

# The ribosomal binding domain for the bacterial release factors RF-1, RF-2 and RF-3

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The *Escherichia coli* ribosomal proteins, L7/L12, are dominant over L11 in modulating the binding of RF-1 and RF-2 to ribosomes. The elevated activity of RF-2 on L11-lacking ribosomes over those containing L11 is abolished by IgG against L7/L12 or by removing the L7/L12 proteins. Adding back L7/L12 restores the original phenotype. The stimulatory factor, RF-3, is active on ribosomes depleted of L7/L12 but on those which lack L11 the stimulatory effects are less pronounced or often not seen. RF-3 cannot restore activity with RF-1 or RF-2 to ribosomes lacking both these sets of proteins. The stimulatory effects of an absence of either L11 or RF-3 on the activity of RF-2 are not additive or synergistic.

*Release factor      Ribosome      Binding domain*

## 1. INTRODUCTION

The *Escherichia coli* ribosomal proteins L7/L12 are required for the binding of the release factors RF-1 and RF-2 [1,2]. They are not absolutely essential, however, since at high concentrations of factor, ribosomes lacking L7/L12 support release factor activity which is insensitive to antibody against the L7/L12 proteins [3]. Recently L11 has been shown to modulate the binding of RF-1 and RF-2 in a reciprocal manner; in the absence of L11 RF-2 has a markedly lower  $K_m$  for binding into a termination complex while RF-1 requires L11 for activity [4,5]. The important domain is in the N-terminal part of protein L11 [5]. Previously RF-2 was shown to crosslink with both L7/L12 and L11 [6]. L11 has been mapped by immunoelectron microscopy at the base of the L7/L12 stalk in the 50 S subunit [7]. The release factor, RF-3, stimulates the activities of RF-1 and RF-2 also by lowering their  $K_m$  for binding into a termination complex with the ribosome [8,9].

We have examined how the stimulatory factor, RF-3, is affected by the ribosomal proteins L7/L12 and L11 and the relative contribution of these proteins to the binding domain of RF-1 and RF-2.

## 2. EXPERIMENTAL

Ribosomes and release factors were isolated from *E. coli* MRE600 and purified as in [10,11] or from the L11-lacking *E. coli* mutant AM 68 [5]. Ribosomal core particles and the supernatant proteins L7/L12 derived from the 70 S ribosome were prepared as reported [3]. The formation of a substrate complex and the assay in vitro of termination dependent upon codon and release factor was essentially as described [11]. A typical reaction contained in 0.05 ml: 50 mM Tris-OAc, pH 7.2, 30 mM Mg(OAc)<sub>2</sub>, 75 mM NH<sub>4</sub>OAc, 1–2 µg RF-2 or 5 µg RF-1 (purification stage V-11), 70 µg RF-3, as indicated, 3–5 pmol of f[<sup>3</sup>H]Met-tRNA/AUG/ribosome complex (4000 cpm/pmol), 0.08 A<sub>260nm</sub> units of UAA, UGA or UAG. Incubations were for 30 min at 20°C. The f[<sup>3</sup>H]Met was extracted into ethyl acetate at pH 1. RF-3 activity was assayed essentially as in [8]. A typical reaction contained in 0.05 ml: 50 mM Tris-OAc, pH 7.2, 30 mM Mg(OAc)<sub>2</sub>, 50 mM KCl, 3–5 pmol f[<sup>3</sup>H]-Met-tRNA/AUG/ribosome substrate (4000 cpm/pmol), 1 µg RF-2, 5 µg RF-1, 70 µg RF-3 (purification fraction III-8), 0.05 A<sub>260nm</sub> UAA or UAG or UGA. The IgG against L7/L12 was prepared as

previously described [3].

### 3. RESULTS AND DISCUSSION

Ribosomes lacking L7/L12 were prepared from normal 70 S ribosomes and from ribosomes derived from an *E. coli* L11-lacking mutant [5]. This gave a choice of ribosomes: (a) with a full complement of proteins, (b) with all proteins except L7/L12, (c) with all proteins except L11 and (d) with all proteins except L7/L12 and L11. The relative activities of these ribosomes with RF-1 and RF-2 are shown in fig.1. The control ribosomes showed activity with both factors which reflected the specific activity of each factor (panel a). The L7/L12 depleted ribosomes gave greatly reduced activity with both factors (panel b) while the L11-lacking ribosomes showed the expected highly elevated activity with RF-2 and low activity with

