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mRNA 5′-Cap Binding Activity in Purified Influenza Virus Detected by Simple, Rapid Assay

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Reovirus mRNA 5′-terminal caps were 3′-radiolabeled with ^{32}pCp and used as affinity probes for proteins with cap binding activity. A rapid, simple, and sensitive blot assay was devised that could detect cellular cap binding protein in a complex polypeptide mixture. By using this method, cap binding activity was found in detergent-treated influenza virus but not in reovirus or vaccinia virus. Preincubation of capped reovirus mRNA with purified cellular cap binding protein reduced its primer effect on influenza transcriptase, whereas priming by ApG was not affected. The results indicate that influenza transcriptase complexes include cap-recognizing proteins that are involved in the formation of chimeric mRNAs.

Many eucaryotic viruses contain RNA polymerases that catalyze the synthesis of functional viral mRNAs (2). In purified reoviruses (7) and vaccinia virus (14), additional activities are associated with the transcriptase complex. They include enzymes that modify the initiated 5′-ends of nascent transcripts to form caps with the general structure, m^{7}GpppN. In contrast to these examples of de novo cap synthesis, influenza virus mRNA caps are derived preformed from heterologous capped mRNAs that act as primers of the virion transcriptase (11). This finding suggested that influenza virus contains a protein(s) that recognizes cap structures. Proteins that bind to the 5′-terminal m^{7}GpppN of eucaryotic mRNAs have been found in several organisms ranging from brine shrimp to mammalian species (1, 6, 21). In addition, a cap binding protein of M_r ~24,000 (CBP-24) that stimulates translation of capped viral and cellular mRNAs in vitro has been isolated from rabbit reticulocytes (20). In view of the unusual mechanism for obtaining 5′ caps on influenza virus mRNA, it was of interest to test purified virions for cap binding activity.

Previously, it was shown that cellular cap binding proteins can be detected by chemical cross-linking to the radiolabeled 5′ end of oxidized capped mRNA (20, 21). In this procedure, proteins that bound to mRNA and contained free amino groups near the 5′-cap formed covalent, Schiff base linkages. They were stabilized by reduction with cyanoborohydride, and the resulting radiolabeled proteins were analyzed by polyacrylamide gel electrophoresis. Cap specificity was determined by competitive inhibition of cross-linking in the presence of an excess of m^{7}GDP or other cap analogs. We used this technique to test a variety of purified animal viruses including reo, vaccinia, avian leukemia, and influenza viruses for cap binding activity. Although each virus yielded a distinct pattern of cross-linked proteins, the interactions were apparently not cap specific; i.e., they were not inhibited by m^{7}GDP. Therefore, we devised a different method for detecting cap binding activity in a complex protein mixture. For this test, we prepared the 5′-terminal mRNA fragment, m^{7}GpppG^{m}pC, by enzymatic digestion of reovirus mRNA with RNase T2 and alkaline phosphatase (7). The capped oligonucleotide was 3′-end-labeled with [^{32}P]pCp by incubation with RNA ligase (5). After repurification, m^{7}GpppG^{m}pC*pC was used as a probe for detecting cap binding proteins by the following simple procedure. Protein samples were spotted on nitrocellulose strips, exposed to the radiolabeled cap structure, washed extensively, and analyzed by autoradiography. The utility and sensitivity of this rapid technique can be illustrated by results obtained with a mixture of cellular proteins containing CBP-24 (Fig. 1). An intermediate step in the isolation of CBP-24 from rabbit erythrocyte proteins is fractionation in a sucrose gradient (22). When equal amounts of protein in successive gradient fractions 1 through 8 were spot tested, m^{7}G-specified cap binding activity was detected in a position (<6S) previously shown to be enriched in CBP-24 (Fig. 1A, spots 4 and 5) (1, 22). These gradient fractions contained ~0.05% CBP-24 and were usually pooled for the next step in the purification scheme, i.e., affinity chromatography on a column of m^{7}GDP-Sepharose (20). Samples that eluted from the column in buffer containing m^{7}GDP (>90% of protein was CBP-24 by polyacrylamide gel

