

Feedback Regulation of the *rplJL-rpoBC* Ribosomal Protein Operon of *Escherichia coli* Requires a Region of mRNA Secondary Structure

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The *rplJ-rpoBC* (L10) operon of *Escherichia coli* is regulated in part through translational repression (feedback regulation) by ribosomal protein L10 or a complex of ribosomal proteins L10 and L7/L12 (L10-L7/L12). We have constructed mutants in the untranslated leader region of a *rplJ-lacZ* fusion by oligonucleotide-directed mutagenesis. The mutations include several deletions and a number of single base changes, all of which fail to exhibit normal feedback regulation. Chemical probing of part of the *rplJ* mRNA leader in the mutagenized region confirms that all of the mutations lie in a stem structure located 140 nucleotides upstream from the translation start-site. The structure includes a 12 base-pair stem, a four base stem-loop, and a six base bulge-loop. Point mutations that abolish feedback regulation are presumed to disrupt this stem structure. Pseudorevertants of selected point mutations were constructed by combining pairs of single base mutations. In these cases, both the secondary structure of the RNA and feedback regulation were restored. The results allow us to define a region of secondary structure in the *rplJ* mRNA leader that is necessary for feedback regulation.

1. Introduction

Regulation of ribosomal protein (r-protein[†]) synthesis in *Escherichia coli* occurs in part at the level of translation by an autogenous feedback mechanism that involves at least one of the gene products of each r-protein operon (Dennis & Fiil, 1979; Lindahl & Zengel, 1979; Dean *et al.*, 1981; Yates & Nomura, 1981; Yates *et al.*, 1981). Experiments both *in vivo* and *in vitro* support the idea that this regulation occurs as the result of an interaction between a regulatory r-protein and the untranslated leader region of its own mRNA. The result of such an interaction is a decrease in the level of translation of some, or all of the r-proteins encoded by that polycistronic mRNA (for reviews, see Lindahl & Zengel, 1982, 1986).

Previous studies have demonstrated that the *rplJ*

and *rplL* genes of the *rplJL-rpoBC* operon are regulated by a feedback mechanism similar to that described above (Dennis & Fiil, 1979; Holowachuk *et al.*, 1980; Brot *et al.*, 1980; Fukuda, 1980; Yates *et al.*, 1981). It has been shown that the addition of protein L10 or a complex of one L10 and four L7/L12 molecules (L10-L7/L12) to an *in-vitro* DNA-directed protein synthesis system can effect feedback regulation (Brot *et al.*, 1980; Yates *et al.*, 1981; Johnsen *et al.*, 1982). It has been shown that the L10-L7/L12 complex can bind specifically to the untranslated leader region of the *rplJ* mRNA and protect a region of that RNA from ribonuclease digestion (Johnsen *et al.*, 1982). Additional studies have shown that the central part of the 375 nucleotide leader is important for translation of the mRNA and that mutations in this region can abolish feedback regulation (Fiil *et al.*, 1980; Friesen *et al.*, 1983). Furthermore, mRNA encoded by these non-feedback mutants shows a reduced ability to form a stable complex with L10-L7/L12 (Christensen *et al.*, 1984).

A model to describe the regulation of the *rplJL-rpoBC* operon has been proposed (Christensen *et al.*,

[†] Abbreviations used: r-protein, ribosomal protein; tRNA, transfer RNA; bp, base-pair(s); DMS, dimethyl sulfate; DEP, diethylpyrocarbonate; X-gal, 5-bromo-4-chloro-3-indolyl β -galactosidase; EMB, eosin/methylene blue; R.F, replicative form; DTT, dithiothreitol.

1984). It is thought that the leader region of the *rplJ* mRNA can assume two alternative secondary structure configurations termed form I and form II. Under repressing conditions, it is thought that L10-L7/L12 binds to form I about 180 nucleotides upstream from the translation start-site. It is suggested that this binding causes a change in the structure of part of the *rplJ* mRNA such that the Shine-Dalgarno sequence becomes involved in the formation of a stem structure that prevents translation of the message. According to the model, the structure of the mRNA at the L10-L7/L12 binding site remains essentially unchanged regardless of the extent of protein binding. So, the secondary structure of the L10-L7/L12 binding site might provide important structural features that are required for protein binding while the sequences located downstream are involved in a conformational realignment of the secondary structure. Accordingly, the alternative secondary structure model accounts for the effects of the mutations described above and it allows one to make predictions about the regulatory mechanism.

