Proteins associated with rabbit reticulocyte mRNA caps during translation as investigated by photocrosslinking

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ABSTRACT

This laboratory previously detected by UV crosslinking a number of proteins associated with cytoplasmic mRNA in mammalian cells, and the data suggested that they are involved in translation. To find out which proteins are associated with caps we made use of reticulocyte mRNA specifically labeled in the cap with ³²P together with a cell-free translation system and UV crosslinking. Approximately 8 bands corresponding to proteins crosslinked to the cap itself have been detected by polyacrylamide gel electrophoresis after UV crosslinking and digestion with RNases or tobacco pyrophosphatase. All but one were specific for methylated caps. One was similar in size and partial peptide map to a cap-binding protein, CBP I, previously identified in other laboratories, and most of the others corresponded to proteins previously known to be associated with mRNA but not known to be associated with caps. The results suggest that most mRNA-associated proteins are associated with caps or poly(A). Also, the number of cap-associated proteins may be greater than previously suspected.

INTRODUCTION

Most mRNAs in eukaryotes have post-transcriptionally added caps at their 5' termini, and caps function in initiation of translation (reviewed in refs. 1,2). It is reasonable to suppose that the role of caps in translation is mediated by proteins that bind to them, and considerable effort has been devoted to identifying such proteins, especially in the laboratories of Sonenberg, Shatkin, and Merrick (3-8). Previous attempts to identify cap-binding proteins made use of chemical crosslinking to periodate-oxidized caps. This procedure relied on competitive inhibition of crosslinking by cap analogs to distinguish specific cap-binding proteins from ones non-specifically crosslinked to caps, and it has been especially useful for detecting cap-binding proteins in isolated protein fractions (4-8). It has also had limited use in complete translation systems (3). It is now generally agreed that at least 3 cap-binding proteins function in initiation (reviewed in ref. 9). One of these, CBP I (also known as eIF-4E), has a M_r of 24 kDa, and it constitutes a subunit of a larger complex called CBP II or eIF-4F. This com-

plex also contains a cap-binding protein of 46 kDa which may be identical to initiation factor eIF-4A (9). A protein of 80 kDa identical to eIF-4B can also be crosslinked to caps (7,8). Some additional proteins which were crosslinked to caps in a seemingly specific manner in initiation complexes have been described (3). However, they have not been further characterized.

We have previously described a number of proteins which were associated with mRNA in mammalian cells by the criterion of UV crosslinking (10,11). They associate in the cytoplasm with already existing mRNA and exchange there with a pool of proteins not bound to mRNA (12). Recently, we found that rabbit reticulocyte extracts capable of protein synthesis are also capable of reconstituting mRNA-protein complexes (mRNP) indistinguishable from those of intact cells, starting from protein-free mRNA (13). These findings are consistent with the idea that the mRNP proteins are involved in translation. However, except for one protein associated with poly(A), their binding sites were not known. Because the cap is one of the few features common to most kinds of mRNA it seemed a likely protein binding site. Also, the work on cap-binding proteins mentioned above suggested that some mRNP proteins might be associated with caps. To identify proteins associated with caps we utilized reticulocyte mRNA specifically labeled in the cap together with UV crosslinking and a cell-free translation/mRNP reconstitution system. We found that a number of proteins become associated with caps in the course of translation. Most seem to correspond to previously described mRNP and cap-binding proteins, but a few may not.

MATERIALS AND METHODS

Materials

Vaccinia guanylyl transferase, tobacco acid pyrophosphatase (TAP) and T4 polynucleotide kinase were obtained from BRL. RNA ligase was from New England Biolabs, and RNases were purchased from Sigma. RNasin was obtained from Promega Biotec, and calf intestine alkaline phosphatase was obtained from Boehringer-Mannheim. Gold label grade cyclohexylamine was obtained from Aldrich. Marker nucleotides for thin layer chromatography (TLC) were from Sigma and Pharmacia/P-L. PEI-cellulose TLC plates were obtained from Sybron/Brinkmann.

