Vol. 96, No. 1, 1980
September 16, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 400-407

IMMUNOSPECIFIC BINDING OF CAPPED mRNA OF REOVIRUS BY AN ANTIBODY TO A CAP STRUCTURE; $m^7G(5')pppGm$.

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Received August 4,1980

SUMMARY: Antibody specific for $m^7G(5')pppGm$ was elicited in rabbits. Binding of capped RNA by the antibody and specificity of the binding were assessed by radioimmunoassay using capped and uncapped mRNA of reovirus. More than 50% of capped mRNA, whereas less than 2% of uncapped mRNA were bound by the antibody. The ratios of the amount of competitor to that of $m^7G(5')pppGm$ required for 50% inhibition of binding of capped mRNA was 1; 180; 430; 28,600; and 114,000 for $m^7G(5')pppG$, $G(5')pppGm$, $G(5')pppG$, yeast tRNA and rabbit rRNA respectively. Poly(A) showed no competition. By immunoaffinity chromatography, a 15-fold enrichment of capped mRNA was attained from the mixture of the RNA in a vast excess of rRNA.

The presence of poly(A) tracts on the 3'-end of most eukaryotic RNA has facilitated the isolation and fractionation of intact mRNA (1 & 2), heterogeneous nRNA (3), and large RNA fragments which contain the 3'-end of poly(A) containing RNA (4) by affinity chromatography.

The finding of blocked and methylated cap# structures of the general type $m^7G(5')pppN$ on the 5' terminus of many eukaryotic cellular and viral RNAs (see 5 for review) opened the possibility of isolating capped intact RNA or 5'-end fragments of RNA by exploiting this unique structure.

In this report, the successful production of an antibody to a cap structure, which binds to capped mRNA, characterization of the antibody by RIA

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Abbreviations used: cap, blocked, methylated 5' terminus of RNA; m^7G, 7-methylguanosine; Gm, 2'-O-methylguanosine; $m^7G(5')pppN$, 7-methylguanosine 5'-triphosphoryl-5'-nucleoside; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; RIA, radioimmunoassay; RIgG, non-immune rabbit IgG; TBS, 0.01 M Tris·HCl, pH 7.4 containing 0.15 M NaCl; NETS, 0.01 M Tris·HCl, pH 7.2 containing 0.1 M NaCl, 0.01 M EDTA and 0.5% $NaDodSO_4$; Buffer A, TBS containing 2 mM $MnCl_2$, 1 mg/ml of heparin and 727 μg/ml of ovalbumin.

0006-291X/80/170400-08$01.00/0

using radiolabeled reovirus mRNA and the immunoaffinity chromatography of capped and uncapped RNA will be described.

METHODS

Antibody Preparation. $m^7G(5')pppGm$ was conjugated to BSA via periodate oxidation essentially following the published procedure (6). On the average, 80 µg of the cap was bound to 1 mg of BSA. Three New Zealand white rabbits were immunized intramuscularly with 0.6 mg of the conjugate emulsified in three volumes of complete Freund's adjuvant. One month later, booster injections were given using half the amounts of the initial dosage. Sera were collected weekly thereafter for 6 weeks. The gamma globulin fraction twice precipitated from the pooled antisera by 40% saturated $(NH_4)_2SO_4$, was rendered RNase free by treatment with Macaloid (7). Coupling of proteins to CNBr-activated Sepharose 4B was done following the recommended procedure (Pharmacia).

RIA of mRNA. A reaction mixture (0.55 ml of buffer A) contained radiolabeled mRNA and 23 µg of the gamma globulin fraction of antibody and/or RIgG. Where indicated, a varying amount of competitor was added to the reaction before antibody addition. After 30 min at 37°C, the reaction tube was put on ice and 55 µl of TBS containing 240 µg of goat anti-RIgG antibody was added. After 40 min on ice, the precipitate was collected on a GF/F glass microfiber filter and processed as reported previously (8).

