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Identification of nuclear cap specific proteins in HeLa cells

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ABSTRACT

Two polypeptides of apparent molecular mass of 20 and 115 kilodaltons in nuclear fractions from HeLa cells were shown to recognize and be crosslinked to the cap structure of eukaryotic mRNAs in a cap-dependent fashion. Crosslinking of the 20 and 115 kDa polypeptides was sensitive to inhibition by low concentrations of the cap analogue m^7GDP and resistant to inhibition by high KCl concentrations. In addition, crosslinking of these polypeptides to the cap structure occurred in nuclear extracts prepared from poliovirus-infected cells, under conditions where cytoplasmic cap binding proteins were incapable of interacting with the mRNA cap structure. The possible function of nuclear cap binding proteins is discussed.

INTRODUCTION

All eukaryotic cellular mRNAs analyzed to date (except for organelle mRNAs) are blocked at their 5' end by the cap structure $m^7G(5')ppp(5')N$ (where N is any nucleotide: refs. 1,2). It has been shown that the cap structure enhances translational efficiency by facilitating ribosome binding to mRNA (1,2) and protects the mRNA against 5' exonucleolytic degradation (3). More recent studies suggest that the cap structure plays a role in pre-mRNA splicing (4,5) and 3' end processing (6,7). It was shown that capped pre-mRNA transcripts were spliced more efficiently than uncapped transcripts (4,5). In addition, the latter studies demonstrated that cap analogues inhibited splicing and 3' end processing *in vitro* whereas the unmethylated counterparts had no effect.

Cap function in translation is mediated by a distinct set of proteins capable of interacting with the cap structure (for a recent review see ref. 8). Three polypeptides in initiation factor (IF) preparations from several species were shown to interact specifically with the cap structure of mRNA by using chemical crosslinking to $[^3H]$-cap labeled oxidized reovirus mRNA . Of these proteins, a 24 kDa polypeptide (termed 24K-CBP, CBP I or eIF-4E) crosslinks specifically to the 5' cap of mRNA in an ATP-Mg^{2+} independent manner whereas crosslinking of the 50 (eIF-4A) and 80 kDa (eIF-4B) polypeptides is ATP-Mg^{2+} dependent (8,9). More recently crosslinking of the 24K-CBP and eIF-4B was reported using a photochemical crosslinking technique (10). eIF-4E can be

isolated by affinity chromatography as a homogeneous protein or in association with two other polypeptides having molecular masses of 50- and 220 kDa and termed CBP II, eIF-4F or CBP complex (11-13); the 50 kDa polypeptide was shown to be very similar to a previously characterized initiation factor eIF-4A (12,13). The role of the 220 kDa polypeptide is still unknown (8).

If cap function in pre-mRNA splicing or other events in the nucleus is mediated by cap interacting proteins, they should be localized in the nucleus and detectable by similar methods previously used to identify cytoplasmic cap binding proteins. One such study used a photoreactive cap analogue to identify three polypeptides in nuclear fractions having molecular masses of 80-, 89- and 120 kDa (14). It is possible however, that additional signals, apart from the cap structure, exist on the mRNA and are required for CBP binding and therefore missed detection in this study. In addition, the nuclear fractions used in the latter study are not those commonly used in studies of nuclear activities such as splicing and 3' end processing, and might not contain one or more of the putative CBPs required for these events (4-7). Consequently we chose to use a ^{32}P-cap labeled mRNA to identify proteins capable of interacting with the cap structure in nuclear extracts using UV light-induced crosslinking.

Here, we identify two polypeptides of molecular masses of 20 and 115 kDa in HeLa nuclear extracts, which crosslink in a cap specific fashion to the cap structure. The results demonstrate that the 20 and 115 kDa polypeptides are different in several respects from the 24, 50 and 80 kDa cytoplasmic cap binding proteins previously characterized (8).

