Functional Characterization of Eukaryotic mRNA Cap Binding Protein Complex: Effects on Translation of Capped and Naturally Uncapped RNAs†

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ABSTRACT: We examined the effects of a eukaryotic mRNA cap binding protein (CBP) complex purified by cap analogue affinity chromatography [Edery, I., Humebelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H., & Sonenberg, N. (1983) *J. Biol. Chem. 258*, 11398–11403], on translation of several capped and naturally uncapped mRNAs in extracts prepared from poliovirus-infected or mock-infected HeLa cells. The CBP complex has activity that restores capped mRNA (globin, tobacco mosaic virus, and others) function in extracts from poliovirus-infected HeLa cells. Translation of two naturally uncapped RNAs (poliovirus and mengovirus RNAs), the translation of which is not restricted in extracts from poliovirus-infected cells, is also not stimulated by the CBP complex. Translation of several capped eukaryotic mRNAs (vesicular stomatitis virus, reovirus, and tobacco mosaic virus) in extracts from mock-infected cells is inhibited when the potassium ion concentration is increased. However, translation of capped AMV-4 RNA, which has negligible secondary structure at its 5′ end, is resistant to this inhibition. Furthermore, the CBP complex reverses the high salt induced inhibition of translation of the former mRNAs. Since mRNA secondary structure is more stable at elevated salt concentrations, these data are consistent with a model in which the CBP complex has a role in melting mRNA secondary structure involving 5′-proximal sequences, to facilitate ribosome binding.

The cap structure, $m^7GpppN(m)$, is found at the 5′ terminus of almost all eukaryotic mRNAs analyzed to date, certain viral mRNAs being exceptions [for reviews see Shatkin (1976) and Banerjee (1980)]. Numerous studies have demonstrated that the cap structure facilitates stable complex formation between 40S ribosomal subunits and mRNA during translation initiation (Banerjee, 1980). However, the degree of dependence on the cap structure for translation is influenced by various parameters including temperature (Weber et al., 1978), the concentration of IF[1] (Held et al., 1977), and ionic strength (Weber et al., 1977; Chu & Rhodes, 1978) and also varies among different mRNAs (Lodish, 1976; Walden & Thach, 1982).

It was anticipated that cap function is mediated by a cap-specific mRNA–protein interaction, and subsequently the polypeptides involved have been detected by specific chemical cross-linking to oxidized cap structures of several viral mRNAs (Sonenberg et al., 1978; Sonenberg, 1981) and purified by m^7GDP affinity chromatography (Sonenberg et al., 1979; Tahara et al., 1981). By use of the cross-linking technique, it has been shown that in crude rabbit reticulocyte IF, polypeptides of 24, 28, 50, and 80 kDa specifically interact with the cap structure, and with the exception of the 24-kDa polypeptide, the cross-linking is dependent on ATP–Mg^{2+} (Sonenberg, 1981; Sonenberg et al., 1981). Using purified initiation factors, Grifo et al. (1982) have suggested that the 50- and 80-kDa polypeptides correspond to eIF-4A and eIF-4B, respectively. In the case of eIF-4A, this suggestion has been verified (Edery et al., 1983).

The fact that the 28-, 50-, and 80-kDa polypeptides interact with mRNA in an ATP-dependent, cap-specific manner implies that cap function and ATP hydrolysis are related aspects of translation initiation. This along with several other observations led to the proposal that a factor involved in cap recognition may function, in part, by utilizing the energy from ATP hydrolysis to melt capped mRNA secondary structure involving 5′-proximal sequences and consequently facilitate ribosome binding (Sonenberg, 1981; Sonenberg et al., 1981). This model is consistent with the findings that mRNAs with reduced secondary structure are less dependent on both the cap structure and ATP for binding of 40S ribosomal subunits (Kozak, 1980; Morgan & Shatkin, 1980) and that some naturally uncapped RNAs exhibit a reduced dependence on ATP for the same function (Jackson, 1982).

Poliovirus RNA is one of the few naturally uncapped eukaryotic mRNAs (Hewlett et al., 1976; Nomoto et al., 1976), and cell extracts from poliovirus-infected HeLa cells have altered initiation factor activity such that they no longer translate cellular (capped) mRNAs but can translate naturally uncapped RNAs (Rose et al., 1978). It has long been an attractive hypothesis that inactivation of a cap binding protein explains this discrimination, and Tahara et al. (1981, 1982) have isolated two forms of cap binding protein by m^7GDP affinity chromatography of rabbit reticulocyte initiation factors. These are termed CBPI and CBPII, CBPI being identical with the 24K CBP originally isolated by Sonenberg et al. (1979) and CBPII comprising CBPI plus major polypeptides of 48 and 225 kDa. The 48-kDa polypeptide of CBPII is eIF-4A (Grifo et al., 1983; Edery et al., 1983). The activities of CBPI and CBPII are distinct in that the ability to restore translation of capped mRNAs in poliovirus-infected cell ex-

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[1] Abbreviations: IF, initiation factor(s); eIF, eukaryotic initiation factor; kDa, kilodalton; CBP, cap binding protein; AMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; VSV, vesicular stomatitis virus; STNV, satellite tobacco necrosis virus; EMCV, encephalomyocarditis virus; PMSF, phenylmethanesulfonyl fluoride; Cl_3CCOOH, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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tracts copurifies with CBPII but does not reside in CBPI (Tahara et al., 1981, 1982). Recently, Grifo et al. (1983) have shown that a reconstituted protein synthesis system can be stimulated by a factor purified by a different procedure and having very similar polypeptide composition to that of the CBPII previously described. This factor can also restore translation of capped mRNAs in extracts from poliovirus-infected HeLa cells and is therefore assumed to be identical with CBPII. In light of the fact that it is required for maximal translation in a reconstituted system, it has been given bona fide initiation factor status as eIF-4F.