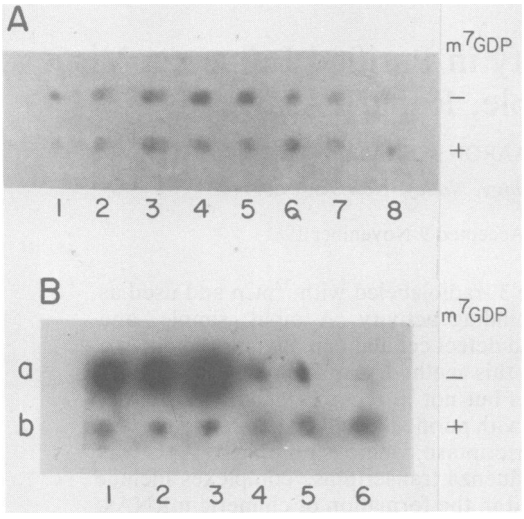

FIG. 1. Detection of cellular cap binding protein by filter binding. m^7GpppG^mpC was prepared from reovirus mRNA by digestion with RNase T2 and alkaline phosphatase as described (4). The caps were purified by high-voltage paper electrophoresis, radiolabeled to a specific activity of 1 to 2 μCi $pmol^{-1}$ with [^{32}P]pCp, using T4-RNA ligase (5), treated again with phosphatase, and repurified by electrophoresis. The structure of the $m^7GpppG^mpC^*pC$ product was verified by electrophoretic analyses in parallel with authentic m^7GpppG^mpCp after digestion with various enzymes, including RNase T2, nuclease P1, and nucleotide pyrophosphatase or alkaline phosphatase or both. Protein samples (2 μl) were spotted on strips of cellulose nitrate paper (BA85; Schleicher & Schuell, Inc.) which had been soaked for 10 min in binding buffer consisting of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.4]), 70 mM KCl, 3 mM Mg acetate, and 1 mM dithiothreitol and air dried. On top of each protein spot was applied 2 μl of an aqueous solution of $m^7GpppG^mpC^*pC$ with or without 0.5 mM m^7GDP. The paper strip was soaked for 5 min at room temperature and washed three times in 100 ml each of binding buffer. After drying, ^{32}P-labeled bound caps were detected by autoradiography or scintillation counting or both. (A) Samples of fractions (2 μg each) from a sucrose gradient of erythrocyte proteins (1, 22) were tested with 0.03 pmol (27,000 cpm) of caps (autoradiogram exposure, 5 h). Radioactivity in spots 2, 4, 5, and 7 in the absence (presence) of m^7GDP: 370 (300), 1,080 (485), 825 (390), and 255 (290) cpm, respectively. (B) Samples (2 μl) from a m^7GDP-Sepharose affinity column eluted with m^7GDP (spots 1 through 3) or 1 M KCl (spots 4 through 6) (20) were tested with 0.04 pmol (27,000 cpm) of caps. Protein applied to spots: 1, 360 ng; 2, 140 ng; 3, 340 ng; 4, 560 ng; 5, 590 ng; 6, 580 ng (exposure, 20 h). Radioactivity bound in spots 3a and b was 6,300 and 140 cpm, respectively.

analysis [20, 22]) effectively and specifically bound ^{32}P-labeled caps (Fig. 1B, spots 1 through 3). Binding was also inhibited strongly by $m^7GpppG^{(m)}$ but not by GpppG, indicating cap specificity (data not shown). Samples eluted subsequently from the same affinity column in buffer with 1 M KCl (no detectable CBP-24 by polyacrylamide gel analysis) showed little if any specific cap binding activity even at higher protein levels (spots 4 through 6). In other experiments, 1 to 5 ng of affinity-purified CBP-24 could be readily detected by this method.

The blot assay was similarly employed to test several animal viruses for cap binding activity. Purified influenza virus was incubated in transcriptase reaction mixtures and spot tested. Cap binding activity was observed at 10 min at 31°C in incubation mixtures containing nonionic detergent to permeabilize virions (Fig. 2). Activity was detected at 5 min but declined after 30 min, presumably owing to instability of the putative cap-recognizing protein(s). Specificity of the interaction was indicated by cap analog inhibition. m^7GDP at a level of 0.1 mM (2,000-fold molar excess over $m^7GpppG^mpC^*pC$) decreased binding by 80% (890 to 190 cpm); the same concentration of GDP, G^mDP, and m^7G did not cause inhibition (Fig. 2). Partial inhibition observed with GTP (50% at 1.1 mM) may indicate an overlap between GTP binding site(s) and putative cap binding protein(s) in the influenza transcriptase complex. Although the detergent-activated influenza virus bound caps specifically in the filter assay, similar amounts of purified reovirus and vaccinia virus under the same conditions did not (Table 1).

The results indicate that a putative cap binding protein(s) in influenza virus and purified CBP-24 share the ability to bind reovirus mRNA 5'-terminal cap structures. We therefore tested

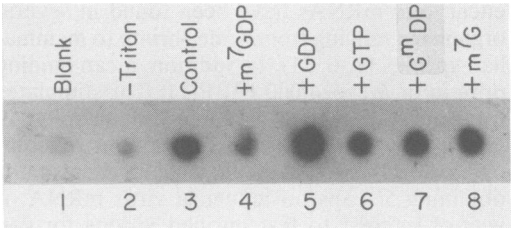

FIG. 2. Detection of cap binding activity in influenza virus. Purified influenza A virus strain WSN (11 μg of protein) was incubated in a transcription reaction mixture (25 μl) containing 50 mM Tris-hydrochloride (pH 7.8); 1 mM dithiothreitol; 120 mM K acetate; 5 mM Mg acetate; and 1 mM each ATP, GTP, UTP, and CTP with or without 0.4% Triton N101. After 10 min at 31°C, samples of 2 μl were spotted and assayed as described in the legend to Fig. 1 but with 0.11 pmol (46,000 cpm) of caps and 200 pmol of the compound indicated. The reaction mixture included: spot 1, Triton and no virus; spot 2, virus and no Triton; spots 3 through 8, Triton plus virus. Autoradiogram exposure, 24 h.