In order to study further the regulatory site on the *rplJ* mRNA leader, we have constructed a set of deletion, single base change, and double base change mutations in the leader region of a *rplJ-lacZ* fusion plasmid and have determined the effect of these mutations on feedback regulation. The mutations are within the L10-L7/L12 binding site (Johnsen *et al.*, 1982) and all lie in a region of the mRNA that was predicted to include a stem structure with a bulged loop (Friesen *et al.*, 1983; Christensen *et al.*, 1984). We have examined the secondary structure of the *rplJ* mRNA in this region by chemical modification and find that it forms a structure similar to that predicted on the basis of theoretical considerations. Mutations that abolish feedback regulation are presumed to disrupt this structure. Additional mutations that restore base-pairing also restore feedback regulation. These results allow us to define a region of secondary structure in the mRNA that is necessary for feedback regulation. Furthermore, it is the overall secondary structure of this region and not the primary sequence *per se* that is required for this regulation.

2. Materials and Methods

(a) Materials

[γ - 32 P]ATP (spec. act. 3000 Ci/mmol) was obtained from New England Nuclear. Dimethyl sulfate (DMS) was from Eastman Chemical Co., and diethylpyrocarbonate (DEP) was from Sigma. Chemicals for electrophoresis were purchased from Eastman. Restriction endonuclease, bacteriophage T4 DNA ligase, and DNA polymerase (Klenow) were supplied by New England Biolabs or Bethesda Research Laboratories and were used as recommended by the manufacturer; reverse-transcriptase was from Life Sciences. Chemicals for DNA synthesis were purchased from Bachem, Beckman, and Applied Biosystems.

(b) Oligonucleotide synthesis and construction of mutant plasmids

Oligonucleotides were synthesized by 2 different methods. Four oligonucleotides were synthesized manually using phosphate triester chemistry (Miyoshi *et al.*, 1980) using fully protected nucleotide dimers purchased from Bachem. The remaining 6 oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer using phosphite triester chemistry (Beaucage & Carruthers, 1981). Oligonucleotides were purified by electrophoresis on denaturing polyacrylamide gels followed by passage over a Waters Sep-Pack cartridge.

Oligonucleotide-directed mutagenesis was essentially as described by Zoller & Smith (1983), except that strain BMH71-18*mutL* (Carter *et al.*, 1985) was used as a recipient for mutagenized DNA. Use of this strain increased the efficiency of mutagenesis and allowed us to omit the sucrose gradient enrichment for RFI DNA. Mutagenesis was done using JF3833-M13 as a single-stranded DNA template. JF3833-M13 is an M13mp8 derivative that carries a 922 bp *Pst*I fragment that includes the 3' 331 nucleotides of the *rplA* gene, the *rplJ* regulatory region (including the promoter and the 375 nucleotide untranslated leader region) and the first 75 nucleotides of *rplJ* (see Fig. 1). Mutant clones were plaque-purified, single-stranded DNA was isolated and the entire 922 bp insert was sequenced by the dideoxy method (Sanger *et al.*, 1977) using a combination of universal and mutagenic oligonucleotide primers. The mutagenized 922 bp *Pst*I fragments were purified from JF3833-M13 derivatives and cloned into the unique *Pst*I site of plasmid pGA200 (Friesen *et al.*, 1983) to create the *rplJ-lacZ* fusions described below and shown in Fig. 1. Double mutants were constructed as described above, except that the JF3833-M13 derivative used as template already had a single base change either at position 1547 (G \rightarrow C) or 1552 (G \rightarrow C).