RF-1 (panel c). Ribosomes lacking L7/L12 and L11 had essentially no activity with RF-1 and poor activity with RF-2 (panel d). This indicates that the increased affinity of RF-2 for ribosomes lacking L11 [5] is drastically reduced when L7/L12 are removed and suggests that the modulation of RF-1 and RF-2 binding at the domain by L11 is dependent upon an initial interaction for which L7/L12 are necessary. A general conformational change in the binding domain for the factor rather than from a specific requirement of L7/L12 for the binding site could also cause this effect on removing L7/L12 from the L11-lacking ribosome. However, anti-L7/L12 had a similar effect on the activities of RF-1 and RF-2 with L11-lacking ribosomes as shown in fig.2A, arguing against a non-specific conformational change at the domain when L7/L12 are removed. The loss of activity of L11-lacking ribosomes was reversible since it could be restored by titration of the L7/L12 proteins back onto the depleted ribosomal particle as shown in fig.2B.

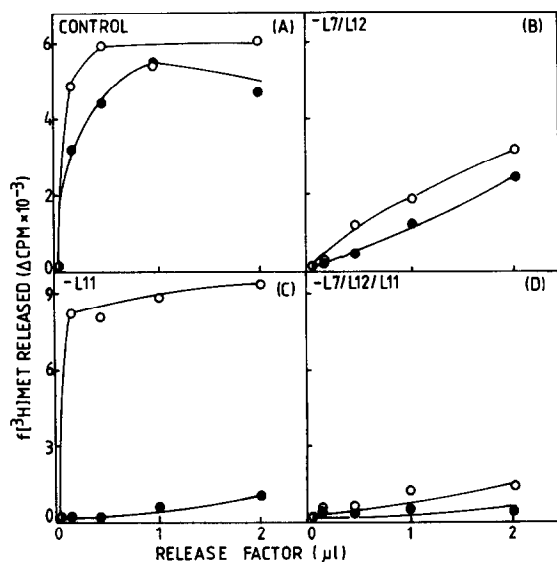


Fig.1. Activity of ribosomes depleted in L7/L12, or L11 or L7/L12/L11 with RF-1 and RF-2. Ribosomes were prepared as described in section 2 and used to make substrates for in vitro termination. Each was assayed with increasing amounts of (●—●) RF-1 (7 μg/μl) or (○—○) RF-2 (2 μg/μl). Backgrounds in the absence of release factor varied from 300–3000 and were subtracted in each case. (A) Control ribosomes, (B) ribosomes lacking L7/L12, (C) ribosomes lacking L11, (D) ribosomes lacking L7/L12 and L11.

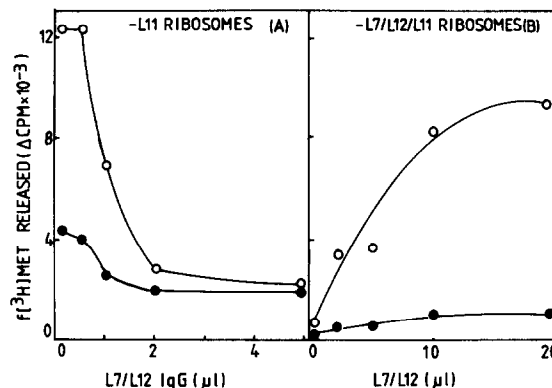


Fig.2. Activity of ribosomes lacking L11 dependent upon L7/L12. (A) Ribosomes from the L11-lacking mutant AM68 were used to make substrate for in vitro termination and assayed with (●—●) RF-1 and (○—○) RF-2 in the presence of increasing amounts of IgG against L7/L12. A background in the absence of release factor (2000 cpm) was subtracted in each case. (B) Ribosomes from the L11-lacking mutant AM68 were depleted in L7/L12. The resulting cores were used to make substrate for in vitro termination and assayed with (●—●) RF-1 and (○—○) RF-2 with increasing amounts of the L7/L12 protein fraction. A background in the absence of release factor (5000 cpm) was subtracted in each case.

