Identification and Characterization of Cap-binding Proteins from Yeast*

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Photochemical cross-linking of Saccharomyces cerevisiae ribosomal salt wash preparations to cap-labeled mRNA reveals, in addition to the previously characterized 24-kDa cap-binding protein (eIF-4E), the presence of two novel cap-binding proteins (CBPs) of apparent molecular masses of 96 and 150 kDa. Cross-linking of the 96-kDa CBP was found to occur spontaneously without UV light induction. Based on the ATP/Mg** requirements, the three CBPs can be subdivided into two classes: 1) ATP/Mg** independent (24- and 150 kDa) and 2) Mg** dependent (96 kDa). The co-purification of the 24- and 150-kDa CBPs through several different chromatographic steps is consistent with the existence of a yeast CBP complex, possibly analogous to mammalian eIF-4F.

The binding of eukaryotic ribosomes to mRNA is a complex process involving at least three initiation factors and ATP (for recent reviews, see Moldave, 1985; Rhoads, 1988). This step is considered to be rate-limiting in translation initiation (Jaggs et al., 1981) and is consequently a key target for translational regulation (for reviews, see Edery et al., 1987; Sonenberg, 1988).

An important feature in ribosome binding to mRNA is the prior interaction of CBPs with the 5' cap structure, m'GpppX (where X is any nucleotide). Several approaches have been used to purify and characterize cytoplasmic proteins that interact with the cap structure in mammals (Sonenberg et al., 1979; Trachsel et al., 1980; Hellmann et al., 1982; Webb et al., 1984), wheat-germ (Lax et al., 1985; Seal et al., 1986), and yeast (Altmann et al., 1985). Proteins that could either specifically cross-link to the cap structure or be purified by cap-analog affinity chromatography were termed cap-binding proteins. Numerous studies indicate that these proteins interact to mediate cap binding. In mammals these proteins include: eIF-4A (50 kDa) (Grifo et al., 1983; Edery et al., 1983), eIF-4B (80 kDa) (Grifo et al., 1983; Edery et al., 1983; Pelletier and Sonenberg et al., 1985; Milburn et al., 1988), eIF-4E (24 kDa) (Sonenberg et al., 1978), and eIF-4F (CBP complex: eIF-4E, eIF-4A, and p220) (Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983). Using energy derived from ATP hydrolysis, eIF-4F in conjunction with eIF-4A and eIF-4B is thought to mediate the denaturation of 5' proximal mRNA secondary structure, thereby enhancing 40 S ribosomal subunit binding (Sonenberg, 1981; Ray et al., 1985).

eIF-4E is present in limiting amounts in mammalian cell extracts, relative to other initiation factors (Hiremath et al., 1985; Duncan et al., 1987). Since eIF-4F is an integral component of eIF-4F, it most probably renders eIF-4F limiting in the cell. This is consistent with the translational discriminatory activity of eIF-4F in several in vitro translation systems (Ray et al., 1983; Sarkar et al., 1984).

Functional studies of CBPs in yeast should greatly benefit from a genetic approach that is not available in mammals. However, biochemical characterization of CBPs in yeast is lagging behind that of mammalian systems. Yeast eIF-4E has been purified and its gene cloned (Altmann et al., 1985; 1987). In addition, a putative yeast homolog of the mammalian eIF-4A has been cloned (Linder and Slonimski, 1988). eIF-4B and eIF-4F homologs have not yet been identified in yeast. Here, we describe the characterization and isolation of cap-binding proteins from Saccharomyces cerevisiae, including a probable homolog of the mammalian eIF-4F.

**EXPERIMENTAL PROCEDURES**

Materials—Hexokinase and S-adenosyl-L-methionine were purchased from Boehringer Mannheim. DNase, EcoRI, and vaccinia virus guanylyltransferase were from Bethesda Research Laboratories. Rabbit anti-rat antibody was supplied by Dako Corp. [5-3H]CTP (23.7 Ci/mmol), [α-32P]GTP (3000 Ci/mmoll), [α-32P]ATP (3000 Ci/ mmoll), [γ-32P]ATP (3000 Ci/mmoll), and SP6 polymerase were obtained from Du Pont-New England Nuclear. m'GDP and GDP were obtained from P-L Biochemicals. RNasin and goat anti-rabbit alkaline phosphatase-conjugated antibody and the color development system were purchased from Promega Biotech. 2-Deoxyglucose, AMP-P(CH3)P, AMP-P(NH)P, and AMP(CH3)P-P were from Sigma, and polyethyleneimine-cellulose 3000 thin layer chromatography plates were purchased from Kodak.

