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Structural dissection of human translation elongation factor 1Bγ (eEF1Bγ): expression of full-length protein and its truncated forms

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Aim. *To gain more insights into properties of the human translation elongation factor eEF1Bγ and its interaction with partners we intended to produce the full-length protein and its truncated forms.* ***Methods***. *cDNAs encoding truncated forms of eEF1Bγ were generated by PCR amplification with respective primers and cloned into vectors providing polyhistidine, glutathione S-transferase or maltose binding protein tags. The recombinant proteins were expressed in Escherichia coli and purified by affinity chromatography. An aggregation state of the proteins was analyzed by analytical gel filtration.* ***Results***. *The expression, purification and storage conditions for the full-length recombinant His-eEF1Bγ were optimized. Several truncated forms of eEF1Bγ were also expressed and purified to homogeneity. Two short variants of C-terminal domain comprising amino acids 264–437 or 229–437 were obtained in monomeric state. Two short variants of N-terminal domain comprising amino acids 1–33 or 1–228, fused with glutathione S-transferase, were obtained and estimated to be dimers by gel filtration. The mutants of N-terminal domain comprising amino acids 1–92 or 1–163, fused with maltose binding protein, were obtained as soluble high molecular weight aggregates only.* ***Conclusions***. *The purified recombinant His-eEF1Bγ and several truncated forms of the protein were obtained and characterized. These protein variants will be used for further studies on the protein-protein interaction.*

Keywords: eEF1Bγ, protein structural domains, expression of recombinant proteins, protein purification.

Introduction. Elongation factor 1 (eEF1) is one of the major players during the elongation cycle of eukaryotic protein synthesis [1]. eEF1 consists of two functionally distinct parts: eEF1A and eEF1B. eEF1A is a G-protein ensuring the delivery of aminoacylated tRNA to the ribosome. eEF1B acts as a guanine nucleotide exchange factor which catalyzes the conversion of inactive eEF1A*GDP into active eEF1A*GTP bound form. In higher eukaryotes, the eEF1B complex consists of three proteins eEF1Bα, eEF1Bβ and eEF1Bγ.

Both eEF1Bα and eEF1Bβ are catalytic guanine nucleotide exchange subunits which interact with eEF1Bγ, a structural component of eEF1B complex. Beside the presence in the complex a functional role of eEF1Bγ is not clear. This subunit itself does not possess any exchange activity, but eEF1Bγ isolated from *Artemia salina* enhanced the catalytic activity of eEF1Bα *in vitro* [2]. eEF1Bγ was found to interact with tubulin and based on this observation the role of this protein in anchoring eEF1B complex to the membranes and/or cytoskeleton was proposed [2]. Moreover, eEF1Bγ was shown to bind and bundle specifically the keratin intermediate filaments [3]. The interaction between eEF1Bγ and keratin regulates protein synthesis in the epithelial cells, but the physiological relevance of this bidirectional relationship remains to be defined [3]. eEF1Bγ was suggested to possess nonspecific RNA binding properties [4]. eEF1Bγ subunit might be also a regulatory

element within the eEF1B complex. This subunit was found to be a substrate for the cell cycle protein kinase CDK1/cyclineB [5]. The phosphorylation of eEF1Bγ seems to be necessary for effective translation of valine-rich proteins [6]. The overexpression of eEF1Bγ subunit was observed in some gastric and esophageal carcinomas [7, 8]. Nuclear localization of eEF1Bγ in both normal and cancer tissues suggests an unknown nucleus-specific role in human cells [9]

eEF1Bγ consists of two independently folding structural domains connected by a lysine-rich linker [6, 10]. The C-terminal domain is a highly conserved exceptionally protease resistant domain which consists of five stranded, anti-parallel β-sheets surrounded by five α-helices [10]. A functional significance of this domain remains to be deciphered. By contrast, the N-terminal domain of eEF1Bγ is homologous to Theta class glutathione S-transferases (GST) [11] and interacts with eEF1Bα [2]. The crystallographic structure of yeast eEF1Bγ N-terminal domain was solved and found to be very similar to those of GST enzymes [12]. However, the activity of this domain toward the GST model substrate 1-chloro-2,4-dinitrobenzen (CDNB) was not detected [12].

To gain more insights into the properties of eEF1Bγ and its interaction with partners we elaborated a purification procedure of the full-length protein, designed and expressed its truncated forms. We demonstrate that the recombinant eEF1Bγ can be purified as a monomer and stored in the conditions preventing its multimerization. We show that individual N- and C-terminal domains of this protein could be also expressed in bacteria and purified under the conditions mostly preventing the formation of aggregates. By contrast, the attempt to express and obtain isolated subdomains from the eEF1Bγ N-terminal domain was unsuccessful.

Materials and methods. pET16b/eEF1Bγ plasmid containing ORF of human eEF1Bγ protein was kindly provided by Dr. G. Janssen (Leiden University, The Netherlands).

Supplementary information can be found on Web site http://www.biopolymers.org.ua.

Design of eEF1Bγ truncated forms. The conservative domains in the structure of eEF1Bγ proteins were defined by multiple sequences alignment of eEF1Bγ *Homo sapiens* (NP_001395.1), *Rattus norvegicus* (NP_001004223.1), *Bos taurus* (NP_001035577.1), *Xenopus laevis* (AAB29958.1), *Carassius auratus* (BAB64568.1), *Bombyx mori* (AEE28213.1), *Artemia* sp. (AAC83401.1) amino acid sequences using ClustalW program (Fig. S1).

Construction of the eEF1Bγ truncated forms with N- and C-terminal His-tag. To produce the constructs carrying C-terminal His-tag, the cDNA fragments encoding different truncated forms of eEF1Bγ were produced by PCR with following pairs of primers: appropriate 5' forward primers indicated below and common reverse 5'-aactcgagcttgaatattttgccctgattgaaggctttgccca primer. Forward primer for eEF1Bγ (34–437) fragment was 5'-ttccatggcaccaccccacttccattttggcc, for eEF1Bγ (93–437) – 5'-ttccatggcccaggtggtgcagtgggtga, for eEF1Bγ (229–437) – 5'-aaccatggacacaccacggaaagagaa gggttca, for eEF1Bγ (264–437) – 5'-aaccatggatgaatgtga gcaggcgct. PCR was conducted with DreamTaq or Pfu DNA Polymerase («Thermo Scientific», USA) using pET16b/eEF1Bγ plasmid as a template. PCR products were purified and digested by *XhoI* and *NcoI* («Thermo Scientific») and ligated into pET28b («Novagen». USA) expression vector digested by the same enzymes. Resulted recombinant plasmids pET28b/eEF1Bγ (Nr-437)-His encode truncated forms of eEF1Bγ with C-terminal His-tag.