(c) Bacterial strains, culture media and β -galactosidase assays

Strain MC1000 (*araD139 ara[ABOIC]⁻ leu-7679, Δ lac[*I*POZY]X74, *galU*, *galK*, *rpsL*, *relA*, *spoT*; Casadaban & Cohen, 1980) was used as the host strain for all of the plasmid-mediated transformations and β -galactosidase assays. MC1000 was also used as the source of RNA for the structure mapping experiments. Cells were grown in L-broth (Lennox, 1955) or in MOPS-glucose medium supplemented with 0.4% glucose and 50 μ g L-leucine/ml (Neidhardt *et al.*, 1974). Tetracycline was present at a concentration of 4 μ g/ml and chloramphenicol at 5 μ g/ml. β -Galactosidase activity was measured as described by Miller (1972), on cultures growing exponentially in MOPS-glucose medium. Strain BMH71-18*mutL* (Δ *lac-proAB*), *supE*, *thi*; [*F'**lacI*^q *Z* Δ M15, *proA*⁺*B*⁺]; Kramer *et al.*, 1984) was used as the recipient for mutagenized M13 (JF3833-M13) DNA. M13 clones were then propagated in strain JM101 (Messing, 1983).*

(d) RNA preparation and structure mapping

(i) RNA preparation

RNA was prepared from 500-ml cultures of strain MC1000 grown at 37°C in L-broth. When the optical density of the culture reached 0.4 ($A_{600} = 0.4$) the cells were poured into centrifuge bottles containing 100 g of crushed ice and 10 mg of chloramphenicol. The cells were

then pelleted by centrifugation in a Sorvall GS-3 rotor (8000 revs/min, 10 min). Cell pellets were suspended in 2 ml of lysis buffer (10 mM Tris·HCl (pH 7.3), 10 mM KCl, 5 mM-MgCl₂). Lysozyme was added to a concentration of 3 mg/ml and then cells were frozen in a solid CO₂/ethanol bath. The suspension was thawed and RNA was purified by repeated extraction with hot phenol as described by Barry *et al.* (1980). Phenol-extracted RNA was precipitated by the addition of 3 vol. 95% ethanol. RNA pellets were suspended in 0.3 M-sodium acetate then re-precipitated with 95% ethanol. These pellets were dissolved in water and the RNA concentration was determined by spectrophotometry.

(ii) Chemical modification of RNA

RNA was chemically modified using the DEP and DMS reactions described by Peattie & Gilbert (1980). Samples (60 µg) of RNA were precipitated by the addition of 0.1 vol. 3 M-sodium acetate and 3 vol. 95% ethanol. RNA pellets were suspended in 300 or 400 µl of buffer A or buffer B (see below) and allowed to renature by incubating at 37°C for 20 min prior to modification. Control (unmodified) samples were treated the same throughout, except that they were not treated with modifying reagents.

RNA was chemically modified under native, semi-denaturing, and fully denaturing conditions. Modifications done under native conditions were incubated at 30°C or 37°C in buffer A (10 mM-MgCl₂, 50 mM-sodium cacodylate, pH 7.0). Modifications done under semi-denaturing or fully denaturing conditions were in buffer B (1 mM-EDTA, 50 mM-sodium cacodylate, pH 7.0) at 30°C, 37°C, 55°C, 70°C or 85°C.

For dimethyl sulfate (DMS) modifications, 1 µl of DMS (Eastman) was added to 300-µl samples of RNA in buffer A or buffer B and the samples were incubated at 37°C or 90°C for 6 min or 1 min, respectively. The reactions were stopped by adding 75 µl of DMS stop buffer (1 M-Tris-acetate (pH 7.5), 1 M-2-mercaptoethanol, 1.5 M-sodium acetate, 0.1 mM-EDTA, 0.2 mg tRNA/ml) and the samples were precipitated by adding 900 µl of cold 95% ethanol. Modified RNA samples were precipitated with ethanol 2 more times and modified bases were detected by primer-extension as described below.

DEP modifications were done by adding 10 µl of DEP (Sigma) to 399-µl samples of RNA in buffer A or buffer B. These samples were incubated for various periods of time, as described in the Figure legends. The reactions were stopped by adding 150 µl of 3 M-sodium acetate and the samples were precipitated by adding 1 ml of cold 95% ethanol. The modified RNA was precipitated 2 more times as described above and modified sites were detected by primer-extension.

