A Cap Binding Protein That May Mediate Nuclear Export of RNA Polymerase II-transcribed RNAs

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Abstract. It has previously been shown that efficient export of U1 snRNA or of microinjected, in vitro synthesized, RNA transcripts from the nucleus of Xenopus oocytes is facilitated by their monomethyl guanosine cap structures. Nuclear exit of these transcripts could be competitively inhibited by microinjection of an excess of a cap analog, the dinucleotide m7GpppG (Hamm, J., and I. W. Mattaj. 1990. Cell. 63:109-118). We have now analyzed the ability of several other related cap analogs to inhibit the export of U1 snRNA from the nucleus. The results define the recognition specificity of a factor(s) involved in RNA transport, and indicate that the cap binding activity (CBA) involved in RNA export is different from cap binding proteins (CBPs) involved in the initiation of translation. A CBP, whose specificity for different analogs correlates with the ability of the analogs to inhibit U1 snRNA export, is identified in nuclear extracts prepared from HeLa cells. We propose that this protein may have a role in the export of capped RNAs from the nucleus.

The mechanism of nuclear export of RNAs transcribed by RNA polymerase II (U snRNAs and mRNAs) is not well understood. However, recent progress in this area has been significant. Sequences and structural features of RNAs which are either required for export or which result in nuclear retention have been identified (for recent reviews see Maquat, 1991; Izaurralde and Mattaj, 1992). These are presumed to interact with components of the transport machinery or with anchoring nuclear factors, respectively. Several of the pre-mRNA sequences that affect transport are also required for RNA processing events.

Direct evidence for the role of introns in the nuclear retention of pre-mRNAs came from a series of experiments performed in yeast (Legrain and Rosbash, 1989), mammalian cultured cells (Chang and Sharp, 1989) or Xenopus oocytes (Hamm and Mattaj, 1990). Mutated pre-mRNAs that could not be spliced but that could form defective splicing complexes were anchored in the nucleus, presumably in abortive complexes. Conversely, mutant pre-mRNAs unable to form detectable splicing complexes were exported rapidly into the cytoplasm. Thus the formation of splicing complexes and RNA export from the nucleus are antagonistic, or competing processes.

The effect of 3′ end processing on the export of several RNA transcripts has also been analyzed (Eckner et al., 1991). In contrast to the situation with intron splicing, evidence for a positive mechanistic linkage between 3′ end formation and the export of histone mRNA transcribed was obtained. Moreover, at least for histone mRNAs, specific recognition of sequences or structures within the processed RNA seemed also to be required for export from the nucleus (Eckner et al., 1991).

Sequence or structural features are limited to particular RNAs or to subclasses of RNA, thus they cannot be signals involved in the transport of all RNAs. One of the structural features common to all polymerase II-transcribed RNAs, and thus a good candidate for recognition by factors generally involved in export of these RNAs, is the monomethyl guanosine cap structure m7G(5′)ppp(5′)N. Indeed, it has recently been shown that efficient export of both U1 RNA and microinjected, in vitro synthesized, RNAs from the nucleus of Xenopus oocytes is dependent on their having an m7GpppN cap structure. Furthermore, nuclear exit of U1 snRNA transcripts is specifically inhibited by microinjection of an excess of a cap analog, the dinucleotide m7GpppG (Hamm and Mattaj, 1990).

These results imply the existence of a cap binding activity (CBA)1 involved in export of U1 snRNA, and probably also of other pol II-transcribed RNAs, that binds directly to the monomethyl cap structure. Several cap binding proteins (CBPs), present either in the cytoplasm or in the nucleus, have previously been described (Sonenberg et al., 1978; Sonenberg, 1981; Patzelt et al., 1983; Rozen and Sonenberg, 1987; Ohno et al., 1990), the best studied being the eukaryotic translation initiation factor 4E (elF-4E; for reviews see Shatkin, 1985; Rhoads, 1988; Sonenberg, 1988). In this study we have examined whether any of these CBPs might

1. Abbreviations used in this paper: CBA, cap binding activity; CBP, cap binding protein.

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The Journal of Cell Biology, Volume 118, Number 6, September 1992 1287-1295 1287
be the factor that mediates U1 snRNA export. A CBP whose specificity for different cap analogs correlates well with the ability of the analogs to inhibit U1 snRNA export was identified in nuclear extracts from HeLa cells. Analysis of the biochemical properties and cap-binding specificity of this protein suggests strongly that it is the CBP previously identified and purified by Ohno et al. (1990). The properties of this protein are consistent with it being an effector of nuclear export.

