A nuclear cap binding protein from HeLa cells

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ABSTRACT

We have identified a cap binding protein in a HeLa nuclear extract using a gel mobility shift assay probed with capped RNA. Subcellular fractionation of HeLa cells revealed that the majority (about 70%) of the cap binding activity is present in the nuclear extract, about 20% is in the cytoplasmic S100 fraction, and almost none in the ribosome-high salt wash fraction, indicating that the protein in active form localizes mainly in the nuclei. Competition experiments with various cap analogues showed that the G(5')ppp(5')N- blocking structure as well as the methyl residue at the N7 position of the blocking guanosine is important for the binding of this protein, and that the trimethylguanosine cap structure which exists at the 5' termini of many snRNAs is not recognized by this protein. Immunoprecipitation experiments using various anti-snRNP antibodies suggested that this protein is partially associated with U2 snRNP. We purified this protein to near homogeneity from a HeLa nuclear extract by several chromatographic procedures including capped RNA-Sepharose chromatography. The purified protein shows molecular weight of 80 kilodaltons, as judged by SDS gel electrophoresis, and binds specifically to the cap structure.

INTRODUCTION

It has been well accepted that a cap structure, $m^7G(5')ppp(5')N$, blocking the 5' termini of many eukaryotic mRNAs is important for cellular functions. The cap structure is known to stimulate translation in eukaryotic cells by facilitating ribosome binding to mRNA. A few cytoplasmic proteins have been reported that specifically interact with the cap structure. A 25 kilodalton cap binding protein (CBP-I or eIF-4E) binds to the cap structure during an early step in the initiation of protein synthesis, and stimulates translation of capped but not uncapped mRNAs (1). Two other initiation factors [eIF-4A (46 kilodalton) and eIF-4B (80 kilodalton)] are known to interact with the cap structure of mRNA in an ATP-Mg^{2+} dependent manner (2,3). CBP-I was purified to near homogeneity (4–7), and its cDNA has also been isolated and sequenced (8).

There has been cumulative evidence indicating that the cap structure also plays an important role in a nuclear event, mRNA splicing (9–14). The *in vitro* splicing reaction is inhibited by cap analogues or if uncapped mRNA precursor (pre-mRNA) is used as a substrate (9–13). The formation of the spliceosome was shown to be cap dependent (13). The positive effect of the cap structure on pre-mRNA splicing has been unequivocally demonstrated in the microinjection experiments using *Xenopus* oocytes (14). We have shown both *in vitro* and in *Xenopus* oocyte nuclei that when pre-mRNAs contain two introns within a single molecule, the enhancing effect of the cap structure seems to be restricted to the splicing reaction of the intron proximal to the cap structure (11,14). We have also shown that the methyl residue at the N7 position of the blocking guanosine of the cap structure is important for the splicing reaction (11,12). It is highly likely that some cap binding factor(s) is involved in mRNA splicing as is the case with translation. However, very little is known about such nuclear cap binding factors.

In HeLa cells, Patzelt *et al.* previously identified, by photoaffinity labeling, three nuclear cap binding proteins having molecular mass of 120, 89 and 80 kilodaltons, respectively, which are associated with the nuclear matrix (15). More recently, Rozen and Sonenberg identified 115 and 20 kilodalton cap binding proteins in a HeLa nuclear extract by UV crosslinking (16). However, the biochemical properties and functions of these cap binding proteins remain to be elucidated.

We developed a gel mobility shift assay to detect cap binding activity. Using this method we identified a cap binding protein in a HeLa nuclear extract. Complete purification of this protein revealed that this protein has molecular weight of 80 kilodaltons as judged by SDS gel electrophoresis. Purification of this protein may provide a vital clue to understand the functions of the cap structure in nuclei. In this paper we describe purification and some properties of the 80 kilodalton nuclear cap binding protein.

MATERIALS AND METHODS

Chemicals and Enzymes

[α-^{32}P]GTP (410 Ci/mmol) was obtained from Amersham. Other nonradioactive nucleotides including cap analogues were from Pharmacia. SP6 RNA polymerase and restriction enzymes were purchased from Takara Shuzo Co. Micrococcal nuclease and proteinase K were obtained from Boehringer-Mannheim and Merk, respectively. $m^{2,2,7}$GpppG was kindly gifted by Dr. Yasumi Oshima (Kyushu University).

Preparation of probes

A capped RNA probe (58 nucleotides long including the blocking guanosine, $2-10\times10^6$ cpm/μg) was synthesized by m^7GpppG-

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primed transcription of the plasmid pSP64 (17) linearized with EcoRI using SP6 RNA polymerase in the presence of [α-^{32}P]GTP as described previously (9). An uncapped probe was synthesized similarly without the dinucleotide primer. The RNA probes were purified on a 5% polyacrylamide gel containing 8 M urea, dissolved in water, and stored at −20 °C.

