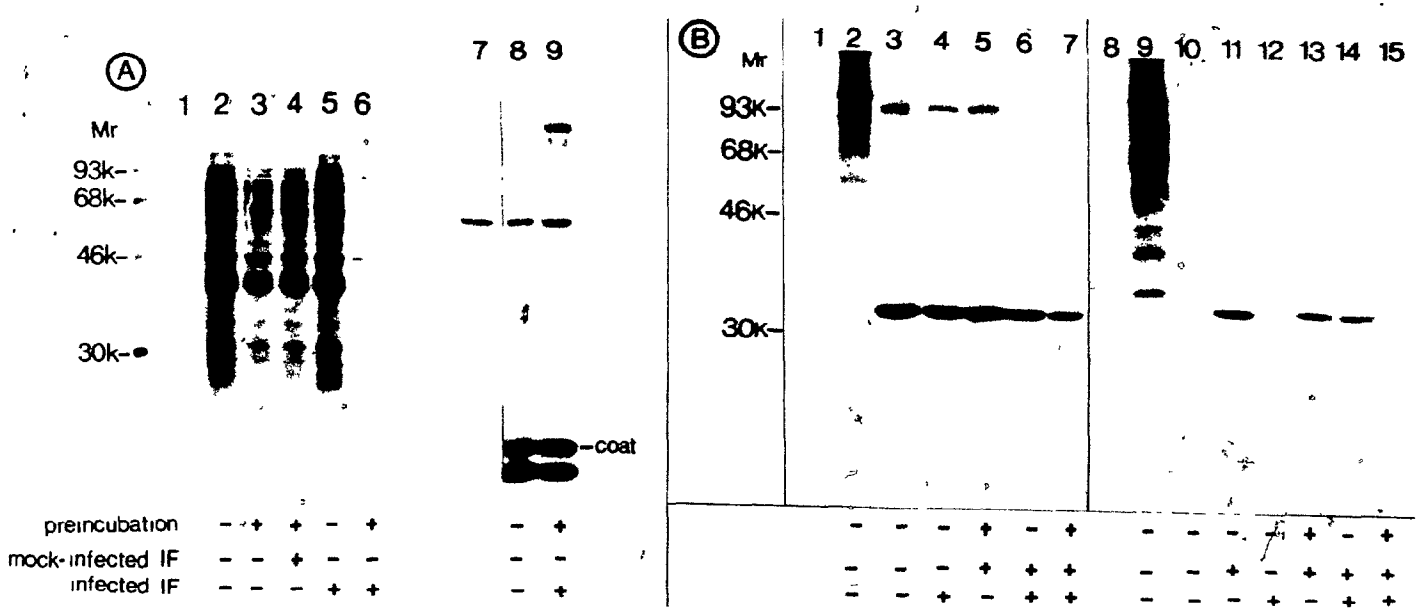
Crosslinking was also performed with $(NH_4)_2SO_4$-fractionated IF in the presence of Mg^{2+}/ATP. The level of detectable CBPs was again markedly reduced in preparations from infected cells (data not shown). Most of the 24K-CBP fractionated in the 0-40% saturated $(NH_4)_2SO_4$ cut, consistent with previous findings that the 24K-CBP copurifies with initiation factors eIF-3 and eIF-4B (12,14,15), both of which fractionate in the 0-40% $(NH_4)_2SO_4$ cut of IF preparations (33). More recently, Hansen and Ehrenfeld have demonstrated that the 24K-CBP is present in the 0-40% $(NH_4)_2SO_4$ cut of IF from HeLa cells (27).

Rose et al. (9) have reported that cell-free extracts from poliovirus-infected cells contain an activity that slowly reduces the ability of uninfected extracts to translate capped mRNAs <u>in vitro</u>. It was of interest, therefore, to determine whether IF preparations from infected

cells had such an activity and also whether these IFs could impair the crosslinking ability of the different CBPs. To this end, we preincubated IF from mock-infected cells with IF from poliovirus-infected cells prior to the cross-linking assay. Incubation of IF from mock-infected HeLa cells with [^{3}H]methyl-labeled oxidized reovirus mRNA resulted in crosslinking of several proteins (Fig. 3, lane 1). Addition of m^{7}GDP to the incubation mixture decreased the crosslinking of the previously described 24, 32, 50, and 80-kDal CBPs (lane 2; note that the 24 and 28-kDal polypeptides were not resolved in this experiment). Preincubation of IF from mock-infected cells for 15 min (lane 3) or 30 min (lane 5) did not impair their specific crosslinking ability. Simple mixing of IF from poliovirus-infected cells with IF from mock-infected cells did not diminish the crosslinking ability of the various CBPs in the latter fraction (lane 7). However, preincubation of this mixture for 15 min (lane 9) drastically diminished the ability of the CBPs to crosslink to mRNA. Nonspecific crosslinking of polypeptides, for example the 92-kDal protein, was not affected even after 30 min preincubation (lane 11). Again, it is apparent that there is residual crosslinking of the 50 and 80-kDal polypeptides that is no longer inhibited by m^{7}GDP (compare lanes 9 and 10), indicating that the residual level is probably due to nonspecific crosslinking of polypeptides with molecular weights similar to those of CBPs (see also Fig. 2). In contrast, after preincubation a fraction of the 24K-CBP could still crosslink to mRNA, and this crosslinking was sensitive to m^{7}GDP (compare lane 10 to lane 9). This residual amount of 24K-CBP was not abolished even after 30 min preincubation with IF from poliovirus-infected cells (lane 11). Lane 13 represents the crosslinking pattern of IF from poliovirus-infected cells that

FIGURE 4

Effect on protein synthesis of preincubation of HeLa cell extracts with IF from mock-infected or poliovirus-infected cells. Micrococcal nuclease-treated HeLa cell extracts prepared from poliovirus-infected or mock-infected cells were preincubated at 37°C with the indicated amounts of IF from poliovirus-infected or mock-infected cells in 25 µl incubation mixtures containing the components required for protein synthesis except for [^{35}S]methionine and mRNA. At the times indicated, mRNA and [^{35}S]methionine were added and incubation was continued for 60 min at 37°C. (A) Translation in mock-infected HeLa cell extracts with no added RNA (lanes 1 and 7), 2 µg of reovirus mRNA (lanes 2-6), or 1 µg of STNV RNA (lanes 8 and 9). IFs (20 µg) were added where indicated in the figure and preincubation time was: lane 3, 25 min; lane 4, 25 min; lane 4, 25 min; lane 6, 12 min; and lane 9, 20 min. The autoradiograph for STNV translation products was exposed for a longer period than the one for reovirus translation products. (B) Translation in extracts of mock-infected cells (lanes 1-7) or poliovirus-infected cells (lanes 8-15) was programmed by 0.5 µg of EMC RNA (lanes 2 and 9) or ≈ 1 µg of Sindbis RNA (lanes 3-7 and 10-15). No RNA was added in lanes 1 and 8. IFs (90 µg) from mock-infected or poliovirus-infected cells were included as indicated and preincubation time was 20 min.


A
1 2 3 4 5 6
Mr
93k-
68k-
46k-
30k-
7 8 9
coat
preincubation
mock-infected IF
infected IF
B
Mr
93K-
68K-
46K-
30K-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

do not contain cap-specific crosslinkable proteins. Preincubation of this preparation had no effect on the crosslinking pattern (lane 15). These results indicate that IF from poliovirus-infected cells contain an activity that rapidly impairs the ability of the various CBPs to recognize the cap structure of the mRNA and would presumably effect a reduction in cellular protein synthesis. These results also exclude the possibility that IF from poliovirus-infected cells contain a preformed inhibitor of CBP function, because no effect could be observed without preincubation.

An important question to address was whether IF preparations from poliovirus-infected cells could also mediate the reduction in the ability of extracts from mock-infected cells to translate capped mRNAs that is observed after preincubation with extracts from poliovirus-infected cells (9). Fig. 4A is an autoradiograph of the translation products encoded by reovirus and satellite tobacco necrosis virus (STNV) mRNAs in extracts from mock-infected HeLa cells. Lanes 1 and 7 represent the endogenous translation products in the nuclease-treated extracts. Translation of reovirus mRNA yielded the various reovirus structural polypeptides (lane 2). Preincubation of the extract in the absence or presence of IF from mock-infected cells (lanes 3 and 4, respectively) slightly reduced the extent of translation, and addition of IF from infected cells without preincubation had no effect on translation of reovirus mRNA (lane 5). However, preincubation of the same mixture for 12 min dramatically curtailed translation (lane 6). STNV RNA was translated in mock-infected extracts to yield the 22-kDal coat protein and an 18-kDal prematurely terminated translation product (34) (Fig. 4A, lane 8), and could also be translated in poliovirus-infected extracts (data not shown). In contrast

to the distinct inactivation of reovirus mRNA translation there was no detectable inhibition of STNV translation in extracts from mock-infected cells that were preincubated with IF from poliovirus-infected cells (compare lane 9 to lane 8). An unexplained synthesis of a 94-kDal polypeptide was observed in the preincubated cell extract (lane 9).

In a second set of experiments we attempted to determine whether IF from poliovirus-infected cells could also reduce the restoring activity of IF from mock-infected cells. In the data shown, EMC RNA was translated with higher efficiency in infected than in mock-infected cell extracts (Fig. 4B, lanes 9 and 2, respectively). Sindbis mRNA was translated efficiently in extracts from mock-infected cells to yield the coat protein and the 93-kDal B_1 precursor polypeptide (31) (lane 3), while 1/10th as much translation (as determined by densitometry tracing of the coat protein band) was observed in poliovirus-infected cell extracts (lane 10). Addition of IF from mock-infected cells with 30 min preincubation had no effect on translation of Sindbis mRNA (lane 5), whereas addition of IF from infected cells or a mixture of IF from mock-infected and infected cells without preincubation slightly reduced translation (lanes 4 and 6). However, addition of the mixture to extracts from mock-infected cells followed by a 20 min preincubation resulted in approximately 60% inhibition of translation of Sindbis mRNA (compare lane 7 to lane 3). Thus, the slight inhibition observed in lanes 4 and 6 could be explained by the inhibitory effects of the infected IF during the translation incubation. In the translation system from poliovirus-infected cells, IF from mock-infected cells restored the ability to translate Sindbis mRNA (lane 11) but preincubation of these extracts with IF from mock-infected cells partially reduced the restoring activity by ≈ 30%

(lane 13). In contrast, IF from poliovirus-infected cells did not exhibit significant restoring activity (lane 12), and addition of IF from poliovirus-infected cells to the infected cell extract supplemented with IF from mock-infected cells without preincubation only partially inhibited the translation (40% inhibition, lane 14). This partial inhibition could reasonably be explained by inactivation of the restoring activity during the translation incubation. However, preincubation of extracts supplemented with IF from mock-infected cells with IF from infected cells resulted in a complete loss of the restoring activity of the mock-infected IF (lane 15). This result indicates that the IF from poliovirus-infected cells contain an activity that neutralizes the restoring activity. This activity is probably related to the activity that impairs the crosslinking ability of CBPs, thus lending support to the belief that the restoring activity resides in the CBPs.

DISCUSSION

It has been suggested that the 24K-CBP is inactivated during infection by poliovirus and that this inactivation mediates the shut-off of host protein synthesis. More recently it has been shown that other polypeptides (28,50, and 80 kDal) can specifically recognize the cap structure (17,19), and that higher molecular weight polypeptides purified by m^7GDP affinity chromatography are essentially required for restoring translation of capped mRNAs in poliovirus-infected cell extracts (16). The 50- and 80-kDal polypeptides may correspond to eIF-4A and eIF-4B, respectively, on the basis of the crosslinking characteristics of these factors (35).

In this study, we attempted to determine whether a change in the 24K-CBP, the other CBPs or both, accompanies the shut-off of host protein synthesis exerted by poliovirus, by using the crosslinking assay (14). Our finding that all of the polypeptides capable of recognizing the cap structure lose their binding activity as a result of poliovirus infection is consistent with what would be expected if the various CBPs in HeLa cells are structurally related, as is the case with CBPs of rabbit reticulocytes (18). In addition, the apparently coordinate inactivation of all the CBPs suggests that they are functionally related.

The reduction in the amount of detectable CBPs during poliovirus infection correlates both with the inability of extracts from infected cells to translate capped mRNAs and with the absence of restoring activity in IF of infected cells. This strongly suggests that functional CBPs are a vital component of the restoring activity. This suggestion is supported by data demonstrating the existence of activity that can, upon preincubation, impair both the restoring activity of IF from mock-infect#

ed cells and the ability of CBPs to recognize and crosslink to the cap structure. This activity resides in IF preparations from infected cells and rapidly impairs the ability of CBPs to crosslink to the cap structure. These findings are also consistent with the findings of Brown and Ehrenfeld (36), who demonstrated an activity in IF preparations from poliovirus-infected cells that specifically restricted the translation of capped mRNAs in reticulocyte lysate.

Our results appear to be at variance with those of Hansen and Ehrenfeld (27), who concluded that the cap-binding ability of 24K-CBP as assayed by the crosslinking technique is not reduced during poliovirus infection. However, careful examination of their data reveals that the extent of crosslinking of the 24K-CBP is distinctly lower in preparations from infected cells (figures 2 and 3 in ref. 27). In addition, we performed cross-linking analysis with $(NH_4)_2SO_4$-fractionated IF under similar conditions to these authors in the absence of ATP, and again we found substantially reduced amounts of 24K-CBP in preparations from poliovirus-infected cells (data not shown).

The mechanism by which CBPs are inactivated during poliovirus infection is not clear, nor is it known whether the inactivating factor(s) is virally coded or induced. It is possible that CBPs become modified or degraded in poliovirus-infected cells. It may be possible to differentiate between these different possibilities by using monoclonal antibodies directed against CBPs.

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CHAPTER 3

Capped mRNAs with Reduced Secondary Structure Can Function in Extracts from Poliovirus-Infected Cells

SUMMARY

Extracts from poliovirus-infected HeLa cells were used to study ribosome binding of native and denatured reovirus mRNAs and translation of capped mRNAs with different degrees of secondary structure. Here, we demonstrate that ribosomes in extracts from poliovirus-infected cells can form initiation complexes with denatured reovirus mRNA, in contrast to their inability to bind native reovirus mRNA. Furthermore, the capped alfalfa mosaic virus 4 RNA, which is most probably devoid of stable secondary structure at its 5' end, could be translated at much higher efficiency than other capped mRNAs in extracts from poliovirus-infected cells.

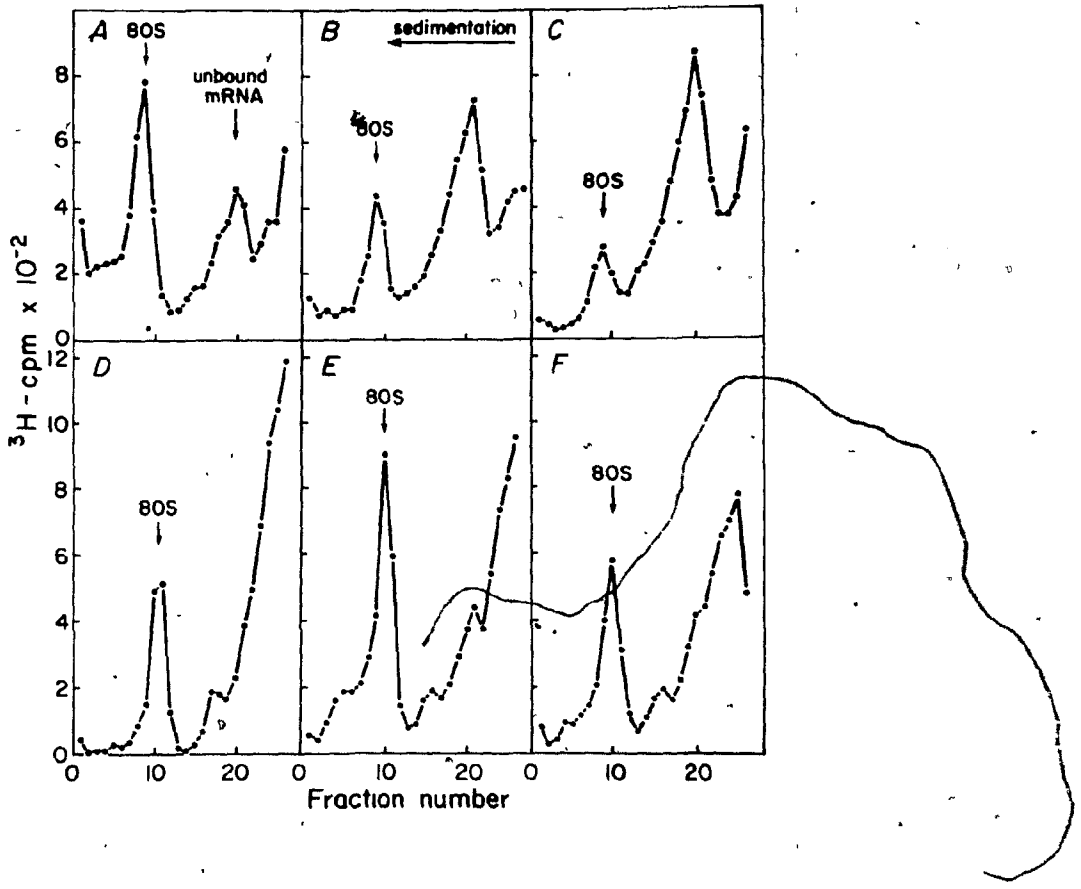
INTRODUCTION, RESULTS AND DISCUSSION

The cap structure at the 5' terminus of almost all eucaryotic mRNAs, $m^7GpppN(m)$ (30), has been shown to facilitate translation initiation complex formation (for a recent review see reference 2). However, the degree of dependence on the cap structure for translation varies among different capped mRNAs, as indicated by variable extents of decrease in translation due to decapping (24,27) or addition of cap analogs (13,38). Moreover, the extent of dependence on the cap structure for translation has been shown to be a function of salt concentration (6,39,40), temperature (38), and the concentration of initiation factors (IF) (12). It was believed that the function of the cap structure is mediated by a cap-binding protein (CBP), and consequently, a 24-kilodalton (Kd) polypeptide was identified by specific cross-linking to the 5' oxidized cap structure of reovirus mRNA (33) and purified to apparent homogeneity by m^7GDP-Sepharose 4B affinity chromatography (34). Subsequently, it has been demonstrated that additional polypeptide with molecular masses of 28, 50, and 80 Kd can be specifically cross-linked (as indicated by m^7GDP inhibition) to the oxidized cap structure, although cross-linking of these polypeptides is absolutely dependent on ATP-Mg^{2+} (31). Whether each of these polypeptides interacts directly with the cap structure or whether they exist in a complex containing a cap recognition element is still an open question. In any event, we will refer to polypeptides that can be specifically cross-linked to the cap structure as CBPs. Recent, cross-linking experiments with purified IF have suggested that the 50- and 80-Kd polypeptides correspond to eIF-4A and eIF-4B, respectively (9).

It has been suggested that a CBP(s) facilitates ribosome binding by melting the secondary structure of the mRNA (32). This hypothesis is

FIGURE 1

Binding of native and m^7I-capped inosine-substituted reovirus mRNA to ribosomes in extracts from mock-infected cells as a function of K^+ concentrations. HeLa S3 cells were grown in media supplemented with 5% calf serum. Cell extracts were prepared as described by Lee and Sonenberg (22), except that extracts were not dialyzed. Native reovirus [methyl-^{3}H]mRNA (~20,000 cpm/μg) and m^7I-capped inosine-substituted mRNA (~35,000 cpm/μg) were prepared as described by Muthukrishnan et al. (245) and Morgan and Shatkin (23), respectively. For ribosome binding, native mRNA (10,500 cpm) or inosine-substituted mRNA (13,000 cpm) was incubated in 50 μl of an S10 HeLa cell extract at 30°C for 10 min in buffer containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5), 20 amino acids (10 μM each), 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phsophate, 4 μg of creatin phosphokinase, 3 mM magnesium acetate, 40 μg of rabbit reticulocyte rRNA, 200 μM sparsomycin to inhibit polypeptide chain elongation, and potassium acetate as indicated below. Initiation complexes were analyzed in glycerol gradients by centrifugation for 90 min at 48,000 rpm and 4°C in an SW50.1 rotor (4,35). The final concentrations of potassium acetate (excluding 20 mM KCl contributed by the HeLa cell extract) and the percentages of input mRNA bound were as follows: (A) 70 mM, 36%; (B) 105 mM, 18%; (C) 145 mM, 11%; (D) 70 mM, 30%; (E) 105 mM, 35%; and (F) 145 m, 31%.


A
80S
unbound mRNA
B
sedimentation
80S
C
80S
D
80S
E
80S
F
80S
^{3}H-cpm x 10^{-2}
Fraction number

consistent with observations that the irreversibly denatured, inosine-substituted reovirus mRNA is less dependent on the cap structure for initiation complex formation (19,20,23). In addition, a monoclonal antibody with anti-CBP activity can inhibit initiation complex formation with native reovirus mRNA but not with inosine-substituted mRNA (32).

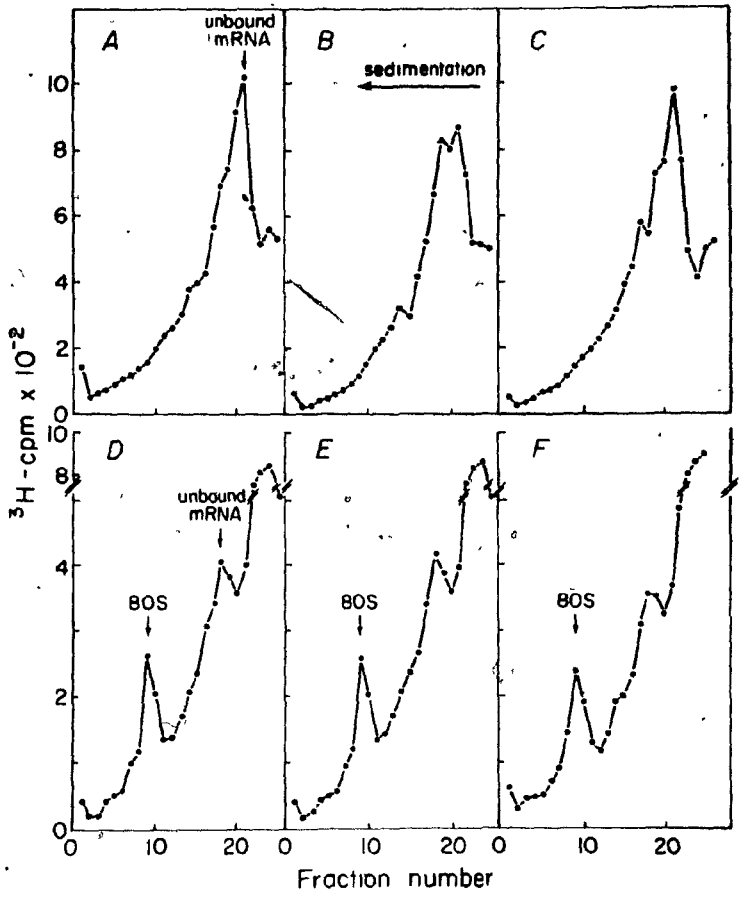
In poliovirus-infected HeLa cells, the translational machinery of the host is modified in such a way that it will direct the synthesis of virus proteins only (8). The uncapped poliovirus RNA (14,25) must be translated independently of the cap structure, and indirect evidence has suggested that inactivation of a factor involved in cap recognition is responsible for the shut-off of host protein synthesis and subsequent preferential translation of poliovirus RNA (28). Later work has indicated that this cap recognition factor(s) resides in unstable form in the 24-Kd CBP (37) and in stable form in a fraction containing high-molecular-weight polypeptides in addition to the 24-Kd CBP (36). Most recently, it has been demonstrated that cap recognition ability in poliovirus-infected cells is impaired in such a way that the cap-specific polypeptides can no longer be cross-linked to the cap structure (22). Other investigators have found that the 24-Kd CBP is dissociated from eIF-3 in ribosomal salt wash preparations from poliovirus-infected cells (10,11). Consequently, we used extracts from poliovirus-infected cells to study the function of cap recognition and present evidence which is consistent with the contention that a CBP(s) facilitates ribosome binding by melting secondary structures of the mRNA involving 5' sequences proximal to the initiation codon.

Reovirus mRNA can form initiation complexes in HeLa cell extracts (Fig. 1), and the binding of mRNA to ribosomes decreases as the K^+

concentration increases (from 36% binding at 90 mM K^+ (Fig. 1A) to 11% binding at 165 mM K^+ (Fig. 1C). It is possible that this inhibition is due to an effect of a high salt concentration on an interaction among components of the initiation machinery. For example, an elevated K^+ ion concentration might directly impair the activity of a CBP(s). Another reasonable explanation is based on the observation that mRNA assumes a more compact structure at higher salt concentrations (15). Hence, if a CBP(s) is required to melt the secondary structure at the 5' end of mRNA and the melting step limits initiation complex formation, one would expect that ribosome binding to native capped reovirus mRNA should have a greater dependence on CBP(s) at higher K^+ concentrations. In light of these considerations, the binding of m^7I-capped inosine-substituted reovirus mRNA, which contains less secondary structure than native mRNA (19,23), should be less susceptible to variations in salt concentrations because the secondary structure of m^7I-capped RNA should not be altered as significantly as that of native mRNA under these circumstances. Indeed, the extent of binding of this mRNA remained constant (~ 30 to 35% of input mRNA bound) when the K^+ concentration was increased from 90 to 165 mM (Fig. 1D through F). The binding of inosine-substituted mRNA to ribosomes is resistant to inhibition by m^7GDP (15% decrease at 0.2 mM), as has been reported before in the wheat germ system (23). We also analyzed ribosome binding of bromouridine-substituted reovirus mRNA, which possesses enhanced secondary structure (19), at increasing K^+ concentrations and found that binding was more sensitive to inhibition by high salt concentrations than was native mRNA binding (data not shown). Thus, inhibition of initiation complex formation by increased salt concentrations appears to be directly related to the degree of secondary

FIGURE 2.

Binding of native and m^7I-capped inosine-substituted reovirus mRNAs to ribosomes in extracts from poliovirus-infected cells as a funtion of K^+ concentration. Poliovirus (Mahoney 1 strain) infection fo HeLa cells was performed as previously described with 10 to 20 PFU per cell, and preparation of cell extracts was as described previously (22,28). Native reovirus [methyl-^{3}H]mRNA (13,000 cpm) or inosine-substituted mRNA (18,000 cpm) was incubated for ribosome binding in 50 µl of an extract from poliovirus-infected cells as described in the legend to Fig. 1, and initiation complex formation was analyzed as described in the legend to Fig. 1 and elsewhere (4,35). (A through C) Native mRNA; (D through F) inosine-substituted mRNA. The final concentrations of potassium acetate (excluding 20 mM KCl contributed by the HeLa cell extract) and the percentages of input mRNA bound were as follows: (A) 70 mM, 3%; (B) 105 mM, 3%; (C) 145 mM, 3%; (D) 70 mM, 15%; (E) 105 mM, 16%; and (F) 145, 15%.