(iii) Identification of modified sites

Modification of RNA with DEP and DMS at specific bases can act as a block to the polymerizing activity of AMV reverse-transcriptase at the site of modification (Inoue & Cech, 1985; Moazed *et al.*, 1986; Lempereur *et al.*, 1985; Youvan & Hearst, 1979). Such pause-sites on the RNA were detected by primer-extension using a (5'-³²P) end-labeled oligonucleotide whose 3' end hybridized to the RNA at position 1633 (Post *et al.*, 1979) of the DNA sequence. 60 pmol of the synthetic oligonucleotide 5' CGTCTTAATTACGGTGAGC 3' were labeled in a 50-µl reaction that included 100 mM-Tris·HCl (pH 8.0), 8 mM-MgCl₂, 4 mM-dithiothreitol (DTT), 50 µCi of [γ-³²P]ATP (New England Nuclear), and 10 units of polynucleotide

kinase (Bethesda Research Laboratories). The reactions were incubated at 37°C for 30 min then extracted with phenol once and with ether twice. Water (200 µl) was then added to the labeled DNA. From 0.5 to 1.0 pmol of the labeled oligonucleotide was added to 100 µl (60 µg) of modified RNA that had been suspended in 0.3 M-sodium acetate, and this mixture was precipitated by the addition of 350 µl of 95% ethanol. The resulting pellet was suspended in 7.5 µl of reverse-transcriptase reaction buffer (50 mM-Tris·HCl (pH 8.3), 40 mM-NaCl, 0.5 mM-EDTA) and incubated at 50°C for 20 min. The mixture was cooled slowly to room temperature and the primer-extension reaction was initiated by adding 4 µl of R mix (2.5 mM-deoxynucleoside triphosphates, 0.6 µg actinomycin D/ml, 3 mM-DTT, 30 mM-MgCl₂) and 5 units of AMV reverse-transcriptase (Life Sciences). The mixture was incubated at 42°C for 1 h, and the reaction was stopped by precipitation with 40 µl of 95% ethanol after adding 1.5 µl of 3 M-sodium acetate. The resulting pellet was suspended in 6 µl of water and 6 µl of loading dye. The samples were boiled for 3 min prior to loading on a 5% sequencing gel (Maxam & Gilbert, 1977). A DNA sequencing ladder was generated by the Sanger dideoxy method (Sanger *et al.*, 1977) using the same oligonucleotide primer and was used for size reference. Autoradiography was done at -80°C with an intensifying screen using Kodak XAR-5 film.

3. Results

Previous studies on the regulation of the *rplJ* and *rplL* genes revealed that a region of the mRNA located between 80 and 200 nucleotides upstream from the translation start-site was important for translation of the RNA and for feedback regulation (Fiil *et al.*, 1980; Christensen *et al.*, 1984). Secondary structures were predicted for this region on the basis of theoretical considerations (Friesen *et al.*, 1983; Christensen *et al.*, 1984) and in both cases a stem structure with a bulged loop was predicted to be located at position 1548-1581 of the DNA sequence reported by Post *et al.* (1979). This presumed stem structure is located within the region of the mRNA found to be protected from nuclease digestion by ribosomal protein L10-L7/L12 (Johnsen *et al.*, 1982) and it is also the location of one of the non-feedback mutants that was isolated previously (Friesen *et al.*, 1983). Figure 1 shows the proposed secondary structure of a portion of the *rplJ* mRNA leader region (Christensen *et al.*, 1984).

