

In Vitro Translation in Reovirus- and Poliovirus-infected Cell Extracts

EFFECTS OF ANTI-CAP BINDING PROTEIN MONOCLONAL ANTIBODY*

(Received for publication, December 23, 1980, and in revised form, February 24, 1981)

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Monoclonal antibodies directed against rabbit reticulocyte cap binding proteins were used as probes to study cap-dependent translation initiation in mock-infected and virus (poliovirus or reovirus)-infected cells. The antibodies inhibited capped mRNA translation, but did not have any effect on the translation of naturally uncapped mRNAs in uninfected cell-free extracts prepared from either L-cells or HeLa cells. Cell-free extracts prepared from either reovirus-infected L-cells or poliovirus-infected HeLa cells did not support capped mRNA translation. However, translation of naturally uncapped mRNAs in the latter extracts was comparable to that observed in extracts from uninfected cells. Thus, translation of mRNAs in extracts from either reovirus- or poliovirus-infected cells proceeds most probably via a cap-independent mechanism, since it is not inhibited by antibodies directed against cap binding proteins.

A cap structure, m⁷GpppN_m, is present at the 5' end of all cellular mRNAs analyzed to date (1, 2). The only eukaryotic mRNAs which do not contain a 5' cap structure are a few viral mRNAs, notably the picornaviruses (3-7) and two plant viruses: satellite tobacco necrosis virus (8) and cowpea mosaic virus (9).

The cap structure was shown subsequently to play an important role in initiation of protein synthesis (1, 2). To elucidate the mechanism by which the cap structure functions in initiation of translation, Sonenberg and Shatkin (10) developed a method to identify the proteins situated in the vicinity of the 5' cap, by cross-linking the proteins to [³H]methyl-labeled oxidized reovirus mRNA. Using this technique it was found that a M_r = 24,000 polypeptide present in the high salt wash of eukaryotic ribosomes and which copurifies with eu-

karyotic initiation factors 3 and 4B, recognized specifically the 5' cap of eukaryotic mRNAs (11). The 24,000 cap binding protein was subsequently purified using affinity chromatography techniques (12) and was shown to stimulate preferentially the translation of capped mRNAs in *in vitro* protein synthesizing extracts from HeLa cells (13).

Poliovirus infection of HeLa cells results in an early shut-off of host protein synthesis (14). The lesion has been shown to occur at the level of initiation of translation (15). Fractions obtained by washing ribosomes isolated from poliovirus-infected HeLa cells with 0.5 M KCl did not stimulate translation of host mRNA, but could stimulate translation of poliovirus RNA (16). Moreover, Rose *et al.* have prepared cell-free extracts from poliovirus-infected or mock-infected HeLa cells and demonstrated that poliovirus RNA could be translated in both cell-free systems whereas capped mRNAs (vesicular stomatitis virus and globin) could be translated only in mock-infected cell extracts (17). More recent work demonstrated that a protein with a M_r = 24,000, which was shown to be identical with the 24,000 CBP,¹ could restore the ability of the infected cell-free extracts to translate capped mRNAs (18). Thus, it was concluded that the 24,000 CBP is modified in poliovirus-infected cells. Since poliovirus RNA is uncapped (3-5), it must bypass the cap-dependent mechanism of protein synthesis and by inactivating the CBP, poliovirus takes over the host protein synthesis machinery resulting in the shut-off of host protein synthesis.

The mechanism by which a virus shuts-off protein synthesis via inactivation of a CBP seems not to be restricted to poliovirus, but apparently occurs also with reovirus which is known to contain capped plus strands in the double-stranded RNA present in the viral particles (19). Although mRNA made *in vitro* by reovirus core particles (20, 21) and early in infection *in vivo*² is capped, recent reports have shown that reovirus progeny subviral particles, which are responsible for the synthesis of the majority of mRNAs late in infection, synthesize uncapped (pG...terminated) mRNAs (22, 23). These findings are consistent with the report that cell-free extracts prepared from reovirus-infected L-cells late in infection could translate only pG...terminated reovirus mRNAs and not capped reovirus mRNAs, suggesting a transition from cap-dependent to cap-independent initiation of protein synthesis (24).