A
unbound mRNA
B
sedimentation
C
D
unbound mRNA
80S
E
80S
F
80S
^{3}H-cpm x 10^{-2}
Fraction number

structure of the mRNA, which is consistent with the contention that mRNA secondary structure is a significant determinant in inhibition of initiation complex formation at elevated K^+ concentrations.

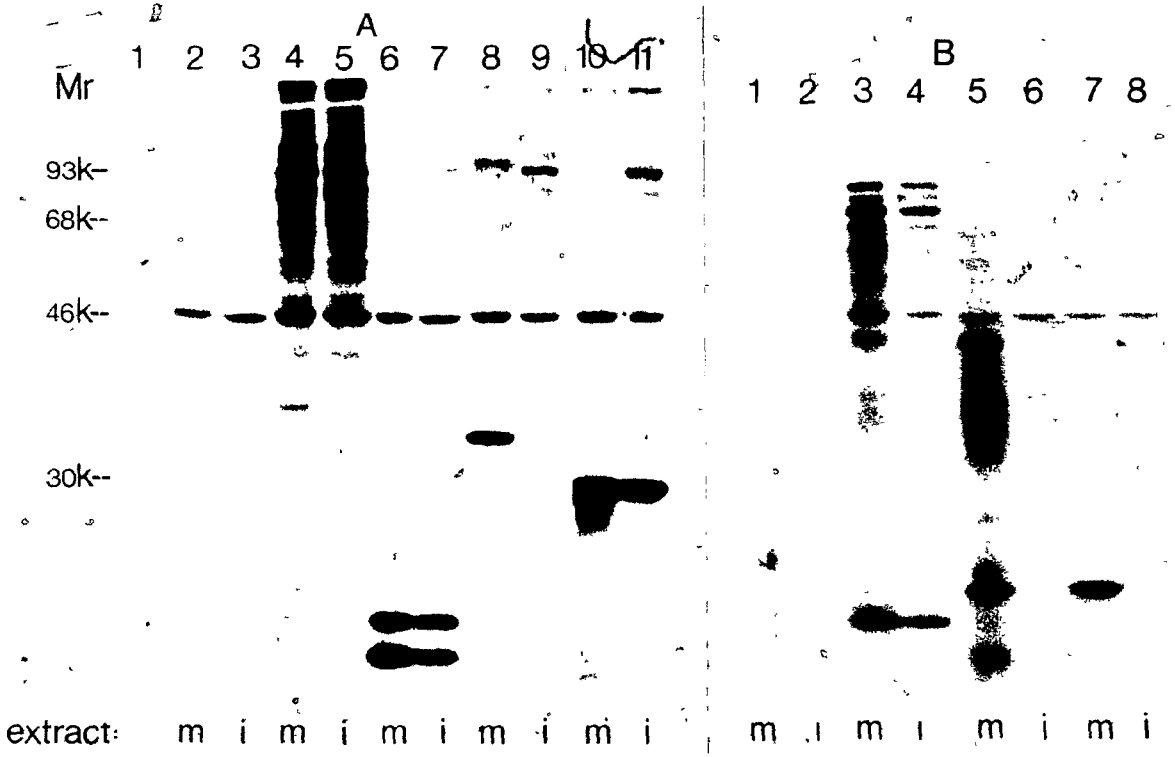
Based on the observation that extracts from poliovirus-infected cells are unable to initiate translation with capped mRNAs (8), native reovirus mRNA should not form initiation complexes in these extracts. Indeed, native reovirus mRNA did not bind to ribosomes in extracts from poliovirus-infected cells with the different K^+ concentrations used (Fig. 2A through C). However, these extracts were able to promote binding of inosine-substituted mRNA to a significant extent (~ 15% of mRNA input bound at all salt concentrations, as compared with 30 to 35% in the extracts from mock-infected cells), and binding was resistant to m^7GDP inhibition, as was the binding in extracts from mock-infected cells (data not shown). These data indicate that impairment of cap recognition ability in poliovirus-infected cells prohibits initiation complex formation only with mRNAs containing significant secondary structure.

To further test the idea that only mRNAs with considerable secondary structure are dependent on a cap recognition function for initiation of translation, we analyzed the translation of mRNAs with various degrees of secondary structure at their 5' ends in extracts from poliovirus- and mock-infected cells. Figure 3A show the [^{35}S]methionine-labeled translation products from different mRNAs resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Extracts from poliovirus-infected HeLa cells are able to efficiently translate the naturally uncapped RNA from encephalomyocarditis (EMC) virus (3,22). In this experiment, the translation of EMC virus RNA in extracts from infected cells was about 90% as efficient as translation in extracts of mock-infected cells (Fig.

FIGURE 3

Translation of capped and naturally uncapped mRNAs in extracts from mock-infected and poliovirus-infected cells. Translation in HeLa cell extracts was carried out essentially as previously described (28,34). Reaction mixtures (25 µl) contained the following: 130 mM potassium acetate, 0.4 mM magnesium acetate, 20 mM HEPES (pH 7.5), 1 mM ATP, 54 µM GTP, 9 mM creatine phosphate, 22 µg of creatine phosphokinase per ml, 2.5 mM dithiothreitol, 0.2 mM spermidine, 19 amino acids (10 µM each; no methionine), 20 µCi of [^{35}S]methionine (1,195 Ci/mmol, New England Nuclear Corp.), and mRNA in the amounts indicated. Incorporation of [^{35}S]methionine was assayed after 60 min at 37°C by spotting 5-µl aliquots on Whatmann 3 MM filter paper disks, which were processed for liquid scintillation counting as described previously (34), the rest of the reaction mixture being used to analyze the ^{35}S-labeled products by polyacrylamide gel electrophoresis and fluorography. Translation in extracts from mock-infected cells (m) and translation in infected cell extracts (i) are shown. (A) Reaction mixtures included no added RNA (lanes 2 and 3) or 1 µg of each of the following RNAs: EMC virus (7) (lanes 4 and 5), STNV (lanes 6 and 7), Sindbis virus (5) (Lanes 8 and 9), and AMV-4 (lanes 10 and 11). Lane 1 contained relative molecular weight markers. (B) Reaction mixtures included no added RNA (lanes 1 and 2) or 1 µg of each of the following RNAs: EMC virus (7) (lanes 3 and 4), reovirus (24) (lanes 5 and 6), and rabbit globin (21) (lanes7 and 8). The synthesis of radioactive polypeptides was quantified by densitometric

tracing of autoradiographs from gels exposed for short times to ensure quantitative estimates. The relative synthesis of the major polypeptides (for EMC virus, in the region between molecular masses 70 and 115 Kd) directed by the various mRNAs in the extracts from infected versus mock-infected cells was as follows. (A) EMC virus, 90%; STNV, 50%; Sindbis virus, no detectable synthesis in extracts from poliovirus-infected ells; AMV-4; 40%. (B) EMC virus, 50%; reovirus and globin, no detectable synthesis in extracts from poliovirus-infected cells.


A
1 2 3 4 5 6 7 8 9 10 11
Mr
93k--
68k--
46k--
30k--
extract: m i m i m i m i m i
B
1 2 3 4 5 6 7 8
m i m i m i m i

3A, cf. lanes 5 and 4). A lower relative efficiency (~ 50% in extracts from infected cells, as compared with extracts from mock-infected cells) was observed for the translation of satellite tobacco necrosis virus (STNV) RNA, which is also naturally uncapped (16) (Fig. 3A, cf. lanes 7 and 6). Thus, in these experiments, extracts from infected cells were able to support translation of naturally uncapped RNAs, albeit with lower efficiency than extracts from mock-infected cells (50 to 90% in infected, as compared with mock-infected). We believe that this reduction is due to a nonspecific loss of translational activity, since we and other have obtained extracts from poliovirus-infected cells which could translate EMC virus and STNV RNAs at the same efficiency as extracts from mock-infected cells (see references 3 and 22 for examples). Translation of Sindbis virus RNA (consisting of the 26S and 42S RNA species-both capped) yielded mainly the coat protein (~ 33 Kd polypeptide) and its B_1 precursor protein (~ 95 Kd polypeptide) coded by the 26S RNA species (3) (Fig. 3A, lane 8). In contrast to the partial decrease of translation (Fig. 3A, lanes 4 through 7) observed with naturally uncapped RNAs, translation of the capped Sindbis virus RNA was totally restricted in extracts from infected cells (lane 9). Translation products were also observed which were endogenous to the cell extracts. Extracts from mock-infected cells yielded a prominent polypeptide of ~ 46 Kd and a minor polypeptide of ~ 93 Kd whereas endogenous translation in extracts from infected cells produced polypeptides of ~ 93.5 and 85 Kd in addition to the ~ 46 Kd polypeptide. The 93.5 and 85 Kd polypeptides are most obvious in Fig. 3A, lanes 9 and 11, and probably represent the polioviral precursor polypeptides NCVP1a and NCVP1b, respectively (29).

To further establish that the infected lysate had a reduced capacity

for translating capped mRNAs, we analyzed translation of capped mRNAs other than Sindbis virus RNA (Fig. 3B). In this experiment, EMC virus RNA translation in extracts from infected cells was about 50% as efficient as translation in extracts from mock-infected cells. (Fig. 3B, cf. lanes 4 and 3). However, the translation of reovirus and rabbit globin mRNAs was reduced to undetectable levels (Fig. 3B, lanes 6 and 8). These results indicate that the infected-cell extracts used were indeed not functional in translation of these capped mRNAs. Nucelotide sequence analysis of 5' terminal portions of rabbit globin mRNAs has allowed computer-aided prediction of stable secondary structure in these regions (1,26). In view of our hypothesis that dependence on the cap structure for translation initiation is related to degree of mRNA secondary structure, these predictions are in accord with the inability of extracts from poliovirus-infected cells to translate globin mRNAs.

To further test our model, we analyzed the translation of the capped alfalfa mosaic virus 4 (AMV-4) RNA, which contains an adenosine-uracil-rich 5' leader region (128) and hence cannot forms stable secondary structure, as predicted by computer-aided anlaysis (P. Auron, personal communication). Consequently, although this mRNA is capped, we might expect its translation to be less dependent on the cap structure,. Indeed, translation of AMV-4 RNA in poliovirus-infected extracts was only partially reduced (~ 60%) relative to translation in mock-infected extracts (Fig. 3A, cf. lanes 11 and 10), and this reduction was comparable to that observed with naturally uncapped STNV RNA. This result is also consistent with previous data showing that translation of AMV-4 RNA is resistant to inhibition by the cap analog m^7GDP and a monoclonal antibody with anti-CBP acitivity (32), indicating that the cap structure is

less essential for AMV-4 RNA translation. It might be argued that AMV-4 and STNV RNAs are plant RNAs which would normally be translated at a lower temperature and possibly by a slightly different mechanism, compared with mammalian mRNAs, and might therefore not be appropriate for study in the mammalian system. However, in this respect it is significant that plant cellular mRNAs are dependent on the cap structure for translation, as are mammalian cellular mRNAs (see, for example, reference 13). Furthermore, translation of the plant viral RNA of tobacco mosaic virus in a reticulocyte lysate has been shown to exhibit characteristics similar to those of rabbit globin mRNA with respect to optimal salt concentrations and cap requirement (40). In addition, the ability of STNV and AMV-4 RNAs to translate in extracts from poliovirus-infected cells is most likely not attributable to their plant origin, since tobacco mosaic virus RNA behaved like the capped mammalian mRNAs studied here in that it was efficiently translated in extracts from mock-infected cells but not in extracts from poliovirus-infected cells (data not shown).

In summary, we have used extracts from poliovirus-infected HeLa cells to examine the requirements for cap-dependent translation, since evidence has recently been provided to indicate that this system is specifically impaired in a cap recognition function required for translation of capped mRNAs (10,11,22). The results described here are consistent with a model in which a CBP(s) destabilizes the secondary structure of capped mRNAs in an energy-dependent process to facilitate binding of 40S ribosomal subunits. This model is based on several reported observations. First, inosine-substituted capped reovirus mRNA which has reduced secondary structure is less dependent on both the cap

structure and ATP hydrolysis for initiation compex formation (19,20,23). Second, some naturally uncapped RNAs, such as cowpea mosaic virus and EMC virus RNAs, are less dependent on ATP for initiation complex formation than are capped mRNAs (17), again indicating that the requirement for the cap structure and for ATP are related aspects of translation initiation. Finally, the observations that a monoclonal antibody with anti-CBP activity does not inhibit ribosome binding to inosine-substituted reovirus mRNA (32) and that cap recognition by some CBPs requires ATP-Mg^{2+} (31) have implicated CBPs as effectors of the ATP-dependent step in ribosome binding.

At the present time, it is not clear which structural features are responsible for allowing the cap-independent translation of naturally uncapped RNAs. Whether the translation initiation mechanism for these mRNAs is entirely independent of a CBP(s) remains to be determined.

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CHAPTER 4

mRNA Secondary Structure as a Determinant in Cap Recognition and Initiation Complex Formation

SUMMARY

Polypeptides of M_r = 50,000 and 80,000 in rabbit reticulocyte initiation factor preparations can be specifically cross-linked to the oxidized 5' cap structure of native reovirus mRNA in an ATP-Mg^{2+}-dependent manner (Sonenberg, N., Guertin, D., Cleveland, D., and Trachsel, H. (1981) Cell 27, 563-572). However, specific cross-linking of these polypeptides can occur in the absence of ATP-Mg^{2+} when m^7I-capped inosine substituted mRNA, (which contains less secondary structure than native reovirus mRNA), is used. We also found, using wheat germ extract that inhibition of initiation complex formation by high salt concentrations is directly related to the degree of secondary structure of the mRNA. Binding of ribosomes to bromouridine-substituted reovirus mRNA is severely inhibited at high K^+ concentrations, while binding to inosine-substituted mRNA is only slightly inhibited and binding of native reovirus mRNA is inhibited to an intermediate degree. These results are consistent with the hypothesis that cap recognition factors mediate an ATP-dependent melting of secondary structures involving 5' proximal sequences to the initiation codon in order to facilitate binding of ribosomes during translation initiation.

INTRODUCTION

Polypeptides that interact with the 5' terminal cap structure m^7GpppN, of eukaryotic mRNAs have been identified in IF[1] preparations from rabbit reticulocytes (1-3) and several other mammalian cells (1,4,5) by specific cross-linking to the oxidized cap structure of viral mRNAs. Cross-linking of an ~ 24 kDa polypeptide (termed the 24-kDa cap binding protein, 24K- CBP) is not dependent on ATP-Mg^{2+} (1-3), while cross-linking of 28-,50-, and 80-kDa polypeptides has an absolute requirement for ATP-Mg^{2+} which is probably hydrolyzed, since nonhydrolyzable analogues of ATP do not substitute in this reaction (3). The latter polypeptides have been termed "cap binding proteins" (CBPs, Refs. 3 and 6) because their cross-linking is inhibited by cap analogues. Hydrolysis of ATP is required for initiation of protein synthesis in eukaryotes but not in prokaryotes (7-9), and other observations have implicated ATP as a mediator of cap function. (a) Morgan and Shatkin (10) and Kozak (11,12) have shown that reovirus mRNA with reduced secondary structure is less dependent on both the cap structure and ATP for initiation complex formation than native reovirus mRNA. (b) Jackson (13) has reported that naturally uncapped mRNAs such as those of cowpea mosaic virus and EMC virus are less dependent on ATP for initiation complex formation than capped mRNAs. In addition, a monoclonal antibody with anti-CBP activity was found to inhibit ribosome binding to native reovirus mRNA but did not inhibit binding to inosine-substituted mRNA (6). Thus, we were prompted to propose that cap recognition factors are involved in an ATP-Mg^{2+}-

[1]The abbreviations used are: IF, initiation factor; eIF, eukaryotic initiation factor; CBP, cap binding protein; EMC, encephalomyocarditis; AMV-4, alfalfa mosaic virus-4; kDa, kilodalton.

dependent melting of secondary structure involving mRNA 5' proximal sequences to facilitate ribosome attachment. If the requirement for ATP hydrolysis is to melt the secondary structure of the mRNA, then cross-linking of the 28-, 50-, and 80-kDa polypeptides to inosine-substituted reovirus mRNA should be less dependent on ATP-Mg^{2+}, since this mRNA has lower potential to form secondary structure as compared to native mRNA. In an attempt to verify this prediction, we analyzed the ability of reovirus mRNAs with different degrees of secondary structure to cross-link to cap recognition factors in the presence and absence of ATP-Mg^{2+}. In addition, we examined the extent of inhibition of initiation complex formation induced by increasing salt concentration in relation to the degree of secondary structure of the mRNA, and found a direct relationship between the degree of secondary structure and the extent to which ribosome binding is inhibited by high salt concentration.

MATERIALS AND METHODS

Preparation of reovirus mRNAs- [^{3}H]-methyl-labeled reovirus mRNA was synthesized with viral cores in the presence of [^{3}H]methyl-S-adenosyl-methionine (AdoMet, specific activity, 70 Ci/mmol, New England Nuclear), as described by Muthukrishnan et al. (14), to a specific activity of ~ 2 x 10^4 cpm/μg. m^7I-capped inosine-substituted reovirus mRNA was synthesized according to Morgan and Shatkin (10) to a specific activity ~ 3 x 10^4 cpm/μg, and bromouridine-substituted reovirus mRNA was prepared, according to Kozak (11), to a specific activity of ~ 1.5 x 10^4 cpm/μg. Oxidation of mRNA was performed as described by Muthukrishnan et al. (14) and Sonenberg and Shatkin (15).

Preparation of Cell Extracts and Initiation Factors - Wheat-germ S23 extract and reticulocyte lysate were prepared as previously described (Refs. 16 and 17, respectively). A 0.6M KCl wash of ribosomes from rabbit reticulcoyte lysate prepared as described (5) was used as a source of initiation factors.

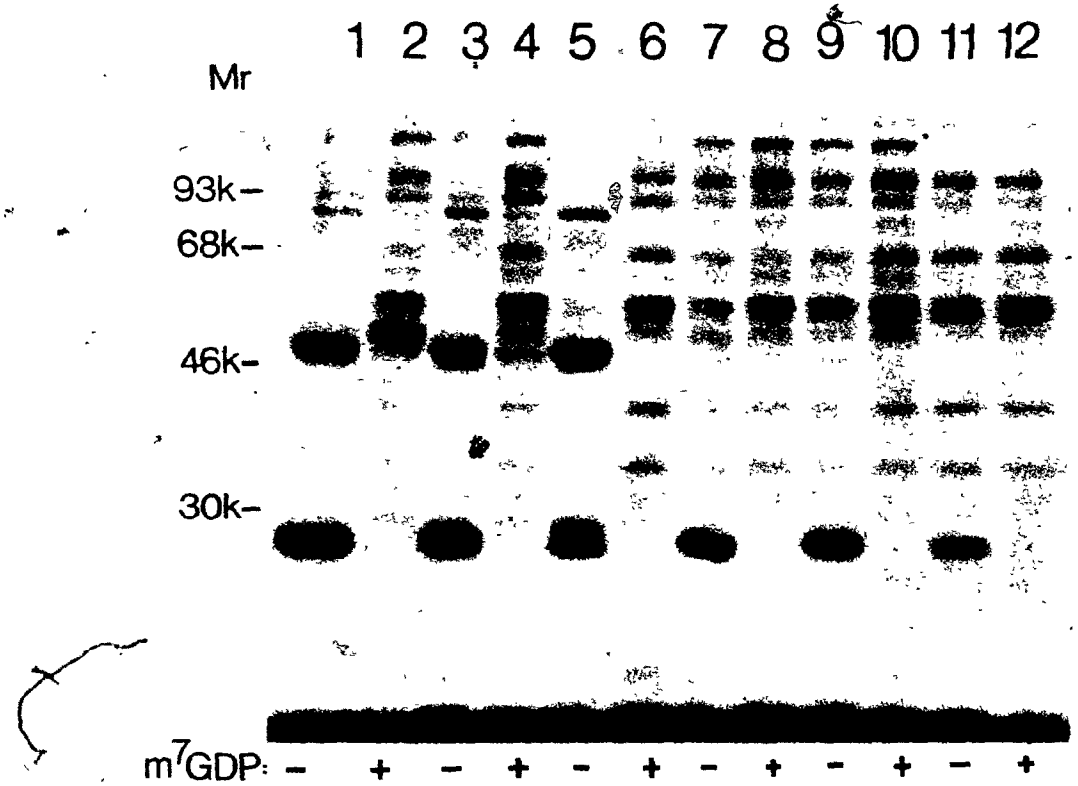
Cross-linking of Oxidized mRNA to Initiation Factors- Cross-linking was performed as described by Sonenberg and Shatkin (15) with the modification of Hansen and Ehrenfeld (4). Reaction mixtures (30 μl) contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 0.9 mM ATP, 70 μM GTP, 9 mM creatine phosphate, 22 μg/ml of creatine phosphokinase, 11 μM of each of 19 amino acids (minus methionine), 2 mM dithiothreitol, 0.2 mM spermidine, 60 μM phenylmethylsulfonyl fluoride, 0.5 mM $Mg(OAc)_2$, ~ 100 μg of initiation factors, and mRNA in the amounts specified in the legend. m^7GDP was included at 0.7 mM and the salt concentration was adjusted by the addition of KCl, as indicated in the figure legends. Incubation was for 10 min at 30°C followed by the

addition of 3 µl of 0.2 M $NaBH_3CN$. The incubation mixture was left overnight at 4°C followed by the addition of 2 µl of RNase A (5 mg/ml) and incubation for 30 min at 37°C to degrade the mRNA (4). Cross-linked proteins were analyzed by polyacrylamide gel electrophoresis and fluorography as previously described (5,15).

Ribosome Binding- Ribosome binding was performed with the indicated amount of [^{3}H]methyl-labeled reovirus mRNA and incubation was for 10 min at 25°C in 50 µl reaction mixtures that were 50% (v/v) wheat-germ S23 extract and contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 10 µM each of 20 amino acids, 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 4 µg of creatine phosphokinase, 3 mM $Mg(OAc)_2$, and 200 µM sparsomycin to inhibit chain elongation. The salt concentration was adjusted by the addition of KOAc to the endogenous KCl (45 mM, final concentration of KCl) contributed by the wheat germ extract. Ribosome binding was analyzed as previously described (15, 16) by glycerol gradient centrifugation for 90 min at 48,000 rpm and 4°C in an SW 50.1 rotor.

FIGURE 1

Effect of K^+ concentration on the cross-linking of rabbit reticulocyte cap binding proteins to native reovirus mRNA. [^{3}H]methyl-labeled reovirus mRNA (5 x 10^4 cpm) was incubated with ~ 100 μg of crude initiation factors from rabbit reticulocytes for 10 min at 30°C and samples were processed for electrophoresis and fluorography as described under "Materials and Methods" (x-ray film was exposed at -70°C for 1 week). Incubation was performed in presence of ATP (lanes 1-6) or absence of ATP (lanes 7-12). KCl was added to give the following final concentrations: lanes 1,2,7, and 8, 30 mM; lanes 3,4,9, and 10, 65 mM; lanes 5, 6,11, and 12, 140 mM. m^7GDP (0.7 mM) was added as indicated.


1 2 3 4 5 6 7 8 9 10 11 12
Mr
93k–
68k–
46k–
30k–
m7GDP: – + – + – + – + – + – +

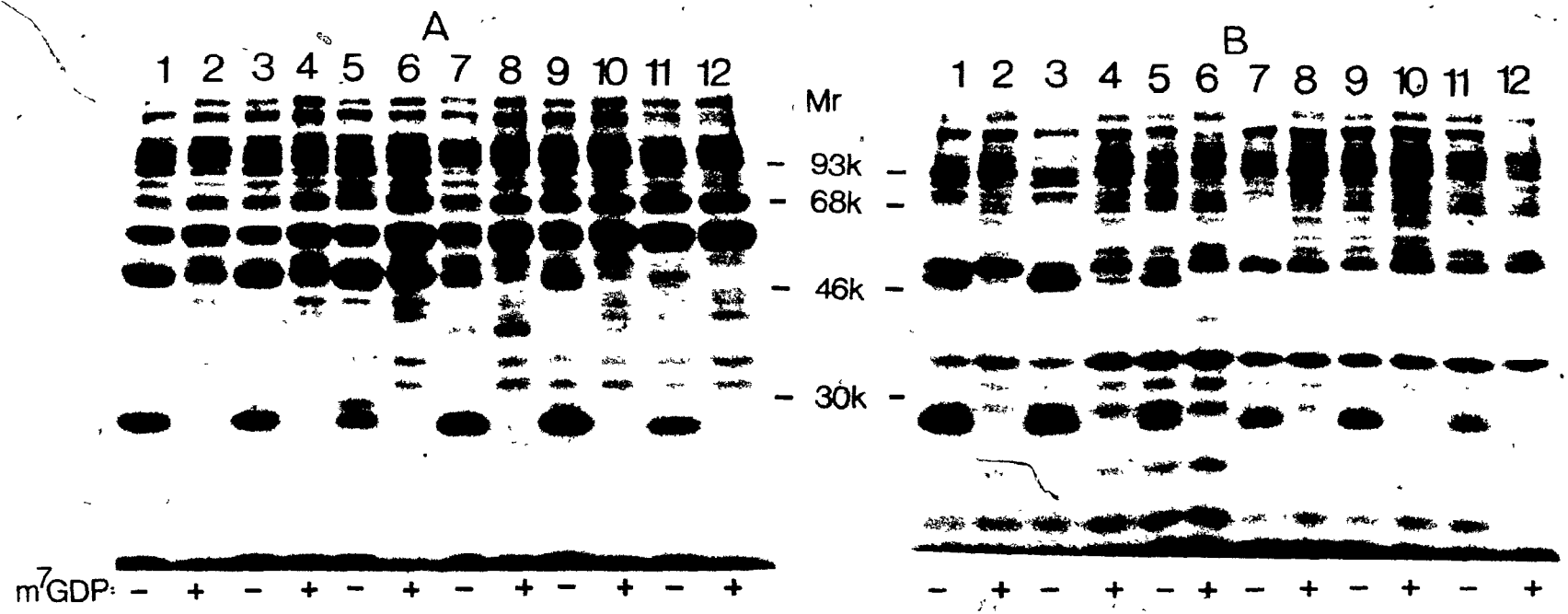
RESULTS

To study the relationship between ATP-dependent cross-linking of cap recognition factors and the secondary structure of the mRNA, we analyzed polypeptides in initiation factor preparations from rabbit reticulocytes for specific cross-linking to reovirus mRNAs with different degrees of secondary structure in the presence and absence of ATP. If ATP hydrolysis is required to provide energy to melt secondary structures, then we might expect that cross-linking of the 28-, 50-, and 80-kDa polypeptides to inosine-substituted mRNA would be less dependent on ATP than cross- linking to native reovirus mRNA. Since the degree of secondary structure of mRNA is also a function of salt concentration (18), we performed this cross-linking analysis at different salt concentrations.

