Studying the Copy Number of Ribosomal Protein L7/L12

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Studying the copy number of ribosomal protein L7/L12 was performed using monoclonal antibody 3G9 to the linear epitope on the C-terminal domain of this protein from *Escherichia coli*. Immunohistochemical study showed that *Agrobacterium tumefaciens* ribosomes include 6 copies of protein L7/L12. Our results suggest that the copy number of this protein has an evolutionary role.

Key Words: *ribosomal protein L7/L12; copy number; monoclonal antibodies*

Long-term evolution of bacteria was accompanied by the development of various factors for survival and protection. The ribosome is a complex conservative apparatus (2.3 MD), which remains practically unchanged in microorganisms. This structure is responsible for protein synthesis in the cell due to activity of three major functional sites and several domains. One of the ribosomal sites, GTP-associated region (guanosine triphosphate), stimulates hydrolysis with GTP factors of elongation and initiation. In prokaryotic organisms, this site is composed by a fragment of 23S RNA and ribosomal proteins L11, L10, and L7/L12 and represents a flexible lateral rod-like stem [8,9].

E. coli L7/L12 contains 120 amino acid residues. The molecular weight of this protein is 12.2 kDa (Fig. 1). It consists of 2 proteins, L7 and L12. These proteins are identical, the only difference is acetylated N-terminal Ser in L7. Protein L7/L12 forms a complex with the C-terminal domain of protein L10. The N-terminal domain of protein L10 is bound to rRNA. Four copies of protein L7/L12 appear as 2 dimers on protein L10 of the *E. coli* ribosome. They form a major part of the rod-like stem in subunit 50S [11]. L7/L12 occurs as a dimer in the solution. It can be incorporated into the ribosome, but preserves its dimeric structure. Therefore, the L7/L12 dimer is a structural and functional unit of this protein [3].

Protein L7/L12 plays an important role in translation. This protein interacts with factors IF2, EF-Tu, EF-G, and RF3 and stimulates the GTPase activity [10,14]. Protein L7/L12 is involved in the regulation of ribosomal translation. High flexibility of the L7/L12 stem, which determines its functional activity, make its difficult to study the structure of this complex [4]. L7/L12 is the only ribosomal protein, which does not interact with rRNA and consists of more than one molecule per ribosome. It was hypothesized that the stem is formed by 4 copies of L7/L12 (as reported for *E. coli* ribosomes), which does not depend on the type of the organism. Studying the protein composition of ribosomes from *T. maritima* and *T. thermophilus* bacteria showed that the ribosome of these microorganisms contains 6 copies of the protein [4,5]. The number of L7/L12 copies is determined by the length of the C-terminal domain in protein L10. It also depends on the presence of helix α_8, which is longer in microorganisms with 3 dimers of L7/L12 (by 10 amino acids). Hydrophobic interactions play the major role in the relationship between helix α_8 of L10 and N-terminal domain of protein L7/L12 [4,5].

Previous experiments showed that 6 copies of ribosomal protein L7/L12 occur only in 2 micro-

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organisms, *T. maritima* and *T. thermophilus* [4,5]. A common feature of these microorganisms is their survival at high temperatures. The biological role of increasing the number of L7/12 copies (from 4 to 6 copies) remains unclear.

Here we studied the copy number of ribosomal protein L7/L12 in prokaryotic organisms.

MATERIALS AND METHODS

Experiments were performed with the following bacteria: *Agrobacterium tumefaciens*, *Rhizobium meliloti*, *Deinococcus radiodurans*, *Thermus thermophilus*, *Escherichia coli*, and *Thermotoga maritima*. These microorganisms were studied in pairs. *D. radiodurans* and *T. thermophilus* belong to the family *Deinococci*. *Agrobacterium tumefaciens* and *Rhizobium meliloti* are more close to each other and belong to the genus *Agrobacterium*. The copy number was studied in microorganisms that live under normal and extreme conditions. Immunohistochemical study of the copy number of ribosomal protein L7/L12 was performed using monoclonal antibody (monoAB) directed to a linear epitope on the C-terminal domain of *E. coli* protein L7/L12. A special test system was constructed for the detection of ribosomal protein L7/L12.

The study was conducted on 70S ribosomes, recombinant protein, and C-terminal domain of *E. coli* L7/L12 (Prof. M. V. Rodnina, Institute of Physical Biochemistry, Witten/Herdecke University, Witten), goat antibodies to mouse immunoglobulins (GAMI), conjugates of streptavidin and GAMI with horseradish peroxidase (Sigma), and bacterial mass of microorganisms (Institute of Genetics and Selection of Industrial Microorganisms).

