The messenger RNA cap-binding protein (CBP) was isolated from human erythrocyte, rabbit erythrocyte, and rabbit reticulocyte lysate by affinity chromatography on 7-methylguanosine 5'-triphosphate-Sepharose. The specific activity of binding to capped oligonucleotides was similar for the human erythrocyte and rabbit CBPs. Isoelectric focusing of human and rabbit preparations revealed that each was composed of up to five species. The pI values of human and rabbit CBPs ranged from 5.7 to 6.5. The predominant form in erythrocytes had a pI of 6.3 while in reticulocytes, two major species, having pI values of 5.9 and 6.3, were present. Labeling of rabbit reticulocytes with [32P]orthophosphate revealed that the pI 5.9 but not the pI 6.3 form contained phosphate. All of the phosphate was found in phosphoserine residues.

The amino acid compositions of human erythrocyte and rabbit reticulocyte CBPs were quite similar. Both proteins had 7 tryptophan and 6 cysteinyl residues. Labeling with [1-14C]iodoacetic acid under native and denaturing conditions provided evidence that 2 of the cysteinyl residues are present in the reduced form and 4 in disulfide bridges. Species of CBP with faster or slower electrophoretic mobilities could be generated by treatment of the protein either with O2 in the presence of a catalyst or with dithiorthreitol. The predominant form of the untreated protein migrated between these two forms.

Cap-binding protein (CBP) specifically binds to the 5'-terminal 7-methylguanosine moiety (cap) of eukaryotic messenger RNA and stimulates translation of capped but not uncapped mRNAs (for reviews, see Banerjee, 1980; Rhoads, 1985; Shatkin, 1985). The recognition of the mCAP structure apparently represents the entry point for mRNA into the protein synthesis cycle and thus may be the committed step of protein synthesis. Recent estimates suggest CBP is present at only 2-5% of the molar concentration of ribosomes, considerably below that of other initiation factors (Hiremuth et al., 1985). Thus, cap binding may also be the rate-limiting step for initiation. In keeping with this, Thach and co-workers (Ray et al., 1983) have presented evidence that CBP, in a complex with other polypeptides (Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983; Lax et al., 1985), acts as a messenger RNA discriminatory factor.

These considerations suggest that CBP may serve a regulatory role in the initiation of protein synthesis. Accordingly, we have developed a method of rapidly purifying large amounts of CBP in order to study its chemical structure. In this report we describe the presence of phosphate residues and the existence of sulfhydryl groups and disulfide bonds in the protein.

MATERIALS AND METHODS

RESULTS

Purification of Human CBP.—The affinity chromatography method of Webb et al. (1984), which consists of passing the postribosomal supernatant fraction of reticulocytes over a column of mGTP-Sepharose twice, was modified to shorten it to one day procedure. Since purified CBP loses activity rapidly (Trachsel et al., 1980; Hellmann et al., 1982), the dialysis step in which the eluting ligand, mGTP, was removed caused reduced yields for the second affinity chromatography. To alleviate this problem, CBP was eluted from the first mGTP-Sepharose column in buffer containing 0.5 M KCl, diluted to 0.1 M KCl, and immediately applied to a second mGTP-Sepharose column. CBP was eluted specifically from the second column using mGTP.

The existence of CBP in rabbit erythrocyte lysate was first shown by Tahara et al. (1981). We have extended this observation to human erythrocytes. When applying this method to erythrocyte lysates, it was not necessary to carry out the ultracentrifugation step to remove ribosomes. However, the purity of CBP at subsequent stages was improved if lysates were diluted in low ionic strength buffer and centrifuged to remove precipitated protein prior to the first affinity chromatography step. In order to compare the activities of erythrocyte versus reticulocyte CBP, the oligonucleotide binding assay of Hellmann et al. (1982) was chosen. The specific activity of affinity-purified human CBP preparations was found to be 270 pmol/mg, similar to that of affinity-purified rabbit reticulocyte CBP (282 pmol/mg; Webb et al., 1984). For chemical analysis of CBP, when it was not necessary...
to have active material, preparations of the highest purity could be obtained by reverse phase HPLC. CBP purified by either one or two passages over the affinity column were analyzed by HPLC. Fig. 1 shows the HPLC profile of human erythrocyte CBP purified by a single passage over m7GTP-Sepharose. A number of minor peaks are seen; these were substantially reduced in CBP preparations passed twice over m7GTP-Sepharose (data not shown). The major species eluting at 22 min was shown by gel electrophoresis to be CBP (inset). No contaminating polypeptides were detectable. (The faint bands one-fourth of the way down the gel are an artifact of the silver staining method.)