Fig. 1 shows the cross-linking pattern obtained with native reovirus mRNA and a rabbit reticulocyte IF preparation at different salt concentrations (30, 65 and 140 mM K^+) in the presence and absence of ATP. Cap specific cross-linking was indicated by inhibition upon addition of the cap analogue, m^7GDP. In the presence of ATP at a relatively low salt concentration (30 mM), cross-linking of three polypeptides of approximate molecular masses 24, 50 and 80 kDa was inhibited by m^7GDP (compare lane 1 to 2). Cross-linking of the 50- and 80-kDa polypeptides increased gradually with increasing salt concentration (lanes 3 and 5, ~ 1.5- and 2-fold increase for the 50- and 80-kDa polypeptides, respectively, when concentration was increased from 30 to 140 mM K^+) while cross-linking of the 24-kDa polypeptide was decreased by ~ 40% at the highest salt concentration relative to 30 and 65 mM K^+ (lane 5). In addition, specific cross-linking of a 28-kDa polypeptide was observed at 140 mM K^+ (lane 5).

FIGURE 2

Cross-linking of reticulocyte cap binding proteins to reovirus mRNA with altered secondary structure. A, [^{3}H]methyl-labeled m^{7}I-capped inosine-substituted mRNA (4 X 10^4 cpm) or B, bromouridine-substituted mRNA (4 X 10^4 cpm) was incubated with ~ 100 μg of crude initiation factors from rabbit reticulocytes for 10 min at 30°C and samples were processed for electrophoresis and fluorography as described under "Materials and Methods" (x-ray film was exposed at -70°C for 4 weeks). Incubation was performed in presence of ATP (lanes 1-6) or absence of ATP (lanes 7-12). KCl was added to give the following final concentrations: lanes 1,2,7, and 8, 30 mM; lanes 3,4,9, and 10, 65 mM; lanes 5,6,11, and 12, 140 mM. m^{7}GDP (0.7 mM) was added as indicated.


A
1 2 3 4 5 6 7 8 9 10 11 12
Mr
93k
68k
46k
30k
m7GDP: – + – + – + – + – + – +
B
1 2 3 4 5 6 7 8 9 10 11 12
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Cap specific cross-linking of the 24-, 28-, 50-, and 80-kDa polypeptides to native reovirus mRNA has been demonstrated previously (3,6), that of the 28-, 50-, and 80-kDa polypeptides having an absolute requirement for ATP-Mg^{2+} in strict contrast to the 24-kDa polypeptide. Cross-linking analysis in the absence of ATP yielded results consistent with this latter observation (Fig. 1, lanes 7-12). The only specific cross-linking in the absence of ATP was of the 24-kDa polypeptide and this was reduced by 60% at the highest, relative to the lowest, salt concentration (compare lane 11 to lane 7), as was the case in the presence of ATP. This suggests that the reduced amount of cross-linked 24-kDa polypeptide at the high salt concentration is due to an effect of the salt per se and not due to competition between CBPs for a limited amount of mRNA.

The cross-linking profile obtained in the presence of STP with inosine-substituted reovirus mRNA (Fig. 2A) is essentially the same as that for native reovirus mRNA (Fig. 1) except for the fact that the total amount of specific cross-linking is reduced by approximately 2- to 3-fold (as determined by densitometric tracing) because of greater extent of nonspecific cross-linking. (Note that exposure of gels in Figs. 1 and 2 for autoradiography was four times longer for the experiments performed with inosine-substituted mRNA than for those with native reovirus mRNA to enable better visualization of the cross-linked CBPs). Cross-linking of the ATP-dependent CBPs is stimulated (~ 1.5-1.2-fold) by increased salt concentrations while the converse is true for the 24-kDa polypeptide (2-fold reduction at the highest, as compared to the lowest, salt concentration). It is also noteworthy that cross-linking of the ATP-dependent 50- and 80-kDa polypeptides is proportionally greater, relative to the 24-CBP with the inosine-substituted mRNA as compared to native

reovirus mRNA, at the two lower salt concentrations. This might be due to the diminished secondary structure of the former mRNA as will be considered later.

When cross-linking of IF preparations to inosine-substituted mRNA was performed in the absence of ATP (Fig. 2A, lanes 7-12), the 24-kDa polypeptide was cross-linked, but in sharp contrast to the case with native reovirus mRNA there was also specific cross-linking of the 50- and 80-kDa polypeptides, albeit to a lesser extent than in the presence of ATP (compare lanes 7 and 9 in Fig. 2A to those in Fig. 1). The relative amount of cross-linked 50-kDa CBP in the absence of ATP was 45 and 54% at 30 and 65 mM potassium ion concentration, respectively, of that obtained in the presence of ATP at the same K^+ concentration as determined by densitometry tracing. Cross-linking of the 28-kDa polypeptide, which only occurs to a significant extent at 140 mM K^+ concentration in the presence of ATP (Fig. 2A, lane 5), did not occur in the absence of ATP except possibly at 65 mM K^+ concentration. This could be due to the generally reduced level of cross-linking of the different CBPs at the elevated K^+ concentration (Fig. 2A, lane 11). Cross-linking of the 50- and 80-kDa polypeptide is optimal at 65 mM salt as compared to 140 mM K^+ optimum in the presence of ATP, a situation which could be explained if the inosine-substituted mRNA has some secondary structure at the higher concentration, which in the absence of ATP prevents cross-linking of ATP-Mg^{2+}-dependent cap specific polypeptides to the mRNA.

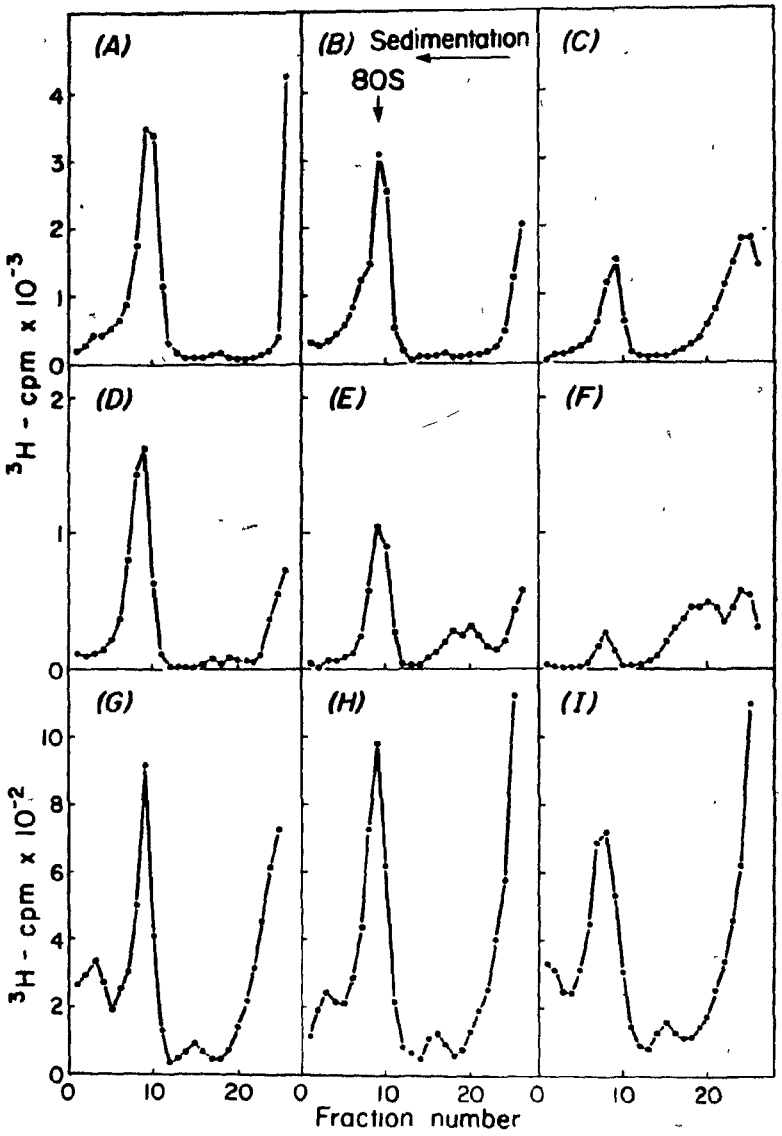
To ensure that the observed effects were due to changes in secondary structure and were not simply a result of inosine substitute per se, we performed identical experiments with bromouridine-substituted mRNA since in this case the secondary structure should be more stable than in native

mRNA (11). As can be seen in Fig. 2B, in the presence of ATP the extent of cap-specific cross-linking of this mRNA to the various polypeptides was similar to that of inosine-substituted mRNA and approximately 3-fold lower than that of native mRNA. However, as with the native mRNA, the proportion of cross-linked polypeptides at low potassium concentrations was in favor of the 24-CBP. Maximum cap-specific cross-linking of the 50- and 80-kDa polypeptides was achieved at 65 mM potassium ion concen tration (Fig. 2B, lane 3), in contrast to the 140 mM optimum for inosine-substituted and native mRNA. In the absence of ATP-Mg^{2+}, the only cap-specific cross-linking was of the 24-CBP (lanes 7-12) and maximal cross-linking was achieved at 65 mM potassium (lane 9) as for the cross-linking in the presence of ATP-Mg^{2+}. These results further indicate that mRNA with secondary structure requires ATP-Mg^{2+} for cross-linking to the 28-, 50-, and 80-kDa cap-specific polypeptides.

It has been shown that salt concentrations have an effect on the degree of cap dependence exhibited by capped mRNAs for translation (19-21), which might be related to the fact that high salt concentrations confer more stable secondary structure on mRNA (18). In light of our proposed model in which mRNA secondary structure is melted by a CBP(s) as a prerequisite for binding of ribosomes, one significant prediction is that, under conditions in which the melting step limits initiation complex formation, increasing salt concentrations will eventually inhibit formation of initiation complexes. This inhibition should be less pronounced with the relaxed, inosine-substituted mRNA since the stability of its secondary structure is considerably reduced (10,11). To verify this prediction, we analyzed the effect of K^+ concentration on binding of wheat germ ribosomes to reovirus mRNAs with different degrees of

FIGURE 3

Effect of K^+ concentration on translation initiation complex formation. Ribosome binding to native reovirus mRNA (24,000 cpm: A-C), bromouridine-substituted reovirus mRNA (26,000 cpm; D-F), or inosine-substituted reovirus mRNA (15,000 cpm; G-I) was performed as described under "Materials and Methods". The final concentrations of K^+ in the reaction mixtures including 45 mM KCl contributed by the wheat germ extract and added KOAc was a follows: A,D and G, 45 mM; B,E, and H, 90 mM; C.F, and I, 180 mM. The per cent of radioactivity bound to ribosomes was the following: A, 72%; B, 76%; C, 30%; D, 72%; E, 56% F, 9%; G, 60%; H, 63%; I, 55%.


(A)
(B) Sedimentation
80S
(C)
(D)
(E)
(F)
(G)
(H)
(I)
^{3}H - cpm x 10^{-3}
^{3}H - cpm x 10^{-2}
Fraction number
0
10
20

secondary structure (binding of these mRNAs to ribosomes appears to be functional since polysomes accumulated in the absence of the chain elongation inhibitor, sparsomycin). Fig. 3 (A-C) shows that binding of native reovirus mRNA to ribosomes is reduced when the K^+ concentration is increased. Binding is decreased from 72% of input mRNA bound at 45 mM K^+ (A) to 30% bound at 180 mM K^+ (C). The binding of bromouridine-substituted mRNA, which contains more stable secondary structure than native mRNA (11), should be inhibited to a greater extent than native mRNA when K^+ concentrations are increased, assuming again that the melting step limits formation of initiation complexes in the cell extracts. The results (D-F) indicate that this is the case: about 72% of the input mRNA was bound at 45 mM K^+ (D) similarly to native mRNA. However, at the highest K^+ concentration (180 mM, F) the binding was reduced to ~ 10% of that at 45 mM K^+, in comparison to an ~ 60% reduction observed with native reovirus mRNA. To further test our prediction, we carried out ribosome binding experiments with inosine-substituted reovirus mRNA. Since it does not contain significant secondary structure (10-12), it is predicted that an increase in K^+ concentrations will not affect ribosome binding to this mRNA. Fig. 3 (G-I) shows that the binding of inosine-substituted mRNA decreased only slightly (~ 10%) from 62% input mRNA bound at 45 mM (G) to 56% at 180 mM (I).

DISCUSSION

Several observations have led to our hypothesis that cap recognition factors facilitate ribosome binding by melting secondary structures of eukaryotic mRNAs involving 5' sequences proximal to the initiation codon (3,6). Firstly, it has been demonstrated that the irreversibly denatured, inosine substituted reovirus mRNA is less dependent on both the cap structure and ATP hydrolysis for initiation complex formation than is native reovirus mRNA (10-12). Secondly, a monoclonal antibody with anti-CBP activity was shown to inhibit binding of ribosomes to native reovirus mRNA but had no such effect when inosine-substituted mRNA was used (6).

Since high ionic strength most likely confers more stable secondary structure on mRNA (18), we analyzed the effect of salt concentration on ribosome binding to reovirus mRNAs with different degrees of secondary structure. We found a direct relationship between the degree of secondary structure of the mRNA and the extent to which initiation complex formation is inhibited by high salt concentration (Fig. 3). A reasonable interpretation of these results is that the increased stability of the mRNA secondary structure under high salt concentrations prevents the factors involved in melting the mRNA from functioning. An alternative explanation is that the activity of a factor(s) involved in melting of the mRNA secondary structure is directly inhibited by high salt concentrations. A concerted effect of these two possibilities is also not excluded by our results. These results are in accord with the observations that translation of some capped mRNAs is inhibited at high salt concentrations (19) whereas translation of the naturally capped AMV-4 mRNA, which has little potential for forming stable secondary structure

at its 5' end (22), is not sensitive to high salt concentrations (19).

The precise molecular mechanism by which cap recognition factors mediate ribosome binding is not clear. The results presented here indicate that the 50- and 80-kDa polypeptides are able to interact with the cap structure in an ATP-independent manner only when the secondary structure of the mRNA is reduced. This observation provides evidence that mRNA secondary structure determines the accessibility of the cap structure to the different cap recognition factors and is consistent with the idea that ATP hydrolysis is required to melt the secondary structure of eukaryotic mRNA, although it yields no further indication as to precisely which factor might effect this process.

Recently, Grifo et al. (23) have demonstrated that preparations of eIF-4A and eIF-4B can be specifically cross-linked to the 5' oxidized cap structure in an ATP-Mg^{2+}-dependent manner, suggesting that the 50- and 80-kDa polypeptides might correspond to eIF-4A and eIF-4B, respectively. Cross-linking of each of these purified factors required the presence of the other and since the 24K-CBP was invariably present in preparations of eIF-4B, it is possible that cap recognition by eIF-4A and eIF-4B is also dependent on the 24K-CBP. These results together with previous observations (3,6) suggest that functional cap recognition factors exist as a complex, containing both cap recognition and secondary structure melting functions. Interaction of this complex with the cap structure might be a sequential process in which the 24K-CBP recognizes the cap structure, followed by ATP-dependent melting of the secondary structure and subsequent interaction of the 50- and 80-kDa polypeptides with the cap structure. In a more recent publication, Tahara et al. (24) have reported that the cap specific cross-linking of purified eIF-4A and

eIF-4B to m^7I-capped inosine-substituted reovirus mRNA is dependent on ATP-Mg^{2+}. The apparent difference between this result and those reported here is probably due to the fact that we have used a crude system that may contain, in addition to eIF-4A and eIF-4B, other components of importance to the cap recognition process.

In summary, our results are consistent with the possibility that ATP-dependent melting of the secondary structure of mRNA is a prerequisite for interaction between certain cap recognition factors and the 5' terminus of the mRNA. This interaction may then facilitate binding of 40S ribosomes to the 5' terminus of the mRNA. Elucidation of the molecular mechanisms involved in such a process await a direct demonstration of melting activity. One approach which should prove valuable in this respect is the use of mRNA secondary structure mapping techniques to determine alterations in mRNA secondary structure in the presence of purified cap recognition factors.

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CHAPTER 5

Involvement of Eukaryotic Initiation Factor 4A in the Cap Recognition Process

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 an 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 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+} (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^{2+}, 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^{2+} (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 the 24K-CBP (termed CBPI) 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 an 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 initiation factors), and subfractionation of the latter fraction to a 0-40% ammonium sulfate 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% ammonium sulfate 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 h at 38,000 rpm in an SW40 rotor at 4dC. 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 m^7GTP-agarose affinity column (1 x 0.7 cm) (preparation of this column will be described elsewhere) equilibrated in Buffer A containing 0.1 M KCl and 10% glycerol. Proteins which bound to the column nonspecifically 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 1M KCl and 10 glycerol was used to elute the

remaining adsorbed material.

Cross-linking of mRNA to Proteins Synthesis Factors - [^{3}H]Methyl-labeled, oxidized reovirus mRNA was incubated with initiation factor preparations for 10 min at 30°C 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).

Prepartion 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 with 200 µl of eIF-4A (30 µg) in TBS/complete Freund's adjuvant (1:1). The injection was repeated after 2 weeks, with incomplete Freund's adjuvant. Four weeks later a final injection was given (400 µl of TBS/incomplete Freund's adjuvant containing 125 µg of 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 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 h followed by addition of 200 µl/well of 2.5% BSA in TBS for 1 h 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 h, plates were washed 4-5 times with TBS and incubated for 3 h with 1 µg/ml of peroxidase-conjugated

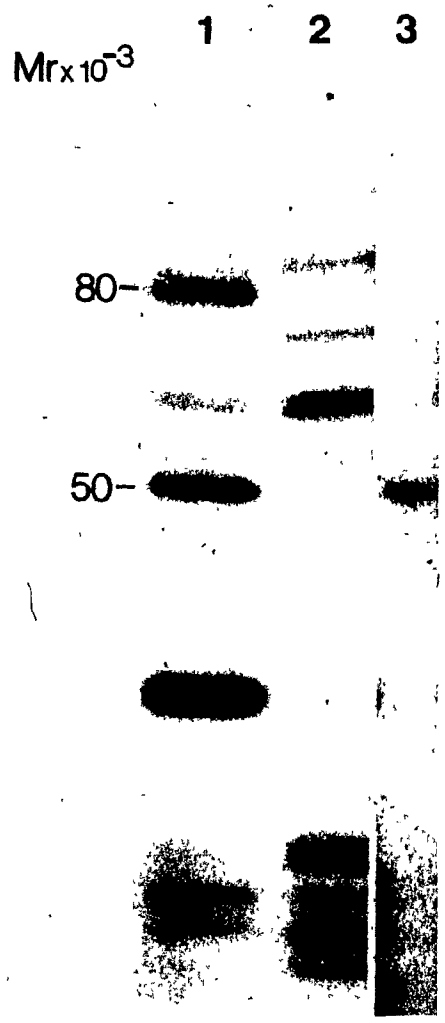
rabbit anti-mouse immunoglobulin (Dako) in TBS containing 0.5% BSA. The plates were washed again and stained with 0.4 mg/ml of 5-amino-2-hydroxybenzoic acid, 0.003% H_2O_2 in TBS (50 µl/well). For preparation of purified antibody, cells (1-2 X 10^7) of the clone were injected intraperitoneally in BALB/cJ mice. About 2 weeks later the mice were killed 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 incubation 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 h at 25 V and 1A. The nitrocellulose paper was incubated for 1 h 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 h, washing in TBS, and development by a color reaction with diaminobenzidine (19). For the experiment described in Fig. 3 (lanes 2 and 3) the procedure of Meyer et al. (18) for immunoblotting was followed.

FIGURE 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 under "Materials and Methods" and in Ref. 3) in a final volume of 300 µl for 10 min at 30°C, in absence (lanes 1 and 3) or presence of 0.7 mM m^7GDP (lane 2). Following the addition of 30 µl of 0.2 M $NaBH_3CN$ and incubation overnight at 4°C, RNase A (20 µl, 10 mg/ml) was added and incubation was continued for 30 min at 37°C. 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°C, 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 h and addition of 20 µl of rabbit anti-mouse immunoglobulin (Dako) at equivalence. After incubation overnight at 4°C, 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-

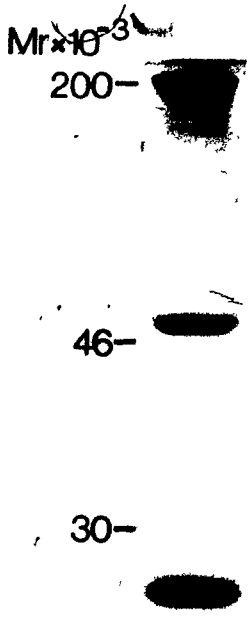
RESULTS

We attempted to determine whether the 50-kDa polypeptide present in rabbit reticulocyte crdue initiation factor 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 jmmunoprecipitate total initiation factors 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 initiation factors 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 preivously purified the 24K-CBP by centrifugation of a 0-40% ammonium sulfate fraction of rabbit reticulocyte ribosomal high salt wash on sucrose gradients in 0.1 M KCl and application of the slow sedimenting fractions on an 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 from an 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 an m^7GTP-agarose column for affinity chromatography in the final step of purification. Fig. 2 shows a typical Coomassie blue-staining pattern of

FIGURE 2

Analysis of the CBP complex polypeptides by SDS-polyacrylamide gel electrophoresis. CBP complex (~ 2 μg), purified as described under "Materials and Methods", was resolved on a 12.5% SDS-polyacrylamide gel followed by Coomassie blue staining.


Mr×10-3
200–
46–
30–

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 wit observations of Tahara et al. (9), and the 24 kDa polypeptide comigrates with purified 24-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 polypeptides, since polyclonal antibodies affinity purified with 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 polypeptide 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 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

FIGURE 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 under "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. Lane 3 contained 1 μg each of eIF-4A and eIF-4B.


1
2
3
eIF-4A

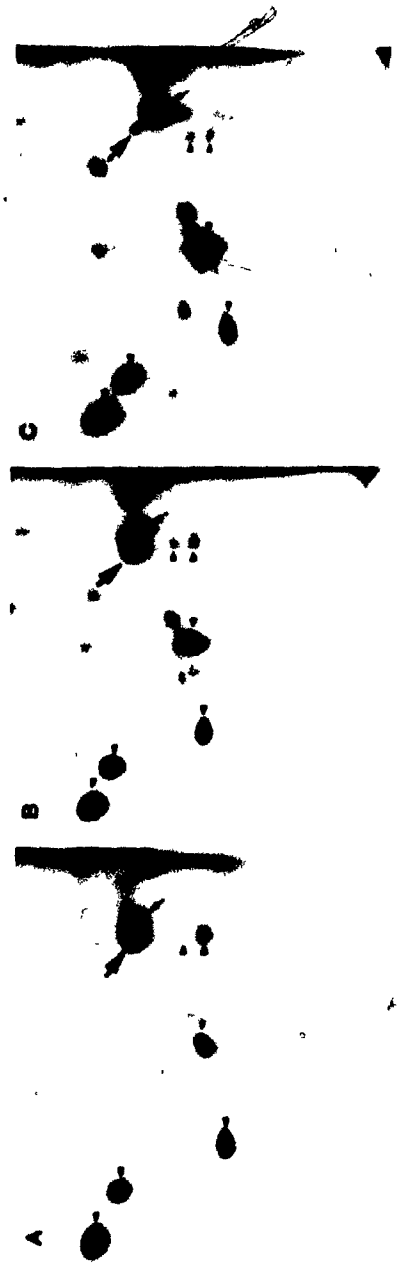
presence of both eIF-4A and eIF-4B in the CBP complex, but no reaction with a polypeptide corresponding to eIF-4B was detected (lane 2). In a control experiment, it can be seen that the mixture of antibodies against eIF-4A and eIF-4B reacted strongly with their cognate antigens (lane 3; streaking of 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^{2+}-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 (A), 50 kDa polypeptide (C), and a mixture of eIF-4A and the 50 kDa polypeptide (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 be prominent in the eIF-4A preparation (Fig. 4A), the peptide indicated with the thin arrow is prominent in the 50 kDa polypeptide of the CBP complex (Fig. 4C). 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

FIGURE 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 (1.5×10^5 to 2×10^5 cpm) were analyzed by electrophoresis in the first dimension and chromatography on cellulose-coated thin layer plates (Brinkmann) 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 V. Chromatography was in n-butyl alcohol/acetic acid/water/pyridine (15:3:12:10, v/v) for 5-6 h. 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 poly peptide of CBP complex; C, 50 kDA polypeptide of the CBP complex.



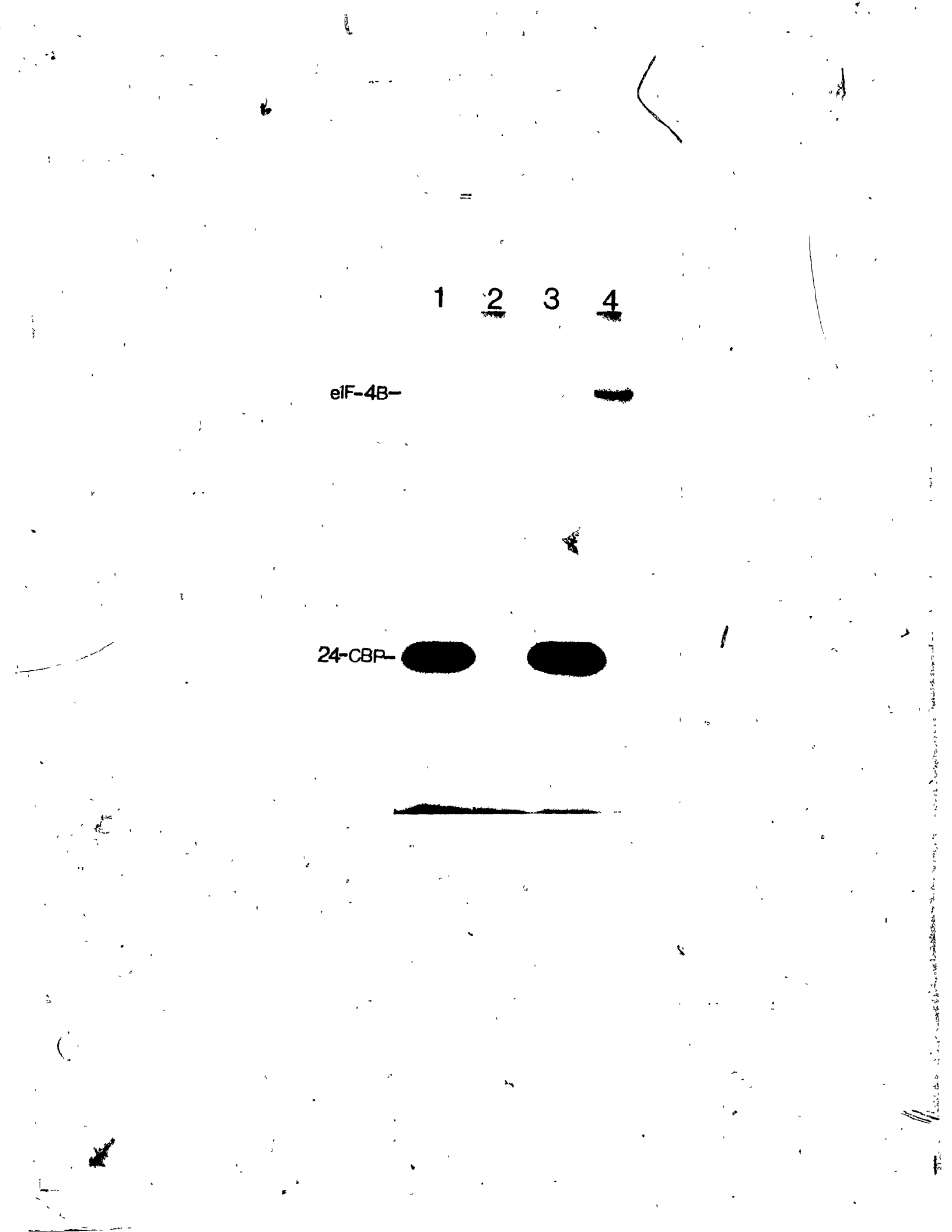
the free form and the CBP complex. In order to gain insight into this question we analyzed the cross-linking characteristics of the different species containing eIF-4A, and since eIF-4B has been implicated in the cap recognition process (4), we examined its involvement here.

