Volume 9 Number 7 1981 Nucleic Acids Research

ATP/Mg^{++}-dependent cross-linking of cap binding proteins to the 5' end of eukaryotic mRNA

Nahum Sonenberg

Department of Biochemistry, McGill University, McIntyre Medical Sciences Building, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6

Received 10 December 1980

ABSTRACT

Two proteins of apparent molecular weights of 28,000 and 50,000 daltons were shown to recognize and cross-link specifically to the 5' cap end of oxidized reovirus mRNA. Cross-linking of these proteins to mRNA was ATP/Mg^{++} dependent, in sharp contrast to cross-linking of a 24K cap binding protein which was purified and characterized previously (Sonenberg, N., Rupprecht, K.M., Hecht, S.M. and Shatkin, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 4345-4349). Non-hydrolyzable analogues of ATP as well as other nucleotides did not substitute for ATP in the cross-linking reaction and Mg^{++} was significantly preferred over other divalent cations in cross-linking of the 28K and 50K dalton proteins. A model involving the function of the latter proteins in recognition and unwinding of the 5' end structure of capped eukaryotic mRNAs is suggested.

INTRODUCTION

Subsequent to its discovery, the cap structure, m^7GpppN, at the 5' end of eukaryotic mRNAs has been shown to play an important role in initiation of translation (1). Numerous studies have shown that the cap structure facilitates ribosome binding to mRNA and the subsequent translation of mRNA in *in vitro* protein synthesis systems (1). In addition, the cap structure also confers protection against 5' exonucleolytic degradation (2).

In our attempt to determine the mechanism by which the cap structure facilitates binding of eukaryotic ribosomes to mRNA, we developed a method to identify proteins that bind near the 5' capped end of mRNA (3). Using this method, which involves cross-linking of oxidized mRNA to proteins, we detected in initiation factor preparations a 24K polypeptide that interacts specifically with the cap structure of mRNA (4). We have recently isolated the 24K cap binding protein (5) and reported on its discriminatory effect in favor of capped mRNA and against naturally uncapped mRNA in protein synthesizing extracts from HeLa cells (6).

It has been shown that ribosomal high salt wash from poliovirus-infected

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HeLa cells did not stimulate the translation of host mRNA (7). However, these fractions could stimulate translation of poliovirus RNA (8). In other experiments, Rose *et al.* showed that globin mRNA and vesicular stomatitis virus mRNA, which are capped mRNAs, could not be translated in extracts prepared from poliovirus-infected HeLa cells (9 see also 5). However, naturally uncapped mRNAs such as those of poliovirus, encephalomycarditis virus and satellite tobacco necrosis virus could be translated in these cell extracts (5,9). These results were interpreted to suggest that a cap binding protein, which is indispensable for translation of capped mRNAs but not for naturally uncapped mRNAs, became inactive following the infection by poliovirus. This notion was recently supported by the finding that purified 24K cap binding protein could restore the ability of poliovirus-infected HeLa cell extracts to translate capped mRNAs (10).

In this report I present evidence that in addition to the 24K cap binding protein, two other proteins can be cross-linked to the 5' cap of mRNA. Cross-linking of the latter is dependent on the presence of ATP and Mg^{++}. The relevance of these polypeptides to eukaryotic protein synthesis initiation and to the 24K cap binding protein is discussed.

MATERIALS AND METHODS

Viral mRNA. Reovirus mRNA containing 5'-terminal m^7GpppG^m was synthesized with viral cores in the presence of [^{3}H]-S-adenosylmethionine (specific activity 70 Ci/mmol- New England Nuclear) essentially as described before (11). The specific activity of the [^{3}H]-methylated mRNA was $\sim$ 80,000 cpm/μg. Periodate oxidation was performed as previously described (11).