**Isoelectric Points—Affinity-purified human erythrocyte CBP resolved into five species during two-dimensional separation by isoelectric focusing and denaturing electrophoresis (Fig. 2).** The isoelectric points of the various species were 5.7, 5.9, 6.1, 6.3, and 6.5. CBP from rabbit erythrocytes contained the same number of species, but that from reticulocytes consisted predominantly of species of pI 5.9 and 6.3 (Fig. 3A).

The presence of several forms of CBP differing in isoelectric point suggested that the protein could be post-translationally modified. To test whether this might be due to phosphorylation, rabbit reticulocytes were labeled with [32P]orthophosphate. CBP was purified and the various forms separated by one-dimensional isoelectric focusing. The gel was stained with silver and subjected to autoradiography (Fig. 3B). Only one radioactive band was found, corresponding to the CBP species with pI 5.9. As a more quantitative determination, the regions where each of the species should be located were excised from the gel and the radioactivity determined. The band at pI 5.9 contained 85% of the total radioactivity while the gel regions for the pI 6.1, 5.7, and 6.3 species contained, respectively, 10%, 4%, and <1%. These results indicate that, of the major forms of CBP present, the pI 5.9 species is phosphorylated while the pI 6.3 species is not. Although the pI 6.1 and 5.7 species were barely detectable by silver staining (Fig. 3B, SIL), the finding of radioactivity associated with them suggests that they may be also phosphorylated. The reason why they differ in pI from the pI 5.9 form has not been determined.

The nature of the phosphorylated amino acids was determined by hydrolyzing the [32P]-labeled protein and separating amino acids by thin layer electrophoresis (Fig. 4). The only labeled phosphoamino acid present was phosphoserine. The other labeled species present corresponds to orthophosphate. Under the hydrolysis conditions used, approximately 40% of the phosphoserine is expected to be degraded to serine and orthophosphate (Hunter and Sefton, 1980).

**Fig. 3. Separation of rabbit CBP by isoelectric focusing.** A, the protein, isolated from erythrocyte (ERY) or reticulocyte (RETIC) lysate, was separated on a two-dimensional gel as described in the legend to Fig. 2. B, HPLC-purified [32P]-labeled CBP from reticulocytes was separated on a one-dimensional isoelectric focusing gel as described in the Miniprint Supplement, except that a slab gel was used. Visualization was by silver staining (SIL) and by [32P]-autoradiography (32P).

**Fig. 2. Twice affinity-purified human CBP separated on a two-dimensional gel in denaturing conditions.** The first dimension was isoelectric focusing in Pharmalyte 5-8, the second, a 12% sodium dodecyl sulfate-polyacrylamide gel (O'Farrell, 1975). Staining was with silver (Wray et al., 1981).

| pH | 5.5 | 6.0 | 6.5 | 7.0 |
|----|-----|-----|-----|-----|
| A. ERY | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) |
| B. RETIC | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) |
| SIL | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) |
| 32P | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) | ![Silver staining](image) |

**Fig. 1. Elution profile of affinity-purified human CBP.** The protein (300 µg) was eluted from the first m7GTP-Sepharose column (2.5 × 3.3 cm) with 30 ml of 50 µM m7GTP and loaded onto an Ultrapore RPSC C3 column. The column was developed as described in the Miniprint Supplement. Inset, denaturing polyacrylamide gel electrophoresis of the major peak seen on HPLC. The single band observed migrated with a molecular weight of 28,000.
iodoacetic acid was performed (i) on the native protein, (ii) after addition of DTT, (iii) after denaturation with 6 M guanidine HCl, and (iv) after addition of guanidine HCl and DTT. The results (Table II) indicate that no cysteinyl residues were available for reaction without denaturation of the protein. Two cysteinyl residues could be labeled after treatment with guanidine HCl, whereas 6 were labeled after denaturation in the presence of DTT, in agreement with the amino acid analysis.