Recently, Trachsel *et al.*³ prepared monoclonal antibodies directed against cap binding proteins. One of the clones produced and characterized was directed against a high molecular weight protein and its breakdown products, all of which shared tryptic and chymotryptic peptides with the 24,000 CBP. It was of interest to study the effects of this monoclonal antibody on the translation of capped or naturally uncapped mRNAs in *in vitro* protein-synthesizing systems prepared from poliovirus- or reovirus-infected cells and mock-infected cells.

MATERIALS AND METHODS

Viral and Cellular mRNAs—Reovirus mRNA containing 5'-terminal m⁷GpppG_p was synthesized with viral cores in the presence of

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[¶] Supported by a grant from the Medical Research Council of Canada.

¹ The abbreviations used are: CBP, cap binding protein; EMC, encephalomyocarditis virus.

² D. Skup, H. Zarbe, and S. Millward, submitted for publication.

³ Trachsel, H., Staehli, C., Sonenberg, N., Staehelin, T., Fessler, R., Kuster, H., and Shatkin, A. J. (1981) *Proc. Natl. Acad. Sci. U. S. A.*, submitted for publication.

10 μ M S-adenosylmethionine as described previously (26). For preparing 5' ppG...ended reovirus mRNA, S-adenosylmethionine was replaced by 250 μ M S-adenosylhomocysteine and 0.5 mM inorganic sodium pyrophosphate (27). Encephalomyocarditis virus RNA was isolated as described previously (28), and L-cell high molecular weight RNA according to Harding *et al.* (29).

Cell-free Translation—Growth of HeLa cells, their infection with poliovirus, and preparation of cell-free extracts from infected and mock-infected cells were as described previously (17). Cell-free extracts from L-cells infected with reovirus or mock-infected cells were prepared according to Skup and Millward (24). Translation in these extracts was performed essentially as described by Zarbl *et al.* (23), and translation products were analyzed by polyacrylamide gel electrophoresis as described (11).

Preparation of Monoclonal Antibodies—Monoclonal antibodies were prepared as described in detail by Trachsel *et al.*³ by injecting mice with crude eukaryotic initiation factor # 3 preparations which contain the 24,000 CBP. Hybridomas were screened for anti-CBP clones in radioimmunoassays with 7-methyl-GDP-Sepharose-purified 24,000 CBP. The monoclonal antibody used in these studies belonged to the IgM class and contained μ type heavy chain and κ type light chains³. Anti-CBP antibody was partially purified from ascitic fluid collected from mice injected with hybridoma clones producing the anti-24,000 CBP by precipitation with 50% $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in buffer containing 50 mM sodium phosphate (pH = 8.2) and dialyzed against the same buffer. The latter fraction was then applied on a DEAE-cellulose column equilibrated in 50 mM sodium phosphate buffer (pH = 8.2). IgM antibodies did not bind to the column and were collected in the void volume. Immunoglobulin fractions that served in control experiments were obtained from Ehrlich ascitic fluid of mice injected with Ehrlich ascites cells, using the same procedures as described above for the anti-CBP preparations.

RESULTS

Capped reovirus mRNA can be translated very efficiently in L-cell extracts (24). When a mixture of reovirus-capped mRNAs was incubated in L-cell extracts, the main mRNA species translated was the s_4 class mRNA (Fig. 1, lane 1). Addition of a partially purified anti-CBP monoclonal antibody preparation inhibited the translation of s_3 by approximately 70% (Fig. 1, lane 2). The other reovirus-coded proteins (σ , μ , and λ classes) were made in smaller amounts and were inhibited to a similar degree. In control experiments, immunoglobulin fractions from Ehrlich ascitic fluid of mice not injected with hybridoma cells did not inhibit translation of reovirus mRNA (Fig. 1, lane 3). Encephalomyocarditis virus RNA contains at its 5' end a small polypeptide which is covalently linked to the RNA (30), instead of the common 5' cap structure. Nevertheless, EMC RNA is readily translated in *in vitro* protein-synthesizing systems (Fig. 1, lane 4, see also Ref. 12). EMC translation may, therefore, by-pass cap-dependent translation by analogy to poliovirus (17). If this were the case, then anti-CBP monoclonal antibody should not inhibit the translation of EMC RNA in cell-free extracts. Indeed, when anti-24,000 CBP monoclonal antibodies were added to L-cell extracts translating EMC RNA, no inhibition of EMC translation was observed (Fig. 1, lane 5). As has been shown before (24), capped reovirus mRNA could not be translated in cell extracts from reovirus-infected L-cells (Fig. 1, lane 8), and the presence of antisera did not change this result (Fig. 1, lanes 9 and 10). In contrast, ppG...ended