We have purified a cap binding protein complex by m^7GDP affinity chromatography, comprising CBPI, eIF4A, and an ~220-kDa polypeptide (Edery et al., 1983). Here we show that this complex (referred to throughout this paper as the CBP complex) is able to restore translation of several capped mRNAs in extracts from poliovirus-infected HeLa cells, indicating functional analogy with the CBPII purified by Tahara et al. (1981, 1982). We also report that naturally uncapped RNAs or a capped mRNA (AMV-4 RNA) with no significant 5′-proximal secondary structure shows a decreased dependence on the CBP complex for translation and that the CBP complex is able to reverse the inhibition of translation of several capped mRNAs, caused by high salt concentrations, in extracts from mock-infected HeLa cells.

Materials and Methods

Cell, Virus, and mRNAs. HeLa S3 cells were grown in media supplemented with 5% calf serum and infected with poliovirus type 1 (Mahoney strain), at a multiplicity of infection of 10–20, as previously described (Lee & Sonenberg, 1982). The sources of different mRNAs were as follows: TMV, AMV-4, and VSV mRNAs were kind gifts from A. Asselin, J. Bol, and L. Poliquin, respectively. Globin and reovirus mRNAs were prepared according to published procedures (Krystosek et al., 1975; Muthukrishnan et al., 1976). Poliovirus and mengovirus RNAs were prepared according to Villa-Komaroff et al. (1975) and Eggen & Shatkin (1972), respectively.

Preparation of CBP Complex. Purification of the CBP complex was as described by Edery et al. (1983). The concentration of the CBP complex preparation was 0.15 mg/mL in 20 mM Hepes buffer (pH 7.5) containing 100 mM KCl, 0.2 mM EDTA, 7 mM β-mercaptoethanol, 0.5 mM PMSF, and 10% glycerol.

Cell-Free Translation. Cell extracts from mock-infected or poliovirus-infected (3-h postinfection) HeLa cells were prepared as described by Rose et al. (1978) and modified by Lee & Sonenberg (1982). In vitro protein synthesis was carried out essentially as described by Rose et al. (1978). Incubation mixtures at a final volume of 12.5 μL (or 25 μL) contained 4.5 μL (or 9 μL) of micrococcal nuclease treated S10 extract in 20 mM Hepes buffer, pH 7.6, 0.8 mM $Mg(OAc)_2$, 1 mM ATP, 55 μM GTP, 9 mM creatine phosphate, 24 μg/mL creatine kinase, 2 mM dithiothreitol, 0.2 mM spermidine, 11 μM each of 19 amino acids (minus methionine), 24 μg/mL yeast tRNA (Boehringer), 15 μCi (or 30 μCi) of [^{35}S]methionine (>1000 Ci/mmol), KOAc (given as final concentration adjusted by addition of KOAc as indicated to the 30 mM KOAc contributed by the extract), and mRNA in the amounts indicated in figure legends. Incorporation of [^{35}S]methionine into acid-precipitable products was assayed after 60 min at 32 °C by spotting 5-μL aliquots on Whatman 3MM filter paper disks followed by boiling for 15 min in 5% Cl_3CCOOH and successive washes with cold 5% Cl_3CCOOH ethanol–ether (1:1), and finally ether. Radioactivity was measured in toluene-based scintillation fluid. Background incorporation in the absence of exogenous mRNA at one salt concentration was subtracted from values obtained at all salt concentrations, since we found that it was not affected by salt concentration. ^{35}S-Labeled products (7.5 μL of translation mixture) were resolved on 12.5% SDS/polyacrylamide gels followed by treatment with ENHANCE (New England Nuclear) and fluorography. Quantitiation of labeled bands was performed by scanning the autoradiogram with a soft laser scanning densitometer (LKB).

Results

In initial attempts to characterize the translational function of the CBP complex purified in our laboratory (Edery et al., 1983), we analyzed its effect on translation of several eukaryotic mRNAs (both capped and naturally uncapped) in extracts prepared from either mock-infected or poliovirus-infected HeLa cells. In light of observations which show that salt concentration influences the degree of cap dependence exhibited by capped mRNAs for translation (Weber et al., 1977; Chu & Rhodes, 1978), we assessed the activity of the CBP complex at different salt concentrations. In all experiments we employed subsaturating amounts of mRNA in order not to limit any other component essential for translation and analyzed the translation products by SDS/PAGE.