Cross-linking of oxidized mRNA to protein. [^{3}H]methyl-labeled ozidized reovirus mRNA (0.75 μg) was incubated with partially purified initiation factors from rabbit reticulocytes (4). This latter fraction consisted of a 0-40% ammonium sulfate cut of total initiation factors, which was subsequently sedimented in a 10-40% glycerol gradient (Beckman SW40 rotor, 40,000 rpm, 20 hr) containing 0.1 M KCl in 20 mM 4-(2-hydroxyethyl)-1-piperazin-ethanesulfonate (Hepes), pH 7, 1 mM dithiothreitol, 0.2 mM EDTA, 10% (vol/vol) glycerol. The upper half of the gradient was pooled for cross-linking experiments. Incubation was carried out for 10 min at 30°C in one of two different mixtures in a total volume of 25 μl: mixture A, contained 20 mM Hepes, pH 7.6, 75 mM KCl and 1 mM dithiothreitol and mixture B, is essentially the medium used for ribosome binding (11), which consisted of 20 mM Hepes pH 7.3, 10 μM each of twenty amino acids, 2 mM dithiothreitol, 1 mM

ATP, 0.2 mM GTP, 5 mM creatine phosphate, 4 μg of creatine phosphokinase, 70 mM KCl and Mg acetate as indicated in each experiment. Reversible complexes which formed between proteins and mRNA were stabilized by reduction with $NaBH_3CN$. Reaction mixtures were then treated with a mixture of RNases, and the radioactive proteins were analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis and fluorography as described previously (3). Radioactive molecular weight markers (NEN) included phosphorylase B (93000), bovine serum albumin (68000), ovalbumin (46000) and carbonic anhydrase (30000).

Materials. $NaBH_3CN$ was purchased from Aldrich. m^7GDP was from P-L Biochemicals, Inc. and AMP-P(CH_2)P and AMP-P(NH)P were from Boehringer Mannheim.

RESULTS

Cross-linking of oxidized reovirus mRNA to crude initiation factor preparations from rabbit reticulocytes or other mammalian systems demonstrated that a 24K dalton polypeptide is located in close proximity to, and interacts with the 5' cap structure of eukaryotic mRNA (4). Cross-linking experiments were performed previously (4,5) in incubation mixtures which were deficient in various components needed for the formation of eukaryotic protein initiation complexes, namely ATP, Mg^{++}, creatine phosphate etc. (12,13). In order to assess the possible involvement of these components in cap recognition, cross-linking experiments were performed in the presence of all the components required for initiation complex formation except those contributed by the cell-free extract.

Fig. 1 shows the cross-linking pattern obtained in mixture B. The 24K cap binding protein became cross-linked to the mRNA (lane 1) as was shown before when cross-linking was performed in buffer A (4). However, in mixture B two additional major proteins, with apparent molecular weights of 50K and 28K daltons, were cross-linked to the 5' cap structure (lane 1). In order to determine whether the cross-linking of the 50K and 28K dalton proteins to the 5' cap structure is cap specific, incubations were carried out in presence of m^7GDP or GDP. As shown in lane 2, m^7GDP clearly inhibited the cross-linking of the 50K and 28K dalton proteins in addition to the 24K cap binding protein. Some non-specific proteins were also labeled and they increased in intensity as a consequence of adding m^7GDP. By contrast, GDP did not have any effect on the cross-linking of the 24K, 28K and 50K dalton proteins, this result being consistent with their cap binding speci-

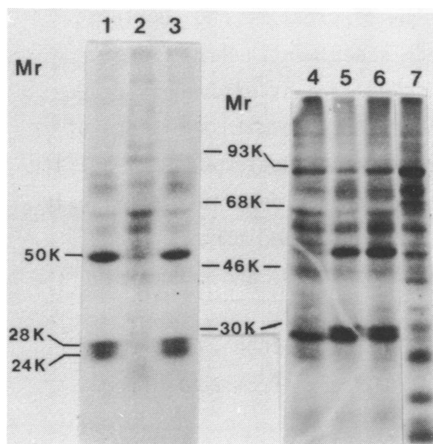

Figure 1. Polyacrylamide gel profile of initiation factor preparations cross-linked in different incubation mixtures. Partially purified initiation factors (96 μg) were cross-linked in mixture B (see Materials and Methods) to oxidized [^{3}H]-methyl reovirus mRNA in the presence of the indicated additions and run on polyacrylamide gels which were fluorographed as described. Samples 1-3 each contained 0.6 mM Mg^{++} (1) control (2) 0.4 mM m^{7}GDP (3) 0.4 mM GDP (4) Mg^{++} omitted (5) 1 mM Mg^{++} (6) 2 mM Mg^{++} (7) 1 mM Mg^{++} plus 0.2 mM m^{7}GDP.