Methods

Translation of protein-free rabbit reticulocyte mRNA in a homologous micrococcal nuclease-treated cell-free system was done as described (13). Caps were removed from mRNA by β -elimination by a modification of the pro-

cedures described by Lockard et al. (14) and Keith and Gilham (15). Cyclohexylamine, Hepes, and EDTA were mixed at final concentrations of 0.8 M, 0.2 M, and 2 mM respectively. The pH was adjusted to 8 with HCl. 30 μ g of mRNA purified by chromatography on oligo(dT)-cellulose as described (13) were dissolved in 300 μ l of freshly prepared 1 mM NaIO₄ containing 0.5% sodium dodecylsulfate (SDS) and incubated in the dark for 1 hr at ambient temperature. The oxidized mRNA was ethanol precipitated after addition of RNase-free glycerol to 10 mM. The dried precipitate was dissolved in 100 μ l of water and mixed with an equal volume of the cyclohexylamine solution. Decapping was accomplished by incubating for 45 min at 45°C. De-capped mRNA (about 10 μ g per reaction) was re-capped by guanylyl transferase as described by Green et al. (16) in the presence of 0.1-1 mCi of [α -³²P]GTP (410-3000 Ci/mMole). In one experiment radioactive GTP was replaced by non-radioactive GTP at 1 mM. In another S-adenosyl-L-methionine (SAM) was replaced by S-adenosyl-L-homocysteine (SAH) to block methylation of caps.

mRNA was 3' end-labeled with T4 RNA ligase and [³²P]pCp (17) or 5' end-labeled with polynucleotide kinase and [γ -³²P]ATP (18) as described. Nucleotides incorporated into RNA were analyzed by PEI-cellulose TLC after digestion with appropriate enzymes. Chromatography was done in 0.6-1.2 M LiCl to separate methylated and unmethylated guanine nucleotides and by the procedure of Randerath and Randerath (19) to separate other nucleotides. If the TLC samples contained glycerol the dried plates were washed with 95% ethanol and dried again before chromatography to remove the glycerol.

mRNA was analyzed by electrophoresis in a 10 cm 5% polyacrylamide gel containing 7 M urea in Tris-borate-EDTA buffer (pH 8.3) for 2 hr at 200V. After electrophoresis the gels were stained with ethidium bromide, photographed with UV illumination, dried, and autoradiographed. Labeled and unlabeled mRNAs were translated in micrococcal nuclease-treated reticulocyte lysate at a concentration of 50 μ g/ml. After translation the reactions were chilled on ice and layered on 11 ml 15-45% linear sucrose gradients which were centrifuged in a Beckman SW41 rotor for 135 min at 36 krpm at 4°C. The gradients contained 10 mM NaCl, 10 mM Tris-HCl (pH 7.6), 2 mM EGTA, and 2 mM MgSO₄. They were fractionated into polysomes, 40-80S particles (containing ribosomal subunits, monoribosomes, and initiation complexes), and material sedimenting slower than 40S with the aid of a ISCO density gradient fractionator/UV monitor. Proteins were crosslinked to mRNA by irradiating gradient fractions having volumes of 1.5-7 ml in 6 cm petri dishes with 254 nm UV light at doses of 2-3 X 10⁵ ergs/mm² (10). Proteins crosslinked to

mRNA were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) in 10% gels after digestion with RNases A, T1, and T2 as described (10). In some cases the crosslinked complexes were purified by chromatography on oligo(dT)-cellulose (10) to eliminate protein not crosslinked to mRNA. Other samples were dissolved in oligo(dT)-cellulose binding buffer and heated to 60°C to denature RNA-protein complexes. They were then ethanol precipitated before digestion. In some experiments RNase digestion was replaced by digestion with TAP under the same conditions as RNase digestion except that RNasin was present at 1 unit/ μ l. Gels usually contained 14 C-labeled marker proteins as well as 32 P-labeled proteins. They were impregnated with EN 3 HANCE (New England Nuclear) before drying to aid in detecting 14 C and exposed with an intensifying screen to aid in detecting 32 P. Kodak XAR-5 film was used.

Purification of CBP I from rabbit reticulocyte postribosomal supernatant was done by a slight modification of the procedure described by Sonenberg et al. (5). m 7 GDP was coupled to agarose by the procedure of Lamed et al. (20). Postribosomal supernatant from 10 ml of lysate was applied directly to a 0.5 ml column containing 2 μ mole of bound nucleotide. After washing with binding buffer and 1 mM GDP cap-binding proteins were eluted with 100 μ M m 7 GDP. CBP I was UV crosslinked to cap-labeled mRNA as described by Pelletier and Sonenberg (21). Peptide mapping of 32 P-labeled proteins with protease V8 or N-chlorosuccinimide was done as described (22,23).