MATERIALS & METHODS

Materials: RNase A and S-adenosyl-L-methionine were purchased from Boehringer Mannheim Biochemicals. RNase-free DNase I and vaccinia virus guanylyltransferase were from Bethesda Research Laboratories, Inc. Human placenta RNase inhibitor was from Promega Biotec. GDP and 7-methyl-GDP (m^7GDP) were purchased from P-L Biochemicals, Inc. [5-^{3}H] CTP (23.7 Ci/mmol), [α-^{32}P] GTP (> 3,000 Ci/mmol), and SP6 RNA polymerase were obtained from New England Nuclear Corp.

Cell extracts: HeLa S3 cells were grown in suspension in 10% calf serum at 5 x 10^5 cells/ml. Nuclear extracts were prepared as described by Dignam *et al.* (15). Preparation of post-ribosomal supernatant (S-100), post mitochondrial supernatant (S-10) and high salt wash of ribosomes was according to Lee and Sonenberg (16). Poliovirus type 1 (Mahoney strain) infection of HeLa cells was as described by Lee and Sonenberg (16), and cell extracts were prepared 3h after infection.

In vitro transcriptions and capping reactions: The construction of pMc-*myc*/X was described (17). Uncapped transcripts were synthesized from Eco RI-linearized DNA templates according to Pelletier and Sonenberg (18), and the level of RNA

synthesis was determined by [5-^{3}H] CTP incorporation into RNA. Capping reactions with α-^{32}P-GTP using vaccinia guanylyl transferase were performed as described by Pelletier and Sonenberg (10).

Photochemical Crosslinking of mRNA to Proteins: Photochemical crosslinking reactions were performed as described by Pelletier and Sonenberg (10) with minor changes. 2 x 10^4cpm of ^{32}P-mRNA (cap labeled; ~ 6-15 x 10^5 cpm/μg) was incubated with 70-140 μg protein of nuclear extract (5-10 mg/ml protein) in 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, buffer containing 0.5 mM $Mg(OAc)_2$, 1 mM ATP, 2 mM DTT and 3% glycerol at 30°C for 10 min in a volume indicated in the figures. Reaction mixtures were irradiated at 254 nm at 4°C at a distance of 4 cm with a 15-W General Electric G15T8 germicidal lamp for 1 h. The samples were digested for 30 min. at 37°C with 20 μg of RNase A, boiled for 5 min. in electrophoresis sample buffer, and subjected to electrophoresis on 10 to 18 % gradient SDS-polyacrylamide gels, followed by autoradiography. Quantitation of labeled bands was performed by scanning autoradiograms in the linear range with a soft laser scanning densitometer (LKB).

RESULTS

We employed the UV light induced crosslinking technique to identify proteins in the nucleus that are capable of specifically interacting with the cap structure. HeLa nuclear extracts capable of performing cap-dependent nuclear functions, such as splicing were used. ^{32}P-capped labeled transcripts were synthesized from two cloned cDNAs (mouse c-myc and human β globin) inserted into the pSP64 vector. Both transcripts yielded similar crosslinking patterns and therefore only those obtained by using the c-myc transcripts will be shown here.

HeLa cells were fractionated into nuclear and cytoplasmic fractions, and equal cell equivalents were UV crosslinked to cap labeled ^{32}P-mRNA. The crosslinking pattern is shown in Figure 1: several polypeptides in the nuclear extract became crosslinked to the cap structure (lane 1), but only three polypeptides of molecular masses of 20-, 80-, and 115 kDa, became crosslinked in a cap specific manner as assessed by the inhibition of their crosslinking by the addition of 300 μM of the cap analogue m^{7}GDP (compare lane 2 to 1). Addition of GDP had no effect on the crosslinking pattern (data not shown: see fig. 3). The crosslinking of the 80 kDa polypeptide was not detected in most nuclear extract preparations (Figs. 2-5), consistent with it being a contaminating cytoplasmic CBP, most probably eIF-4B (see below). Crosslinking was also performed with other cellular fractions: the post-nuclear fraction was further fractionated into a ribosomal and post-ribosomal (S-100) fraction. Some of the 80 kDa was also detected in the S-100 fraction (lane 3), but most of the 80 kDa polypeptide was confined to the high salt wash fraction of ribosomes (lane 5). The