Tobacco mosaic virus (TMV) RNA is a capped plant viral RNA which is dependent on the cap structure for translation (Wodnar-Filipowicz et al., 1978) and consequently should not be efficiently translated in extracts from poliovirus-infected cells (Ehrenfeld, 1982). Figure 1A shows [^{35}S]methionine incorporation into Cl_3CCOOH-precipitable polypeptides in a nuclease-treated extract from poliovirus-infected cells programmed with TMV RNA, at different K^+ concentrations. The results show that translation of TMV RNA is restricted (~90%) compared to that obtained by using extracts from mock-infected cells (compare Figure 1A to Figure 5C). Addition of the CBP complex stimulated TMV RNA translation significantly and resulted in faithful translation of the RNA, as determined by SDS/polyacrylamide gel electrophoresis (data not shown). This stimulatory activity (in extracts from poliovirus-infected cells) will be referred to as restoring activity throughout the text. The restoring activity is also influenced by the K^+ concentration as evidenced by enhanced activity at low salt concentration (increase of 100 000 cpm at 75 mM K^+ vs. 40 000 cpm at 180 mM K^+). To further characterize the effect of salt concentration on the restoring activity, we measured the stimulation of protein synthesis as a function of the level of added CBP complex. The results from two salt concentrations (125 and 180 mM final K^+ concentration) are shown, as the total incorporation of [^{35}S]methionine into TMV polypeptides (Figure 1B) and SDS/PAGE of the labeled polypeptides (Figure 1C). As can be seen from Figure 1B the slope of the line expressing incorporation of [^{35}S]methionine as a function of added CBP complex is steeper at the lower salt (125 mM K^+) as compared to the higher salt concentration (180 mM K^+). The stimulation ratio (defined as the slope obtained at 125 mM K^+ divided by the slope obtained at 180 mM K^+ for a particular increment in CBP complex) ranges between 1.6 and 1.7 as shown in Table I. Thus, addition of a given amount of CBP complex at two different salt concentrations resulted in a greater increase of TMV RNA translation at the lower salt concentration.

We performed similar analyses using other capped mRNAs, and the results in Figure 2 show the effect of CBP complex on translation of globin mRNA (a cellular mRNA) at the two different salt concentrations used before (125 and 180 mM

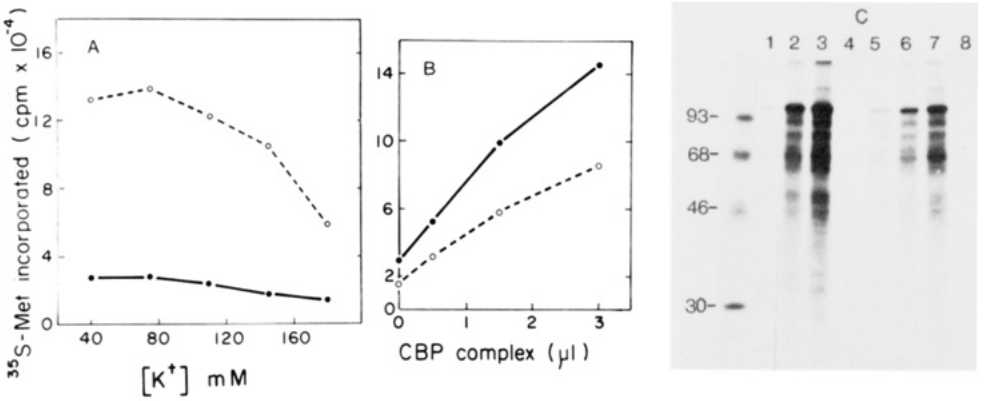

FIGURE 1: Effect of CBP complex on translation of TMV RNA in extracts from poliovirus-infected HeLa cells. Translation was performed as described under Materials and Methods. Reaction mixtures containing 15 μCi of [^{35}S]methionine and 0.5 μg of TMV RNA in a final volume of 12.5 μL (reaction mixture for panel A was scaled up 2-fold except that 2 μg of TMV was used) were incubated for 60 min at 32 °C, and aliquots of 5 μL were withdrawn to determine incorporation of [^{35}S]methionine into Cl_3CCOOH-precipitable polypeptides. Panel A, translation in the absence (●) or presence of 1 μL of CBP complex (○). Panel B, translation at final salt concentration of 125 mM K^+ (●) or 180 mM K^+ (○) with different amounts of CBP complex. Panel C, fluorogram of translation products of panel B. Lanes 1–3, at 125 mM K^+; lanes 4–7, at 180 mM K^+; lane 8, in the absence of exogenous mRNA at 125 mM K^+. CBP complex was added in the following volumes: lanes 1 and 4, none; lane 5, 0.5 μL; lanes 2 and 6, 1.5 μL; lanes 3 and 7, 3 μL. Background incorporation in the absence of exogenous mRNA (panel A, 7670 cpm; panel B, 19 600 cpm) was subtracted from all values. The molecular masses of standard proteins are indicated in kilodaltons on the left of panel C.

Table I: Effect of Salt Concentration on Stimulation of TMV Translation by the CBP Complex

range of added CBP complex (μL)	stimulation of translation (cpm)[a] per μL of CBP complex		stimulation ratio, 125/180 mM K^+
	125 mM K^+	180 mM K^+	
0–0.5	48 000	30 000	1.6
0.5–1.5	46 000	28 000	1.6
1.5–3.0	31 000	18 000	1.7

[a] Values were taken from Figure 1B.