Yeast Strain, Plasmid, and General Methods—The protease-deficient strain R2101 (Jones, 1977) and the SP6 construct p51A2 (Ruskin et al., 1986), containing a derivative of the yeast ribosomal protein gene rp51A, were generous gifts from Dr. M. Rosbash (Brandeis University). Preparation of plasmid DNA, DNA restrictions, and agarose gel electrophoresis were performed by standard methods.
Buffers—Buffer A: 20 mM HEPES, pH 7.5, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 7 mM β-mercaptoethanol; buffer B: 20 mM HEPES, pH 7.0, 0.2 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, and 20% (v/v) glycerol; buffer C, 20 mM HEPES, pH 7.5, 0.1 mM GTP, 2 mM dithiothreitol.

Yeast Extracts—S-100 was prepared according to Lin et al. (1985). Following centrifugation at 37,000 rpm for 90 min (Beckman Ti-60), the ribosomal pellet was resuspended in one-fifth the original volume of buffer A containing 500 mM KCl and 0.1 mM ATP. The mixture was stirred on ice overnight. The ribosomal pellet was collected and the ribosomal high salt wash (RSW) containing the initiation factors was dialyzed for 3 h against buffer B. The extract was centrifuged at 17,000 rpm for 20 min (Sorvall SS-34) to remove insoluble material and the supernatant aliquoted and frozen at -70 °C. When RSW was prepared for chromatographic studies, the dialysis step was omitted, and the RSW was stored at -70 °C in 10% (v/v) glycerol.

ATP Depletion of RSW—ATP depletion was performed essentially as described (Tahara et al., 1983). Briefly, RSW (0.8 mg) was adjusted to 2 mM MgCl₂ and 10 mM 2-deoxyglucose and incubated with 34 units of hexokinase for 30 min at 30 °C. The deoxyglucose was removed by Sephadex G-25 spun column (Pharmacia LKB Biotechnology Inc.) pre-equilibrated in buffer B containing 1% DNase-free BSA (Bethesda Research Laboratories). To assess ATP depletion, the incubation was performed in the presence of [γ-32P]ATP and 1 mM cold ATP. Hydrolysis of [γ-32P]ATP into 32P was measured by chromatography on a polyethyleneimine-cellulose TLC plate in 0.5 M KH₂PO₄.

In Vitro Transcription and Capping Reactions—The methods used were according to Pelletier and Sonenberg (1985). For in vitro transcriptions, the SP6p51Δ2 construct (Ruskin et al., 1986) was linearized with EcoRI, phenol-chloroform extracted twice, and passed over a Sephadex G-50 spin column (Pharmacia LKB Biotechnology Inc.) before precipitation.

Photochemical Cross-linking of Yeast Proteins to mRNA—The UV-induced cross-linking reactions were performed according to Pelletier and Sonenberg (1985) with minor modifications. 5-10 × 10⁶ cpm of [γ-32P]cap-labeled mRNA (20-10 10⁶ cpm/μg) was incubated with 4 μl of RSW (5-10 mg/ml) in buffer C in a total volume of 30 μl. Mixtures were incubated for 10 min at 30 °C and irradiated at 4 °C at a distance of 2.5 cm for 30-45 min (Pelletier and Sonenberg, 1985). The samples were then treated with 20 μg of RNase A for 30 min at 37 °C, resolved on a 12.5% SDS-polyacrylamide gel (Laemmli, 1970), and analyzed by autoradiography.

Preparation of Rat Anti-CBP Polyclonal Antibody—Two female rats (Sprague-Dawley, 2 months old) were injected intraperitoneally with a 1:1 mixture of affinity-purified yeast cap-binding proteins (25 μg) (Altman et al., 1985) and Freund's complete adjuvant. The immunization was repeated with 20 μg and CBPs and Freund's incomplete adjuvant after 4, 8, and 12 weeks. Blood was collected from the tail vein, allowed to clot, and clarified by centrifugation and the serum stored in aliquots at -20 °C. For standard immunoreactions sera dilutions of 1:1000 in 1% BSA/TBS, pH 7.6, 150 mM NaCl were used.

