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RNA secondary structure and translation inhibition: analysis of mutants in the *rplJ* leader

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We have carried out measurements of the stable binding of the ribosomal protein (r-protein) complex L10–L7/L12 to mutant forms of the mRNA leader of the *rplJ* operon of *Escherichia coli*. One of the point mutations, base 1548, which lies within the L10–L7/L12-protected region, almost completely abolishes *in vitro* formation of a stable complex of L10–L7/L12 with *rplJ* mRNA leader, and a second point mutation, base 1634, strongly reduces it. These observations constitute strong support for the proposition that L10–L7/L12 binds to the *rplJ* leader in bringing about translational feedback. To account for the action of these and other mutations, and to explain the mechanism of translation feedback inhibition, we suggest a secondary structure model involving alternate forms of the *rplJ* mRNA leader.

Key words: ribosomal protein complex/mRNA/*rplJ* operon/secondary stucture model

Introduction

In *Escherichia coli* ribosomal protein (r-protein) structural genes are biosynthetically regulated, at least in part, by a feedback mechanism that is presumed to involve the binding of one of the gene products of each r-protein operon to its own polycistronic mRNA leader, thereby reducing the translational efficiency of itself and other genes in the same operon (Lindahl and Zengel, 1982). The *rplJ* operon is one that is regulated in this manner (Dennis and Fiil, 1979; Fiil *et al.*, 1980). It comprises genes which encode two r-proteins, L10 (*rplJ*) and L12 (*rplL*), as well as two genes encoding subunits of RNA polymerase (Fiil *et al.*, 1979; Post *et al.*, 1979). *rplJ* and possibly *rplL* are translationally feedback regulated by L10 (Brot *et al.*, 1980; Yates *et al.*, 1981), or by the L10–L7/L12 (Pettersen and Liljas, 1979) combination (Fukuda, 1980; Yates *et al.*, 1981; Johnsen *et al.*, 1982). Two recent observations have shed some light on the mechanism of translation regulation in the *rplJ* operon. First, L10–L7/L12 binds to a defined region of the *rplJ* mRNA leader (Johnsen *et al.*, 1982). Second, point mutations and deletions have been isolated in the *rplJ* leader which abolish feedback regulation (Friesen *et al.*, 1983). These observations have in common the striking characteristic that they define a region of the mRNA leader that lies some 80–200 bases upstream from the translation initiation site of *rplJ*. Thus an understanding of the mechanism of *rplJ* translational regulation must take into account this long-range effect. The two observations referred to above suggest, but do not prove, that L10–L7/L12 binding is essential for *rplJ* regulation. Proof of this requires a demonstration that non-feedback mutants are also deficient in L10–L7/L12 binding. Here we present experimental evidence to show this to be true, and suggest a model for translation regulation involving alternate forms of the *rplJ* mRNA leader.

Results and Discussion

We have previously used a *rplJ-lacZ* fusion carried on a low-copy plasmid to isolate two point mutants in the *rplJ* leader mRNA which are unable to respond to feedback inhibition (Friesen *et al.*, 1983). *In vivo* these mutants produce as much L10-β-galactosidase fusion protein activity as the wild-type but, in contrast to the wild-type, fail to exhibit reduced synthesis of the fusion protein when placed in the same cell as a plasmid that is producing a slight excess of L10–L7/L12. Figure 1 shows data from an experiment whose aim was to establish whether these mutants are also incapacitated in their translation inhibition when analyzed in an *in vitro* coupled transcription-translation system. Since these experiments were carried out with *lacZ* gene fusions, we determined β-galactosidase activity as a measure of r-protein synthesis. Since some degree of non-specific inhibition of translation has been reported (Johnsen *et al.*, 1982), a parallel experiment was conducted with a plasmid carrying a *lacZ* fusion to the unrelated r-protein gene, *rpsA* (S1) (Christiansen and Pedersen, 1981). When this non-specific effect was subtracted (Figure 1d), the results showed that addition of L10–L7/L12 inhibited synthesis of the fusion protein from the wild-type *rplJ-lacZ* fusion by 48% (Figure 1a) and from the two mutants by only 13% (JF3239; Figure 1b) or 15% (JF3241; Figure 1c). These results are consistent with the *in vivo* characteristics of the mutants (Friesen *et al.*, 1983), although not as marked. It should be noted that for reasons that are not yet understood even with the wild-type *rplJ* mRNA leader, the degree of translation inhibition *in vivo* is higher than that observed *in vitro*.