In order to study the regulatory role of the 1548-1581 stem structure, a series of mutations was constructed by oligonucleotide-directed mutagenesis. The regulatory effects of these mutations were examined using a *rplJ-lacZ* fusion on a low copy-number plasmid described by Friesen *et al.* (1983). Mutated DNA fragments were recovered from M13 clones and transferred to plasmid pGA200 to create *rplJ-lacZ* fusions as shown in Figure 2. The fusion plasmids containing the mutations were used to transform strain MC1000 to chloramphenicol resistance. High levels of β-galactosidase activity were directed by these fusions in the otherwise Lac⁻ host strain as determined using X-gal and EMB-lactose indicator plates

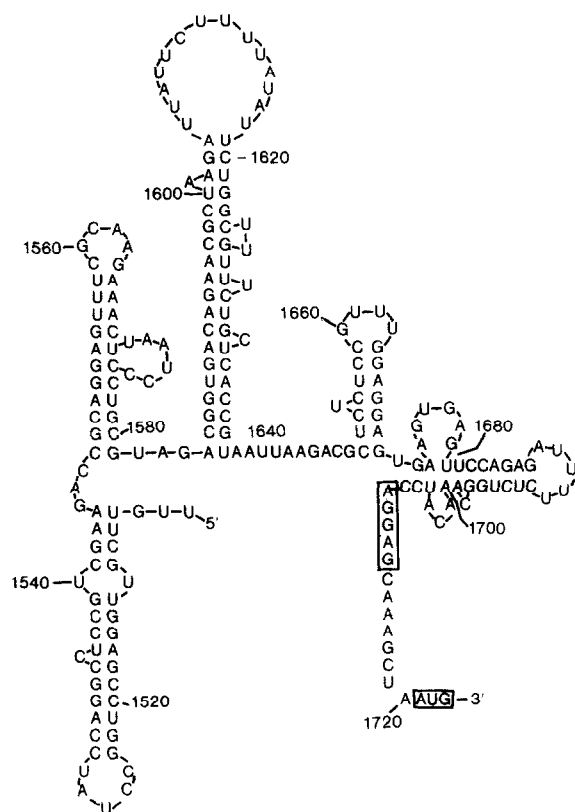


Figure 1. Proposed secondary structure of a portion of the *rplJ* mRNA including the 1548–1581 stem and the translation start-site. From Christensen *et al.* (1984). Nucleotides are numbered as by Post *et al.* (1979).

(data not shown). The regulatory effects of the mutations were examined by co-transforming the fusion-containing strains with a second plasmid, pNF1337 (termed a donor plasmid), that carries the genes for L10 and L7/L12 along with its entire regulatory region. This high copy-number plasmid directs the synthesis of L10-L7/L12 that can bring about feedback regulation of a wild-type fusion plasmid, and thus reduce *lacZ* expression as described previously (Friesen *et al.*, 1983). As a control, the fusion-containing strains were co-transformed with pBR322 rather than pNF1337 as a donor plasmid. When pNF1337 is used as the donor with a wild-type fusion plasmid (pGA189), the level of β -galactosidase activity is at least 5.4-fold lower than when pBR322 is used as the donor (i.e. the ratio is 5.4). For the fusion plasmids in which feedback regulation has been abolished completely, this ratio is 1.0 or lower. This is referred to as the feedback ratio, a measure of the ability of a given fusion plasmid to respond to feedback regulation.