Materials and Methods

Materials

T3 RNA polymerase and RNAsin were from Promega Biotec (Madison, WI). BSA (fraction V), Calf Intestine Alkaline Phosphatase (type I), and Ferritin from Horse Spleen (type I) were from Sigma Chemical Co. (St. Louis, MO). Pepstatin A, benzamidine and DTT were from Sigma Chemical Co., PMSF from Serva Biochemicals (Paramus, NJ), Trasylol (10000 KIE/ml) from Bayer and thiodyglycol from Pierce Chemical Co. (Rockford, IL). Ammonium sulfate (nucleate and protease free) was from Sigma Chemical Co. All column chromatography resins were from Pharmacia Fine Chemicals (Piscatway, NJ). Triton X-100, Tween 20, and polyethylene glycol (PEG 4000) were from Merck & Co. (Rahway, NJ). The cap analogs m^7GpppG and GpppG were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and Sigma Chemical Co. respectively, GDP was from Sigma Chemical Co.乙GpppG, m^7GpppG, m^GMP, mGDP and mGTP were synthesized and purified as described (Darzynkiewicz et al., 1985, 1988, 1989, 1990). Labeled nucleoside triphosphates were from Amersham Corp (Arlington Heights, IL).

Recombinant Plasmids and In Vitro Synthesis of RNA Transcripts

The UIAD mutant was described by Hamm et al., 1987. For native gel assays, a 77-nucleotide RNA fragment was used. This fragment was obtained by in vitro transcription of plasmid USII-Scherly et al., 1988) using T3 RNA polymerase. The in vitro transcription reactions were performed according to the manufacturer (Promega-Biotec) and included the incorporation of (a-32P)GTP and, as indicated, a cap analog. Unlabeled competitor RNA was synthesized as described above with the following modifications. The reaction volume was increased to 800 µl and incubation was performed for 3 h. No cap analog was included in the reaction. All RNA products were phenol extracted and purified by gel filtration on Sephadex G-50 spin columns equilibrated with 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. Unlabeled RNAs were concentrated by ethanol precipitation and quantified spectrophotometrically. Labeled RNAs were further purified by electrophoresis on an 8% denaturing polyacrylamide gel, the RNA was localized by autoradiography, excised from the gel, and eluted overnight at room temperature in 0.5 M Tris-HCl, pH 7.4, 1 mM MgCl2, 0.1% SDS (wt/vol), 0.1 mM EDTA and 10 µg/ml of yeast RNA. The eluted RNA was ethanol precipitated and resuspended in H2O at 2 x 10^6 cpm µl^-1 of yeast RNA. The integrity of the transcribed products was examined by analysis on denaturing 6% polyacrylamide gels. Yeast RNA (Sigma Chemical Co.), (type XI; further purified by phenol extraction) was used as an unlabeled competitor.

Oocyte Injections

Oocyte injections were performed as described by Hamm and Mattaj (1990). To transcribe UIAD plasmid RNA, 20 nl of a solution of the plasmid carrying the corresponding gene was microinjected into oocyte nuclei. The concentration of microinjected DNA was 250 µg ml^-1. After a 16-h incubation at 20°C, (α-32P)GTP and cap analogs were coinjected into oocyte cytoplasms. The preinjection of DNA templates was carried out to allow the assembly of transcription complexes, and thus to prevent any delay in labeling of the transcripts whose transport was being studied. Cap analogs were dissolved in H2O and injected at a final concentration of 10 or 50 mM together with 0.25 µCi µl^-1 of (α-32P)GTP (specific activity 400 Ci mmole^-1). The volume microinjected was 20 nl. After four additional hours oocytes were dissected and analyzed as described by Hamm and Mattaj (1990).

Electric Field Mobility Retardation Assay

Binding reactions were carried out in BB buffer (10 mM potassium phosphate, pH 8.0, 0.25 M KCl, 2 mM EDTA, and 5% glycerol). All samples contained 8 µg of yeast RNA (with the exception of samples shown in Fig. 3A, 1 µg/µl of RNasin and ~4 x 10^6 cpm of the radiolabeled RNA fragments described above). Final sample volumes were 10 µl. Nuclear extract or fractions were added to the reaction mixtures from a diluted stock solution in the same buffer. After 30 min at 25°C, 3 µl of Sample Buffer (20% glycerol and 0.005% bromophenol blue in BB buffer) was added to each sample. Samples were applied to a 6% non-denaturing polyacrylamide gel (29:1, acrylamide/bisacrylamide ratio). Electrophoresis was carried out at a constant voltage of 10 V cm^-1 at room temperature in TBE buffer. Gels were fixed in 10% acetic acid after drying. Complexes were visualized by autoradiography.

