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Cloning of cDNA for Cathepsin B mRNA 3′-Untranslated-Region–Binding Protein (CBBP), and Characterization of Recombinant CBBP[1]

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Previously, we purified the cathepsin B mRNA 3′-untranslated-region–binding protein (CBBP) from *Sarcophaga* and suggested its participation in the translational regulation of cathepsin B mRNA in this insect. In this study, we isolated a full length cDNA for CBBP. CBBP was an RNA-binding protein that contained four RGG boxes and four zinc finger motifs required for RNA binding. CBBP was shown to be localized in both the nuclei and cytoplasm of *Sarcophaga* hemocytes. Recombinant CBBP bound to the entire region of cathepsin B mRNA and repressed its translation *in vitro*.

Key words: cathepsin B, insect, RNA-binding protein, tissue remodeling, translational regulation.

In holometabolous insects, most larval tissues decompose and adult structures develop from the imaginal discs during metamorphosis (*1*). In *Sarcophaga peregrina* (flesh fly), cathepsin B is known to participate in decomposition of the larval fat body (*2–6*). At an early pupal stage, cathepsin B is secreted from hemocytes on their interaction with the larval fat body (*4*) and digests its basement membrane, resulting in disintegration of the fat body into trophocytes.

In our previous study, it was suggested that the synthesis of hemocyte cathepsin B is regulated at the translational level (*7*). Namely, whereas larval hemocytes stored an appreciable amount of cathepsin B mRNA, their cathepsin B content was significantly low. When the larvae began to pupate, cathepsin B started to accumulate in the hemocytes. Subsequently, we purified a protein with a molecular mass of 90 kDa that specifically binds to the 3′-untranslated-region (3′-UTR) of cathepsin B mRNA from an embryonic *Sarcophaga* cell line (*8*). This protein (CBBP) was shown to repress the translation of cathepsin B mRNA *in vitro* in a 3′-UTR dependent manner. We found that the original molecular mass of CBBP was 110 kDa, but that the 110-kDa protein was partially degraded during purification, a 90-kDa protein being yielded.

This paper reports the cDNA cloning of CBBP and some characteristics of the recombinant CBBP. The amino acid sequence of CBBP showed high similarity with that of *Drosophila* PEP (protein on ecdysone puffs) (*9*), a member of the heterogeneous nuclear ribonucleoproteins (hnRNPs) (*10*). The recombinant CBBP inhibited the translation of cathepsin B mRNA *in vitro* regardless of its 3′-UTR.

MATERIALS AND METHODS

Cells, CBBP, and Antibody against CBBP—Embryonic *Sarcophaga* cell line NIH-Sape-4 was cultured in M-M medium at 25°C as described previously (*11*). *Sarcophaga* hemocytes were prepared from third instar larvae. CBBP (90-kDa protein) was purified from NIH-Sape-4 cells and anti-CBBP antibody was raised against it as described previously (*8*).

Cloning and Sequencing of CBBP cDNA—About 40,000 clones from an NIH-Sape-4 cDNA library were screened with anti-CBBP antibody and alkaline phosphatase–conjugated secondary antibody (Bio-Rad). For nucleotide sequencing of the cDNA, the insert DNA was subcloned into pBluescript (Stratagene) and then sequenced by the dideoxy chain termination method using a Taq dye terminator sequencing kit (Applied Biosystems). The nucleotide sequences of both strands were determined.

Preparation of Recombinant CBBP—To obtain C-terminal histidine-tagged CBBP, CBBP cDNA was first amplified by PCR using 5′-CTAGCTAGCATGGCATTCCGCAATCAAA-3′ and 5′-TTTCTCGAGATAACGGTTGTAACGACCAC-3′ as N-terminal and C-terminal side primers, respectively. The PCR product was digested with *Nhe*I and *Xho*I, and then cloned into the *Nhe*I- and *Xho*I-digested pET-23a(+) vector (Novagen). *E. coli* BL21(DE3)pLysS cells were transformed with the construct and the transformed bacteria were grown in 2 liters of LB medium at 37°C. The recombinant CBBP was induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside when OD_{600} reached 0.6–1.0. The cells were disrupted by sonication in 80 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9, containing 25 μg/ml aprotinin, 12.5 μg/ml leupeptin, 25 μg/ml pepstatin A, 1.25 mM phenylmethylsulfonyl fluoride, 25 μg/ml phosphoramidon, and 25 μg/ml E-64). After centrifugation for 20 min at 39,000 ×*g*, the supernatant was loaded on a His·Bind Resin (Novagen) column.

