THE REQUIREMENT FOR THE *ESCHERICHIA COLI* RIBOSOMAL PROTEINS L7 AND L12 IN RELEASE FACTOR-DEPENDENT PEPTIDE CHAIN TERMINATION

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1. Introduction

The *Escherichia coli* ribosomal proteins L7 and L12 play an important role in the factor-associated reactions of protein synthesis. A stringent requirement for the proteins was suggested at least for EF-G and EF-Tu [1–4] and RF-1 and RF-2 [5,6] in studies using either selective removal of L7/L12 from the ribosome or antibodies against these proteins. More recently however, it has been shown that the elongation factors EF-Tu and EF-G can interact with a functioning ribosome and carry out their reactions in the absence of L7/L12, although at a reduced rate [7,8]. The requirement for the proteins depends on the ionic conditions [7].

During studies characterising the role of L11 in polypeptide chain termination in vitro [9] significant codon-dependent activity independent of L7/L12 was observed in some experiments. Since these observations were contrary to reports suggesting a strict requirement for L7/L12 in termination in vitro [5], we have investigated this requirement further. We report that while ribosomes lacking L7 and L12 are active, these ribosomal proteins allow the in vitro termination reactions to occur at lower concentrations of both termination codon and release factor.

2. Experimental

Ribosomes and release factors were isolated from *E. coli* B and purified as in [10,11]. Ribosomal subunits were isolated from sucrose gradients or from heat-shocked bacteria [12]. Ribosomal core particles derived from the 70 S or 50 S were prepared by the method in [1] using the conditions of [5].

Following the extraction with ethanol/NH_4Cl the ribosome subparticles were pelleted by centrifuging and taken up in a Tris buffer (20 mM Tris–HCl (pH 7.8), 10 mM $Mg(OAc)_2$, 30 mM NH_4Cl, 0.5 mM EDTA, 1 mM DTT). After dialysis against the same buffer the solution of ribosome cores was clarified by centrifuging at 12 000 × *g* for 10 min and then stored at −80°C.

The supernatant proteins extracted from the ribosome by the above procedure were dialysed against 10 mM Tris–HCl (pH 7.5) containing 3 mM DTT and lyophilized. The protein composition of the cores and split proteins was analysed by the electrophoretic system in [13]. All gels were stained with 0.2% (w/v) Brilliant blue R in 50% (v/v) methanol, 7% (v/v) acetic acid and destained with 5% (v/v) methanol, 7% (v/v) acetic acid.

The ability of the cores of the 50 S subunit to bind fMet-tRNA was assayed according to [11], supplementing the reaction with 30 S subunits. Ribosomes and fMet-tRNA were incubated in 20 mM Tris–HCl (pH 7.5), 30 mM $Mg(OAc)_2$, 160 mM NH_4Cl for 30–60 min at 30°C. The extent of complex formation was determined by the retention on Millipore filters of samples from the reactions. In vitro termination was measured as in [11]. The codon-dependent release of f[^{3}H]Met from a f[^{3}H]Met-tRNA/AUG/ribosome complex was determined in reactions incubated for 5 min at 24°C containing in 0.05 ml: 50 mM Tris–HOAc (pH 7.2), 30 mM $Mg(OAc)_2$, 75 mM NH_4OAc, 11 μg RF-1 or 4–6 μg RF-2 or as indicated, 1–3 pmol of f[^{3}H]Met-tRNA/AUG/ribosome intermediate and UAA, 0.1 A_{260} unit or amounts as indicated. Codon-independent reactions were incubated for 20 min at 4°C and contained the same components with 20% (v/v) ethanol in place of

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terminator codon. The f[^{3}H]Met released was extracted into ethyl acetate at pH 1.

An antiserum against the ribosomal proteins L7/L12 was raised in rabbits. About 600 μg protein in complete Freund's adjuvent (containing only L7/L12 as determined by gel electrophoresis) was injected into the rabbit initially and the animal was boosted with further injections of ~100 μg protein in 6 M urea on days 15, 42, 71 and 116. The serum obtained was made 33% in glycerol for storage at −20°C. After dialysis into 50 mM Tris–HCl (pH 8.5), 0.9% NaCl an IgG fraction was prepared by $(NH_4)_2SO_4$ precipitation (33%). The precipitate was dissolved in the same buffer, dialysed and clarified by centrifuging at 10000 × *g* for 20 min. A small sample of the resulting IgG preparation was dialysed into the buffer used for in vitro termination assays at 4°C immediately before use. The IgG fraction formed single precipitin lines with only L7/L12 or ribosomal protein mixtures containing L7/L12 when tested by double immunodiffusion, or immunoelectrophoresis.