Immunoblotting Analysis—Immunoblotting was performed essentially as described (Towbin et al., 1979). Proteins were resolved on a 10% polyacrylamide-SDS gel and transferred onto nitrocellulose paper (Schleicher & Schuell) for 2 h at 25 volts and 1 amp. All incubations were done at room temperature and all washes were for 15 min in TBS. The nitrocellulose paper was incubated in TBS containing 1% BSA (Sigma) for 15 min and incubated with rat anti-CBP antibody diluted 1:1000 in TBS, 1% BSA for 90 min. Incubation with the second (rabbit anti-rat IgG, 1:1000 in TBS, 1% BSA) and third antibody (goat anti-rabbit alkaline-phosphatase-coupled, 1:5000 in TBS, 1% BSA) were performed for 30 min. After the final wash the paper was blot-dried and the color development carried out according to the supplier's specifications (Promega Bio-tech).

RESULTS

To identify yeast S. cerevisiae initiation factors capable of cap-specific interaction with mRNA, we used a photochemical cross-linking assay (Pelletier and Sonenberg, 1985). Initiation factor preparations obtained by a high salt wash of ribosomes (RSW) were mixed with the yeast rp51Δ2 cap-labeled mRNA and UV cross-linked. A number of polypeptides became labeled (Fig. 1A), but only 3 polypeptides of apparent molecular masses of 24, 96, and 150 kDa cross-linked in a cap-specific manner (lane 1; indicated by arrowheads). The cap specificity of the interaction was assessed by performing parallel cross-linking experiments in the presence of m7GDP or GDP. The presence of the cap analog m7GDP totally inhibited the cross-linking of the aforementioned polypeptides (lane 2). Conversely, GDP at the same concentration only marginally affected the cross-linking of the 24- and 150-kDa while inhibiting that of the 93-kDa polypeptide by 3-fold (as determined by scanning densitometry (lane 3); based on several experiments, the average inhibition was 2-fold). Similar cross-linking patterns were obtained with mouse c-myc or human β-globin transcripts, indicating that the cross-linking of these proteins is not message specific (data not shown). The 24-kDa polypeptide has previously been identified as the homolog of mammalian eIF-4E (Altman et al., 1985; 1987). The 96- and 150-kDa polypeptides, however, represent two newly identified yeast CBPs. UV cross-linking experiments using a post-ribosomal supernatant (S-100) gave a similar cross-linking pattern (data not shown). We consequently used the RSW and S-100 fractions interchangeably. A control experiment mixing RSW with cap-labeled mRNA, without UV irradiation, was performed followed by PAGE analysis. Unexpectedly, the 96-kDa CBP cross-linked spontaneously to the cap structure in a cap-specific manner (Fig. 2).

