

## STRUCTURE AND FUNCTIONS OF BIOPOLYMERS

# Far western blot as a tool for identification of interaction between ribosomal proteins and translation elongation factor 1A

T. V. Budkevich, B. S. Negrutskii, A.V. El'skaya

Institute of molecular biology and genetics NAS of Ukraine  
Academician Zabolotnog str., 150, Kyiv, 03680 Ukraine

negrutskii@imbg.org.ua

---

*We have used Far Western blot technique to identify several proteins of 60S ribosomal subunit from rabbit liver which are capable of interacting with homologous translation elongation factor 1A. They belong to phosphoprotein P0.P1/P2 complex situated near A site region of 80S ribosome. It was also shown that eEF1A interacts with several proteins of small subunit of 80S ribosomes. It is important since none of 30S subunit proteins binds elongation factor EF-Tu. Identification of the above-mentioned proteins is the aim of future investigations.*

*Key words: ribosomal proteins, elongation factor 1A, eukaryotic translation*

---

**Introduction.** Systematic investigation of prokaryotic ribosomes in the course of recent decades resulted in the accumulation of an enormous massive of knowledge on the structure and functions of such molecular conveying machines. Particularly, the mechanisms of decoding [1] and peptidyl transferase reaction [2–4] were studied for 70S ribosomes, including the detailed study on the atomic level.

The main efforts in regards of eukaryotic 80S ribosome are directed to the investigation of transla-

tion initiation – a more complex process, compared to similar stage of biosynthesis of protein in bacteria (over 30 polypeptides participating in eukaryotic initiation vs. 3 bacterial factors have been discovered). Unlike the case of initiation, general scheme of translation elongation cycle is very similar in pro- and eukaryotes. The existence of three tRNA binding sites was proven for ribosomes of *Escherichia coli* [5], yeasts [6], and rabbit liver [7]. Besides, allosteric link between A and E ribosomal sites, initially discovered in bacterial system [5], was discovered in yeast ribosomes as well [6]. Noteworthy is the fact that our ideas on structure and functions of

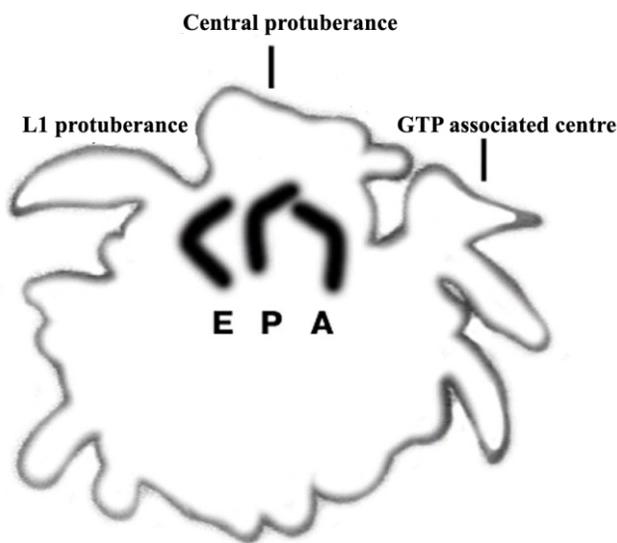


Fig.1 Scheme of eukaryotic 60S ribosomal subunit with marked basic structural elements: L1 and central protuberance, GTP associated centre, which includes phosphor-proteins P0.P1/P2, protein eL12 and sarcin/ricin rRNA loop. Figure also shows the places of localisation of three tRNA-binding sites – A, P, and E.

eukaryotic ribosome are mainly founded on the experimental data on bacterial and archaea ribosomes. At the same time eukaryotic ribosome is 50% larger than its bacterial analogue, it contains additional proteins and numerous insertions into rRNA sequence. Preliminary results of investigation on the structure of 80S ribosome by the method of cryo-electron microscopy [8] reveal differential conformational dynamics of pro- and eukaryotic ribosomes. Also, the conformational changes in 80S ribosome during translation elongation are distinct, unlike the case of 70S ribosome. The study on the location of matrix inside human placenta ribosome, carried out using the method of chemical cross-linking [9], revealed the content of mRNA-containing channel to differ in human and bacterial ribosomes (in the former case mRNA-containing channel consisted of proteins and rRNA, in the latter one – of rRNA only).