SDS-PAGE

Protein gels were carried out according to Laemmli (1970). Gels were stained with Coomassie brilliant blue R or with silver nitrate. Molecular mass standards used were the Sigma Chemical Co., MW-SDS-200, kit composed of: Rabbit Muscle Myosin (205 KD), Escherichia coli β-galactosidase (116 KD), Rabbit Muscle Phosphorylase b (97 KD), BSA (66 KD), Ovalbumin (45 KD), and Bovine Erythrocyte Carbonic Anhydrase (29 KD).

HeLa Nuclear Extract

HeLa nuclear extracts were prepared from ~10^10 cells as described by Dignam et al. (1983) with the following modifications. Buffer C contained 0.6 M KCl instead of 0.42 M NaCl and 0.05% Tween 20, 300 µg ml^-1 benzamidine, 2 µg ml^-1 pepstatin A, 0.5% (vol/vol) thiodyglycol, and 1% (vol/vol) Trasylol. Protease inhibitors and DTT were added to the buffers just before use. The viscous nuclear lysate was centrifuged at 35,000 rpm for 60 min in a Beckman type 50.2 Ti rotor (Beckman Instruments, Inc., Fullerton, CA). The supernatant was used immediately or stored at ~70°C without performing the dialysis step. Final protein concentration was between 8 and 10 mg/ml^-1.

Purification of Nuclear Cap Binding Protein from HeLa Nuclei

Buffer I contained 10% (vol/vol) glycerol, 20 mM Hepes, pH 7.9, and 0.2 mM EDTA. Buffer II contained 10% (vol/vol) glycerol, 20 mM Hepes, pH 7.9, 0.5 M NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 0.5% (vol/vol) thiodyglycol and 1% (vol/vol) Trasylol. All columns were run at 4°C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Cambridge, MA) with BSA as a standard.

HeLa nuclear extract was adjusted to 4 M NaCl, centrifuged for 30 min at 10,000 rpm in a Sorvall SS-34 (Sorvall Instruments, Newton, CT) rotor and applied to a Phenyl-Sepharose CL-4B column of 18 x 2 cm, equilibrated in buffer I plus 4 M NaCl. The column was washed with several volumes of buffer I containing 4 M NaCl and eluted in a stepwise manner with buffer II containing 2.5 M, 1 M, and 50 mM NaCl, respectively. Elution was performed at 0.8 ml min^-1 and monitored at 280 nm. The cap binding activity eluted during the 1 M NaCl step. The 1 M NaCl eluate was diluted threefold with buffer I, adjusted to 50% saturation with solid ammonium sulfate and stirred 30 min on ice. After centrifugation for 30 min at 10,000 rpm (Sorvall HB-4 rotor) the precipitate was dissolved in buffer I containing 2.5 M NaCl. The suspension was applied to a Sephacryl S-300 column of 60 x 2 cm, equilibrated in buffer I plus 2.5 M NaCl. Elution was performed with the same buffer at 0.3 ml min^-1. 2-ml fractions were assayed, pooled, and dialyzed 1 h against 1 M of buffer II. Dialysis was continued in the same buffer containing 15% (wt/vol) PEG 4000 until the sample volume was reduced to ~5 ml.

Affinity Chromatography

In vitro synthesized, capped or uncapped RNAs (~0.7 mg) with a specific activity of 10^9 cpm µg^-1 were coupled to 1.2 g of CNBr-activated Sepharose 4B according to the recommendations of the manufacturer (Pharmacia Fine Chemicals). Coupling efficiency was determined by comparing the input RNA solution with the remaining free RNA in the supernatant and washes. Coupling efficiency was ~75%. The resin was prewashed with 6
**Results**