Effect of the Reduction and Oxidation of CBP on Its Electrophoretic Mobility—If CBP contains internal disulfide bridges, its electrophoretic mobility is likely to be affected by the state of reduction of its cysteine residues. The protein which had not been treated with reducing agents migrated as a diffuse band containing a major species at 27 kDa (Fig. 5A, lane N). Oxidation in the presence of a copper catalyst produced a sharp band with mobility corresponding to a 26-kDa species (lane O). Treatment of the oxidized protein with DTT resulted in a species at 28 kDa (lane O, DTT). The latter species migrated the same as the native protein treated with only DTT (lane DTT), indicating that oxidation did not produce irreversible chemical changes which affected mobility. Treatment of CBP with the weaker reducing agent 2-mercaptoethanol under the standard conditions described by Laemmli (1970) produced only partial reduction, resulting in a doublet at 27 and 28 kDa (data not shown). Although we previously isolated CBP from rabbit reticulocyte ribosomes and assigned it a molecular mass of 24,000 (Hellmann et al., 1982), CBP from this source behaved identically with rabbit and human erythrocyte CBP on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, migrating at 28 kDa after treatment with DTT (data not shown).

DISCUSSION

The fact that CBP can exist in phosphorylated form is shown by its heterogeneity with respect to PI (Figs. 2 and 3A), the ability to label some but not all species of CBP with ^32P (Fig. 3B), and the presence of phosphoserine in CBP (Fig. 4). Approximately 50% of the CBP in reticulocytes is phosphorylated (Fig. 3B). A number of other components of the protein synthetic apparatus are phosphorylated: certain ribosomal proteins, the best studied being S6, and certain subunits of the initiation factors eIF-2, eIF-3, and eIF-4B (reviewed by Traugh, 1981). In the case of eIF-2, phosphorylation of the α subunit prevents eIF-2B-mediated guanine nucleotide exchange, a step which is involved in the recycling of eIF-2 to a form which is active in initiation (reviewed by

### Table I

| Amino acid | Human CBP | Rabbit CBP |
|------------|-----------|------------|
|            | residues/mol ± S.E. | residues/mol ± S.E. |
| Aspartic acid | 28.5 ± 0.7 (29) | 26.7 ± 0.5 (27) |
| Threonine | 18.5 ± 0.6 (19) | 14.9 ± 0.7 (15) |
| Serine | 13.5 ± 1.0 (14) | 16.0 ± 1.6 (16) |
| Glutamic acid | 27.6 ± 0.5 (28) | 28.0 ± 1.8 (28) |
| Proline | 16.8 ± 1.4 (17) | 17.0 ± 0.4 (17) |
| Glycine | 14.7 ± 1.1 (15) | 15.8 ± 3.5 (16) |
| Alanine | 11.4 ± 1.0 (11) | 16.0 ± 1.0 (16) |
| Cysteine | 6.0 ± 0.5 (6) | 5.8 ± 0.2 (6) |
| Valine | 13.4 ± 0.6 (13) | 12.1 ± 1.2 (12) |
| Methionine | 3.6 ± 0.4 (4) | 5.4 ± 0.7 (5) |
| Isoleucine | 11.7 ± 0.7 (12) | 10.6 ± 0.7 (11) |
| Leucine | 22.2 ± 1.6 (22) | 19.8 ± 1.1 (20) |
| Tyrosine | 6.5 ± 0.3 (7) | 7.4 ± 0.7 (7) |
| Phenylalanine | 9.4 ± 0.3 (9) | 9.4 ± 0.8 (9) |
| Histidine | 5.8 ± 0.4 (6) | 5.9 ± 0.5 (6) |
| Tryptophan | 7.4 ± 0.2 (7) | 7.2 ± 0.8 (7) |
| Lysine | 15.3 ± 0.8 (15) | 15.4 ± 0.9 (15) |
| Arginine | 13.4 ± 0.8 (13) | 14.4 ± 0.9 (14) |

* Determined as cysteic acid.
* Protein was hydrolyzed in the presence of 2% thioglycolic acid; values are corrected for tryptophan breakdown.