Constructs with N-terminal His-tag were prepared in the same way. Forward primer for eEF1Bγ (34–437) fragment was 5'-tttcatatggcaccaccccacttccattttggcc, for eEF1Bγ (93–437) – 5'-tttcatatggcccaggtggtgcagtgggtga, for eEF1Bγ (229–437) – 5'-aaacatatggacacaccacggaaa gagaagggttca, for eEF1Bγ (264–437) – 5'-aaacatatggat gaatgtgagcaggcgct. Reverse primer 5'-aactcgagcttgaat attttgccctgattgaaggctttgccca was the same for all constructs. PCR was conducted with DreamTaq DNA Polymerase using pET16b/eEF1Bγ plasmid as a template. PCR products were purified and digested by *XhoI* and *NdeI* and ligated into *pET28a* («Novagen») expression vector digested by the same enzymes. Resulted recombinant plasmids pET28a/His-eEF1Bγ (Nr-437) encode truncated forms of eEF1Bγ with N-terminal His-tag.

Construction of the eEF1Bγ truncated forms with N-terminal GST-tag. The cDNA fragments encoding different truncated forms of eEF1Bγ were produced by PCR with following primers: 5' forward tttggatccatg gcggctgggaccctgtacac was the same for all mutants, reverse primer for eEF1Bγ (1–33) was 5'-tttctcgagtcag

gagagcacgcggacct, for eEF1Bγ (1–92) – 5'-tttctcgagtcat gctgcctctggagtactt, for eEF1Bγ (1–163) – 5'-tttctcgagtc atgtgatgtcagccaatgtcac, for eEF1Bγ (1–228) – 5'-tttctcg agtcacttttaggttgggtctctgc. PCR was conducted with Pfu DNA Polymerase using pET16b/eEF1Bγ plasmid as a template. PCR products were purified and digested by *BamHI* and *XhoI* and ligated into *pGEX6P-1* («GE Healthcare», USA) expression vector digested by the same enzymes. Resulted recombinant plasmids pGEX6P/GST-eEF1Bγ (1-Nr) encode truncated forms of eEF1Bγ in frame with GST sequence.

Construction of the eEF1Bγ truncated forms with N-terminal MBP-tag. The cDNA fragments encoding different truncated forms of eEF1Bγ were produced by PCR with following primers: 5' forward aagttctgtttcagggccc ggcggctgggaccctgtacacg was the same for all mutants, reverse primer for eEF1Bγ (2–33) was 5'-atggtctagaaa gctttaggagagcacgcggacctgag, for eEF1Bγ (2–92) – 5'-at ggtctagaaagctttaggctgctgcctctggagtacttcc, for eEF1Bγ (2–163) – 5'-atggtctagaaagctttagacaactgtgatgtcagccaat gtcac, for eEF1Bγ (2–228) 5'-atggtctagaaagctttatgtgtc ctttttaggttgggtctctgc. PCR was conducted using pET16b/eEF1Bγ plasmid as a template. PCR products were purified and introduced into pOPINM (Addgene plasmid 26044) expression vector by site independent ligation cloning procedures [13]. Resulted recombinant plasmids pOPINM/MBP-eEF1Bγ (1-Nr) encode truncated forms of eEF1Bγ in frame with MBP sequence. Constructs were prepared during the 5th EMBO Practical Course on High throughput protein production and crystallization (16–24 May 2013, Harwell, United Kingdom).

All constructs described above were verified by DNA sequencing.

Test of protein overexpression in Escherichia coli. Respective producing strains containing plasmid DNA of interest were grown in 100 ml of LB medium supplemented with appropriate antibiotic at 37 °C to an A_{600} = = 0.5. The expression was induced by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 3–4 h. Total bacterial extract (TE) was prepared as follows: 1 ml of culture was harvested by centrifugation and bacterial pellet was dissolved in 150 µl of sample buffer Laemmli with 6 M urea (SBLU1×) and boiled for 10 min. Extract of soluble proteins (SE) was prepared from the rest of culture. Bacteria were pelleted, washed twice with extraction buffer (30 mM Tris-HCl, pH 8.0, 30 mM KCl, 0.1 mM EDTA, 10 % glycerol, 2 mM DTT) followed by centrifugation. Pellet was resuspended in 1 ml of extraction buffer supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF), sonicated and centrifuged at 16000 *g* for 20 min (4 °C). 10 µl of supernatant was added to 200 µl SBLU1×, boiled for 10 min. Equal volumes of total and soluble extracts were used for analysis on SDS-PAGE.

Expression of full-length His-eEF1Bγ and its truncated forms in bacteria and their purification by affinity chromatography. The protein encoded by pET16b/eEF1Bγ plasmid was expressed in *E. coli* BL21(DE3) pLysS grown on LB medium supplemented with ampicillin (100 µg/ml). Culture (0.3 L) was grown at 37 °C to an A_{600} = 0.5, transferred at 20 °C and grown till A_{600} = = 0.8. The expression was induced by addition of 0.8 mM IPTG for 16 h. Cells were washed twice with icecold extraction buffer (30 mM Tris-HCl, pH 7.5, 30 mM KCl, 0.1 mM EDTA, 10 % glycerol, 5 mM 2-mercaptoethanol), resuspended in 8 ml of the same buffer containing 1 mM PMSF, and sonicated. All subsequent steps were conducted at 4 °C. After centrifugation at 18000 × *g* for 30 min, the clear supernatant was recovered and concentration of NaCl was adjusted to 500 mM, imidazole pH 8.0 to 20 mM and Tween-20 to 0.01 %. The obtained solution was mixed with Ni-NTA resin (2 ml of 50 % slurry, «Qiagen», USA), preequilibrated with the same buffer, and incubated on the orbital shaker for 1.5 h. The resin was washed with buffer A (25 mM Tris-HCl, pH 7.5, 500 mM KCl, 20 mM imidazole, pH 8.0, 10 % glycerol, 5 mM 2-mercaptoethanol) and then with buffer A containing 1 M NaCl and packed into column. eEF1Bγ was eluted from the column by 220 mM imidazole, pH 8.0 on buffer A. Fractions were collected and analyzed by SDS-PAGE. The purest fractions were combined and dialyzed against storage buffer (30 mM Tris-HCl, pH 7.5, 150 mM KCl, 55 % glycerol, 2 mM DTT). Protein was stored at –20 °C. Protein concentrations were determined using a calculated absorption coefficient: 1.74 A_{280} units $\cdot$ $mg^{-1} \cdot cm^{-1}$.