**Nuclear Export of U1 snRNA Is Not Inhibited by the Same Set of Cap Analogs That Inhibit Protein Synthesis**

After transcription, U1 snRNAs are exported into the cytoplasm where they assemble into U1 snRNPs before migrating back into the nucleus. This remigration requires the presence of a functional Sm binding site, the region recognized by the common U snRNP proteins (Hamm et al., 1990). A U1 mutant unable to bind these proteins will therefore accumulate in the cytoplasm, being unable to move back to the nucleus. Recently, however, it has been shown that in the presence of the cap analog m'G(5')ppp(5')G, such a mutant is localized predominantly in the nucleus since the dinucleotide inhibits the nuclear exit of U1 transcripts (Hamm and Mattaj, 1990). Fig. 1 shows that this inhibition is concentration dependent. Genes encoding a U1 mutant in which the Sm binding site was replaced by an artificial sequence (U1AD) were injected into the nuclei of oocytes. 16 h later (α-32P)GTP was injected either alone or together with mTGpppG. After a further 4-h incubation oocytes were dissected, and RNA from nuclear and cytoplasmic fractions or from total oocytes was analyzed. In control oocytes (Fig. 1, lanes 1–3) U1AD transcripts were present almost exclusively in the cytoplasmic fraction (lane 2). In contrast, in oocytes injected with 50 mM mTGpppG (Fig. 1, lanes 7–9), ~50% of U1AD transcripts were retained in the nucleus (lane 9). The inhibitory effect is concentration dependent, since it was much reduced when a 10 mM solution was injected (Fig. 1, lanes 4–6) and was partial when solutions of intermediate concentration were used (data not shown). Note that injection of the dinucleotide reduces the overall level of transcription by a factor of roughly twofold compared with control oocytes. This effect was observed with all nucleotides tested, independent of their effect on transport (see below), and cannot therefore be the cause of the transport inhibition.

Together with previous results (Hamm and Mattaj, 1990) this suggests that a trans-acting factor which binds to the...
Figure 3. A CBP is present in HeLa nuclear extracts. An electrophoretic mobility retardation assay was carried out using a radioactively labeled RNA probe of 77 nucleotides with either an inverted 7-methyl guanosine cap structure or a triphosphate 5'-end. Yeast RNA or the same 77-nucleotide-long (unlabeled) RNA with a 5'-triphosphate were used as competitors. HeLa nuclear extract (0.3 μl) was added to the RNA mixtures and incubated for 30 min at 25°C. After the incubation period, samples were loaded onto a 6% polyacrylamide gel in TBE buffer. The star indicates the altered mobility of the capped RNA due to a CBP present in the nuclear extract. The filled circles indicate two major shifts due to unidentified RNA binding proteins. (A) Increasing amounts of competitor yeast RNA were tested as indicated above the lanes. (Lanes 1-7) Gel retardation assay using a capped RNA probe; (lanes 8-14) gel retardation assay using an uncapped RNA probe; (lanes 1 and 8) free RNAs. (B) Effect of unlabeled RNA as competitor. HeLa nuclear extract was added to mixtures containing capped or uncapped labeled 77-nucleotide RNA (lanes 1-4 or 5-8, respectively) in the presence of 8 μg of yeast RNA and 0.2 μg (lanes 3 and 7) or 1 μg (lanes 4 and 8) of unlabeled uncapped 77-nucleotide RNA. (Lanes 1 and 5) Free RNAs; (lanes 2 and 6) no unlabeled RNA.

monomethyl cap structure is involved in U1 snRNA export. Several nuclear and cytoplasmic CBPs have been described in extracts of mammalian, plant and yeast cells (see introduction). To investigate if the CBA involved in nuclear transport might be related to the translation initiation factor elF-4E (reviewed by Shatkin, 1985; Rhoads, 1988; Sonenberg, 1988), we tested a series of cap analogs, whose affinity for human elF-4E has previously been determined, for their ability to inhibit nuclear export of U1ΔD transcripts in vivo.

In initial experiments five dinucleotide cap analogs, m^2GpppG, EtGpppG, m^2m^2GpppG, and the mononucleotide m^2GMP, were tested. In oocytes injected only with (α-3^2P)GTP (Fig. 2, lanes 1-3) U1ΔD transcripts were present almost exclusively in the cytoplasmic fraction (Fig. 2, lane 2). In contrast, in oocytes injected either with 50 mM m^2GpppG (Fig. 2, lanes 7-9) or 50 mM EtGpppG (Fig. 2, lanes 10-12), >60% of U1ΔD transcripts were retained in the nucleus. Injection of either m^2GpppG (Fig. 2, lanes 13-15) or m^2m^2GpppG (data not shown) at 50 mM did not affect the cellular distribution of U1ΔD transcripts which accumulate, as in the control, exclusively in the cytoplasm. The dinucleotide GpppG seems to be slightly more efficient as competitor than the dimethyl dinucleotide since in its presence a small fraction of U1ΔD transcripts were retained in the nucleus (Fig. 2, lanes 4-6). It is possible that this inhibitory effect is due to a partial methylation of the dinucleotide after injection. The mononucleotide m^2GMP did not inhibit nuclear export (data not shown).