K^+). At either salt concentration, there is very little globin synthesis in the absence of added CBP complex. As with TMV RNA, the restoring activity of the CBP complex is better manifested at the lower salt concentration (stimulation ratio between 125 and 180 mM K^+ is 4, when 0.5 μL of CBP complex is added). Addition of more CBP complex saturates the system more readily than is the case for TMV RNA, and therefore, stimulation of translation is not linear. The reason for this behavior, which is different from that observed with TMV translation, is not clear, but it is possible that another factor which is required more for globin synthesis becomes limiting in the system. In terms of restoring activity, the CBP complex had the same effect on translation of capped reovirus or VSV mRNAs (data not shown) as was observed with TMV and globin mRNAs, indicating that the activity is generally required for translation of capped mRNAs and is influenced by the salt concentration.

It has been proposed that some form of cap binding protein functions by melting mRNA secondary structure in order to facilitate binding of ribosomes to mRNA (Sonenberg, 1981; Sonenberg et al., 1981). Any such melting activity might reside in or be directly dependent on the CBP complex and therefore might be impaired as a consequence of poliovirus infection. This notion gains support from the observations that the capped plant viral RNA, AMV-4 RNA, which is devoid of any stable secondary structure in its 5′-noncoding region (Gehrke et al., 1983), can be translated with relative efficiency in extracts from poliovirus-infected cells (Sonenberg et al., 1982). A likely prediction that follows is that addition of CBP complex to extracts from poliovirus-infected cells should not markedly stimulate the translation of AMV-4 RNA. The

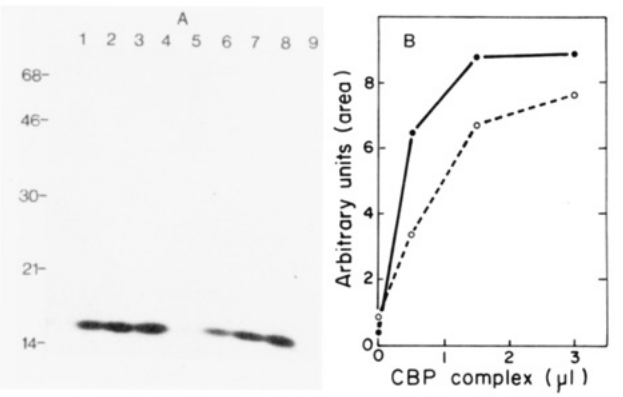

FIGURE 2: Effect of CBP complex on translation of globin mRNA in extracts from poliovirus-infected HeLa cells. Translation was performed as described under Materials and Methods with 0.5 μg of rabbit reticulocyte globin mRNA. Panel A, lanes 1–4, fluorogram of translation products at a final K^+ concentration of 125 mM; lanes 5–8, at 180 mM; lane 9, in the absence of exogenous mRNA at 125 mM K^+. CBP complex was added in the following volumes: lanes 1 and 5, none; lanes 2 and 6, 0.5 μL; lanes 3 and 7, 1.5 μL; lanes 4 and 8, 3 μL. Panel B, quantitative analysis of the labeled globin bands in the fluorogram of panel A at 125 mM K^+ (●) or 180 mM K^+ (○). The molecular masses of standard proteins are indicated in kilodaltons on the left of panel A.

results shown in parts A (SDS/PAGE of translation products) and B (densitometric analysis of AMV coat protein synthesis) of Figure 3 verify this prediction. Synthesis of the AMV coat protein (M_r 29 000) was stimulated by only 10% when the amount of CBP complex was increased by 6-fold at 125 mM K^+ (Figure 3A,B, compare lanes 4 and 2). Translation was stimulated more (about 2-fold) at 180 mM K^+. It is of interest that endogenous poliovirus RNA is translated (polypeptides of M_r ~85 000–95 000 which correspond to poliovirus proteins P1-1a and P3-1b) with AMV-4 RNA when CBP complex is not present in the reaction mixture (Figure 3, lanes 1 and 5). Addition of CBP complex reduces the translation of poliovirus RNA especially when the highest amount of CBP complex (3 μL) is added (Figure 3, compare lanes 4 and 7 to lanes 1 and 5, respectively).

It is clear that the activity which is impaired during poliovirus infection and which copurifies with the CBP complex is not required or is very much less limiting for the translation

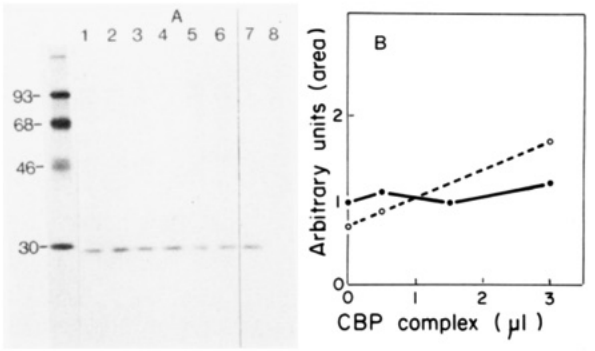

FIGURE 3: Effect of CBP complex on translation of AMV-4 RNA in extracts from poliovirus-infected HeLa cells. Translation was performed as described under Materials and Methods with 0.5 μg of AMV-4 RNA. Panel A, fluorogram of translation products: lanes 1–4, translation at a final K^+ concentration of 125 mM; lanes 5–7, at 180 mM; lane 8, in the absence of exogenous mRNA at 125 mM K^+. CBP complex was added in the following volumes: lanes 1 and 5, none; lanes 2 and 6, 0.5 μL; lane 3, 1.5 μL; lanes 4 and 7, 3 μL. Panel B, quantitative analysis by densitometric tracing of the AMV-4 coat protein band in the fluorogram shown in panel A, at 125 mM K^+ (●) or 180 mM K^+ (○). The molecular masses of standard proteins are indicated in kilodaltons on the left of panel A.