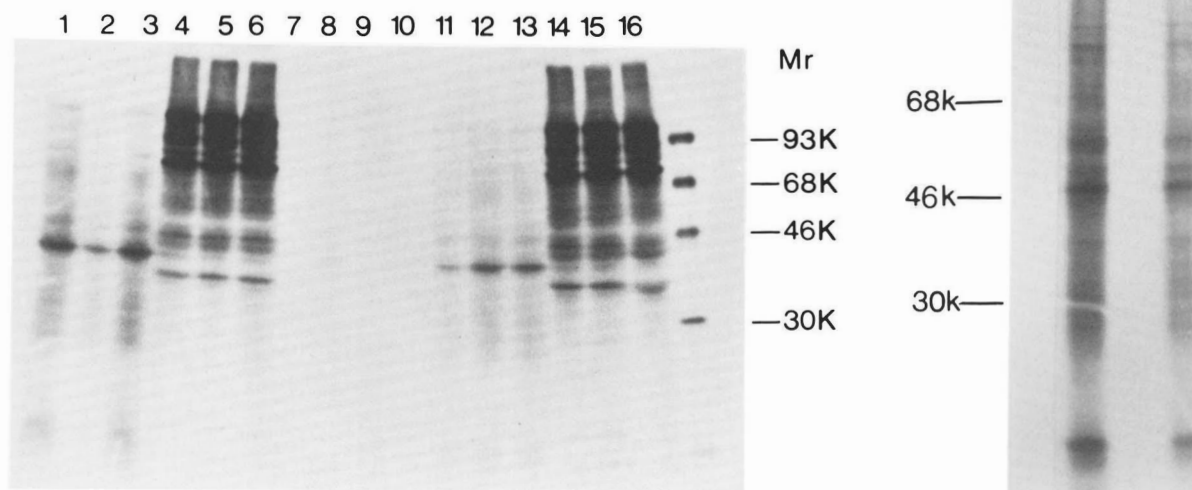


FIG. 1 (left). Polyacrylamide gel electrophoresis of proteins synthesized in reovirus-infected or mock-infected L-cell-free extracts and the effect of anti-CBP antibody. Translation was done in 25- μ l reaction mixture containing 1 μ g of EMC RNA or 5 μ g of reovirus mRNA under conditions described before (24). S-Adenosylhomocysteine (250 μ M) was included to prevent capping and methylation of ppG...ended reovirus mRNA during incubation. Anti-CBP or control antibody (8 μ g) was present where indicated. Incubation was carried out for 60 min at which time 2 μ l of incubation mixture were withdrawn, mixed with sample buffer, and run on 10–18% polyacrylamide gradient gels which were dried and autoradiographed (13). Lanes 1–6, translation in uninfected L-cell extracts. Lanes 7–16, translation in reovirus-infected L-cell extracts. Lanes 1 and 8, capped reovirus mRNA; lanes 2 and 9, capped reovirus mRNA

and anti-CBP antibody; lanes 3 and 10, capped reovirus mRNA and control antibody; lanes 4 and 11, EMC; lanes 5 and 12, EMC and anti-CBP antibody; lanes 6 and 13, EMC and control antibody; lane 7, no mRNA added; lane 14, uncapped reovirus mRNA; lane 15, uncapped reovirus mRNA and anti-CBP antibody; lane 16, uncapped reovirus mRNA and control antibody.