In the absence of ATP, incubation of oxidized reovirus mRNA with the CBP complex alone results in cross-linking of the 24 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^{2+} 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 nonspecific 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 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. 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. 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

FIGURE 5

Cross-linking profile of CBP complex to [^{3}H]methyl-labeled oxidized reovirus mRNA in the absence of ATP-Mg^{2+}. Cross-linking was performed as described under "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.

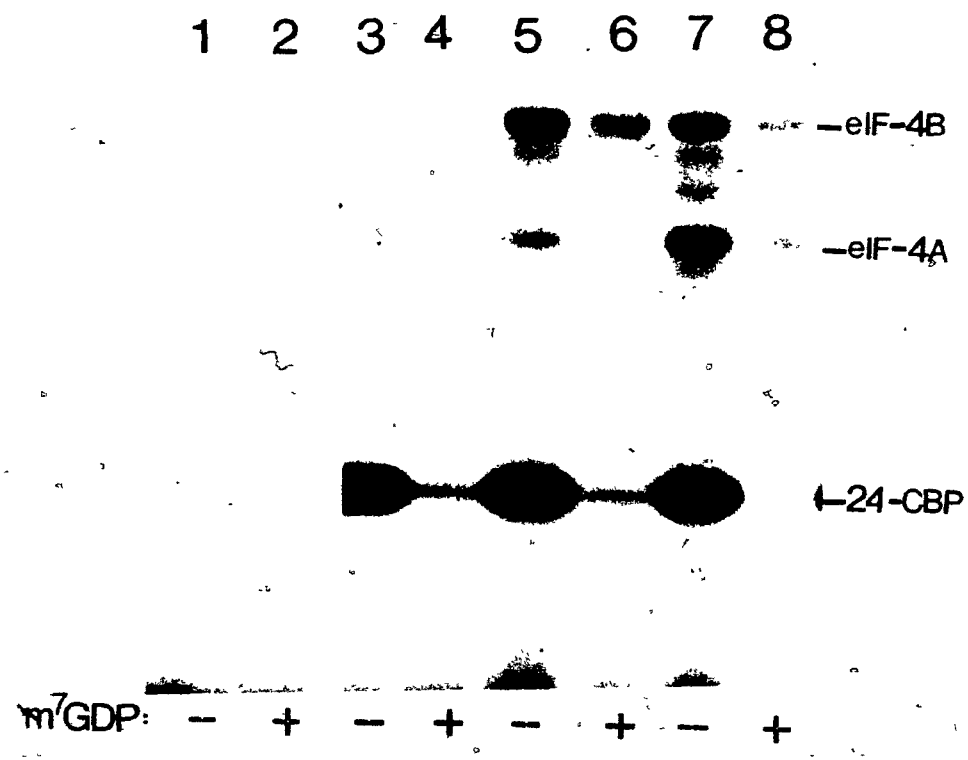

1 2 3 4
eIF-4B–
24-CBP–

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 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 with 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) (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 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.

FIGURE 6

Effect of CBP complex on cross-linking of eIF-4A and eIF-4B to 5' [^{3}H]methyl-labeled oxidized mRNA in the presence of ATP-Mg^{2+}.

[^{3}H]Methyl-labeled oxidized reovirus mRNA was incubated with initiation factors and samples processed for SDS-polyacrylamide gel electrophoresis and fluorography as described under "Materials and Methods" and in Fig. 5. Cross-linking was performed in the presence of 1 mM ATP and 0.5 mM Mg^{2+} and in the presence or absence of 0.7 mM m^7GDP as indicated on 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
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 with 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. 2). However, peptide analysis shows that eIF-4A and the 50 kDa polypeptide which reacts with the anti-CBP antibody are distinct polypeptides (H. Trachsel, 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% ammonium sulfate

fraction of initiation factors (14,15), while cross-linkable eIF-4A is found in the 0-40% ammonium sulfate fraction. This suggests that cross-linking of eIF-4A is dependent on factors present in the 0-40% ammonium sulfate fraction which could be complexed with eIF-4A. Fractionation of the complexed portion of eIF-4A in the 0-40% ammonium sulfate 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 observations 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^{2+} 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^{2+} 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 the CBP complex is required for a 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. lee, I. Edery and N. Sonenberg, 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. Edery, K.A.W. Lee and N. Sonenberg, 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 (corresponding to the 55 kDa polypeptide in Ref. 9) is not an integral component of the CBP complex in agreement with our data.

ACKNOWLEDGEMENTS

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CHAPTER 6

Isolation and Structural Characterization of Cap Binding Proteins from Poliovirus-Infected Hela Cells

SUMMARY

In poliovirus-infected HeLa cells, poliovirus RNA is translated at times when cellular mRNA translation is strongly inhibited. It is thought that this translational control mechanism is mediated by inactivation of a cap binding protein complex [comprising polypeptides of 24 (24-kilodalton cap binding protein), 50 and ~220 kilodaltons]. This complex can restore the translation of capped mRNAs in extracts from poliovirus-infected cells. We have previously shown that the virally induced defect prevents interaction between cap recognition factors and mRNA. Here, we show that the cap binding protein complex (and not the 24-kilodalton cap binding protein) has activity which restores cap specific mRNA-protein interaction when added to initiation factors from poliovirus-infected cells. Thus, the activity which restores the cap specific mRNA-protein interaction and that which restores the translation of capped mRNAs in extracts from poliovirus- infected cells, copurify. The results also indicate by an alternative assay, that the cap binding protein complex is the only factor inactivated by poliovirus. We also purified cap binding proteins from uninfected and poliovirus-infected HeLa cells. By various criteria, the 24-kilodalton cap binding protein is not structurally modified as a result of infection. However, the 220 kilodalton polypeptide of the cap binding protein complex is apparently cleaved by a putative viral (or induced) protease. By in vivo labeling and m^7GDP affinity chromatography, we isolated a modified cap binding protein complex from poliovirus-infected cells, containing proteolytic cleavage fragments of the 220 kilodalton polypeptide.

INTRODUCTION

The cap structure, $m^7GpppX(m)$ is found at the 5' terminus of almost all eucaryotic mRNAs, picornaviral and some plant viral RNAs being notable exceptions (27). Many studies have indicated that the cap structure facilitates 40S ribosome attachment to mRNA during initiation of translation (3,27) and it was anticipated that this function is mediated by a cap specific mRNA-protein interaction.

By chemical cross-linking to [^{3}H]-labeled oxidized capped viral mRNAs, it has been shown that polypeptides of 24, 50 and 80 kDa present in crude initiation factors (IF) from rabbit reticulocytes and several other mammalian sources (12, 19, 28 and KL & NS unpublished observations) specifically interact with the cap structure. The identity of two of these polypeptide is known: the 24 kDa polypeptide corresponds to the 24K cap binding protein (refs. 28, 29; see below) and the 50 kDa corresponds to eIF-4A based on the fact that it can be immunoprecipitated with a monoclonal antibody against eIF-4A (6) and that purified eIF-4A can be cross-linked to mRNA with similar characteristics to that of the 50 kDa polypeptide (6,10). The identity of the 80 kDa polypeptide is not established, but several results strongly suggest that it is eIF-4B. It was shown that this factor can specifically cross-link to the 5' oxidized reovirus mRNA only in the presence of ATP-Mg^{++} (6,10) as demonstrated for the 80 kDa polypeptide in preparations of crude IF. In addition cross-linking of eIF-4B requires the presence of other initiation factors. (eIF-4A in ref. 10 and CBP complex in ref. 6).

Polypeptides with affinity for the cap structure have been purified from rabbit reticulocytes by m^7GDP affinity chromatography. Originally a 24K cap binding protein (24K-CBP, CBPI or eIF-4E) was purified by

Sonenberg et al. (30) and subsequently a high molecular weight complex comprising the 24K-CBP and major polypeptides of 50 kDa and 220 kDa, was purified by several groups independently (6, 11, 31). This complex is referred to as CBP II, eIF-4F or the CBP complex (throughout this paper). The 50 kDa polypeptide is very similar to eIF-4A as determined by 2-D gel analysis (11), peptide map analysis (6) and immunoreactivity (6). Furthermore, both polypeptides exhibit similar mRNA cross-linking characteristics and therefore, we will refer to this polypeptide as eIF-4A. Experiments with purified factors have shown that the CBP complex, eIF-4B and mRNA are sufficient to reconstitute the cap specific mRNA-protein interaction observed by the chemical cross-linking assay when using crude initiation factors (6). These results suggest that interaction of the CBP complex and eIF-4B with the cap structure somehow facilitates attachment of 40S ribosomal subunits to capped mRNAs.

Poliovirus infection of HeLa cells results in a rapid and apparently quantitative inhibition of cellular protein synthesis, such that viral RNA is almost exclusively selected for translation (1,8). It was shown that crude IF from poliovirus-infected cells could stimulate translation of poliovirus RNA in vitro but had no such effect on translation of cellular mRNAs (16). Consequently, various groups were led to ask which particular initiation factor was inactivated by poliovirus. Using different approaches Helentjaris et al. (16) and Rose et al. (25) obtained evidence that eIF-3 and eIF-4B were inactivated, respectively.

These apparently conflicting observations were soon to be reconciled. On discovery that poliovirus RNA is naturally uncapped (17,22) it was an attractive hypothesis that inactivation of some form of CBP actually explains the shut-off of host protein synthesis. In accord with

this idea, Tahara et al. (31) purified a protein complex (~ 8-10S) by m^7GDP affinty chromatography which comprised major polypeptides of 24 (24K-CBP) 50, 94 and ~ 220 kDa and which could restore translation of capped mRNAs in extracts from poliovirus-infected cells. This latter activity is referred to as restoring activity. Recently, we have described a CBP complex (the CBP complex, ref. 6) comprising the 24K-CBP and polypeptides of 50 (eIF-4A) and ~ 220 kDa, which also has restoring activity (7). The 24K-CBP can also be detected in preparations of eIF-3 and eIF-4B (29), as can other CBP complex components (9,11), thus most likely explaining the effects previously attributed to these factors (16,25). By a different approach (19), we analyzed CBPs following poliovirus infection using the chemical cross-linking assay and showed that the cap binding activity of the 24, 50 and 80 kDa cap specific polypeptides was almost completely abolished following infection. In contrast to this, Hansen and Ehrenfeld (12), by using the cross-linking assay, reported no change in the amount of the 24 kDa polypeptide, but did find that the 24 kDa polypeptide no longer cosediments with eIF-3 following poliovirus infection (13). These results suggested a modification to CBP which possibly prevents a functional association between eIF-3 and CBP.

Taken together these results engender the belief that some form of CBP is indeed inactivated by poliovirus and a report from Etchison et al. (9) pointed to the likely mechanism. Using antisera against a 220 kDa polypeptide (P220) present in preparations of eIF-3 (under conditions in which the restoring activity fractionates with eIF-3), these authors showed that P220 is degraded in poliovirus-infected cells. The anti-P220 antibody also recognizes the ~ 220 kDa polypeptide of the CBP complex (9) and so it was proposed that proteolysis of

P220 by a poliovirus-dependent protease results in the shut-off of host protein synthesis.

Here we show that addition of the CBP complex to IF from poliovirus-infected HeLa cells (I-IF) can restore the interaction between the 80 kDa polypeptide (present in I-IF) and the cap structure, as assayed by the chemical cross-linking technique. The 24K-CBP has no such activity, strongly suggesting that restoration of the 80 kDa cap binding activity and restoration of capped mRNA function in extracts from poliovirus- infected cells, are due to the same activity. In an attempt to demonstrate directly the defect in the CBP complex caused by poliovirus, we isolated cap binding proteins from uninfected and poliovirus-infected HeLa cells. By various criteria, the 24K-CBP is unaltered by poliovirus infection, whereas the CBP complex is structurally modified. By in vivo labeling of cells and subsequent m^7GDP-affinity purification of CBPs, we obtained a CBP complex from poliovirus-infected HeLa cells which contains proteolytic cleavage fragments of P220.

MATERIALS AND METHODS

Cells and virus - Mouse L-929 cells and HeLa S3 cells were grown in suspension in 10% calf serum. Infection of L cells with reovirus type 3 (Dearing strain; 10 PFU/cell) and virus purification were performed as previously described (2). Infection of HeLa cells with poliovirus type 1 (Mahoney strain; 10-20 PFU/cell, except where otherwise indicated) was according to Rose et al. (25).

Preparation of [^{3}H]methyl-labeled oxidized reovirus mRNA - Synthesis of [^{3}H]methyl-labeled reovirus mRNA to a specific activity of ~ 8 x 10^4 cpm/μg with viral cores in the presence of S-adenosylmethionine (specific activity: 70 Ci/mmol, New England Nuclear; 1 Ci = 3.7 x 10^{10} becquerels) and periodate oxidation were according to Muthukrishnan et al. (21).

Preparation of crude protein synthesis initiation factors - Preparation of rabbit reticulocyte lysate, high salt wash of ribosomes (as a source of initiation factors) and subfractionation to a 0-40% ammonium sulphate fraction were as described by Schreier and Staehelin (26). Preparation of HeLa cell extracts and crude initiation factors was according to Lee and Sonenberg (19).

Cross-linking of mRNA to protein synthesis factors - [^{3}H]methyl-labeled oxidized reovirus mRNA was incubated with crude IF and/or purified CBPs (as described in the Figure Legends) for 10 min at 30°C essentially as described before (19), followed by addition of $NaBH_3CN$ (Aldrich, freshly prepared solution) overnight and RNase A to digest the mRNA. The samples were resolved on SDS/polyacrylamide gels and labeled bands detected by fluorography. All cross-linking incubations were carried out in the presence of ATP/Mg^{2+} as previously described (19).

Two dimensional gel electrophoresis - This was performed exactly as described by O'Farrell (23).

Purification of cap binding proteins - (a) Purification of rabbit reticulocyte CBP complex was essentially as described by Edery et al. (6). A 0-40% ammonium sulphate fraction of ribosomal high salt wash was layered on 12 ml, 10-35% linear sucrose gradients in Buffer A (20 mM Hepes pH 7.5, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 7 mM β-mercaptoethanol) containing 0.5 M KCl. Centrifugation was for 24 hours at 38,000 rpm in an SW40 rotor at 4°C. The top half of the gradient, excluding the fast sedimenting eIF-3 (>10S), was pooled and dialyzed against buffer A containing 0.1 M KCl and 10% glycerol. The dialyzed material was then loaded directly onto an m^7GDP-agarose affinity column (6,9) equilibrated in buffer A containing 0.1 M KCl and 10% glycerol. Non-specifically bound proteins were eluted by washing the column in 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 then eluted with 75 μM m^7GTP in buffer A containing 0.1 M KCl and 10% glycerol.

(b) Rabbit reticulocyte 24K-CBP was purified from the S100 fraction by a modification of the procedure of Tahara et al. (31). S100 was mixed with DEAE-cellulose (3 volumes of S100 and 1 volume of swollen DEAE-cellulose) equilibrated in low-column buffer (LCB;20 mM Tris pH 7.5, 0.2 mM EDTA, 7 mM β-mercaptoethanol) containing 80 mM KCl. The 24K-CBP binds to DEAE-cellulose under these conditions. The resin was then washed extensively with LCB containing 80 mM KCl to remove excess hemoglobin, followed by batch elution of bound material with LCB containing 250 mM KCl. The eluate was then concentrated by 0-50% ammonium sulphate fractionation, the precipitate dialyzed against LCB containing 200 mM KCl

and the dialyzed material diluted 2-fold before loading on to the m^7GDP-affinity column.

(c) Purification of CBP complex from HeLa cells was very similar to the purification of rabbit CBP complex, with the exception that the crude IF were not fractionated by ammonium sulphate. For each purification, not less than 20 liters of log phase HeLa cells at a cell density of 5×10^5 cells/ml, were used to prepare crude IF. In the case of infected lysates, infection was verified according to various criteria e.g. the presence of viral antigens by immunoblotting and the translational specificity (capped vs naturally uncapped mRNA translation) of cell extracts in in vitro translation.

(d) Purification of 24K-CBP from HeLa cells was achieved by passing total post- ribosomal supernatant (S100) over the m^7GDP-affinity column, followed by elution as described above for the CBP complex,

Purification of CBP complex from in vivo labeled HeLa cells - HeLa cells (13 ml at 4×10^5 cells/ml) were pelleted and resuspended in 8 ml of methionine free media containing 20% dialyzed fetal calf serum and 200 µCi/ml of [^{35}S]methionine (>1000 Ci/mmol, New England Nuclear). Labeling was for 6 hrs at which time, the cells were split equally in two. Half were infected with poliovirus in a volume of 400 µl at a multiplicity of infection of 50 PFU/cell and the other half were mock-infected. The conditions of adsorption and infection were as described by Rose et al. (25). At 3 hrs post-infection, cells were pelleted and resuspended in 180 µl of lysis buffer containing 150 mM NaCl, 20 mM Tris pH 7.5, 0.5% Nonidet-P40 and 2 mM phenylmethylsulfonyl fluoride. The suspension was adjusted to 600 mM KOAc and then left to stir on ice for 30 minutes. Crude IF (~ 200 µg) from uninfected cells was then added as carrier and

the cell extracts were sedimented through a linear 10-30% sucrose gradient in buffer A containing 0.5 M KOAc in an SW 50.1 rotor at 39,000 rpm for 15 hours. Catalase (11S) was run on a separate gradient and the material migrating slower than catalase in the gradient was pooled for m^7GDP-affinity chromatography. The pooled fractions were diluted with water to a final KOAc concentration of 100 mM and this material was loaded directly onto the affinity column. The column was then washed with 50 ml of LCB containing 100 mM KCl followed by 20 ml of 100 μM GDP in LCB containing 100 mM KCl. The first 1 ml of GDP eluate was collected. The affinity resin was then transferred to an Eppendorf tube and the cap specific polypeptides batch eluted with 100 μM m^7GDP in LCB containing 100 mM KCl.

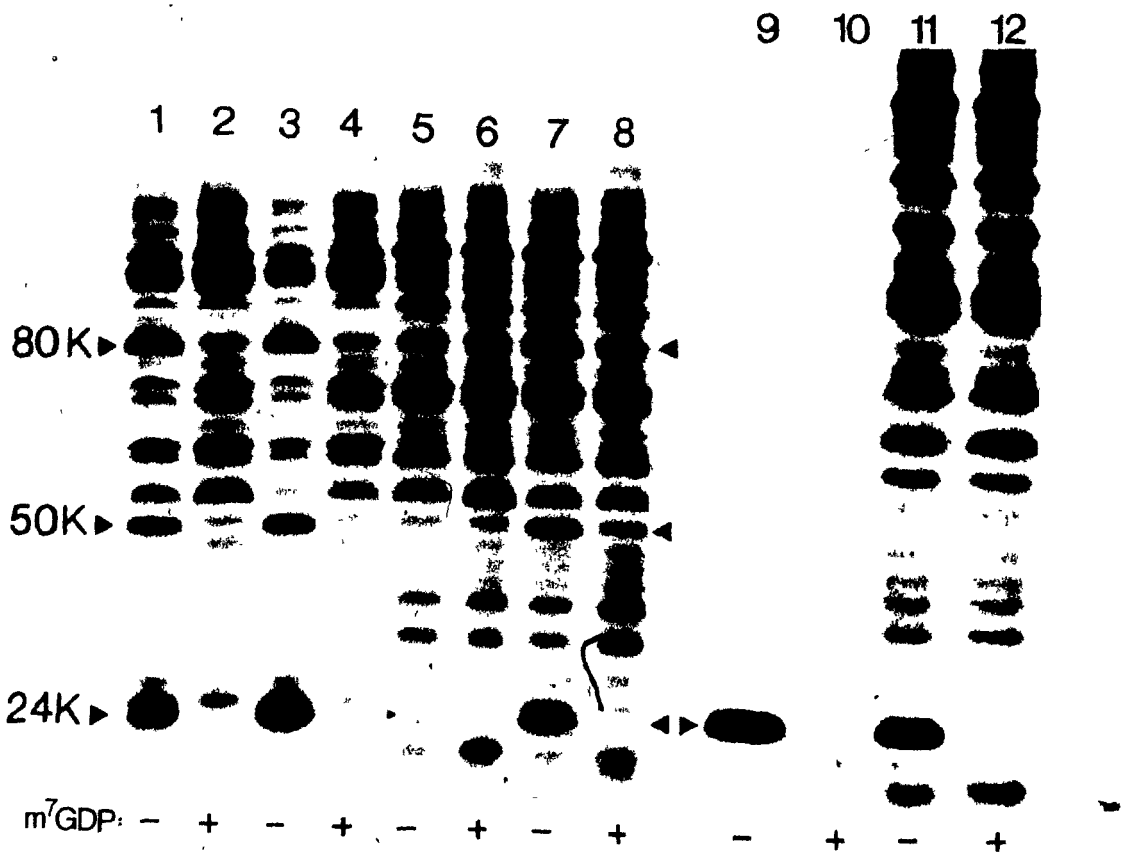
Preparation of polyclonal antisera against sheep CBP complex - This was carried out according to Vaitukaitis (34). The CBP complex was purified from sheep erythrocytes according to the protocol described for the purification of the 24K-CBP from rabbit reticulocyte S100 (see above). CBP complex (20 μg) in LCB containing 500 mM KCl was mixed with 1.2 volumes of complete Freund's adjuvant. This material was injected intradermally into the back of a rabbit in about twenty different spots. Four months later the rabbit was boosted subcutaneously with 20 μg of antigen injected in 3 different places in the back. One week later the rabbit was bled through the ear and serum prepared.

Immunoblot analysis (Western blotting) - This was performed essentially as described by Edery et al. (6). Polypeptides were resolved on a 10% SDS/polyacrylamide gel, transfered to nitrocellulose paper and

the blot was then incubated in Tris-buffered saline (TBS) pH 7.5 containing 1% BSA for one hour. This was followed by incubation overnight in TBS containing 1% BSA and the anti-CBP complex antibody. Blots were washed in TBS, followed by incubation with peroxidase conjugated goat anti-rabbit IgG and visualization of immunoreactive species by colour development with diaminobenzidene (32). Antisera against P220 (prepared as described above) was diluted 1:330 in TBS containing 1% BSA before incubation with nitrocellulose blots.

FIGURE 1

Effects of purified cap binding proteins on chemical cross-linking of crude initiation factors from poliovirus-infected cells. Crude IF, purified CBPs or mixtures of the two, were incubated under cross- linking conditions with [^{3}H]-oxidized reovirus mRNA as described in Materials and Methods. Labeled polypeptides were then resolved on 10% SDS/polyacryl amide gels followed by autoradiography. Lanes 1 and 2 contained ~ 100 μg of crude U-IF. Lanes 3 and 4, ~ 100 μg of crude U-IF plus 1.5 μg (containing ~ 0.3 μg of 24K-CBP) of rabbit reticulocyte CBP complex. Lanes 5 and 6, ~ 100 μg of crude I-IF. Lanes 7 and 8, ~ 100 μg of crude I-IF plus 1.5 μg (containing ~ 0.3 μg of 24K-CBP) of rabbit reticulocyte CBP complex. Lanes 9 and 10, 2 μg of rabbit reticulocyte 24K-CBP from the S100 fraction. Lanes 11 and 12, ~ 100 μg of crude I-IF and 2 μg of rabbit reticulocyte 24K-CBP. m^7GDP (0.67 mM) was included as indicated below the figure.


1 2 3 4 5 6 7 8 9 10 11 12
80K
50K
24K
m7GDP: − + − + − + − + − + − +

RESULTS

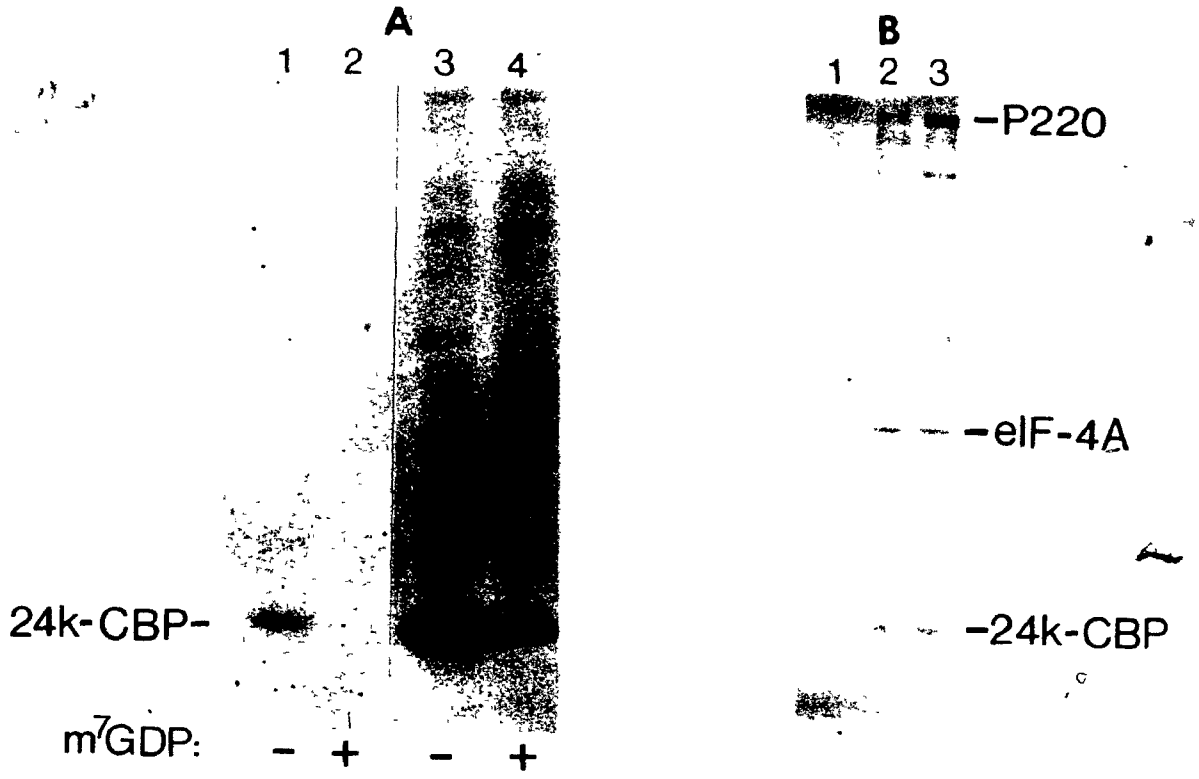
Originally Tahara et al. (31) and more recently Edery et al. (7) have shown that the activity which restores translation of capped mRNAs in extracts from poliovirus-infected HeLa cells (referred to as restoring activity), copurifies with the CBP complex but does not reside in the 24K-CBP (the 24 kDa subunit of the CBP complex). Using a different approach, we analyzed CBPs in IF from poliovirus infected cells (I-IF) by the chemical cross-linking assay and detected reduced levels of the 24, 50 and 80 kDa polypeptides when compared to the levels detected in IF from uninfected cells (U-IF) (19).