Note that although the 24-kDa polypeptide migrates as 29 kDa, it will be referred to as the 24-kDa polypeptide, since it is the size predicted by its amino acid sequence (Altman et al., 1987).
The ATP dependence of 43 S pre-initiation complex binding to the mRNA in eukaryotes (Marcus, 1970; Trachsel et al., 1977; Benne and Hershey, 1978) has been correlated with the ATP/Mg\textsuperscript{2+} requirement for the cap-specific interaction of eIF-4A (50 kDa) and eIF-4B (80 kDa) (Sonenberg, 1981; Grifo et al., 1982, 1983; Edery et al., 1983). Eukaryotic eIF-4F (CBP complex) in concert with eIF-4B and eIF-4A, is believed to destabilize the secondary structure at the 5′ end of mRNAs thereby facilitating 40 S ribosomal subunit attachment (for a recent review, see Sonenberg, 1988). It was therefore important to analyze the ATP/Mg\textsuperscript{2+} requirements of the yeast CBPs. To this end, RSW preparations were ATP-depleted by hexokinase treatment and used in photochemical cross-linking experiments (Fig. 2). Lanes 1 and 2 show the cross-linking pattern in the presence of ATP/Mg\textsuperscript{2+} and in the absence or presence of m\textsuperscript{7}GDP, respectively. When ATP/Mg\textsuperscript{2+} was added back to depleted preparations, the original cross-linking pattern was obtained (compare lane 3 to 1), indicating that the cross-linking ability of the RSW preparation was not irreversibly impaired by the hexokinase treatment. In the absence of ATP and Mg\textsuperscript{2+}, the cross-linking of the 96- and 150-kDa CBPs was drastically reduced (compare lane 4 to 3). The inability of the 150-kDa polypeptide to cross-link in the absence of ATP and Mg\textsuperscript{2+} was not always as dramatic as shown in Fig. 2 (see, for example, Fig. 3, lane 1). The reason for the presence of the strong background of radioactivity is unclear although it seems to be related to the UV irradiation (see below). Addition of ATP in the absence of Mg\textsuperscript{2+} restored the cap-specific cross-linking of the 150-kDa polypeptide while maintaining that of the 24 kDa at the control level (compare lanes 6 and 7 to 3). The cap-specific interaction of the 96-kDa CBP, however, is not restored under these conditions (lanes 6 and 7). Addition of Mg\textsuperscript{2+} in the absence of ATP restores the cross-linking ability of the 96-kDa CBP to intermediate levels (data not shown). The presence of 10 mM of EDTA in the photochemical cross-linking reaction completely abolished the 96-kDa cross-linking without affecting that of the 24 or 150-kDa (data not shown). The cap-specific interaction of the 96-kDa CBP therefore requires Mg\textsuperscript{2+}. The ATP requirement for 150-kDa cross-linking was further investigated by the use of AMP-P(CH\textsubscript{2})\textsubscript{P}, a non-hydrolyzable analog of ATP (lanes 8 and 9). AMP-P(CH\textsubscript{2})\textsubscript{P} substituted for ATP in the reaction and permitted cap-specific cross-linking of the three CBPs. Consequently, the hydrolysis of ATP does not appear to be required for the cap-binding activity of the 3 yeast CBPs. The ATP/Mg\textsuperscript{2+} independent cross-linking property of yeast eIF-4E is therefore identical to that of its mammalian homolog (Sonenberg, 1981). Interestingly, the cap-specific cross-linking of the 96-kDa CBP was stimulated in the presence of AMP-P(CH\textsubscript{2})\textsubscript{P} (compare lane 8 to 2; and see below).

The nucleotide requirements for cross-linking of the CBPs, was further investigated by substituting ATP with a number of different nucleotides and non-hydrolyzable analogs of ATP (Fig. 3). In the absence of ATP (lane 1) the characteristic inefficient cross-linking of the 150-kDa protein is observed (see also Fig. 2, lanes 4 and 5). However, all ATP substitutions that were tried, including ADP, AMP, and adenosine (lanes 4–6), other nucleotide triphosphates (lanes 7–9), or non-hydrolyzable ATP analogs (lanes 10–12) allowed efficient cross-linking of the 150 kDa. The extent of cross-linking of the 24-kDa CBP was generally unaffected by the different cross-linking conditions. The reasons for the increase in cross-linking of the 96-kDa CBP, when ATP is substituted by ADP, AMP, or adenosine (lanes 4–6), will be alluded to under “Discussion.” GTP substitution resulted in diminished cross-linking of the 96-kDa polypeptide (lane 8), consistent with the inhibition observed when cross-linking was performed in the presence of GDP (Fig. 1). UTP also seems to act as a poorer substitute of ATP, both for the 96 and to some extent for the 150 kDa (lane 9). The reasons for this remain unclear. Taken together, these results indicate that the 96- and 150-kDa CBPs cross-link in an ATP-independent fashion.

It has been shown in the mammalian system that two mRNA recognition factors are required for cap function: eIF-4A and eIF-4B. These factors do not possess a bona fide cap recognition site but can interact with it subsequent to the interaction of eIF-4F (Edery et al., 1983; Abramson et al., 1987). Accordingly, eIF-4A and eIF-4B by themselves do not cross-link to the cap structure nor bind to an m\textsuperscript{7}GDP-agarose column (Edery et al., 1983). It was therefore of interest to determine whether the yeast polypeptides that cross-linked to the mRNA cap structure were capable of binding specifically to an m\textsuperscript{7}GDP-agarose column. The yeast eIF-4E has been previously purified by such a method (Altmann et al., 1985). However, some minor polypeptides were also observed (Altmann et al., 1985). Fig. 4A shows a characteristic Coomassie Blue stain of a purification profile of CBPs from S. cerevisiae that of yeast eIF-4E has been previously purified by such a method (Altmann et al., 1985).