Johnsen *et al.* (1982) have demonstrated the stable binding of the L10–L7/L12 complex to the *rplJ* mRNA leader. The region of the RNA that was protected by L10–L7/L12 includes one of the point mutants (base 1548) that fail to show translation inhibition and lies near the other (base 1634). We determined whether these two mutations affected the formation of stable RNA-protein complex. Figure 2 shows that the C to T change at base 1548 (plasmid pJF3241) almost entirely abolishes formation of the stable complex, reducing it to the same level as a deletion (plasmid pNF1776) that removes almost the entire *rplJ* mRNA leader. The C to T change at base 1634 (plasmid pJF3239) results in an ~50% reduction in stable complex formation. These results provide the first evidence that binding of a regulatory r-protein to its mRNA leader is directly involved with regulation of its own biosynthesis.

We have attempted to account for the results reported above, as well as earlier observations, in a model in which the

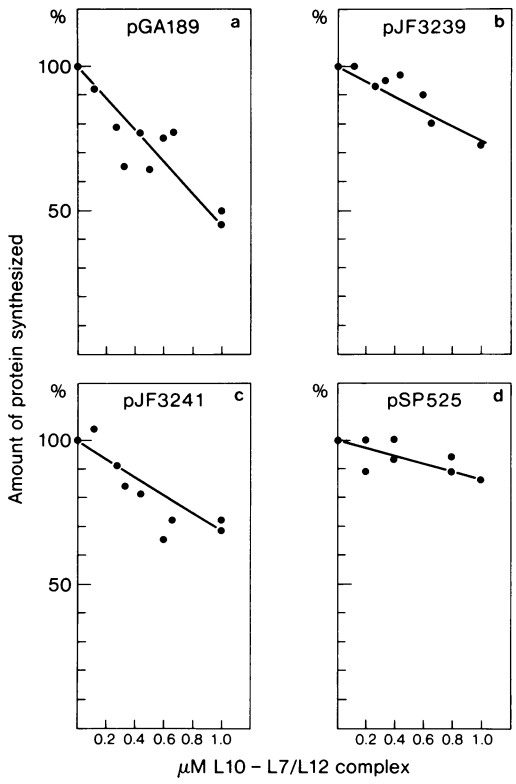

Fig. 1. Effect of L10–L7/L12 complex on *in vitro* synthesis of L10-β-galactosidase fusion protein. DNA templates: **(a)** pGA189 (wild-type *rplJ* leader), **(b)** pJF3239 (C to T mutation at base 1548), **(c)** pJF3241 (C to T mutation at base 1634) were described previously (Friesen *et al.*, 1983). **(d)** pSP525, which is included as a control, carries a translation fusion between *E. coli rpsA* and *lacZ* (J. Skou and S. Pedersen, personal communication). *In vitro* protein synthesis conditions were as described in Materials and methods. Regression analysis indicates a variance of ± 15%.

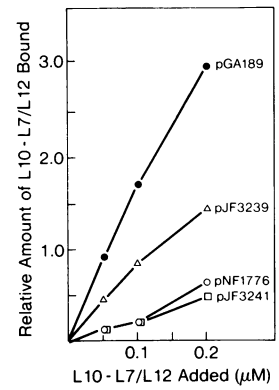

Fig. 2. Binding of L10–L7/L12 complex to the *rplJ* mRNA leader. The experiments were performed with *in vitro* synthesized RNA as described in Materials and methods.

rplJ mRNA leader is capable of assuming two alternate configurations. In formulating this scheme, we have taken into account the following observations. (i) There are two kinds of *rplJ* leader mRNA mutants that fail to register feedback, one which almost completely abolishes formation of stable complex and a second which reduces it by half (Figure 2). (ii) There are mutants lying some 80–200 bases upstream from the translational initiation site of *rplJ* that reduce the translation efficiency of *rplJ* and *rplL* (Fiil *et al.*, 1980; Friesen *et al.*, 1983). (iii) L10–L7/L12 binds stably to the *rplJ* mRNA leader in a specific location (Johnsen *et al.*, 1982). (iv) A 96-base deletion extending from base 1497 to base 1593 entirely abolishes expression of *rplJ* (Friesen *et al.*, 1983). (v) The secondary structure of mRNA in the region of the translation initiation site (Shine and Dalgarno, 1974) can influence the efficiency of translation (Hall *et al.*, 1982).