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The column was washed with wash buffer (45 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9) and then eluted with elution buffer (300 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9). The eluate was dialyzed against buffer B (20 mM Hepes, pH 7.5, 70 mM 2-mercaptoethanol, 0.05% Nonidet P-40) and then applied to a Mono S HR 5/5 column. After washing of the column with buffer B containing 450 mM NaCl, the recombinant CBBP was

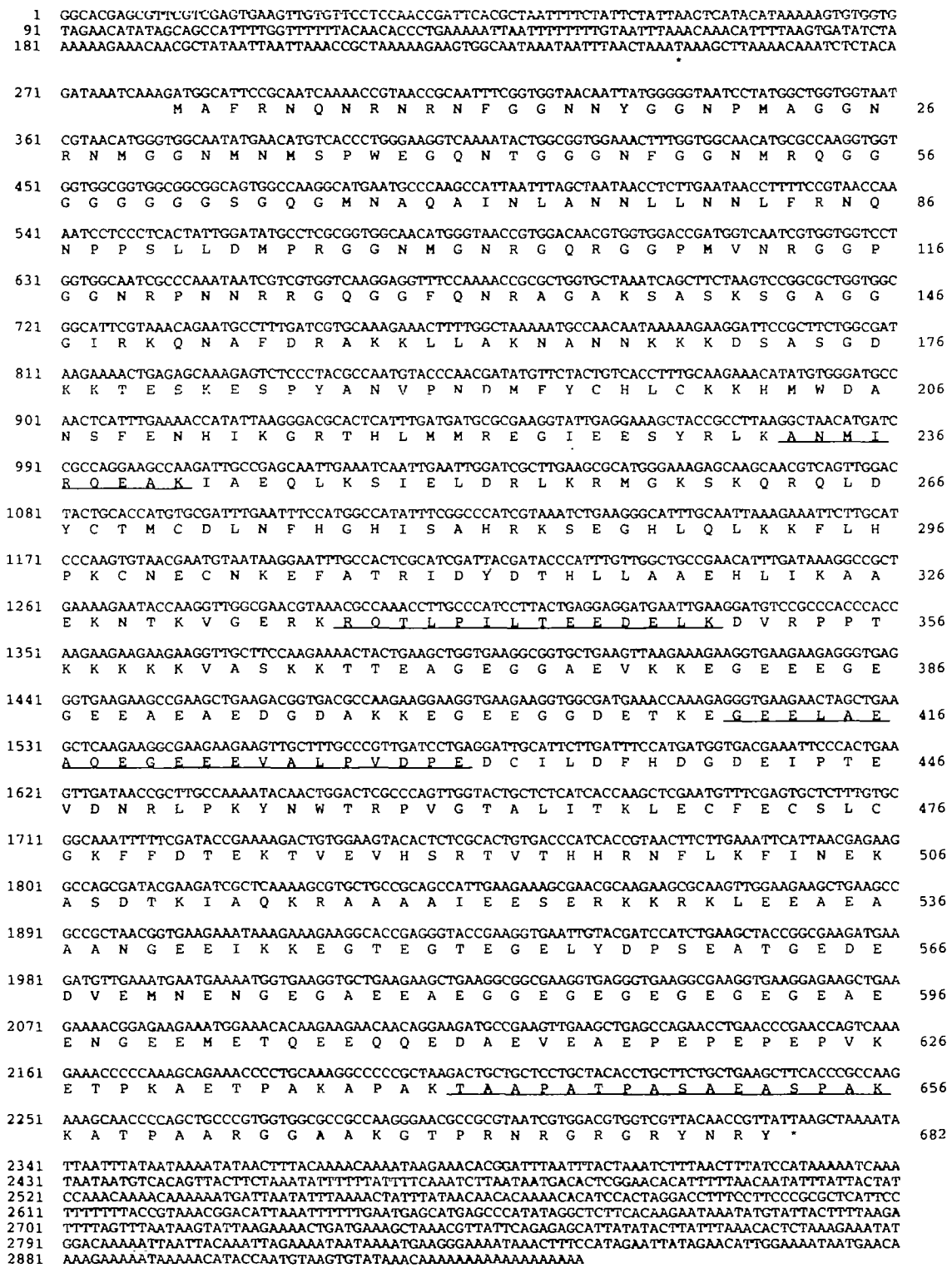

Fig. 1. **Nucleotide sequence of CBBP cDNA.** The deduced amino acid sequence is shown below the nucleotide sequences. Nucleotide numbers are given at the left and amino acid numbers starting from the first Met are given at the right of each line. Amino acid sequences determined in advance are underlined. The poly(A) addition signal is boxed. Asterisks indicate in-frame termination codons.

eluted with buffer B containing 1 M NaCl. The eluate was dialyzed against 10 mM Tris/HCl, pH 7.6, containing 2 mM dithiothreitol.

Cell Fractionation—All procedures were carried out at 4°C. Hemocytes were suspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 10% sucrose, 0.2% Triton X-100, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, 4 mM dithiothreitol) at a density of 1×10^8 cells/ml and then homogenized. Nuclei were pelletted by centrifugation at 1,200 ×*g* for 5 min and the resulting supernatant was used as the cytoplasmic fraction. The pellet was resuspended in 10 volumes of 0.25 M sucrose/3.3 mM $CaCl_2$, and then the suspension was layered onto 0.88 M sucrose and centrifuged at 1,200 ×*g* for 8 min. The pellet was resuspended and sonicated in lysis buffer, and then the suspension was centrifuged at 7,500 ×*g* for 5 min. The supernatant was used as the nuclear fraction.