3. Results and discussion

A codon-dependent in vitro termination activity independent of the ribosomal proteins L7/L12 of ≤40% of that observed with complete ribosomes was observed in some experiments with ribosome cores prepared as in [1] in contrast to [5]. We have eliminated the trivial possibility that this finding is a result of the core preparations retaining significant amounts of L7/L12. Firstly we have examined the ribosomal proteins of the cores by two dimensional gel electrophoresis using the gel system in [13] as modified [14]. In this system, in contrast to that in [15], complexes between L7/L12 and L10 [16,17] are broken down during electrophoresis and no L8 or L8′ is observed [18]. No L7/L12 was detectable in the cores from this analysis. The low activity of the cores in termination in vitro could conceivably have been due to a small percentage of the ribosomes having retained L7/L12, despite the fact that the proteins were not observed on gels. In order to exclude rigorously this possibility studies were carried out using anti L7/L12 IgG. Antisera prepared against these proteins were known to inhibit termination [6]. At the level of antibody chosen, anti L7/L12 significantly inhibited codon-dependent and codon independent in vitro termination with untreated ribosomes (60–70%) but had little or no effect on the same activities exhibited by the ribosomal cores (table 1). This indicated that the in vitro termination activity of the cores was indeed independent of L7/L12.

Some of the properties of the in vitro termination reactions have been explored with ribosomes, and core particles which completely lack L7/L12. Conditions for the initial studies were optimal for the control ribosomes. The rate of codon-dependent in vitro termination with ribosomes containing or lacking L7/L12 was studied. While there is a marked difference in rate of release of fMet between the two preparations, both exhibit in vitro termination activity. The reaction is a pseudo-second order reaction and in

Table 1
Inhibition of in vitro termination by anti L7/L12 immunoglobulin

Ribosomes	Additions	f[^{3}H]Met-released (Δpmol) anti-L7/L12 IgG		% Inhibition
		−	+	
70 S	UAA	1.03	0.32	69
70 S (−L7/L12)	UAA	0.31	0.33	0
70 S	Ethanol	1.36	0.53	61
70 S (−L7/L12)	Ethanol	0.68	0.62	9

In vitro termination assays were carried out as in section 2 except that 5 μl anti-L7/L12 IgG dialyzed against the assay buffer before use was pre-incubated with f[^{3}H]Met-tRNA/AUG/ribosome complex for 15 min at 4°C. Each reaction contained 2 pmol f[^{3}H]Met-tRNA/AUG/ribosome complex and 2 μg RF-2. Radioactivity extracted in the absence of release factors (0.1–0.15 pmol) was subtracted in each case

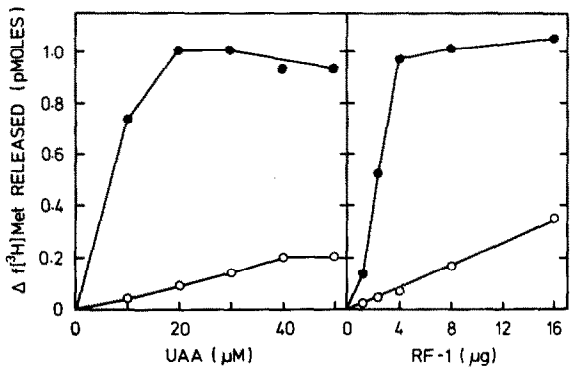

Fig.1. Requirements for codon-dependent in vitro termination. The UAA and RF requirements for release of fMet from f[^{3}H]Met-tRNA/AUG/ribosome complexes found with 70 S ribosomes (•——•) or cores lacking L7/L12 (○——○) were determined as in section 2. Each reaction contained 2 pmol f[^{3}H]Met-tRNA, ribosomes or cores at a concentration of 4 A_{260} and either (a) 6 μg RF-2 and indicated concentrations UAA or (b) 30 μM UAA and indicated amounts of RF-2. Radioactivity extracted in the absence of either codon (0.06 pmol) or RF-2 (0.08 pmol) was subtracted in each case.

our studies the control ribosomes had a rate constant of 0.6 l . mol^{-1} . min^{-1}, whereas the reaction on cores had a rate constant of 0.004 l . mol^{-1} . min^{-1}. However, the reaction rate for in vitro termination is dependent upon the concentration of both codon and release factor in the reaction (fig.1). The rates of reaction obtained with ribosomes lacking L7/L12 increase significantly with increasing concentrations of both UAA (fig.3a) and release factor (fig.3b). These concentrations are considerably higher than those required by unextracted ribosomes for maximum rates of in vitro termination. As a consequence the optimal conditions of fMet release from substrates containing ribosomes with L7/L12 and from substrates with ribosomes lacking L7/L12 are different. It is probably for this reason that in [5] and the cores in some of our experiments exhibited very low levels of codon-independent in vitro termination activity. Although the studies suggest that ribosomes can interact with the release-factors independent of L7/L12, L12, these ribosomal proteins clearly facilitate the codon-directed termination event by allowing the factor to function at a lower concentration. In vitro, the conditions can be manipulated to produce a less stringent requirement for L7/L12 in the termination event. It is probable however, that in vivo the presence of L7/L12 at its correct ribosomal site is critical for efficient functioning of the termination event during translation of a messenger RNA.