In the model we consider that the portion of *rplJ* leader mRNA which lies between base 1505 and base 1721 (the start codon for *rplJ*) exists normally in Form I (Figure 3, left), in which the region of the *rplJ* ribosome-binding site (Shine and Dalgarno, 1974) is not base-paired and *rplJ* is thus open for translation. Form I is stabilized by stems A, B and C (Figure 3, left). We suggest that stem A and at least a part of stem C is important for recognition by L10–L7/L12 (see below). When L10–L7/L12 binds to stem A, the structure is caused to shift to Form II (Figure 3, right). Stems A and B′ of Form II comprise the stable L10–L7/L12 binding site since they are protected *in vitro* by L10–L7/L12 (Johnsen *et al.*, 1982). The transition between Form I and Form II is brought about by a shift in base pairing (Figure 3, lower) such that bases 1584 through 1589, which formerly were in stem C of Form I, now base pair with bases 1541–1537 in stem B′ of Form II; bases 1519–1508 in stem B of Form I are thereby excluded from base pairing. The consequence of weakening stem C of Form I by pairing the region of bases 1584–1589 in an alternate structure is to favour the formation of stem D of Form II (Figure 3, right); the displaced portion of Form I stem C, interacts with the mRNA region near the ribosome-binding site of *rplJ* to sequester it and to reduce the *rplJ* translational efficiency. This is the mechanism whereby binding of L10–L7/ L12 in the central region of the *rplJ* leader signals the inhibition of *rplJ* translation.

Stems A and B′ of Form II coincide almost exactly with the RNA sequence which is protected by L10–12 against nuclease attack (Johnsen *et al.*, 1982). An interesting feature of this composite structure is the sequences including bases 1576–1585 and bases 1534–1543 (bold face, Figure 3). These constitute a region of dyad symmetry with eight of ten base matches, part of it (bases 1577–1582) is homologous to a 23S rRNA sequence (Noller, 1980). These structural features might be important for L10–L7/L12 recognition.

The model presented here can explain the phenotypes of the two feedback-insensitive mutations. The first kind of mutation, the C to T transition mutation at base 1548, results both *in vivo* (Friesen *et al.* 1983) and *in vitro* (Figure 1) in reduction of translation feedback; the formation of a stable L10–L7/L12 complex with the *rplJ* mRNA leader of this mutant is undetectable above background (Figure 2). Base 1548 lies within the region, spanning bases 1523–1579, to which L10–L7/L12 has been shown *in vitro* to bind (Johnsen *et al.*, 1982). Both Form I and Form II are dependent for their structure on base pairing in stem A. The mutation at base 1548 destroys one G-C pair, thus weakening the hydrogen bonding at the foot of stem A and shortening the stem. This abolishes either binding or recognition, or both, of L10–L7/L12 to the *rplJ* mRNA leader. Thus it seems clear that the length of stem A is crucial for these functions.

The C to T transition mutation at position 1634 is the second kind of non-feedback mutant. It is also deficient in feedback translation inhibition, both *in vivo* (Friesen *et al.*, 1983) and *in vitro* (Figure 1), yet retains ~50% of the normal ability to form stable L10–L7/L12–mRNA complex (Figure 2). This mutation lies outside of the region of the