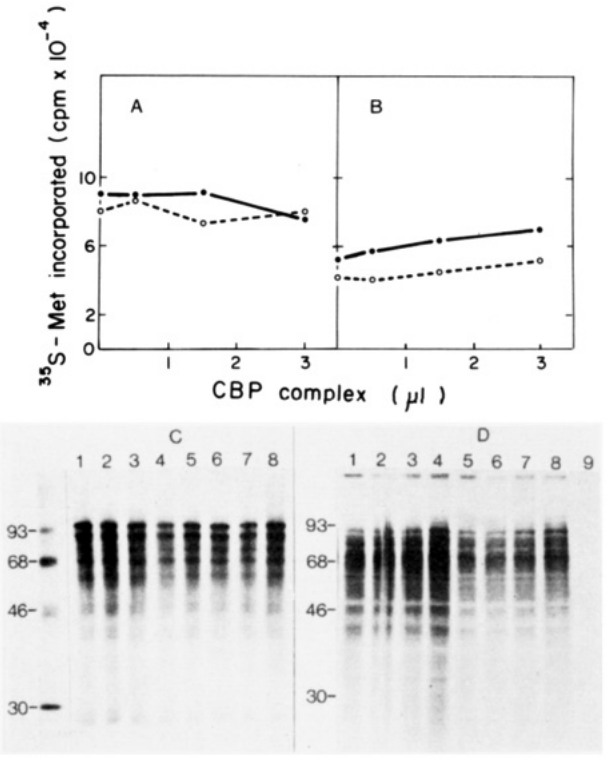

FIGURE 4: Effect of CBP complex on translation of poliovirus and mengovirus RNAs in extracts from poliovirus-infected HeLa cells. Translation was performed as described under Materials and Methods with ~0.5 μg of poliovirus RNA (panels A and C) or mengovirus RNA (panels B and D) and final K^+ concentrations of 125 (●) or 180 mM (○). Incorporation of [^{35}S]methionine into Cl_3CCOOH-precipitable polypeptides is expressed as cpm per 5-μL aliquot (panels A and B), and background incorporation in the absence of exogenous mRNA (panel A, 19600 cpm; panel B, 13200 cpm) was subtracted. Panels C and D, fluorogram of translation products. Lanes 1–4, at a final K^+ concentration of 125 mM; lanes 5–8, at 180 mM; lane 9, in the absence of exogenous mRNA at 125 mM K^+. CBP complex was added in the following volumes: lanes 1 and 5, none; lanes 2 and 6, 0.5 μL; lanes 3 and 7, 1.5 μL; lanes 4 and 8, 3 μL. The molecular masses of standard proteins are indicated in kilodaltons on the left of panels C and D.

of naturally uncapped RNAs (poliovirus, EMCV, and STNV RNAs) since translation of these mRNAs is not impaired in extracts from poliovirus-infected cells [see Rose et al. (1978), Bonatti et al. (1980), and Lee & Sonenberg (1982), respectively]. However, it remains an open question as to whether or not these mRNAs can utilize a modified CBP complex (if such an entity exists) or whether their translation proceeds independently of or is much less dependent on the CBP complex. Consequently, it was pertinent to examine the translational dependence exhibited by naturally uncapped RNAs, on the CBP complex in HeLa cell extracts. Figure 4A shows the labeled translation products (resolved by SDS/PAGE) obtained in extracts from poliovirus-infected cells programmed with poliovirus RNA in the presence of different amounts of the CBP complex. The gel pattern shows that translation of poliovirus RNA occurs faithfully in these extracts and is accompanied by correct processing of the poliovirus polyprotein, as evidenced by the appearance of bands with similar mobility to authentic viral polypeptide (e.g., VP-3, ~25 kDa). Addition of increasing amounts of the CBP complex did not stimulate poliovirus RNA translation at the two concentrations of K^+ used (lanes 1–4 at 125 mM K^+ and lanes 5–8 at 180 mM K^+) and was even slightly inhibitory at 125 mM K^+ (compare lane 4 to lane 1 in Figure 4C). Quantitation of radioactivity incorporated is shown in Figure 4A. Similar results were obtained with mengovirus RNA (Figure 4, panels B and D), although in contrast to poliovirus RNA, addition of CBP complex did not have any inhibitory effect on mengovirus RNA translation and was slightly stimulatory (~20%) when the highest amount of CBP complex was added (compare lanes 4 to 1 and 8 to 5 in Figure 4D). It is possible that the lack of stimulatory activity of the CBP complex when naturally uncapped RNAs are used reflects a mechanism of initiation which does not involve the CBP complex.