Immunoblotting—Proteins separated by electrophoresis (*12*) were transferred electrophoretically from the gel onto a polyvinylidene difluoride membrane (Millipore). The filter was immersed in a blocking solution (PBS containing 5% skim milk and 0.1% Tween 20) for 1 h at room temperature. Then the filter was successively treated with anti-CBBP or anti–*Sarcophaga* histone H1 serum and horseradish peroxidase–conjugated anti–rabbit IgG. Signals were detected with an ECL Western blotting detection reagent (Amersham) according to the manufacturer's instructions.

Affinity Purification of Anti-CBBP Antibody—Affinity purification of anti-CBBP antibody was achieved in the following way. Recombinant CBBP (200 μg) was electrophoresed on a 7.5% SDS polyacrylamide gel, blotted onto a polyvinylidene difluoride membrane (Millipore), and then stained with Ponceau S. The band of recombinant CBBP was excised and incubated with the 6-fold diluted anti-CBBP antibody at 4°C for 40 h. Then the membrane was rinsed in a rinse solution (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.25% skim milk, 0.01% sodium azide), and the antibody was extracted from the membrane with 0.2 M glycine/HCl (pH 2.5). The extract was neutralized with 1 M KOH and then BSA was added to the extract to a final concentration of 1%.

Immunofluorescence Study—Hemocytes were suspended in insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$) at a density of 10^6 cells/ml, and 15 μl of the suspension was placed in each well of a 10-well multititest slide. The hemocytes were allowed to settle for 15 min at room temperature and then fixed for 2 min with acetone at room temperature. The slides were blocked in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 1% BSA for 1 h, and then successively treated with affinity-purified anti-CBBP antibody (1.3 μg/ml) and fluorescein isothiocyanate (FITC)–labeled secondary antibody (DAKO).