Along with functions, connected with association of ribosome and translation of mRNA, many ribo-

somal proteins are specific for functions not associated with ribosomes. Being outside of ribosome, they take part in various cell processes, for instance, splicing regulation of its own pre-mRNA (L30 of *Saccharomyces cerevisiae*, human S13 and S26) [10] or the development of viral infection (proteins L18 and L24 bound with transactivator of cauliflower mosaic virus, *i.e.* protein, regulating translation re-initiation on IRES of polycistronic mRNA) [11].

Tumour cells are known for the changed level of gene expression of various ribosomal proteins, *e.g.* large intestine cancer cells are specific for the increased gene expression of a number of ribosomal proteins, namely S3, S6, S8, S12, S19, P0, L5, L18, L31, and L37; during liver cancer the similar picture is observed for gene of ribosomal protein P0; in lymphoid tissue tumours these are S3a, S4, S17, L41; during prostate cancer – genes encoding proteins L7a and L37 [12]. The exact reasons as well as cell mechanisms in the basis of increased gene expression in ribosomal proteins in the course of carcinogenesis have not been determined.

Eukaryotic translation elongation factor 1A (eEF1A) belongs to a large family of GTP-binding proteins, the representatives of which take part in the regulation of crucial cell processes and are specific for high conservatism of primary amino acid sequences [13]. eEF1A catalyses GTP-binding of aminoacyl-tRNA (aa-tRNA) to ribosomal acceptor (A) site.

Ternary complex of eEF1A\*GTP\*aa-tRNA binds to GTPase associated centre of ribosome, which includes pentameric complex of L10.L7/L12 (eukaryotic homologues – phosphorylated proteins P0.P1/P2), L11 (eukaryotic homologue – eL12), and conservative sarcin/ricin rRNA loop [14] (Fig.1). Productive codon-anticodon recognition accelerates GTP hydrolysis by eEF1A molecule and dissociation of eEF1A in GDP-bound form leads to accommodation of tRNA in A site. The views on the sequence of events in the course of aa-tRNA binding as a part of ternary complex with eEF1A\*GTP as well as on the list of participants of this process are rather confine at the absence of structural data on eukaryotic decoding complex as these ideas reside on the similarities on decoding complex of *E. coli* [15]. Therefore, the lack

of information on the organisation and functioning of eukaryotic apparatus of translation elongation is evident.

Due to the fact that obtaining of information on structural organisation of 80S ribosome of higher eukaryotes remains the hardest experimental task, the central role in the study on eukaryotic ribosomes is given to biochemical methods of investigation. The latter supply to the bulk of data, coming to be helpful later for interpretation of results, obtained using structural methods. The aim of current work is to identify eukaryotic ribosomal proteins, capable of interacting with homologous translation elongation factor eEF1A using Far Western blot technique. The data of this method presuppose that the main 60S proteins of ribosomal subunits, interacting with eEF1A, are phosphoproteins P.0P1/P2, comprising GTPase associated centre of ribosome. Moreover, our data testify that 4 proteins of minor subunit are capable of participating with eEF1A.

Further identification of 40S subunit proteins by mass-spectrometry may possibly allow determining the role of these interactions during translation or other cell processes, involving both eEF1A [16] and ribosomal proteins.

**Materials and Methods.** *Isolation of ribosomal subunits and eEF1A.* Adult rabbit livers were used as the source of preparations of 60S and 40S subunits. The method of obtaining ribosomal subunits, free from endogenous tRNA and mRNA, is based on scheme [17] with some modifications. In particular, to eliminate endogenic RNases from the polysomal preparations at the early stage of purification, post-mitochondrial supernatant was centrifuged through 1 M sucrose cushion, containing 0.5 M of KCl. Concentrations of subunits were determined on the basis of the following correlations: 60 pM/1 optic unit of  $A_{260}$  for 40S and 30 pM/1 optic unit of  $A_{260}$  for 60 S. eEF1A preparation was obtained combining gel-filtration and ion-exchange chromatography, as earlier described in [18]. Protein concentration was determined according to Bradford's method [19].