Gel mobility shift assay probed with capped RNA

The RNA probe was mixed with a subcellular fraction in 10 μl of 10 mM Hepes (pH 7.9), 25−500 mM KCl, 0−1000 μg/ml yeast RNA, 5% glycerol, and incubated at 20 °C for 30 min. Then the reaction mixture was directly loaded on a 6% polyacrylamide gel in 1/4 strength TAE buffer (10 mM Tris-acetate, 0.5 mM EDTA, pH 8.3) which had been pre-run at 10 V/cm for 1 hour. A dye marker (0.05% bromophenol blue) was applied to another lane of the same gel to determine the electrophoretic mobility. The gel was run at 10 V/cm with buffer circulation until bromophenol blue migrated about 8 cm. The gel was then dried and autoradiographed with an intensifying screen.

Preparation of subcellular fractions from HeLa cells

A nuclear extract was prepared as described by Dignam *et al.* (18) with following modifications. When crude nuclei were suspended in buffer C, the buffer containing 0.6 M KCl instead of 0.42 M NaCl was employed, so that the KCl concentration of the mixture was 0.25 M, as determined by the conductivity of the mixture. Cytoplasmic S100 fraction was also prepared as described in the same paper. A ribosome-high salt wash fraction was prepared as follows. The 100,000×g pellet from the step of S100 preparation, which contained the ribosomes, was suspended in 2 ml/10^9 cells of buffer E [20 mM Hepes (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% glycerol] containing 0.1 M KCl and 0.2 mM (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF, Wako Chemical Co.), and then the KCl concentration was adjusted to 0.6 M with 3 M KCl. The mixture was stirred gently at 4 °C for 1 hour, and centrifuged for 1 hour at 100,000×g. The supernatant was designated the ribosome-high salt wash fraction.

Immunoprecipitation with anti-snRNP antibodies

Fifteen μl of protein A-Sepharose (PAS, Pharmacia) equilibrated with NET-2 buffer (40 mM Tris-Cl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.05% Nonidet P-40 (NP-40), 0.5 mM DTT) was mixed with 500 μl of NET-2 buffer and 10−20 μl of anti-snRNP serum in a plastic tube, and gently mixed at room temperature for 2 hours. Antibody-bound PAS was washed 4 times with 500 μl of NET-2. The washed PAS pellet was then mixed with 6 μl of a HeLa nuclear extract and 10 μl of NET-2, and incubated at 4 °C with gentle mixing. The mixture was centrifuged, and the supernatant was transferred into another tube (15 μl, sup fraction). The pellet was washed 4 times with 500 μl of NET-2, and then mixed with 30 μl of elution buffer (1 M KCl, 20 mM Hepes pH 7.9, 5 mM EDTA, 1 mM DTT, 0.2 mM APMSF). After incubation on ice for 5 min, the supernatant (30 μl) was recovered by centrifugation, and designated the ppt fraction. The cap binding activity of 1 μl of the sup fraction and of 2 μl of the ppt fraction was tested by the gel shift assay.

In the case of immunoprecipitation of RNA probes, anti-U2 antibody-bound PAS (15 μl) was mixed with 6 μl of a HeLa nuclear extract, 10 μl of NET-2, 30 unit of RNase inhibitor and 40 ng of RNA probe, and the sup and ppt fractions were prepared by the same procedure as described above. The probe RNA was recovered from the sup and ppt fractions by proteinase K treatment and phenol extraction followed by ethanol precipitation, and subsequently fractionated on a 5% polyacrylamide gel containing 8 M urea. Sera used in this experiment were as follows. 1) anti-U1 snRNP serum; 2) anti-U1 snRNP and anti-Sm sera; 3) anti-Sm serum; 4) anti-U2 snRNP (Ya) serum (19); 5) normal human serum; 6) anti-Sm monoclonal antibody (Y-12) (20); 7) anti-U1 snRNP (Ag) serum (21). These antibodies were kindly gifted by Dr. Tsuneyo Mimori (Keio University) and Dr. Joan A. Steitz (Yale University).

Preparation of capped RNA-Sepharose

Capped RNA, the same molecule used in the gel mobility shift assay, was prepared in a larger scale similarly as above, and covalently attached to cyanogen bromide activated Sepharose CL-2B in the same way as the preparation of sequence-specific DNA affinity resin described previously (22). In short, Sepharose CL-2B (Pharmacia, 5 ml of settled volume) was activated by cyanogen bromide, and 300 μg of the capped RNA was coupled to the resin. The resin was then treated with 1 M ethanolamine-HCl (pH 8.0) to inactivate the unreacted Sepharose, washed extensively and stored as described previously (22).