UV-Crosslinking—Recombinant CBBP (0.5 μg) or BSA (1 μg) and probe RNA (0.5 ng) were incubated in 20 μl of reaction buffer (10 mM Tris/HCl, pH 7.6, 50 mM NaCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol) on ice for 30 min. Then, the reaction mixture was irradiated under an Ultraviolet Crosslinker (wavelength, 254 nm. UVP) for 20 min. Then samples were treated with RNase A (1 mg/ml) and subjected to SDS polyacrylamide gel electrophoresis followed by autoradiography. Probe RNAs were synthesized essentially as described previously (*7*, *8*). Briefly, 3′-UTR, coding region, and 5′-UTR of cathepsin B mRNA were separately amplified by PCR, cloned into the *Xho*I/*Eco*RI-digested pBluescript SK(+) expression vector in an orientation allowing the sense strand to be transcribed by T7 RNA polymerase, and then transcribed in the presence of [α-^{32}P]UTP.

In Vitro Translation—Capped cathepsin B mRNAs (full length mRNA, 3′-UTR–deleted mRNA, 5′-UTR–deleted mRNA, and 3′- and 5′-UTR–deleted mRNA) were synthesized with T7 RNA polymerase as described previously (*7*, *8*). Firefly luciferase mRNA was synthesized by transcribing pSP-luc$^+$ (Promega) using SP6 RNA polymerase. The translation reaction was performed in a rabbit reticulocyte lysate (Pharmacia) in the presence of [^{35}S]methionine essentially as described previously (*8*).

RESULTS

Cloning of CBBP cDNA—To identify the CBBP cDNA, we determined the amino acid sequences of four peptides derived from the purified CBBP. They were RQTLPIL-TEEDELK, ANMIRQEAK, XGEELAEAQEGEEEVALPV-DPE (X indicates an unidentified residue) and TAAPA-TPASAEASPAK. The CBBP cDNA isolated from an NIH-Sape-4 cDNA library contained an open reading frame consisting of 682 amino acid residues (Fig. 1). The amino acid sequence contained the four peptide sequences presented above. Moreover, an in-frame termination codon was present upstream of the first Met codon, indicating that this cDNA is the full length cDNA for CBBP.

The molecular mass of CBBP in larval hemocytes was estimated to be 110 kDa on SDS polyacrylamide gel electrophoresis (Fig. 2B), whereas that of CBBP calculated from the putative amino acid sequence was 74 kDa. To explain this discrepancy, we produced the recombinant CBBP as a C-terminally histidine-tagged fusion protein. The purified recombinant CBBP gave two bands corresponding to molecular masses of 110 and 105 kDa (Fig. 2A). Both bands reacted with anti-CBBP antibody on immunoblotting (Fig. 2B). Thus, we assume that CBBP comprising 682 amino acid residues behaves as a 110-kDa protein on electrophoresis, and that the 105-kDa protein is its degradation product.

Fig. 2. **Electrophoresis and immunoblotting of recombinant CBBP.** (A) Recombinant CBBP (0.5 μg) was subjected to 7.5% SDS polyacrylamide gel electrophoresis. (B) A hemocyte lysate of third instar larvae which contained 110-kDa CBBP and recombinant CBBP were subjected to immunoblotting with anti-CBBP antibody. Lane 1, hemocyte lysate (5 μg); lane 2, recombinant CBBP (40 ng). The gels were calibrated with molecular mass markers.

CBBP Is a Homologue of Drosophila PEP—Database searches revealed that the primary sequence of CBBP is highly homologous to that of *Drosophila* PEP (*9*). The amino acid identity between CBBP and PEP was 72%, when the sequences were arranged to attain maximum matching (Fig. 3). Like *Drosophila* PEP, CBBP contained four C_2H_2 type zinc fingers (*13*) and four RGG boxes (*14*), which are known as RNA binding motifs, nuclear localization signals, acidic domains, and a Gly- and Asn-rich domain at its N-terminal region. Thus, we concluded that CBBP is the *Sarcophaga* homologue of *Drosophila* PEP.

Drosophila PEP was reported to be a component of hnRNP complexes, and was shown to associate preferentially with active ecdysone-inducible puffs on polytene chromosomes (*9*, *10*). However, the function of *Drosophila* PEP is not clear, although it is speculated to be involved in the transcription or post-transcriptional regulation of ecdysone-inducible genes.