Identification of a CBP in HeLa Nuclear Extracts

The results described in the above section suggest that the CBA involved in U1 snRNA export is not elF-4E. We therefore decided to search for CBPs whose binding specificity for cap analogs correlated with the ability of the analogs to inhibit RNA transport. A simple assay that has previously been used to detect complexes between RNA and various specific RNA-binding proteins (Leibold and Munroe, 1988; Query et al., 1989; Scherly et al., 1989) including CBPs (Ohno et al., 1990) is native gel electrophoresis. Nuclear or whole cell extracts prepared from different sources were assayed for CBPs using this assay. For the analysis, a 3^2P-labeled RNA 77-nucleotides in length with either a triphosphate or a m^2GpppG 5'-end was incubated with nuclear extract and resulting complexes were separated by nondenaturing polyacrylamide gel electrophoresis. Yeast RNA was included in the reactions to minimize nonspecific interactions between proteins and the probe RNA.

Of the extracts tested, including nuclear extracts from mammalian and frog cultured cells and whole cell extracts from Xenopus oocytes or Saccharomyces cerevisiae, the clearest positive results were obtained using nuclear extracts of HeLa cells, where an abundant specific complex was ob-
obtained with the capped probe (indicated by a star on Fig. 3A, lanes 2–7; see also Ohno et al., 1990). This complex was absent when the uncapped control RNA was used (Fig. 3A, lanes 9–14). Two additional major complexes were observed for both RNAs (indicated by dots) and were competed to different extents by increasing concentrations of yeast RNA. These bands were consistently stronger for the uncapped lanes absent when the uncapped control RNA was used (Fig. 3A, lane 4 in the same conditions as Fig. 3B, lanes 4 and 8. The ability retardation assay was carried out in which the same experiments described in the following sections were carried out. A competitor allowed exclusive detection of the complex due to the CBP in the extract (see lanes 3, 4, 7, and 8). The experiments described in the following sections were carried out in the same conditions as Fig. 3B, lanes 4 and 8. The CBP that gave rise to the retarded complex was also detected in S-100 cytoplasmic extracts, although the specific activity was at least five times lower than in nuclear extracts (data not shown). Whether this cytoplasmic activity reflects the cellular distribution of the protein in vivo or results from nucleic leakage during cell homogenization is not yet established.

The buffer conditions for optimal complex formation were analyzed. The CBP does not require magnesium or ATP for binding and is insensitive to salt concentration up to 1 M KCl. Binding is not inhibited by nonionic detergents such as NP-40, Tween 20, Triton X-100, or digitonin up to 2%. However, ionic detergents such as SDS or sarkosyl inhibited binding at concentrations as low as 0.05% even in the presence of an excess of nonionic detergent. All of the properties of the CBP described thus far were similar to those reported by Ohno et al. (1990), suggesting that we were most likely detecting the protein investigated by those authors (see below).

The Binding Specificity of HeLa Nuclear CBP

The specificity of the CBP observed in HeLa nuclear extracts was investigated by testing different cap analogs for their ability to compete for its binding to a capped RNA probe. Native gel electrophoresis was carried out under conditions where only the CBP was observed (see Fig. 3B, lane 4). Increasing amounts of different cap analogs were added to the reaction mixtures and their effects on cap-dependent complex formation assayed. Fig. 4A shows a competition experiment with the cap analogs m7GpppG (Fig 4A, lanes 3–7) and Et7GpppG (Fig 4A, lanes 10–14). These dinucleotides were able to inhibit 50% of the binding at concentrations between 5 and 10 μM. Dinucleotides methylated in position 2 in addition to position 7 were ~1,000-fold less efficient as competitors than the analogs substituted exclusively in position 7. Fig. 4B shows that the m2′GpppG (Fig 4B, lanes 10–13) and m2′,2″GpppG (Fig 4B, lanes 15–18) were able to inhibit 50% of the shift at concentrations between 2.5 and 5 mM whereas the same inhibition was observed with m7GpppG at 5 μM (Fig 4B, lane 3). The unmethylated dinucleotide GpppG was a more efficient competitor than the di- or tri-methylated dinucleotides since it inhibited complex formation to the same extent at a fivefold lower concentration (Fig. 4B, lanes 5–8). Competition experiments were also performed with mononucleotides methylated at position 7 (Fig. 4C). The mononucleotide di- and tri-phosphates were able to inhibit >50% of the shift at 1 mM concentration (Fig 4C, lanes 10 and 13, respectively) while m7GMP inhibited more weakly and GMP not at all at this concentration (Fig 4C, lanes 7 and 4, respectively).