ficity (lane 3). It should be pointed out that the cross-linking of a minor protein of molecular-weight of ∿ 75-80K daltons was also inhibited by the addition of m^{7}GDP and not GDP (compare lane 2 with lanes 1 and 3). Cross-linking of this protein was more prominent when total initiation factors were used (unpublished observations). Since the cross-linking of the 28K and 50K dalton proteins to the 5' cap structure of mRNA is cap specific, I will refer to them henceforth as cap binding proteins.

Further experiments were designed to identify the components in mixture B which were required for the cross-linking of the 50K and 28K dalton polypeptides to the cap structure. Omission of Mg^{++} from the cross-linking incubation buffer resulted in a sharp decrease in the cross-linking of the 28K and 50K dalton proteins (Fig. 1, lane 4). When Mg^{++} was added back to the incubation mixture, at a final concentration of 1 or 2 mM, the cross-linking of the 28K and 50K proteins was dramatically stimulated (Fig. 1, lanes 5 and 6 compared to lane 4). High concentrations of Mg^{++} (8 mM) inhibited cross-linking (data not shown). Different preparations of partially purified initiation factors were used for the experiments depicted in lanes 1-3 and 4-7. This might have been the reason for the cross-linking of proteins at the molecular weight range of ∿ 55-60K and 90K daltons in the preparation used in lanes 4-7. Cross-linking of mRNA to these proteins is not cap dependent since addition of m^{7}GDP did not prevent their cross-linking (lane 7).

The requirement for Mg^{++} in the cross-linking of the 28K and 50K cap binding proteins indicated that Mg^{++} may be functioning via complex formation

with ATP, and that an ATP/Mg^{++} complex might be required for recognition of the cap structure by the 28K and 50K cap binding proteins. To investigate this possibility, cross-linking was performed in the presence and absence of ATP by supplementing mixture A, which included KCl, dithiothreitol and Hepes (pH 7.6), with the individual components of mixture B. The results are shown in Fig. 2. In mixture A, in the absence of ATP and Mg^{++}, the major proteins labeled were the 24K cap binding protein and a 52K dalton protein (lane 1), which was shown previously to correspond to elongation factor 1 (EF-1) (4). Cross-linking of the 52K EF-1 protein had been shown to be prevented by the addition of GDP or GTP but not by m^7GDP or m^7GTP (ref. 4 and also lane 2). This is in sharp contrast to cross-linking of the 50K dalton protein in mixture B which is dramatically inhibited by m^7GDP but not by GDP (see Fig. 1, lanes 2 and 3). Addition of 2 mM ATP and 0.4 mM GTP did not stimulate the cross-linking of the 24K cap binding protein (lane 2) but GTP prevented the cross-linking of the EF-1 as discussed above (4). However, when Mg^{++} was included in the reaction mixture in addition to ATP, cross-linking of the 28K and 50K dalton polypeptides became apparent (lane 3). Under the same conditions the extent of cross-linking of the 24K cap binding protein and EF-1 to mRNA was reduced. The simplest explanation for this finding is that mRNA was limiting in these reaction mixtures. Preferential binding of the mRNA to the 28K and 50K cap binding proteins reduced the availability of the