Intracellular Localization of CBBP in Larval Hemocytes—We originally detected CBBP in the lysate of larval hemocytes (*7*), and purified it from a cytoplasmic fraction of NIH-Sape-4 cells (*8*). However, as *Drosophila* PEP was reported to be localized in the nucleus (*9*, *10*), we examined the intracellular localization of CBBP in larval hemocytes. For this, we fractionated larval hemocytes into cytoplasmic and nuclear fractions, and then subjected these fractions to immunoblotting. CBBP was detected in both the cytoplasmic and nuclear fractions under the conditions where histone H1 was detected only in the nuclear fraction (Fig. 4A). These results were confirmed by an immunofluorescence study. The fluorescence of affinity-purified anti-CBBP antibody was detected in both the nuclei and cytoplasm, al-

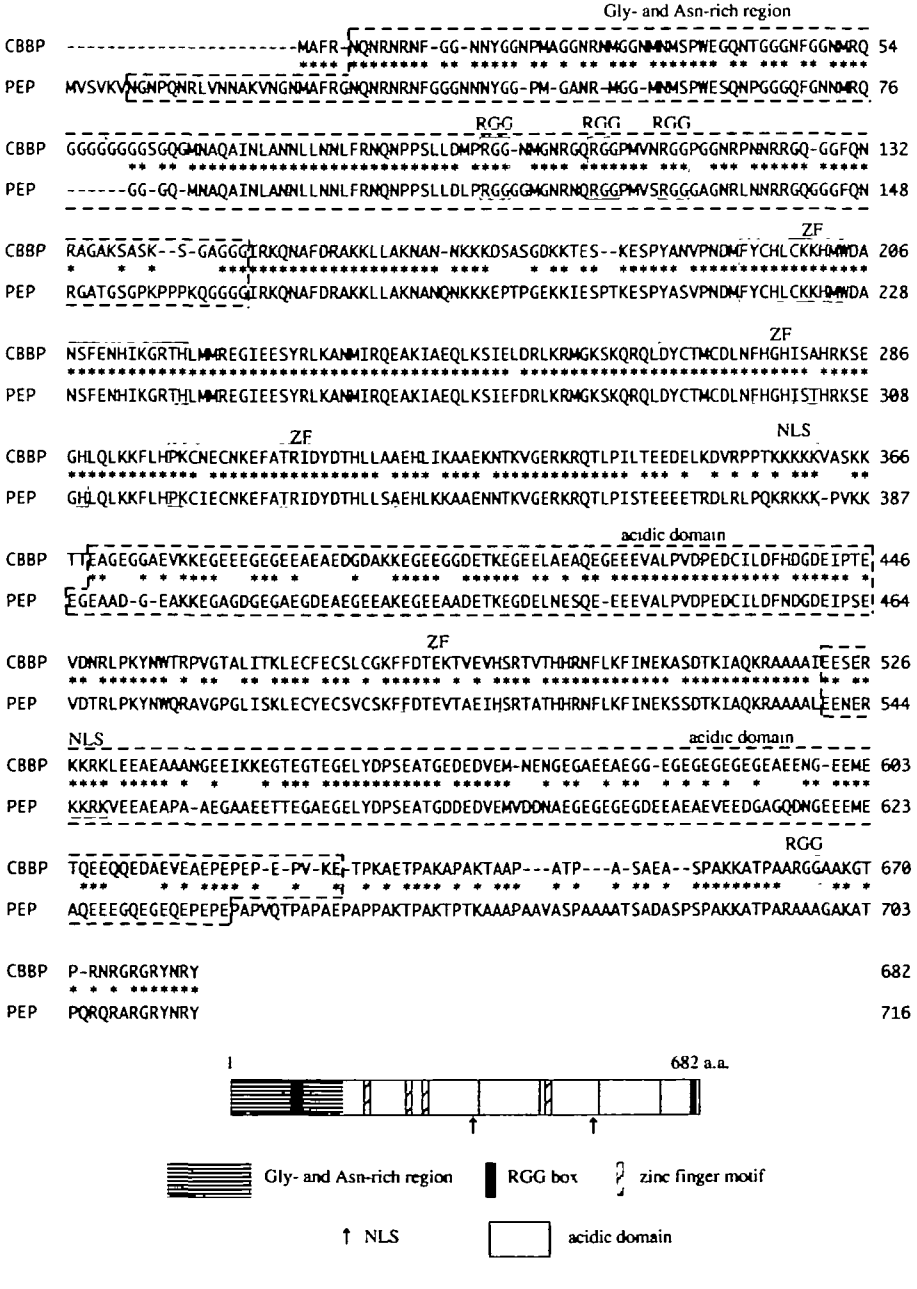

Fig. 3. **Sequence comparison of CBBP with *Drosophila* PEP.** Gaps were introduced to obtain maximal matching. Identical amino acid residues are indicated by asterisks. RGG box motifs (RGG), zinc finger motifs (ZF), and putative nuclear localization signals (NLS) are boxed. The N-terminal Gly- and Asn-rich region and acidic domains are boxed with dotted lines. A schematic illustration of CBBP is presented at the bottom.