Message-dependent extracts from mock-infected HeLa cells are poor in translation of many exogenous mRNAs (Jagus et al., 1981), and it has been shown that CBPI (Sonenberg et al., 1980) and CBPII (Tahara et al., 1981, 1982) can stimulate translation of capped mRNAs but not naturally uncapped RNAs in extracts from uninfected HeLa cells. In light of these observations we wanted to examine the dependence of different mRNAs on the CBP complex for translation in extracts from mock-infected cells. The results in Figure 5 show [^{35}S]-methionine incorporation directed by VSV mRNA (panel A), reovirus mRNA (panel B), and TMV mRNA (panel C) at different salt concentrations and in the presence of increasing amounts of the CBP complex. Several noteworthy observations arise from this experiment. (A) The translation of all three mRNAs is better at the lower salt concentration (75 mM K^+) than at the higher salt concentrations (145 and 215 mM K^+), when no exogenous CBP complex is added. Translation of the three mRNAs is inhibited to a different extent by high salt concentrations; at 215 mM K^+, translation of VSV, reovirus, and TMV mRNAs is inhibited by 63%, 42%, and 30% respectively, relative to translation at 75 mM K^+. (B) The CBP complex did not have any significant stimulatory activity at 75 mM K^+, while in contrast, it stimulated translation of all three mRNAs at the higher salt concentration, thus alleviating the inhibitory effect of the salt.

It has been shown that translation of AMV-4 RNA is more resistant to inhibition by high salt concentrations as compared to that of several other capped mRNAs (Herson et al., 1979). In addition, many other lines of evidence have indicated that the translation of AMV-4 RNA is not strongly dependent on the cap structure (Sonenberg et al., 1982; van Vloten-Doting et al., 1977). Thus, it was pertinent to examine the dependence of AMV-4 RNA on the CBP complex for translation at different salt concentrations. As has been shown before in the

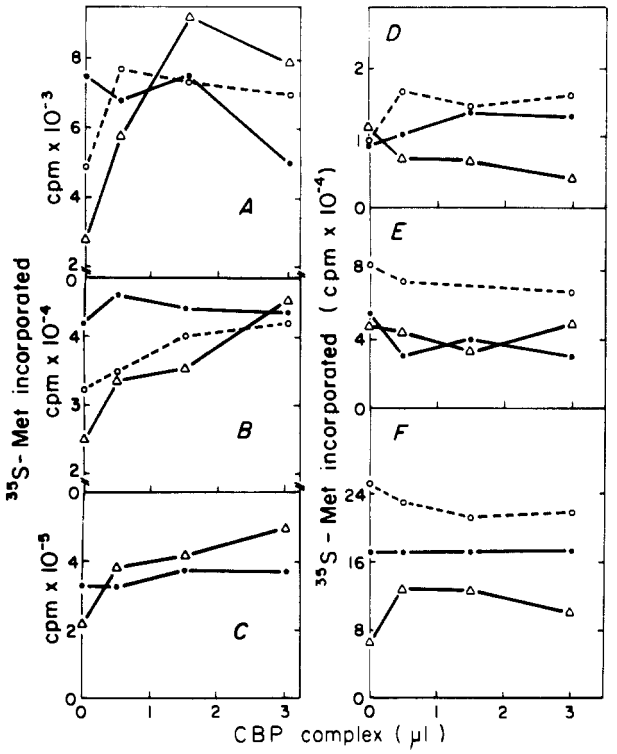

FIGURE 5: Effect of CBP complex on the translation of capped and naturally uncapped mRNAs in extracts from mock-infected HeLa cells. Translation was performed as described under Materials and Methods with 0.5 μg of each of the following mRNAs: VSV (panel A), reovirus (B), TMV (C), AMV-4 (D), poliovirus (E), and mengovirus (F), in a total incubation volume of 12.5 μL containing final K^+ concentration of 75 mM K^+ (●), 145 mM K^+ (○), and 215 mM K^+ (△). Background incorporation (cpm) in the absence of exogenous mRNA (panel A, 4730; panel B, 4730; panel C, 23 900; panel D, 14 800; panel E, 5613; panel F, 21 051) was subtracted.

wheat germ system (Herson et al., 1979), translation of AMV-4 RNA is not inhibited by high salt concentrations (Figure 5D). Addition of the CBP complex stimulated translation of AMV-4 RNA by ~1.5-fold at both 75 and 145 mM K^+ but inhibited translation (~3-fold in the presence of 3 μL of the CBP complex) at 215 mM K^+. These effects are strikingly different than those observed with VSV, reovirus, and TMV mRNAs, in which cases translation was best stimulated at high salt concentrations.

Finally, we determined the effect of the CBP complex on translation of the naturally uncapped poliovirus and mengovirus RNAs in extracts from mock-infected HeLa cells, and the results were consistent with those obtained when extracts from poliovirus-infected cells were used. For poliovirus RNA, [^{35}S]methionine incorporation is not affected by addition of the CBP complex either at low (75 mM K^+) or at high (215 mM K^+) salt concentration (Figure 5, panel E). Similarly, translation of mengovirus RNA (panel F) is also not significantly affected by the addition of the CBP complex at the three different salt concentrations (translation at 215 mM K^+ is stimulated 2-fold by addition of 0.5 μL of the CBP complex but then decreases at higher concentrations of the complex). It is also worth noting that the translation of these two naturally uncapped mRNAs appears to have a higher K^+ optima for translation than the capped mRNAs we used, in the absence of added CBP complex. Translation of poliovirus and mengovirus RNAs is better at 145 mM than at 75 mM K^+ (Figure 5E,F) while translation of VSV and reovirus mRNAs is better at 75 mM than at 145 mM K^+. Inhibition of translation of poliovirus and mengovirus RNAs is observed at high salt concentrations (compare 215 mM K^+ to 145 mM K^+ in Figure 5E,F), but significantly, it is not overcome by the addition of the CBP complex. This indicates that the inhibitory effect of high salt concentrations on the translation of naturally uncapped RNAs is not due to the same mechanism(s) as that which results in inhibition of translation of capped mRNAs.