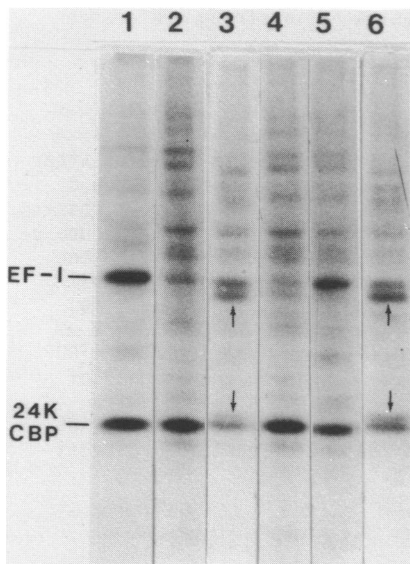

Figure 2. Effect of various components on cross-linking pattern of proteins. Cross-linking was done in mixture A with the following additions. (1) control (2) 2 mM ATP + 0.4 mM GTP (3) 2 mM ATP + 1 mM Mg^{++} (4) 0.4 mM GTP + 1 mM Mg^{++} (5) 10 μg/ml creatine phosphokinase + 10 mM creatine phosphate + 1 mM Mg^{++} (6) as in (5) + 2 mM ATP. The 50 K dalton protein is indicated by the arrow pointing upward and the 28K dalton protein by the arrow pointing downward.

mRNA for interaction with the 24K cap binding protein and the EF-1 protein. Addition of GTP and Mg^{++} without ATP did not allow cross-linking of the mRNA to the 28K and 50K dalton proteins (compare lane 4 with lane 3). Addition of creatine phosphokinase and creatine phosphate had no effect on the extent of cross-linking in the absence of ATP (compare lane 5 with lane 1), nor did it have an additive effect when added in the presence of ATP (lane 6). The results in Figures 1 and 2 demonstrate that Mg^{++} and ATP are required in approximately equimolar amounts for binding of the 28K and 50K cap binding proteins to the 5' cap structure, the step preceding their cross-linking to the 5' end of the labeled mRNA.

To test the divalent cation specificity of the binding reaction, Mg^{++} was replaced by Mn^{++}, Zn^{++} or Ca^{++}. As shown in Fig. 3, none of these divalent cations employed were efficient substitutes for Mg^{++} in stimulating cross-linking of the 28K and 50K cap binding proteins. In the presence of 2 mM Mn^{++} there was some cross-linking of the 50K dalton protein but not of the 28K cap binding protein, and cross-linking of the 24K cap binding protein also was decreased (Fig. 3, lane 2). Similar results were also obtained with Zn^{++} (lanes 3 and 4), which allowed some cross-linking of the 50K dalton protein at 2 mM but not of the 28K dalton protein. Calcium did not substitute for Mg^{++} in the cross-linking of the 28K and 50K cap binding proteins, and even reduced 24K cap binding protein cross-linking to mRNA as compared to Mg^{++} (compare lanes 5 and 6 with lane 1). Thus, ATP dependent cap binding activity of the 28K and 50K cap binding proteins has a strong preference for Mg^{++} as compared to the other divalent cations tested. As noted

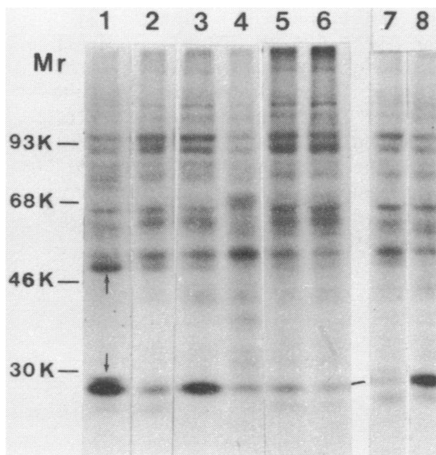

Figure 3. Polyacrylamide gel profiles of labeled proteins after cross-linking in the presence of various divalent cations. Cross-linking was performed in mixture B (Materials and Methods) with the following cations (1) 2 mM Mg^{++} (2) 2 mM Mn^{++} (3) 2 mM Zn^{++} (4) 4 mM Zn^{++} (5) 2 mM Ca^{++} (6) 4 mM Ca^{++} (7) 2 mM Mg^{++} + 0.4 mM m^7GDP (8) 2 mM Mg^{++} in the absence of ATP. The upward and downward pointing arrows indicate the 50K and 28K dalton proteins respectively.