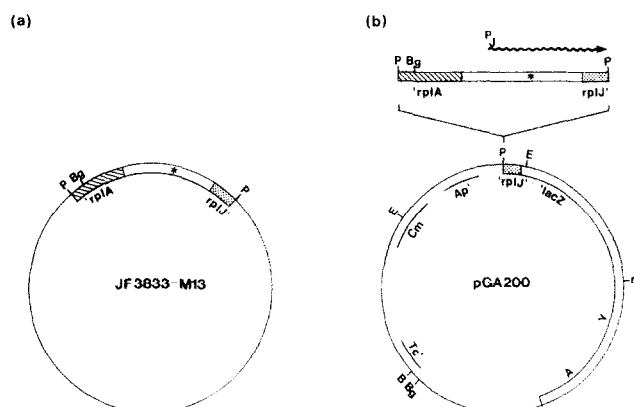


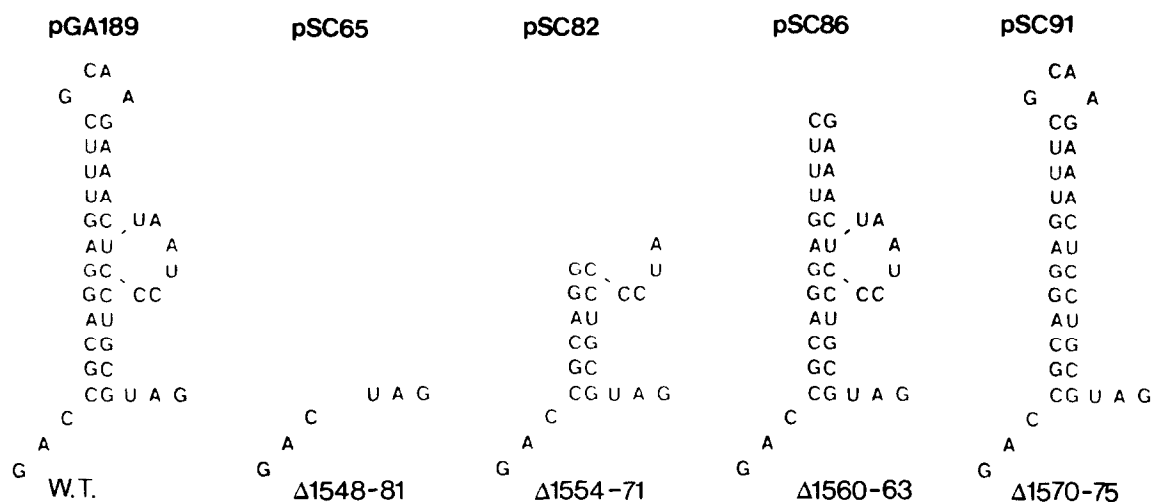
Figure 2. Construction of mutant *rplJ-lacZ* fusion plasmids. (a) The M13 clone JF3833-M13 was used as the single-stranded DNA template for oligonucleotide-directed mutagenesis. JF3833-M13 carries a 922 bp *Pst*I fragment that includes the 3' end of the *rplA* gene, the *rplJ* regulatory region, and the 5' end of the *rplJ* structural gene. An asterisk shows the approximate position of the mutations that were introduced. Oligonucleotides used for mutagenesis were as follows: Δ 1548–1581 5' CGTCGAAGACTAGACGGTGA 3'; Δ 1554–1571 5' GACCGCAGGATCCCTGCG 3'; Δ 1560–1563 5' CAGGAGTTTCGAAACTTAATCC 3'; Δ 1570–1575 5' GCAAGAAACTCCTGCGTAG 3'; 1549 G to C 5' CGAAGACCCCAGGAGTTTC 3'; 1580 C to G 5' CTTAATCCCTGGGTAGACGG 3'; 1581 C to G 5' CCCCTGCATAGACGGTGAC 3'; 1552 G to C 5' CGAAGACCGCACGAGTTTC 3'; 1577 C to G 5' CTTAATCCCGTGCGTAGACG 3'. (b) The mutagenized 922 bp *Pst*I fragment was excised from JF3833-M13 clones and transferred into plasmid pGA200 (Friesen *et al.*, 1983) to create the *rplJ-lacZ* fusions (pSC plasmids) used to examine feedback regulation. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; Cm, chloramphenicol acetyltransferase gene; Tc, tetracycline resistance gene; Ap, β -lactamase gene. The symbol P next to the arrow represents the promoter *rplJp*, which is located at position 1336 (Post *et al.*, 1979). The start-site for L10 translation is at position 1721 (Post *et al.*, 1979). A prime (e.g. *rplJ'*) indicates a partial gene.

(a) Deletion mutations abolish feedback regulation

Four deletion mutants were constructed and the regulatory effects were examined as described above. Plasmid pSC65 carries a 34 bp deletion that removes the entire stem structure from position 1548–1581, as shown in Figure 3(a). This plasmid directs the synthesis of a near wild-type level of β -galactosidase activity in the host strain (as compared to pGA189) when pBR322 is used as the donor plasmid. This confirms that the mutated mRNA is translated efficiently. When pNF1337 is

Figure 3. The position of mutations in the untranslated leader region of the *rplJ* mRNA. (a) The region of the *rplJ* leader from nucleotides 1545 to 1584 (Post *et al.*, 1979) is shown for the wild-type (pGA189) and 4 deletion mutants. (b) Same region of the RNA showing the positions of single base mutations. Single base changes are shown in bold type. The secondary structure of mutated mRNAs has not been determined experimentally. The secondary structure representations serve only to illustrate differences from the wild-type structure, which has been examined by chemical modification (see Figs 4 and 5).