Purification of a cap binding protein

Unless otherwise stated, all operations were performed at 4 °C. A nuclear extract prepared from 2×10^{10} HeLa cells (20 ml, 23.4 mg/ml protein) was loaded onto a DEAE-cellulose column (Whatman, 2.5×18 cm) previously equilibrated with buffer E [20 mM Hepes (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% glycerol] containing 0.1 M KCl. The column was washed with 300 ml of buffer E containing 0.15 M KCl. The column was then washed with buffer E containing 0.3 M KCl, and fractions were collected. Fractions having an A_{280nm} greater than 1.0 were pooled (36 ml, 3.6 mg/ml protein). Solid ammonium sulfate (47.6 g/100 ml, 75% saturation at 0 °C) was added to the pooled fraction, and the mixture was gently stirred on ice for 1 hour. The precipitate was collected by centrifugation for 20 min at 25,000 rpm in the type 60 rotor (Beckman), and then dissolved in 4 ml of buffer E containing 0.1 M KCl and 0.2 mM APMSF. This protein solution was mixed with 2 ml of 3 M KCl and 0.5 ml of 50% glycerol, and fractionated by gel filtration on a Bio-Gel A-1.5 m column (Bio-Rad, 2.5×92.5 cm) previously equilibrated with buffer E containing 1 M KCl and 0.2 mM APMSF. Active fractions were pooled (27 ml, 414 μg/ml protein) and dialyzed for 5 hours against 1 liter of buffer E containing 50 mM KCl with one change, and applied to a DEAE-Toyopearl 650 M column (Toyo Soda Co., 1.8 ml of the resin in a Bio-Rad polypropylene column) previously equilibrated with buffer E containing 50 mM KCl. The column was washed with 10 ml of the same buffer, and eluted with buffer E containing 0.2 M KCl. Fractions having A_{280nm} greater than 0.8 were pooled (2 ml, 3.5 mg/ml protein). The pooled fraction was then adjusted to 0.1% NP-40 and 0.2 mM APMSF, and mixed with 3 ml of capped RNA-Sepharose previously equilibrated with buffer E containing 0.2 M KCl, 0.2% NP-40 and 0.2 mM APMSF in a screw-capped polypropylene tube. The mixture was incubated at 4 °C for 1 hour with gentle mixing on a rotating platform, and then transferred into a polypropylene column (Bio-Rad). The column was washed with 40 ml of buffer E containing 0.2 M KCl and 0.2 mM APMSF, and then washed with 40 ml of buffer E containing 0.7 M KCl and 0.2 mM APMSF. The column was then washed with buffer E containing 0.2 M KCl, 4 M urea and 0.2 mM APMSF, and 0.5 ml-fractions were collected. Active

fractions were pooled, and dialyzed for 5 hours against 1 liter of buffer E containing 20% glycerol, 0.1 M KCl and 0.2 mM APMSF with two changes. This preparation was designated the purified cap binding protein fraction (1.5 ml, 14.4 μg/ml).

RESULTS AND DISCUSSION

Identification of a nuclear cap binding protein

We have previously shown that the cap structure plays a positive role in mRNA splicing *in vitro* and in *Xenopus* oocyte nuclei (11,14). The stimulatory effect of the cap structure on mRNA splicing appears to be mediated by a factor(s) which specifically recognizes the cap structure of pre-mRNA. It was anticipated that characterization of such a factor(s) may provide a vital clue to understanding the mechanism of mRNA splicing. Attempts were made, therefore, to identify a cap binding factor in HeLa nuclear extracts.

We developed a gel mobility shift assay to detect the cap binding activity. The rationale behind this assay is that if a cap binding protein binds to the cap structure of RNA, the electrophoretic mobility of the bound RNA becomes slower than that of the unbound RNA, and that the mobility shift of the RNA due to the cap binding protein is sensitive to the presence of cap analogues such as m^7GpppG or m^7GTP in the binding mixture. Although binding of other proteins to the RNA moiety may also shift the electrophoretic mobility, the shift of RNA band would not be sensitive to cap analogues, and should occur even with an uncapped counterpart used as a probe.