These results demonstrated that the CBP found in HeLa cell nuclear extract exhibited the same cap analog recognition specificity as the CBA involved in U1 snRNA nuclear export, since the only dinucleotides shown to efficiently inhibit CBP binding and U1 RNA export were m7GpppG and Et7GpppG. Furthermore, the observation that the dinucleotides tested in Fig. 4B and the mononucleotides tested in Fig. 4C were inefficient competitors strengthens the argument that the activity is different from eIF-4E. The concentration dependence for the inhibition of CBP binding in vitro was much lower than for transport inhibition in vivo. However, we have no way to measure the effective in vivo concentration of the injected nucleotides.

Purification of CBP from HeLa Nuclear Extracts

The correlation between the results obtained by competition of CBP binding in vitro and of U1 snRNA nuclear export in vivo with different cap analogs suggest that the CBP present in HeLa nuclear extracts could be involved in nuclear export. To obtain more definitive information on the identity of the CBP, and in particular whether it was identical to the CBP previously described (Ohno et al. 1990), we undertook its purification.

Initial attempts to duplicate the purification procedure of Ohno et al. (1990) resulted in disappointingly low recovery efficiencies, and the initial two chromatographic steps of their procedure were therefore changed as follows. The CBP was purified from HeLa nuclear extracts by column chromatography using sequentially Phenyl-Sepharose, Sephacryl S-300 and affinity chromatography (for details, see Materials and Methods). The activity was monitored by the native gel electrophoresis assay described above. Several experiments showed that ~90% of the detectable CBP eluted in the 1 M NaCl elution step of the Phenyl-Sepharose column. Next, this fraction was loaded onto a Sephacryl S-300 column after concentration by ammonium sulfate precipitation. At high salt, about ~60% of the input CBP eluted from the Sephacryl column between the 100- and 66-kD molecular mass markers, corresponding to Calf Intestine Alkaline Phosphatase and BSA, respectively (data not shown). Additional activity was found consistently between the 466- (Ferritin) and 100-kD molecular mass markers, but these fractions were discarded. Note that at salt concentrations below 1 M NaCl the CBP was found predominantly in the exclusion volume of the Sephacryl column. The nature of the high molecular mass complexes formed at lower salt concentration was not investigated further.

Fig. 5A shows the pattern of proteins in the active fractions eluted from the Phenyl Sepharose and Sephacryl columns (lanes 3 and 4, respectively) compared to proteins from nuclear extract (Fig. 5A, lane 2). In parallel, quantitation of specific cap binding after the two first steps of purification was performed. Fig. 5B shows an assay of the active fractions obtained after Phenyl Sepharose or Sephacryl columns (Fig. 5B, lanes 3 and 4, respectively) compared with the total extract (Fig. 5B, lane 2). The CBP-containing fractions eluted from the Sephacryl column were dialyzed to reduce the salt concentration, concentrated, and loaded onto an affinity resin made by coupling
Figure 4. Inhibition of cap-binding by different analogs. A capped RNA probe was incubated with 0.3 μl of HeLa nuclear extract in the presence of 8 μg of yeast RNA and 1 μg of unlabeled uncapped 77-nucleotide RNA as competitors. Increasing amounts of different cap analogs were added to the reaction mixtures which were processed as described in Fig. 3. Final concentrations of cap analogs in the binding reactions are indicated above the lanes. Lane 1 in all panels shows the free RNA. The star indicates the altered mobility of the capped RNA due to the CBP. (A) Competition with the dinucleotides mTGpppG (lanes 3-7) and EtTGpppG (lanes 10-14). (Lanes 2, 8, and 9) No dinucleotides. (B) Competition with the dinucleotides mTGpppG (lane 3), GpppG (lanes 5-8), m2TGpppG (lanes 10-13) and m2,7TGpppG (lanes 15-18). (Lanes 2, 4, 9, and 14) No dinucleotide. (C) Competition with the following mononucleotides: GMP (lanes 3 and 4), mTGMP (lanes 6 and 7), mTGDP (lanes 9 and 10), and mTGTP (lanes 12 and 13). (Lanes 2, 5, 8, and 11) No nucleotide.