The stimulation of RF-1 and RF-2 activity by RF-3 previously reported [8] was confirmed as shown in fig.3 for both RF-1 and RF-2 on ribosomes containing a full complement of proteins. When the ribosomes were depleted of L7/L12, RF-3 could stimulate the remaining activity of RF-2, and RF-1 activity but reproducibly to a lesser degree. This indicates that RF-3 can still function on L7/L12 depleted ribosomes and supports the assertion that there is a difference in the binding characteristics of RF-1 and RF-2 at the domain. Previously one fraction of an IgG against L11 was shown to affect RF-1 but not RF-2 while another fraction of the same antibody preparation affected RF-2 and not RF-1 [5]. RF-3 was not able to restore activity to L11-lacking ribosomes with RF-1 and reproducibly had little or no effect on the activity of these ribosomes with RF-2. When L7/L12 and L11 were all missing from the ribosome there was no activity with either RF-1 or

RF-2 and RF-3 had no effect (fig.3). The optimum conditions used here to observe the RF-3 stimulation of the in vitro termination reaction require  $K^+$  rather than  $NH_4^+$  in the assay. Under these conditions L11-lacking ribosomes had no activity with RF-1 and L7/L12/L11 depleted ribosomes had no activity with RF-2 in contrast to the low activities seen with these ribosomes in the presence of  $NH_4^+$  as in fig.1 and fig.2.

The lack of an RF-3 effect seen with L11-lacking ribosomes could indicate a requirement for this protein to enable RF-3 to bind at the domain. We have investigated this possibility in two ways: first at low concentrations of factor RF-2, and second in the presence of anti-L7/L12. As shown in fig.4 there was a small but reproducible stimulation of RF-2 activity with RF-3 at low concentrations of RF-2 when assayed with  $K^+$  (fig.4A—lower curves) although it is significantly lower than that seen with normal ribosomes (fig.3). No stimulation oc-

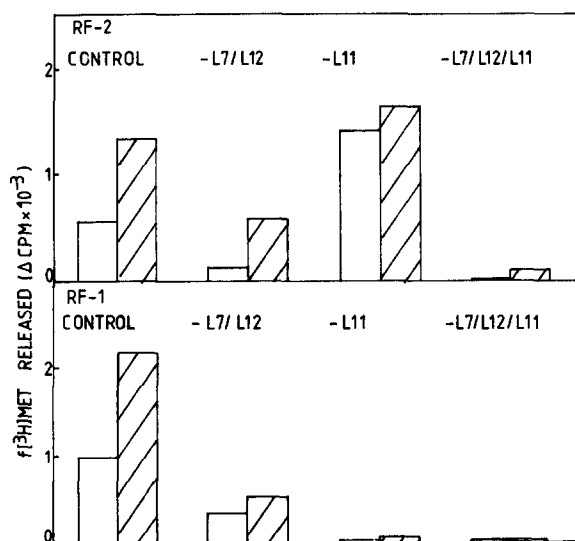


Fig.3. Effect of RF-3 on activity of ribosomes depleted in L7/L12, or L11 or L7/L12/L11. Ribosomes were prepared as described in section 2 and used to make substrate for in vitro termination. Each was assayed as described in a codon-dependent reaction with RF-2 (2  $\mu$ g) (upper panel) or RF-1 (14  $\mu$ g) (lower panel) where 50 mM  $K^+$  replaced the  $NH_4^+$  of the normal assay either with partially purified RF-3 (70  $\mu$ g) (hatched bars), or without (open bars). Backgrounds in the absence of release factor (300–400 cpm) were subtracted in each case.

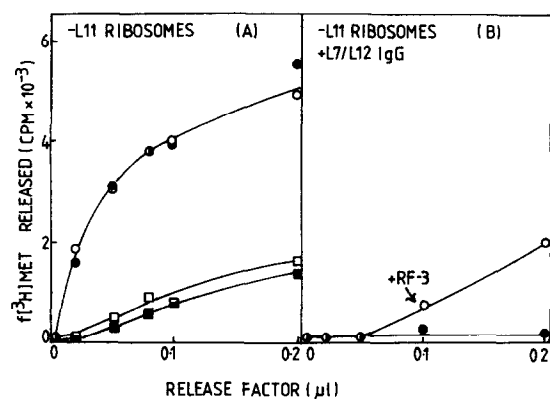


Fig.4. The effect of RF-3 on RF-2 activity on L11-lacking ribosomes. (A) L11-lacking ribosomes were used to form substrate for codon-dependent in vitro termination and assayed at low concentrations of RF-2 as indicated either under normal buffer conditions (75 mM  $NH_4^+$ ) in the presence of RF-3 (○—○) or in its absence (●—●) or under conditions optimal for RF-3 stimulation (50 mM  $K^+$ ) in the presence of RF-3 (□—□) or in its absence (■—■). Backgrounds in the absence of release factor (600 cpm) were subtracted in each case. (B) Codon dependent termination was measured with L11-lacking ribosomes at low concentrations of RF-2 is indicated in the presence of IgG against L7/L12 in the presence of RF-3 (○—○) or its absence (●—●) under conditions optimal for RF-3 stimulation. Backgrounds in the absence of release factor (2000 cpm) were subtracted in each case.