Fig. 2. ATP and Mg\textsuperscript{2+} requirements for the UV induced cross-linking. UV induced cross-linking was performed as described under “Experimental Procedures.” ATP depletion of the RSW (lanes 3–9) was achieved by hexokinase treatment in the presence of 2-deoxyglucose at 30 °C for 30 min as described under “Experimental Procedures.” Cross-linking was performed in the presence of 100 mM KOAc and where indicated, 1 mM ATP or 1 mM of AMP-P(CH\textsubscript{2})\textsubscript{P}, and 0.5 mM Mg\textsuperscript{2+} were added to buffer C. The cap analog m\textsuperscript{7}GDP was added at 0.65 mM where indicated. The molecular mass standards expressed in kilodaltons are indicated on the left.

I. Edery and N. Sonenberg, unpublished results.
Fig. 3. Effects of nucleotide substitutions on UV induced cross-linking of RSW. UV cross-linking was performed as described in the legends to Figs. 1 and 2. The RSW was ATP depleted as described in the legend to Fig. 2 and under "Experimental Procedures." The cross-linking mixture, in buffer C, containing 100 mM KOAc, was supplemented with 0.5 mM Mg<sup>2+</sup> (except for lane 1) and 1 mM of the compounds indicated above each lane. m'GDP was added at 0.65 mM where indicated. The cross-linked cap-specific polypeptides are indicated by arrowheads and the molecular weight standards (kDa) are indicated on the left.

Fig. 4. Purification of CBPs by m'GDP-agarose affinity chromatography. Approximately 500 mg of S-100 extract were applied to the m'GDP-coupled agarose matrix according to Edery et al. (1983, 1988). The column was washed with 100 volumes of buffer A containing 100 mM KCl and 0.1 mM ATP followed by 30 volumes of 0.1 mM GDP in the same buffer. Cap-specific proteins were eluted with 10 volumes of 0.1 mM m'GDP in the same buffer. A fraction (1/20) of the m'GDP eluate was analyzed on a 7.5–12.5% SDS-polyacrylamide gel. The gel was either Coomassie Blue-stained (Panel A) or processed for Western blotting as described under "Experimental Procedures" (Panel B). A: lane 1, load; 2, flow-through; 3, buffer A containing 100 mM KCl; 4, 0.1 mM GDP wash; 5–12, 0.1 mM m'GDP elutions; 22, molecular weight standards (kDa). B: lanes 2 and 3 are as in A; 3, is lane 5 in A. The CBPs are indicated by arrowheads.

(Fig. 4B, lane 1). Several of these polypeptides did not bind the column (see, for example, the 70-kDa polypeptide; lane 2). Washing of the column resulted in the loss of some of the 24- and 150-kDa CBPs (data not shown). However, the bulk of these polypeptides was specifically eluted by m'GDP (lane 3). It is noteworthy that the 93-kDa polypeptide detected by staining (Fig. 4A, lane 6) did not react with the antibody indicating that it is most probably not related to the 150-kDa polypeptide (Fig. 4B, lane 3). The m'GDP elution profile suggests that most of the cross-linkable polypeptides can be specifically eluted from the cap-analog resin. To determine whether this is the case, we performed a cross-linking experiment on yeast RSW followed by immunoprecipitation with the rat anti-CBP polyclonal antibody. Immunoprecipitation analysis of the photochemically cross-linked proteins is presented in Fig. 5. Lanes 1 and 2 show the cross-linking profile...
of a RSW fraction in the absence or presence of m^7GDP, respectively. The antibody specifically and quantitatively immunoprecipitated the 24- and 150-kDa polypeptides (compare lane 1 to lane 3). The 96-kDa CBP is not immunoprecipitated by the antibody consistent with the immunoblot analysis (Fig. 4B). A control experiment using pre-immune rat serum is shown in lane 4. Thus, the 24- and 150-kDa polypeptides that elute specifically from the m^7GDP-agarose column correspond to the 24- and 150-kDa polypeptides that cross-link to the mRNA 5' cap structure.