though the fluorescence in the cytoplasm was fainter than that in the nuclei (Fig. 4B). The fluorescence in the nuclei was not homogeneous, suggesting the heterogeneous distribution of CBBP in ecdysone-responsive loci, as previously reported for *Drosophila* PEP (*9*, *10*). From these results, we concluded that CBBP is present in both the nuclei and cytoplasm of larval hemocytes.

Inhibition of Translation by Recombinant CBBP In Vitro—Previously we showed that the 90-kDa CBBP preferentially repressed the translation of cathepsin B mRNA having a 3′-UTR *in vitro* (*8*). We examined whether or not recombinant CBBP retained the same character. As shown in Fig. 5A, recombinant CBBP repressed the translation of cathepsin B mRNA in a dose-dependent manner. However, unlike the 90-kDa CBBP, this inhibition did not necessarily depend upon the 3′-UTR (Fig. 5B). Recombinant CBBP repressed the translation of cathepsin B mRNA irrespective of the presence of 3′-UTR and/or 5′-UTR. It also repressed the translation of firefly luciferase mRNA. From these results, we concluded that recombinant CBBP nonspecifically inhibits the translation reaction *in vitro* in a rabbit reticulocyte lysate.

We examined whether or not recombinant CBBP has RNA-binding activity. For this, we separately synthesized the 5′-UTR, coding region and 3′-UTR of cathepsin B mRNA, and then examined their binding to recombinant CBBP by means of UV cross-linking. As shown in Fig. 6,