A capped RNA probe (58 nt) was synthesized by m^7GpppG-primed transcription of the plasmid pSP64 linearized with EcoRI using SP6 RNA polymerase in the presence of [α-^{32}P]GTP. An uncapped probe was synthesized similarly without the dinucleotide primer. A HeLa nuclear extract was adjusted with 3 M KCl to 1 M KCl and subjected to gel filtration on a Cellulofine column. Each fraction was assayed for gel mobility shift activity with both the capped and uncapped RNA probes. As shown in Fig. 1, the appearance of at least several bands was observed with both probes in a broad molecular size range. Although the majority of the bands were present with both probes, the band designated B was only detectable with the capped probe. The cap binding factor corresponding to this band was eluted from the column in the vicinity of 100 kilodalton. We could not detect other cap binding factors by this method.

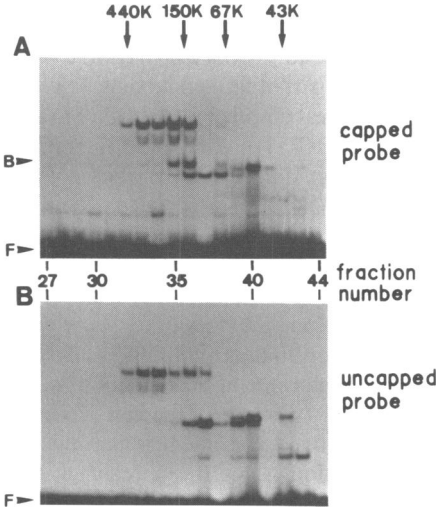


Fig. 1. Identification of a cap binding factor. A HeLa nuclear extract (0.6 ml, 23.4 mg/ml protein) was adjusted with 3 M KCl to 1 M KCl-buffer E and loaded onto a Cellulofine GCL-2000 gel filtration column (Seikagaku-kogyo; 1.5×36 cm) equilibrated with the same buffer, and 1 ml-fractions were collected. Cap binding activity of each fraction was tested using the gel mobility shift assay probed with capped RNA (Panel A) and uncapped RNA (Panel B). The RNA bands not shifted (free RNA) and shifted specifically by the cap binding factor are indicated by arrowheads designated F and B, respectively. The positions of protein size markers are indicated on the top of the figure.

Localization of the cap binding factor in cells

Next we examined the localization of this cap binding factor. HeLa cells were fractionated into three fractions; nuclear extract, cytoplasmic S100 fraction, and ribosome-high salt wash fraction, as described in MATERIALS AND METHODS. Each fraction was assayed for cap binding activity. As shown in Fig. 2, the majority (about 70%) of the cap binding activity was in the nuclear extract, about 20% in the cytoplasmic fraction, and almost none in the ribosome wash fraction, indicating that the active factor is located mainly in the nuclei. The binding of this factor to the capped probe was inhibited by m^7GTP (lane 5), which confirmed that this factor binds to the cap structure of the probe. The band indicated by an asterisk in the figure is visible even with gel-purified probe alone (lanes 1 and 2), and its intensity varies according to the salt and protein concentrations in the reaction mixture. This band appears to represent the free probe of either an alternative secondary structure or a dimeric structure formed by intermolecular base pairing.

Characterization of the cap binding factor

The cap binding activity in the nuclear extract was resistant to micrococcal nuclease treatment but sensitive to proteinase K treatment, indicating that this is a protein factor (Fig. 3A).

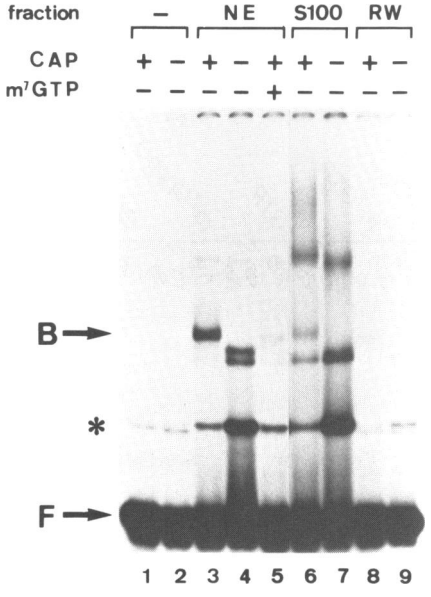


Fig. 2. Localization of the cap binding factor. HeLa cells were fractionated into three fractions; nuclear extract (NE), cytoplasmic S100 fraction (S100), and ribosome-high salt wash fraction (RW), as described in MATERIALS AND METHODS. The cap binding activity of each fraction corresponding to 2×10^4 of HeLa cells was assayed with capped probe (lanes 1, 3, 5, 6 and 8) and uncapped probe (lanes 2, 4, 7 and 9). In the case of lane 5, the nuclear extract was preincubated with 150 μM m^7GTP at 20 °C for 10 min before the assay. In lanes 1 and 2, no subcellular fraction was added. The bands B and F are the same as those in Fig. 1. See the text for the band indicated by an asterisk.