A CBP complex (eIF-4F) has been identified and characterized in both mammalian (Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983) and wheat-germ (Lax et al., 1985; Seal et al., 1986) systems. Gel filtration studies of crude post-ribosomal supernatant from yeast have shown that a small proportion of the 24-kDa CBP eluted in a fraction corresponding to a molecular weight of approximately 130,000 (Altmann et al., 1985). This suggested the presence of a high molecular weight CBP complex in yeast. We wished to determine whether the affinity-purified CBPs might be associated in a complex. Unfortunately, we repeatedly lost the 150-kDa polypeptide in further purification steps, possibly due to its instability. This occurred only when purification steps were conducted on a cap-affinity purified complex, but not when cruder preparations were subjected to chromatography purification (see below). As an alternative, we performed sucrose sedimentation analysis of yeast RSW preparations followed by immunoblotting of the different fractions with the rat anti-CBP antibody. A characteristic immunoblot of a 10–30% sucrose gradient containing 100 mM KCl is shown in Fig. 6A. The 24-kDa polypeptide sedimented in two regions of the gradient (lanes 3 and 4 and 6–9), whereas the 150-kDa CBP migrated only in one peak (lanes 6–9). Thus, co-sedimentation of the 24- and 150-kDa polypeptides occurred in fractions 6–9 (at approximately 6 S). Sedimentation analysis in the presence of 500 mM KCl gave a similar immunoblot profile although the respective fractions were shifted towards the top of the gradient (data not shown). In order to confirm the identities and cap-binding properties of the co-sedimenting polypeptides and to investigate the possible presence of other CBPs, we performed a photochemical cross-linking assay on fractions 6–8 (Fig. 6B). Cap-specific cross-linking of the 24- and 150-kDa CBPs occurred as expected from the immunoblotting analysis.

To further substantiate the physical interaction between the 24- and 150-kDa polypeptides, we performed ion-exchange chromatography. The elution profile of yeast RSW was analyzed after DEAE-cellulose chromatography by immunoblotting of the different fractions with the yeast anti-CBP antibody. Fig. 7 shows that the 24- and 150-kDa CBPs (indicated by arrowheads) co-eluted in lanes 3–5 at a concentration of 135–175 mM KCl. Similarly, these two CBPs co-fractionated by phosphocellulose chromatography at 380–540 mM KCl (data not shown). UV cross-linking of the peak fractions eluted from the DEAE-cellulose and containing the 24- and 150-kDa CBPs, confirmed the immunoblotting results (data not shown). Taken together, these results indicate that as in mammals and plants, eIF-4E associates with another polypeptide to form a complex. Based on this analogy the 150-kDa CBP is the potential homolog of the p220 subunit of mammalian and plant eIF-4F.

**DISCUSSION**

Mammalian and plant cells contain a high molecular weight CBP complex (eIF-4P) that can be purified by cap-affinity chromatography (Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983; Lax et al., 1985; Seal et al., 1986). In mammals, eIF-4P consists of three subunits of 24, 50, and 220 kDa, whereas the 24-kDa eIF-4P and the 50-kDa is eIF-4A. The eIF-4A subunit can be separated from the complex by phosphocellulose chromatography, suggesting a weak association between this subunit and the complex (Ray et al., 1985). This is consistent with other reports of eIF-4F preparations devoid of the eIF-4A subunit (Lee et al., 1985). Furthermore, in wheat germ all eIF-4P preparations obtained failed to show the presence of the eIF-4A subunit (Lax et al., 1985; Seal et al., 1986). Thus, the absence of an eIF-4A subunit in the CBP complex that we describe here is analogous to that of the plant system. The absence of an eIF-4A like subunit in the yeast complex is not due to a lack of this initiation factor in yeast, since a recently cloned gene was shown to contain extensive homology to mouse eIF-4A (Linder and Slonimski, 1988). There are several differences between the high molecular weight subunit from yeast and that of the mammalian or wheat germ-CBP complex. The yeast high molecular weight subunit has a smaller molecular weight; 150,000 as compared to 220,000 in mammals and wheat germ (Sonenberg, 1988). However, in plants there is a second CBP complex that contains an ~80-kDa polypeptide as the high molecular weight subunit (Lax et al., 1985; 1987; Seal et al., 1986). A second difference is the ability of the 150 kDa to photochem-