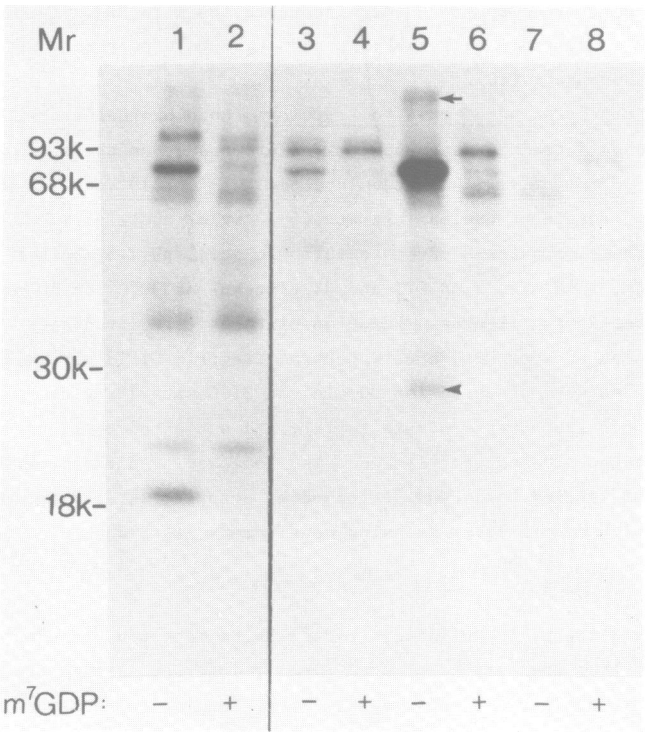

Fig. 1. Subcellular localization of CBPs from HeLa cells. [^{32}P] c-myc mRNA was incubated with fractions prepared from ≈ 2 x 10^6 cells in a total volume of 30 µl, followed by UV light irradiation as described in Materials and Methods. Samples were resolved on an SDS-polyacrylamide gel that was dried and exposed against XAR-5 film (Kodak) at -70°C for 24 h. Lanes 1 and 2, nuclear extract; lanes 3 and 4, S-100; lanes 5 and 6, ribosomal high salt wash; lanes 7 and 8, washed ribosomal pellet. m^7GDP (0.3 mM) was added where indicated in the figure.

characterization of the 80 kDa polypeptide as the major ATP-Mg^{2+} dependent crosslinkable polypeptide in the ribosomal high salt wash fractions was described before (10), and it was identified as eIF-4B (S. Milburn *et al*, manuscript in preparation). The 80 kDa polypeptide sometimes found in the nuclear extract did not become crosslinked in the absence of ATP- Mg^{2+} or at higher salt concentrations ($\succ$ 150 mM KCl), consistent with it being eIF-4B (data not shown). In addition to eIF-4B there are several other polypeptides in the ribosomal high salt wash fraction that became crosslinked less extensively to mRNA in a cap-specific manner: the 24K-CBP (eIF-4E, indicated by an arrowhead), and a high molecular weight polypeptide (~ 200 kDa, indicated by an arrow) that might correspond to the p220 large subunit of the eIF-4F. The washed ribosomes

contained a weakly crosslinked polypeptide of ~ 60 kDa, whose identity is not known (compare lane 8 to 7). Thus, the results in Fig. 1 indicate that two polypeptides of 20 and 115 kDa are confined primarily to the nuclear extract, and that the 80 kDa polypeptide in this fraction is most probably the cytoplasmic eIF-4B that cofractionated with the nucleus. It should be emphasized that the crosslinking assay measures activity and not amount of protein. Thus, it cannot be excluded that the 20- and 115 kDa CBPs might be present in the cytoplasm but are not active for some reason.

To ascertain that crosslinking of the 20 and 115 kDa nuclear CBPs occurred via the m^7G group as opposed to adjacent bases downstream from the cap structure, we performed the following experiment. Following UV irradiation, samples were digested with tobacco acid pyrophosphatase; this treatment yielded enhanced crosslinking as compared to that obtained without enzyme treatment (data not shown). Since tobacco acid pyrophosphatase cleaves only the pyrophosphate bond in the cap structure, these results indicate that the crosslinking of the 20 and 115 kDa occurs to the m^7G residue. Consequently, we will refer to these polypeptides as nuclear CBPs.