Hereinafter, this factor is arbitrarily designated NCBP (Nuclear Cap Binding Protein).

KCl in the concentration range of 50−500 mM did not significantly affect the activity, and substantial binding occurred even in the presence of 1M KCl (Fig. 3B) or 2M urea. The protein-RNA complex once formed is resistant to at least 700 mM KCl, and requires >4 M urea to be dissociated. ATP and Mg^{2+} are not at all required for the cap binding activity.

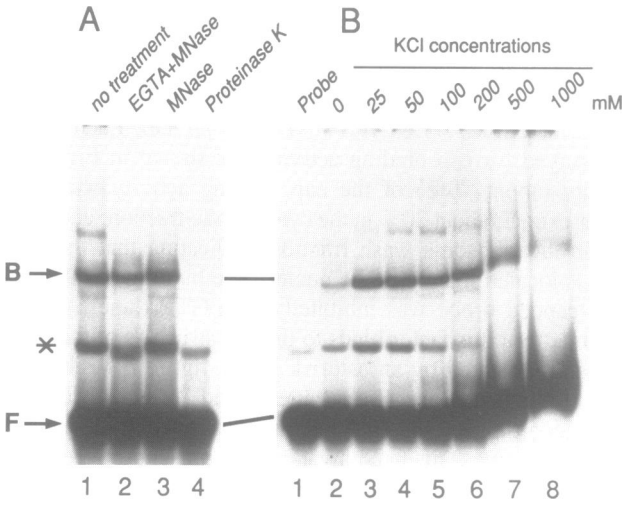


Fig. 3. Characterization of the nuclear cap binding factor. (A) The gel shift assay was carried out with capped probe using a nuclear extract treated as follows. Lanes: 1, untreated nuclear extract; 2, a nuclear extract was treated with 70 U/ml micrococcal nuclease in the presence of 1 mM $CaCl_2$ at 30 °C for 30 min, and the reaction was terminated by the addition of 3 mM EGTA.; 3, a nuclear extract was treated with 70 U/ml micrococcal nuclease in the presence of 1 mM $CaCl_2$ and 3 mM EGTA at 30 °C for 30 min.; 4, a nuclear extract was treated with 1 mg/ml proteinase K at 30 °C for 30 min. The bands B, F and * are the same as those in Fig. 2. (B) The gel shift assay was carried out with capped probe using a nuclear extract in various KCl concentrations indicated on the top of each lane. In lane 1, no extract was added.

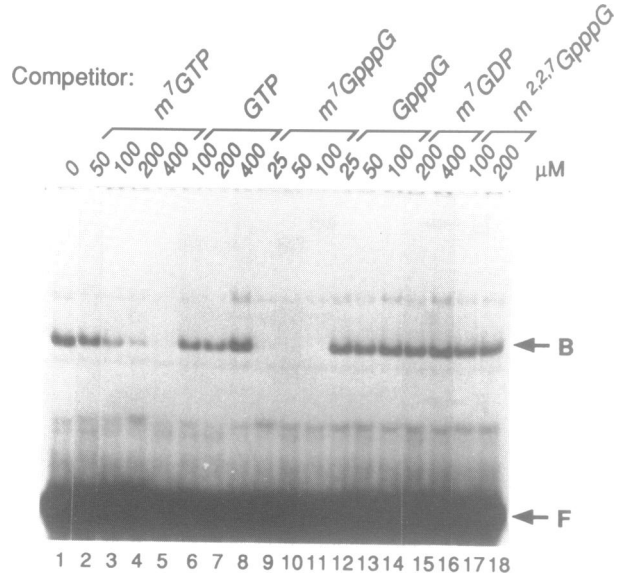


Fig. 4. Competition with cap analogues. A nuclear extract was pre-incubated with various cap analogues indicated on the top of each lane at 20 °C for 15 min, and its cap binding activity was assayed. The bands B, F and * are the same as those in Fig. 2.

Competition with cap analogues

To examine the binding specificity of NCBP, we carried out competition experiments with various cap analogues. A nuclear extract was pre-incubated with various cap analogues at 20 °C for 15 min, and its cap binding activity was assayed. The results are shown in Fig. 4. The cap binding activity was inhibited by m^7GTP and m^7GpppG, but not by GTP and GpppG. This clearly indicates that the methyl residue at the N7 position of the blocking guanosine is important. The result that both m^7GDP and m^7GMP had no strong inhibitory effect (data not shown) suggests that the presence of triphosphate is important. Moreover, m^7GpppG was more inhibitory than m^7GTP, implying that the G-5′-ppp-5′-N- blocking structure is recognized more efficiently by NCBP. Also noted was that $m^{2,2,7}$GpppG did not inhibit the activity. It appears, therefore, that NCBP does not to recognize the trimethylguanosine cap structure which exists at the 5′ termini of many snRNAs. In fact, NCBP did not bind to a $m^{2,2,7}$GpppG-primed probe as judged by the gel mobility shift assay (data not shown).