![Fig. 5. Immunoprecipitation of UV cross-linked CBPs with rat anti-yeast CBP polyclonal antibody.](image-url)
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**Fig. 6. Sucrose gradient sedimentation analysis of yeast cap-binding proteins.** A: approximately 10 mg of RSW were loaded on a 12-ml linear 10–30% sucrose gradient in buffer A containing 100 mM KCl. The gradient was centrifuged at 38,000 rpm for 24 h (Beckman SW-40). Fractions of ~600 µl were collected from the top and 50 µl of the indicated fractions were resolved by SDS-PAGE. Immunoblotting using the rat anti-CBP antibody was performed according to “Experimental Procedures.” The control experiment (lane C) contained 75 µg of RSW of the loaded material. The CBPs are indicated by arrowheads. The position of molecular mass markers (kDa) are indicated on the left. B: 150 µl of fractions 6–8 were dialyzed against 500 ml of buffer A for 2 h and 10 µl of the indicated fractions were used in the photochemical cross-linking assay in buffer C containing 100 mM KOAc, 1 mM ATP, and 0.5 mM Mg++. The fraction number is indicated above the lanes. m7GDP (0.65 mM) was present where indicated. The CBPs are indicated by arrowheads. The molecular mass standards (kDa) are shown on the left.

**Fig. 7. DEAE-cellulose elution profile of yeast cap-binding proteins.** Approximately 20 mg of RSW were loaded on a DE-52 column (0.5 × 37.5 cm) equilibrated in buffer A containing 100 mM KCl, 0.1 mM ATP, and 5% (v/v) glycerol according to Grifo *et al.*, (1983) with slight modifications. Elution was carried by a linear 100–600 mM KCl gradient in loading buffer at a flow rate of 6 ml/h (60 ml total). Fractions of 1 ml were collected and 50 µl of the indicated fractions were resolved on a 7.5–12.5% SDS-polyacrylamide gel. Immunoblotting using rat anti-CBP antibody was performed as previously described. Lane 1, fractions 5 and 6; lane 2, fractions 8 and 9; lane 3, fractions 12 and 13; lane 4, fractions 15 and 16; lane 5, fractions 18 and 19; lane 6, fractions 21 and 22; lane 7, fractions 24 and 25; lane 8, fractions 27 and 28; lane 9, fractions 30 and 31. The CBPs are indicated by arrowheads. Molecular mass standards (kDa) are shown on the right.

ribosome binding (Edery *et al.*, 1984; Ray *et al.*, 1985; Pelletier and Sonenberg, 1985; Lawson *et al.*, 1986; Sonenberg, 1988). This process requires ATP hydrolysis and is dependent on two additional initiation factors, eIF-4A and eIF-4B (Ray *et al.*, 1985; Abramson *et al.*, 1987). The latter initiation factors can interact with the mRNA only after prior interaction of eIF-4F with the cap structure (Edery *et al.*, 1983; Abramson *et al.*, 1987). Interaction of eIF-4F with the cap structure is ATP independent, whereas the interaction of eIF-4A and eIF-4B requires ATP hydrolysis (Sonenberg, 1981; Grifo *et al.*, 1983; Edery *et al.*, 1983; Abramson *et al.*, 1987). Thus, a reasonable scenario is that the first step in mRNA recruitment for translation is binding of eIF-4F to the cap structure in an ATP-independent manner followed by ATP-dependent binding of eIF-4A and eIF-4B with concomitant unwinding of the secondary structure (Sonenberg, 1981; Edery *et al.*, 1987; Abramson *et al.*, 1987). These steps pave the way for ribosome binding. It is likely that a similar pathway occurs in yeast. However, we have not been able to cross-link, in an ATP-dependent fashion, an eIF-4A-like polypeptide from yeast preparations. This is probably not due to a lack of eIF-4A, as mentioned above. In mammalian systems, ATP-dependent cross-linking of eIF-4A was obtained using oxidized cap-labeled reovirus mRNA (Sonenberg, 1981). However, UV-induced cross-linking of eIF-4A is very inefficient (Pelletier and Sonenberg, 1985). It is therefore probable that the same holds true for yeast. In the case of eIF-4B it is not clear what its yeast homolog might be. Yet it is unlikely that the 96-kDa polypeptide is the eIF-4B homolog, since cross-linking of the 96 kDa is ATP-independent and has distinct features from that of eIF-4B (Lee *et al.*, 1983; Pelletier and Sonenberg, 1985).