*Far Western blot technique.* The method is based on the ability of protein partners to interact on the surface of nitrocellulose membrane [20]. After electrophoresis at the denaturing conditions, gel was in-

cubated in re-naturing buffer (50 mM tris-HCl, pH 7.4, 20% glycerol) for 1 hour at room temperature to eliminate SDS and consequently for partial re-naturation of ribosomal proteins [21]. Electrotransfer of proteins from gel to nitrocellulose membrane was carried out in carbonate buffer (10 mM  $\text{NaHCO}_3$ , 7 mM  $\text{Na}_2\text{CO}_3$ , pH 9.9, 20% ethanol) at 30–40 Wt in a cold room (4°C) in the course of a night. Coomassie brilliant blue and Ponceau S were applied on gel and membrane respectively in order to control the transfer. The next stage was the incubation of membrane with eEF1A in the concentration of 25–50 nM in PBS buffer (1.7 mM  $\text{KH}_2\text{PO}_4$ , 5.2 mM  $\text{Na}_2\text{HPO}_4$ , 150 mM NaCl), 3% BSA or 5% of dried milk, 15–20% of glycerol at room temperature for 1 hour constantly stirring. The use of high ionic force buffer solution allowed avoiding possible non-specific electrostatic protein contacts on membrane. Proteins, capable of interacting with eEF1A, were determined by monoclonal anti-eEF1A antibodies (*Upstate Group Inc.*) (1:1000).

For second time hybridisation with antibodies against C-terminal domain of yeast ribosomal proteins P0.P1/P2 (kindly presented by Professor J. Ballesta, Spain) the membrane was additionally purified from earlier bound antibodies by 30 min incubation at 60°C in the buffer (2% SDS, 100 mM  $\nu$ -mercaptoethanol, 62.5 mM tris-HCl, pH 6.8). Then the membrane was treated with primary anti-P0.P1/P2 antibodies (1:3 dilution) and exposed with secondary anti-mouse HRP conjugate.

**Results and Discussion.** *Determination of ribosomal subunit 60S proteins, capable of interacting with eEF1A.* Far Western blot was mentioned above to be based on observation of protein partners interacting with each other on the surface of nitrocellulose membrane after electrophoresis of protein mixture at denaturing conditions, renaturation of proteins in gel and electrotransfer (see Materials and Methods). Thus, we performed SDS-PAAG electrophoresis of 60S subunit proteins (Fig.2, a). Then the proteins were partially renatured and transferred to the membrane, incubated with eEF1A mixture, in order to allow this protein to form a complex with ribosomal proteins. Next the membrane was thoroughly washed from unbound eEF1A. eEF1A molecules,