These results strongly suggest that NCBP binds specifically to the cap structure of pre-mRNAs in nuclei. In higher eukaryotes, the majority of the cap structure of mRNAs exists as the type I cap structure (m^7GpppNm) in which the ribose of the first nucleotide N is 2′-O-methylated (23). As the result of a competition experiment with the type 1 cap analogue (m^7GpppGm) used as competitor, m^7GpppGm was found to be a little bit less inhibitory than m^7GpppG (data not shown), suggesting that the methyl residue of the ribose moiety is not important for binding of NCBP.

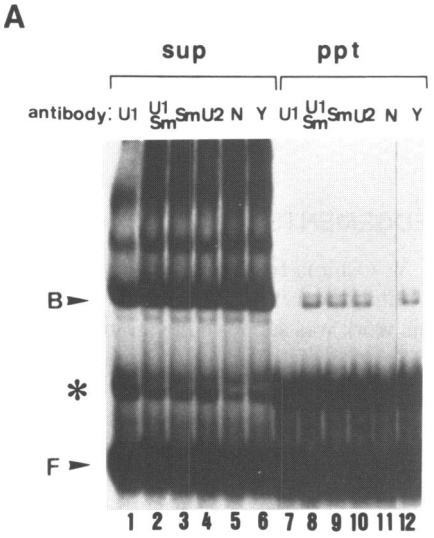
Immunoprecipitation with anti-snRNP antibodies

Since NCBP is a candidate for the cap binding factor involved in mRNA splicing, it is of interest to examine whether NCBP is associated with any known splicing factor(s), such as snRNP. Specific snRNP in the nuclear extract was immunoprecipitated with various anti-snRNP antibodies and protein A-Sepharose, and the cap binding activity of the precipitate and supernatant fractions was assayed as described in MATERIALS AND METHODS. As shown in Fig. 5A, part of the cap binding activity (about 10%) in the nuclear extract was co-precipitated with Sm and U2 snRNP, but not with U1 snRNP. Another anti-U1 snRNP serum, Ag serum (21), did not precipitate NCBP (data not shown). These results suggested that NCBP is associated at least partially with U2 snRNP in the nuclear extract. As NCBP is dissociated from the precipitate fraction by in 300 mM NaCl (data not shown), the interaction between NCBP and U2 snRNP appears to be rather weak. We cannot exclude the possibility, however, that NCBP is associated with other unidentified minor snRNPs which have the same antigenic determinant as U2 snRNPs.

The fact that only 10 percent of the cap binding activity was precipitated in the above experiment is not due to low titer of the antibody preparation, since the percentage did not change when the amount of antibody was increased. The percentage did not change, even if the nuclear extract was pre-incubated with ATP and Mg^{2+} (the standard conditions for the *in vitro* splicing reaction). It is worth noting that essentially the same results were obtained with the whole cell sonicates of HeLa cells (data not shown). It is unlikely that NCBP itself is the anti-U2 and anti-Sm antigen for the following reasons. First, these antibodies cannot detect NCBP in the Western blot. Second, only part of NCBP is precipitated even by sufficient amount of antibody. Third, the interaction between NCBP and U2 snRNP is much

weaker than antigen-antibody interaction as described above.

It should be pointed out that these results do not necessarily show that NCBP is directly associated with U2 snRNP. U2 snRNP is known to bind the branch point sequence of pre-mRNA (24), and NCBP can bind to the cap structure of the same pre-mRNA. Accordingly, it is possible to assume that NCBP is associated with U2 snRNP via pre-mRNA which exists in the nuclear extract endogenously. To test this possibility, the following experiment was carried out. The capped RNA gel shift probe was mixed with a nuclear extract, and U2 snRNP in the mixture was immunoprecipitated as above. If NCBP is directly associated with U2 snRNP, the capped probe is expected to be co-precipitated with U2 snRNP. On the other hand, if the association is via endogenous pre-mRNA, the capped probe should not be co-precipitated because the cap binding site on NCBP is already occupied by the cap structure of the pre-mRNA.