The cross-linking requirements of the 96-kDa polypeptide are very different from those of the other CBPs in that it can spontaneously form a covalent bond with mRNA, without the...
induced generation of a chemically reactive group (Fig. 1). This cross-linking is dependent on the presence of Mg\(^{2+}\). These features are very similar to those required for the formation of the guanylytransferase-[\(^{32}\)P]GMP intermediate in the capping reaction of eukaryotic mRNAs (Mizumoto and Kaziro, 1987). Nevertheless, there are several reasons that argue against the possibility that the 96-kDa polypeptide is guanylytransferase. First, yeast guanylytransferase has been purified and has a molecular weight of 52,000 (Itoh et al., 1987). Second, since guanylytransferase uses GTP as a substrate, it is anticipated that the GDP would inhibit the cross-linking of the 96-kDa polypeptide to the mRNA. However, GDP inhibited cross-linking by only 2- to 3-fold, whereas m\(^{7}\)GDP dramatically inhibited cross-linking. In addition, incubation of [\(^{\alpha}\)-\(^{32}\)P]GTP with RSW failed to yield a labeled polypeptide of molecular weight 96,000. Alternatively, an attractive possibility is that the 96 kDa is involved in degradation of the cap structure. In this regard, a yeast degrading enzyme has been partially purified and has a calculated molecular weight of 79,000 and a strict requirement for Mg\(^{2+}\) (Stevens, 1988). The nucleotide effects on cross-linking of the 96-kDa CBP (Fig. 3) are also consistent with its Mg\(^{2+}\) requirement: an increase in the number of phosphates causes a decrease in the extent of cross-linking. Our interpretation of this behavior is that an increase in phosphates results in better chelation of Mg\(^{2+}\) bringing about a reduction in 96-kDa CBP cross-linking. If indeed the 96-kDa polypeptide is involved in cap metabolism, it would be possible to study the mechanisms of cap turnover which are currently moot.

The p220 component is the least characterized of eIF-4F and its function is enigmatic. The only available functional data is derived from studies using poliovirus-infected cells (for review, see Sonenberg, 1987). The 220-kDa polypeptide is cleaved during poliovirus infection thereby inhibiting cap-dependent translation (Etchison et al., 1982; Lee et al., 1985). A causal relationship has been demonstrated between the cleavage of p220 and the shut-off of host protein synthesis after poliovirus infection (Bernstein et al., 1985), indicating that the integrity of p220 is important for eIF-4F function. However, at present, the mechanism of action of p220 is unknown. Cloning of the 150-kDa gene of S. cerevisiae has been achieved and genetic studies are underway to assess the function of this large CBP. This will aid in elucidating the mechanisms of action of eIF-4F in mRNA-ribosome binding.

Mammalian eIF-4F possesses mRNA discriminatory activity (Ray et al., 1983; Sarkar et al., 1984), consistent with it being present in limiting amounts in the cell relative to other initiation factors (Hiremath et al., 1985; Duncan et al., 1987). The recent finding that the cdc 33 is an allele of eIF-4E, which can differentially affect the expression of two mRNAs (Brenner et al., 1988), supports a translational discriminatory function for the yeast CBP complex.

In summary, we have purified a yeast CBP complex. In addition, we identified and characterized a CBP of apparent molecular weight 96,000 which can cross-link in a UV independent fashion. Based on the presence of eIF-4E and a large polypeptide of 150 kDa in the yeast CBP complex and their ATP/Mg\(^{2+}\) independent cross-linking properties, we have apparently identified the yeast eIF-4F.

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\(^{a}\) C. Goyer and N. Sonenberg, unpublished results.

\(^{b}\) C. Goyer, N. Sonenberg, and M. Altmann, unpublished results.
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