The truncated mutants of eEF1Bγ with N-terminal His-tag encoded by pET28a/His-eEF1Bγ (34–437), pET28a/His-eEF1Bγ (93–437), pET28a/His-eEF1Bγ (229–437), pET28a/His-eEF1Bγ (264–437) were expressed in *E. coli* Rosetta (DE3) grown on LB medium

supplemented with kanamycin (50 μg/ml). Cultures (0.4 L) were grown at 37 °C to an A_{600} = 0.5, transferred at 20 °C and grown till A_{600} = 0.8. The expression was induced by addition of 0.5 mM IPTG for 16 h. Cells were washed twice with ice-cold extraction buffer (25 mM Tris-HCl, pH 7.5, 20 mM imidazole, 150 mM NaCl, 10 % glycerol, 5 mM 2-mercaptoethanol), resuspended in 18 ml of the same buffer containing 1 mM PMSF, and sonicated. All subsequent steps were conducted at 4 °C. After centrifugation at 18000 × *g* for 30 min, the supernatants were recovered and mixed with Ni-NTA resins (2 ml of 50 % slurry, «Qiagen»), pre-equilibrated with the same buffer, and incubated on the orbital shaker for 1.5 h. The resins were washed with buffer A (25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole, pH 8.0, 10 % glycerol, 5 mM 2-mercaptoethanol) and then with buffer A containing 1 M NaCl. To get rid of contaminating proteins the resins with different mutants were additionally washed with buffer A, containing higher imidazole concentrations: His-eEF1Bγ (34–437) – 75 mM imidazole, His-eEF1Bγ (93–437) – 75 mM, His-eEF1Bγ (229–437) – 30 mM, His-eEF1Bγ (264–437) – 30 mM. Finally, all resins were packed into columns and eEF1Bγ N-truncated forms were eluted by buffer A containing 220 mM imidazole. Fractions were collected and analyzed by SDS-PAGE. The purest fractions were combined and dialyzed against storage buffer (25 mM Tris-HCl, pH 7.5, 20 mM imidazole, 150 mM NaCl, 55 % glycerol, 5 mM 2-mercaptoethanol). Proteins were stored at –20 °C. Protein concentrations were determined using a calculated absorption coefficients: 1.62 A_{280} units · mg^{-1} · cm^{-1} for eEF1Bγ (34–437), 1.8 – for eEF1Bγ (93–437), 1.92 – for eEF1Bγ (229–437) and 2.28 – for eEF1Bγ (264–437).

The truncated mutant of eEF1Bγ with N-terminal GST-tag encoded by pGEX6P-1/GST-eEF1Bγ (1–230) was expressed in *E. coli* Rosetta (DE3) grown on LB medium supplemented with ampicillin (100 μg/ml). Cultures (0.3 L) were grown at 37 °C to an A_{600} = = 0.5, transferred at 20 °C and grown till A_{600} = 0.8. The expression was induced by addition of 0.1 mM IPTG for 16 h. Cells were washed twice with ice-cold extraction buffer (PBS pH 7.4, 0.5 mM EDTA, 10 % glycerol, 5 mM 2-mercaptoethanol), resuspended in 18 ml of the same buffer containing 1 mM PMSF, and sonicated. All subsequent steps were conducted at 4 °C. After centrifugation at 18000 × *g* for 30 min, the clear supernatant was recovered and mixed with glutathione-agarose (8 ml, 50 % slurry, «Sigma», USA), pre-equilibrated with the same buffer, and incubated on the orbital shaker for 2.5 h. The resin was washed with buffer A (PBS pH 7.4, 0.5mM EDTA, 10 % glycerol, 5 mM 2-mercaptoethanol) and packed into column. GST-eEF1Bγ (1–230) was eluted from the column by 200 mM glutathione pH 8.0 on buffer A. Fractions were collected and analyzed by SDS-PAGE. The purest fractions were combined and dialyzed against storage buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 55 % glycerol, 5 mM 2-mercaptoethanol). Protein was stored at –20 °C. Protein concentrations were determined using a calculated absorption coefficient: 1.74 A_{280} units · mg^{-1} · cm^{-1} for eEF1Bγ (1–33), 1.51 – for eEF1Bγ (1–92), 1.52 – for eEF1Bγ (1–163), 1.54 – for eEF1Bγ (1–228).

The truncated mutants of eEF1Bγ with N-terminal MBP-tag encoded by pOPINM/MBP-eEF1Bγ (1–92) and pOPINM/MBP-eEF1Bγ (1–163) were expressed in *E. coli* Rosetta (DE3) grown on LB medium supplemented with ampicillin (100 μg/ml). Cultures (0.3 L) were grown at 37 °C to an A_{600} = 0.5, transferred at 20 °C and grown till A_{600} = 0.8. The expression was induced by addition of 1 mM IPTG for 16 h. Cells were washed twice with ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 % glycerol, 5 mM 2-mercaptoethanol), resuspended in 18 ml of the same buffer containing 1 mM PMSF, and sonicated. All subsequent steps were conducted at 4 °C. After centrifugation at 18000 × *g* for 30 min, the clear supernatant was recovered and applied onto MBPTrapTMHP (1 ml, «GE Healthcare»), pre-equilibrated in the extraction buffer. Column was washed and MBP fused protein was eluted by 10 mM maltose on extraction buffer. Fractions were collected and analyzed by SDS-PAGE. The purest fractions were combined and dialyzed against storage buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 55 % glycerol, 5 mM 2-mercaptoethanol). Proteins were stored at –20 °C. Protein concentrations were determined by using a calculated absorption coefficients: 1.5 A_{280} units · mg^{-1} · cm^{-1} for eEF1Bγ (1–92), 1.5 – for eEF1Bγ (1–163).

Protein denaturation-renaturation procedure. The truncated forms of eEF1Bγ obtained as soluble aggregates were subjected to denaturation-renaturation pro-

cedure. Two different approaches were tested: matrix assisted (Ni-NTA) denaturation-renaturation and denaturation of the protein in solution with subsequent renaturation by stepwise dialysis. For matrix assisted denaturation the protein was first immobilized on Ni-NTA and then denatured by addition of 10 mM Tris, 100 mM NaH_2PO_4 pH 8.0, 8 M urea (pH 8). Matrix was washed by the same buffer with pH 6.3. Denatured protein was subsequently eluted by the same buffer with pH 5.9 (monomers) and pH 4.5 (multimers). For renaturation the eluted protein was applied onto Superose 6 HR 10/30 column equilibrated with 20 mM imidazole HCl, pH 7.5, 150 mM NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol. This allowed also estimating the molecular weight of renatured proteins.

