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Expression of a Synthetic Gene for Initiation Factor 4E-Binding Protein 1 in *Escherichia coli* and Its Interaction with eIF-4E and eIF-4E·m^{7}GTP Complex[1]

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An artificial gene coding for the human initiation factor (eIF) 4E-binding protein 1 (4E-BP1) was chemically synthesized and cloned. Although the expression of the 4E-BP1 gene alone has not yet been accomplished, the gene was expressed in *Escherichia coli* [BL21(DE3)] as a fusion gene with the glutathione-S-transferase (GST) gene using a prokaryotic gene fusion vector (pGEX-4T-2), which contains a gene sequence coding the cleavage site for a specific protease, α-thrombin. The fusion gene product was purified to homogeneity by glutathione Sepharose-4B affinity column chromatography. It was shown by m7GTP- and glutathione-affinity chromatography that the binding ability of 4E-BP1 to eIF-4E is nearly the same as that to the eIF-4E·m^{7}GTP complex, implying different binding sites of eIF-4E and its nonallosteric obligation for 4E-BP1 and mRNA cap structure. In contrast with the binding of eIF-4E to the mRNA cap structure, where some functional amino acids play an important role in the binding, the binding to 4E-BP1 was suggested to occur *via* multiple nonspecific interactions.

Key words: 4E-BP1, eIF-4E, expression, interaction, synthetic gene.

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For the efficient translation of mRNA, systematic interaction is required between the mRNA and eukaryotic initiation factor (eIF)-4 proteins, eIF-4A, eIF-4B, eIF-4E, and eIF-4G (*1*). Among these proteins, eIF-4E plays a key role in the initial step of mRNA translation (*2-4*), which is initiated by the binding of eIF-4E to the capped structure (characterized by the presence of 7-methylguanosine-5′,5′-triphosphate) of eukaryotic mRNA. Recent studies have demonstrated that the biological function of eIF-4E is controlled by its phosphorylation (*5*) and by the eIF-4E binding proteins 1 and 2 (4E-BP1 and 4E-BP2) (*6, 7*). These conclusions are based on the observations that the cap binding ability of eIF-4E is increased three to fourfold by the phosphorylation of Ser-209 (*8*), and both 4E-BP1 and 4E-BP2 inhibit cap-dependent mRNA translation through complex formation with eIF-4E and inhibition of the formation of eIF-4F complex (assembly of eIF-4A, eIF-4E, and eIF-4G) (*9-11*). Since 4E-BP1 is a phosphoprotein, also known as PHAS-I, which exhibits a reduced level of complex formation with eIF-4E when the Ser-64 is phosphorylated (*12, 13*), a close relation between phosphorylation and protein synthesis has been suggested.

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Abbreviations: 4E-BP1, initiation factor 4E-binding protein 1; GST, glutathione-S-transferase; eIF-4E, eukaryotic initiation factor 4E; m^{7}GTP, 7-methylguanosine 5′-triphosphate; PHAS-I, phosphorylated heat- and acid-stable protein-I; m^{7}GDP, 7-methylguanosine 5′-diphosphate; IPTG, isopropyl-β-D-thiogalactopropyranoside.

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In contrast to the progressive clarification of the biological functions of eIF-4E in the initiation and regulation of protein synthesis, detailed mechanisms by which the specific bindings of eIF-4E to the mRNA cap structure and to 4E-BPs take place are far from being fully understood, although such information is indispensable for understanding the molecular mechanisms of protein biosynthesis at the atomic level. Thus, we have been investigating the mode of interaction of eIF-4E with mRNA cap analogues by spectroscopic and X-ray structural methods (*14-18*); very recently, the results of the X-ray crystal analysis of the eIF-4E·m^{7}GDP complex have been reported (*19*). As a part of the structural evaluation of the biological function of eIF-4E, we report, in this paper, on the expression of a synthetic 4E-BP1 gene in *Escherichia coli*, the purification of the recombinant protein, and its interaction with wild-type and mutant eIF-4Es in the presence or absence of m^{7}GTP (a model molecule for capped mRNA).

MATERIALS AND METHODS

Materials—Enzymes (T4 DNA ligase, T4 polynucleotide kinase, Taq DNA, T7 RNA polymerase, restriction endonucleases, RNase A, α-thrombin, and so on) were purchased from Takara Shuzo, Toyoboseki, Bethesda Research Laboratories, P-L Biochemicals, or Novagen. The bacterial strain, *E. coli* BL21(DE3) (Novagen), was used for the transfections. pGEX-4T-2 [a glutathione-S-transferase (GST) gene fusion vector], m^{7}GTP- and glutathione-Sepharose 4Bs (the affinity resins for 4E-BP1 purification), and the AutoCycle Sequencing™ Kit were purchased from Pharmacia Biotech. The markers for molecular weight

estimation were obtained from Bio-Rad. Other commercially available materials used were of reagent grade or higher quality.