before (Fig. 1 see also ref. 4), there is non-specific cross-linking of reovirus mRNA to high molecular weight proteins (∿ 55-60K and 90K molecular weight). Cross-linking of these polypeptides is not prevented by the addition of m^7GDP, whereas the cross-linking of the 24K, 28K and 50K cap binding proteins is sharply reduced (lane 7). Moreover, cross-linking with these proteins was not affected by omission of ATP while the cross-linking with the 28K and 50K cap binding proteins was decreased (compare lane 8 with lane 1, see also Fig. 2). Non-specific cross-linking became more apparent when Zn^{++} or Ca^{++} replaced Mg^{++}, probably as a result of the inability of the cap binding proteins to cross-link to the mRNA which became more available for non-specific cross-linking.

The specific requirement for ATP was also tested by using different analogues of ATP. Fig. 4 (lane 1) shows the pattern of a control sample which contained standard cross-linking mixture A plus 2 mM ATP and 2 mM Mg^{++} (GTP was also present in order to reduce the cross-linking of EF-1). Under these conditions all three cap binding proteins (24K, 28K and 50K daltons) were cross-linked. When ATP was replaced by the non-hydrolyzable analogues AMP-P(CH_2)P and AMP-P(NH)P, cross-linking of the 28K and 50K cap binding proteins did not occur (lanes 2 and 5 respectively) implying that ATP hydrolysis is required for the binding and subsequent cross-linking of these proteins to the 5' end of mRNA. ADP (lane 4), also did not replace ATP in promoting the cross-linking reaction of the 28K and 50K cap binding proteins. Another analogue, dADP, had a non-specific inhibitory effect on the cross-

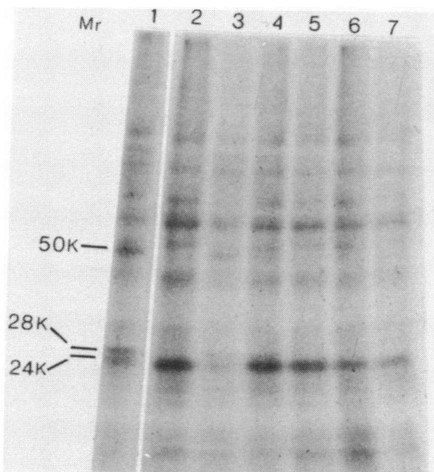

Figure 4. Cross-linking of proteins to mRNA in presence of analogues of ATP. Cross-linking was performed in mixture A (Materials and Methods) in the presence of 2 mM Mg^{++}, 1.6 mM GTP and the following additions at a final concentration of 2 mM (1) ATP (2) AMP-P(CH_2)P (3) dATP (4) ADP (5) AMP-P(NH)P (6) CTP (7) UTP.

linking of all the cross-linkable proteins, regardless of their cap specificity, for some unknown reason (lane 3). Thus, its ability to serve as an analogue of ATP in binding of the cap binding proteins to the cap structure could not be evaluated. Two other nucleotides, CTP (lane 6) and UTP (lane 7), did not substitute for ATP. These results clearly demonstrate the high level of nucleotide specificity in the binding of the 28K and 50K cap binding proteins to the 5' end of the mRNA. Moreover, when 4 mM AMP-P(NH)P was added to a cross-linking reaction which contained 1 mM ATP, cross-linking of the 50K dalton protein was inhibited completely (Fig. 5, compare lane 2 to lane 1). Cross-linking of the 28K dalton protein was resistant to inhibition under these conditions. At 8 mM AMP-P(NH)P (Fig. 5, lane 3), cross-linking of the 28K protein was also prevented, but in this case the cross-linking of the 24K and some non-specific proteins in molecular weight range of 70-90K daltons was also diminished. Very similar results were obtained when ADP was added to the cross-linking mixture (Fig. 5, lanes 4 and 5). These results may suggest that both AMP-P(NH)P and ADP can bind to the 50K cap binding protein. An alternative interpretation of these results, namely that ADP and AMP-P(NH)P chelate the Mg^{++} and prevent it from interaction with ATP seems unlikely, since cross-linking of the 28K dalton protein is not inhibited by 4 mM ADP and 4 mM AMP-(NH)P, although cross-linking of this protein is Mg^{++} dependent.