Discussion

A high molecular weight CBP complex has been described by several groups (Tahara et al., 1981, 1982; Ray et al., 1983; Grifo et al., 1983; Edery et al., 1983), and thus far its activity has been assessed by various translational assays or by examining its role in the binding of initiation factors to capped mRNAs. Consequently, the following activities have been demonstrated: (a) restoration of capped mRNA function in extracts from poliovirus-infected cells (Tahara et al., 1981, 1982); (b) stimulation of globin mRNA translation in a reconstituted protein synthesis system from rabbit reticulocytes (Grifo et al., 1983); (c) relief of translational competition and possible mRNA discriminatory activity (Ray et al., 1983); (d) activity which is required for an ATP-dependent cap-specific mRNA–protein interaction between eIF-4A, eIF-4B, and mRNA (Edery et al., 1983).

In this report we describe the translational activity of the CBP complex purified by cap analogue affinity chromatography in our laboratory (Edery et al., 1983). Structurally, this complex comprises three major polypeptides, which correspond to the 24K CBP [CBPI, originally purified by Sonenberg et al. (1979)], eIF-4A (Edery et al., 1983), and an ~220-kDa polypeptide. Consistent with previous observations (Tahara et al., 1981, 1982) we show that the activity which can restore translation of capped mRNAs in extracts from poliovirus-infected cells copurifies with the CBP complex (Figure 1 and 2) but does not reside in the 24-kDa component (unpublished observations). Using an antibody against a 220-kDa polypeptide, which copurifies with eIF-3 under low ionic strength conditions and also recognizes the 220-kDa polypeptide of the CBP complex, Etchison et al. (1982) have shown that the 220-kDa polypeptide is proteolyzed by a poliovirus-dependent protease activity. Consequently, they have proposed that proteolysis of the 220-kDa polypeptide subunit of the CBP complex explains the shut-off of cellular protein synthesis exerted by poliovirus.

Several observations led to the proposal that some form of CBP functions by melting mRNA secondary structure in order to enable 40S ribosomes to bind capped mRNAs (Ilan & Ilan, 1977; Morgan & Shatkin, 1980; Kozak, 1980; Sonenberg, 1981; Sonenberg et al., 1981). This hypothesis received support from the observation that certain capped mRNAs with reduced secondary structure can function in extracts from poliovirus-infected cells (Sonenberg et al., 1982), which might reflect the fact that these mRNAs are less dependent on a secondary structure melting activity, residing in the CBP complex and impaired upon poliovirus infection. The experiments in this paper are supportive of this latter idea in two ways: (a) The CBP complex stimulated the translation of several capped mRNAs (TMV, globin, VSV, and reovirus mRNAs) in extracts from poliovirus-infected cells. However, it did not stimulate the translation of AMV-4 RNA, which is devoid of stable secondary structure in its 5′-proximal sequences (Gehrke et al., 1983). (b) It is possible that inhibition of translation of capped mRNAs in extracts from mock-infected cells, at high salt concentrations, is due to increased stability of mRNA secondary structure under these conditions (Holder & Lingrel, 1975). Consequently, addition of a factor which can denature the template might overcome high salt induced inhibition of translation. The data in Figure 5, indeed, show that the CBP complex can alleviate this inhibitory effect.

Another reasonable interpretation is that high salt concentrations directly inhibit the activity of the CBP complex, maybe by reducing its affinity for mRNA. However, one still has to explain why the translation of AMV-4 RNA is resistant to inhibition by high salt concentrations. In light of our observation that the amount of inhibition of ribosome binding to reovirus mRNAs at high salt concentrations is directly related to the degree of secondary structure of the mRNA (Lee et al., 1983), it seems a likely possibility that inhibition of capped mRNA translation at high salt concentrations is related (directly or indirectly) to the degree of secondary structure of the mRNA.

At low input levels of exogenous CBP complex, translation of capped mRNAs is best stimulated at high salt concentrations in the mock-infected lysate (Figure 5), whereas for the infected lysate, stimulation is higher at low salt concentrations (Figures 1 and 2). This apparent discrepancy may be explained if one assumes that the CBP complex is always absolutely required for translation regardless of the ionic environment but is, however, less limiting for translation under low salt concentrations. Consequently, in the infected lysate (where the endogenous activity of CBP complex is lower), exogenous CBP will stimulate translation at all salt concentrations, while in the mock-infected lysate stimulation is only observed at high salt since the endogenous level of CBP is saturating under low salt conditions. It follows from this rationale that infected lysates should resemble mock-infected lysates, at increased input levels of CBP complex. This is true for translation of globin mRNA, in which case the fold stimulation for a given amount of CBP complex is greater at the higher salt concentration (180 mM K^+) when added CBP complex approaches saturating levels (above 0.5 μL of CBP complex; Figure 2).