RESULTS

To assess the binding capacity and specificity of the antibody, [^{32}P]GMP labeled capped mRNA ($m^7G(5')pppGm$-RNA) and [^{3}H]GMP labeled uncapped mRNA (ppG-RNA) synthesized by reovirus (Dearing strain) core enzymes (9) were used. As can be seen in Fig. 1, binding of the RNA was clearly dependent on the presence

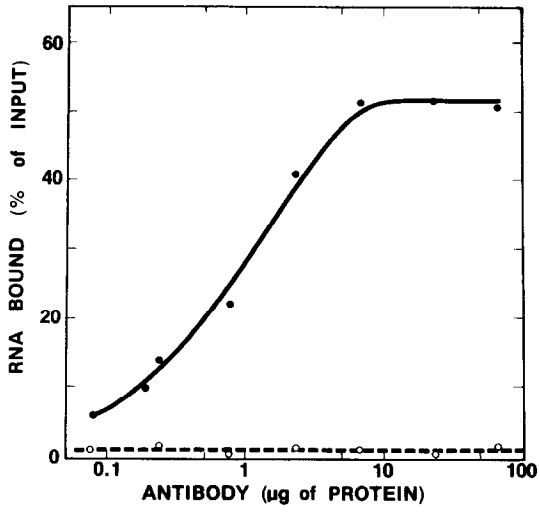

Figure 1. Binding of capped and uncapped reovirus mRNA by antibody. Capped [^{32}P]mRNA (0.63 pmoles of GMP, 2420 cpm) and uncapped [^{3}H]mRNA (0.046 pmoles of GMP, 2170 cpm) were mixed and incubated with varying amounts of antibody. The binding of RNA and antibody was assayed as described in METHODS. Nonspecific binding of ^{32}P and ^{3}H by 23 µg of RIgG was 7.3% and 2.5% of input respectively and was subtracted from the total binding of each reaction. ●——●, capped [^{32}P]mRNA; ○- -○, uncapped [^{3}H]mRNA.

of a cap on the RNA. In the presence of saturating amounts of antibody, a little more than 50% of capped RNA was specifically bound. The binding of uncapped RNA over background was less than 2% at all concentrations of the antibody. Similar results were obtained several times with different preparations of the RNA. The reaction mixture for the binding assay contained ovalbumin and heparin as they enhanced the binding of capped mRNA in preliminary experiments. Divalent cation increased the net binding slightly although background was also increased to a lesser extent. It could be left out if desired. EDTA was inhibitory.

As shown in Fig. 2, the binding of capped mRNA was most effectively inhibited by $m^7G(5')pppGm$ and $m^7G(5')pppG$, showing again that the binding of the RNA is via the cap moiety. Fig. 2 also shows that the 2'-O-methyl portion of the guanosine moiety has little to do with the inhibition of binding by the cap structure in this case. On the other hand, the importance of the 7-metyl group on a m^7G residue was illustrated by the inhibition obtained using $G(5')pppGm$ and $G(5')pppG$, where 180-fold and 430-fold, respectively, more com-

