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

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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 could 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 could other capped mRNAs in extracts from poliovirus-infected cells.

The cap structure at the 5′ terminus of almost all eucaryotic mRNAs, m^{7}GpppN(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^{7}GDP-Sepharose 4B affinity chromatography (34). Subsequently, it has been demonstrated that additional polypeptides with molecular masses of 28, 50, and 80 Kd can be specifically cross-linked (as indicated by m^{7}GDP 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. Recently, 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 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 viral 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 shutoff 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 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 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

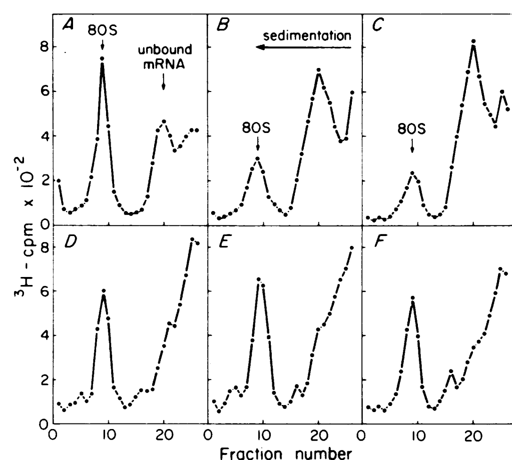

FIG. 1. Binding of native and m^7I-capped inosine-substituted reovirus mRNA to ribosomes in extracts from mock-infected cells as a function of K^+ concentration. HeLa S3 cells were grown in media supplemented with 5% calf serum. Cell extracts were prepared as described by Lee and Sonenberg (22), except that extracts were not dialyzed. Native reovirus [*methyl*-^3H] mRNA (~20,000 cpm/μg) and m^7I-capped inosine-substituted mRNA (~35,000 cpm/μg) were prepared as described by Muthukrishnan et al. (24) 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 phosphate, 4 μg of creatine 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 mM, 31%.

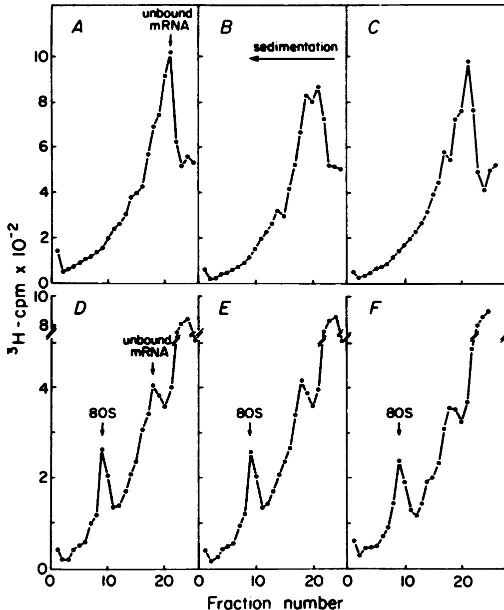

FIG. 2. Binding of native and m^{7}I-capped inosine-substituted reovirus mRNAs to ribosomes in extracts from poliovirus-infected cells as a function of K^+ concentration. Poliovirus (Mahoney 1 strain) infection of HeLa cells was performed as previously described with 10 to 20 PFU per cell, and preparation 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 mM, 15%.

mRNAs with various degrees of secondary structure at their 5′ ends in extracts from poliovirus- and mock-infected cells. Figure 3A shows 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. 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 others 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. Nucleotide 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.

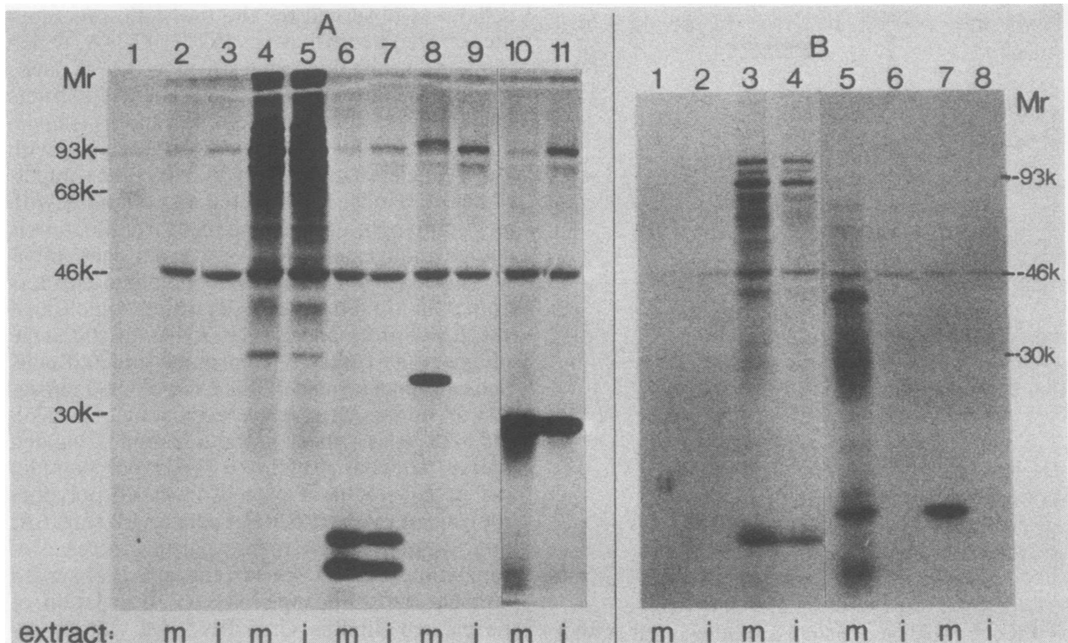

FIG. 3. Translation of capped and naturally uncapped mRNAs in extracts from mock-infected and poliovirus-infected cells. Translation in HeLa cell extracts was carried out essentially as previously described (28, 34). Reaction mixtures (25 μl) contained the following: 130 mM potassium acetate, 0.4 mM 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 Whatman 3MM 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) (lanes 7 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 cells; AMV-4; 40%. (B) EMC virus, 50%; reovirus and globin, no detectable synthesis in extracts from poliovirus-infected cells.

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 (18) and hence cannot form a stable secondary structure, as predicted by computer-aided analysis (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^{7}GDP and a monoclonal antibody with anti-CBP activity (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 complex 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) or whether it employs a modified CBP(s) remains to be determined.

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