FIG. 2 (right). Polyacrylamide gel electrophoresis of proteins synthesized in L-cell-free extract in response to L-cell high molecular weight RNA and the effect of anti-CBP antibody. Translation was carried out in 25- μ l reaction mixtures containing 7.5 μ g of L-cell high molecular weight RNA as described before. Reaction mixtures included no added RNA (lane 1), L-cell RNA (lane 2), L-cell RNA and 8 μ g of anti-CBP antibody (lane 3), L-cell RNA and 8 μ g of control antibody (lane 4).

reovirus mRNA was translated efficiently in this extract (Fig. 1, lane 11). Translation of the latter mRNA was shown before not to be inhibited by 7-methyl-GTP (24), a cap analogue which specifically inhibits initiation of translation of capped eukaryotic mRNAs. Consistent with these findings, the anti-CBP antibody did not inhibit translation of ppG...ended mRNA in reovirus-infected cell extracts, and even stimulated synthesis of reovirus proteins (Fig. 1, compare lane 12 to lane 11). A similar effect was exerted by control antibodies from Ehrlich ascitic fluid (Fig. 1, lane 13).

It was of interest to determine if reovirus-infected L-cell extracts could translate naturally uncapped mRNAs in addition to ppG...terminated reovirus mRNAs. To this end, EMC RNA was added to extracts from reovirus-infected L-cells and shown to be translated as well as in mock-infected cell extracts (Fig. 1, compare lane 14 with lane 4). Anti-CBP antibody did not inhibit translation of EMC RNA, nor did the Ehrlich ascitic fractions from control mice (Fig. 1, lanes 15 and 16, respectively). Thus reovirus-infected L-cell extracts behave similarly to those derived from poliovirus-infected HeLa cells (12, 17).

To test further the specificity of the anti-CBP antibody, we examined its effect on the translation of an homologous mRNA, *i.e.* L-cell mRNA, in L-cell extracts. As seen in Fig. 2, translation of L-cell mRNAs is extremely sensitive to the addition of the anti-CBP monoclonal antibody (Fig. 2, compare lane 3 to 2). In a control experiment, immunoglobulin from control mice did not significantly inhibit translation of L-cell proteins (Fig. 2, lane 4). This complete inhibition of cellular mRNA translation by anti-CBP antibody was also observed with globin mRNA (data not shown). Increased sensitivity of translation of L-cell and globin mRNAs to anti-CBP antibody as compared to reovirus mRNA translation, may reflect a more stringent dependence of cellular mRNAs on the cap structure and CBP's as compared with viral mRNAs. This notion should however be substantiated by more experiments.

Further experiments were conducted with poliovirus-infected HeLa cell extracts in an attempt to demonstrate effects of the anti-CBP antibody analogous to those described above with reovirus-infected cells. Reovirus mRNAs did translate efficiently in HeLa cell extracts prepared from uninfected cells (Table I). Translation products were analyzed on sodium dodecyl sulfate-polyacrylamide gels and were found to correspond to authentic reovirus proteins. Addition of the anti-

CBP antibody inhibited translation to a level of about 25% as compared to the control experiment, while immunoglobulin fractions from control mice had no effect on the incorporation of [³⁵S]methionine radioactivity into proteins. In contrast to reovirus mRNA translation, EMC RNA-directed translation was not affected by addition of the anti-CBP antibody in striking similarity to the antibody action in reovirus-infected L-cells. Translation of capped reovirus mRNA in poliovirus-infected HeLa cell extracts was reduced to about 25% of the level obtained in mock-infected extracts, in agreement with previous results. This trichloroacetic acid-insoluble radioactivity was not incorporated into authentic reovirus proteins (data not shown) and was not diminished by the addition of anti-CBP antibody (Table I). As reported previously (12), translation of EMC RNA in poliovirus-infected extracts was not reduced compared to translation in uninfected extracts (Table I). These results suggest that cell extracts prepared from poliovirus- or reovirus-infected cells share similar properties concerning their ability to preferentially discriminate against capped mRNAs.

DISCUSSION

The purpose of the studies reported here was to employ monoclonal antibodies directed against cap binding proteins to study the mode of translation of capped and naturally uncapped mRNAs in extracts prepared from virus-infected and uninfected eukaryotic cells.

It was previously shown that the monoclonal antibodies used in these studies³ react with a number of proteins having molecular weights higher than the 24,000 CBP. These proteins were shown to share tryptic and chymotryptic peptides with the 24,000 CBP³, which has been isolated by affinity chromatography on 7-methyl-GDP-coupled Sepharose columns (12). This raises the interesting possibility that precursor polypeptides of the 24,000 CBP do exist in the cell, and that these precursor proteins may function in recognition of the mRNA cap structure.