Synthesis of the 4E-BP1 Gene—For the construction of the gene, optimal codons for *E. coli* were used in the expectation that this would generate a high level of expression, and many restriction sites were incorporated into the gene to facilitate insertion into the vector and mutation. Oligodeoxyribonucleotides typically 70 nucleotides in length (U1 to U6, and L1 to L6 in Fig. 1) were synthesized on a 5 nmol scale using a DNA synthesizer. The purification of the respective oligodeoxynucleotides and their ligation to the 4E-BP1 gene by T4 DNA ligase were carried out using the method reported for the construction of the human eIF-4E gene (*20*). The purity of the synthetic gene was checked by electrophoresis on a polyacrylamide gel.

Construction of the Expression Vector and Cloning—The synthetic gene of 4E-BP1 was digested with *Eco*RI and *Sal*I, isolated by agarose gel electrophoresis, and subcloned into the *Eco*RI and *Sal*I sites of pGEX-4T-2 vector. Calcium chloride-treated *E. coli* [BL21(DE3)] cells (100-200 μl), which were prepared according to the Morrison method (*21*), were transformed with the recombinant plasmid (10-20 μl) and were plated on ampicillin (20-50 μg/ml)-containing LB (Luria-Bertani's broth) plates. After the selection of clones carrying the synthetic 4E-BP1 gene, the gene sequence was confirmed using the standard dideoxy-sequencing procedure.

Expression of the Synthetic Gene as a Fusion Gene with the GST Gene—The transformed *E. coli* cells [BL21(DE3)/pGEX-4T-2-4E-BP1] were grown in LB medium containing 100 μg/ml ampicillin at 37°C in a jar fermenter.