RESULTS

Properties of De-capped and Re-capped mRNAs

Since the goal of this work was to investigate proteins associated with the caps of natural mRNA under translation conditions it was necessary to examine the effects of de-capping and re-capping on translation activity. For this purpose the mRNA was de-capped, then re-capped with a non-radioactive cap. Fig. 1 shows that de-capping eliminated 80% of the ability of the mRNA to direct [3 H]leucine incorporation in micrococcal nuclease-treated lysate, and re-capping restored about half of the translation activity. Thus, the mRNA retained a substantial portion of its biological activity despite the extensive manipulations to which it was subjected. In agreement with previous results from other laboratories (reviewed in refs. 1,2) our data showed that translation of reticulocyte mRNA (mostly globin mRNA) is at least partly dependent on the presence of a cap.

Typical capping experiments produced mRNAs having specific activities of 1.1-2.2 X 10 5 dpm/pmole with labeled precursor having a specific activity of

Fig. 1. Translation and electrophoretic analysis of de-capped and re-capped mRNAs. A. 20 μ g of reticulocyte mRNA were de-capped by β -elimination. Half was re-capped by vaccinia guanylyl transferase in the presence of 1 mM unlabeled GTP and 50 μ M SAM. The remainder was incubated similarly except that enzyme was omitted. After incubation the samples were chloroform-phenol extracted, ethanol precipitated, and translated in micrococcal nuclease-treated reticulocyte lysate. In all translations with added mRNA the nominal concentration was 50 μ g/ml. However, there were no corrections for possible handling losses of the de-capped and re-capped RNAs. Curve 1: No mRNA. Curve 2: De-capped mRNA. Curve 3: Re-capped mRNA. Curve 4: Untreated mRNA. B. mRNA was de-capped and re-capped as in A except that 250 μ Ci of [α - 32 P]GTP (3000 Ci/mmol) were used in place of unlabeled GTP. The labeled mRNA was translated as in A. After translation a 4% aliquot of the reaction mixture was set aside. The remainder was sedimented in a sucrose density gradient, and fractions corresponding to polysomes, 40-80S particles, and <40S material were collected. The RNAs in the samples were deproteinized by digestion with proteinase K and phenol-chloroform extraction and analyzed by electrophoresis in a urea-containing 5% polyacrylamide gel which was dried and autoradiographed. The entire 4% aliquot and 20% of the RNA from each gradient fraction were analyzed. A sample of untranslated RNA was also analyzed. The position of the major labeled band corresponds to the position of a globin mRNA marker in a stained portion of the gel. Lane 1: Untranslated RNA. Lane 2: RNA which was translated but not centrifuged. Lanes 3-5: RNA which was translated and centrifuged (40-80S, <40S, and polysomes respectively). O: Origin of gel. G: Globin mRNA. XC: Xylene cyanole. BPB: Bromphenol blue.

1.7 $\times 10^6$ dpm/pmol. Electrophoretic analysis suggested that the mRNA was about 30% pure (data not shown). Therefore, about 22-44% of the molecules were capped. Capping was not quantitative, perhaps because the radioactive GTP concentration was too low for optimum capping (24). When the cap-labeled mRNA was analyzed by PAGE one major band corresponding to globin mRNA

Fig. 2. Nucleotides incorporated into RNA by guanylyl transferase. De-capped mRNA was re-capped with [α - 32 P]GTP in the presence of either SAM (lanes 1-5) or SAH (lanes 6,7). The mRNAs were translated, and the translation reactions were fractionated by sucrose density gradient centrifugation. The mRNAs in the various fractions and total mRNAs were deproteinized and analyzed by PEI-cellulose TLC in 0.6 M LiCl with or without TAP digestion. The digestion products were mixed with unlabeled GMP and m⁷GMP markers. After chromatography the locations of the markers were determined by inspection under short wave UV light, and the chromatograms were autoradiographed. The amounts of radioactivity and exposure times were adjusted to accurately show the relative amounts of GMP and m⁷GMP in each lane, and they do not necessarily reflect the amounts of RNA in the fractions. The spot marked with an asterisk in lane 7 was due to an unidentified impurity or digestion product.

was detected (Fig. 1B, lane 1). The mRNA remained mostly intact after translation and centrifugation of the translation reaction in a sucrose density gradient (Fig 1B, lanes 2-5). However, some degradation could be detected with longer autoradiographic exposures (data not shown).