The ability of the anti-CBP antibody, used in this study, to inhibit specifically the translation of capped mRNAs strongly supports the belief that these antibodies are directed against cap binding proteins, and that cap binding proteins play a crucial role in translation of capped eukaryotic mRNAs.

It is generally believed that naturally uncapped mRNAs are translated via a cap-independent mechanism (12, 17, 18). However, it was alternatively suggested that naturally uncapped mRNAs like EMC, might have a strong affinity for a cap binding protein, thus affording EMC RNA the ability to outcompete capped mRNAs in *in vitro* translation systems (31). The results in Fig. 1 (lanes 4-6 and 14-16) strongly suggest that EMC translation does not require the participation of cap binding proteins since translation of this mRNA is not inhibited by the anti-CBP antibody. Similar results were also obtained with satellite tobacco necrosis virus, another naturally uncapped mRNA.⁴

Redirection of host protein synthesis machinery toward cap-independent translation following infection with poliovirus (15-17) and reovirus (22-24) has been reported. Consequently, extracts prepared from poliovirus- or reovirus-infected cells should not support translation of capped mRNAs but should support translation of naturally uncapped RNAs. This phenomenon can be accounted for, at least in part, by inactivation of a cap binding protein (18). The findings that anti-CBP antibody did not inhibit translation of ppG...ended reovirus mRNA in cell-free extracts prepared from reovirus-infected L-cells (Fig. 1) lend support to the notion that this

TABLE I

Effect of anti-CBP antibody on translation in poliovirus-infected or mock-infected HeLa cell extracts

Translation was carried out as described (24) in 25- μ l reaction mixtures containing 1 μ g of EMC RNA or 5 μ g of reovirus mRNA. Mixtures were incubated for 60 min at 30 °C, 2 μ l were spotted on Whatman No. 3MM paper and processed as described. Anti-CBP or control antibody (8 μ g) were present when indicated.

Extract	mRNA	Antibody	cpm
Uninfected	(-)		7,755
	Capped reovirus		162,534
	Capped reovirus	Anti-CBP	42,588
	Capped reovirus	Control	142,599
	EMC		140,960
	EMC	Anti-CBP	121,709
Infected	EMC	Control	110,064
	(-)		4,472
	Capped reovirus		39,930
	Capped reovirus	Anti-CBP	41,266
	Capped reovirus	Control	37,893
	EMC		140,693
	EMC	Anti-CBP	104,229
	EMC	Control	99,211

⁴ Unpublished observations.

mRNA does not require cap binding proteins for its translation either, and in this sense is similar to natural, uncapped mRNAs. However, the efficiency of translation of poliovirus or EMC RNAs in infected cell extracts is similar to that expressed in mock-infected extracts and is in direct contrast to translation of ppG...ended reovirus mRNA which is restricted to reovirus-infected L-cell extracts prepared late in infection (24).

Translation of reovirus capped mRNA in poliovirus-infected HeLa cell extract was reduced to about 25% as measured by trichloroacetic acid-precipitable incorporation of [³⁵S]methionine (Table I) but no authentic reovirus polypeptides were detected by polyacrylamide gel electrophoresis (data not shown). This result corresponds to that reported for translation of the same mRNA in extracts of reovirus-infected L-cells (24) (see also Fig. 1, lane 8). The same report (Ref. 24) also showed that this 25–30% incorporation of [³⁵S]methionine was resistant to inhibition by 7-methyl-GTP suggesting that this may have resulted from initiation of translation at spurious sites in cleaved mRNAs which did not contain the cap structure. This is supported by the present report which shows that addition of anti-CBP antibody does not diminish the residual 25–30% incorporation of [³⁵S]methionine in extracts of poliovirus-infected HeLa cells (Table I). Taken together, these data strongly indicate that this 25–30% translation of capped reovirus mRNA in reovirus-infected L-cells and in poliovirus-infected HeLa cells is not a cap-dependent process.

More experiments are in progress to elucidate the mechanism by which the cap binding protein(s) is inactivated following infection by poliovirus or reovirus. Specific inactivation of a cap binding protein, as a strategy for taking over host protein synthesis machinery, could be a general mechanism used by many viruses.

Acknowledgments—We acknowledge the skillful technical assistance of Denise Guertin and Charlotte Légaré.

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