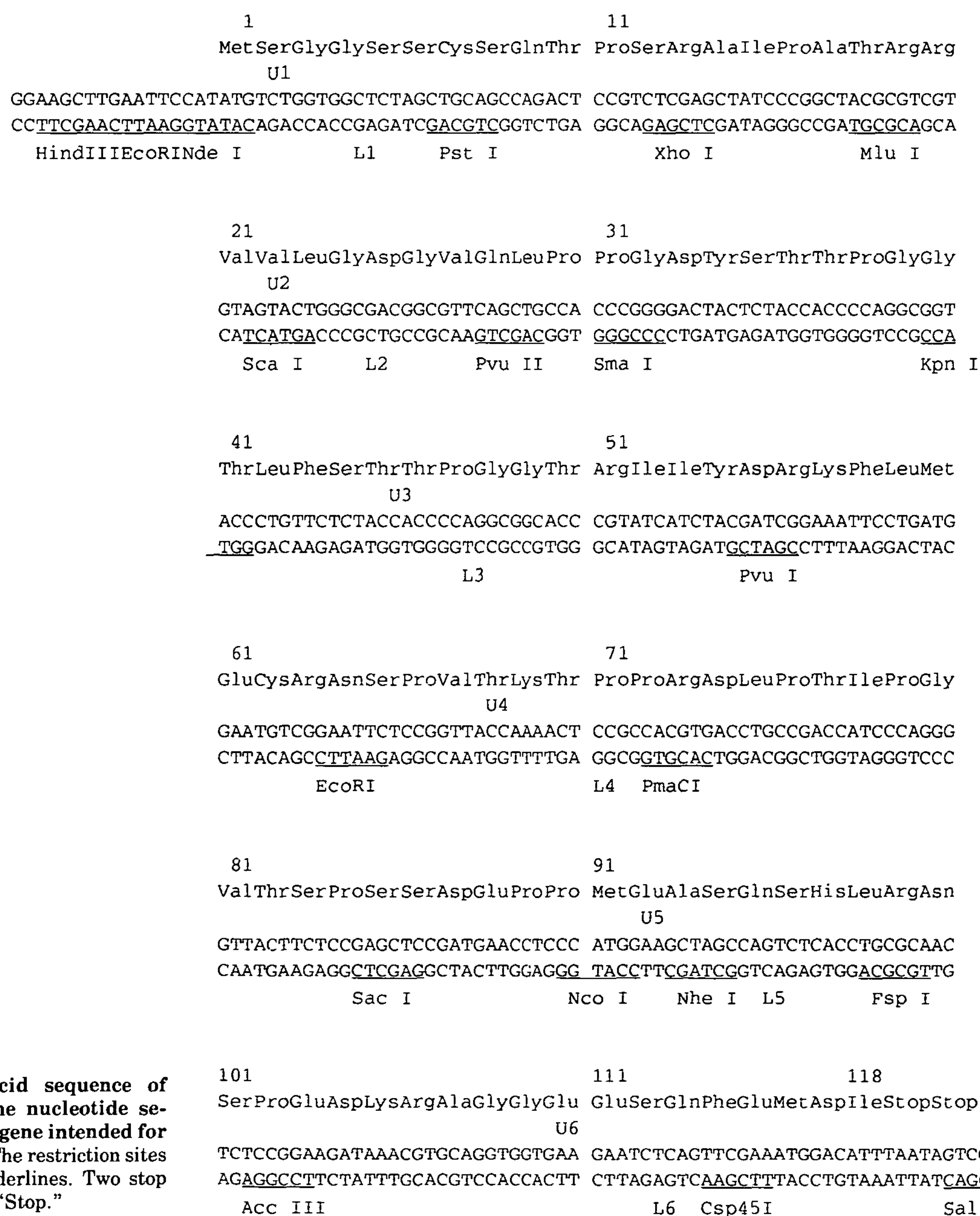

Fig. 1. **The amino acid sequence of human 4E-BP1 and the nucleotide sequence of its synthetic gene intended for expression in *E. coli*.** The restriction sites are indicated by the underlines. Two stop codons are indicated by "Stop."

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Expression of the gene was induced by addition (0.1 mM) of isopropyl-β-D-thiogalactopropyranoside (IPTG) to the medium. Induction continued for 12 h, after which the cells were harvested and disrupted by sonication at 20 kHz and 100 W (30 s each, 10 times, Model UD-200, Tomy). Pellets and supernatants were separated by centrifugation ($15,000 \times g$ at 4°C for 30 min), and aliquots from each fraction were analyzed by 15% SDS-PAGE.

Purification of the Fusion Protein and Isolation of 4E-BP1—The supernatant was applied to a glutathione Sepharose 4B affinity column equilibrated with buffer A [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4] and the column was washed with the same buffer until the optical density of the eluted buffer returned to the baseline level. The purified GST-4E-BP1 fusion protein was eluted with buffer B [50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione].

In addition, the column to which GST-4E-BP1 was bound was reacted with 0.5% α-thrombin in 20 mM Tris-HCl (pH 8.4) buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ at 25°C for 1 h. The recombinant 4E-BP1 was eluted with 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, and was purified by two-step column chromatography using a Mono S cation exchange column and then a Sephadex G-75 gel filtration system.

Binding Ability of 4E-BP1 to Wild-Type and Mutant eIF-4Es—Wild-type or mutant eIF-4Es, prepared according to a method outlined in a previous paper (*17*), were applied to the GST-4E-BP1-bound glutathione affinity column, which was then washed thoroughly with buffer A. The bound proteins were eluted with buffer B and analyzed by 15% SDS-PAGE. The experiments were repeated five times, and the data obtained were averaged.

RESULTS AND DISCUSSION

Expression of the 4E-BP1 Gene—Since the amino acid sequence of human 4E-BP1 has previously been reported (*6*), the synthetic 4E-BP1 gene was designed on the basis of the frequency of codon usage in *E. coli* with the aim of obtaining a high level of expression of the gene. The nucleotide sequence of the synthetic gene was designed to exclude palindromes and any directly repeated sequence of over seven nucleotides. For ligation of the 4E-BP1 gene to the vector, restriction sites for *Eco*RI and *Sal*I were generated in front of the start codon and next to the stop codon, respectively. The entire gene was divided into 12 DNA fragments of approximately 70 nucleotides in length (Fig. 1).

After investigating many vectors and strains, pGEX-4T-2 and BL21(DE3) were finally used as the plasmid and host bacterial strain for the expression of the 4E-BP1 gene, respectively. Plasmid pGEX-4T2-4E-BP1 for expression of the GST-4E-BP1 fusion protein was constructed (Fig. 2).

Purification and Isolation of Recombinant 4E-BP1—The purification of the fusion protein was carried out using a glutathione-Sepharose 4B affinity column. The supernatant fraction of the cells in which expression of the fusion protein has been induced was applied to the affinity column equilibrated with buffer A. After the column was thoroughly washed with buffer A, the purified fusion protein was obtained by elution with buffer B (Fig. 3, lane 1). In another experiment, the GST-4E-BP1 bound to the column was digested with α-thrombin in the equilibrated affinity column and then 4E-BP1 was eluted with buffer A (Fig. 3, lane 2). The eIF-4E binding ability of the isolated 4E-BP1, which was purified by cation exchange and then gel filtration chromatography, was confirmed by SDS-PAGE analysis after eIF-4E-bound m^7GTP affinity column chromatography (see Fig. 4, lane 2).