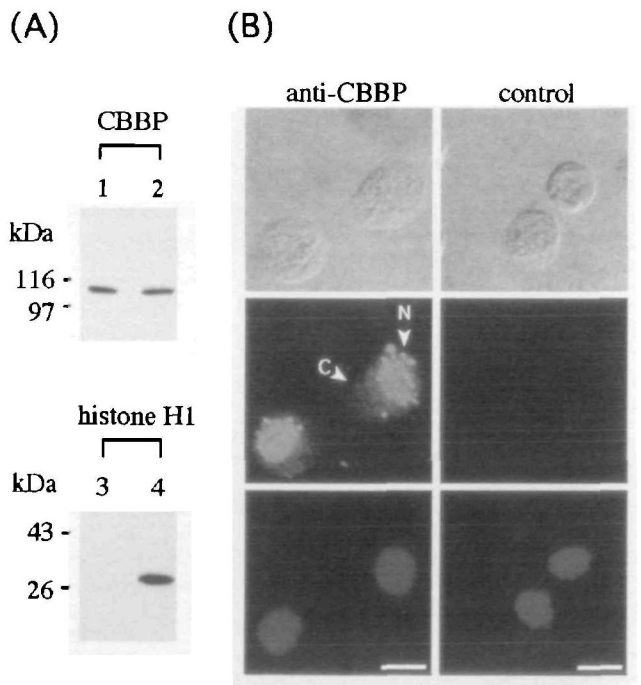

Fig. 4. **Intracellular localization of CBBP in larval hemocytes.** (A) Detection of CBBP in the cytoplasmic and nuclear fractions of larval hemocytes on immunoblotting. Cytoplasmic and nuclear fractions of larval hemocytes were subjected to 7.5% (top) or 12.5% (bottom) SDS polyacrylamide gel electrophoresis followed by immunoblotting with anti-CBBP antibody (top) or anti-*Sarcophaga* histone H1 antibody (bottom). Lane 1, cytoplasmic fraction (5 μg); lane 2, nuclear fraction (1 μg); lane 3, cytoplasmic fraction (50 μg); lane 4, nuclear fraction (10 μg). (B) Immunofluorescence study of CBBP in larval hemocytes. Larval hemocytes were successively treated with anti-CBBP antibody (left) or control antibody (right), and then FITC-conjugated secondary antibody. Top panels, bright field; middle panels, fluorescence microscopy of FITC; bottom panel, fluorescence microscopy of propidium iodide (nuclei). C and N represent cytoplasm and nucleus, respectively. The bar indicates 10 μm.

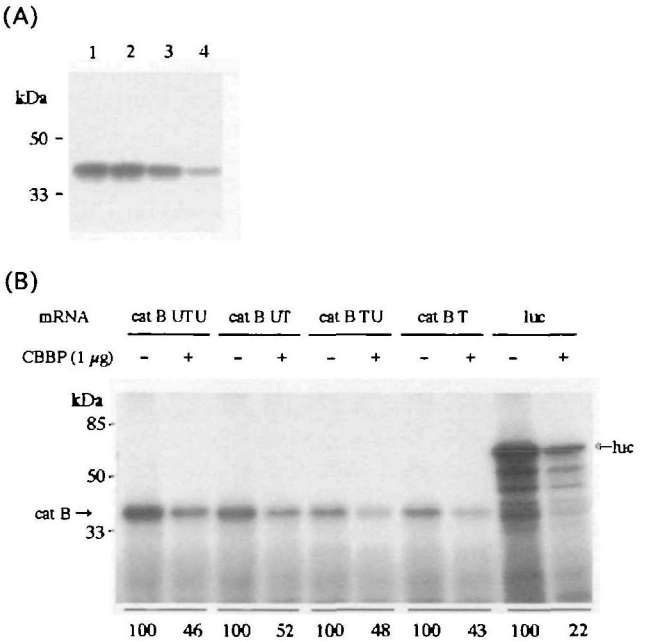

Fig. 5. **Repression of cathepsin B mRNA translation by recombinant CBBP.** (A) Cathepsin B mRNA (0.1 μg) was translated in a rabbit reticulocyte lysate in the presence of increasing amounts of recombinant CBBP. [^{35}S]methionine-labeled products were subjected to 12.5% SDS polyacrylamide gel electrophoresis followed by autoradiography. Amounts of recombinant CBBP added: Lane 1, 0 μg; lane 2, 0.15 μg; lane 3, 0.5 μg; lane 4, 1 μg. (B) Inhibition of the translation of various mRNAs by recombinant CBBP. Each mRNA (0.1 pmol) was translated in a rabbit reticulocyte lysate in the absence or presence of 1 μg recombinant CBBP. cat B and luc denote cathepsin B and firefly luciferase, respectively. UTU, complete cathepsin B mRNA; UT, 3′-UTR–deleted mRNA; TU, 5′-UTR–deleted mRNA; T, 3′- and 5′-UTR–deleted mRNA. Translation of cathepsin B and luciferase mRNAs was quantified by scanning of the bands, and expressed as a percentage of the control level without the addition of recombinant CBBP.