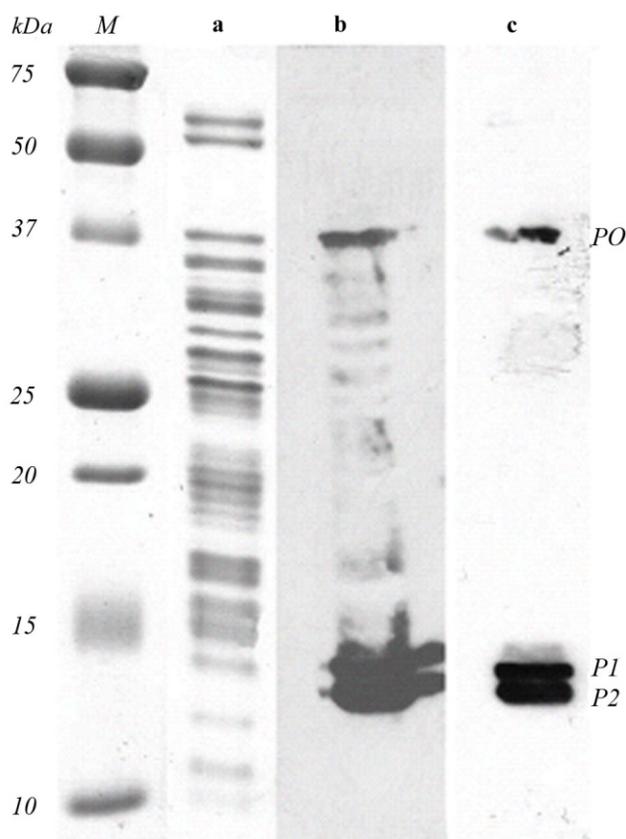


Fig.2 Analysis of large subunit proteins, interacting with rabbit liver eEF1A: *a* – stained with Coomassie brilliant blue; *b* – Far Western blot (exposition by antibodies against eEF1A); *c* – Western blot with antibodies against phospho-proteins P0.P1/P2.

which formed complexes with ribosomal proteins and thus remained on membrane, were localised using anti-eEF1A antibodies. Positive signal was received for 3 proteins (Fig. 2, *b*). On the basis of literature data it is possible to suppose that P0.P1/P2 complex proteins – eukaryotic homologues of prokaryotic proteins L10.L7/L12, associated with GTP associated centre on 50S subunit – are the primary candidates for the role of proteins partners for eEF1A. The mentioned centre is specific for the binding of ternary complex with ribosome during decoding [15].

A series of Toshio Uchiumi's works [14, 22] on *in vitro* reconstruction of a part of GTP associated cen-

tre, performed on bacterial ribosome, revealed the change of bacterial L7/L12 stem for homologous structure of rat liver to result in the loss of capability to recognise prokaryotic EF-Tu (bacterial homologue of eEF1A) and EF-G (bacterial homologue of eEF2) by chimeric 70S ribosomes, and their GTPase and translational activity could be compared with activity of native eukaryotic 80S ribosomes [22].

The data of cross-linking method [23], along with direct visualisation of bacterial decoding complex by cryo-electron microscopy [15] show that proteins L10 and L7/L12 are the primary and, most likely, the only proteins of major subunit, interacting with EF-Tu. In regards of eukaryotic eEF1A, cross-linking method, applied to eukaryotic system ribosome-factor, did not reveal any definite results compared to the case of EF-Tu [24]. The authors identified 3 proteins of 60S subunit (L12, L23, and L39) as those interacting with eEF1A. However, it is worth mentioning that all these proteins are localised on the internal side of major subunit, directed towards minor subunit, which is supposed to result in significant steric obstacles for interactions with eEF1A.

Thus, the reliability of results obtained is rather questionable. It is hard to comprehend why similar method using the same bi-functional cross-linking agent 2-iminotiolane in the case of 70S ribosomes and EF-Tu provided definite result, correlated with the data, obtained using other methods [23], whereas in the case of 80S/eEF1A the conclusion published [24] is most likely to be an artifact. It is possible that the same cross-linking agent can not be used for pro- and eukaryotic ribosomes due to different nature of available cross-links of 70S and 80S ribosomes and elongation factors.

It was possible that the protein eEF1A partners, discovered by us on the major subunit of eukaryotic ribosomes (Fig.2, *b*) could be the analogues of prokaryotic ribosomal proteins L10.L7/L12, interacting with EF-Tu. Eukaryotic analogues of these proteins are acidic phosphorylated proteins P0.P1/P2. It is important to mention also that molecular weights of eEF1A protein partners correspond to the weights of these proteins (34, 12, and 11 kDa). C-terminal domain of mentioned 3 proteins is known

to be highly conservative and the use of antibodies against this domain makes it possible to identify all three proteins. The use of these antibodies in Western blot technique confirmed that the signal obtained was indeed referred to P0.P1/P2 proteins (Fig. 2, *c*). One and the same nitrocellulose membrane was used for identification of eEF1A and P0.P1/P2, which is the clear evidence of the interaction of the same proteins with both antibodies against eEF1A and (upon washing out of the latter) antibodies against P0.P1/P2.