Binding Ability of 4E-BP1 to eIF-4E and the eIF-4E·m^7GTP Complex—As already stated in the introduction, it has been reported that 4E-BP1 regulates the cap-binding activity of eIF-4E by sequestering eIF-4E (*6*). 4E-BP1 is capable of binding eIF-4E, and is thereby able to prevent the assembly of the eIF-4F complex (*9-11*). However, the following questions have not yet been answered: (i) which region of eIF-4E interacts with 4E-BP1? [the region of 4E-BP1 binding to eIF-4E has been proposed but not yet verified (*10, 22*)], and (ii) to what extent is the interaction of eIF-4E with the cap structure affected by the simultaneous binding of 4E-BP1? [although eIF-4E has been reported to be able to bind simultaneously to both 4E-BP1 and the mRNA cap structure (*6, 22*)]. In order to obtain answers to these questions, the binding abilities of 4E-BP1 to eIF-4E and the eIF-4E·m^7GTP complex (a model for the eIF-4E·capped mRNA complex) were investigated using the GST-

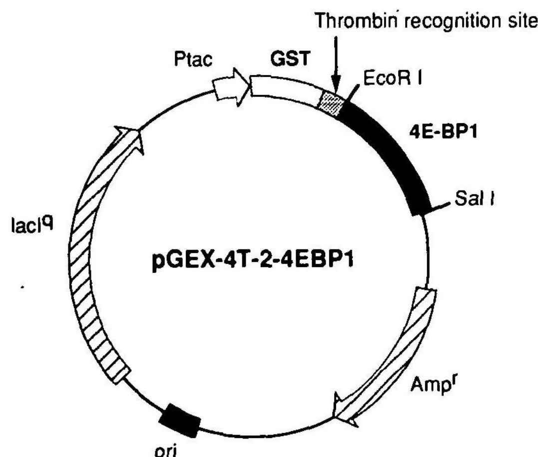

Fig. 2. **Construction of the fusion expression plasmid.** Plasmid pGEX-4T-2 was cleaved with *Eco*RI and *Sal*I, and the phosphorylated 4E-BP1 gene was inserted into the vector by ligation.

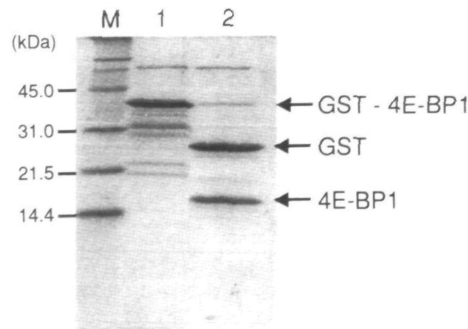

Fig. 3. **SDS-PAGE analyses of the GST-4E-BP1 fusion protein and separation of the protein by digestion with α-thrombin.** Lane 1, fusion protein eluted from the glutathione affinity column; lane 2, fusion protein digested by α-thrombin.

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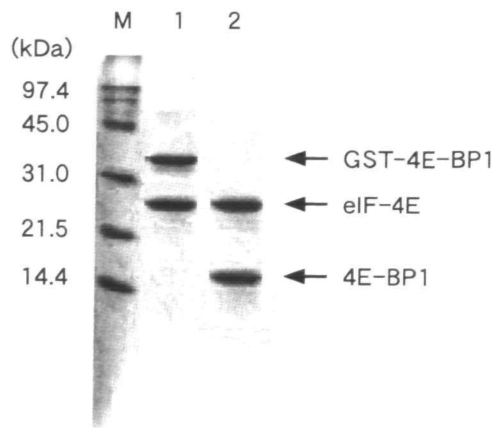

Fig. 4. **SDS-PAGE analyses of the eIF-4E binding ability of 4E-BP1 in the fusion with GST and in the isolated state.** Lane 1, 15% SDS-PAGE of GST-4E-BP1-eIF-4E complex, which was prepared by applying eIF-4E to the GST-4E-BP1-bound glutathione Sepharose 4B affinity column and then eluting with buffer B; lane 2, 15% SDS-PAGE of 4E-BP1-eIF-4E complex, which was prepared by applying the isolated 4E-BP1 to the eIF-4E-bound m^7GTP affinity column and then eluting with buffer C containing 100 μM m^7GTP.