curred when assayed with  $\text{NH}_4^+$  (fig.4A, upper curves). A definite stimulation of RF-2 activity with RF-3 was observed after treatment of the ribosome with anti-L7/L12 however; it indicates that RF-3 can indeed function on L11 depleted ribosomes (fig.4B). This effect is seen because of the dominance of the L7/L12 over L11 in modulating the binding of the release factors. Moreover it provides the first clear separation of the stimulatory effects of RF-3 and of a lack of L11 on the activity of RF-2. When L7/L12 is present then the lowered  $K_m$  for RF-2 binding provided by the absence of L11 [4] masks a similar effect mediated by RF-3.

The inability of the release factors to function on ribosomes lacking L7/L12 and L11, observed under assay conditions optimal for RF-3 stimulation, was reexamined under the usual conditions for the termination assay *in vitro*, i.e., under optimal conditions for RF-1 and RF-2. As shown in table 1 there was stimulation of the other release factor activities by RF-3 with complete ribosomes. Removing L7/L12 resulted in RF-3 stimulating the low activities of RF-1 and RF-2 consistent with the results of fig.3. No stimulation of RF-1 activity on L11-lacking ribosomes was seen with RF-3 but any

possible effect on RF-2 was masked by the highly elevated activity of RF-2 alone. The L11-lacking mutant AM 76, whose ribosomes have higher activity with RF-1 [5] also showed no stimulation with RF-3. Ribosomes lacking L7/L12/L11 had no activity with RF-1, some activity with RF-2, but no stimulation or restoration of activity was seen with RF-3. Given that RF-3 does stimulate RF-2 activity on L11-lacking ribosomes in the presence of anti-L7/L12 the above result may indicate that removal of L11/L7/L12 together from the release factor binding domain results in significant conformational changes which interfere with RF-3 function.

We have examined in a series of preliminary experiments the requirement of other ribosomal proteins for RF-3 activity at the release factor binding domain using 1.5 M LiCl depleted cores and a reconstitution protocol whereby a single protein is omitted [12]. Previously this approach has shown a requirement for L16 in peptidyl-tRNA hydrolysis and in addition L11 and L7/L12 in codon-dependent termination [13,4]. However, to date no additional proteins have been observed to be required for RF-3 function.

Collectively the results suggest that the L7/L12 stalk structure, which is relatively exposed on the 70 S ribosome as determined by immunoelectron microscopy [6] provides an initial recognition site for the release factors 1 and 2. This site may have a high affinity but relatively low specificity for release factors since the other protein synthesis factors, EF-Tu and EF-G, also bind here [14,15]. Once the initial contact has been made then the specificity and affinity at a functional site may be mediated by other components, such as protein L11 in the domain itself or external factors such as RF-3. RF-1 and RF-2 apparently recognise the termination codons at the A site and although their binding domain does not overlap with the tRNA binding site it overlaps with that of EF-Tu [16]. It has not yet been established whether the L7/L12/L11 binding domain and the codon recognition domain are indeed the same. The A site has been inferred to be far from the L7/L12 stalk from a number of studies but recently authors in [17] have argued persuasively that the A site is in a groove quite near the L7/L12 stalk. Our studies on the RF binding domains are more consistent with such a placement. How the release factor influences the peptidyltransferase centre, also

Table 1

The effect of RF-3 on the activities of RF-1 and RF-2 with ribosomes or depleted cores

Ribosomes 70 S	RF-1 (f[ $^3\text{H}$ ]Met released)		RF-2 ( $\Delta\text{cpm}$ )	
Proteins absent	RF-3 —	RF-3 +	RF-3 —	RF-3 +
None	7642	9571	7072	10288
L7/L12	570	1153	623	1257
L11 (AM68)	290	283	11961	11869
L11 (AM76)	1560	1441	—	—
L7/L12/L11	0	0	2109	1992

Ribosomes with either L7/L12 and/or L11 absent as indicated were used to form substrate for *in vitro* termination and assayed in a codon-dependent reaction with RF-1 (5–10  $\mu\text{g}$ ) and RF-2 (1–2  $\mu\text{g}$ ) dependent upon RF-3 (70  $\mu\text{g}$ ); under conditions optimal for RF-1 and RF-2 activity (75 mM  $\text{NH}_4^+$ ) rather than for RF-3 stimulation. Backgrounds in the absence of release factor varied from 300–2200 cpm were subtracted in each case

inferred to be far from the RF binding domain, is unclear at present.

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