We wanted to test the hypothesis that the activities required to restore capped mRNA function in extracts from poliovirus-infected cells and for the interaction of the cap specific polypeptides with the cap structure (as assayed by cross-linking) are identical and reside in the CBP complex. To this end, we assayed the ability of purified CBP complex to restore cap specific cross-linking of the different cap specific polypeptides. We have previously demonstrated that cross-linking of the CBP complex by itself, in the presence or absence of ATP-Mg^{++}, results in cap specific cross-linking of the 24K-CBP only (ref. 6; the same preparation of CBP complex has been used in the current experiments). Addition of purified eIF-4B to the CBP complex (in the presence of ATP-Mg^{++}) results in cap specific cross-linking of eIF-4A and eIF-4B in addition to the 24K-CBP (6). These results strongly suggest that the 80 kDa polypeptide which can be cross-linked in crude IF is eIF-4B. Fig. 1 (Lane 1) shows the cross-linking profile of a total IF preparation from uninfected HeLa cells. Cross-linking of several polypeptides is inhibited by the addition of m^7GDP (compare Lane 2 with Lane 1), as previously reported by

us (19), and include the 24,50 and 80 kDa polypeptides. The 24 kDa polypeptide indicated in the figure is actually a doublet which corresponds to the 26 and 28 kDa cap specific polypeptides described in ref. 19. We also previously reported specific cross-linking of a polypeptide of ~ 32 kDa in U-IF (19) which can also be seen in Lane 1. The amount of this polypeptide varies in different preparations, however, and is often completely absent. Its significance, if any, is therefore not clear. Addition of the CBP complex to U-IF had no stimulatory effect on cross-linking of the 80 kDa cap specific polypeptide present in the IF preparation while there was a small increase in the amount of cross-linked 24 and 50 kDa cap specific polypeptides (2 fold, as determined by densitometry of the labeled bands, compare Lanes 3 and 1). Cross-linking of I-IF resulted in a very small amount of specific cross-linking of the 24 kDa polypeptide only, as previously reported (Lanes 5 & 6, ref. 19). However, addition of the CBP complex to I-IF restored the cross-linking profile to that observed when using U-IF alone (compare lanes 7 and 1; the 24, 50 and 80 kDa cap specific polypeptides are indicated by arrowheads to the right of Lane 8). It is not possible to tell from the cross-linking in Lane 7 whether the cross-linked 24 and 50 kDa polypeptides are contributed by the I-IF or the CBP complex, since both the 24 and 50 kDa polypeptides are present in both fractions. However, it is clear that the activity required for the cap specific cross-linking of the 80 kDa polypeptide is present in the added CBP complex and is lacking in the I-IF preparation. Since the 80 kDa polypeptide is not present in the CBP complex (6), the only interpretation of this experiment is that the 80 kDa polypeptide (probably eIF-4B) is not inactivated in poliovirus-infected cells as assayed by the cross-linking assay, in accord

FIGURE 2

Autoradiograph of cross-linked 24K-CBP as the free polypeptide or as part of the CBP complex and Coommassie blue staining of the two forms of CBP. (A) Cross-linking, SDS/polyacrylamide gel analysis and autoradiography were as described in Materials and Methods. Lanes 1 and 2 contained ~ 0.25 μg of 24K-CBP. Lanes 3 and 4 contained CBP complex containing ~ 0.5 μg of 24K-CBP. m^7GDP (0.67 mM) was included in lanes 2 and 4 as indicated below the figure. (B) SDS/polyacrylamide gel analysis of purified rabbit reticulocyte CBP sedimented through a 0.5 M KCl sucrose gradient. Rabbit reticulocyte CBP purified from ribosomes, was sedimented through a 10-30% linear sucrose gradient in LCB containing 0.5 M KCl to resolve the CBP complex from the free 24K-CBP. Aliquots from across the gradient were then resolved on a 10% SDS/polyacrylamide gel and stained by Coommassie blue. A section of the gradient is shown and sedimentation was from left to right (i.e. lane 1 is towards the top of the gradient). Material shown in lane 1 (30 μl of the fraction from the gradient) was used for cross-linking analysis in Fig. 2A, lanes 1 and 2. Material shown in lane 3 (30 μl of the fraction from the gradient) was used for cross-linking analysis in Fig. 2A, lanes 3 and 4.


A
1 2 3 4
24k-CBP-
m7GDP: - + - +
B
1 2 3
-P220
-eIF-4A
-24k-CBP

with earlier reports (5,16), and that it cannot cross-link to mRNA in I-IF because the CBP complex is inactivated. Our results also indicate (using an assay other than restoration of capped mRNA function in extracts from poliovirus-infected cells) that the CBP complex is the only initiation factor inactivated during poliovirus infection. It was also important to assay the ability of purified 24K-CBP to restore the cap specific cross-linking of the 80 kDa polypeptide in I-IF in light of data showing that it might be required for eIF-4B cross-linking (10). Figure 1 shows that the cross-linking of the 24K-CBP is completely sensitive to m^7GDP (compare Lanes 9 and 10). Addition of 24K-CBP to I-IF resulted in specific cross-linking of this polypeptide, albeit to a somewhat reduced level (compare Lanes 12 and 10), presumably due to competition for labeled oxidized mRNA from the vast excess of non-cap specific polypeptides present in the I-IF. Addition of the 24K-CBP, however, did not enhance the cross-linking of any other polypeptide in the I-IF preparation (Lanes 11 and 12). Thus, the ability of the CBP complex but not the 24K-CBP to restore the cross-linking of the 80 kDa polypeptide (probably eIF-4B), are consistent with previous results demonstrating that the CBP complex is absolutely essential for the cap specific cross-linking of eIF-4B (6) and indicate that this complex is inactivated in poliovirus-infected cells consistent with earlier reports (7,9,31).

In view of the fact that the 24K-CBP by itself does not restore cap specific cross-linking upon addition to I-IF, we wanted to find out why the amount of 24K-CBP detected by the cross-linking assay, is greatly reduced in I-IF compared to that for U-IF. It was also important to address this question in light of a previous report from Hansen et al. (12) that the amount of 24K-CBP detected by the cross-linking assay in

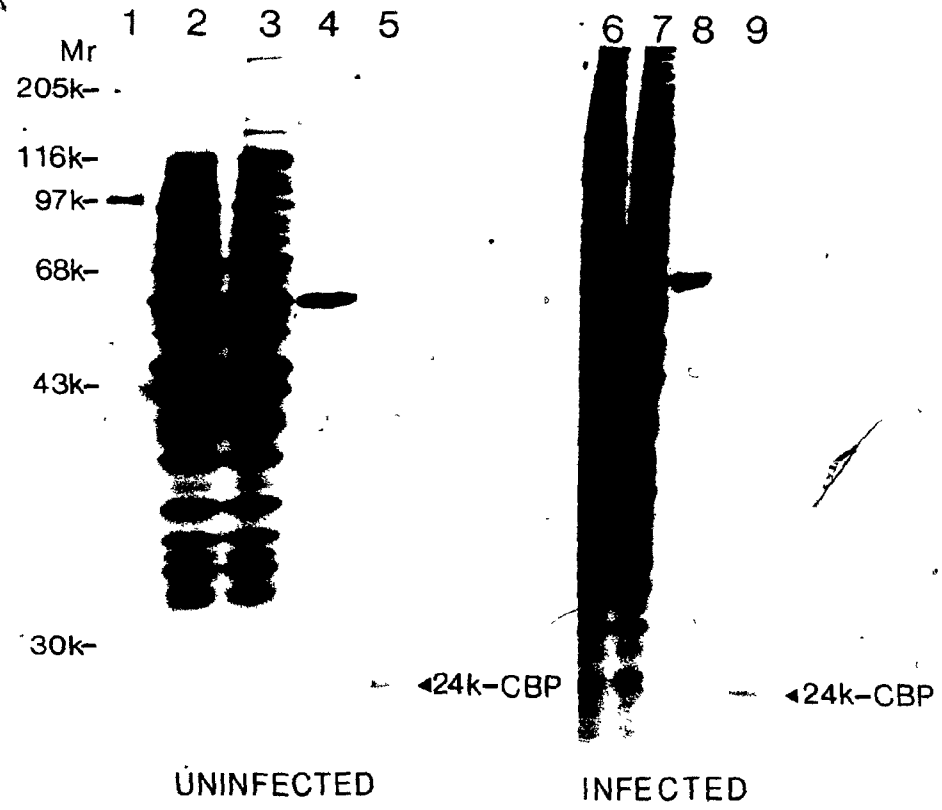
IF, was not reduced as a consequence of poliovirus infection. Since the CBP complex seems to be the factor which is inactivated, we tested the idea that cross-linking of the 24K-CBP to mRNA is more efficient when it is part of the CBP complex as compared to when it is in the free form. If this is true, then a defect in the CBP complex might indirectly affect the cross-linking of the 24K-CBP. We performed cross-linking experiments with purified 24K-CBP and CBP complex (containing an approximately equal amount of 24K-CBP). Fig. 2A shows cross-linking of 24K-CBP which is completely m^7GDP sensitive (lanes 1 and 2). It should be noted that approximately 8 fold less 24K-CBP was used here compared to the amount used in Fig. 1, lanes 9 and 10 and that the exposure time is different. This explains the substantial difference in the amount of cross-linked 24K-CBP observed in the two cases. Using the CBP complex there is a much higher amount of m^7GDP sensitive cross-linked 24K-CBP (compare lanes 3 and 4 to lanes 1 and 2). [Note that the autoradiogram is over exposed to show the cross-linked polypeptide in Lane 1.] Fig. 2B shows Coommassie blue staining of the samples used for the cross-linking experiments. m^7GDP-affinity purified rabbit reticulocyte CBP from ribosomes was run on a sucrose gradient in 0.5 M KCl to resolve the free 24K-CBP and the CBP complex which otherwise copurify on the cap affinity column. The gel shows a section of the gradient with lane 1 being towards the top of the gradient. Lanes 1, 2 and 3 represent contiguous gradient fractions. Lane 1 (Fig. 2B) shows a Coommassie blue stain of the material used for cross-linking analysis in Fig. 2A lanes 1 and 2, indicating the presence of the 24K-CBP and a small amount of the 50 kDa polypeptide. Lane 3 (Fig. 2B) is a Coommassie blue stain of the CBP complex used for cross-linking in Fig. 2A lanes 3 and 4, showing approximately two-fold more

24K-CBP than lane 1 (Fig. 2B) and the other major CBP complex polypeptides (50 kDa and ~ 220 kDa, ref. 6). The presence of the 50 kDa polypeptide (eIF-4A, in lane 1) sedimenting slower than the purified CBP complex (lanes 2 and 3) probably means that it tends to dissociate from the CBP complex to a slight degree during centrifugation under high salt (0.5 M KCl) conditions. It should be noted that the amounts of 24K-CBP used in these experiments fall in the linear range for the cross-linking assay (data not shown). Thus, it is clear from this data that the cross-linking efficiency of the 24K-CBP is considerably higher when it is part of the CBP complex. This most likely relates to the reduced level of cross-linked 24K-CBP observed in I-IF in which case the CBP complex is inactivated and indicates that the activity required to stimulate the cross-linking of 24K-CBP is impaired. We have also examined the translational restoring activity of the fractions shown in Fig. 2B and found that it correlates with the presence of the 220 kDa polypeptide (data not shown), consistent with previous observations (7,31). In summary, efficient cap specific cross-linking of the 24K-CBP to mRNA and translational restoring activity are both dependent on the CBP complex.

The results of Etchison et al. (9) which indicate that poliovirus causes proteolysis of the 220 kDa polypeptide (P220) of the CBP complex, provide the first evidence of a particular structural defect in the CBP complex. Again though, as in all previous attempts to characterize the defect in CBP caused by poliovirus, the approach was indirect. In an attempt to examine directly the abundance, structure and subcellular distribution of CBPs following poliovirus infection we purified them from uninfected or poliovirus-infected cells, using the m^7GDP-affinity chromatography technique.

FIGURE 3

Purification of 24K-CBP from the S100 fraction of uninfected and poliovirus infected cells. m^7GDP-affinity chromatography was performed as described in Materials and Methods. Purified fractions were resolved on a 10% SDS/polyacrylamide gel followed by Coommassie blue staining. Lane 1, 5 µl of molecular weight standards, 1 mg/ml protein (Sigma). Lane 2, ~ 100 µg of material from uninfected cells loaded onto the m^7GDP column. Lane 3, ~ 100 µg of flow through from uninfected material. Lane 4, 40 µl (from a total of 1 ml) of GDP eluate from uninfected cells. Lane 5, 50 µl (from a total of 500 µl) of m^7GDP eluate obtained from uninfected cells. Lane 6, ~ 200 µg of material from poliovirus-infected cells loaded onto the m^7GDP column. Lane 7, ~ 200 µg of flow through from infected cells. Lane 8, 40 µl (from a total of 1 ml) of GDP eluate from infected cells. Lane 9, 50 µl (from a total of 500 µl) of m^7GDP eluate from infected cells. Note that the total amount of protein loaded on the affinity column was the same for uninfected and infected cells.


1 2 3 4 5
6 7 8 9
Mr
205k–
116k–
97k–
68k–
43k–
30k–
◂24k–CBP
◂24k–CBP
UNINFECTED
INFECTED

Hansen et al., (14) were able to detect the 24K-CBP by chemical cross-linking in the S100 fraction of HeLa cells and we have purified homogeneous 24K-CBP from the S100 fraction of rabbit reticulocytes. Consequently, we attempted to purify the 24K-CBP from the S100 fraction obtained from equal amounts of uninfected or infected cells and Fig. 3 shows an SDS/polyacrylamide gel analysis of the purified fractions. Several assays were used to verify that the infected fractions used as starting material for the purification were actually infected e.g. mRNA (capped vs naturally uncapped) specificity of the corresponding cell extracts in translation and the presence of viral antigens by immunoblotting. Most of the polypeptides present in the S100 fraction are not retained during passage through the m^7GDP- coupled resin [e.g. compare lanes 2 and 3, which are the load and flow through respectively, from uninfected material]. Elution with 100 μM GDP shows a single polypeptide of Mr ~ 60,000 that either has affinity for the GDP moiety of the affinity column or, less likely, associates with the 24K-CBP via a GDP sensitive interaction (lane 4). The amount and size of this polypeptide are not affected by poliovirus-infection (compare lanes 4 and 8). Elution with 100 μM m^7GDP, yielded homogenous 24K-CBP (lane 5), which comigrates with the 24K-CBP of rabbit reticulocytes (data not shown). Again, neither the abundance or size of this polypeptide is altered following poliovirus infection (compare lanes 5 and 9).

In light of some speculation that the 24K-CBP becomes phosphorylated during poliovirus infection (18) and to examine the possibility that it undergoes some other kind of covalent modification, we performed two-dimensional (2-D) gel analysis (Isoelectric focussing in the first dimension and SDS/polyacrylamide gel electrophoresis in the

FIGURE 4

2-Dimensional gel analysis of 24K-CBP from uninfected and poliovirus infected cells. Samples of m^7GDP-affinity purified 24K-CBP (from lanes 5 or 9, Fig. 3) were resolved on 2-D gels according to O'Farrel (23) followed by Coommassie blue staining. (A) 0.5 μg of 24K-CBP from uninfected cells (U-24K-CBP); (B) 0.5 μg of 24K-CBP from poliovirus-infected cells (I-24K-CBP); (C) mixture of ~ 0.3 μg of U-24K-CBP and ~ 0.3 μg of I-24K-CBP.

UNINFECTED

B

INFECTED

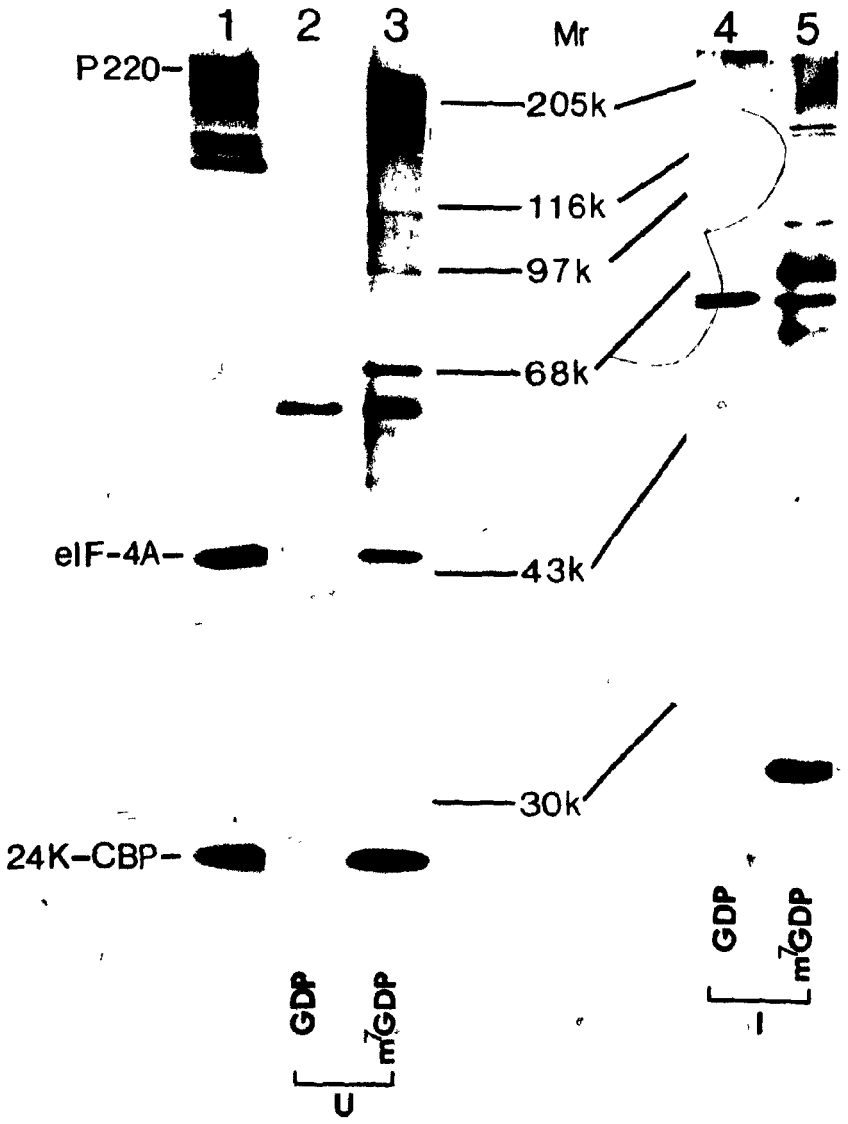
C

MIXING

second dimension) of the m^7GDP-affinity purified protein from uninfected and infected cells. Fig. 4 shows Coommassie blue staining of the 2-D gels. It is clear that the polypeptides from uninfected and infected cells comigrate in both dimensions (Fig. 4C, mixture of 24K-CBP from uninfected and infected cells) and that there is only one species with a slightly acidic isoelectric point of ~ 6.5. Thus, poliovirus infection has no effect on either the size, abundance or net charge of the 24K-CBP isolated from the S100 fraction, neither does it impair its ability to bind to a cap analogue, since it can be retained by and specifically eluted from the m^7GDP-affinity column. It was also important to examine the charge of the 24K-CBP associated with ribosomes in relation to its distribution between the free polypeptide and the CBP complex and hence in relation to the restoring activity. We performed these experiments with CBP isolated from rabbit reticulocytes since more manageable amounts of material are obtained from this source. Analysis of ribosomal 24K-CBP from rabbit reticulocytes (either as the free 24K-CBP or as part of the CBP complex) showed the presence of two major isoelectric variants as previously reported (30). There were however, no obvious differences in the relative abundance of these forms when comparing those associated with the CBP complex to those isolated as the free 24 kDa polypeptide (data not shown). We have not performed this analysis with HeLa ribosomal 24K-CBP, and therefore cannot exclude the possibility that it behaves differently. However, since the activity which can restore translation of capped mRNAs in extracts from poliovirus-infected cells, is associated with the CBP complex and is absent from the 24K-CBP isolated from ribosomes, it seems clear from the latter results that the restoring activity is

FIGURE 5

Purification of CBP complex from the ribosomal salt wash obtained from uninfected and poliovirus-infected cells. Fractions were purified on the m^7GDP-affinity column as described in Materials and Methods and resolved on a 10% SDS/polyacrylamide gel, followed by silver staining (20). Lane 1, 0.5 μg of CBP complex from rabbit reticulocyte ribosomes. Lane 2, 40 μl (from a total of 4 ml) of GDP eluate from uninfected cells. Lane 3, 30 μl (from a total of 1 ml) of m^7GDP eluate from uninfected cells. Lane 4, 40 μl (from a total of 4 ml) of GDP eluate from poliovirus-infected cells. Lane 5, 30 μl (from a total of 1 ml) of m^7GDP eluate from poliovirus-infected cells.


1
2
3
Mr
4
5
P220–
205k
116k
97k
68k
eIF-4A–
43k
30k
24K-CBP–
GDP
m7GDP
U
GDP
m7GDP

not related to a particular isoelectric variant of the 24K-CBP.

The rabbit reticulocyte CBP complex is defined as such by several criteria: 1) co-elution of the different polypeptides from the m^7GDP-affinity column; 2) co-elution and stability to several conventional purification steps including gel filtration; and 3) co-sedimentation of components of the purified CBP complex in sucrose gradients containing 0.5 M KCl. Further indication that the 24, 50 and 220 kDa polypeptides are complexed together comes from the fact that i) the 24 kDa polypeptide is the only polypeptide which, by itself, interacts with cap structures as assayed by chemical cross-linking (6) and ii) the purified 50 kDa polypeptide (eIF-4A) does not bind to the m^7GDP column (unpublished observations). Thus, although the CBP complex has not been rigorously characterized stoichiometrically or biophysically, there is good reason to believe that it represents a homogeneous biological entity.

The CBP complex was previously purified from the high salt wash of rabbit reticulocyte ribosomes by m^7GDP affinity chromatography (6, 9, 31) and we used a similar protocol to purify it from HeLa cells, with the exception that the IF were <u>not</u> fractionated with ammonium sulphate for technical convenience (see Materials and Methods). Fig. 5 shows SDS/polyacrylamide gel analysis of the purified fractions. Lane 1 contains a sample of rabbit reticulocyte CBP complex, showing major bands of 24 (24K-CBP), 50 (eIF-4A) and ~ 220 kDa (P220) as previously shown (6) and some degradation product of P220, identified as such by tryptic peptide mapping (data not shown) and the presence of common antigenic determinants (9). Lane 2 shows the GDP eluate obtained when material from uninfected cells was loaded on the m^7GDP column. Again, as was the case for S100 fractions, a single polypeptide of Mr ~ 60,000 is eluted.

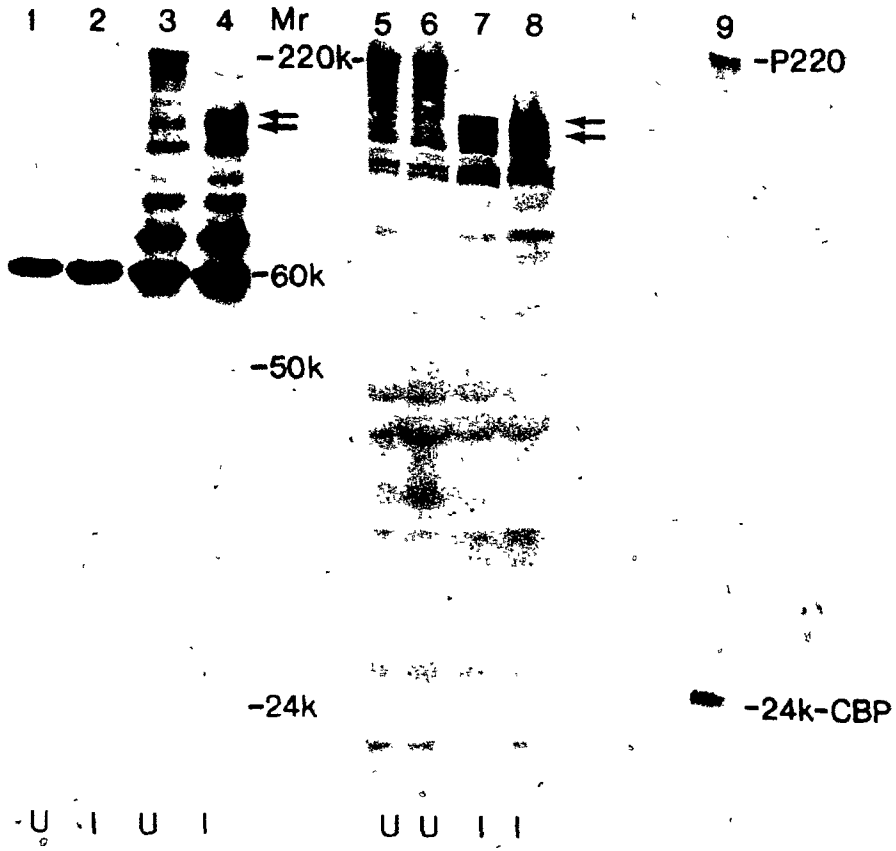
This is presumably the same polypeptide as obtained from the S100 fractions (Fig. 3, lane 4). Lane 3 shows the m^7GDP eluate obtained from uninfected cells. The 24, 50 and ~ 220 kDa polypeptides comigrate with their reticulocyte counterparts, although the ~ 220 kDa polypeptide is a smear, presumably due to proteolysis. In addition, there are bands of ~ 60 and 70 kDa and other minor bands. The 60 kDa polypeptide is not associated with the CBP complex since it can be completely removed by extensive washing of the affinity column with GDP before elution with m^7GDP. The 70 kDa polypeptide is specifically eluted with m^7GDP and may therefore correspond to the ~ 70 kDa polypeptide previously described in preparations of CBP II (11). These results show that the CBP complex from HeLa cells is structurally very similar to the rabbit reticulocyte CBP complex, a result which accords with the high degree of conservation of protein synthesis initiation factors between rabbits and humans (4). Purification of CBP from the ribosomal high salt wash obtained from poliovirus-infected HeLa cells yielded distinctly different results (Fig. 5, lanes 4 and 5). The samples were run on a different gel to that shown in lanes 1-3 and the corresponding molecular weights are indicated in the figure. Lane 4 shows the GDP eluate obtained from infected cells, showing again an ~ 60 kDa polypeptide. The amount and size of this polypeptide are again not changed due to poliovirus infection (compare lanes 4 and 2). There is also staining just either side of the ~ 60 kDa polypeptide which is an artifact of the silver staining procedure (20) and does not represent purified polypeptides. Lane 5 shows the m^7GDP eluate obtained from poliovirus-infected cells. There is no change in either the amount or size of the 24 kDa polypeptide while the remaining m^7GDP specific bands are distinctly different. Firstly, the ~ 220 kDa and 50

kDa (eIF-4A) polypeptides are almost completely absent. Second, there are two new bands of ~ 130 kDa which are not present in the preparation from uninfected cells (compare lane 5 and 3). While it should be borne in mind that silver staining of polypeptides is not necessarily quantitative, it does appear that the amount of the ~ 130 kDa polypeptides is significantly less than the amount of 24 kDa polypeptide, particularly on a molar basis. The presence of 24K-CBP in the m^7GDP eluate obtained from infected cells, serves as a useful internal control and argues strongly against non-specific loss of the other CBP complex polypeptides (50 and ~ 220 kDa). Furthermore, the observation that homogeneous 24K-CBP is obtained by m^7GDP-affinity purification of the post- ribosomal supernatant from infected cells, indicates that the CBP complex is not merely redistributed in the infected cell such that it no longer associates with ribosomes. Thus, a reasonable interpretation of these results is that the CBP complex is modified following poliovirus-infection. The significance of the ~ 130 kDa polypeptides will be addressed later in light of the results presented in Fig. 6 and other data.