If substrates for in vitro termination prepared with cores lacking L7/L12 were assayed in the presence of ethanol or were supplemented with L7/L12 then a marked increase in the rate of reaction was observed (data not shown). The requirement for ethanol was studied further and the effect of different concentrations of ethanol on fMet release is shown in fig.2. In these studies a marked difference was seen not only between 70 S and L7/L12-depleted ribosomes but also between the two release factors, RF-1 and RF-2. RF-1 is less efficient than RF-2 in stimulating the release of fMet from the fMet-tRNA/AUG/ribosome complex in the codon-independent assay. At 20% (v/v) ethanol, fMet release stimulated by RF-1 is ~50% of that by RF-2 (fig.2a,b). Ribosomes lacking L7/L12 are more dependent on added ethanol than complete ribosomes, particularly in RF-1 mediated in vitro termination reactions. In this case the cores are less active than complete ribosomes at all concentrations of ethanol tested (fig.2a). In contrast, at 20% (v/v) ethanol the cores have similar activity to the complete ribosomes for the RF-2 mediated event (fig.2b).

RF-1 preparations can possess tRNA as a contaminant (Caskey, personal communication) and in the presence of ethanol the formation of fMet-ethyl ester is promoted [19]. The RF-1 (or RF-2) used for

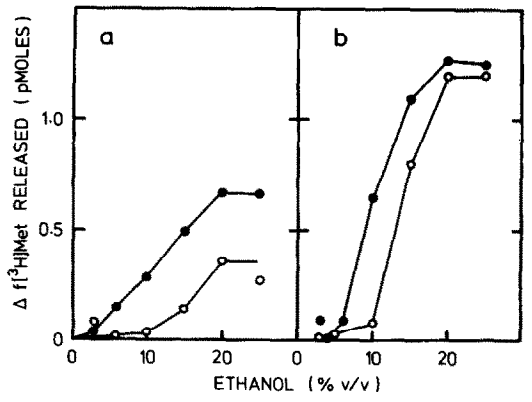

Fig.2. The effect of ethanol concentration on release of f[^{3}H]Met. The release of f[^{3}H]Met at different concentrations of ethanol for (a) RF-1 and (b) RF-2 was determined. Each reaction contained 3 pmol f[^{3}H]Met-tRNA, with 4 μg RF-1 or 2 μg RF-2. Reactions contained 70 S ribosomes (•——•) or cores lacking L7/L12 (○——○). Radioactivity extracted in the absence of release factors were subtracted.

the above studies in the absence of added tRNA did not stimulate the formation of the ester in the presence of ethanol. For this reason we do not think the lower activity of RF-1 for f[^{3}H]Met release (fig.2) results from a depletion of the substrate available for the codon-independent termination, but rather it is a reflection of functional differences between the factors.

The rate of the codon-independent reaction increases significantly both with complete ribosomes and cores lacking L7/L12, at levels of release factors well above that required for a maximum rate of in vitro termination in the codon-dependent reaction. At these higher concentrations of the factor, RF-2 behaves at 10% (v/v) ethanol like RF-1 at 20% (v/v) ethanol. While this difference in activity is difficult to interpret it may reflect slightly different binding sites for the factors on the ribosome or a difference in conformational flexibility between the factors. Methanol at 20% (v/v) has been shown directly to alter the conformation of the 50 S subunit. In our experiments, ribosomes lacking L7/L12 are more dependent on ethanol concentration than control ribosomes (fig.2). This is probably due to conformational effects which result from the loss of L7/L12. Removal of L7/L12 resulted in a localised conformational change within the ribosome [21] and methanol could restore GTPase activity to cores rendered inactive by removal of L7/L12 [22]. In the case of the GTPase activity the methanol allows expression of an activity intrinsic to the ribosome whereas in termination the co-operative interaction of the RF is also required. This requirement is demonstrated by a difference in response between RF-1 and RF-2 in the codon-independent assay.

The ion requirements for in vitro termination were also studied with L7/L12 deficient ribosomes. Both complete ribosomes and cores have a preference for NH_4^+ and a similar optimum at 75 mM. For EF-G dependent functions, the low activities of ribosomes lacking L7/L12 have been reported stimulated by NH_4^+ nearer to the activities of complete ribosomes [7]. This effect was not seen in termination reactions in vitro and no further stimulation was observed.

Our studies, in agreement with [7,8], suggest that L7/L12 do not represent the only part of the ribosome involved in the interaction of the soluble factors EF-Tu, EF-G and RF-1 and RF-2. This conclusion had not been obvious from the antibody studies since as for EF-G [4], L7/L12 were the only proteins implicated in the ribosomal binding of the release factors [6]. To explain this conundrum a role for the rRNA in the binding of the elongation factors has been suggested [7]. It should be noted also that the lack of inhibition by an Fab preparation does not necessarily imply that the antigenic protein is not involved in factor recognition (or any other function being assayed). The Fab sites do not represent the whole protein. The elongated ribosomal proteins sometimes contain functionally distinct domains which would require antibodies to each of the domains to reveal functional involvement. Thus it is possible that several proteins and/or parts of the rRNA form a factor binding site.

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