The capped 77-nucleotide RNA used for the previous assays to a Sepharose support. After extensive washes, proteins bound to the column were eluted with 0.2% SDS. After electrophoresis in SDS–polyacrylamide gels and silver staining a major 80-kD band was observed in the SDS eluted fractions (indicated by a filled circle in Fig. 5 A, lane 5). This band was absent when an affinity column made with uncapped 77-nucleotide-long RNA was used (data not shown). Quantitation of the activity eluted from the affinity column was not possible since after SDS treatment the protein could not be renatured. However, by comparing the binding activity present in the input, flowthrough, and wash fractions, we calculated that ~80% of the CBP bound to the affinity column. Assuming that the CBP is efficiently eluted by SDS-containing buffer, we can estimate that the protein was purified at least 4,000-fold. Elution of the affinity column with urea (4 M) or salt (4 M NaCl) allowed recovery of the binding activity. However, neither of these methods gave efficient elution and the activity spread over several column volumes. Fig. 5 C shows the elution profile of the affinity column when 4 M urea was used for elution. The activity was present in several fractions (Fig. 5 C, lanes 4 to 10) that represented 4 column volumes. The sample in Fig. 5 C, lane 2 is equivalent to that in Fig. 5 B, lane 4. The active fractions were pooled and an amount equivalent to a single fraction was compared with the fraction in which the activity was absent (shown in Fig. 5 C, lane 3) by SDS-PAGE (Fig. 5 D, lanes 1 and 2, respectively). Activity in the native gel electrophoresis assay correlated with the presence of the 80-kD band on a protein gel (Fig. 5 D, lane 1). This protein was absent in the fraction which was inactive in complex formation (Fig. 5 D, lane 2). Proteins observed between the 45-
and 66-kD molecular mass markers were present in all fractions and their presence did not correlate with CBP activity. Together, the above results strongly indicate that the CBP in HeLa nuclear extract is the 80-kD protein. The apparent molecular mass of the purified protein is identical to that of the CBP purified by Ohno et al. (1990), confirming that the two proteins are almost certainly the same.

**Discussion**

We have described the purification of a CBP from HeLa nuclear extracts. The specificity of this protein for cap analogs is different from that of the best characterized cap binding protein, the translational initiation factor eIF-4E. Equilibrium binding constants for complexes of eIF-4E with N-7 alkyl-substituted cap analogs have been determined and vary over a roughly 10-fold range as follows: m'GTP \( \approx \) m'GpppG > m'GMP > m'GpppG > m'GMP > EtGpppG > m'GpppG (Carberry et al., 1989, 1990). The CBP described in this study binds efficiently only to m'GpppG and EtGpppG. The affinity for other dinucleotides is at least 1,000-fold lower. In addition, while eIF-4E binds to N-7 substituted guanosine or diguanosine triphosphate with similar equilibrium binding constants, the nuclear CBP binds to m'GpppG at least 100-fold more efficiently than to m'GTP. These results suggest that the nuclear CBP is not eIF-4E.

We have also shown that the relative affinity of cap analogs for the HeLa CBP correlates with their efficacy as inhibitors of the nuclear export of U1 snRNA in Xenopus oocytes, since the only nucleotides which were able to inhibit U1 snRNA export were m'GpppG and EtGpppG. In summary, our results demonstrate that the CBA involved in RNA export and the nuclear CBP exhibit the same binding specificity for the cap analogs tested, but more direct biochemical evidence is required to show that the same CBP is also present in the nuclei of Xenopus oocytes.

In addition to eIF-4E, several other CBPs have been described. Three, with apparent molecular masses of 120, 89, and 80 kD have been identified by photoaffinity labeling in HeLa nuclear extracts (Patzelt et al., 1983). Rozen and Sonenberg (1987) identified two additional nuclear CBPs of 115 and 20 kD by UV cross-linking. Finally, a CBP of 80 kD has been detected in HeLa nuclear extracts by means of an electrophoretic mobility shift assay (Ohno et al., 1990). Based on most of the reported properties of this protein, e.g., the apparent molecular mass, the resistance of binding to high salt and to competition with GpppG and m'GpppG, it seems likely that it is the same as the protein analyzed here, although we saw similar inhibition of binding in competition experiments with either m'GDP or m'GTP (Fig. 4 C) while the complexes observed by Ohno et al. (1990) were insensitive to competition with m'GDP.

