SYNTHESIS OF RIBOSOMAL PROTEINS IN λrif^{d}18 INFECTED MINICELLS OF *ESCHERICHIA COLI* AND SELECTIVE INCORPORATION INTO RIBOSOMES

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1. Introduction

Recent studies [1,2] on the metabolic stability of the protein components of *Escherichia coli* ribosomes in situ have revealed a subclass of proteins which, in vitro, exchanges rapidly between individual ribosomes. The proteins in this category are S1, S2, S21, L7/L12, L9, L10, L11, L33. Proteins S1 and L7/L12 are known to be relatively loosely bound to the ribosome [3,4] whilst the others are apparently held as strongly as some of the nonexchanging proteins. The biochemical significance of this finding, especially in relation to the functions of these proteins in protein biosynthesis, remains unclear.

We report here results relating to this question which were obtained by the use of a system consisting of *E. coli* minicells infected with the transducing phage λrif^{d}18. Minicells contain all the enzyme systems necessary for transcription and translation but little or no parental DNA. Infection of minicells by bacteriophage results in the synthesis of proteins encoded by the phage genome [5]. Phage λrif^{d}18 contains several bacterial genes belonging to the 88–90 min segment of the genetic map of *E. coli* chromosome including genes for ribosomal proteins L1, L10, L11 and L7/L12 [6,7]. Interestingly, 3 of these 4 proteins belong to the exchanging category whilst protein L1 is a nonexchanger. Therefore the exchange behavior of these proteins newly synthesized in the minicells after infection, should reflect their differing character if the result from the heavy isotope transfer experiments [1,2] is of general validity.

Results reported below show that *E. coli* minicells infected with λrif^{d}18 synthesize proteins L1, L10, L11, L7/L12 as well as the polypeptides β, β' of RNA polymerase and elongation factor Tu (EF-Tu) which are also encoded by this phage [8,9]. Ribosomes isolated from infected minicells contained the bulk of newly synthesized L10, L11 and L7/L12. On the other hand, the major portion of newly synthesized L1 appeared free in the supernatant, unbound to the ribosome.

2. Materials and methods

2.1. *Minicells, λrif^{d}18, protein synthesis and slab-gel electrophoresis*

Minicells were prepared as in [5,10] from *E. coli* DS410 and from *E. coli* DS410 carrying the plasmid KB280. Plasmid KB280 results in the synthesis of large amounts of λ repressor protein [11]. Infection of minicells containing a high concentration of λ repressor permits the synthesis of polypeptides encoded by non-lambda DNA carried by λ-transducing phages and inhibits the concurrent synthesis of λ polypeptides [12]. λrif^{d}18 phage was isolated by thermal induction of the temperature-sensitive lysogenic strain *E. coli* NF910 (kindly provided by N. P. Fiil). The phage was concentrated and separated from the helper phage by CsCl density gradient centrifugation. Minicell infection, incorporation of [^{35}S]methionine, sample preparation, electrophoretic separation of radioactively-labelled polypeptides and autoradiography of electropherograms has been described [5,10].

2.2. *Fractionation of cell extract, two-dimensional gel electrophoresis*

Radioactively-labelled minicell pellets were suspended in 0.5 ml 10 mM Tris–Cl (pH 7.6), 50 mM KCl, 10 mM Mg acetate, 7 mM 2-mercaptoethanol (Tris/KCl/Mg/Me) and lyzed by the freeze–thaw–lysozyme procedure [13]. The lysate was sonicated briefly (15 s, 0°C) to disaggregate polysomes and was layered over a 34 ml 10–30% sucrose gradient in Tris/KCl/Mg (SW 27 rotor) and centrifuged for 14 h at 20 000 rev./min. The gradient was fractionated using a model 640 Isco gradient analyzer with UV monitor. Radioactivity contained in hot trichloroacetic acid-insoluble material was determined, as in [14], in a Nuclear Chicago Isocap scintillation counter. The fractions which contained 70 S ribosomes, 50 S subunits, 30 S subunits and supernatant proteins were separately pooled, dialyzed against 0.05% 2-mercaptoethanol and lyophilized. Proteins were extracted with 67% acetic acid [15] and analysed by two-dimensional gel electrophoresis [16]. Protein spots were cut out, treated with 1 ml Soluene-350 (Packard) and counted in the presence of 0.5% PPO/toluene. Gels 1.5 mm thick were used for autoradiographic analysis.