Thus, the possibility of direct contact between ribosomal proteins P0.P1/P2 with eEF1A has been determined. As P0.P1/P2 and L10.L7/L12 proteins are known to be functional analogues, the interaction described in the current paper testifies to the conservatism of decoding in pro- and eukaryotes.

Intriguing is the fact of the presence of several minor signals on the membrane, which may suggest the presence of additional eEF1A binding sites on 80S ribosome, *e.g.* in the region of E site [16].

*Determination of 40S subunit proteins, capable of interacting with eEF1A.* The procedure, analogous to the aforementioned, was also used to obtain small subunit proteins, capable of contacting with eEF1A (Fig.3). This kind of interactions was not shown for bacterial ribosomes. Regarding eukaryotic ribosomes, the authors of already mentioned work [24] used 2-iminotiolane as a bi-functional cross-linking agent and proposed proteins S23/S24, as well as S26, for eEF1A partners. However, as it has been mentioned before, it is possible that these interactions are the artifacts of the procedure of chemical cross-linking in the eukaryotic system. Recently, eEF1A, bound with small subunit, was discovered to play the role of association factor of yeast ribosomes [25]. Unfortunately, the data on where exactly the site of eEF1A binding on the surface of 40S subunit is and which ribosomal protein may participate in such interaction have not been provided.

We have demonstrated the interaction of eEF1A with four proteins of small ribosomal subunit using the method of Far Western blot technique (Fig.3). Molecular weight of these proteins was shown to be in the range of 37–25 and 20–15 kDa. As protein partners of EF-Tu on small subunit of bacterial ribo-

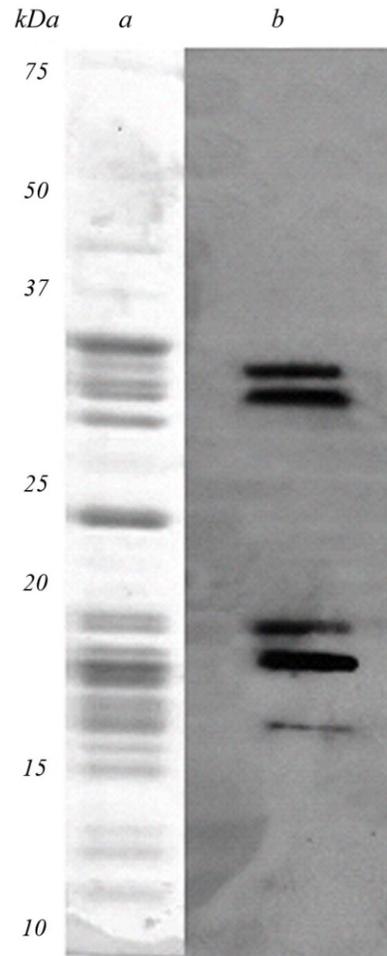


Fig.3 Analysis of small subunit proteins, interacting with rabbit liver eEF1A: *a* – stained with Coomassie brilliant blue; *b* – Far Western blot (exposition by antibodies against eEF1A).

some remain unknown, and moreover, it is quite possible that contacts between EF-Tu and 30S subunit are completely absent, the method of analogies, used for identification of large subunit proteins, can not be applied. Our further investigation will be aimed at identification of discovered proteins using the methods of mass-spectrometry. It is possible to suppose that the interactions, observed between eEF1A and 40S subunit proteins reflect the capability of these proteins to interact while performing functions, not directly connected with synthesis of proteins.