The cytoplasmic cap binding proteins are required for initiation of translation and their interaction with the cap structure is interdicted in fractions from poliovirus-infected cells (for recent reviews see refs. 19,20). Thus, crosslinking of the 24-, 50- and 80 kDa cytoplasmic CBPs is dramatically reduced when examined in fractions from poliovirus-infected cells (10,16). To determine whether the 20 and 115 kDa nuclear CBPs are capable of interacting with the cap structure following poliovirus infection, we performed the UV crosslinking reaction in extracts prepared from poliovirus-infected cells. Infected and mock-infected HeLa cells were fractionated into a nuclear extract, post nuclear supernatant, and S-10 extract. The crosslinking pattern of each fraction is illustrated in Figure 2. The post nuclear supernatant and S-10 fractions from mock-infected cells showed specific crosslinking of the 80 kDa CBP (eIF-4B; compare lanes 1 and 5 to 2 and 6, respectively: crosslinking of the 24k-CBP is not seen on this exposure), whereas the corresponding infected fractions showed a significant reduction in crosslinking of eIF-4B (the 80 kDa polypeptide), in accordance with previous results (refs. 10, 16: lanes 3 and 7). In contrast, the crosslinking profile of the infected and mock-infected nuclear extract was identical: the 20 and 115 kDa nuclear CBPs became crosslinked with equal intensity in both extracts (lanes 9 and 11). Note that this nuclear extract was apparently not contaminated by eIF-4B, since crosslinking of this polypeptide was not detected. These results are consistent with the notion that the cap-specific 20- and 115 kDa polypeptides are functionally different from the 24-, 50- and 80 kDa CBPs, which are inhibited from interaction with the cap

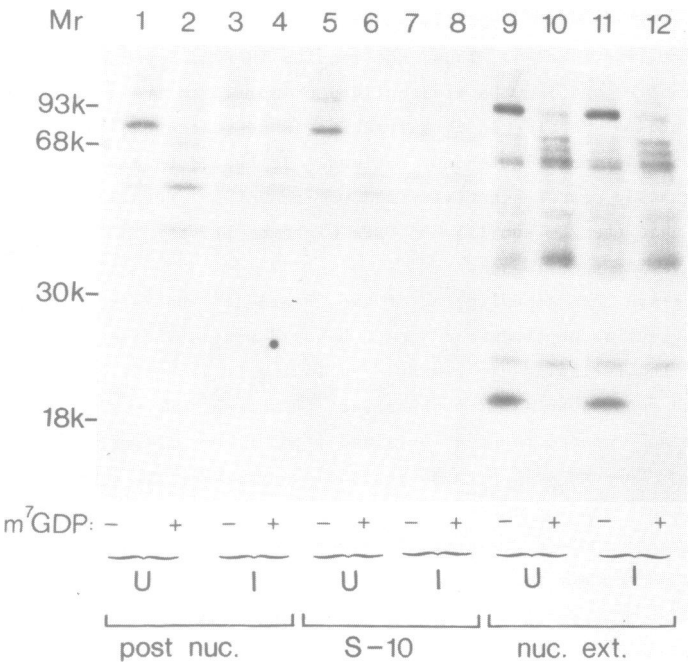

Fig. 2. Effect of poliovirus infection on UV light induced crosslinking of CBPs to mRNA. [^{32}P] c-myc mRNA was incubated with ≈ 4 x 10^6 HeLa cell equivalents in a total volume of 30 µl, followed by UV light irradiation and SDS-polyacrylamide gel analysis of the crosslinked products as described in Materials and Methods. U-mock-infected fractions; I-poliovirus-infected fractions. Post nuc.- post nuclear fraction. Nuc. ext.- nuclear extract. m^7GDP (0.3mM) was present where indicated in the figure.

structure in extracts from poliovirus-infected cells (10,16).