The renaturation of immobilized on Ni-NTA protein was also carried out in another manner [14] using linear 6–1 M urea gradient on buffer 20 mM Tris HCl pH 7.4, 500 mM NaCl, 20 % glycerol at low flow rate (0.8 ml/min) during 1.5 h. After renaturation the protein was eluted by 250 mM imidazole pH 8.0.

The denaturation of protein in solution was performed in 20 mM Tris HCl, pH 7.5, buffer with 8 M urea. The renaturation was carried out by stepwise dialysis in the buffer solutions containing 20 mM imidazole HCl pH 7.5, 150 NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol and 6, 4, 2 M urea during 6–8 h in each solution. The last dialysis was performed in the same buffer solution without urea. The molecular weight of renatured protein was estimated by analytical gel filtration.

Analytical gel filtration of proteins. To assess the aggregation state of the purified proteins size-exclusion chromatography on a Superose 6 HR 10/30 column (24 ml, «GE Healthcare») was performed. The column was equilibrated with 25 mM imidazole-HCl, pH 7.5, 150 mM NaCl, 10 % glycerol, 5 mM 2-mercaptoethanol. All samples were loaded in 0.1 ml and run at 0.4 ml/min; the elution was monitored at 280 nm. Elution of particular protein from column was described in term of corresponding K_{av} value. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of individual protein, V_0 is the void volume of the column, and V_t is its total bed volume. V_0 and V_t were determined with blue dextran (2 MDa) and 2-mercaptoethanol (78,1 Da), respectively. Following standard proteins were used for column calibration: thyroglobulin – 669 kDa, ferritin – 450 kDa, catalase – 232 kDa, alcohol dehydrogenase – 150 kDa, bovine serum albumin – 67 kDa, ovalbumin – 45 kDa, chymotrypsinogen A – 25 kDa, cytochrome c – 12,4 kDa. The K_{av} value of each protein standard (on the liner scale) was plotted against the corresponding molecular weight (on the logarithmic scale). The straight line that best fits the points on the graph was drawn and used for molecular weight determination of the protein of interest (Supplement Fig. S2).

Antibodies and Western Blot Analysis. Mouse monoclonal antibodies for the detection of His-tagged recombinant proteins («Roche», Germany) were used at 0.5 μg/ml working concentration. Mouse anti-GST monoclonal antibody («Pierce», USA) was used at 0.5 μg/ml working concentration. Anti-mouse secondary antibodies conjugated with peroxidase («Sigma», USA) were used at 1:10000 working dilution. Proteins were separated on 10 or 12 % SDS-PAGE and then transferred onto 0.45 μm nitrocellulose membrane («Bio-Rad», USA) using Trans-Blot Turbo transfer system («Bio-Rad») according to the manufacture recommendations. The membranes were treated subsequently with primary and secondary antibodies diluted in PBS containing 5 % non-fat dry milk and 0.1 % Tween-20 followed by extensive washing with PBS-0.1 % Tween-20 solution. The immune complexes were detected by Immobilon Western Chemiluminiscent HRP Substrate («Millipore», USA) on ChemiDoc system («Bio-Rad»).

Results and Discussion. *Domain structure of the eEF1Bγ protein and design of its truncated forms*. The eukaryotic translation elongation factor eEF1Bγ (Fig. 1) consist of two rather hydrophobic domains of about 200 amino acids each (N-terminal or Domain 1 and C-terminal or Domain 2), which are linked through a highly polar central lysine-rich stretch of about 30–60 residues [3, 6, 10, 15]. In order to understand the interaction between eEF1Bγ and its partners we prepared a set of truncated forms of this protein fused to different tags. Based on the multiple sequence alignment we prepared four N-terminal deletion mutants without 33, 92, 228 (C-terminal domain with lysine-rich linker) and 263 (C-terminal domain alone) amino acids (Fig. 1, Supplement Fig. S1). A polyhistidine sequence was attached either to the N-end or to the C-end of the protein. To overcome possible problems with the solubility of the N-terminal truncated forms we also prepared the

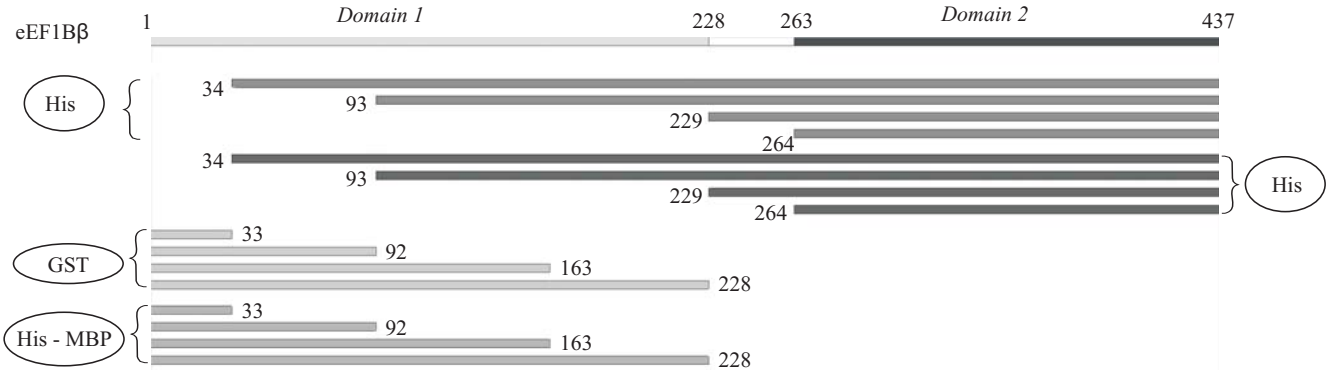

Fig. 1. Schematic representation of the eEF1Bγ truncated forms

C-terminal truncated forms: amino acid fragments 1–33, 1–92, 1–163 and 1–228 (N-terminal domain) were attached to GST or MBP tags (Fig. 1). All deletion mutants were expressed in *E. coli* and, if soluble, purified by affinity chromatography as described below.

Expression and purification of the full-length eEF1Bγ in the conditions preventing its multimerization. The expression of eEF1Bγ with N-terminal His-tag was successfully carried out in *E. coli*. After overnight IPTG induction at 20 °C His-eEF1Bγ was recovered in the fraction of soluble proteins and purified by affinity chromatography to an apparent homogeneity (Fig. 2).