3. Results

3.1. *Expression of λrif^{d}18 in infected minicells*

Autoradiograms of the electrophoretic separation of radioactively-labelled polypeptides synthesized in λrif^{d}18-infected minicells are shown in fig.1. The minicells which contain λ repressor synthesize virtually only those polypeptides which are encoded by the transduced fragment of DNA, namely β and β′ subunits of RNA polymerase, elongation factor Tu (EF-Tu) and ribosomal proteins L1, L7/12, L10 and L11 [6–9]. These polypeptides do not exhibit detectable chain-length heterogeneity (fig.1) nor do they change in molecular weight over extended periods of incubation. The molecular weights of L10, L11 and

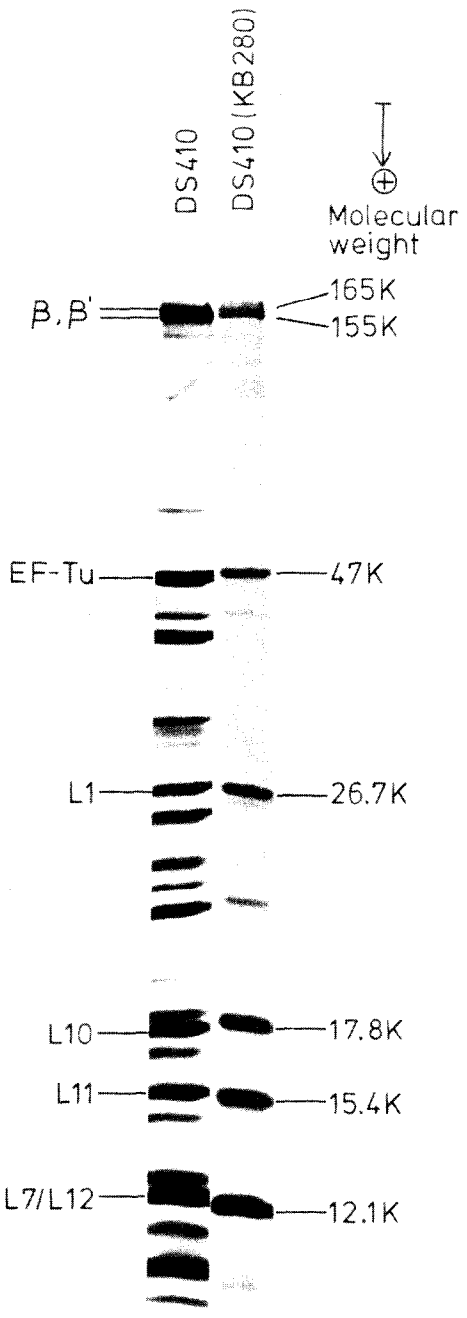


Fig.1. Autoradiogram of the electrophoretic separation of polypeptides synthesized in λrif^{d}18-infected minicells. Infected minicells (2×10^{10}/ml; m.o.i. = 5) were incubated for 45 min at 37°C in minimal medium, [^{35}S]methionine was added [10] and incubation continued for 120 min at 37°C. Samples were prepared for electrophoresis and analysed on 11–20% polyacrylamide–SDS gradient gels as in [5,10]. Minicells were prepared from *E. coli* DS410 or DS410 (pKB280) and exposed to ultraviolet irradiation prior to infection to prevent the expression of entrapped plasmid pKB280 [12].

L7/12 determined in this system (fig.1) agree very well with those derived from their amino acid sequences [17–20]. Thus these proteins are not apparently synthesized in detectable precursor forms in a system which should be incapable of assembly of mature ribosomes.

3.2. *Attachment of newly-made proteins to ribosomes*

Having demonstrated the synthesis of ribosomal proteins in infected minicells we wished to know whether the newly-synthesized ribosomal proteins occurred free in the cytoplasm or particle-bound to the ribosomes. The results of sucrose gradient analysis shown in fig.2 indicate that a fraction of the total counts occurs bound to 70 S ribosomes and to 50 S subunits. Very little of the radioactivity was bound to 30 S subunits and there was no radioactivity appearing as distinct peaks of intermediate sedimentation values.

Two-dimensional gel electrophoretic separation of the radioactivity in the different regions of the gradient showed (fig.3) that the ribosome-bound radioactivity

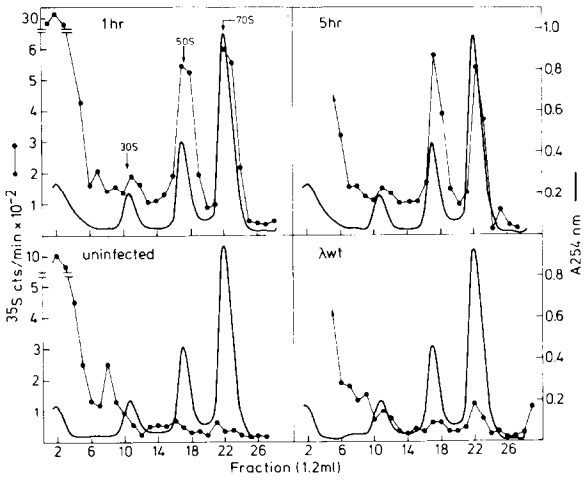


Fig.2. Sucrose gradient separation of ribosome-bound and free labelled polypeptides synthesized in λrif^{d}18-infected minicells. Minicells (DS410) infected with λrif^{d}18 and incubated with [^{35}S]methionine for 1 h or 5 h were lysed by the freeze–thaw–lysozyme procedure [13]. Intact ribosomes, ribosomal subunits and free proteins were separated by sucrose gradient centrifugation (section 2). Control minicell preparations (i.e., uninfected or infected with a wild-type λ phage) were processed in the same manner. (•–•) Newly-synthesized polypeptides; (——) $A_{254\ nm}$.