To analyze the incorporated label the RNA was digested with TAP, and the digestion products were analyzed by PEI-cellulose TLC. This enzyme removes caps but leaves the mRNA otherwise intact (25). Without digestion all of the radioactivity remained at the origin (Fig. 2, lane 1). Therefore, there was no unincorporated label. After TAP digestion about 5% of the radioactivity remained at the origin, and the remainder migrated as GMP and m⁷GMP (lane 2). Therefore, at least 95% of the label was incorporated into caps. By cutting

out the spots and scintillation counting it was determined that 75% of the caps were methylated.

Since the goal was to identify proteins associated with caps during translation it was essential that the cap-labeled mRNA be incorporated into polysomes with high efficiency. Also, since some of the caps were not methylated, it was of interest to determine the extent to which methylated and unmethylated mRNAs were incorporated into polysomes. The cap-labeled mRNA was translated, and the translation reactions were sedimented in sucrose density gradients. The gradients were fractionated into polysomes, 40-80S particles, and <40S material. The RNAs in these fractions were digested with TAP, and the digestion products were analyzed by TLC. The results are shown in Fig. 2, lanes 3-5. 57% of the label were present in polysomes, 23% in 40-80S particles, and 20% in <40S material. The mRNA in the 40-80S particles was presumably associated with monoribosomes or ribosomal subunits as initiation complexes. TLC revealed that the <40S mRNA was almost completely unmethylated (lane 3), whereas the polysomal mRNA was over 80% methylated (lane 5). The majority of the 40-80S RNA was unmethylated, but some methylated RNA was present (lane 4). Thus, methylated mRNA was preferentially incorporated into polysomes. It has been reported that unmethylated mRNA can be translated in reticulocyte lysate with lower efficiency than methylated mRNA, particularly at the relatively low salt concentration (80 mM KCl) used in our experiments (26,27). Therefore, it is likely that at least some of the unmethylated mRNA present in the polysomes and 40-80S particles was translatable.

Proteins Associated with Caps

To identify proteins associated with caps the cap-labeled mRNA was translated, and the translation reactions were fractionated by sucrose density gradient centrifugation. Proteins were crosslinked to mRNA by irradiating with UV light and analyzed by SDS PAGE after digestion with RNases or TAP. In several experiments about 10% of the radioactivity in crosslinked mRNP was trichloroacetic acid insoluble after RNase digestion. This result suggested that about 10% of the caps were crosslinked to protein. To find out if proteins were crosslinked to the cap itself or to a site downstream from the cap the crosslinked complexes were digested separately with TAP and RNases. RNasin was present during TAP digestion as a precaution against possible low levels of RNase activity in the TAP. Proteins crosslinked to the cap itself should be detectable as bands in a gel after digestion with TAP. However, proteins crosslinked to sites downstream from the cap should be unlabeled

Fig. 3. Detection by PAGE of proteins crosslinked to caps after digestion with TAP and RNases. mRNA cap-labeled in the presence of SAM was translated, and the translation reaction was centrifuged in a sucrose density gradient. The gradient fractions were irradiated with UV light to crosslink proteins to mRNA. The RNAs in the 40-80S and polysome regions were recovered by pelleting in a Beckman 75Ti rotor for 16 hr at 48 krpm. After denaturing the mRNP by heating at 60°C in SDS the samples were digested either with a mixture of RNases (5 µg of A, 50 units of T1, and 12.5 units of T2, lanes 2,4), or with 5 units of TAP (lanes 3,5). Digestion was done for 60 min at 37°C. The samples were analyzed by SDS PAGE in a 10% gel which was dried and autoradiographed for 2 days with a screen. The numbered lines indicate the M_r s of the proteins. Lane 1 contained ^{14}C -labeled marker proteins which (from top to bottom) were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome C.

after TAP digestion, but labeled after RNase digestion if the crosslink site is in a cap-containing oligonucleotide. If the proteins are crosslinked directly to the cap the same bands should be seen after digestion with RNases or TAP. The results of this experiment are shown in Fig. 3. Samples with proteins crosslinked to mRNA in 40-80S particles (lanes 2,3) showed 2 major bands having M_r s of 52 and 65 kDa. These bands were seen after digestion with RNases or TAP. Samples with proteins crosslinked to polysomal mRNA also

contained the 52 and 65 kDa bands, but several additional bands ranging in size from 15 to 93 kDa were seen (lanes 4,5). Again, these bands were seen with either RNase or TAP digestion. Therefore, they appear to represent proteins crosslinked at least in part to the cap itself, although some crosslinking to sites downstream from the cap cannot be ruled out. The bands were not seen without RNase or TAP digestion. In the absence of digestion most of the radioactivity remained at the origin of the gel (data not shown). Therefore, the bands do not represent RNA fragments or proteins not crosslinked to RNA. The same bands were seen if RNases A, T1, and T2 were used separately as well as in combination. These bands were no longer seen after digestion with proteinase K (data not shown). This is the result expected if they were proteins. Also, they were bound to oligo(dT)-cellulose along with mRNA under conditions in which proteins not covalently attached to mRNA are not bound (data not shown).