In microinjection experiments in Xenopus oocytes, it has been shown that the cap structure of a pre-mRNA can have an effect on both the efficiency of splicing of an intron in the pre-mRNA and on the selection of cap-proximal versus cap-distal introns (Inoue et al., 1989). Furthermore, earlier work in which cap analogues were used as inhibitors of in vitro splicing (Konarska et al., 1984; Edery and Sonenberg, 1985) or of splicing complex formation (Patzelt et al., 1987) had...
suggested the existence of a CBP which had a role in pre-mRNA processing. On this basis, but without more direct evidence, Ohno et al., (1990) proposed that the 80-kD CBP which they identified might be involved in splicing. The results presented in this paper suggest a different possibility, that the 80-kD CBP may play a role in the export of capped RNAs from the nucleus. It is of course possible that the protein might be bifunctional. In addition, the other nuclear CBPs mentioned above might have roles in either splicing or RNA transport. Moreover, it may be that, while both RNA polymerase II-transcribed U snRNAs and mRNAs require cap structures for export from the nucleus (Hamm and Mattaj, 1990), that the CBP mediating export of these two classes of RNA could be different.

It is likely that transport out of the nucleus will involve RNPs rather than naked RNAs. The very large Balbiani ring mRNAs, made in Chironomus tentans salivary glands, have been convincingly shown to be associated with proteins on both sides of the nuclear membrane during export (Mehlin et al., 1991). While our data (Hamm and Mattaj, 1990; this study) show that cap recognition by a CBA is required for export, they do not rule out the possibility that other RNA binding proteins might also be required for export of either specific pol II RNAs or of RNAs in general.

Recently, suggestive evidence for a role of the hnRNP A1 protein in mRNA export has been provided (Piñol-Roma and Dreyfuss, 1992). mRNA precursors associate in the nucleus with the abundant hnRNP proteins to form hnRNPs (reviewed by Dreyfuss, 1986). Until recently, it was believed that these proteins dissociate from the RNA before or during transport, but the new data suggest that this may not be the case for all the hnRNP proteins. In particular, it was shown that hnRNP A1 protein shuttles between the nucleus and cytoplasm during interphase. Cross-linking experiments demonstrated that the cytoplasmic pool of A1 protein was associated with poly (A)^+ RNA (Piñol-Roma and Dreyfuss, 1992). This suggests that mRNA may be exported from the nucleus in association with the A1 protein, and potentially also other shuttling hnRNPs.

Proteins involved in the export of other specific RNAs have been described. Nuclear export of 5S RNA is probably mediated by either the L5 ribosomal protein, TFIIIA, or another protein with similar RNA binding specificity, since mutated 5S RNAs unable to interact with these proteins remain in the nucleus (Guddat et al., 1990). The influenza virus matrix protein M1 is required for nuclear export of viral RNPs (Martin and Helenius, 1991). In the absence of M1, the RNPs accumulate in the nucleus. Finally, the Human Immunodeficiency Virus (HIV) Rev protein is required for accumulation of unspliced late viral RNAs in the cytoplasm (reviewed by Chang and Sharp, 1990) and the suggestion has been made that it might directly stimulate the nuclear export of these RNAs. However, evidence has been presented that the mode of action of this protein is to dislodge viral RNAs from spliceosome components which anchor them to the nucleus, rather than to stimulate export directly (Chang and Sharp, 1989).

Definite proof that any of the above-mentioned proteins has a role in mRNA export is lacking and, as yet, there is no indication of how any protein might mediate RNA export at the molecular level. Our results suggest that the CBP examined here may form an essential part of RNP export sub-

strates formed on U snRNAs and possibly other pol II-transcribed RNAs, the first such component to be identified. Further characterization of the nuclear CBP will allow us to establish its role more definitively.

We wish to thank Caroline McGuigan for technical assistance, Jordi Ber-
nues, Matthias Hentze, Eduard Hurt, Angus Lamond, Bertrand Séraphin, Kenneth Simmen, and David Tollervey for comments on the manuscript and Maryka Kimmins for secretarial help.

E. Izaurralde is a recipient of a fellowship from the Swiss National Science Foundation. E. Darzynkiewicz and J. Stepinski were supported by the Polish Committee for Scientific Research, Project Nr. 4 0800 91 01.

Received for publication 20 March 1992 and in revised form 4 June 1992.

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