The fractions eluted from the Ni-NTA column were analyzed by gel filtration on Superose 6 HR. We observed that the aggregation state of His-eEF1Bγ depended on its concentration. When His-eEF1Bγ was loaded on the column at 0.3 mg/ml, it came out as a single sharp peak (Fig. 3, *A*), whereas at 1.6 mg/ml some high molecular weight aggregates appeared (Fig. 3, *B*). At higher concentration the ratio of aggregates increased significantly (Fig. 3, *C*). Thus, to avoid the aggregation the Ni-NTA column should not be overloaded by the His-eEF1Bγ protein. We estimated that the extract of soluble proteins obtained form as much as 300 ml of His-eEF1Bγ producing culture may by loaded on 1 ml of packed Ni-NTA matrix at the conditions described in Material and methods.

The molecular weight of human recombinant His-eEF1Bγ was estimated by gel filtration to be about 200 kDa that is 4 fold higher than the theoretical value of the monomer (52 kDa). A sedimentation equilibrium analysis of purified His-eEF1Bγ demonstrated that this

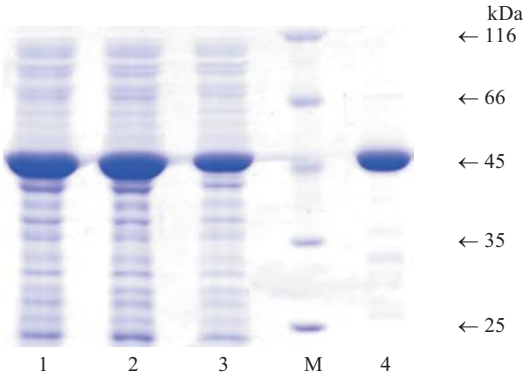

Fig. 2. Expression and purification of His-eEF1Bγ by Ni-NTA affinity chromatography. 10 % SDS-PAGE analysis of fractions: *1* – total bacterial extract; *2* – soluble extract; *3* – fraction of proteins not bound to Ni-NTA agarose; *4* – purified His-eEF1Bγ protein (4 μg). *M* – molecular weight markers. Corresponding molecular weights are depicted on the right (kDa)

protein behaves in solution as a monomer with a small portion of multimers (V. F. Shalak, unpublished results). Thus, recombinant His-eEF1Bγ is not a globular protein and most probably has extended shape in solution.

The large soluble aggregates of recombinant rabbit [16], *B. mori* [17] and multimers of the human eEF1Bγ [10] were previously observed. The aggregates of rabbit eEF1Bγ could be dissociated by 500 mM NaCl and at this salt concentration the molecular weight of eEF1Bγ was estimated by gel-filtration to be about 140 kDa [16]. The preparation of the *B. mori* eEF1Bγ was separated by gel filtration chromatography at 200 mM KCl into two fractions: large aggregates migrated close to the column void volume, whereas another fraction migrated as a single peak of about 150 kDa [17]. In contrast, in our experiments with His-eEF1Bγ the high salt con-

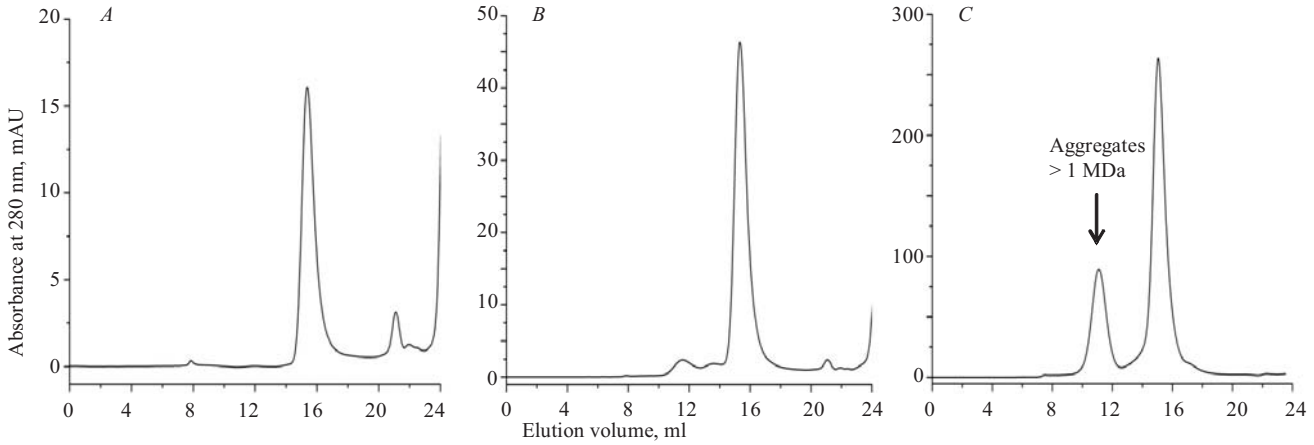

Fig. 3. Analysis of the full-length His-eEF1Bγ by gel filtration on Superose 6 HR column. Concentration of His-eEF1Bγ applied on column was: *A* – 0.3 mg/ml; *B* – 1.6 mg/ml; *C* – 8 mg/ml

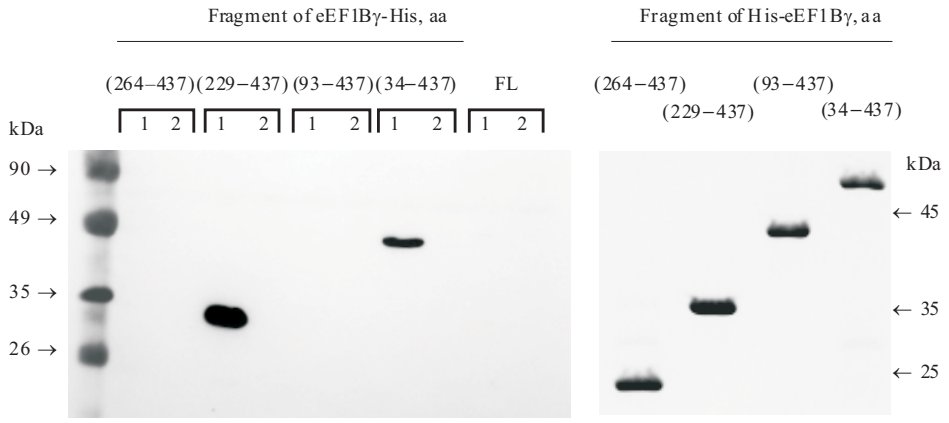

Fig. 4. Expression of the eEF1Bγ truncated forms with C- or N-terminal His-tag in *E. coli*. Left panel: western-blot analysis with anti-His6 antibody of total (*1*) and soluble (*2*) protein extracts for the presence of eEF1Bγ deletion mutants. FL – full-length eEF1Bγ-His. Right panel: 12 % SDS- PAGE of affinity purified eEF1Bγ truncated forms with the N-terminal His-tag. Position of marker proteins is indicated by arrows (kDa)

centration (up to 1 M NaCl) did not reduce the amount of large aggregates. The addition of Tween-20 or Triton X-100 to the buffer solution also had no effect (data not shown). This disagreement with the literature data can be explained by the presence of N-terminal polyhistidine sequence absent in the rabbit eEF1Bγ [10] that may influence the protein folding or its tendency for aggregation. It's worth mentioning that we did not succeed to express the full-length eEF1Bγ with C-terminal His-tag in *E. coli*.