To further characterize the affinity of the nuclear CBPs to the mRNA cap structure we titrated the cap analogue m^7GDP to determine the concentration that is required to inhibit the crosslinking reaction. Fig. 3 shows that 50% inhibition of crosslinking of the 20 and 115 kDa nuclear CBPs protein was achieved with 10 µM m^7GDP (lane 4). At 300 µM of m^7GDP crosslinking of the nuclear CBPs was completely inhibited (lane 6), whereas GDP at the same concentration had no effect, whatsoever (lane 7).

An important aspect of cap recognition by cytoplasmic CBPs, is the requirement of ATP-Mg^{2+} for crosslinking of eIF-4A and eIF-4B to the cap structure (8,13,21). The effect of ATP-Mg^{2+} on the crosslinking of the 20 and 115 kDa nuclear CBPs is shown in Fig. 4. ATP had no significant effect on the extent of crosslinking of the 20 kDa nuclear CBP either in the presence or absence of Mg^{2+} (compare lane 1 with lanes 3, 5 or 7, note that crosslinking of

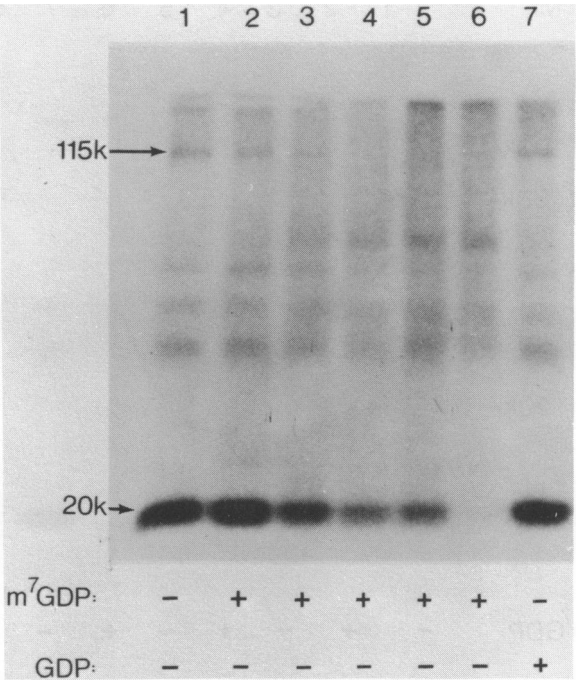

Fig. 3. Effect of m^7GDP on photochemical crosslinking of CBPs to mRNA. [^{32}P] c-myc mRNA was incubated with 14 µl (140 µg) of nuclear extract in a total volume of 30 µl, followed by UV light irradiation and SDS-polyacrylamide gel analysis of the crosslinked products as described in Materials and Methods. The amounts of m^7GDP added were as follows. Lanes 1 to 6, 0, 1, 5, 10, 20 and 300 µM m^7GDP, respectively. Lane 7, 0.3 mM GDP.

the 20 kDa nuclear CBP is reduced by ~2 fold in the presence of Mg^{2+}). However, ATP had a stimulatory effect on the crosslinking of the 115 kDa nuclear CBP. In the absence of Mg^{2+}, ATP slightly stimulated (1.5 fold) the crosslinking of the 115 kDa nuclear CBP (compare lane 3 to 1). ATP exhibited a stronger stimulatory effect (3 fold) on the 115 kDa polypeptide crosslinking when Mg^{2+} was present (compare lane 7 to 5). Additional experiments showed, however that the stimulatory effect is non-specific because several other nucleotides including CTP, UTP, dATP, AMP and ADP or even adenosine could stimulate the crosslinking of the 115 kDa polypeptide (data not shown). We have no explanation for this phenomenon but we think that the addition of nucleotides or nucleosides might somehow stabilize the interaction between the mRNA and the 115 kDa polypeptide. To examine the possibility that the endogenous ATP levels are sufficient to promote an ATP-dependent crosslinking reaction, we have performed this reaction in nuclear extracts that were depleted of ATP by hexokinase treatment. We found