Experimental mice were immunized with the C-terminal domain of *E. coli* protein L7/L12. The quality of immunization was estimated from antibody titer in serum samples (enzyme immunoassay, EIA). Hybridomas were obtained by fusion of splenocytes from immune mice (Balb/C) with Sp2/0 myeloma in PEG_{4000} (polyethylene glycol) [12].

During EIA, ribosomes and isolated protein L7/L12 of *E. coli* were sorbed to a plate (5 μg/ml). The interaction of biotin-labeled monoAB (10 μg/ml-10 ng/ml) with antigens was recorded. Signal detection was performed on a Multiscan vertical spectrophotometer (Labsystems) at 492 nm.

Sandwich EIA was performed in the presence of antigen in the solution. Sorption of monoAB to the plate (10 μg/ml) was followed by incubation with antigens (1000-0.3 ng/ml) and biotin-labeled secondary monoAB (1 μg/ml).

The cell extracts containing ribosomes were obtained for the following four types of bacteria: *Agrobacterium tumefaciens*, *Deinococcus radiodurans*, *Rhizobium meliloti*, and *T. thermophilus*. The S30 extract was prepared from bacterial cells on ice. To isolate the S30 extract, a weighted sample of the biomass from bacterial cells was ground in a mortar for 2 min, mixed with aluminum oxide powder (2-fold greater weight), and thoroughly ground for 30 min. The mass was mixed with DNAase I (up to 3 μg/ml) not containing RNase and ground for 10 min. The sample was suspended in a buffer to destroy bacterial cells (20 mM Tris-HCl ([pH 7.6), 100 mM NH_4Cl, 10.5 mM magnesium acetate, 0.5 mM EDTA, and 3 mM 2-mercaptoethanol) and centrifuged (3000 rpm, 10 min; 9000 rpm, 30 min; 14,000 rpm, 1 h). The ribosome-containing supernatant received the name "S30 extract". The concentration of ribosomes was measured on a Thermo spectrophotometer at 260 nm.

Proteins and ribosomes were separated by denaturing electrophoresis using a Tris-Tricine buffer system. Proteins from 2 identical gels were transferred to a 0.45-μ nitrocellulose membrane (Schleicher & Schuell) and incubated with biotinylated monoAB 2E2 or 3G9 (antibody concentration 100 ng/ml) and incubated with streptavidin-horseradish peroxidase conjugate (working dilution 1:20,000). The membrane was developed with a SuperSignal luminescence set (Pierce). Membrane chemiluminescence was recorded using a ChemiDoc XRS camera (BioRad).

Ribosomal protein sequences from various bacteria were subjected to multiple comparison [6]. Amino acid sequences of helix α_8 in ribosomal proteins L10 were compared for the following six species of bacteria: *Agrobacterium tumefaciens* (Q8UE06), *Escherichia coli* (POA7J3), *Thermotoga maritima* (P29394), *Deinococcus radiodurans* (Q9RSS9), *Rhizobium meliloti* (Q92QH9), and *Thermus thermophilus* (Q72GS1). Sequence alignment procedure allowed us to determined the location of binding sites of L7/L12 protein dimers with protein L10.

RESULTS

The number of bound dimers of protein L7/L12 is determined by the length of helix α_8 in protein L10. The length of helix α_8 in *T. maritima* and *T. thermophilus* is greater than that in *E. coli* by 10 amino acid residues [4,5]. This insertion on protein L10 is required for binding of the third dimer in protein L7/L12 to the ribosome [1]. Amino acid alignment of protein L10 from 6 microorganisms showed that helix α_8 in *T. maritima*, *A. tumefaciens*, *R. meliloti*,

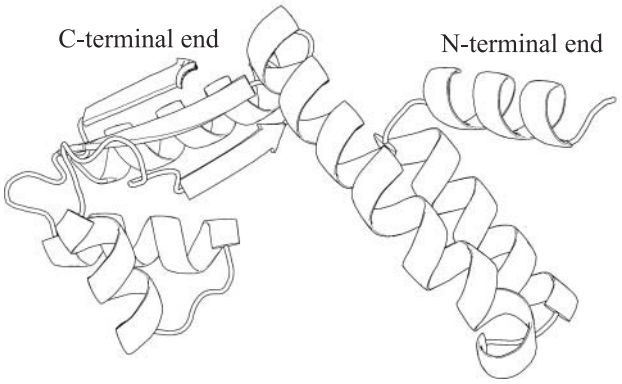

Fig. 1. Structure of *E. coli* protein L7/L12.