The pattern of proteins crosslinked to the cap was different from that obtained by direct protein labeling (13, see also Fig. 5), and TAP digestion established that the proteins were indeed crosslinked to the cap. However, it seemed desirable to further investigate the specificity of the assay. One control for specificity consisted of labeling the mRNA at the 3' end with T4 RNA ligase and [³²P]pCp. Since there is a 78 kDa protein associated with poly(A) (11,13), this protein, if any, should be detected by crosslinking to 3' end-labeled mRNA. The 3' end-labeled mRNA was competent for translation, and the majority was incorporated into polysomes. The results are shown in Fig. 4A. After UV crosslinking, RNase T2 digestion and PAGE mainly one band was seen having the expected size of 78 kDa. Thus, the pattern of proteins crosslinked to 3' end-labeled mRNA was different from the pattern crosslinked to cap-labeled mRNA. In agreement with previous data (11,13) this band was seen in 40-80S particles and <40S material as well as in polysomes. It was bound to oligo(dT)-cellulose, and it was not seen without RNase T2 digestion. When the 3' end-labeled RNA was analyzed by PAGE most of the label was incorporated into material having the size expected for globin mRNA. Digestion with RNase T2 gave 3'-AMP as the only detectable product upon TLC, and digestion with nuclease P1 gave 5'-CMP as the only detectable product (data not shown). These are the results expected if labeling was specific for the 3' end of mRNA.

Another control for specificity consisted of investigating proteins crosslinked to de-capped mRNA which was 5' end-labeled with polynucleotide kinase and [γ -³²P]ATP after digestion with alkaline phosphatase. In con-

Fig. 4. Detection by PAGE of proteins crosslinked to mRNA 3' ends and de-capped 5' ends. **A.** Reticulocyte mRNA was 3' end labeled with [32 P]pCp and RNA ligase. It was translated, and the translation reaction was centrifuged in a sucrose density gradient. After irradiation the samples were digested with RNases A, T1, and T2 and analyzed by PAGE. Lane 1: Marker proteins. Lanes 2-4: Proteins crosslinked to mRNA in the <40S, 40-80S, and polysomal fractions. **B.** De-capped mRNA was treated with alkaline phosphatase and 5' end labeled with [γ - 32 P]ATP and polynucleotide kinase. It was translated, and the proteins crosslinked to the RNA after irradiation were analyzed as in **A.** The positions of marker proteins are indicated on the left. In this experiment the markers were (from top to bottom) myosin heavy chain, phosphorylase B, bovine serum albumin, ovalbumin, β -lactoglobulin, α -chymotrypsinogen, and lysozyme. Lanes 1-3: Proteins crosslinked to mRNA in the <40S, 40-80S, and polysomal fractions.

trast to the situation with cap-labeled mRNA, 82% of the radioactivity sedimented at less than 40S, and only 4% was incorporated into polysomes when this mRNA was translated. Two major bands with sizes of 65 kDa and 52 kDa were crosslinked to it (Fig. 4B). Therefore, these proteins do not bind exclusively to caps. However, the other proteins detected with cap-labeled mRNA were faint or undetectable with mRNA that was not capped, and by this criterion they are specific cap-binding proteins.

Cap analogs such as m⁷GDP efficiently inhibit protein synthesis and mRNA binding to ribosomes in the wheat germ cell-free translation system (27-29), and inhibition of chemical crosslinking by cap analogs has been widely used

as a criterion for specific binding of proteins to caps (3-8). This approach has been most useful in a simple in vitro binding assay, since it may be assumed that in such a system inhibition of crosslinking is due to competitive binding. It is less useful in a complete translation system, since it is not clear if inhibition of crosslinking is a primary effect due to competitive binding or a secondary effect due to inhibition of protein synthesis by cap analogs. Furthermore, several laboratories have reported that translation in the reticulocyte system is not absolutely dependent on the presence of methylated caps, and cap analogs may not completely inhibit translation and mRNA binding to ribosomes, particularly if the system is not treated with nuclease (26,27,30-34). We found that a high dose of m⁷GDP (0.8 mM) inhibited [³H]leucine incorporation in a translation reaction by about 80%, whereas GDP had little or no effect. In the presence of m⁷GDP the majority of the mRNA radioactivity was present in 40-80S particles, whereas in the presence of GDP the majority was present in polysomes (data not shown). Thus, in agreement with the results of Suzuki (33,34), mRNA binding to ribosomes was inhibited less than expected from the overall inhibition in protein synthesis. Similarly, we observed a partial inhibition of crosslinking of proteins to labeled caps. For example, as determined by densitometry of autoradiograms, crosslinking of the 15 kDa band and the 24 kDa band were inhibited by 67% and 40% respectively relative to a GDP control (data not shown).