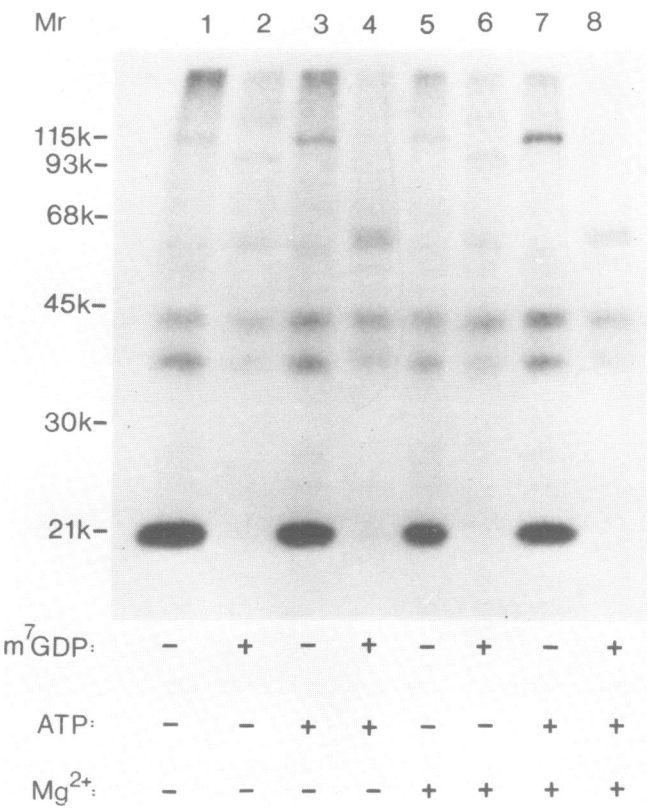

Fig. 4. Effects of ATP and Mg^{2+} on UV light-induced crosslinking of CBPs to mRNA. [^{32}P] c-myc mRNA was incubated with 14 µl (140 µg) of nuclear extract in a total volume of 30 µl, followed by UV light irradiation as described in Materials and Methods. Samples were resolved on an SDS-polyacrylamide gel that was dried and exposed against Fuji X-ray film for 18 h at -70°C. Reaction mixtures included 0.3 mM m^7GDP, 1 mM ATP and 0.5 mM $Mg(OAc)_2$ where indicated in the figure.

that the crosslinking profile in these extracts was similar to that of untreated extracts, indicating that endogenous ATP is not required for the interaction of the 20- and 115 kDa nuclear CBPs with the cap structure (data not shown). Thus, we conclude that energy derived from ATP hydrolysis is not required for the crosslinking of the 20- and 115 kDa polypeptides.

We have also studied the salt requirements for the crosslinking of the 20 and 115 kDa polypeptides. The crosslinking pattern as a function of KCl concentrations from 250 to 1550 mM is shown in Fig. 5. Varying the KCl concentrations from 50 to 250 mM in the crosslinking reactions had no effect on