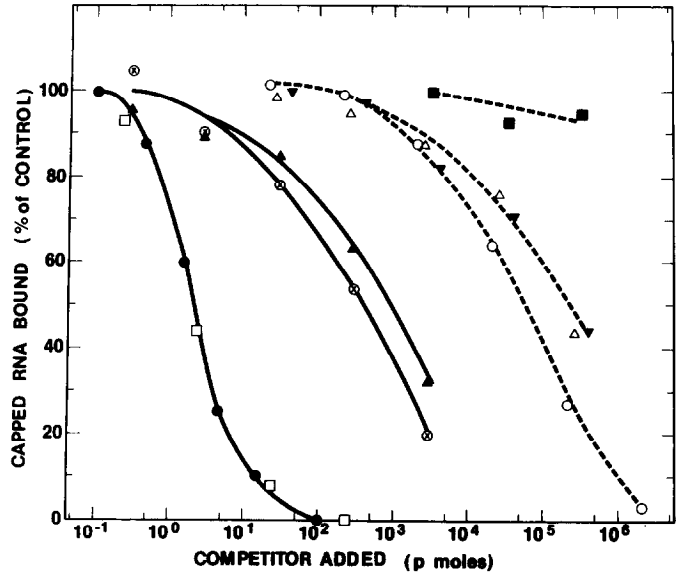

Figure 2. Competition RIA of capped reovirus [^{32}P]mRNA and anti-cap antibody. Capped [^{32}P]mRNA (0.63 pmoles of GMP, 2080 cpm) was competed with $m^7G(5')pppGm$ (●—●), $m^7G(5')pppG$ (□—□), $G(5')pppGm$ (⊗—⊗), $G(5')pppG$ (▲—▲), yeast tRNA (○--○), rabbit liver rRNA (△--△), E. coli rRNA (▼--▼) and poly(A) (■--■) for 23 µg of anti-cap gamma globulin fraction. Without a competitor, 980 cpm over nonspecific binding (200 cpm) was bound.

petitor than $m^7G(5')pppGm$ were required to attain 50% inhibition. The 2'-O-methyl portion of the guanosine residue has some effect in this case, suggesting some contribution by 2'-O-methyl in the binding of cap structure by the antibody. For 50% inhibition of binding, 28,600 and 114,000 times more yeast tRNA and E. coli or rabbit liver rRNA, respectively, were required than $m^7G(5')pppGm$ (Fig.2). Although both RNA species are known to contain m^7G as a minor component (see 10), it is hardly the major cause of the competition. It is known that m^7G in tRNA is protected against reduction by $NaBH_4$ (11). Also, anti-m^7G antibody apparently is not reactive with the m^7G residue in intact tRNA (12) and rRNA (10). The weak cross-reactivity of G and poly(G) in the [^{3}H]Lys-cap/anti-cap antibody RIA system (manuscript in preparation) indicated that the inhibition is exerted by G residues in these RNAs. The in-

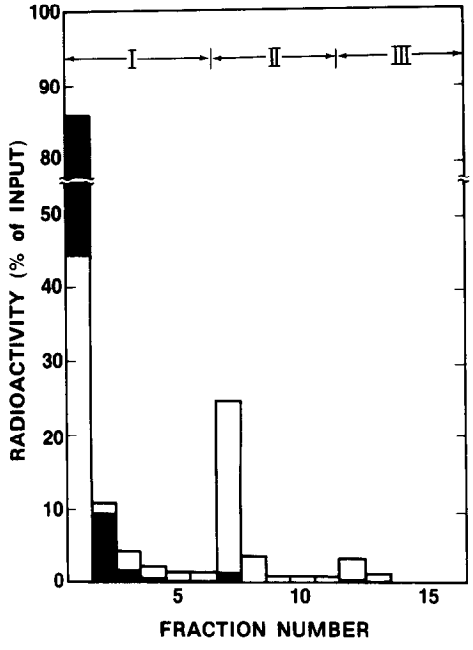

Figure 3. Immunoaffinity chromatography of capped mRNA and rRNA. Capped reovirus [^{32}P]mRNA (13.3 pmoles of GMP, 27,200 cpm; □) and E. coli [^{3}H]rRNA (7.5 nmoles of GMP, 214,000 cpm; ■) were mixed, loaded on an anti-cap Sepharose 4B column (0.7x0.6 cm), which had been washed with TBS containing poly(A) (0.1 mg/ml). After 30 min at 23°C, unbound RNA was eluted with six 1.4 ml portions of TBS (fractions I). The bound RNA was eluted with NETS after treating the column with 0.02 M Tris·HCl buffer, pH 7.4 containing 5% $NaDodSO_4$ and 1 M 2-mercaptoethanol for 20 min (fractions II) or NETS containing pronase (1 mg/ml) for 30 min (fractions III). TCA precipitable radioactivities of the aliquots were measured for quantitation.

ability of poly(A) (Fig. 2) and poly(U) (data not shown) to inhibit the binding supports a similar conclusion in this case.