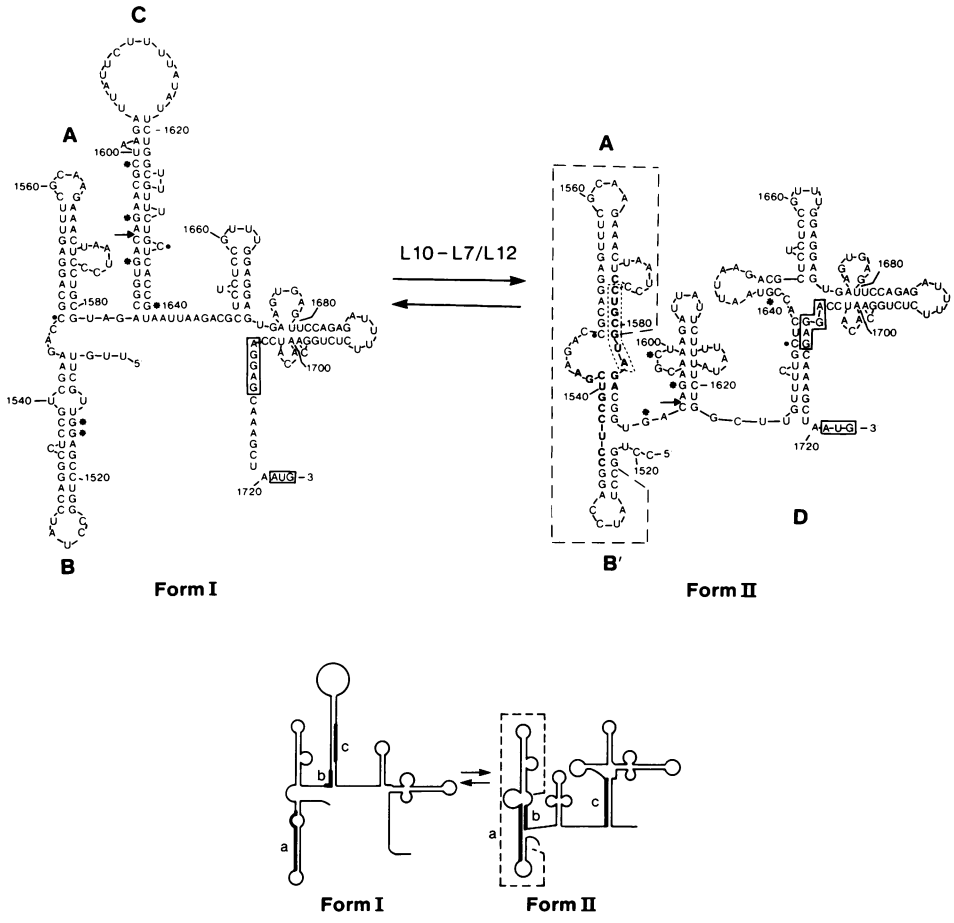

Fig. 3. Upper. Possible secondary structures of the *rplJ* leader mRNA. Base numbers are as in Post *et al.* (1979). Mutations that inhibit translation of *rplJ* (Fiil *et al.*, 1980) (*) or escape translation inhibition (Friesen *et al.* 1983) (●) are indicated. The rightward end-point of a 96-base deletion (Friesen *et al.*, 1983) (the leftward end-point does not appear in this diagram) is shown by an arrow between base 1592 and base 1593. In Form I, the calculated free energies (Tinoco *et al.*, 1973) of the A, B and C stems are −25.8, −21.8 and −28.0 kcal, respectively. In Form II, a 7-base homology to 23S rRNA is shown in a dashed box, and a direct repeat (8 out of 10 bases), is shown in boldface. The calculated free energy of the A−B′ structure of Form II is −38.4 kcal. Stem D, Form II, blocks the *rplJ* ribosome-binding site; the Shine-Dalgarno (1974) sequence and the initiator codon are enclosed in a box. The free energy of stem D, Form II, is −12.2 kcal. The C to T mutation at base 1634 lowers the free energy of this stem to −5 kcal. **Lower.** A diagram showing the main features of Forms I and II. The thick lines labelled a, b and c indicated the regions in which base pairing is shifted by the binding of L10−L7/L12. In both the upper and lower diagrams, the large dashed box in Form II encloses the area that is protected *in vitro* by L10−L7/L12 (Johnsen *et al.*, 1982).

mRNA leader that is protected by L10−L7/L12 (Johnsen *et al.*, 1982), but within the region that has potential for base pairing near the *rplJ* ribosome binding site (stem D, Form II). We suggest that base 1634 has two functions. (i) It is part of a recognition site (but not a binding site) for L10−L7/L12; hence, mutation results in reduced, but not abolished, binding. (ii) It is part of the region which, in base pairing near the ribosome-binding site, inhibits *rplJ* translation; hence mutation weakens base pairing in this region (−12.2 kcal for the wild-type; −5 kcal for the mutant) and renders *rplJ* translation permanently open, indifferent to L10−L7/L12 binding elsewhere in the mRNA leader.