The data presented here clearly demonstrate that the activity of the CBP complex is significantly affected by the ionic environment although the cause(s) of the observed effects is (are) unclear. At low salt concentrations, translation of capped mRNAs is less dependent on the CBP complex, but this is not to imply that the CBP complex is dispensable for translation under these conditions. Instead, it is possible that the CBP complex is multifunctional and may, for example be required for direct interaction with other components of the initiation machinery.

A major reservation concerning the use of in vitro protein synthesizing systems from nucleated cells (as used in this study) concerns the relatively poor activity of these systems when compared to the in vivo situation. One approach that should prove useful in overcoming this limitation and elucidating more directly the role of the CBP complex in translation initiation is the use of antibodies to inhibit function in an efficient in vitro assay system such as reticulocyte lysate or in vivo by microinjection into cells.

Added in Proof

In agreement with our data, Daniels-McQueen et al. (1983) have recently reported that translation of the unnaturally uncapped poliovirus and EMCV RNAs is not stimulated by exogenous CBP II (CBP complex) in a fractionated translation system.

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Isolation of a Heme-Controlled Inhibitor of Translation That Blocks the Interaction between Messenger RNA and Eukaryotic Initiation Factor 2†

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ABSTRACT: A heme-controlled inhibitor of translation was isolated from the S-100 of rabbit reticulocytes by a novel procedure including chromatography on double-stranded ribonucleic acid (dsRNA)–cellulose. The inhibitor thus purified is extremely active and functionally resembles previously studied heme-controlled inhibitor preparations in terms of kinetics and extent of inhibition of translation, relief of inhibition by eukaryotic initiation factor 2 (eIF-2), relief of inhibition by 2-aminopurine, and preferential inhibition of α- over β-globin synthesis. The action of this inhibitor on translation is resistant to treatment with bacterial alkaline phosphatase, micrococcal nuclease, or trypsin and to incubation at 95 °C, pH 2 or pH 12. The inhibitor not only is retained on DEAE-cellulose, phosphocellulose, and dsRNA–cellulose but also exhibits a high affinity for the dye Cibacron Blue, properties that suggest that it may be a protein. Unlike previously described heme-controlled inhibitor preparations, or preparations that did not pass over dsRNA–cellulose, the inhibitor recovered upon dsRNA–cellulose chromatography does not exhibit eIF-2 kinase activity. The inhibitor does not block ternary complex formation between eIF-2, methionyl-$tRNA_f^{Met}$, and GTP but inhibits the ability of eIF-2 to form a complex with labeled globin mRNA. In the presence of inhibitor, the formation of mRNA/eIF-2 complexes can be restored effectively by an excess of eIF-2 but not by an excess of mRNA. The inhibitor thus appears to block the interaction between eIF-2 and mRNA not by competing with eIF-2 for a binding site on mRNA but, instead, by acting on eIF-2 itself. Since there is evidence for a specific interaction between eIF-2 and mRNA during initiation of protein synthesis, inhibition of this interaction by the inhibitor described here may be involved in translational control by heme.

Initiation of translation in lysates from reticulocytes (Bruns & London, 1965) or other eukaryotic cells (Weber et al., 1975) is inhibited in the absence of added heme. A similar inhibition is observed in reticulocyte lysates in the presence of dsRNA[1] or oxidized glutathione [reviewed by Revel & Groner (1976), Safer & Anderson (1978), Hunt (1979), Ochoa & de Haro (1979), and Austin & Clemens (1980)]; translation in extracts of interferon-treated cells is also sensitive to dsRNA (Kerr et al., 1974). In each case, the inhibition can be reversed by high levels of eIF-2 (Kaempfer, 1974; Clemens et al., 1975; Kaempfer et al., 1979a), the initiation factor that forms a ternary complex with Met-$tRNA_f$ and GTP and promotes the binding of Met-$tRNA_f$ to 40S ribosomal subunits, a necessary prerequisite for the subsequent binding of mRNA (Darnbrough et al., 1973; Trachsel et al., 1977). In addition, eIF-2 itself binds specifically to mRNA (Kaempfer, 1974; Barrieux & Rosenfeld, 1977, 1978; Kaempfer et al., 1978a,b, 1979b; Rosen & Kaempfer, 1979), recognizing a site essential for translation that has been shown to be virtually identical with the ribosome binding site sequence (Kaempfer et al., 1981; Perez-Bercoff & Kaempfer, 1982). The relevance of this interaction for protein synthesis is indicated by the finding that a direct correlation exists between the affinity of an mRNA species for eIF-2 and its ability to compete in translation (Di Segni et al., 1979; Rosen et al., 1982; Kaempfer, 1982).

Incubation of reticulocyte lysates in the absence of heme or in the presence of dsRNA leads to the appearance of translation inhibitory activity. Purified preparations of in-

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[1] Abbreviations: eIF-2, eukaryotic initiation factor 2; dsRNA, double-stranded RNA; Met-$tRNA_f$, methionyl-$tRNA_f^{Met}$; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, diethylaminoethylcellulose; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IN, inhibitor; AP, 2-aminopurine; BAP, bacterial alkaline phosphatase; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

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