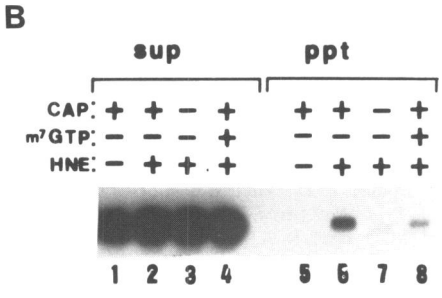


Fig. 5. Immunoprecipitation with anti-snRNP antibodies. (A) specific snRNPs in a HeLa nuclear extract were immunoprecipitated using various anti-snRNP sera, and the cap binding activity in the supernatant (lanes 1−6) and pellet (lanes 7−12) was compared, as described in MATERIALS AND METHODS. Sera used in this experiment were as follows. Lanes 1 and 7, anti-U1 snRNP serum; lanes 2 and 8, anti-U1 snRNP and anti-Sm sera; lanes 3 and 9, anti-Sm serum; lanes 4 and 10, anti-U2 snRNP serum; lanes 5 and 11, normal human serum; lanes 6 and 12, anti-Sm monoclonal antibody (Y-12). The bands B, F and * are the same as those in Fig. 2. (B) anti-U2 snRNP antibody-bound protein A-Sepharose was mixed with a HeLa nuclear extract (in lanes 1 and 5, buffer E containing 0.1 M KCl and 20% glycerol was used instead of the extract) and either capped probe (lanes 1, 2, 4, 5, 6 and 8) or uncapped probe (lanes 3 and 7) in the presence (lanes 4 and 8) or absence (lanes 1−3 and 5−7) of 150 μM m^7GTP. The sup and ppt fractions were prepared as described in MATERIALS AND METHODS. RNA probe was recovered from each fraction, electrophoresed on a 5% polyacrylamide gel, and autoradiographed.

As shown in Fig. 5B, the capped probe was co-precipitated with U2 snRNP (lane 6), and the co-precipitation was inhibited by cap analogue (lane 8) or if uncapped probe was used (lane 7). These results strongly suggest that the association between NCBP and U2 snRNP is not via endogenous pre-mRNA, but is rather direct. However, the possibility that association between the two molecules via other factor(s) is not completely ruled out.

It is unlikely that NCBP binds to the trimethylguanosine cap structure of U2 RNA for two reasons. First, if such binding does occur, the capped RNA should not be co-precipitated with U2 snRNP, because the cap binding site on NCBP has already been occupied by the trimethylguanosine cap structure. Second, NCBP is not co-precipitated with U1 RNA which also has the trimethylguanosine cap structure. The argument described above was made on the assumption that NCBP has only one cap binding site. However, we cannot exclude the possibility that NCBP has multiple cap binding sites and that it can bind to both endogenous pre-mRNA and the capped probe at the same time.

Purification of NCBP

As NCBP is associated with U2 snRNP, this protein is supposed to be a strong candidate for the cap binding factor involved in mRNA splicing. We purified NCBP from a HeLa nuclear extract as described in MATERIALS AND METHODS. In short, nuclear extracts prepared from 2×10^{10} HeLa cells were applied onto a DEAE-cellulose column, and the step-wise elution with 150 and 300 mM KCl was performed. The eluate with 300 mM KCl was then fractionated by a Bio-Gel A gel filtration column in the presence of 1 M KCl. Active fractions were pooled, concentrated in a small DEAE-Toyopearl column, and then applied onto a capped RNA-Sepharose column. The column was then washed extensively with the buffer containing 0.7 M KCl. Most of NCBP remains bound to the resin even after washing with 0.7 M KCl, whereas proteins which bind nonspecifically to the RNA moiety are eluted. NCBP was eluted with 4 M urea, and immediately dialyzed. This fraction was designated the