The purification of large amounts of the CBP complex from HeLa cells is a somewhat cumbersome and time consuming activity, yielding only around 50 μg of CBP complex from 10^{10} log phase HeLa cells (20 liters of cells at 5 x 10^5 cells/ml). There are in addition, many steps between the cell harvest and the m^7GDP-affinity purification, possibly contributing to artifactual disintegration of the native CBP complex as it exists in the cell. Consequently, we decided to label mock and poliovirus-infected cells with [^{35}S]methionine and attempt to isolate the CBP complex by a faster protocol. Cells were labeled for 6 hours with [^{35}S]methionine. At the end of this time, the cells were divided equally

FIGURE 6

Purification of in vivo labeled CBP complex from uninfected and poliovirus-infected HeLa cells. Fractions were purified on an m^7GDP-affinity column as described in Materials and Methods and resolved on a 10% SDS/Polyacylamide gel followed by autoradiography. Lane 1, 40 µl (from a total of 1 ml) of GDP eluate obtained from uninfected cells. Lane 2, 40 µl (from a total of 1 ml) of GDP eluate from poliovirus-infected cells. Lane 3, 50 µl (from a total of 500 µl) of m^7GDP eluate from uninfected cells. Lane 4, 50 µl (from a total of 500 µl) of m^7GDP eluate from poliovirus-infected cells. Polyclonal antisera against the CBP complex was used to probe extracts from uninfected and poliovirus-infected HeLa cells for P220 related antigens. S10 extracts were resolved on a 10% SDS/polyacrylamide gel followed by western blotting as described in Materials and Methods. The figure shows immunoreactive species in lanes 5-9. Lanes 5 and 6, 150 µg of protein from different S10 extracts from uninfected cells. Lanes 7 and 8, 150 µg of protein from different S10 extracts from poliovirus-infected cells. Lane 9, 5 µg of rabbit reticulocyte CBP complex.


1
2
3
4
Mr
5
6
7
8
9
-220k-
-P220
-60k
-50k
-24k
-24k-CBP
U
I
U
I
U
U
I
I

in two and half were infected with poliovirus while the other half was mock-infected. In order to monitor the infection we performed a mock-labeling experiment in which [^{35}S]methionine was added at 2.5 hours post infection to previously unlabeled cells, in the presence and absence of poliovirus. The in vivo labeling pattern observed between 2.5 and 3 hours post-infection (at which time the cell extracts were prepared) confirmed that shut-off of cellular protein synthesis was complete and that virus specific proteins were being synthesized (data not shown).

Fig. 6 shows the results of the purification of [^{35}S]methionine labeled CBP from uninfected and poliovirus-infected HeLa cells. Elution of the m^{7}GDP column with 100 μM GDP yielded a single polypeptide of Mr ~ 60,000 (lane 1, uninfected). This is presumably the same polypeptide observed when unlabeled material was used for the purification of 24K-CBP from the S100 fraction (e.g. Fig. 3, lane 4). Again, the amount and size of this polypeptide are not affected by poliovirus infection (compare lane 2 to lane 1). Lane 3 shows the m^{7}GDP eluate obtained from uninfected material (including the 60K polypeptide which is particularly abundant and is not completely washed off during the elution with GDP in this experiment). The material eluted has polypeptides comigrating with the 24, 50, 220 kDa polypeptides of rabbit reticulocyte CBP complex (indicated by molecular weight to the right of lane 4). However, we have no evidence at present to prove that the 24 and 50 kDa polypeptides are indeed the 24K-CBP and eIF-4A respectively. The other bands (Mr = 35,70 kDa, etc.) are either related to the CBP complex since they are specifically eluted with m^{7}GDP (compare lane 3 and 1) or alternatively are other proteins which bind specifically to the column (the criterion for specificity being elution with m^{7}GDP and not with GDP). Since the

load onto the column contains total soluble cell protein and also proteins solubilized by 0.5 M KCl and 0.5% Nonidet-P40 it is likely that some, at least, of the additional polypeptides are not related to the CBP complex. For example, likely candidates are putative nuclear cap binding proteins as reported (24). Another possibility is that the additional polypeptides are loosely associated with the CBP complex but are lost during the purification protocols previously employed (6, 11, 31). It should be noted that the relative labeling intensities of the 24, 50 and ~ 220 kDa polypeptides is not equal. This might be accounted for, in part, by the size of the polypeptides (assuming an average methionine content for each polypeptide) but may also reflect different rates of entry of the newly synthesized components into the CBP complex. In addition, the relative labeling intensity of the 24, 50 and 220 kDa polypeptides varies among different preparations from uninfected cells (unpublished observations). When material from poliovirus-infected cells was loaded onto the column, the m^7GDP eluate obtained was distinctly different from that obtained for uninfected material (lane 4). There was no change in either the abundance or size of the 24 and 50 kDa polypeptides, while in contrast, there was no detectable ~ 220 kDa polypeptide. Instead there are additional bands of Mr ~ 130 kDa (indicated by arrows) which are completely absent from the m^7GDP eluate obtained from uninfected cells (Compare lane 4 to lane 3). The size and abundance of all the other bands present in the m^7GDP eluate are also <u>not</u> affected by poliovirus infection (compare lane 4 to lane 3).

These results demonstrate that P220 is cleaved by a putative viral (or induced) protease but that the presumed cleavage products (~ 130 kDa polypeptides) are still retained and can be specifically eluted from the

m^7GDP affinity resin. This suggests that the presumed cleavage products remain associated with a cap binding component (most probably the 24K-CBP) in the form of a modified CBP complex. It seems to us extremely unlikely, although admittedly not precluded, that the cleavage products derived from P220 would have a cryptic m^7GDP binding site and thus bind directly to the m^7GDP affinity resin. The difference in the amounts of the ~ 130 kDa cleavage products obtained in Fig. 6 (lane 4) as compared to Fig. 5 (lane 5) suggest that the putative modified CBP complex is not stable to the purification protocol employed for the experiments in Fig. 5. This may also explain the absence of the 50 kDa polypeptide in Fig. 5.

The existence of proteolytic cleavage fragments of P220 of Mr ~130 kDa, accords with the original observation of Etchison et al. (9), who demonstrated the appearance of such polypeptides in crude HeLa cell extracts, following poliovirus infection. In this case, the cleavage products observed are thought to be related to P220 of the CBP complex by virtue of common antigenicity. We have recently raised polyclonal antibodies to the purified sheep CBP complex which bind strongly to the 24 and 220 kDa polypeptides of rabbit CBP complex (Fig. 6, lane 9). Consequently, we probed HeLa cell extracts with this antisera and obtained very similar results to those of Etchison et al. (9). In lanes 5 and 6, different extracts from uninfected cells and in lanes 7 and 8, different extracts from poliovirus-infected cells, were probed with the anti-CBP complex antisera. The antisera does not react with the HeLa cell 24K-CBP (lanes 5-8). However, the antisera clearly reacts with the ~ 220 kDa polypeptide present in extracts from uninfected cells (lanes 5 and 6), and this antigen comigrates with P220 of the rabbit CBP complex

(compare lane 5 to lane 9). There is no detectable 220 kDa polypeptide in extracts from poliovirus-infected HeLa cells, while there are putative degradation products of Mr ~ 130 kDa(lanes 7 and 8, indicated by arrows). These latter bands comigrate with the cleavage products present in the putative modified CBP complex isolated by m^7GDP-affinity chromatography from poliovirus-infected cells (compare lanes 7 or 8 with lane 4), strongly suggesting that they are identical.

DISCUSSION

Analysis of eucaryotic mRNA CBPs by the chemical cross-linking assay indicates a complex interaction primarily between polypeptides of 24, 50 and 80 kDa, ATP and mRNA. This interaction is presumed to facilitate 40S ribosomal subunit attachment to cellular mRNA during translation and is very discretely prevented upon poliovirus infection of HeLa cells, thus resulting in shut-off of cellular protein synthesis. Recently, Edery et al. (6) have demonstrated that the cap specific mRNA-protein interaction observed between crude initiation factors and mRNA, can be reconstituted using the CBP complex (containing eIF-4A as a subunit), eIF-4B and mRNA. These factors appear to intereact with mRNA in close concert, since the m^7GDP sensitive cross-linking of eIF-4A as part of the CBP complex is strictly dependent on eIF-4B and the cross-linking of eIF-4B is likewise dependent on the CBP complex. This idea accords with the simultaneous loss of the cross-linking ability of all the cap specific polypeptides following poliovirus infection (19), again consistent with a close functional relationship between them. Thus, the virally induced lesion in the CBP complex is probably sufficient, by itself, to prevent interaction of eIF-4A and the 80 kDa polypeptide (probably eIF-4B) with the cap structure and consequently block 40S ribosome attachment to cellular mRNAs. The CBP complex has activity which restores the specific cross-linking profile when added to IF from infected cells. This activity is not present in the 24K-CBP and thus copurifies with the translational restoring activity, strongly suggesting that the two activities are identical. The fact that eIF-4A and eIF-4B are neither structurally modified (5) nor functionally impaired (16) following poliovirus infection, is consistent with our observation that

the 80 kDa polypeptide (probably eIF-4B) is present and active in IF from infected cells, at least, as assayed by chemical cross-linking to mRNA in the presence of exogenous CBP complex.

The 24K-CBP from infected cells, either as the free polypeptide or as part of the putative modified CBP complex, can recognize the cap structure, as indicated by the fact that it can be purified by m^7GDP-affinity chromatography (Figs. 3 and 5). However, the amount of cross-linked 24K-CBP in I-IF is considerably lower than that from U-IF. In light of our finding that the cross-linking of the 24K-CBP from rabbit reticulocytes is greatly enhanced when it is part of the CBP complex as compared to the free polypeptide, it seems likely that the 24K-CBP in the putative modified CBP complex from poliovirus-infected cells behaves like the free 24 kDa polypeptide in terms of cross-linking to mRNA. This again points to a significant role for P220 in mediating the interaction between the 24K-CBP and mRNA.

We have presented direct evidence that the 24K-CBP is not structurally modified following poliovirus infection. Furthermore, the subcellular distribution of the 24K-CBP is not changed. In contrast, the native CBP complex cannot be purified from any fraction obtained from poliovirus-infected cells. These results demonstrate directly that the native CBP complex is modified by poliovirus infection. The in vivo labeling experiments indicate that a modified CBP complex exists in infected cells which contains the proteolytic cleavage products of P220, and possibly eIF-4A. Similar results are obtained for purification of unlabeled ribosomal CBP from infected cells, although in this case eIF-4A is definitely absent and the amount of the ~ 130 kDa polypeptides seems significantly reduced. Thus, the exact structure of the modified CBP

complex is uncertain. In other experiments we have obtained the 24K-CBP in free form from ribosomal high salt of infected cells, which suggests that the modified CBP complex is unstable. In addition, the eIF-4A component of the CBP complex is apparently not as strongly associated with the CBP complex as the other components as indicated by the observation that a small amount of eIF-4A dissociates from the CBP complex under high salt conditions (see Fig. 2B). This might well explain the lack of eIF-4A in the ribosomal CBP isolated from infected cells, in which case P220 is cleaved, possibly resulting in decreased affinity of eIF-4A for other complexed components. In any event, the results presented here suggest that an intact P220 is essential for CBP complex function. First, efficient cap specific cross-linking of the 24K-CBP, eIF-4A and eIF-4B is dependent on the CBP complex and does not occur following poliovirus infection. Second, restoring activity is likewise a property of the CBP complex and correlates with the presence of P220 as opposed to the 24K-CBP or eIF-4A. Whether or not the association of the modified CBP complex with ribosomes from infected cells reflects an involvement in translation of poliovirus RNA remains to be determined. Indeed, the mechanism by which poliovirus RNA initiates translation is something of a mystery, both in terms of any possible role of CBP(s) (modified or otherwise) and concerning which structural features of the viral messenger allow efficient translation in the absence of the cap structure.

The question concerning the relationship between the viral dependent protease which cleaves P220 and the poliovirus replicative cycle now challenges. One possibility is that the activity which processes the poliovirus primary cleavage products (P3-7C) is also responsible for

cleavage of P220. However, this appears unlikely, in light of our recent data that antibodies directed against poliovirus protein P3-7C do not inhibit cleavage of P220 in vitro (K.A.W.L., I.E., R. Hanecak, E. Wimmer and N.S. submitted for publication). Therefore, there might be another viral protease involved in this cleavage or induction of a cellular function, possibly one which is involved in regulation of protein synthesis in a broader sense. The availability of mutants derived from infectious cloned poliovirus DNA should aid in approaching these problems.

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CHAPTER 7

Poliovirus Protease P3-7c Does Not Cleave P220 of the Eukaryotic mRNA Cap Binding Protein Complex

ABSTRACT

Infection of HeLa cells by poliovirus results in proteolysis of the large subunit (P220) of the cap binding protein complex. This is believed to cause the rapid shut-off of host protein synthesis during poliovirus infection. In this communication we examined the possible involvement of poliovirus proteins P3-7C (a proteinase) and P2-X in cleavage of P220. Using antisera against these two viral polypeptides we were unable to inhibit proteolysis of P220 in an in vitro assay. These results indicate that viral proteins P3-7C and P2-X are not directly involved in cleaving P220 and hence causing shut-off of cellular protein synthesis.

INTRODUCTION

The mechanism by which poliovirus inhibits HeLa cell protein synthesis, a subject of intense study for several years (4), has recently been clarified in some respects. In vivo, poliovirus causes a rapid and extensive inhibition of cellular (capped) mRNA translation, whereas translation of the naturally uncapped poliovirus RNA proceeds with high efficiency (1). Many lines of evidence have demonstrated that the failure of capped mRNAs to enter polysomes is due to a virally induced defect in the translation initiation machinery of the host cell (for a recent review see ref. 4). The fact that cell extracts prepared from poliovirus-infected cells are also specifically deficient in an activity required for capped mRNA translation (3,10,11,13) and thus faithfully mimic the in vivo situation, provided an assay for the factor which is inactivated. Consequently, it has been shown that the cap binding protein (CBP) complex (also termed eIF-4F or CBP II) can restore translation of capped mRNAs in extracts from poliovirus-infected cells (3,13) or in a reconstituted translation system from poliovirus-infected cells (6) and thus it is thought that poliovirus achieves inhibition of cellular protein synthesis by somehow inactivating the CBP complex.

The CBP complex consists of three polypeptides, the 24K-CBP (also termed CBP I or eIF-4E), eIF-4A and an ~ 220 kDa polypeptide (2,7). Etchison et al. (5) have presented evidence which indicates that the 220 kDa polypeptide is cleaved by a viral dependent protease, yielding cleavage fragments of ~ 130 kDa. Subsequently, we have isolated a modified CBP complex (by using m^7GDP affinity chromatography) from poliovirus-infected HeLa cells, which contains proteolytic fragments of P220, with apparent molecular weights of ~ 130 kDa (Lee et al., J. Virol. In

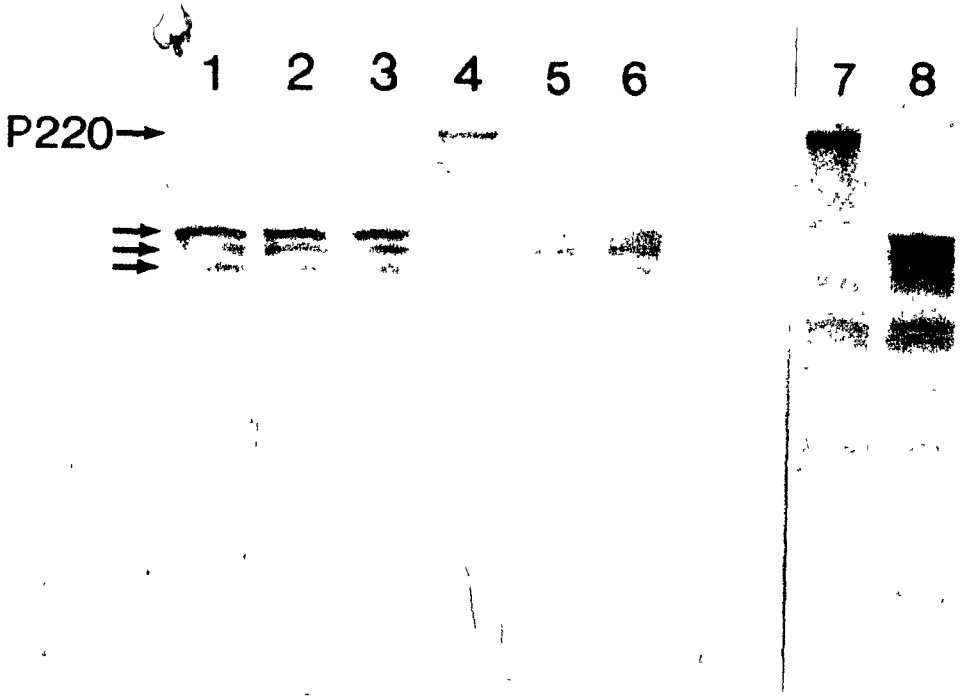
Press). While it remains to be demonstrated directly that proteolysis of P220 results in loss of activity of the CBP complex it is clearly most likely that proteolysis of P220 is the cause of inhibition of cellular protein synthesis.

It is currently not known whether the protease activity which cleaves P220 is virally encoded or whether it is an induced cellular activity. The poliovirus genome encodes at least one protease activity (P3-7C, ref. 8) and may have protease activities mapping elsewhere in the genome. Protein P3-7C is known to process the viral polyprotein to produce most of the viral polypeptides by cleavage between Gln-Gly amino acid pairs (8). There are, however, other cleavage sites (1 Asn-Ser and 2 Tyr-Gly) that are not cleaved by P3-7C. The protease(s) responsible for these other cleavage events is unidentified. It has been shown (8), however, that the activity does not appear to reside in P2-X as had been previously claimed (9).

Several studies have shown that the virus dependent activity which is responsible for inactivating the CBP complex and consequently for the inhibition of cellular translation, can be assayed in vitro. Originally, Rose et al. (11) showed that translational restoring activity (i.e. the activity which can restore capped mRNA function in extracts from poliovirus-infected HeLa cells) can be slowly inactivated upon incubation with a cell extract from poliovirus-infected cells. We have confirmed these results (10) and have also shown that crude initiation factor preparations from infected cells have an activity which can slowly impair the cap binding activity of polypeptides present in crude initiation factors from uninfected cells (10). These observations are consistent with the contention that the mechanism by which cellular protein synthesis is

FIGURE 1

In vitro assay for the protease which cleaves P220 of the cap binding protein complex. HeLa S3 cells were grown in media supplemented with 5% calf serum. Poliovirus type 1 (Mahoney Strain) infection of HeLa cells was performed as previously described with 10-20 plaque forming units per cell, and preparation of cell extracts was as previously described (10,11). Extracts were mixed (as indicated below) and incubated for 30 minutes at 37°C. Reactions were stopped by addition of electrophoresis sample buffer and resolved on 10% polyacrylamide gels containing SDS. Following electrophoresis, polypeptides were transferred to nitrocellulose paper according to Towbin et al., (14). Nitrocellulose blots were pre-saturated with 1% bovine serum albumin in TBS (10 mM tris pH 7.5, 150 mM NaCl) for 30 minutes at room temperature. Blots were incubated with anti-P220 antiserum in 1% BSA in TBS for 3 hrs at room temperature. The antisera was raised in rabbits against sheep CBP complex injected intradermally (15) as described elsewhere (K.L. et al., J. Virol. in press) and was diluted 2000 fold in 1% BSA in TBS before use. Blots were subsequently washed with six changes of TBS over a period of 30 minutes followed by incubation with peroxidase conjugated goat anti-rabbit IgG (Boehringer Mannhein) diluted 1000 fold in 1% BSA in TBS for 1 hour. Immunoreactive species were then visualized by staining with diaminobenzidene as described elsewhere (14). Lane 1 contained 10 µl of S10 extract from uninfected cells (U-S10) and 5 µl of S10 extract from poliovirus-infected cells (I-S10). Lane 2, 10 µl of U-S10 and 2.5 µl of I-S10. Lane 3, 10 µl of U-S10 and 1 µl of I-S10. Lane 4, 10 µl of U-S10 only. Lane 5, 10 µl of U-S10 and 5 µl of I-S10 which were not incubated at 37°C for 30 minutes. Lane 6, an aliquot of the reaction mixture used in Lane 5, following 30 minutes incubation at 37°C. Lanes 7 and 8, U-S10 and I-S10 respectively, which were not incubated.


1 2 3 4 5 6 7 8
P220

inhibited in poliovirus-infected cells is catalytic in nature and is not a result of steric hindrance by some viral protein. Finally, Etchison et al. (5) have shown that the protease activity which cleaves P220 of the CBP complex can be detected in crude initiation factors from poliovirus-infected cells.

Using polyclonal antisera against P220 of sheep erythrocytes we probed extracts from either uninfected (U-S10) or poliovirus-infected (I-S10) cells and obtained the same results as Etchison et al. (5). Fig. 1 is an immunoblot showing that the anti-P220 serum reacted mainly with a 220 kDa polypeptide present in U-S10 (lane 7) whereas this polypeptide was absent in I-S10 (lane 8). Instead, the antisera recognized in I-S10, polypeptides that are presumably cleavage products of P220 with molecular weights between 110-130 K (lane 8). In an attempt to assay the protease activity in vitro, we mixed U-S10 and I-S10 and monitored proteolysis of P220 by probing with anti-P220. As a control we incubated U-S10 alone for 30 minutes, and found that P220 is stable under these conditions (lane 4). We have repeated this experiment with many different cell extracts and have never detected degradation of P220, even after longer incubation times (data not shown), an observation suggesting that P220 is not intrinsically unstable. Lanes 1-3 show mixtures of U-S10 with decreasing amounts of I-S10. The results show that a ratio of U-S10:I-S10 of 2(10 µl of U-S10 and 5 µl of I-S10, in which both extracts contained equal protein concentration as determined by A_{280}/A_{260} readings) is sufficient to completely proteolyze P220 after 30 minutes of incubation (lane 1). The same bands of 110-130 kDa in lane 8 are also seen in lanes 1-3. It is reasonable to assume that the disappearance of P220 from U-S10 upon mixing with I-S10 occurred because the P220 was

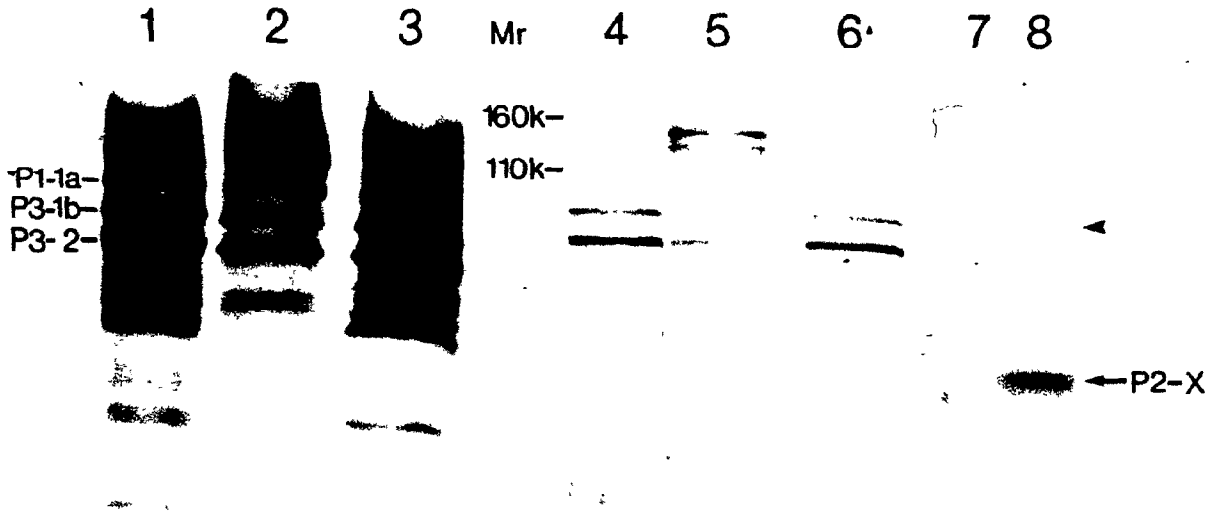
cleaved to yield the smaller products. We have, however, not yet purified the cleavage activity from I-S10 and incubated it with U-S10 to directly document the products of proteolysis. For a U-S10:I-S10 ratio of 4, there is almost complete proteolysis of P220 after a 30 minute incubation (lane 2). For a U-S10:I-S10 ratio of 10 there is clearly someP220 remaining after 30 minutes, (lane 3). Thus, a U-S10:I-S10 ratio of 4 is approximately the end point for titration of the I-S10 against the protease activity under our assay conditions. Lanes 5 and 6 show that loss of P220 is time dependent. Lane 5 shows P220 related antigens after a simple mixing of U-S10 and I-S10 without incubation and lane 6 shows an aliquot of the same sample after 30 minutes incubation. It should be noted that the incubations shown in lanes 5 and 6 are from a different experiment to those in lanes 1-4. Thus the absolute amount of P220 is less in lane 5 than in lane 4 due to variation in staining intensity between experiments. In summary, the results presented in Fig. 1, confirm previous reports (5) and demonstrate that there is a protease activity present in extracts from poliovirus-infected HeLa cells which can degrade P220.

We next wanted to determine whether poliovirus proteins P3-8C or P2-X are involved in P220 proteolysis. Protein P3-7C is a most likely candidate for such a protease activity since it is the viral protein involved in most of the cleavages of viral precursor polypeptides to yield both structural and non structural proteins (8). In the case of P2-X it has been reported that this protein has protease activity involved in processing of poliovirus protein precursors (9), but this was not verified in a more recent study (8).