When a mixture of capped reovirus [^{32}P]mRNA and a vast excess of [^{3}H]-rRNA was applied to an anti-cap antibody-Sepharose 4B column (ca. 1.8 mg of gamma globulin), 35% of [^{32}P]mRNA and 2% of the [^{3}H]rRNA was retained on the column (Fig. 3). About 85% of the bound fraction was effectively released by

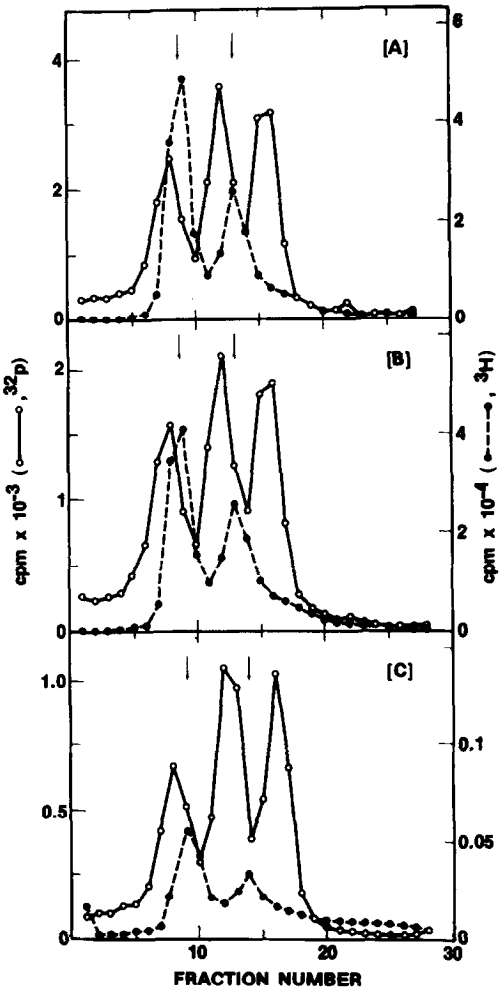

Figure 4. Sedimentation pattern through sucrose density gradients of capped reovirus mRNA and E. coli rRNA before and after immunoaffinity chromatography. Aliquots of the original mixture of capped [^{32}P]mRNA and [^{3}H]rRNA, [A]; fractions I, [B]; and fractions II, [C]; of Fig. 3 were centrifuged through 15 to 30% linear sucrose density gradients in 0.01 M Tris·HCl, pH 7.2 containing 0.1 M NaCl, 10 mM EDTA and 0.2% NaDodSO$_4$ for 14 hrs at 30,000 rpm at 21°C in a Spinco SW41 rotor. Radioactivity in each fraction was counted after adding 0.5 ml of H_2O and 10 ml of Aquasol (NEN). ○—○, ^{32}P; ●---●, ^{3}H; ↓, 23S and 16S rRNA peaks. Note the difference in scales.

treatment with 5% $NaDodSO_4$ and 2-mercaptoethanol and the rest was released by pronase digestion of the antibody. Apparently, rRNA at relatively low concentration somewhat inhibited the binding of capped [^{32}P]mRNA. As rRNA is a poor competitor of the capped mRNA (Fig. 2), this result may be explained by the decreased capacity of the antibody after immobilization, most likely because of steric hindrance. The specificity of the antibody column was confirmed when less than 1% of RNA were bound to non-immune RIgG-Sepharose columns.

Fig. 4 depicts the sucrose density gradient sedimentation patterns of reovirus [^{32}P]mRNA and E. coli [^{3}H]rRNA before and after immunoaffinity chromatography. It is apparent that mRNAs of all size were equally bound by the antibody and little, if any, degradation of the RNAs occurred during the whole procedure.

DISCUSSION

Although Munns et al (13) reported the inhibition of translation of chorion mRNA by anti-m^7G antibody, it was not unequivocally shown that the antibody had bound specifically to the cap structure in intact mRNA. From the report of the same authors (14), it is apparent that the same antibody was not useful in isolating intact adenovirus mRNA although it could be used to purify cap structures from nuclease-digested RNA. The apparent lower affinity of the anti-m^7G antibody for cap structures in intact mRNA suggests the interaction of the m^7G moiety with other parts of the RNA molecule. Because of its positive charge, the m^7G moiety may bind to the negatively charged phosphate group of RNA or it may be involved in formation of some other structure, e.g., "base triple" (15).