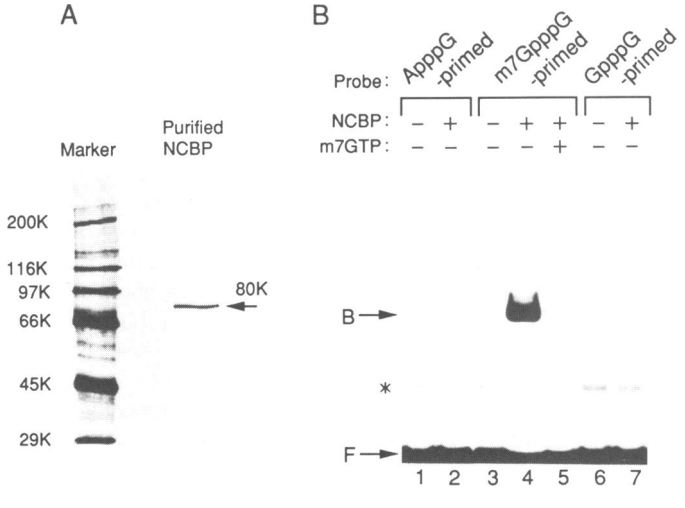


Fig. 6. (A) The purified NCBP (70 ng) and protein size markers were subjected to 10% SDS/PAGE followed by silver staining. The molecular size of each marker was shown on the left side of the figure. (B) The gel shift assay with purified NCBP. The bands B, F and * are the same as those in Fig. 2. Lanes: 1, ApppG-primed probe alone; 2, ApppG-primed probe + purified NCBP (70 ng); 3, m^7GpppG-primed probe alone; 4, m^7GpppG-primed probe + purified NCBP (70 ng); 5, the same as lane 4 except for the addition of 300 μM m^7GTP; 6, GpppG-primed probe alone; 7, GpppG-primed probe + purified NCBP (70 ng).

Table 1. Purification of NCBP.

	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)
HeLa nuclear extract	468	156000*	333	100
DEAE-cellulose	130	112000	862	71
Bio-Gel A	11.2	32400	2890	20
DEAE-Toyopearl	7.0	33400	4770	21
Capped RNA-Sepharose	0.02	5400	270000	3.4

*The activity that shifts 1 ng of the capped probe in the standard reaction mixture containing 20 ng of the probe is arbitrarily defined as one unit.

purified NCBP fraction. The protein was purified approximately 810-fold with the overall yield of 3.4% as compared with the nuclear extract (Table 1).

NCBP purified in this way was near homogeneity as judged by SDS/PAGE (25) followed by silver staining as shown in Fig. 6A, a single protein band of approximately 80 kilodaltons was visible. It is worth noting that although the gel region below 28 KD molecular weight is not shown in Fig. 6A which represents the electrophoretic pattern in a 10% SDS/PAGE, we could not detect any bands below 28 KD when the same NCBP preparation was electrophoresed in a 15% SDS/polyacrylamide gel (data not shown). As shown in Fig. 6B, the purified NCBP was active in binding to the cap structure and its binding was strongly inhibited by m^7GTP. The protein bound weakly to the unmethylated cap structure (GpppG-primed probe) and not at all to a pseudo-cap structure in which the blocking nucleoside is adenosine instead of guanosine (ApppG-primed probe). These results are consistent with the suggestion from the competition experiment that not only the methyl residue at the N7 position of the blocking guanosine but the G-ppp-N- blocking structure itself is important for the binding of NCBP.

NCBP shows molecular mass of 80 kilodaltons, as judged by SDS/PAGE. This molecular mass is very similar to that of eIF-4B which is known to interact with the cap structure of mRNA during initiation process of protein synthesis (3). It might be argued that NCBP is the same as eIF-4B, which was possibly contaminated in the nuclear extract during extract preparation. This possibility is excluded by the following reasons. First, ATP and Mg^{2+} do not at all affect the binding activity of NCBP, whereas ATP and Mg^{2+} are required for efficient interaction of eIF-4B with the cap structure (2,3). Second, substantial binding occurs even in the presence of 1 M KCl in the case of NCBP, whereas the interaction of eIF-4B with the cap structure cannot be detected in the presence of >150 mM KCl (16). Third, the cap binding activity cannot be detected by the gel shift assay in the ribosome-high salt wash fraction in which eIF-4B is most abundant (16).

Patzelt *et al.* previously identified in HeLa cells, by photoaffinity labeling, three nuclear cap binding proteins having molecular mass of 120, 89 and 80 kilodaltons, which are associated with the nuclear matrix (15). More recently, Rozen and Sonenberg identified 115 and 20 kilodalton cap binding proteins in a HeLa nuclear extract by UV crosslinking (16). NCBP is presumably the same protein that Patzelt *et al.* identified as 80 kilodalton cap binding protein. Although these investigators reported the presence of other cap binding proteins in HeLa nuclear extracts, we could not detect those proteins by the gel shift assay. This is probably due to the difference in the assay methods for detection of cap binding proteins. In this respect, our gel shift assay cannot detect the 25-kilodalton cap binding protein (eIF-4E) in the ribosome-high salt wash fraction. The interaction between eIF-4E and the cap structure is known to be weak or transient (3). Presumably our assay method would not be able to detect such interactions.

Although NCBP is a candidate of the cap binding protein involved in mRNA splicing, we have so far no direct evidence indicating the involvement of NCBP in mRNA splicing. The biological function of NCBP remains to be clarified. We have also detected similar cap binding activity in nuclear extracts of chicken brain, mouse lymphoma cell M12 and myeloma cell P3, suggesting that the function of NCBP is widely conserved.

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