Т. В. Будкевич, Б. С. Негруцкий, А. В. Ельская

Использование метода Far Western гибридизации для определения рибосомных белков, взаимодействующих с фактором элонгации трансляции 1A

Резюме

Методом Far Western гибридизации определены белки 60S рибосомной субчастицы из печени кролика, взаимодействующие с гомологичным фактором элонгации трансляции eEF1A. Это фосфобелки P0.P1/P2, расположенные в районе А сайта 80S рибосомы. Показано, что eEF1A взаимодействует также с несколькими белками малой субчастицы 80S рибосом. Этот факт является весьма важным, поскольку ни один из белков малой субчастицы бактериальных рибосом не взаимодействует с фактором элонгации EF-Tu. Точная идентификация вышеупомянутых белков является предметом дальнейших исследований.

Ключевые слова: рибосомные белки, фактор элонгации 1A, эукариотная трансляция.

Т. В. Будкевич, Б. С. Негруцкий, Г. В. Ельська

Використання методу Far Western гібридизації для визначення рибосомних білків, які взаємодіють з фактором елонгації трансляції 1A

Резюме

Методом Far Western гібридизації визначено білки 60S рибосомної субчастинки з печінки кроля, які взаємодіють з гомологічним фактором елонгації трансляції eEF1A. Це фосфобілки P0.P1/P2, розташовані неподалік А сайту 80S рибосоми. Показано, що eEF1A взаємодіє також з декількома білками малої субчастинки 80S рибосом. Цей факт є дуже важливим, оскільки жоден з білків малої субчастинки бактеріальних рибосом не взаємодіє з фактором елонгації EF-Tu. Точна ідентифікація вищезгаданих білків є метою подальших досліджень.

Ключові слова: рибосомні білки, фактор елонгації 1A, еукариотна трансляція.