### Table II

| Conditions of carboxymethylation | Radioactivity | [14C]Carboxymethylcysteine |
|----------------------------------|--------------|---------------------------|
| cpm/μg protein | mol Cys/mol protein |
| Native | 68 | <0.2 |
| Native + DTT | 52 | <0.2 |
| Denatured | 958 | 2.2 |
| Denatured + DTT | 2624 | 6.0 |

* Where indicated, the protein was preincubated with 1 mM DTT for 40 min at 37 °C. CBP was carboxymethylated as described in the Miniprint Supplement with [1-14C]iodoacetic acid in buffer A-100 containing 50 μM mGTP, pH 8.5, with pH adjusted with N-ethylmorpholine (native conditions) or in 6 M guanidine HCl, 0.2 M N-ethylmorpholine-HCl, pH 8.5 (denatured conditions). Reactions were terminated with 20 mM DTT, dialyzed against 2 mM ammonium bicarbonate, and the protein purified by HPLC as described under “Materials and Methods.” Radioactivity was determined by liquid scintillation spectrometry.
74 Structural throline and 0.5 mM CuSO$_4$ as a catalyst at electrophoretic mobility. Chromatography in the absence of DTT, was incubated for about 100 ng of CBP were loaded on each lane except lane DTT, where 200 ng were loaded. Samples were separated on a 12% polyacrylamide gel and detected by silver staining. B, estimation of molecular weight of human CBP. Reduced, native, and oxidized forms of CBP migrated as polypeptides of 28,3, 27.3, and 26.0 kDa, respectively. The points represent the migration of protein molecular mass standards (see Miniprint Supplement).

Safer, 1983; Ochoa, 1983). With the other phosphorylated initiation factors, to which we now add CBP, the effect of phosphorylation is not known. Cap-binding per se may not be altered, since both phosphorylated and nonphosphorylated forms of CBP bind to the mGTP-Sepharose column (Fig. 3B). However, we have not yet measured the relative affinities of capped oligonucleotide for the phosphorylated and nonphosphorylated forms. By analogy with eIF-2, one might also expect that some other aspect of CBP’s action, e.g., binding to ribosomes, release of the cap, or recycling, could be affected by phosphorylation.

The existence of approximately two sulfhydryl groups in CBP is supported by several lines of evidence. First, amino acid analysis indicated a total of 6 cysteiny1 residues (Table I), but only two of these could be labeled with [1-14C]iodoacetic acid in the absence of DTT (Table II). Second, the labeling of two tryptic peptides with this reagent was insensitive to DTT while that of other peptides was increased (data not shown). Third, untreated CBP migrates at a position intermediate between the forms of CBP in which cysteinyl residues are fully oxidized and fully reduced (Fig. 5), presumably due to a more compact structure of internally cross-linked forms. We have frequently obtained preparations in which CBP migrated as a doublet and have also demonstrated that 2-mercaptoethanol is insufficient to convert all of the CBP to the fully reduced form. Sonenberg (1981) showed that cross-linking of caps to CBP generated a doublet when ATP and Mg$^{2+}$ were included. Also, Lax et al. (1985) isolated two forms of a polypeptide with the properties of CBP in complexes with other polypeptides, one having a molecular weight of 26,000 and the other, of 28,000. These observations may be related to conformational changes or incomplete reduction of disulfides.

A number of other RNA-binding proteins have been shown to contain essential thiol groups. The coat protein of bacteriophage R17 contains two thiol groups (Romeniuk and Uhleneck, 1985). Reaction with 5-bromouridine as well as unmodified cytidine nucleotides inactivates one of these, suggesting transient formation of a Michael addition product between the thiol and a pyrimidine residue in the RNA. Schimmel and co-workers (Starzyk et al., 1982) have also presented evidence that Ala-tRNA synthetase, which is activated by classical thiol modifying reagents, forms a transient covalent adduct with its cognate tRNA by Michael addition to a pyrimidine base. Thymidylate synthetase also appears to form a Michael adduct between the 5-carbon of dUMP and a cysteinyl residue (Wataya et al., 1980). Finally, an analogy between CBP and the pp12 protein of Rous sarcoma virus can be drawn. Like CBP, this protein (i) binds single-stranded, (+)-sense RNA, (ii) can exist in both phosphorylated and nonphosphorylated forms (Leis et al., 1984), (iii) contains 6 cysteinyl residues (Misono et al., 1980), (iv) is modified by alkylating agents, and (v) these are in the form of sulfhydryls (Veigl et al., 1979). In the case of pp12, phosphorylation increases RNA binding affinity.