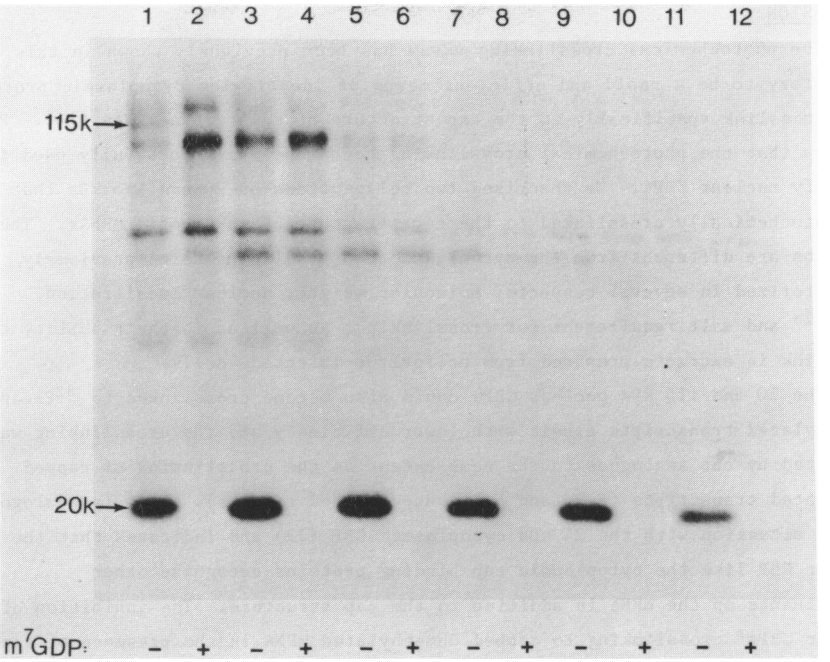

Fig. 5. Effect of K^+ concentration on UV light-induced crosslinking of CBPs to mRNA. [^{32}P] c-myc mRNA was incubated with 10 µl (70 µg) of nuclear extract in a total volume of 30 µl, followed by UV irradiation and SDS-polyacrylamide gel analysis of the crosslinked products as described in Materials and Methods. The amounts of KOAc in each reaction mixture were as follows: Lanes 1 and 2, 250 mM; lanes 3 and 4, 550 mM; lanes 5 and 6, 750 mM; lanes 7 and 8, 1050 mM; lanes 9 and 10, 1250 mM; lanes 11 and 12, 1550 mM. m^7GDP (0.3 mM) was added as indicated in the figure.

the extent of crosslinking of the 115 and 20 kDa CBPs (data not shown). Increasing the KCl concentration from 250 to 550 mM abolished the crosslinking of the 115 kDa nuclear CBP, but the extent of crosslinking of the 20 kDa was augmented two fold (compare lane 3 to 1). Increasing the concentration of KCl up to 1250 mM had no significant effect on the crosslinking (compare lanes 5, 7 and 9 to lane 3). Crosslinking efficiency was decreased by ~ 70% only when KCl concentration was increased to 1550 mM (compare lane 11 to lane 9). Thus, we conclude that the interaction between the 20 kDa nuclear CBP and the mRNA cap structure is of very high affinity since it can occur at KCl concentrations higher than 1 M. The interaction of the 115 kDa nuclear CBP with the cap structure is less refractory to inhibition by high KCl concentrations, than the 20 kDa nuclear CBP.

DISCUSSION

The photochemical crosslinking assay has been previously shown in this laboratory to be a rapid and efficient means of identifying cytoplasmic proteins that crosslink specifically to the cap structure of eukaryotic mRNAs (10). Here we show that the photochemical crosslinking technique was successfully used to identify nuclear CBPs. We described two polypeptides of 20 and 115 kDa that can be photochemically crosslinked to the cap structure of eukaryotic mRNAs. These proteins are different from the cytoplasmic cap binding proteins previously characterized in several respects: molecular weight, nuclear localization, ATP-Mg^{2+} and salt requirement for crosslinking, as well as in their ability to crosslink in extracts prepared from poliovirus-infected cells.

The 20 and 115 kDa nuclear CBPs could also become crosslinked to ^{32}P-capped unmethylated transcripts albeit with lower efficiency and the crosslinking was inhibited by cap analogues to the same extent as the crosslinking of capped methylated transcripts (F.R. and N.S., unpublished results). This is analogous to the situation with the 24 kDa cytoplasmic CBP (22) and indicates that the nuclear CBP like the cytoplasmic cap binding proteins recognize other determinants on the mRNA in addition to the cap structure. The inhibition of nuclear CBPs' crosslinking to capped unmethylated mRNA in the presence of cap analogues suggest that the proteins have a single site available for crosslinking and this is the cap binding site.

It was previously shown that all of the cytoplasmic CBPs that interact with mRNA are incapable of crosslinking to the mRNA cap structure in poliovirus-infected cell extracts (16). This is a result of the inactivation of the CBP complex and since the interactions of the other CBPs (eIF-4A and eIF-4B) with the mRNA is dependent on prior binding of the CBP complex, they can not associate with the mRNA (19,20). Consequently, the resistance of the 20 and 115 kDa nuclear CBPs to inactivation by poliovirus-infection is consistent with their preferential nuclear localization and suggest that they are not involved in mRNA translation. It is also of interest that a nuclear function such as splicing is not impaired in poliovirus-infected cells (I. Edery, unpublished observations).