(a)



(b)

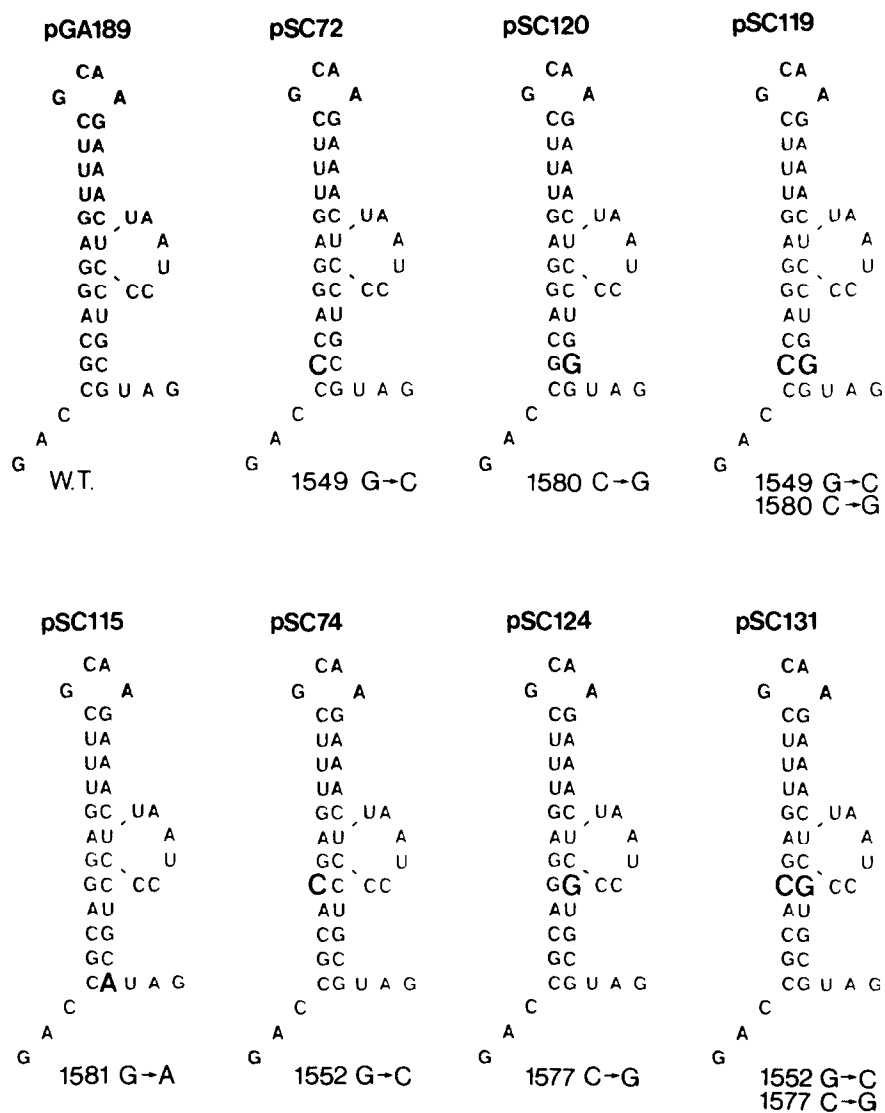


Table 1
Feedback regulation of *rplJ-lacZ* fusion plasmids

Fusion plasmid	Donor plasmid	β -Galactosidase act.	Feedback ratio
pGA189 (w.t.)	pBR322	79.9	5.4
	pNF1337	14.7	
pSC65 (Δ 1548-81)	pBR322	71.5	1.0
	pNF1337	71.3	
pSC82 (Δ 1554-71)	pBR322	83.7	1.0
	pNF1337	84.3	
pSC86 (Δ 1560-63)	pBR322	44.4	1.5
	pNF1337	30.2	
pSC91 (Δ 1570-75)	pBR322	83.7	1.2
	pNF1337	69.4	
pSC72 (1549 G to C)	pBR322	32.8	1.2
	pNF1337	28.3	
pSC74 (1552 G to C)	pBR322	78.1	1.2
	pNF1337	64.3	
pSC124 (1577 C to G)	pBR322	68.9	0.9
	pNF1337	80.8	
pSC120 (1580 C to G)	pBR322	77.0	1.2
	pNF1337	62.5	
pSC115 (1581 G to A)	pBR322	72.3	1.7
	pNF1337	42.0	
pSC119 (1549 G to C)	pBR322	61.8	7.4
(+ 1580 C to G)	pNF1337	8.4	
pSC131 (1552 G to C)	pBR322	66.8	4.0
(+ 1577 C to G)	pNF1337	16.5	

Cells were grown exponentially at 30°C in minimal MOPS medium (Neidhardt *et al.*, 1974) plus 0.4% glucose, 50 μ g l-leucine/ml, 5 μ g chloramphenicol/ml and 4 μ g tetracycline/ml. β -Galactosidase activity was measured as described by Miller (1972). At least 3 samples were taken when the optical density (A_{450}) was between 0.2 and 0.7. Results from several experiments were averaged to yield the final value. The doubling time for the cultures was 90 (\pm 10) min and the variation among samples was \pm 10%. The feedback ratio is defined as the ratio of β -galactosidase activity produced by a fusion plasmid in the presence of pBR322 to that produced in the presence of pNF1337; it is a measure of the ability of a fusion plasmid to respond to feedback regulation.

used as the donor plasmid, pSC65 continues to direct a high level of β -galactosidase activity, which indicates that the mutated leader region does not respond to normal feedback regulation. The feedback ratio for pSC65 is about 1.0. These results are summarized in Table 1.