Thus, depending on the concentration, the recombinant His-eEF1Bγ can form soluble and irreversible under physiological conditions high molecular weight aggregates. To prevent the aggregation the purified His-eEF1Bγ protein was kept at –20 °C in the storage buffer with 55 % glycerol at 2 mg/ml concentration.

Expression, purification and aggregation state of the N-terminally truncated forms of eEF1Bγ fused with C- terminal or N-terminal His-tag. The expression of the N-terminally truncated forms fused with C-terminal or N-terminal His-tag (Fig. 1) is presented in Fig. 4. The expression of all variants with C-terminal His-tag was unsatisfactory: full-length eEF1Bγ-His, truncated proteins eEF1Bγ (93–437)-His and eEF1Bγ (264–437)-His were not detected at all by western-blot analysis of corresponding bacterial extracts. The deletion mutants eEF1Bγ (34–437)-His and eEF1Bγ (229–437)-His were detected in total cell extracts only which suggested that these two proteins were in the insoluble aggregates (Fig. 4, left panel).

Further optimization of the expression conditions didn't improve the expression of these truncated forms (data not shown).

In contrast, the same truncated forms of eEF1Bγ fused with the N-terminal His-tag (Fig. 1, *A*) were expressed as soluble proteins. All of them were purified

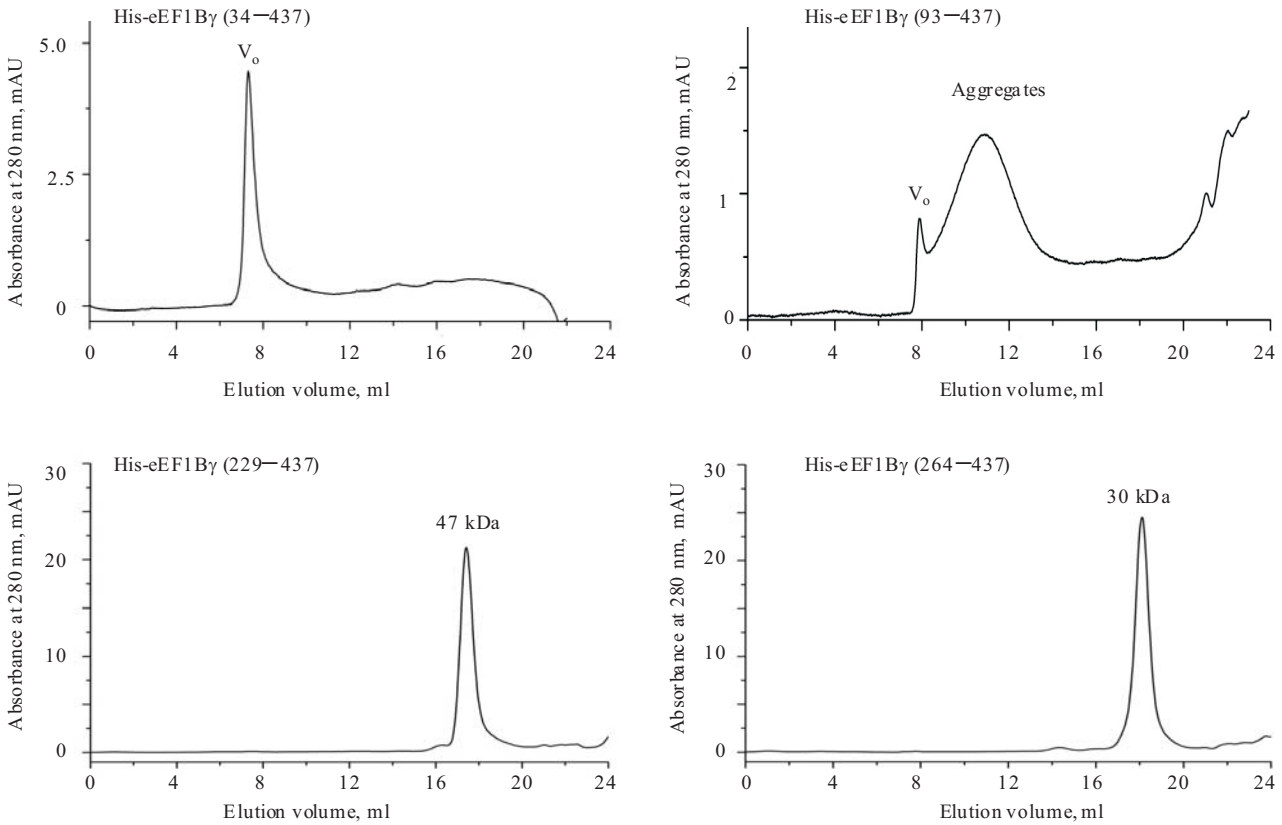

Fig. 5. Analysis of the eEF1Bγ truncated forms by gel filtration on Superose 6 HR column. Each protein was applied on the column at 10 μM concentration

by affinity chromatography to homogeneity (Fig. 4, right panel) as described in Material and methods.

The aggregation state of all truncated forms obtained was estimated by gel filtration (Fig. 5). The His-eEF1Bγ (34–437) and His-eEF1Bγ (93–437) proteins formed large soluble aggregates eluted in the column void volume or close to it (> 1 MDa). The shorter forms His-eEF1Bγ (229–437) and His-eEF1Bγ (264–437) migrated as a sharp single peaks of 47 and 30 kDa, respectively (Fig. 5). The calculated molecular weight of His-eEF1Bγ (264–437) is 21.4 kDa that is in agreement with the obtained experimental value, whereas the molecular weight of His-eEF1Bγ (229–437) is 25.4 kDa that is almost two fold lower as compared to the experimental result. The structure of 19 kDa C-terminal domain of the human eEF1Bγ (residues 276–437) was solved by NMR [10]. This domain behaves mostly as a monomer in solution and has a contact lens shape. This should be true for the truncated mutant His-eEF1Bγ (264–437), which is only 13 amino acids longer. By contrast, His-eEF1Bγ (229–437) is most probably a non-globular protein: due to the presence of lysine rich linker (amino acids 228–263, Fig. 1) its shape might became considerably extended. Thus, both His-eEF1Bγ (264–437) and His-eEF1Bγ (229–437) variants are suitable for further studies with interacting partners.