and *T. thermophilus* is longer than in *E. coli* and *D. radiodurans* (Fig. 2). *T. thermophilus* and *D. radiodurans* belong to the class *Deinococci*. Both microorganisms survive under extreme conditions. However, *D. radiodurans* did not contain the insertion in helix α_8. These data illustrate the presence of only 4 copies of protein L7/L12. *A. tumefaciens* and *R. meliloti* bacteria are more close to each other than the previous pair of microorganisms. They belong to the genus *Agrobacterium*. The protein sequence of these bacteria differs only by 2 amino acid resides. Amino acid sequences of L10 were compared in *A. tumefaciens* and *T. maritima*. Helix α_8 of protein L10 from *A. tumefaciens* has 3 amino acids, which are similar to the corresponding amino acids in helix α_8 of protein L10 from *T. maritima*. Helix α_8 of proteins L10 from *A. tumefaciens* and *T. maritima* are characterized by low homology. Similarly to the terminal end of helix α_8 in protein L10 from *T. maritima* (two Leu and Val), the terminal end of helix α_8 in protein L10 from *A. tumefaciens* contains several hydrophobic amino acids (three Val). This hydrophobic fragment is probably involved in binding of the third (additional) dimer of protein L12 to the ribosome. We conclude that *A. tumefaciens* contains 6 copies of protein L7/L12 (similarly to *T. maritima*).

Antibody titer after the 4th immunization was more than 1:100,000. The fusion of immunized mouse splenocytes and myeloma line sp2/0 allowed us to obtain 200 populations. Thirty of these populations were able to produce antibodies against *E. coli* L7/L12 protein. Two populations (2E2 and 3G9) were selected and cloned. EIA and immunoblotting assay showed that antibodies of these populations interact with the isolated protein. Moreover, these antibodies interact with the ribosomal protein. The interaction of proteins L7/L12 from 6 microorganisms with antibodies was studied by means of immunoblotting. Antibodies 2E2 were shown to interact only with *E. coli* protein L7/L12. Antibodies 3G9 interact strongly with protein L7/L12 from *E. coli* and *A. tumefaciens* and, less significantly, with protein L7/L12 from *R. meliloti* and *T. thermophilus*. The copy number of ribosomal protein L7/L12 from *A. tumefaciens* was studied with antibodies 3G9.

The copy number should not be evaluated by EIA, since protein L7/L12 undergoes dimerization in solution [3]. It can result in misinterpretation of experimental data. As regards the ribosome, there are steric difficulties in the interaction between antibodies and protein. The study was conducted by means of immunoblotting. Under these conditions, the protein is denatured and available for antibodies.

Recombinant antigen of *E. coli* L7/L12 was used in concentrations of 1-20 pM (Fig. 3). In this range of concentrations, the linearity of protein calibration was maintained after Coomassie stai-

TABLE 1. Intensity of Signals Reflecting Interaction of Antibodies 3G9 with the Antigen

Sample	Content of protein L7/L12, pM	Signal intensity, rel. units
E. coli protein L7/L12	1.8	33 934
	3	64 030
	4	254 047
	10	308 425
E. coli 70S ribosome	1	221 715
	2.5	280765
A. tumefaciens 70S ribosome	0.3	33 298
	0.5	60 307

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Agrobacterium tumefaciens Q8UE06 123 LATLPSLDELRAKLLGLLNAPATRVATVVAAPASQLARVFSAYAKK------DEAA 172
Thermotoga maritima       P29394 131 IAKLPSKEELYAMLVGRVKAPITGLVFALSGILRNLVYVLNAIKEK-------KSE 179
Deinococcus radiodurans   Q9RSS9 125 LASLGSKTTLQAELVGVLS----------AHLSNFVGILEAYREKLEGEGGSESA 169
Escherichia coli          P0A7J3 126 LATLPTYEEAIARLMATMK----------EASAGKLVRTLAAVRDA------KEAA 165
Thermus thermophilus      Q72GS1 125 LAELPTMDELRAELVGVLQAPMAELVGVLGGVARELVGILEAYAEK-------KAA 173
Rhizobium meliloti        Q92QH9 123 LATLPSLDELRAKLLGLINAPATRVATVVAAPASQLARVFSAYAKK------DEAA 172
                                                                  helix α8
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Fig. 2. Multiple alignment of amino acid sequences in helix α_8 of ribosomal protein L10 from 6 various microorganisms.

