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

ATP-Mg$^{2+}$ INDEPENDENT CROSS-LINKING OF CAP BINDING PROTEINS TO m$^1$-CAPPED INOSINE-SUBSTITUTED REOVIRUS mRNA

(Received for publication, September 3, 1982)

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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 37, 563–572). However, specific cross-linking of these polypeptides can occur in the absence of ATP-Mg$^{2+}$ when $m^1$-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.

Polypeptides that interact with the $5'$ terminal cap structure, $m^GpppN$, of eukaryotic mRNAs have been identified in IF$^2$ 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, 24 CBP) is not dependent on ATP-Mg$^{2+}$ (1–3), while cross-linking of the 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 was 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$^{2+}$-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: ["H"methyl-labeled reovirus mRNA was synthesized with viral cores in the presence of [3H]methyl-s-adenosymethionine (AdoMet, specific activity, 70 Ci/mmol, New England Nuclear), as described by Muthukrishnan et al. (14), to a specific activity of ~2 × 10$^4$ cpm/fg. m$^1$-capped inosine-substituted reovirus mRNA was synthesized according to Morgan and Shatkin (10) to a specific activity of ~3 × 10$^4$ cpm/fg, and bromouridine-substituted reovirus mRNA was prepared, according to Kozak (11), to a specific activity of ~1.5 × 10$^4$ cpm/fg. 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 0.6-M KCl wash of ribosomes from rabbit reticulocyte 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 modifications of Hansen and Ehrenfeld (4). Reaction mixtures (30 μl) contained 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic 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$^GDP$ 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 NaBH₄CN. 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 amounts of [3H]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 μM 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 spermidine 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.

RESULTS

To study the relationship between ATP-dependent cross-linking of cap recognition factors and the secondary structure of the mRNA, we analyzed polyribosomes 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, m⁷GDP. 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 m⁷GDP (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). 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-Mg²⁺ 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 ATP 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-polypeptides 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 salt concentration, which in the absence of ATP.
FIG. 2. Cross-linking of reticulocyte cap binding proteins to reovirus mRNA with altered secondary structure. A, [3H]methyl-labeled m1-capped inosine-substituted mRNA (4 x 10⁴ cpm) or B, bromouridine-substituted mRNA (4 x 10⁴ 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. m7GDP (0.7 mM) was added as indicated. The positions of the cross-linked CBPs are indicated by dots.

prevents cross-linking of ATP-Mg2+-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 substitution 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 concentration (Fig. 2B, lane 3), in contrast to the 140 mM optimum for inosine-substituted and native mRNA. In the absence of ATP-Mg2+, 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-Mg2+. These results further indicate that mRNA with secondary structure requires ATP-Mg2+ 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 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

FIG. 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 as 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%.
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 cap recognition factor could 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-Mg²⁺-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 24-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 24-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 24-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 mI-capped inosine-substituted reovirus mRNA is dependent on ATP-Mg⁺. 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 40 S 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 may 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.

Acknowledgments—We thank A. J. Shatkin for helpful comments on the manuscript.

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