corresponded to the 4 proteins L1, L10, L11 and L7/12 in the case of both 70 S and native 50 S peaks. In the case of the 70 S pattern (fig.3A) protein L7/12 gave the most intense spot as was expected since this protein is present in at least 4 copies/ribosome (e.g. [21]). Protein L1 showed the weakest spot and, on a relative molar scale using the similar ratio of methionine to total amino acids [22], it must be present in an even lesser amount since the molecular weight of L1 is considerably higher than that of the other ribosomal proteins [23].

The electrophoretic pattern of native 50 S subunits (fig.3B) showed all 4 proteins but the spot of L7/L12 by far dominated the picture. The pattern from native 30 S subunits (fig.3C) contained only faint spots which may have arisen from the slight contamination of this peak by 50 S subunits.

The two-dimensional gel electrophoretic separation of the radioactivity present in the supernatant region (fig.3D) showed a very different picture. Several non-ribosomal proteins synthesized by the infected minicell (DS410) are resolved by the system and some of these proteins obscure the position of protein L7/L12. The pattern in fig.3D shows a faint spot in the position of protein L10 and a strong spot in the position of L1. Control experiments using extracts from minicells either uninfected or infected with wild-type λ phage did not show these two spots. We therefore infer that these spots correspond to proteins L10 and L1.

The amount of radioactivity appearing in proteins L1, L10, L11 and L7/12 in the two-dimensional gels of 70 S ribosomes has been determined after staining the gels and cutting out the spots. The results (fig.4) show that the bound radioactivity increases with time. Protein L1 showed the lowest level of activity whilst protein L7/12 showed the highest level.

We have also determined the radioactivity in proteins L1, L10 and L11 in the supernatant fraction by co-electrophoresis of the samples with carrier ribosomal proteins and cutting out and processing the corresponding spots.

The results for proteins L1 and L10 are shown in fig.4. Protein L10 (and also L11, data not shown) show higher levels of counts in ribosomes while protein L1 showed higher levels of counts in the supernatant.

Proteins L10, L11 and L7/L12, whose primary

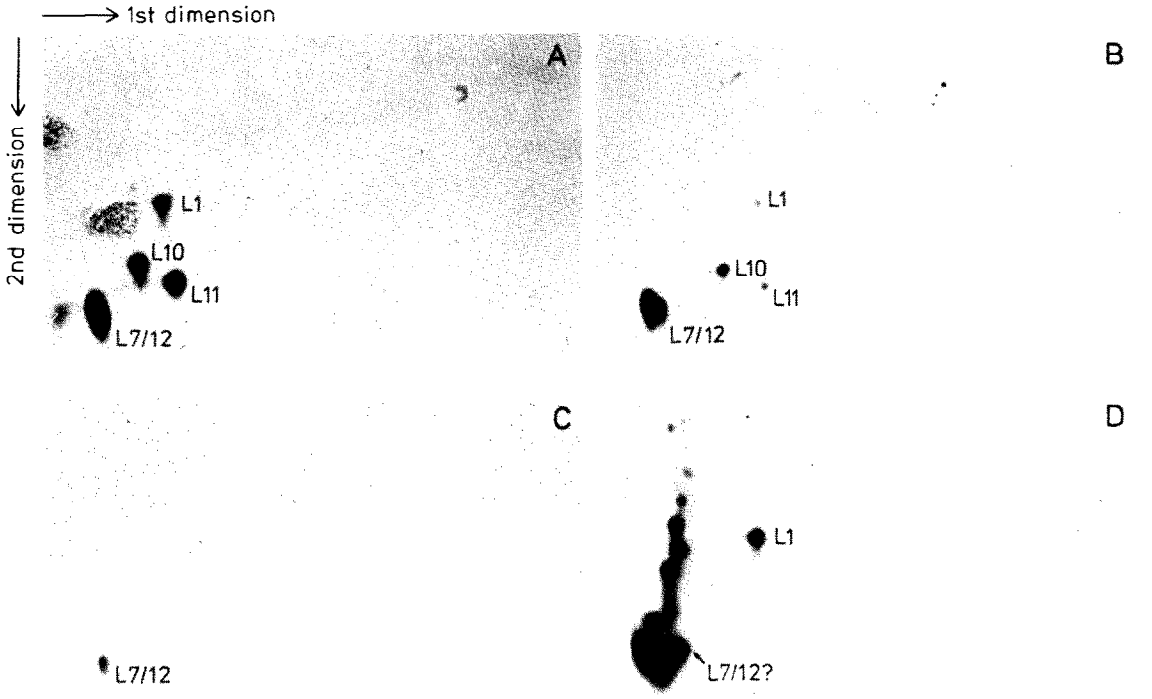
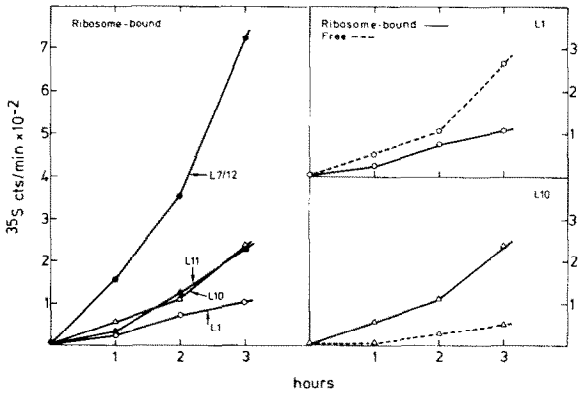