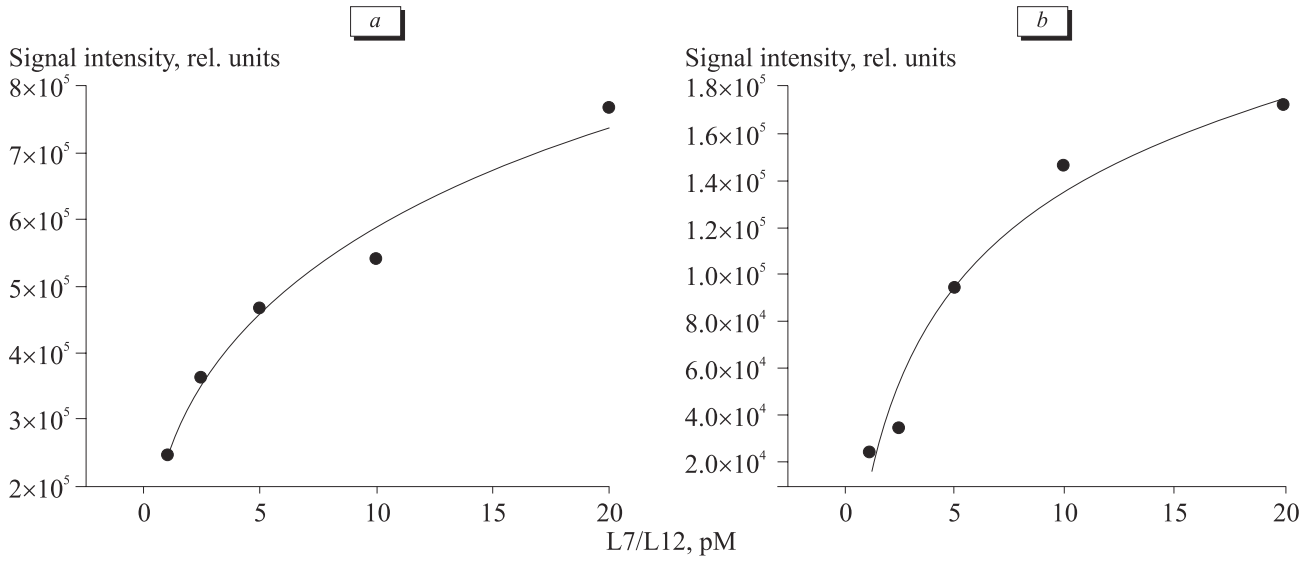

Fig. 3. Calibration curves for studying the copy number of L7/L12 in ribosomes from *E. coli* (*a*) and *A. tumefaciens* (*b*).

ning and immunoblotting procedure. It was probably related to the use of monoclonal, but not polyclonal antibodies. Ribosomes of *E. coli* and *A. tumefaciens* were taken in 2 sites to evaluate the copy number of protein L7/L12 (as described for *T. maritima*; Table 1) [4]. The data were approximated by the logarithmic function. During the interaction of antibodies with 70S ribosomes of *E. coli*, the signal was 3.6 times higher that that observed with the monomeric protein (Table 1).

Our results are consistent with published data that *E. coli* 70S ribosomes include 4 copies of protein L7/L12 [2]. The signal from *A. tumefaciens* 70S ribosomes was 5.8-fold greater than the specific response of the recombinant antigen (Table 1). Similar results were obtained for *T. maritima* [4]. The ribosome of *A. tumefaciens* includes 6 copies of protein L7/L12.

Greater number of protein copies probably provides the resistance of microorganisms to adverse environmental conditions during the early stage of life development on the Earth. It contributes to selection of translation factors from the intracellular medium and increase in the accuracy of translation. These features optimize the process of protein synthesis [4]. The increase in the number of L7/L12 copies results in the formation of a more hydrophobic nucleus in the L7/L12 stem, which contributes to stabilization of ribosomes [13]. Previous experiments showed that the decrease in the number of copies in the C-terminal domain of L7/L12 in the ribosome (from 6 to 4 copies) has only a partial effect, which is related to availability of translation factors [7]. The additional copy of protein L7/L12 is probably used by the microorganism under extreme conditions, which provides the survival [7]. *A. tumefaciens* can exist under normal conditions, but has special proteins to maintain the survival under unfavorable conditions. The genome of *A. tumefaciens* has a large number of ABC transporter genes that are used to obtain nutrient substances, sugars, and amino acids. The elevated content of this protein increases the probability of microorganism survival under competitive conditions. Under some conditions, *A. tumefaciens* can cause severe nosocomial infection.

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