TABLE 1. Binding of $m^7GpppG^mpC\overset{*}{p}C$ to purified viruses

Addition(s)		Binding (cpm) to following type of virus:		
Triton	m^7GDP	Influenza	Vaccinia	Reo
−	−	46	33	47
−	+	24	23	11
+	−	333	17	28
+	+	13	10	6

Purified influenza virus (10), vaccinia virus (9), and reovirus (18) (11, 18, and 25 μg, respectively) were incubated in transcription mixtures (25 μl) as described in the legend to Fig. 2. After 10 min at 31°C, 2-μl samples were tested with 0.06 pmol (23,000 cpm) of ^{32}P-labeled caps with and without 800 pmol of m^7GDP. Spots located by autoradiography were counted in toluene-based scintillant.

the effect of adding CBP-24 to influenza transcriptase reactions primed by reovirus-capped mRNA. Incubation of the primer mRNA with CBP-24 for 10 min at room temperature before addition to the transcriptase mixture reduced the rate of RNA synthesis by 50% as measured by incorporation of radiolabeled nucleotide precursor into acid-insoluble products (Fig. 3A). Without preincubation with primer, CBP-24 had no effect on the rate of RNA synthesis (data not shown). Similarly, when the primer mRNA was preincubated with proteins eluted from the affinity column in 1 M KCl (see Fig. 1B, spots 4 through 6), there was no inhibition (Fig. 3A). Influenza virus transcription can also be primed in a cap-independent reaction by high concentrations of ApG (15, 17). RNA synthesis in this reaction was not diminished by CBP-24 (Fig. 3B). These results rule out the possibility of nonspecific inhibition, for example, due to nucleases or other contaminants in the purified CBP-24. We interpret these results as suggesting that CBP-24 competes with an influenza cap binding activity involved in viral transcription, presumably at the level of generating capped primer mRNA fragments (16). The CBP-24 competed only when prereacted with the primer and inhibited partially even in molar excess (sixfold relative to mRNA caps and to the estimated amount [8] of influenza P proteins). It therefore seems likely that, under these conditions, the influenza transcriptase complex has a greater affinity for caps than does purified CBP-24 or has additional internal binding sites on the primer mRNA (12, 19).

It will be of interest to compare the cap-recognizing properties of influenza virus with cap binding proteins from normal and virus-infected cells. The possible involvement of cellular cap binding proteins in influenza virus gene expression can be explored with specific antibodies. Proteins that interact with one or more

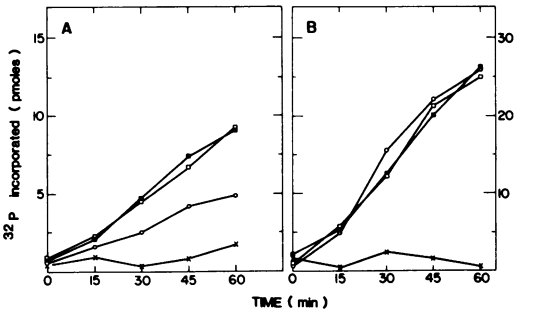

FIG. 3. Effect of cellular cap binding protein on influenza transcription. Purified influenza virus (11 μg) was incubated at 31°C in transcription mixtures (25 μl) containing Triton N101 as described in the legend to Fig. 2, except with 50 μM [α-^{32}P]CTP (specific activity, 160 μCi μmol^{-1}). The primer was capped reovirus mRNA (2 μg ≈ 2.8 pmol) (A) and 0.25 mM ApG (B). Reaction samples of 2 μl were assayed for the incorporation of [^{32}P]CMP into 10% trichloroacetic acid-precipitable material. Symbols: ×, no primer added; ■, plus primer; ○, plus primer and 360 ng of CBP-24; □, plus primer and 360 ng of control protein from 1 M KCl column eluate as described in the legend to Fig. 1B. Protein samples and primer were preincubated for 10 min at room temperature before addition to reaction mixtures containing influenza virus.

forms of caps, e.g., m^7GpppN on mRNAs and $m_3^{2,2,7}GpppN$ on low-molecular-weight nuclear RNAs, may also be implicated in the generation of mature cellular mRNAs (13). Recent concomitant studies in which affinity photolabeling methods were employed indicate that the cap binding activity in influenza virus is associated with a P protein (3; I. Ulmanen, B. A. Broni, and R. M. Krug, Proc. Natl. Acad. Sci. U.S.A., in press).

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