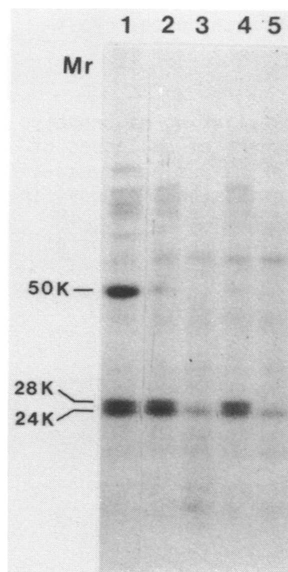

Figure 5. Effect of AMP-P(NH)P and ADP on cross-linking in the presence of ATP. Cross-linking was done in mixture B containing 1 mM Mg^{++} and the following additions. (1) control (2) 4 mM AMP-P(NH)P (3) 8 mM AMP-P(NH)P (4) 4 mM ADP (5) 8 mM ADP.

Furthermore, the affinity of Mg^{++} for ADP is an order of magnitude lower than for ATP (14).

The requirement for ATP and Mg^{++} in the cross-linking and most probably in the binding of the 28K and 50K proteins to the 5' end of eukaryotic mRNA suggested that these proteins may have to be phosphorylated in order to enable cross-linking to occur. Alternatively, the binding of these proteins to mRNA may require energy supplied by the hydrolysis of ATP. In an attempt to distinguish between these two possibilities the following experiment was carried out. Initiation factor fractions were pre-incubated with ATP and Mg^{++} in mixture A to allow putative protein phosphorylation to take place. This was followed by addition of EDTA to prevent further Mg^{++} dependent reactions. Finally, oxidized mRNA was added and subsequently incubated to obtain cross-linking of bound proteins. The results (data not shown) demonstrated that cross-linking was prevented by the addition of EDTA, which presumably prevents ATP/Mg^{++}-dependent mRNA protein complex formation by chelating Mg^{++}. Addition of NaF to the preincubation mixture, in an attempt to block the action of putative phosphatases (15), did not prevent the inhibitory effect of EDTA. The results may suggest that the 28K and 50K cap binding proteins do not have to be phosphorylated prior to their binding and cross-linking to mRNA. Thus, the results indicate that ATP/Mg^{++} functions directly during mRNA binding via an ATPase reaction catalyzed by the 50K and/or 28K cap binding proteins.

In an effort to elucidate the possible relationship between the 28K and 50K proteins on the one hand,and these two proteins and the 24K cap binding protein on the other, we used monoclonal antibodies directed against cap binding proteins. Clones producing these antibodies were screened for with affinity purified 24K cap binding protein using radioimmunoassay techniques. The production and characterization of these antibodies were described in detail by Trachsel *et al.* (16). Most strikingly, these antibodies could react with proteins having higher molecular weights than the 24K cap binding protein (Notably; 210K, 160K, 50K, and 28K dalton proteins) (16). Furthermore, some of the high molecular weight antigens were isolated using monoclonal antibodies covalently bound to Sepharose 4B. The tryptic maps of two of the isolated polypeptides (28K and 50K) were found to be similar to the 24K cap binding protein peptide map, indicating that all the three proteins share common peptides (16). This result suggests that the 24K cap protein might have originated by proteolytic cleavage of a higher molecular weight precursor protein.