Fig.3. Autoradiogram of the two-dimensional gel separation patterns of ribosome-bound and free proteins synthesized in λrif^{d}18-infected minicells. Fractions corresponding to: (A) 70 S ribosomes; (B) 50 S subunits; (C) 30 S subunits; and (D) ribosome-free proteins, were taken from a sucrose gradient (fig.2). Proteins were extracted with acetic acid, electrophoresed [16], and the gel slabs (impregnated with PPO) were used to expose Dupont Cronex 2 X-ray film for 10 days.

structures are known [17–20], contain 5, 5 and 3 residues of methionine, respectively. If we assume that the label [^{35}S]methionine enters into these 3 proteins from a common pool then the molar ratios of the proteins can be calculated from their radioactivity.



The results summarized in table 1 show that newly synthesized L10 and L11 bind to the ribosome at the same molar level, whereas newly synthesized L7/12 binds at a 4–5-fold higher molar level. Further results have indicated that, with increasing time, λrif^{d}18-infected minicells synthesize protein L7/L12 in a stoichiometry (relative to L10 or L11) which is appreciably greater than the 4 copies found in ribosomes.

Fig.4. Kinetics of accumulation of newly synthesized ribosomal proteins in ribosome-bound and free forms. Minicells were infected with λrif^{d}18 and incubated with [^{35}S]methionine for 1 h, 2 h and 3 h. Intact ribosomes (70 S) were separated from free proteins by sucrose gradient centrifugation. Carrier ribosomes were added to both fractions and proteins extracted and electrophoresed [16]. After staining the gel slab, individual protein spots were cut out and radioactivity determined (section 2). Spots by other ribosomal proteins contained only the background level of radioactivity.

Table 1
Amount of newly synthesized ribosomal proteins bound to 70 S ribosomes

Protein	m/mol[a]	^{35}S cpm after (min)[b]			Rel. molar amount after (min)		
		60	120	180	60	120	180
L10	5	57	111	236	1.0[c]	1.0	1.0
L11	5	34	124	230	0.6	1.1	1.0
L7/12	3	155	354	725	4.5	5.3	5.1

[a] From primary structure [17–20]
[b] Radioactivity in blank gel slices (11–13 cpm) subtracted
[c] Taken as standard for calculation

4. Discussion

We have demonstrated here the synthesis of ribosomal proteins L1, L10, L11 and L7/12 by *E. coli* minicells infected with transducing phage λrif^{d}18 (fig.1–3). The data in fig.1 show that β, β′-subunits of RNA polymerase and elongation factor Tu are also expressed. The stable species of rRNA are encoded by λrif^{d}18 [6,24] and preliminary results indicate these molecules are also synthesized in infected minicells (results not shown).

Previous experiments involving heavy isotope transfer methodology have shown that proteins L10, L7/12 and protein L11 to a lesser extent belong to the small subclass of ribosomal proteins which can rapidly exchange between ribosomes while protein L1 belongs to the major subclass of nonexchanging proteins [1,2]. The results from the present experiments confirm the above behavioral pattern of these 4 proteins by analysis in a very different system. Proteins L10, L11 and L7/12 are involved in ribosome-dependent GTP hydrolysis and in the translocation step in peptide chain formation [25]. Further experiments are necessary to determine whether there is an obligatory exchange by the above exchangeable proteins in one or more of the steps in the ribosome cycle.

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