The nuclear localization of CBPs is also consistent with the proposed function of the cap structure in nuclear events such as mRNA splicing (4, 5) and 3' end processing (6,7). In this respect, it is pertinent that splicing of pre-mRNA in *in vitro* systems was inhibited by very low concentrations of cap analogues (~ 90% inhibition by 10 μM m^7GpppG ref. 4 and 50% inhibition by 1 μM m^7GDP, ref. 5), whereas the concentration of cap analogues required to inhibit translation to the same degree is at least one order of magnitude higher (inhibition of translation varies considerably depending on the cell extract, mRNA, temperature and salt concentration, refs. 1,8, but even in the wheat-germ

extract where translation is more susceptible to inhibition by cap analogues 50% inhibition was achieved with ~ 20 μM m^7GpppAm in the study by Lodish and Rose, ref. 23). Furthermore, low concentrations of m^7GDP (10 μM) caused a 50% reduction in crosslinking of the 20 and 115 kDa polypeptides. The strong inhibition of crosslinking by low concentrations of a cap analogue suggest either that very small amounts of these proteins are present in the nucleus, or that the interaction between these proteins and the cap structure is very strong, these possibilities not being mutually exclusive. The latter possibility is also consistent with the ability of the 20 kDa polypeptide (and to a lesser extent the 115 kDa polypeptide) to crosslink at high salt concentrations (as high as 1.55 M KCl for the 20 kDa nuclear CBP, Fig. 5). We have no results to indicate whether the nuclear CBPs are indeed involved in splicing or polyadenylation and that these proteins might be involved in other nuclear functions such as nuclear-cytoplasmic transport or mRNA capping is a valid possibility. Futhermore, it is also important to emphasize the possibility that the 20 and 115 kDa nuclear CBPs recognize in the cell the trimethylated caps of snRNAs and not pre-mRNAs or mature mRNAs.

Patzelt et al. (14) identified three CBPs associated with the nucleus with molecular masses of 80, 89 and 120 kDa. The two higher molecular weight CBPs could be released from the nuclear matrix by a combination of DNase and RNase digestion and detergent extraction, whereas the 80 kDa nuclear CBP remained entirely in the nuclear matrix fraction. It is conceivable that the 120 kDa nuclear CBP identified by Patzelt et al. corresponds to the 115 kDa described here. However, Patzelt et al. did not observe the crosslinking of the 20 kDa polypeptide in their nuclear fractions, and we have not observed the crosslinking of the 80 and 89 kDa CBPs. It is unlikely that the 80 kDa polypeptide they identified is identical to eIF-4B that contaminated some of our nuclear extracts, because they did not detect the crosslinking of eIF-4B in their IF preparation, where it is most abundant. The reasons for the different results could be several fold. a) We have used mRNA for crosslinking rather than a cap analogue used by Patzelt et al. Consequently, it is possible that crosslinking of the 20 kDa nuclear CBP requires an mRNA structure in addition to the cap structure. It is also possible that binding of the 20 kDa CBP to the mRNA is a secondary event (as is crosslinking of the cytoplasmic eIF-4A and eIF-4B), mediated by another CBP that binds directly to the cap structure. b) The method of preparation of nuclear fractions was different in the two studies; we have used nuclear extracts that possess transcriptional and post-transcriptional activity (i.e. splicing, and 3' end processing), but did not contain the nuclear matrix, whereas Patzelt et al. extracted the nucleus by a combination of DNase, RNase and detergent treatment and thus enriched their fraction for nuclear matrix components. In

spite of this treatment, one of their nuclear CBPs (80 kDa) was fractionated with the insoluble nuclear matrix. Therefore, this polypeptide would not be expected to be present in the nuclear extract and thus not be available for crosslinking in our assay.

Purification of the 20- and 115 kDa nuclear CBPs should aid in elucidation of their function.

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