Initially we tested the activity of our preparations of antibodies

FIGURE 2

Effects of anti-P3-7C and anti-P2-X on processing of poliovirus precursor proteins. Translation incubations using extracts from poliovirus-infected HeLa cells were carried out according to Lee et al., (10) except for the fact that extracts were not nuclease treated. The cell extract was preincubated for 60 minutes at 4°C with antibody buffer or the desired antibody. The translation incubations were then performed. Reaction mixtures contained (in a total volume of 25 µl) 9 µl of cell extract, 130 mM potassium acetate, 0.4 mM magnesium acetate, 20 mM Hepes (pH 7.5), 1 mM ATP, 200 µM GTP, 9 mM creatine phosphate, 22 mg of creatine phosphokinase per ml, 2.5 mM dithiothreitol, 0.2 mM spermidine, 19 amino acids (10 µM each, minus methionine), 20 µCi of [^{35}S] methionine (> 1000 Ci/mmol, New England Nuclear) and 6 µl of antibody buffer (10 mM tris pH 8.0, 10 mM KCl) or IgG fractions of the antibodies indicated below. Antisera to poliovirus proteins P3-7C and P2-X and purification of IgG fraction was as described elsewhere (8). Following incubation for 60 minutes at 37°C, samples were resolved on 10% SDS/polyacrylamide gels followed by autoradiography. Lanes 1-3 and lanes 4-6 are different exposures of the same autoradiograph (exposure times were 1 hour and 5 minutes, respectively). Lanes 1 and 4, translation products in the absence of antibody. Lanes 2 and 5, translation products in the presence of 60 µg of anti-P3-7C. Lanes 3 and 6, translation products in the presence of 60 µg of anti-P2-X. Lanes 7 and 8 show an immunoblot of extracts from uninfected (lane 7) and infected (lane 8) cells probed with anti-P2-X. Blotting conditions were as described in the legend to Figure 1.


1
2
3
Mr
4
5
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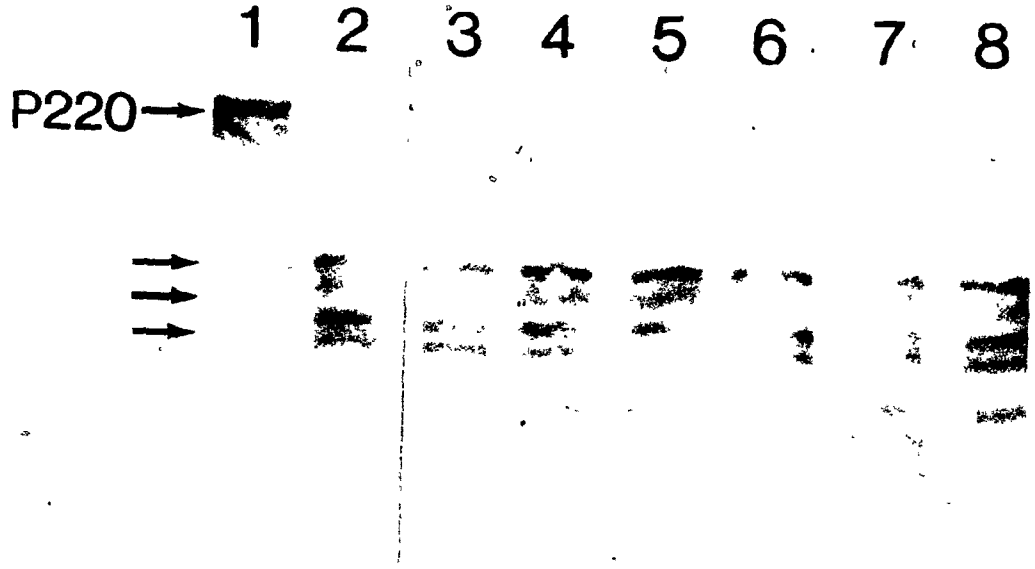
7
8
160k–
110k–
P1-1a–
P3-1b–
P3-2–
←P2-X

against P3-7C and P2-X. Anti-P3-7C is known to inhibit cleavages at glutamine-glycine pairs which occur when processing of viral precursors is assayed during in vitro translation of endogenous poliovirus RNA in extracts from poliovirus-infected cells (11). Consequently, we used this assay to test the activity of anti-P3-7C. Fig. 2 (lanes 1-6) shows the [^{35}S]-methionine labeled proteins produced in an extract from poliovirus-infected HeLa cells. Lanes 1-3 and lanes 4-6 show different exposures of the same gel. Lanes 1 and 4 show endogenous translation products in the absence of antibody. There are major bands which correspond to the three precursor proteins (P1-1a, P3-1b and P3-2, respectively) indicated to the left of lane 1. In addition there are several lower molecular weight bands which correspond to the various viral proteins derived from the higher molecular weight precursors. Addition of anti-P3-7C to the translation incubation results in inhibition of processing as indicated by the disappearance of the lower molecular weight bands and the build up of an ~ 150 kDa polypeptide, lanes 2 and 5. The ~ 150 kDa polypeptide consists of the combined amino acid sequences of P2-3b and P3-1b as previously shown (8). Densitometry of the lower molecular weight bands indicated that under the conditions of our assay, greater than 90% of P3-7C activity was blocked by anti-P3-7C. Addition of anti-P3-7C to the translation incubation had no effect on the total incorporation of [^{35}S]-methionine into TCA precipitable material (data not shown). Lanes 3 and 6 show the effects of anti-P2-X on poliovirus protein processing. It can be seen that anti-P2-X has no effect on processing of poliovirus polypeptides, as previously shown (8). In order to ascertain that the anti-P2-X antibody was active, we probed HeLa cell extracts from uninfected and poliovirus-infected HeLa cells. The immunoblot is shown in lanes 7 and

FIGURE 3

Effects of anti-P3-7C and anti-P2-X on proteolysis of P220. The protease assay was carried out as described in the legend to figure 1, and the figure shows P220 related antigens. Lane 1 contained 4 µl of U-S10 . Lanes 2-8, 4 µl of U-S10 and 2 µl of I-S10. Lanes 3-5, 2 µg, 10 µg and 20 µg of anti-P3-7C, respectively. Lanes 6-8, 2 µg, 10 µg and 20 µg of anti-P2-X, respectively.


1 2 3 4 5 6 7 8
P220

8, representing the uninfected and infected cell extracts, respectively. The antibody reacted with P2-X in extracts from infected cells (lane 8) and gave no reaction with a similar molecular weight polypeptide in extracts from uninfected cells (lane 7). There is also a weak reaction with a higher molecular weight band (indicated by an arrowhead) in extracts from infected cells which most probably corresponds to the precursor polypeptide P2-5b. Thus, the antibodies we are using are active in inhibiting the activity or recognizing their cognate antigens.

We next asked whether anti-P3-7C or anti-P2-X could inhibit the proteolysis of P220. The protease assay was performed under the conditions used for lane 1, Fig. 1, to ensure that efficient proteolysis was achieved but that the protease activity was not in vast excess. Lanes 1 and 2 (Fig. 3) show U-S10 and a mixture of U-S10 and I-S10 respectively, incubated for 30 minutes. In lanes 3-5, increasing amounts of anti-P3-7C were added to the incubation under the same conditions as for the in vitro translation experiments. The highest amount of antibody added (expressed as μg of antibody per μl of I-S10) was in excess of the amount which resulted in greater than 90% of the P3-7C activity (lane 5). It is clear from our data that anti-P3-7C has no effect on the protease activity which cleaves P220 as evidenced by the absence of P220 in lanes 6-8. Lanes 3-6 show that anti-P2-X (added in the same amounts as anti-P3-7C) also has no effect on proteolysis of P220. We conclude that the activity which cleaves P220 is not the same as that (P3-7C) which cleaves poliovirus precursor polypeptides. The data also suggests that P2-X is not directly involved in proteolysis of P220.

The results presented here and in previous reports establish that P220 of the CBP complex is proteolytically cleaved in poliovirus-infected cells. However, it remains to be proven rigorously that the cleavage is indeed the cause for loss of activity of the CBP complex. The identification of a viral protease responsible for the degradation of P220 would lend support to the proposed mechanism of inhibition of host cell protein synthesis. The results shown here indicate that the poliovirus proteinase P3-7C is not involved in the cleavage of P220, because anti-P3-7C antibody does not inhibit P220 cleavage under the same conditions as it inhibits poliovirus protein cleavage. A similar conclusion can be made for polypeptide P2-X, but with some reservations since the only assay we have for the anti-P2-X antibody is immunoreactivity on a nitrocellulose blot and it is possible that anti-P2-X cannot inhibit the enzymatic activity of P2-X.

Our conclusion is in accord with recent results obtained by Lloyd, Etchison and Ehrenfeld (PNAS, in press) which demonstrated that P3-7C activity can be separated from the P220 proteolyzing activity and that antibodies against P3-7C do not inhibit P220 proteolytic cleavage.

If P3-7C and P2-X are not directly responsible for cleavage of P220, then the question of the identity of this protease remains unanswered. It is possible that a hitherto uncharacterized poliovirus encoded protease is involved or, alternatively, that poliovirus infection induces a cellular activity that cleaves P220. If the latter is true it would be interesting to know whether such an activity plays a role in regulation of protein synthesis in situations other than during poliovirus infection of HeLa cells.

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CHAPTER 8

GENERAL DISCUSSION

(a) The role of cap binding proteins in initiation of translation

The initiation phase of protein synthesis is one of the most complex events occuring in the eucaryotic cell. While it shares several features in common with the prokaryotic process, there are salient differences which most likely reflect the existence of regulatory features involving the eucaryotic initiation machinery. Firstly, the number of identifiable diffusable factors required for eucaryotic initiation is about ten compared to just three in the prokaryotic process. Second, there is a requirement for ATP hydrolysis in eucaryotes. Third, eucaryotic ribosomes are much larger and more complex than prokaryotic ribosomes, while catalyzing essentially similar reactions. Lastly, the cap structure 5'm^7GpppX(m)3' is required for efficient translation of eucaryotic mRNAs.

Qualitative control of protein synthesis occurs in many instances in eucaryotes and our lack of understanding of the mechanisms involved is partly due to a corresponding void in our knowledge of the way in which ribosomes bind to mRNA and initiate translation. Consequently, because the cap structure plays a central role in this process we aimed to identify factors involved in the cap recognition process and to elucidate their mechanism of action.

In an attempt to unambiguously identify the components involved in the cap specific mRNA-protein interaction between polypeptides in crude initiation factors and mRNA, we first wanted to structurally characterize the components of the high molecular weight CBP complex originally described by Tahara et al. (CBPII) (119) and later by Grifo et al. (eIF-4F) (128). Using the m^7GDP affinity column we were able to purify a complex of three polypeptides (24,50 and 220 kDa) which is functionally very similar to CBPII and eIF-4F. The 24 kDa polypeptide corresponds to

the 24K-CBP originally isolated by Sonenberg et al., as determined by the ability to specifically crosslink to the cap structure, comigration with the 24K-CBP in 2-D gel systems and the presence of common antigenic determinants. By similar criteria and in addition, by 2-D peptide mapping, the 50 kDa polypeptide of the CBP complex is eIF-4A, although there might be a subtle difference between free eIF-4A and eIF-4A which is associated with the CBP complex. This suggestion results from a difference in the relative amounts of two spots observed in 2-D tryptic peptide maps of the two forms of eIF-4A . The functional significance of this difference is not known. The 220 kDa polypeptides is currently poorly characterized except for evidence that it plays a role in the poliovirus mediated shut-off of host protein synthesis (as will be described later) and is thus apparently indispensable for CBP complex function.

We have not yet performed any physical studies to determine the stoichiometry of the CBP complex but we suggest that the 24, 50 and 220 kDa polypeptides are stably associated and represent a homogenous biological entity. This follows from many observations in this thesis and elsewhere. First, the CBP complex is stable to many purification steps and the purified components cosediment in sucrose gradients containing high salt (0.5M KCl). [If one assumes a 1:1 stoichiometry of the subunits and hence a molecular weight of ~ 300 kDa for the native complex, it has an anomalously small sedimentation coefficient of ~ 6S. This accords with the observations of Grifo et al. (128) who reported similar characteristics for eIF-4F and suggests that the CBP complex is highly asymmetric]. Second, although the CBP complex is lacking one polypeptide compared to CBPII (119) or eIF-4F (128), it appears functionally

equivalent. It can restore translation of capped mRNAs in extracts from poliovirus-infected cells (129) and it allows the interaction of eIF-4A and eIF-4B with the cap structure (157). These observations however, do not imply that the in vivo form of the CBP complex is the same, since it must be remembered that high salt fractionation is employed to purify the complex.

Having obtained a relatively pure preparation of the CBP complex we examined its interaction with mRNA. When the CBP complex alone is used, only the 24K-CBP appears to interact with the cap structure. Because the free 24K-CBP is the only factor which by itself can be specifically crosslinked to mRNA, this most likely means that binding of the CBP complex to mRNA occurs via the 24K-CBP component. On examining the crosslinking efficiency of the 24K-CBP as part of the CBP complex compared to the free 24 kDa polypeptide, we found a dramatic increase for the CBP complex associated form. Both forms (the 24K-CBP and the CBP complex containing the 24K-CBP) have affinity for cap analogues since they are efficiently retained by the m^7GDP-affinity resins used to purify them. However, we have not performed any studies to measure the binding affinities of the two forms and so it remains a possibility that the difference in crosslinking efficiency reflects a difference in affinity for the cap structure and is therefore likely to be biologically significant. Another possibility is that there is a conformational difference in the interaction between free 24K-CBP and mRNA, and CBP complex associated 24K-CBP and mRNA, which simply affects the chemical crosslinking assay. Thus, it should be cautioned that the crosslinking assay is artificial and differences in crosslinking efficiency might not be related to biological activity.

Addition of CBP complex and eIF-4B together results in the cap specific crosslinking of 24K-CBP, eIF-4A (50 kDa) and eIF-4B (80 kDa).This interaction also requires ATP/Mg^{2+} and results in a specific crosslinking profile which is very similar to that observed between polypeptides present in crude initiation factors and mRNA. These results suggested that the 50 and 80 kDa cap specific polypeptides detected in crude factors correspond to eIF-4A and eIF-4B respectively. This was confirmed for eIF-4A but direct evidence to demonstrate that the 80 kDa polypeptide is eIF-4B is lacking at present. In summary, the CBP complex and eIF-4B are sufficient to reconstitute the cap specific mRNA protein interaction observed in the crude system.

The cap structure (and by implication cap binding protein), eIF-4A and eIF-4B are required for formation of intiation complexes between 40S ribosomes and mRNA. The pathway that is currently envisioned for this process is that the CBP complex and maybe eIF-4A and eIF-4B, interact with the mRNA and by some mechanism, subsequently allow binding of the ribosome. We sought to examine the idea that mRNA secondary structure near the 5' terminus of the mRNA is denatured in an active process by factors which interact with the cap structure. To this end we asked whether capped mRNAs with reduced secondary structure could function (either in translation or in partial initiation reactions) in extracts from poliovirus-infected cells. Because the CBP complex is impaired in these extracts, any translation initiation event must presumably occur by a mechanism other than the normal one for capped mRNAs. On the one hand, we found that irreversibly denatured inosine-substituted reovirus mRNA was able to bind to ribosomes in extracts from infected cells, while native reovirus mRNA could not. Binding of ribosomes to inosine

substituted mRNA was insensitive to cap analogues, further suggesting that it occurs independently of a cap recognition step. We also assayed translation of different capped mRNAs in extracts from poliovirus-infected cells and found that translation of capped Alfalfa Mosaic Virus (AMV)-4 RNA occurred with an efficiency comparable to that of naturally uncapped mRNAs (EMCV and Satellite Tobacco Necrosis Virus (STNV) RNAs). Thus the translation of AMV-4 RNA does not require the full activity of the CBP complex, in accord with many earlier observations suggesting that this mRNA is not strongly dependent on the cap structure for translation.

It now seems clear that the 5' region of AMV-4 RNA between the cap and the AUG is devoid of stable secondary structure (351) and in this sense is similar to inosine-substituted reovirus mRNA. Taken together these results suggest that capped mRNAs with reduced secondary structure are less dependent on an activity of the CBP complex for initiation complex formation. Some caveats apply however to the interpretation of the above data. One caution concerns the authenticity of the 80S initiation complexes formed on inosine substituted mRNA. This is indeed a difficult thing to assess, in light of the fact that inosine substituted mRNAs cannot direct synthesis of a protein. Recently, it has been suggested that authentic ribosome binding to inosine-substituted mRNA requires ATP and the cap structure (352). This is in contrast to a previous report which claimed that the cap structure and ATP were not essential for this function when inosine-substituted mRNA is used. The former suggestion is based on the observation that there is a change in the ribosome binding characteristics for inosine substituted mRNA depending on whether ATP is present or not. In the presence of ATP, 80S complex formation seems to occur by a similar mechanism to that for

regular reovirus mRNA, in that it is sensitive to inhibition by cap analogues and the labeled cap structure is protected by the 40S ribosome. However in the absence of ATP, 80S complex formation seems insensitive to cap analogues and the 40S ribosome does not protect the cap structure from nuclease digestion. On the basis of these observations, the authors suggest that 'authentic' ribosome binding to inosine substituted mRNA requires both the cap structure and ATP. This implies that the 80S complexes formed in the absence of ATP are non-functional which, as stated before, is difficult to verify. Two points favor the interpretation that the complexes formed on inosine substituted mRNA would direct synthesis of a protein, given the chance! First, the fact that inosine substituted mRNA forms 80S complexes in the presence of the polypeptide chain elongation inhibitor sparsomycin, strongly suggests that the complexes are formed near the 5' end of the mRNA. If the observed complexes are a result of internal sticking of 80S subunits (producing nonfunctional complexes), then one might well expect the formation of larger entities containing many ribosomes. In addition, if the elongation block is removed, then inosine substituted mRNA can go on to form polysome like complexes which are similar to those formed on native reovirus mRNA.

In the case of AMV-4 RNA, it is clear that its translation is less dependent on the fully active CBP complex since it can be translated faithfully and as efficiently as naturally uncapped RNAs in extracts from poliovirus-infected cells. In other experiments we have shown that AMV-4 RNA translation is not stimulated by addition of CBP complex to extracts from uninfected HeLa cells, under conditions in which other capped mRNAs are stimulated (reovirus, globin and VSV mRNAs) (129). These results show

directly that AMV-4 RNA has a reduced dependence on the CBP complex for translation. The fact that AMV-4 RNA is most probably devoid of secondary structure 5' to the AUG accords with our model but again is only supportive. It cannot be ruled out that AMV-4 RNA contains specific primary sequences which function to allow efficient ribosome binding without any need for the cap structure. Such features, for example, might include binding sites for initiation factors which otherwise depend on the CBP complex to bind mRNA. Alternatively, AMV-4 RNA might be able to directly interact with the 40S ribosomal subunit. In this way, AMV-4 RNA might behave as a naturally uncapped RNA.

Whatever the mechanism of translation of AMV-4 RNA is, the fact that it is not very dependent on the cap structure is particularly noteworthy. Firstly, it demonstrates that the cap structure per se does not dictate a cap dependent mechanism for initiation of translation, which is consistent with the view that other structural features of the template confer such dependence. These features are presumably absent from AMV-4 RNA. This observation is in agreement with the report of Brown et al. (388) who showed that decapped VSV mRNA is not efficiently translated in extracts from poliovirus-infected cells. Thus, the simple absence of the cap structure on poliovirus RNA is not sufficient to allow initiation factors in infected cells to discriminate between capped and uncapped mRNAs. Secondly, it has been clear for some time that the degree of dependence on the cap structure for translation varies among different mRNAs and thus, AMV-4 RNA provides an example displaying very low cap dependence. This might have more general significance, since it raises the possibility that some cellular mRNAs might be efficiently translated either without a cap structure or, in the absence of functional cap bind-

ing protein. If the CBP complex is a target for regulation in situations other than during poliovirus-infection, then this possibility might have significant physiological ramifications. While the shut-off of cellular protein synthesis by poliovirus is generally thought of as being non-discriminatory with respect to cellular mRNAs, I do not know of any study which has rigorously examined this question. Consequently, it would indeed be of interest to probe for cellular mRNAs which might not be 'shut-off' during poliovirus infection.

To examine the effect of mRNA secondary structure on interaction of cap specific polypeptides with the cap structure and on ribosome binding to capped mRNAs, we employed reovirus mRNA transcripts with different degrees of secondary structure. We found a correlation between the stability of mRNA secondary structure and the dependence on ATP for interaction for the 50 and 80 kDa polypeptides with the cap structure. Whether or not the cap specific interaction between the 50 and 80 kDa polypeptides and inosine-substituted mRNA is merely less dependent on ATP or totally independent is not clear from our results, since it remains a possibility that the crude initiation factors contain tightly bound ATP. However, one argument against this is that we have been unable to detect levels of ATP greater than 0.5×10^{-8}M (unpublished observations) in our factor preparations using the highly sensitive luciferase enzyme assay for ATP (353).

We found a direct relationship between the stability/amount of mRNA secondary structure and the extent to which ribosome binding is inhibited by high salt. It is well documented that high salt concentrations inhibit translation of capped mRNAs under conditions in which naturally uncapped mRNAs are not affected (124). Further evidence that this effect

is actually cap related, is that translation is more sensitive to cap analogues at high salt concentrations (354) and that the inhibition of translation can be relieved by the addition of CBP complex (129). The above results, obtained from employing mRNAs with varying degrees of secondary structure, suggest that inhibition of translation at high salt concentrations is related to the stability of mRNA secondary structure although the mechanism is not clear. One possibility is that the activity of a factor involved in melting mRNA secondary structure is directly inhibited at high salt concentrations. Alternatively, the increased stability of the secondary structure might prevent the putative melting factors functioning efficiently.

A major reservation concerning a model in which a cap recognition factor melts mRNA secondary structure is that we have thus far been unable to demonstrate any melting activity directly. The best evidence to indicate such an activity comes from experiments in which a monoclonal antibody with anti-CBP activity was found to inhibit the binding of ribosomes to regular reovirus mRNA but had no effect on binding of ribosomes to denatured inosine substituted reovirus mRNA. This antibody was also found to inhibit the cap specific crosslinking of CBP's suggesting that the activities required to denature mRNA and for interaction of CBPs with the cap structure are related. Unfortunately, although the antibody employed in these studies exhibited very striking anti-CBP function (37) it was never determined exactly which antigen was being recognized by the antibody. The availability of purified CBP complex should allow the development of a direct assay for the denaturation activity. One way would be to produce small mRNA transcripts in vitro with well defined, extensive secondary structure and to incubate these with purified CBP's, ATP/Mg^{2+} and to follow denaturation of the mRNA by the hyperchromic shift

in a spectrophotometer. Another possibility would be to map mRNA secondary structure using structure specific RNases in the presence and absence of purified CBP complex. In an initial approach along these lines, there is evidence that eIF-4A and the CBP complex do increase the sensitivity of mRNA to nuclease (R. Thach, personal communication). This could mean that there is less mRNA secondary structure in the presence of these factors although unfortunately, since only single strand specific nucleases were employed in these studies the results are open to interpretation.

Despite the lack of direct evidence, our observations suggest that mRNA must undergo an ATP dependent conformational change which allows interaction of eIF-4A and eIF-4B (probably) with the cap structure. This step may well be necessary although probably not sufficient to allow formation of 40S initiation complexes. Because it is possible to reconstitute the cap-specific mRNA protein interaction observed when using crude initiation factors, by using the CBP complex and eIF-4B only, it appears that these factors are sufficient to bring about such a change in conformation of the mRNA. Whether the CBP complex by itself is competent or whether it acts in concert with eIF-4B cannot be answered at the moment and resolution of this question will require a direct assay for the putative melting activity. A further observation worth noting is that cap specific crosslinking of eIF-4A is enhanced in the purified system by addition of free eIF-4A. The interpretation here is not straightforward. Firstly, we have presented evidence that the eIF-4A associated with the CBP complex is slightly modified compared to the free eIF-4A (as indicated by the slight difference in peptide maps), although the functional significance of this is not known. Secondly, we cannot

tell which form of eIF-4A is being crosslinked when the CBP complex (containing eIF-4A) and free eIF-4A are present together. The stimulation is significant, however and is consistent with the fact that free eIF-4A stimulates other assay systems in which the CBP complex is already present; for example, the reconstituted protein synthesis system of Grifo et al. (128). These observation suggest an involvement of free eIF-4A in initiation, although a unique role is not yet apparent. That is to say, although free eIF-4A stimulates many assay systems, it is not clear whether it acts via association with the CBP complex or by itself and hence in a different way to CBP complex associated form.

The proposed mechanism for the interaction between cap recognition factors and mRNA suggests that one of the components will have an ATPase activity. It is also possible that the requirement for ATP might be due to a phosphorylation event which activates one of the components. There is evidence that eIF-4A has an mRNA dependent ATPase activity which is stimulated by eIF-4B (355). As might be expected, the CBP complex has a similar activity, presumably due to the presence of eIF-4A. It was also claimed that eIF-4A, eIF-4B and the CBP complex act synergistically to hydrolyze ATP, again pointing to a concerted action of these factors in mRNA recognition. This problem deserves much attention because an understanding of the ATPase activity will no doubt prove most illuminating in elucidating the mechanism of ribosome/mRNA attachment. Phosphorylation of factors might also play a role in mRNA recognition by CBP complex, eIF-4A and eIF-4B. In the case of eIF-4B there appear to be multiple phosphorylation sites and phosphorylated eIF-4B seems to be active. Two-dimensional gel analysis of the 24K-CBP and eIF-4A (either as the free polypeptides or as constituents of the CBP complex) indicate the

presence of different isoelectric variants although the structural differences between the variants have not been elucidated to date. Thus, a role for ATP in phosphorylation of these mRNA binding factors is neither demonstrated nor precluded.

Messenger RNA recognition is but one of the many partial reactions in eucaryotic translation initiation, the sum of these reactions terminating in formation of an 80S initiation complex at the AUG codon. It is pertinent to consider how the factors involved in this step might interact with other components of the initiation machinery and therefore how they might function in the overall process of initiation. Firstly, the fact that the CBP complex copurifies with either eIF-3 or eIF-4B, (depending on the fractionation procedure employed), demonstrates that the CBP complex does have affinity for these two factors, although no functional significance has yet been suggested. In the case of eIF-3, it seems that the CBP complex can be removed by washing in high salt (0.5 M KCl) and there is also some evidence that the eIF-3/CBP association is disrupted as a consequence of poliovirus infection (155). This latter observation hints at biological significance and will be discussed further in the second half of this chapter. eIF-3 is a very large multisubunit factor which is involved in mRNA binding to 43S preinitiation complexes and so it might be that eIF-3/CBP association is required for this step. Another mechanism for 43S complex/mRNA interaction could involve binding of the CBP complex, eIF-4A and/or eIF-4B directly to some integral component of the 40S ribosomal subunit. However, to date, attempts to demonstrate binding of radiolabeled factors (eIF-4A, eIF-4B or the CBP complex) to the 40S subunit have not succeeded, suggesting that a direct stable interaction does not occur.

Subsequent to formation of 48S preinitiation complex formation, the 40S ribosome relocates on the mRNA followed by joining of 60S subunits and formation of 80S initiation complex at the AUG codon. Kozak has proposed the scanning mechanism for this step, which postulates that 40S ribosomes attach at or near the 5' cap structure followed by migration along the mRNA until they encounter the AUG codon whereupon 80S initiation complexes are formed (reviewed in reference 67). Since the postulated migration of 40S ribosomes along mRNA requires ATP hydrolysis, it is a possibility that the RNA dependent ATPase of the CBP complex and eIF-4A (alluded to earlier) is involved in migration of 40S ribosomes. This however is pure conjecture at present and awaits further investigation.