We attempted also to perform a denaturation-renaturation procedure for the His-eEF1Bγ (34–437) and His-eEF1Bγ (93 –437) protein variants. The denaturation of both proteins was done in the buffer containing 8 M urea. For the renaturation two approaches were tested: renaturation of proteins immobilized on Ni-NTA matrix by reversed liner gradient of urea and in solution by a step-wise dialysis. Unfortunately, neither approach led to a sufficient recovery of both proteins in a monomeric form (data not shown).

As was mentioned above, we failed to express the truncated and full-length eEF1Bγ with C-terminal His-tag (Fig. 4, left panel), whereas the respective N-terminal His-tag proteins were purified as soluble proteins (Fig. 4, right panel). The result confirms the significance of localization of the polyhistidine sequence for

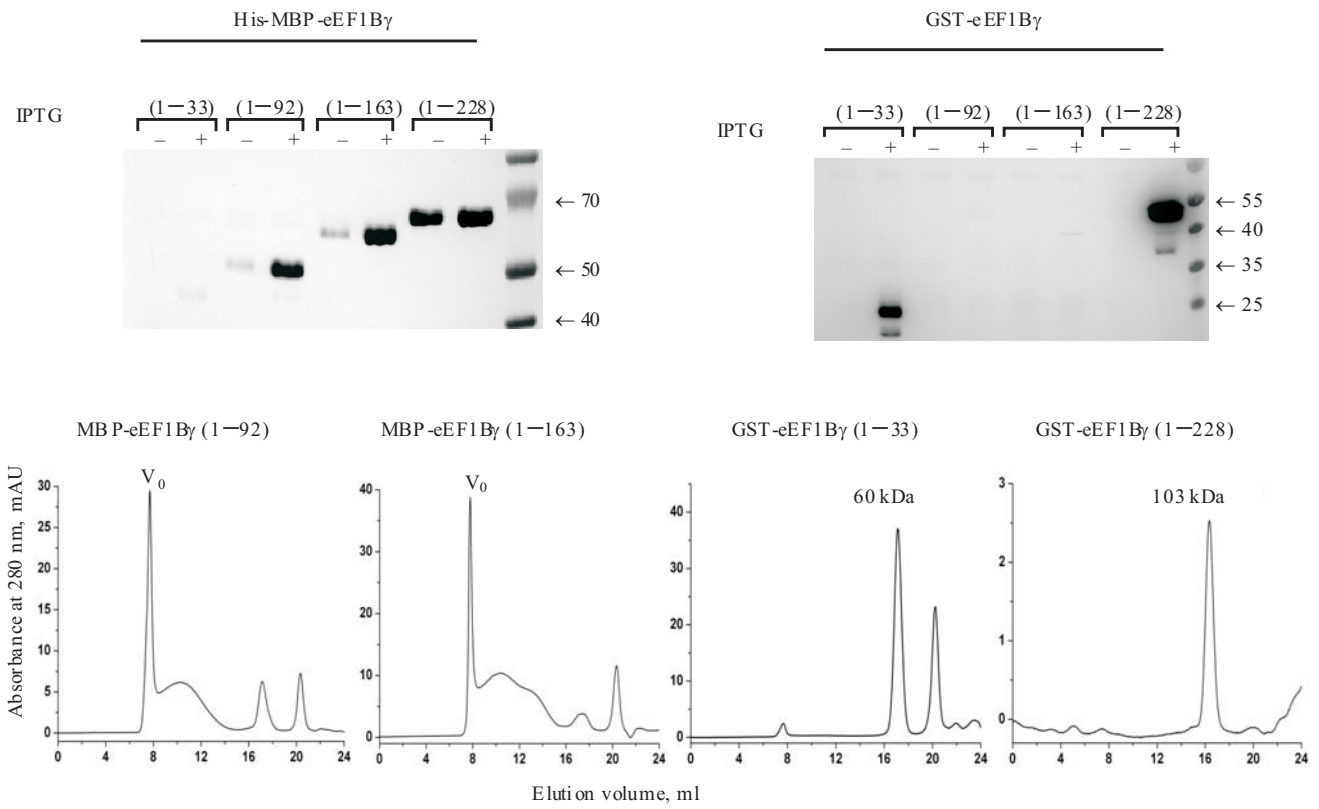

Fig. 6. Expression of the truncated forms of eEF1Bγ fused with MBP or GST in bacteria. Upper panel: western-blot analysis of the soluble protein extracts with anti-His-tag and anti-GST antibodies, respectively. Position of marker proteins is indicated by arrows (kDa).The expression was induced by IPTG (γ, +). Lower panel: gel filtration analysis of the eEF1Bγ purified truncated forms on Superose 6 HR column. GST-eEF1Bγ (1–230) at 4 μM concentration was applied on the column, the other truncated variant – at 15 μM concentration. V_0 indicates the void volume of column

folding and/or solubility of a particular recombinant protein expressing in bacteria [18]. Thus, for successful expression of the recombinant human eEF1Bγ the N-terminal localization of an affinity tag appeared to be crucial.

Expression, purification and aggregation state of the C-terminally truncated forms of eEF1Bγ fused with N-terminal GST- or MBP-tag. The C-terminal eEF1Bγ deletion mutants schematically shown in Fig. 1 were expressed in *E. coli* in the form of MBP and GST chimeric proteins (Fig. 6). Three truncated forms of eEF1Bγ with His-MBP-tag demonstrated good expression, except the eEF1Bγ (1–33) deletion mutant (Fig. 6, upper panel). In contrast, only GST-eEF1Bγ (1–33) and GST-eEF1Bγ (1–228) were expressed in bacteria as soluble proteins. Thus, for further purification we chose two truncated forms with different tags: eEF1Bγ (1–92) and eEF1Bγ (1–163) with His-MBP and eEF1Bγ (1–33) and eEF1Bγ (1–228) with GST-tag. GST-eEF1Bγ (1–33) and GST-eEF1Bγ (1–228) were successfully purified by affinity chromatography to an apparent homogeneity (not shown) and their aggregation state was checked by analytical gel filtration. Both proteins eluted from a column as sharp peaks of 60 and 103 kDa, respectively (Fig. 6). In GST-eEF1Bγ (1–33) preparation a small contaminating protein (< 10 kDa) was detected. Taking into account that the theoretical molecular weight of GST-eEF1Bγ (1–33) is 30.4 kDa and GST-eEF1Bγ (1–228) is 52.5 kDa, both proteins most probably are dimers in solution.