4E-PB1 fusion protein and the glutathione affinity column; the eIF-4E binding ability of 4E-BP1 in the fusion with GST was shown to be almost the same as that in the isolated form (Fig. 4). Recombinant eIF-4E and its complex with m^7GTP were prepared according to the method described in a previous paper (*17*). After equilibration of the mixture of the GST-4E-BP1 fusion protein and eIF-4E or the eIF-4E$\cdot m^7GTP$ complex for 1 h at room temperature, it was applied to the affinity column. The column was washed with buffer A, and the bound materials were eluted with buffer B and analyzed by 17.5% SDS-PAGE; total protein was determined by the Bradford method (*23*). On the other hand, the binding ability of GST-4E-BP1 to eIF-4E$\cdot m^7GTP$ complex was also estimated using the m^7GTP-Sepharose affinity column, to which an equilibrated mixture of GST-4E-BP1 and eIF-4E was applied. After washing of the column with buffer C [20 mM Hepes-KOH (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl], the bound materials were eluted with buffer C containing 100 μM m^7GTP, and the total protein was determined by the Bradford method.

The binding ability was estimated as the ratio of eIF-4E bound to GST-4E-BP1 with respect to the applied (total) quantity of eIF-4E. An average value of 0.7 was obtained for eIF-4E, which was not different from that for the eIF-4E$\cdot m^7GTP$ complex, 0.7 ± 0.1; the ratio of 0.8 for the 4E-BP1-eIF-4E$\cdot m^7GTP$ pair was obtained from the m^7GTP affinity chromatography data. These results indicate that the binding of 4E-BP1 does not significantly affect the interaction of eIF-4E with the mRNA cap structure (nonallosteric behavior of eIF-4E).

Comparison of the Binding of 4E-BP1 to Wild-Type and Mutant eIF-4Es—In order to investigate possible binding regions and modes of interaction of eIF-4E with 4E-BP1, the binding abilities of various eIF-4E mutants were compared; a series of eIF-4E mutants had previously been prepared to identify the amino acid residues responsible for the recognition of the mRNA cap structure, in which each of 15 conserved and functional amino acids was replaced with a nonpolar Leu or Ala residue (*17*). After the expression quantities of all mutant genes in *E. coli* were confirmed to be nearly the same within the experimental errors, the supernatant fractions of the induced cells were equilibrated with the GST-4E-BP1 fusion protein for 1 h at 37°C, and the respective mixtures were applied to the glutathione-Sepharose affinity column, and then the bound proteins were eluted with buffer B. The binding ability to GST-4E-BP1 was estimated as the total quantity of each bound mutant with respect to the wild type eIF-4E. The results are given in Table I, in which the binding ability to the mRNA cap affinity column of each eIF-4E mutant (*17*) is also given for comparison.

TABLE I. **Binding ability of 4E-BP1 to wild-type and mutant eIF-4Es.** The cap-binding ability of each mutant form is also given for comparison.

Mutant protein	Binding to 4E-BP1[a]	Binding to m^7GTP[b]
Wild-type	+++	+++
W43L	+++	−
W46L	+++	−
W56L	+++	+
W73L	+++	−
W102L	+++	−
W113L	+++	+
W130L	+++	−
W166L	+++	−
E103A	+++	−
D104A	+++	++
E105A	+++	++
H33A	+++	+++
H37A	+++	+
H200A	+++	−

[a,b]Binding to 4E-BP1 or m^7GTP is shown as: +++, comparable to wild-type cap-binding ability; ++, 40-80% of wild-type cap-binding ability; +, 20-40% of wild-type cap-binding ability; and −, less than 20% of wild-type cap-binding ability.

As is clear from this table, a significant difference can be seen between the modes of binding of eIF-4E to the cap structure and to 4E-BP1. Obviously, the recognition of the mRNA cap structure by eIF-4E is dependent on some amino acids, which can interact specifically with the cap structure, *e.g. via* π-π stacking or hydrogen bond interaction (*17*). In contrast, no notable difference was observed in the ability of 4E-BP1 to form complexes with eIF-4E mutants. Although it could not be discounted that in these mutants the mutated amino acid is not located at the 4E-BP1 binding region, the results suggest that the binding of 4E-BP1 to eIF-4E is *via* multiple nonspecific interactions between the structurally complementary regions of both molecules; after the submission of this paper, it was reported by Matsuo *et al.* (*24*), based on an NMR study, that the 4E-BP binding site spreads over the α-helix, β-sheet, and several regions of non-regular secondary structure of eIF-4E, and this is consistent with our present result. In any event, it could be said that the conserved Trp residues in eIF-4E, although necessary for the recognition of the mRNA cap structure (*17, 19*), are not necessary in 4E-BP1 binding.

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