Since competition of crosslinking by cap analogs was unsatisfactory we also crosslinked proteins to mRNA having unmethylated caps as an alternative method for identifying specific cap-binding proteins. This approach has been used by Pelletier and Sonenberg in a simpler system with results comparable to competition by cap analogs (21). Labeled mRNA having unmethylated caps was made by replacing SAM in the capping reaction with SAH, and it contained no detectable methyl groups (Fig. 2, lane 7). When the unmethylated cap-labeled mRNA was analyzed by PAGE it was found to be indistinguishable in size and homogeneity from methylated cap-labeled mRNA. Also, it remained intact upon cell-free translation (data not shown). Only 18% of the unmethylated mRNA was incorporated into polysomes, and only 2 bands having sizes of 52 kDa and 65 kDa were detected in association with unmethylated caps (Fig. 5, lanes 8-10). The 52 kDa band was by far the most prominent one. Thus, the results obtained by crosslinking proteins to unmethylated caps were similar to those obtained by crosslinking to 5' ends of uncapped mRNA. The proteins crosslinked to methylated caps but not to unmethylated ones are taken to be specific cap-binding proteins. They include all of the

Fig. 5. Proteins associated with unmethylated caps and caps of edeine pre-initiation complexes. mRNAs were cap-labeled in the presence of SAM or SAH and translated with or without 1 μ M edeine. The proteins associated with caps in different fractions of sucrose density gradients were analyzed as described above. mRNP proteins labeled directly with [14 C]formaldehyde (13) were included in the gel for comparison. Lane 1: Marker proteins. Lanes 2-4: 14 C-labeled mRNP proteins. Lanes 5-7: Proteins associated with caps made in the presence of SAM. Lanes 8-10: Proteins associated with caps made in the presence of SAH. Lanes 11-13: Proteins associated with caps made in the presence of SAM and translated with edeine.

proteins crosslinked to caps except the 52 kDa band. The 65 kDa protein is a special case, since its crosslinking to caps was greatly reduced, but not completely eliminated by demethylation, and it is still not completely clear if it is a specific cap-binding protein.

It is generally believed that cap-binding proteins are involved in the pre-initiation step of translation (9). To find out to what extent the cap-associated proteins we detected were specific to the pre-initiation step we examined proteins associated with caps in the 48S pre-initiation complexes formed in the presence of edeine (35) as well as proteins associated with 40-80S and polysomal mRNA. The results are shown in Fig. 5. This figure also shows the mRNP proteins detected by direct labeling of the UV cross-linked reticulocyte mRNP with [14 C]formaldehyde (lanes 2-4). They are inclu-

ded for the purpose of comparison with the cap-labeled proteins. There was very little protein associated with caps or total mRNA in <40S mRNP (lanes 5 and 2). Only a small amount of the 52 kDa band was detectable. This was also the major protein associated with caps in 40-80S mRNP, and it was detectable in polysomes as well (lanes 6,7). It corresponded in size to a protein detected by direct protein labeling (lanes 2-4). The second most prominent band associated with ³²P-labeled caps in 40-80S mRNP was the 65 kDa one (lane 6). It was the most prominent band in edeine mRNP (lane 12). It was not seen in the 40-80S or polysomal mRNP proteins detected by direct protein labeling (lanes 3,4). However, we previously detected a prominent band of this size in edeine mRNP by direct protein labeling (13). Some other proteins associated with caps were seen in 40-80S mRNP and edeine mRNP, including ones of 24 and 15 kDa (lane 6). However, these bands were most prominent in polysomes (lane 7). The 15 kDa band was prominent in polysomal mRNP proteins detected by direct protein labeling (lane 4), but the 24 kDa band could not be detected by direct protein labeling in the exposure shown in Fig. 5. However, it could be detected with very long exposures (data not shown). Some bands corresponding to cap-associated proteins were seen predominantly if not exclusively in polysomes. These included ones of 93, 47, and 34 kDa (lane 7). Others were seen mainly in edeine pre-initiation complexes. They included one of about 98 kDa, and possibly one of even larger size (lane 12). A band of 98 kDa was found in edeine mRNP by direct protein labeling (13). Thus, the 65 kDa and 98 kDa proteins previously detected in association with mRNA in edeine pre-initiation complexes by direct protein labeling may be associated with caps, although the 65 kDa band does not bind exclusively to methylated caps (Figs. 4A and 4B, Fig. 5, lanes 8-10), and it has not been determined if the 98 kDa one does. Some bands seen by direct protein labeling were not found in association with caps. They included bands of 78, 68, 62, and 29 kDa (lane 4).