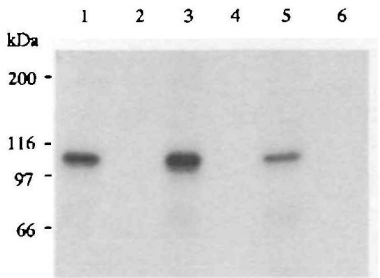

Fig. 6. **Binding of recombinant CBBP to cathepsin B mRNA.** Recombinant CBBP (0.5 μg) was incubated and UV-crosslinked with ^{32}P-labeled cathepsin B mRNA 3′-UTR, 5′-UTR, and coding region probes. Cross-linked RNA was detected by 7.5% SDS polyacrylamide gel electrophoresis followed by autoradiography. As a negative control, 1 μg of BSA was used instead of recombinant CBBP. Lane 1, recombinant CBBP and cathepsin B mRNA 3′-UTR; lane 2, BSA and cathepsin B mRNA 3′-UTR; lane 3, recombinant CBBP and cathepsin B mRNA 5′-UTR; lane 4, BSA and cathepsin B mRNA 5′-UTR; lane 5, recombinant CBBP and cathepsin B mRNA coding region; lane 6, BSA and cathepsin B mRNA coding region.

CBBP was shown to bind to all of these regions, but no appreciable binding was detected with BSA. Thus, recombinant CBBP seems to bind to various RNAs through its RNA-binding motifs.

DISCUSSION

CBBP was originally purified from the cytoplasmic fraction of NIH-Sape-4 cells as a 90-kDa protein that specifically binds to the 3'-UTR of cathepsin B mRNA (*8*). The 90-kDa CBBP was shown to repress the translation of cathepsin B mRNA in a 3'-UTR dependent manner in a rabbit reticulocyte translation system. However, the 90-kDa CBBP was found to be produced through partial proteolysis of the 110-kDa protein during the course of its purification. So far, we have characterized the artificially produced 90-kDa CBBP, not the intact 110-kDa CBBP.

In this study, we demonstrated that the 110-kDa CBBP is a *Sarcophaga* homologue of *Drosophila* PEP, and that it is located in both the nuclei and the cytoplasm. *Drosophila* PEP is a member of the hnRNP family. Recently, Hamann and Strätling reported that *Drosophila* PEP specifically binds to hsp70 transcripts by mainly recognizing two sequences, GAU and GRRCG (R = purine) (*15*). However, we could not identify specific binding sequences for the 110-kDa CBBP.

Although many hnRNPs are located in nuclei (*16*), some are known to function in the cytoplasm. For example, hnRNP A1 is involved in the transport of mRNA from the nucleus to the cytoplasm (*17*, *18*), and hnRNP K and E1 are involved in mRNA silencing in the cytoplasm during erythroid differentiation (*19*). Thus, CBBP is assumed to be involved in the translational regulation of a certain mRNA species, such as cathepsin B mRNA, in the cytoplasm.

We found that recombinant CBBP inhibited the translation of cathepsin B mRNA and firefly luciferase mRNA, indicating that it has the ability to inhibit translation like the 90-kDa CBBP. However, contrary to 90-kDa CBBP, recombinant CBBP inhibited the translation of cathepsin B mRNA regardless of its 3'-UTR. This may be explained by the difference in the number of RNA-binding motifs between the two proteins.

Previously, we determined the partial N-terminal amino acid sequence of the 90-kDa CBBP to be XXXXNKKKDSA, in which X is an unidentified residue (unpublished result). The sequence of NKKKDSA was located between residues 166 and 172 of the 110-kDa CBBP. If we assume that the 90-kDa CBBP was produced through cleavage between residues 161 Ala and 162 Lys of the 110-kDa CBBP, the resulting 90-kDa CBBP should lack the Gly- and Asn-rich region that contains three RGG boxes (Fig. 3). Possibly, deletion of these RNA binding motifs changes the binding specificity of the 90-kDa CBBP so that it binds preferentially to the 3'-UTR of cathepsin B mRNA.

It is questioned that the 110-kDa CBBP in fact participates in the translational control of cathepsin B mRNA in larval *Sarcophaga* hemocytes. Judging from our present results, the inhibition of translation by recombinant CBBP is not specific for cathepsin B mRNA. As the binding of recombinant CBBP to cathepsin B mRNA does not seem to depend upon a specific RNA sequence, it is likely to interefere with the translation of any mRNA used by binding to it nonspecifically. Even so, we cannot exclude the possibility of participation of CBBP in the translational regulation of cathepsin B mRNA in larval *Sarcophaga* hemocytes.

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