Unlike GST fusions, the purification of His-MBP bound truncated forms was more problematic. We decided to purify the His-MBP-eEF1Bγ (1–92) and His-MBP-eEF1Bγ (1–163) mutants because the same truncated forms were not expressed as GST fusions (Fig. 6, upper panel). First, we tried to use the Ni-NTA matrix

for purification, but this approach was not successful. The binding of His-MBP fusions to the Ni affinity matrix was very low. Then, we used the MBPTrap^{TM}HP column that specifically interacts with maltose binding protein. Using this matrix both His-MBP-eEF1Bγ (1–92) and His-MBP-eEF1Bγ (1–163) proteins were purified to an apparent homogeneity (not shown) and their aggregation state was tested by gel filtration. Unfortunately, both proteins were obtained as soluble aggregates eluted in and close to the void volume of column (Fig. 6, lower panel). The denaturation procedure in 8 M urea with subsequent renaturation (described in Material and method) led to the partial dissociation of the aggregates but resulted in extremely low yield of the target proteins (data not shown).

Thus, only two truncated forms GST-eEF1Bγ (1–33) and GST-eEF1Bγ (1–228) were obtained as soluble proteins suitable for further investigation. Despite all our efforts, the heavy aggregates of other forms were not possible to disrupt. In our opinion the reason for this failure lays in highly hydrophobic nature of the eEF1Bγ N-terminal domain (41.9 % of hydrophobic residues). Most probably the removing of the relatively long amino acid fragments from this protein may lead to the incorrect folding followed by its irreversible aggregation via exposed hydrophobic regions. Presumably, the N-terminal domain of eEF1Bγ could not be divided into separate subdomains.

Conclusions. The recombinant His-eEF1Bγ and its truncated variants His-eEF1Bγ (264–437), His-eEF1Bγ (229–437), GST-eEF1Bγ (1–33) and GST-eEF1Bγ (1–228) were expressed in *E. coli* and purified under the conditions mostly preventing the formation of aggregates. For the successful expression of the recombinant full-length eEF1Bγ and its truncated forms the affinity tag should be attached to the N-end of the protein. All the obtained protein variants will be used for further study on the protein-protein interaction.

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Структурний поділ фактора елонгації трансляції 1 Bγ (eEF1Bγ) людини: експресія повнорозмірного білка та його вкорочених форм

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Резюме

Мета. *Для детального вивчення властивостей фактора елонгації трансляції eEF1Bγ людини і його взаємодії з партнерами оптимізувати експресію кДНК повнорозмірного білка та його вкорочених форм.* ***Методи***. *кДНК, які кодують вкорочені форми eEF1Bγ, синтезували методом ПЛР-ампліфікації з відповідними праймерами і клонували у вектори, що містять які афінну мітку полігістидинову послідовність, глутатіон S-трансферазу або білок, який зв'язує мальтозу. Рекомбінантні білки експресували в бактеріях і очищували афінною хроматографією. Агрегатний стан отриманих білків аналізували з використанням аналітичної гель-фільтрації.* ***Результати***. *Оптимізовано експресію, очищення та умови зберігання повнорозмірного рекомбінантного His-eEF1Bγ. Також експресовано і очищено до гомогенного стану кілька вкорочених форм eEF1Bγ. Дві вкорочені форми С-кінцевого домену, які містять амінокислотні залишки 264–437 і 229–437, отримано у вигляді розчинних мономерних білків. Дві вкорочені форми N-кінцевого домену, що містять амінокислотні залишки 1–33 і 1–228, злиті з глутатіон S-трансферазою, одержано у вигляді димерів згідно з результатами гель-фільтрації. Інші делеційні мутанти, які містять амінокислотні залишки 1–92 і 1–163 N-кінцевого домену та злиті з білком, що зв'язує мальтозу, можуть бути отримані лише у вигляді розчинних високомолекулярних агрегатів.* ***Висновки***. *Отримано і охарактеризовано рекомбінантний білок His-eEF1Bγ та його чотири вкорочені форми. Ці форми в подальшому буде використано для вивчення білково-білкових взаємодій.*

Ключові слова: eEF1Bγ, білкові структурні домени, експресія рекомбінантних білків, хроматографічне очищення білків.

Структурное разделение фактора элонгации трансляции 1Bγ (eEF1Bγ) человека: экспрессия полноразмерного белка и его усеченных форм

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Резюме

Цель. *Для более детального изучения свойств человеческого фактора элонгации трансляции eEF1Bγ и его взаимодействия с партнерами оптимизировать экспрессию кДНК полноразмерного белка, а также его усеченных форм.* ***Методы***. *кДНК, кодирующие укороченные формы eEF1Bγ, генерировали методом ПЦР-амплификации с соответствующими праймерами и клонировали в векторы, содержащие в качестве аффинной метки полигистидиновую последовательность, глутатион S-трансферазу или белок, связывающий мальтозу. Рекомбинантные белки экспрессировали в бактериях и очищали аффинной хроматографией. Агрегатное состояние полученных белков анализировали с помощью аналитической гель-фильтрации.* ***Результаты***. *Оптимизированы экспрессия, очистка и условия хранения полноразмерного рекомбинантного His-eEF1Bγ. Также експрессированы и очищены до гомогенного состояния несколько усеченных форм eEF1Bγ. Две укороченные формы С-концевого домена, содержащие аминокис-*

лотные остатки 264–437 и 229–437, получены в виде растворимых мономерных белков. Две укороченные формы N-концевого домена, содержащие аминокислотные остатки 1–33 и 1–228, слитые с глутатион S-трансферазой, получены в виде димеров по результатам гель-фильтрации. Другие делеционные мутанты, содержащие аминокислотные остатки 1–92 и 1–163 N-концевого домена и слиты с белком, связывающим мальтозу, могут быть получены только в виде растворимых высокомолекулярных агрегатов. ***Выводы****. Получены и охарактеризованы рекомбинантный фактор элонгации трансляции His-eEF1Bγ и его четыре усеченные формы, которые в дальнейшем будут использованы для изучения белково-белковых взаимодействий.*

Ключевые слова: eEF1Bγ, белковые структурные домены, экспрессия рекомбинантных белков, хроматографическая очистка белков.

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