While the cap structure is required for efficient translation of most capped mRNAs, the requirement is not absolute and furthermore, varies among mRNAs. The cap structure functions during the rate limiting step of initiation and this step occurs at different rates for different mRNAs. Consequently, this step is likely to be important in allowing competitive inhibition of translation of some mRNAs by others. The site of competition is now thought to involve a step just prior to binding of 40S ribosomes to mRNA in which mRNAs compete for a limiting component of the translation machinery. This limiting component is referred to as 'mRNA discriminatory factor' and mRNAs with high affinity will be translated at the expense of mRNAs with low affinity. Because the CBP complex (CBPII, reference 160) can alleviate competition between reovirus mRNAs, it has been suggested that the CBP complex is a mRNA discriminatory factor (160). Sarkar et al. (356) have obtained similar results. They found that the CBP complex (used for the experi

ments in this thesis) was able to relieve translational competition between α and β globin mRNAs in rabbit reticulocyte lysate. Translation of α globin was preferentially stimulated by addition of the CBP complex under conditions in which total protein synthesis remained constant. This is diagnostic of a mRNA discriminatory effect and strongly supports the contention that the CBP complex is a mRNA discriminatory factor.

The structural features of mRNA which determine their intrinsic translational efficiencies remain to be elucidated. In contrast to prokaryotes, in which case the Shine and Dalgarno consensus sequence can affect translational efficiency, there seems to be no generalized eucaryotic counterpart (371). A role for specific primary sequences in certain cases is not yet excluded however, and it is noteworthy that the 3' end of 18S ribosomal RNA (the eucaryotic equivalent of prokaryotic 16S ribosomal RNA which interacts with the Shine and Dalgarno sequence) does appear to be juxtaposed to the 5' end of mRNA in eucaryotic initiation complexes (82). Whether or not base pairing occurs remains to be determined. If it does, then mRNA sequences which are complementary to the 18S ribosomal RNA might clearly serve to increase the efficiency of translation.

The observation that the CBP complex can alleviate translation between uncapped reovirus mRNAs (160) prompted the suggestion that the CBP complex binds to features of the mRNA other than the cap structure. These features would vary among different mRNAs, resulting in differential affinity for the CBP complex and thus, different translational efficiencies. Two points would appear difficult to reconcile however, if this suggestion provides the whole explanation. First, there is a wide spectrum of translational efficiencies among even a relatively small popula-

tion of mRNA (for example the 10 mRNAs of reovirus) (160). Second, in light of the apparent lack of conserved primary structure near the 5' end of eucaryotic mRNAs the putative sequences recognized by the CBP complex would be of the order of a few bases only. Hence, it is difficult to envision how such a broad spectrum of translational efficiencies could arise from such limited potential sequence variation. The fact that the CBP complex does interact with uncapped mRNAs and affects their translation to different extents, implies that there is differential recognition of a binding site on the mRNA, which might well partly explain the discriminatory activity. However, the rationale outlined above suggests that other features of the mRNA must also be significant in determining the affinity for discriminatory factor.

It follows from our model for the function of the CBP complex, that mRNA secondary structure might contribute to the translational efficiency of mRNAs and hence their ability to compete for discriminatory factor. If the putative denaturation step is a relatively inefficient process, then mRNAs with extensive 5' secondary structure will be more dependent on the CBP complex and will therefore be discriminated against. Conversely, mRNAs with little secondary structure will have a low requirement for the CBP complex and will be preferentially translated. Thus, we have proposed the following mechanism to account for the discriminatory activity of the CBP complex (356). Binding of the CBP complex to mRNA occurs via interaction of the 24K-CBP with the cap structure. Subsequently, the CBP complex (possibly in conjunction with eIF-4B as described earlier) migrates along the mRNA in the 5'→3' direction and denatures mRNA secondary structure. The degree of mRNA secondary structure will determine the efficiency of this step and hence

the dependence on the CBP complex. This suggestion is compatible with the available kinetic data which indicate that the CBP complex does have different affinities for different mRNAs (160) and that the mRNA discrimination step occurs just prior to binding of the ribosome to mRNA. A critical test of this proposed mechanism would be to examine the effects of CBP complex (in vitro) on competition between mRNAs which have well defined 5' secondary structure but are otherwise identical. Until this data is available the postulated effects of mRNA secondary structure in influencing mRNA discrimination remain speculative.

The significance of mRNA discrimination in contributing to qualitative control of protein synthesis in vivo is likewise uncertain. The observation that the hierarchy of translational efficiencies among reovirus mRNAs in vitro and in vivo, is the same (196) augurs well for the validity of the in vitro system as a measure of translational efficiency. However, it tells us nothing of whether the in vivo translational efficiencies have any physiological significance. Teleologically, given the variation in translational efficiencies observed in vivo, one might expect the cell to have evolved ways of exploiting this. To date though, the only examples in which translational competition appears to play a role in allowing expression of particular genes, occur in virally infected cells which contain enormously high amounts of specific viral mRNAs (383). In other cases (for example, during the cell cycle (223)) when total protein synthesis is significantly reduced, it is likely that translational competition is increased and thus 'weaker' mRNAs will be inhibited to a greater extent than 'stronger' ones. Again, however, there might be other controls operating in these cases (eg., sequestering of mRNA in inactive form) and so it is difficult to assess the signifi-

cance of mRNA discrimination in these cases.

Despite the difficulties in elucidating the reasons for the proposed negative effect of mRNA secondary structure on translation in vitro, it is nonetheless important to address the in vivo situation. Pelletier and Sonenberg have constructed a series of mutants of the HSV-1 thymidine kinase gene, in which differing degrees of secondary structure were introduced into the 5' non-coding region of the mRNA sequence. These mutant genes were analyzed for their ability to transform tk minus cells to tk plus and it was found that excessive secondary structure of the mRNA decreases the transformation efficiency (87). Measurement of ribosome binding to the various mRNAs derived from the mutant genes indicated that excessive secondary structure also impedes this process (87). These results provide compelling evidence that 5' proximal mRNA secondary structure can influence efficiency of expression in vivo, which is a most significant observation because it suggests a number of ways in which genes might be amenable to translational control. For example, production of different mRNAs from the same gene (either by differential splicing or by utilization of alternative promoters) is known to occur in a tissue specific manner (357) or during development (358). In some cases, this results in mRNAs which differ only in their 5' non-coding regions, thus possibly affecting translation of the two types of mRNA. One might envision, for example, that loss of non-coding exon containing stable secondary structure might release a gene from negative translational control. This kind of mechanism has recently been proposed to explain activation of the c-myc gene in some Burkitts lymphomas (359). Another possibility is that gene rearrangements at the DNA level, eg. by transposition events, might result in production of mRNAs with different

translational properties. Finally RNA: RNA duplexes might be formed between mRNAs and putative 'anti-sense' (complementary) mRNAs, thus potentially inhibiting translation of the former. The existence of such anti-sense transcription units has been described in E. coli (234). Moreover, transfection of cloned genes into eucaryotic cells has indicated that anti-sense genes can inhibit expression of their cognate 'sense genes' in a sequence specific manner, although the site of inhibition was not determined (360). These observations suggest an extremely useful and highly selective way of controlling expression of eucaryotic genes. It will be of interest to see whether such translational control mechanism are indeed operative.

(b) Poliovirus induced shut-off of cellular protein synthesis

At the onset of the work described in this thesis there was evidence to indicate that the initiation factor inactivated by poliovirus was the CBP complex. This conclusion was based on the observation that the purified CBP II (reference 119) could restore translation of capped mRNAs in extracts from poliovirus-infected HeLa cells (119). Edery et al. (129) confirmed this result for the CBP complex which was used for the experiments reported in this thesis.

In order to gain further insight into the defect in the CBP complex caused by poliovirus infection, we employed two approaches to directly compare CBPs from uninfected and poliovirus-infected HeLa cells. Firstly, we examined CBPs in crude IF, using the chemical crosslinking assay and secondly, we purified CBPs using the m^7GDP-affinity chromatography technique. The crosslinking analysis demonstrated that the 50 and 80 kDa cap specific polypeptides are unable to interact with the cap structure after poliovirus infection while the crosslinking of the 24K-

CBP is also significantly reduced. The crosslinking data presented in this thesis concerning the 24K-CBP is not in agreement with that obtained by Hansen et al. (155,372). These authors reported that the 24K-CBP is present in preparations of initiation factors from infected cells (I-IF) (0-40% ammonium sulphate fraction) to the same level as uninfected cells. Furthermore, they examined the sedimentation of the 24K-CBP in factors from infected cells and reported again, that the 'amount' of 24K-CBP was not reduced as a consequence of infection but that it no longer cosedimented with eIF-3. From these results it was concluded that a putative eIF-3-24K-CBP complex is disrupted during poliovirus infection. We have repeated the experiments performed by Hansen et al. exactly, with the exception that we employed [^{3}H]-oxidized reovirus mRNAs and Hansen et al. used [^{3}H]-oxidized vesicular stomatitis virus (VSV) mRNA for the crosslinking assay. Consistent with our previous results, we found that crosslinking of the 24K-CBP is significantly reduced following poliovirus infection (373). We also analyzed the sedimentation of the 24K-CBP in sucrose gradients but, consistent with our other observations, were unable to detect significant amounts of the 24K-CBP in preparations from infected cells (K.A.W.L. and N.S., unpublished observations). Thus it is difficult to reconcile the conflicting data at present. One possibility is that the 24K-CBP interacts slightly differently with VSV versus reovirus mRNAs in a way which affects the chemical crosslinking assay. That is to say, there might be sequences near the cap which influence the interaction between the 24K-CBP-mRNA interaction. Such a situation might then simply affect the chemical crosslinking assay by adventitiously bringing a primary amino group of the 24K-CBP in close proximity to the reactive dialdehyde groups of the oxidized cap structure. If this is

the case, a modification of the 24K-CBP (indirectly, because of its presence in the CBP complex) might affect its crosslinking ability to reovirus but not to VSV mRNAs. It should be emphasized however, that the most striking effect of poliovirus-infection as detected by the crosslinking assay is the complete loss of a cap specific interaction between mRNA and the 50 and 80 kDa polypeptides. This interaction requires ATP and the data in this thesis demonstrate conclusively that the CBP complex is also required. Thus, the fact that Hansen et al. did not use ATP in their analyses precludes a more telling comparison of results, particularly in relation to the CBP complex.

The fact that crosslinking of the 24, 50 and 80 kDa polypeptides is simultaneously lost following infection suggested a close relationship between these polypeptides, although at the time it was not clear whether this relationship was structural or functional. The results presented here and by others demonstrate that the latter possibility is true. First, the 24K-CBP is the only purified factor which can bind specifically to cap structures, suggesting that interaction of the 50 and 80 kDa polypeptides with the cap is mediated indirectly through an interaction involving the 24 kDa polypeptide. Second, the 50 kDa which becomes crosslinked is eIF-4A and the 80 kDa polypeptide is most likely eIF-4B; these two factors appear not to be structurally related (to each other or to the 24K-CBP) but are known to function in mRNA binding to ribosomes. Lastly, the cap-specific crosslinking of the 80 kDa polypeptide (probably eIF-4B) present in initiation factors from infected cells (I-IF) can be restored by addition of the CBP complex to I-IF. Since (as described earlier) the CBP complex and eIF-4B are sufficient to reconstitute the

cap-specific mRNA protein interaction, the latter observation suggests that eIF-4B is present and active in I-IF (at least in terms of the crosslinking assay) and that it can mediate interaction of eIF-4A with the cap structure when exogenous CBP complex is supplied. Addition of the 24K-CBP to I-IF was not sufficient to allow crosslinking of the endogenous 80 kDa polypeptide (probably eIF-4B) again indicating the requirement for the CBP complex (as opposed to the 24K-CBP) in the cap recognition process. The fact that the activities required to reconstitute cap specific crosslinking and to restore capped mRNA translation in extracts from poliovirus-infected cells, copurify, strongly suggests that they are identical activities. In summary, the crosslinking and restoring activity data indicate that the CBP complex is the only factor inactivated by poliovirus, since addition of this factor results in full restoration of the cap specific mRNA protein interaction and capped mRNA translation. In accord with this, the initiation factors which had previously been implicated in the shut-off (eIF-3 and eIF-4B) have been isolated from poliovirus-infected cells and shown to be neither structurally modified nor functionally impaired (361-363).

To characterize the virally induced defect of the CBP complex we isolated cap binding proteins directly from uninfected and poliovirus infected cells, using m^7GDP affinity chromatography. We obtained a CBP complex from uninfected cells which is structurally very similar to the rabbit reticulocyte CBP complex. [The 24K-CBP was identified by specific elution from an m^7GDP column and chemical crosslinking to mRNA (data not shown). The 50 kDa polypeptide comigrates on SDS gels with the 50 kDa polypeptide from rabbits (eIF-4A) and reacts with a monoclonal antibody to eIF-4A (data not shown). The 220 kDa polypeptide(s) (P220) comigrate

with rabbit P220 and reacts with polyclonal antisera to P220 from rabbits (data not shown)]. This was expected and accords with the highly conserved nature of initiation factors between HeLa cells and rabbit reticulocytes (46).

It was previously suggested that the CBP complex is physically disrupted following poliovirus infection (155) and experiments with antibody against a 220 kDa polypeptide present in eIF-3 preparations showed that the 220 kDa polypeptide is proteolyzed during poliovirus infection. The antibody employed also reacted with the ~ 220 kDa polypeptide of the CBP complex thus suggesting that proteolysis of this polypeptide by a viral dependent protease is the mechanism by which the CBP complex is inactivated (374). The results described in chapter 6 of this thesis strongly support this suggestion and furthermore, indicate that the CBP complex in infected cells contains the cleavage products of P220. Unfortunately, owing to the nature of the protocol we employed to isolate the modified CBP complex, the precise structure and subcellular location are not clear. Essentially, we isolated labeled proteins from a whole cell extract, with a sedimentation coefficient of $\leq$ 11S under high salt conditions (0.5M KCl). Thus, while all the polypeptides present in the material eluted from the m^7GDP-column interact directly or indirectly with the cap analogue, the relationship between them is not immediately obvious. Furthermore, extent of [^{35}S] labeling does not necessarily correlate with the abundance of the proteins. However, because the putative degradation products of P220 are specifically eluted from the affinity column this implies that they associated with the 24K-CBP, assuming (with reasonable conviction) that the 24K-CBP is the only polypeptide which interacts directly with the cap structure. The labeled band

which comigrates with the 50 kDa polypeptide (eIF-4A) is not strongly labeled but we believe that it is eIF-4A, and that the amount of this polypeptide in the modified CBP complex is not reduced due to poliovirus infection. This is based on the observation that the 50 kDa polypeptide is sometimes a major band in [^{35}S]-labeled purified CBP and secondly, the extent of labeling is never decreased in preparations from infected versus mock-infected cells (KAWL and NS, unpublished observations). We do not know the reason for the variability in labeling of the 50 kDa polypeptide at present, but it might well reflect variation in the rates at which newly synthesized polypeptide is incorporated into the CBP complex. In any case, we might tentatively conclude at this point that the modified CBP complex in infected cells contains the 24K-CBP, eIF-4A and cleavage products of P220. Polypeptides other than those that comigrate with known CBP complex polypeptides (i.e. 24, 50 and 220 kDa polypeptides) are either (1) 'sticky' contaminants, (2) specific proteins which interact with the complex in vivo or (3) novel cap binding proteins which bind directly to the cap analogue. Because the purification was from whole cell extracts, the latter possibility is particularly noteworthy in view of recent evidence suggesting a role for the cap structure in pre-mRNA splicing (375,376) and hence in nuclear events.

The conclusion from the sum of these results, is that an intact P220 is essential for the function of the CBP complex and that proteolysis of P220 is responsible for inhibition of cellular protein synthesis during poliovirus infection. However, certain questions need to be addressed before this inference can be considered fact. First, it should be demonstrated that the modified CBP complex is unable to function in any of the assays currently used to assess CBP complex function. This might

prove extremely difficult unless a purification scheme is devised which allows purification of measurable amounts of unlabeled CBP complex. Second, although it is clearly likely that proteolysis of P220 would inactivate the CBP complex, it is still open to question as to whether the viral dependent protease is required to shut-off cellular protein synthesis. Two pieces of circumstantial evidence argue that it is. First, the activity appears very early during infection at times when the cells are relatively healthy. Furthermore, the kinetics of proteolysis of P220 roughly correlate with the shut-off of cellular translation. Secondly, the protease appears to be highly specific for P220 since there is no detectable proteolysis of other cellular proteins. The best approach to gain insight into this question will most probably come from the construction of conditional poliovirus mutants in the shut-off, by manipulation of cloned infectious viral DNA. Using this approach one would hope to be able to correlate the inability to shut-off protein synthesis with the inability to proteolyse P220.

How might cleavage of P220 inactivate the CBP complex and consequently prevent efficient binding of ribosomes to cellular mRNAs? The work described here and elsewhere suggests the following mechanism for ribosome binding to mRNA.

Step 1 - Binding of the CBP complex to mRNA through the 24K-CBP, the affinity of which might be influenced by other components of the CBP complex.

Step 2 - Denaturation of the mRNA, possibly requiring eIF-4B, thus allowing interaction of eIF-4A and eIF-4B with the cap structure.

Step 3 - Binding of the 43S preinitiation complex to the mRNA-CBP-eIF-4B complex.

The available evidence seems to favor the interpretation that proteolysis of P220 would prevent the putative denaturation step, although there are many ways in which this could be achieved. First, because the modified CBP complex can be purified from infected cells using the m^7GDP-affinity ligand, this suggests that interaction of the 24K-CBP with mRNA is not perturbed. However, the difference in cross-linking characteristics between 24K-CBP in uninfected and infected preparations indicates otherwise. Thus, it seems a distinct possibility that the putative modified CBP complex does not interact productively with mRNA, which alone might account for the inability of eIF-4A and eIF-4B to interact with the cap structure. Second, the interaction of eIF-4A and eIF-4B with the cap structure might be mediated through a direct interaction with P220, i.e. eIF-4A and/or eIF-4B might only interact with the cap structure via a physical association with the CBP complex (P220 in particular). If it is true that the ATP dependent interaction of eIF-4A and eIF-4B with the cap structure is a prerequisite for ribosome binding then either of the above mechanism would explain the inhibition of cellular protein synthesis. Our observation that denatured mRNA is able to bind to ribosomes in extracts from infected cells suggests that this is the case. However, it remains possible that ribosome binding following denaturation of the mRNA is not a passive process with respect to the requirement for the CBP complex. For example, binding might depend on interaction of a component of the 43S preinitiation complex with the CBP complex. In this respect it is noteworthy that eIF-3 (which is present on the 43S preinitiation complex) appears to have

affinity for the CBP complex and that eIF-3/CBP association is perturbed following infection. Thus, intact P220 might be required for binding to eIF-3 and loss of this capacity might also contribute to the inhibition of cellular protein synthesis.

The identity of the protease which cleaves P220 is unknown as is the significance of this activity to the poliovirus replicative cycle. The viral genome encodes at least one protease activity (P3-7C) which processes the primary cleavage products derived from the viral polyprotein. Proteases mapping elsewhere in the genome have also been reported. The results in chapter 7 provide very strong evidence that P3-7C is not involved in cleavage of P220. (These results agree with those of Lloyd et al., Proc. Natl. Acad. Sci., in press). We can also tentatively conclude that P2-X is not involved but this remains to be rigorously examined. It is possible that there is an as yet unidentified viral protease which cleaves P220. Another (more interesting?) idea is that the protease is an induced cellular activity which might be involved in translational control in cases other than in poliovirus infection. Alternatively, a poliovirus protein might modify P220 and render it a substrate for the putative cellular protease. In any event, the identification of the protease is an intriguing question.

Virus infection in many different eucaryotic setting results in shut-off of host protein synthesis although there appears to be various mechanisms and the time course for shut-off differs, depending on the replication strategy of particular viruses. For example, infection of different types of cell by other picronaviruses does not in every case, elicit such an immediate inhibition of host cell protein synthesis as does poliovirus infection of HeLa cells (382). Thus, poliovirus is not

typical of all picornaviruses. In cases where there is a gradual transition from cellular to viral translation there is good reason to expect that accumulation of high amounts of viral RNA coupled with a limiting initiation capacity in the cell, results in preferential translation of viral mRNAs. This kind of effect is also consistent with the fact that viral mRNAs in general appear to be very efficient messengers and thus can outcompete cellular mRNAs in translation. It is likely that mRNA competition plays an important role in those cases where shut-off occurs late in infection (377-380,386,387). However, in light of the fact that poliovirus RNA is considered a weak messenger (particularly for a viral one (288)) it is possible that the virus had to evolve a specific mechanism for inhibiting cellular translation in order to replicate efficiently. Thus, the capacity of poliovirus to inhibit cellular protein synthesis might be a crucial part of the replication cycle. The lack of viable poliovirus mutants in the shut-off hints at truth in this possibility.

There is a body of evidence to suggest that a transition from a cap dependent to a cap independent mechanism of translation is a more general feature of the shut-off phenomenom, although again there are differences in the mechanism. Reovirus infection of L cells results in a gradual inhibition of cellular translation along with the preferential utilization of uncapped reovirus mRNAs, which predominate late in infection (364). Messenger RNA dependent extracts prepared from infected cells are unable to translate capped mRNAs and thus it seems that reovirus also inactivates a factor involved in mRNA cap recognition. Whether or not the CBP complex is inactivated and the mode of inactivation remains to be answered. Despite the similarity in the mechanism employed by reovirus

and poliovirus to usurp the translational machinery of the host cell there is also a fundamental difference. While translation of poliovirus RNA occurs independently of virus specific factors, the translation of uncapped reovirus mRNAs appears strictly dependent on expression of the viral genome (369) because uncapped reovirus mRNAs are not translated in extracts from uninfected cells. Since the structures of the two types of reovirus mRNAs are identical apart from their 5' termini, it is not surprising that a virus specific (or induced) factor is required for efficient translation of uncapped reovirus mRNA. The identity of this factor and the mechanism by which it acts (maybe by substituting for the CBP complex but specifically for uncapped reovirus mRNAs?) are intriguing qeustions. Yet another mechanism involving the inhibition of cap binding proteins occurs during semliki forest virus (SFV) infection of neuroblastoma cells. In this case, crude initiation factors obtained from infected cells show reduced ability to stimulate capped mRNA translation while the purified CBP obtained from infected cells show no such loss of activity (365). This suggested that a virus specific factor somehow sterically blocks the activity of the CBP and evidence was obtained to indicate that a viral capsid protein is responsible. The restriction is somehow inoperative on late SFV mRNA, either due to discriminatory activity of the capsid protein or alternatively, due to a decreased requirement of late SFV mRNA for CBP. Again, while there are similarities with the poliovirus induced shut-off, there is a distinct difference in that the mechanism employed by SFV is apparently stoichiometric while that employed by poliovirus is almost certainly catalytic. However, it does seem that cap binding proteins might be the 'Achilles heel' of the eucaryotic translation machinery in the case of many viruses.

A neglected aspect of translation of poliovirus RNA concerns the way in which it is efficiently translated without a cap structure. Indeed, poliovirus RNA is a strange one for a eucaryotic ribosome to encounter. Three peculiarities are, the absence of the 5' cap structure, the presence of eight AUG codons 5' to the major translation initiation site and lastly, and the unusually long (743 nucleotides compared to an average of 50-100 for eucaryotic mRNAs) 5' untranslated region.

Whether ribosomes bind near the 5' end of poliovirus RNA and then scan the long leader region (somehow 'ignoring' eight potential translation start sites!) or whether they bind internally and hence nearer the initiation site is a contentious point. A modification of the scanning model rationalizes how ribosomes might successfully scan the leader region of poliovirus RNA as far as the 9th AUG. Analysis of functional AUGs indicates that flanking nucleotides are important and the consensus sequence 5'(G)AXXAUGG3' has been proposed (366). The A in position -3 seems to be particularly well conserved and mutation of this nucleotide can change the initiation site for protein synthesis to a more distal AUG with favorable flanking nucleotides (366). In the case of poliovirus mRNA it is striking that none of the 8 AUGs 5' to the major initiation site have an A in position -3, while the 9th one does. Although this doesn't help to decide whether or not ribosomes scan the leader region of poliovirus RNA, if it is true that they do, then the importance of the A in position -3 is strongly emphasized. Another possibility is that ribosomes do not 'scan' poliovirus RNA but instead bind internally near the major initiation site. Evidence in support of such a mechanism is that under conditions where elongation of protein synthesis is blocked, ribosomes do not accumulate on the long leader region of poliovirus RNA.

The structural features of poliovirus RNA which might enable ribosomes to bind internally are not yet apparent. Possibly it is significant that there is an adenine-uridine rich region just preceding the ninth AUG which could facilitate internal binding by providing a denatured region in the mRNA (264). However, a denatured region would be insufficient, by itself, to allow internal binding (according to Kozak, reference 67) and thus specific primary sequences of poliovirus RNA must be important.

In the case of other naturally uncapped RNAs there are some indications that internal ribosome binding might be a generalized feature of initiation of translation on these templates. Jackson has reported that EMCV and CPV mRNAs exhibit a greatly reduced dependence on ATP for 80S initiation complex formation (184). This suggests that there is no energy dependent migration of 40S ribosomal subunits along these mRNAs prior to 80S complex formation. However, since the position of the 80S complexes relative to the AUG was not ascertained, this suggestion remains speculative. Other studies using mengovirus RNA, in which ribosome binding sites have been identified by nuclease protection experiments have also indicated that ribosomes can bind internally to mengovirus RNA (367). In this case, the putative ribosome binding sites seem to share common sequences with binding sites for eIF-2 and so it is possible that eIF-2 might direct binding of ribosomes to these internal sites. While these data together suggest that naturally uncapped RNAs in general might bind ribosomes internally and thus obviate the need for a free 5' end and the cap structure, it is possible that each naturally uncapped RNA employs a different and maybe unique mechanism to achieve this. The whole problem of the mechanism of translation initiation for

uncapped RNAs deserves much attention, and two approaches should prove enlightening. First, it is of importance to develop more efficient fractionated translation systems so that the individual factor requirement for uncapped RNAs can be realistically determined. Another way in which this might be achieved, is by employing highly specific antisera to initiation factors to inhibit translation in crude cell lysates. Second, it should be possible to construct chimeric mRNAs (derived from, for example, poliovirus RNA and a typical capped mRNA) and consequently map the cis-acting sequences of the uncapped RNA which allow translation by a cap independent mechanism.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Studies with mRNAs containing varying degrees of secondary structure indicated that mRNA secondary structure can impede ribosome binding to mRNA. mRNA secondary structure also prevents interaction of certain cap binding proteins with the cap structure of messenger RNA, thus suggesting an explanation for the negative effect of mRNA secondary structure on ribosome binding.

2. The inability of cap binding proteins to interact with the cap structure following poliovirus infection probably results in the shut-off of cellular protein synthesis.

3. The activity which is impaired during poliovirus infection resides in the CBP complex. The 220 kilodalton polypeptide of the CBP complex is proteolyzed during poliovirus infection, thus probably explaining the inactivation of the CBP complex.

4. The viral protease P3-7C is not involved in proteolysis of the 220 kilodalton polypeptide of the CBP complex.