In comparing the amino acid composition of CBP (Table I) with that of several other initiation factors (eIF-2, -4A, -4B, -4C, and 5; Grifo et al., 1982), it is found that CBP has a low content of basic amino acids, similar to eIF-4A, a factor which, like CBP, is involved in the joining of mRNA to the 40 S initiation complex. The cysteine content, on the other hand, is high, similar to eIF-4D. The most unusual feature of the amino acid composition of CBP, however, is the high tryptophan content; while the initiation factors mentioned above vary from 0 to 1 mol % of tryptophan, CBP contains 4.7 mol % (7 residues). This may be significant in light of the finding of Ishida et al. (1983) that the cap structure has a high affinity for tryptophan, especially when the tryptophanyl residues are surrounded by negatively charged groups. Some of the tryptophanyl residues in CBP may be located in the cap-binding site. Current work in our laboratory is aimed at determining which peptides and amino acid residues are located in the active site of this protein.

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Additional references are found below.

SUPPLEMENTARY MATERIAL TO:
Structural Analysis of the Messenger RNA Cap-Binding Protein.

Preparation of Phosphatase, Sulfhydryl and Disulfide Groups

Mickleth Krychilt, Paul R. Gerber, Thomas G. Vannama, and Robert S. Rhoads

Materials - E. coli-Sephacrose (the g-aminophenyl)-water. Mentioned by buffer uved.

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1970). molecular weight standards included horseradish peroxidase (67 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and succinyl
tryptophan hydrate (20 kDa). Protein was visualized by silver staining (Veigl et al., 1981).

Active acid analysis of CBP - Protein (5 µg) was hydrolyzed in evacuated, acid-washed glass tubes for 10-17 d at 110°C. In 3 µl of 6 M HCI (Veigl et al., 1984), with addition of a small crystal of phenol. Values for amino acid content were not corrected for destructive losses during hydrolysis, except for those of tryptophan. For the determination of tryptophan, hydrolysates were in 6 M HCI with either 2 or 6 M ethylenediamine hydrochloride (Matsuda and Sasahara, 1969) or in 3 M mercaptopropanesulfonic acid (Parker et al., 1974). For determination of cysteine, 5 µl of CBP was oxidized with peroxycetic acid (Hirs, 1967) in a total volume of 10 µl prior to hydrolysis. Amino acids were analyzed on a Beckman System 5000 High Performance Analyzer.

Phosphoprotein acid determination - HPLC-purified, [S-32P]-labeled CBP (3000 cpm, 3 µg) was solubilized in 100 µl of 8 M urea and lyophilized in an evacuated tube for 1 d at 110°C. The hydrolysate was dried using a rotary evaporator and dissolved in 5 µl of water containing 2 µl each of unlabeled phosphoserine, phosphothreonine and phosphotyrosine. This-layer electrophoresis was performed on cellulose plates in 2.36 fmoles acid and 7.6 isocitric acid (White and Kaback, 1976) for 1 h at 1000 V. Amino acids were visualized with ninhydrin (Allan, 1981) followed by autoradiography.

Carrboxylation - Lyophilized, HPLC-purified CBP (300 µg) was dissolved in 0.5 ml of 6 M guanidine-HCl, 0.2 M 6-ethylmercaptoethanol-HCl, pH 0.5, and, unless stated otherwise, 1.0 µl 0T. After incubation at 37°C for 40 min, 1 µl of 0.5 M lisodan acid was added and incubation continued at 75°C for 20 min. The reaction was terminated by addition of 10 µl of 1.0 M EDTA. The protein was dialyzed against 2 ml ammonium bicarbonate and lyophilized.

Labeling of CBP with [35S] - A rabbit was injected with radioactive phosphoprotein to obtain 200 µg of intact CBP with no measurable electrophoretic migration. The radioactivity of the cysteine was added by labeling with chloroacetic acid (Hirs, 1978). The labeling reaction was performed on 10 µl of the following solution: 35 mCi (0.13 µCi) of 35S-labeled cysteine or 35S-labeled phosphorus containing 2 µl of each of unlabeled phosphoserine, phosphothreonine and phosphotyrosine. This-layer electrophoresis was performed on cellulose plates in 2.36 fmoles acid and 7.6 isocitric acid (White and Kaback, 1976) for 1 h at 1000 V. Amino acids were visualized with ninhydrin (Allan, 1981) followed by autoradiography.

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