In an attempt to determine whether the 28K and 50K dalton polypeptides which react with the anti-cap binding protein antibody can recognize the cap structure, rabbit reticulocyte initiation factors were cross-linked in the presence or absence of the anti-cap binding protein antibody (Kindly provided by Dr. H. Trachsel). As shown in Fig. 6 (compare lane 2 to lane 1), the anti-cap binding protein antibody sharply inhibited the cross-linking of the three cap binding proteins (24K, 28K and 50K daltons) to the mRNA, while cross-linking of other proteins was not reduced, in striking similarity to the effect of m^7GDP on cross-linking. Note that also the cross-linking of minor proteins of molecular weights of $\sim$ 80K was also inhibited by addition of the antibody similar to the effect of m^7GDP (compare Fig. 6 with Fig. 1). Immunoglobulin fractions enriched for IgM antibodies from Ehrlich ascitic fluid, which were tested as control antibodies, did not diminish the cross-linking of proteins to the 5' cap structure of mRNA (lane 3). Thus, the higher molecular weight cap binding proteins (28K and 50K proteins) seem to contain the 24K cap binding protein domain and in addition have the ability to react with the 5' cap structure of eukaryotic mRNAs in an ATP/Mg^{++} dependent reaction.

DISCUSSION

The findings reported here have led to the identification of two new putative mRNA cap binding proteins. These proteins differed markedly from

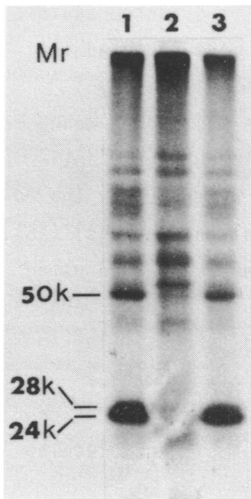

Figure 6. Effect of anti-cap binding protein monoclonal antibody on cross-linking of proteins to reovirus mRNA. Cross-linking was performed in mixture B containing 1 mM Mg^{++}, in the presence of the indicated antibody additions. (1) control (2) 3 μg of anti-cap binding protein antibody (3) 3 μ of immunoglobulin fractions from Ehrlich ascitic fluid. Monoclonal antibodies from ascitic fluid collected from mice injected with a hybridoma clone (producing IgM antibodies) were purified by 50% $(NH_4)_2SO_4$ precipitation and DEAE cellulose chromatography (16). An additional step involved centrifugation in a 10-40% sucrose gradient containing 150 mM KCl and 20 mM Hepes pH 7.6 (Beckman SW40 rotor, 16 hr, 40K rpm), to separate IgM class antibodies ($\sim$ 15S) from lower molecular weight proteins. Immunoglobulin fractions from Ehrlich ascitic fluid were prepared in a similar way.

the 24K cap binding protein in that ATP and Mg^{++} are required for their cross-linking to the oxidized 5' cap of reovirus mRNA. The results suggest that a Mg^{++}-dependent hydrolysis of ATP is needed for the binding of the 28K and 50K proteins to the mRNA.

These findings are consistent with previous reports which documented the requirement for ATP in initiation of protein synthesis in eukaryotes in contrast to lack of this requirement in the prokaryotic systems (12,13). Recent observations (17) using inosine-substituted reovirus mRNA, which has considerably less secondary structure than the native mRNA (17,18) , indicated that this mRNA was much less dependent on ATP for protein synthesis initiation complex formation as compared to normal, guanosine containing mRNA. Moreover, Morgan and Shatkin (17) found that the binding of inosine-substituted mRNA to ribosomes was inhibited by m^7GMP to a lesser extent than guanosine containing reovirus mRNA, indicating that initiation complex formation by the modified mRNA is less cap dependent. This result agrees with earlier observations by Kozak (18), who showed that uncapped inosine substituted reovirus mRNA binds efficiently to ribosomes. These results could be interpreted to indicate that the function of ATP in initiation complex formation is to provide energy for the destabilization of the secondary structure at the 5' end of mRNA, and thus enable the 40S ribosomal subunit to attach to the 5' end of the mRNA. A protein which recognizes the cap structure might function as an unwinding protein. Disruption of the secondary structure of the mRNA as in the case of inosine-substituted reovirus mRNA (17,18) may partially obviate the need for ATP and the cap structure. Thus, it is tempting to suggest that the 28K and 50K proteins use the energy generated by the hydrolysis of ATP to bind to and unwind the 5' end structure of eukaryotic mRNAs. The latter notion, is strongly supported by experiments which showed that monoclonal anti-cap binding protein antibody inhibited the binding of standard reovirus mRNA to ribosomes, but did not inhibit the binding of inosine-substituted reovirus mRNA (N.S. and H. Trachsel, in preparation).