## REFERENCES

- Ogle J. M., Brodersen D. E., Clemons W. M., Jr., Tarry M. J., Carter A. P., Ramakrishnan V. Recognition of cognate transfer RNA by the 30S ribosomal subunit // *Science*.—2001.—**292**.—P. 897–902.
- Schmeing T. M., Huang K. S., Kitchen D. E., Strobel S. A., Steitz T. A. Structural insights into the roles of water and the 2' hydroxyl of the P site tRNA in the peptidyl transferase reaction // *Mol. Cell*.—2005.—**20**.—P. 437–448.
- Trobro S., Aqvist J. Mechanism of peptide bond synthesis on the ribosome // *Proc. Nat. Acad. Sci. USA*.—2005.—**102**.—P. 12395–12400.
- Erlacher M. D., Land K., Wotzel B., Rieder R., Micura R., Polacek N. Efficient ribosomal peptidyl transfer critically relies on the presence of the ribose 2'-OH at A2451 of 23S rRNA // *J. Amer. Chem. Soc.*—2006.—**128**.—P. 4453–4459.
- Hausner T. P., Geigenmuller U., Nierhaus K. H. The allosteric three-site model for the ribosomal elongation cycle. New insights into the inhibition mechanisms of aminoglycosides, thiostrepton, and viomycin // *J. Biol. Chem.*—1988.—**263**.—P. 13103–13111.
- Triana F., Nierhaus K. H., Chakrabarty K. Transfer RNA binding to 80S ribosome from yeast: evidence for three sites // *Biochem. Mol. Biol. Int.*—1994.—**33**.—P. 909–915.
- El'skaya A. V., Ovcharenko G. V., Palchevskij S. S., Petrushenko Z. M., Triana-Alonso F. J., Nierhaus K. H. Three tRNA binding sites in rabbit liver ribosomes and role of the intrinsic ATPase in 80S ribosome from higher eukaryotes // *Biochemistry*.—1997.—**36**.—P. 10492–10497.
- Spahn C. M., Gomez-Lorenzo M. G., Grassucci R. A., Jorgensen R., Andersen G. R., Beckmann R., Penczek P. A., Ballesta J. P., Frank J. Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation // *EMBO J.*—2004.—**23**.—P. 1008–1019.
- Graifer D. M., Karpova G. G., Knorre D. G. Location of template on the human ribosome as revealed from data on cross-linking with reactive mRNA analogs // *Biochemistry (Mosc)*.—2001.—**66**.—P. 585–602.
- Ivanov A. V., Малыгин А. А., Карпова Г. Г. Рибосомные белки эукариот: взаимодействие с собственными пре-мРНК и участие в регуляции сплайсинга // *Молекуляр. биология*.—2006.—**40**.—С. 640–649.
- Park H. S., Himmelbach A., Browning K. S., Hohn T., Ryabova L. A. A plant viral «reinitiation» factor interacts with the host translational machinery // *Cell*.—2001.—**106**.—P. 723–733.
- Vaarala M. H., Porvari K. S., Kyllonen A., Mustonen M. V., Lukkarinen O., Vihko P. T. Several genes encoding ribosomal proteins are over-expressed in prostate-cancer cell lines: conformation of L7a and L37 over-expression in prostate-cancer tissue samples // *Int. J. Cancer*.—1998.—**78**.—P. 27–32.
- Bourne H. R., Sanders D. A., McCormick F. The GTPase superfamily: conserved structure and molecular mechanism // *Nature*.—1991.—**349**.—P. 117–127.
- Hagiya A., Naganuma T., Maki Y., Ohta J., Tohkairin Y., Shimizu T., Nomura T., Hachimori A., Uchiumi T. A mode of assembly of P0, P1, and P2 proteins at the GTPase-associated center in animal ribosome: *in vitro* analyses with P0 truncation mutants // *J. Biol. Chem.*—2005.—**280**.—P. 39193–39199.
- Valle M., Zavialov A., Li W., Stagg S. M., Sengupta J., Nielsen R. C., Nissen P., Harvey S. C., Ehrenberg M., Frank J. Incorporation of aminoacyl-tRNA into the ribosome as seen by cryo-electron microscopy // *Nat. Struct. Biol.*—2003.—**10**.—P. 899–906.
- Negrutskii B. S., El'skaya A. V. Eukaryotic translation elongation factor 1 : expression, functions, and possible role in aminoacyl-tRNA channelling // *Progr. Nucl. Acids Res. Mol. Biol.*—1998.—**60**.—P. 47–78.
- Bommer U., Burkhardt N., Jünemann R., Spahn C. M. T., Triana-Alonso F. J., Nierhaus K. H. Ribosomes and polyosomes // *Subcellular Fractionation. A practical approach* / Eds J. Graham, D. Rickwoods.— Oxford: IRL Press at Oxford Univ, 1996.—P. 271–301.
- Shalak V. F., Budkevich T. V., Negrutskii B. S., El'skaya A. V. A fast and effective method for purification of elongation factor 1 alpha from rabbit liver // *Ukr. Biokhim. Zh.*—1997.—**69**.—P. 104–109.

19. *Bradford M. M.* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding // *Anal. Biochem.*—1976.—**72**.—P. 248–254.
20. *Hall R. A.* Studying protein-protein interactions via blot overlay or far western blot // *Meth. Mol. Biol.*—2004.—**261**.—P. 167–174.
21. *Dunn S. D.* Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies // *Anal. Biochem.*—1986.—**157**.—P. 144–153.
22. *Uchiumi T., Honma S., Endo Y., Hachimori A.* Ribosomal proteins at the stalk region modulate functional rRNA structures in the GTAase center // *J. Biol. Chem.*—2002.—**277**.—P. 41401–41409.
23. *Naq B., Johnson A. E., Traut R. R.* Identification of the elongation factor Tu binding site on 70S *E. coli* ribosomes by chemical cross-linking // *Ind. J. Biochem. and Biophys.*—1995.—**32**.—P. 343–350.
24. *Uchiumi T., Ogata K.* Cross-linking study on localization of the binding site for elongation factor 1 on rat liver ribosomes // *J. Biol. Chem.*—1986.—**261**.—P. 9668–9671.
25. *Herrera F., Correia H., Triana L., Fraile G.* Association of ribosomal subunits. A new functional role for yeast EF-16 in protein biosynthesis // *Eur. J. Biochem.*—1991.—**200**.—P. 321–327.

UDC 576.311.33:57.083.34

Received 01.02.07