The cap-associated proteins found in this study were detected by different methods than the ones used in previous studies by other investigators, and it is therefore important to investigate the relationship of our proteins to the ones identified by others. The 47 and 52 kDa bands are similar in size to eIF-4A. To find out if they are related to eIF-4A we attempted to immunoprecipitate them with a monoclonal antibody against eIF-4A (8). However, under conditions in which we were able to immunoprecipitate eIF-4A from whole lysate we were unable to precipitate any of the proteins UV crosslinked to cap-labeled mRNA under translation conditions.

Therefore, the 47 and 52 kDa bands are probably not related to eIF-4A, and eIF-4A is probably a very minor constituent of UV crosslinked 40-80S and polysomal mRNP, although its possible presence in preinitiation complexes has not been investigated.

The 24 kDa band, which appears to be a major cap-associated protein under translation conditions, is a possible candidate for CBP I. We found that the 24 kDa mRNP protein was identical in electrophoretic mobility to authentic CBP I which had been crosslinked to mRNA (data not shown). CBP I was purified by affinity chromatography on m⁷GDP-agarose and elution by m⁷GDP (5). We also compared the 24 kDa mRNP protein with authentic CBP I which had been crosslinked to cap-labeled mRNA by partial peptide mapping with protease V8 or N-chlorosuccinimide (22,23). The 24 kDa mRNP protein gave digestion products similar to those of CBP I (data not shown). However, the band broadening which resulted from UV crosslinking made it impossible to obtain maps of high resolution. The available data are consistent with the idea that the 24 kDa protein is CBP I. However, proof of identity has not been obtained.

DISCUSSION

Direct Photocrosslinking of Proteins to Caps

We chose to investigate cap-associated proteins by direct photocrosslinking because of our previous experience with photocrosslinking and because this approach might yield information not obtainable by chemical crosslinking to periodate-oxidized mRNA. UV crosslinking detects proteins in close and stable contact with RNA (reviewed in ref. 37). Our approach requires the use of mRNA specifically labeled in the cap. Although such mRNA could have been made by in vitro transcription, we chose to use de-capping and re-capping because the product is identical to natural mRNA except for the presence of a labeled cap and because of our previous experience with globin mRNP. Although the mRNA was contaminated with rRNA and possibly other RNAs, they were not detected upon electrophoretic analysis of the cap-labeled mRNA. They were not labeled because they do not have di- or triphosphate termini (2).

Digestion with TAP and PEI-cellulose TLC showed that nearly all of the label incorporated into RNA was in caps. Also, digestion of the crosslinked mRNP established that the proteins detected were crosslinked mainly to the cap itself, rather than a nucleotide downstream from the cap. The actual site of crosslinking was most likely the guanine base, since the bases are the photochemically reactive components of nucleic acids. Since most of the

proteins were specific for methylated caps they must have been crosslinked to m⁷G. Pelletier and Sonenberg recently described photocrosslinking of proteins to methylated caps (21), and there are other reports of protein photocrosslinking to unmethylated guanine nucleotides (38-40).

Although the proteins detected were crosslinked to the cap, this observation does not by itself establish that they are specific cap-binding proteins. However, additional observations suggest that this may be the case for most of them. All except the 52 kDa band were crosslinked more efficiently to methylated caps than to unmethylated caps, or to uncapped 5' ends, and the 24 kDa band was shown to be similar to CBP I. The 52 kDa protein is evidently not a specific cap-binding protein, since it binds to 5' ends of uncapped mRNA as well as to unmethylated caps. Other data also support the specificity of the photocrosslinking assay. The major protein found in association with the 3' end of the mRNA was the expected poly(A)-associated one, and it was not associated with caps. Also, some of the mRNP proteins detected by direct protein labeling did not appear to be cap-associated.