Ilan and Ilan (19) have reported that a component which copurified with the multicomponent initiation factor eIF-3 had the ability to melt the secondary structure of globin mRNA. Since eIF-3 was subsequently shown to contain the 24K cap binding protein (4,10), it is possible that the unwinding activity in the preparation was due to the 24K cap binding protein.

Some eukaryotic viral mRNAs that are translated efficiently do not contain a 5' terminal cap structure. These include the RNAs of animal

picornaviruses (20-22) and two plant viruses, satellite tobacco necrosis virus (23) and cowpea mosaic virus (24). Initiation of translation of these mRNAs must therefore occur by a cap-independent mechanism. If the hypothesis proposed above is correct, namely that ATP/Mg^{++} is needed in the initiation of protein synthesis for recognition of the cap structure by cap binding proteins, then naturally uncapped mRNAs should by-pass the requirement for ATP/Mg^{++}. Indeed, recent unpublished data from R. Jackson's laboratory have suggested that cowpea mosaic virus RNA forms initiation complexes in the absence of ATP (personal communication). Similar results were obtained by M. Kozak with satellite tobacco necrosis viral RNA (personal communication). These results strongly suggest that the ATP requirement and cap recognition are related aspects of translation initiation.

The ability of the monoclonal anti-cap binding protein antibody to specifically inhibit the cross-linking of the cap binding proteins (Fig. 6), strongly suggests that the 24K, 28K, and 50K cap binding proteins are structurally related. Cleavage of high molecular weight precursors might occur *in vivo* as a process for generating active cap binding proteins. Alternatively, cleavage could have occured *in vitro* during the process of isolation of the initiation factors. Studies done by Trachsel *et al.* (16) indicate that some processing might take place *in vivo*. In their studies they reported that the only polypeptide on endogeneous polysomes from rabbit reticulocytes which was capable of interacting with the anti-cap binding protein antibody was a 50K polypeptide. Furthermore, the latter was one of the major polypeptides which cross-linked to the 5' cap structure of reovirus mRNA in 80S monosomes. The authors interpreted these results to imply that the 50K polypeptide which might have been cleaved from a higher molecular weight precursor (210K or 160K dalton proteins) was the active cap binding protein.

Further experiments to elucidate the functions of the cap binding proteins and the role of ATP hydrolysis during initiation of protein synthesis are currently underway with chemically decapped mRNAs or naturally uncapped mRNAs enzymatically capped *in vitro* with vaccinia capping enzymes (25,26). These experiments should increase our understanding of initiation of protein synthesis and its regulation in normal and virus infected cells.

ACKNOWLEDGEMENTS

This work was supported in part by grant # MA-7214 from the MRC of CANADA and by a grant from the National Cancer Institute of CANADA. I thank

Dr. H. Trachsel for a generous gift of the anti-cap binding protein antibody and for helpful discussions. I am grateful to Dr. A.J. Shatkin for stimulating discussions and valuable comments, and Dr. M. Fraser for critical reading of the manuscript.

ABBREVIATIONS

m^7GDP, 7-methylguanosine-5'-diphosphate. AMP-P(CH_2)P, adenylyl (β-γ-methylene)-diphosphonate. AMP-P(NH)P, adenylyl imidodiphosphonate. eIF-eukaryotic initiation factor. S-100, 100,000xg supernatant fractions from reticulocyte lysate.

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