The binding of anti-cap antibody to capped reovirus mRNA and the competition of the binding by cap analogues and RNAs reported here have unequivocally shown that the antibody bound the RNA via the cap moiety. The success of the production of an antibody which binds to a cap moiety in RNA seems to have depended on the use of a whole cap structure as an antigen. Whether this was because of the production of an antibody which recognized the whole cap structure or an antibody which had a high affinity to a part of the cap structure, e.g. m^7G, remains to be seen. It is worth mentioning here that free m^7G

and the m^7G moiety in cap structures have different conformations and thus may have different antigenicity (16 & 17).

The reason of binding of only a little more than 50% of reovirus mRNA may be either the presence of substantial amount of uncapped RNA in the preparation because of insufficient capping or degradation of RNA, or inaccessbility of the cap in some of the RNA. The apparent intactness of the RNA after immunoaffinity chromatography (Fig. 4) and the reported population of capped RNA synthesized by the procedure used in this study (9) seem to favor the latter explanation. In this connection, it is of interest that almost all of 70S RNA of simian sarcoma associated virus was bound by the antibody (unpublished observation). The more detailed analysis of these observations are now in progress.

Immunoaffinity chromatography of the mixture of capped mRNA and a vast excess rRNA (x560) showed that although a minor fraction of rRNA was bound and eluted with capped RNA, the chemical amount of rRNA in the eluate was 37 times as much as that of the capped RNA. Neverthless, a 15-fold purification of capped RNA was attained. Although it does not seem feasible at this stage to isolate capped RNA of high chemical purity from samples containing vast excesses of uncapped RNA by a single step procedure, the antibody will be very useful in many aspects of nucleic acids research, where vast excess of uncapped RNA is either non-existent or can be neglected because of differential radiolabeling.

ACKNOWLEDGMENT

I am grateful to Drs. Steven Langbert and Bernard Moss for supplying reovirus and helpful discussion and to Dr. Marjorie Robert-Guroff for her help in preparing the manuscript. The support and encouragement of Dr. Robert Gallo is greatfully acknowledged.

REFERENCES

1. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412.
2. Nakazato, H., and Edmonds, M. (1972) J. Biol. Chem. 247, 3365-3367.
3. Nakazato, H., and Edmonds, M. (1974) in Methods in Enzymol., Grossman, L., and Moldave, K., Eds., Vol. XXIX, pp. 431-443, Academic Press, New York.
4. Coffin, J. M., and Billeter, M. A. (1976) J. Mol. Biol. 100, 293-318.
5. Shatkin, A. J. (1976) Cell 9, 645-653.
6. Erlanger, B.F., and Beiser, S. M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 68-74.

7. Meredith, R. D., and Erlanger, B. F. (1979) Nucleic Acids Res. 6, 2179-2191.
8. Nakazato, H. (1979) Anal. Biochem. 98, 74-80.
9. Furuichi, Y., and Shatkin, A. J. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3448-3452.
10. Sawicki, D. L., Beiser, S. M., Srinivasan, D., and Srinivasan, P.R. (1976) Arch. Biochem. Biophys. 176, 457-464.
11. Igo-Kemenes, T., and Zachau, H. G. (1971) Eur. J. Biochem. 18, 292-298.
12. Munns, T. W., Liszewski, M. K., Oberst, R. J., and Sims, H. F. (1978) Biochemistry 17, 2573-2578.
13. Munns, T. W., Morrow, C. S., Hunsley, J. R., Oberst, R. J., and Liszewski, M. K. (1979) Biochemistry 18, 3804-3810.
14. Munns, T. W., Oberst, R. J., Sims, H. F., and Liszewski, M. K. (1979) J. Biol. Chem. 254, 4327-4330.
15. Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C., and Klug, A. (1974) Nature 250, 546-551.
16. Kim, C. H., and Sarma, R. H., (1977) Nature 270, 223-227.
17. Rainen, L., and Stollar, B. D. (1978) Nucleic Acids Res. 5, 4877-4889.