The number of proteins found in association with caps was larger than expected, since only 3 previously detected cap-binding proteins (CBP I, eIF-4A, and eIF-4B) are definitely known to be involved in translation. It seems unlikely that all of these proteins are simultaneously and independently associated with a particular cap. Rather, different proteins may be associated with it at different steps in translation. This is likely, since different but overlapping sets of proteins were associated with caps in the mRNP formed under translation conditions and edeine preinitiation complexes. It is believed that cap-binding proteins function principally in attachment of mRNA to 40S ribosomal subunits. However, the observation that there were some proteins associated with caps in polysomes, but not in edeine preinitiation complexes, suggests that caps and cap-binding proteins may also have some role at a later step in translation. Another possible explanation for the large number of proteins associated with caps is that some are part of a multi-subunit complex which has more than one member in close enough proximity to the cap for crosslinking. Certainly, CBP I and eIF-4A are thought to be part of a larger complex (9).

Although eIF-4B, which has a molecular weight of 80,000 is a cap-binding protein which can be photocrosslinked to caps (21), it was not detected in the present experiments. The reason for this is not known. One possibility is that eIF-4B was extracted from the mRNP by the sucrose density gradient centrifugation step which was done prior to crosslinking.

Relationship of Cap-associated Proteins to Other mRNP Proteins and Translation Factors

Several cap-associated proteins correspond in size to mRNP proteins detected by direct protein labeling and previously identified cap-binding proteins. The 52 kDa protein is the major one associated with caps in <40S mRNP and mRNP having unmethylated caps. Since it binds to uncapped 5' ends it is clearly not a protein that binds exclusively to caps. It is a major mRNP protein detected by direct protein labeling even in the absence of translation (13). Therefore, it may be a relatively non-specific RNA binding protein which can bind to any site on mRNA except poly(A). There is no evidence to suggest that it has a role in translation, but such a role has not been ruled out. A protein of similar size was found to be associated with mRNA even before the discovery of caps (41,42). The 65 kDa protein is the most prominent cap-associated protein in edeine mRNP. Although it can bind to some extent to mRNA having unmethylated caps, it was previously detected by direct protein labeling in edeine mRNP containing unmodified mRNA which presumably had fully methylated caps, but not in other mRNP classes (13). Its behavior is consistent with the idea that it is transiently associated with mRNA during formation of preinitiation complexes. Recently, Thomas and Arnstein reported that a protein of similar size, found predominantly in preinitiation complexes, is involved in the formation of SDS-stable complexes containing both mRNA and 18S rRNA (43). It seems likely that this protein is involved in initiation, although it does not yet have official status as an initiation factor.

A cap-associated protein of 34 kDa was found in polysomes. This protein can be detected by direct protein labeling. It is reduced in amount in 40-80S mRNP and undetectable in <40S mRNP and edeine mRNP. This laboratory previously found that the amount of this protein associated with mRNA is increased by actinomycin D treatment of mouse L cells (12), and a similar observation was made by Dreyfuss et al. with HeLa cells (44). Actinomycin D gradually inhibits initiation of translation in cultured mammalian cells (45). Since caps are involved in initiation, the induction of this protein by actinomycin D may be related to its effect on initiation.

The 24 kDa protein, which was found predominantly in polysomes, appears to be similar to CBP I by the criteria of size and partial peptide mapping. It was the first cap-binding protein discovered, and it seems to have no requirement for binding to mRNA except the presence of a methylated cap. It may be the first protein to bind to caps in the course of translation, and

perhaps other proteins cannot bind until CBP I is bound. Some of the other cap-associated proteins found in this study cannot readily be isolated on cap analog columns, perhaps because they have additional binding requirements. Since we used a complete translation system we may be able to detect cap-associated proteins not detected by others who worked with incomplete systems. If the cap-associated proteins we detected are also involved in initiation, the initiation process in eukaryotes may be even more complex than it previously seemed. Other investigators have shown that mRNA lacking methylated caps can be translated with low efficiency in the reticulocyte system, and we have shown that mRNAs without methylated caps do not bind most of the proteins that bind to methylated caps. The results suggest that these proteins have an enhancing role in translation, but they are not absolutely required, at least under the conditions generally used for cell-free translation.

It now appears that most mRNP proteins are associated either with caps or poly(A). However, some are clearly not, including the 78 kDa protein not associated with poly(A), and the 62 and 29 kDa mRNP proteins. Their binding sites remain to be identified.

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