The model presented in Figure 3 can also explain the action of mutants we have previously isolated (Fiil *et al.*, 1980), whose effect is to reduce *rplJ* expression. The positions of these mutations are indicated by asterisks in Figure 3. Four of them weaken the structure of stem C of Form I and two weaken stem B. These would tend to enhance Form II, the effect of which is to sequester the ribosome-binding site and reduce *rplJ* translation. Since this class of mutants is still subject to translational feedback (Friesen *et al.*, 1983), we conclude that the weakening of stems B or C does not normally affect recognition and/or binding of L10−L7/L12. A 96-base deletion that removes stems A, B and C completely abolishes *rplJ* expression (Friesen *et al.*, 1983). This is to be expected because in the absence of stem C, the formation of stem D (Form II) is strongly favoured, thus reducing *rplJ* translation. As a final comment, we note that, while the assumption of RNA secondary structure can explain many experimental observations, there is no evidence to confirm its existence. Morevoer, the further complication of tertiary RNA interaction has not yet even begun to be considered.

Materials and methods

In vitro protein synthesis

In vitro protein synthesis was carried out as described previously (Johnsen *et al.*, 1982). Total reaction volume was 50 μl, L10−L7/L12 complex (a gift from Andres Liljas, Uppsala, Sweden) was stored and added in a buffer containing the following: 10 mM Tris-HCl, pH 8.0, 40 mM KOAc, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT). Synthesis was stopped after 45 min by addition of 1 ml of O-nitrophenyl-β-D-galactopyranoside (ONPG), and samples were incubated at 37°C. When yellow colour had developed, 5 μl of 66% acetic acid was added. The sample was centrifuged, and the supernatant was mixed with an equal volume of 1 M Na_2CO_3. Absorbance at 436 nm of the samples

was normalized to the absorbance obtained without addition of L10–L7/L12 complex (which was ~0.1/min of incubation with ONPG).

Plasmids

Plasmid pGA189, pJF3239, pJF3241 have been described previously (Friesen *et al.*, 1983), as has pNF1776 (Johnsen *et al.* 1982), which is equivalent to pJF3216 (Friesen *et al.* 1983); the latter two plasmids carry a deletion of the entire *rplJ* leader. Plasmid DNA was prepared and purified according to standard procedures.

In vitro transcription

RNA was transcribed for 60 min at 37°C from ~2 μg DNA template in 100 μl of: 120 mM Tris-HCl, pH 7.8, 8 mM $Mg(OAc)_2$, 0.2 mM DTT and 0.2 mM of each of the four ribonucleotide triphosphates, 50 μCi of [^{3}H]UTP and 1 μg of RNA polymerase (a gift from Koichi Yoshinanga, Kyoto, Japan). The transcribed RNA was extracted with phenol, ethanol precipitated and resuspended in 250 μl of: 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM DTT, 10 μg/ml yeast RNA. The synthesized RNA was used directly in the binding studies without attempts to purify it from the template DNA.

Protein-RNA binding

Binding conditions and the filter binding assay were as described before (Johnsen *et al.*, 1982), using *in vitro* synthesized RNA. The amount of acid-precipitable [^{3}H]RNA present in each of the binding assays was equal to within 10%. For each template, the amount of RNA bound to L10–L7/L12 was normalized to the total amount of mRNA present in the binding reaction. This was determined by hybridization to a 922 base *Pst*I DNA fragment (bases 869–1791) that contains all of the *rplJ* RNA leader and a portion of *rplJ*. The amount of RNA capable of binding and capable of hybridizing to a purified *lacZ* DNA fragment was also determined; this was equal to within ± 10% for all samples, indicating that all transcripts were of approximately equal length. This was confirmed by direct visualization of ^{32}P-labelled transcripts on denaturing gels from related experiments, which also indicated that all transcripts were of approximately equal length (unpublished results).

Nucleotide numbering

The nucleotides are numbered according to Post *et al.* (1979).

Acknowledgements

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