The remaining deletion mutants exhibit a similar phenotype; they direct a high level of β -galactosidase activity when either pBR322 or pNF1337 is used as the donor plasmid, indicating that they have a reduced susceptibility to feedback regulation. The feedback ratio for these mutants in the presence of pBR322 as compared to pNF1337 varies from a value of 1.0 to about 1.5, as shown in Table 1. Plasmid pSC82 carries an 18 bp deletion that removes the upper half of the stem structure as well as two bases in the bulge-loop (see below and Fig. 2), pSC86 carries a 4 bp deletion that removes the stem-loop at position 1560-1563, and pSC91 carries a 6 bp deletion at position 1570-1575 that removes the bulge-loop. The 1560-1563 deletion on pSC86 has a feedback ratio of about 1.5 for the pBR322/pNF1337 donor plasmid pair, indicating that feedback regulation is reduced. However, as seen in Table 1, this mutant produces a lower level of β -galactosidase activity than the wild-type fusion in the presence of pBR322. This indicates that the

mutation also affects transcription, translation or mRNA stability, either singly or in combination. Plasmids pSC82 and pSC91 both direct wild-type levels of β -galactosidase activity and both are feedback-defective. They have feedback ratios of 1.0 and 1.2, respectively. The analysis of these deletion mutants allows us to conclude that all portions of the region from position 1548-1581 are necessary for feedback regulation.

(b) Point mutations abolish feedback regulation

Five different single base mutants were constructed and their regulatory effects were examined using the two-plasmid system as described. All of these mutations are located in the lower part of the 1548-1581 stem, and all have the effect of reducing feedback regulation. The results are summarized in Table 1, and the positions of these mutations are shown in Figure 3(b). Plasmids pSC72 and pSC74 carry G to C transversions at positions 1549 and 1552, respectively. Both mutations have the effect of abolishing feedback regulation. The feedback ratio for these fusions in the presence of pBR322 *versus* pNF1337 is 1.2 in both cases. However, pSC72 shows a reduced level of β -galactosidase activity compared to the wild-type

fusion pGA189 in the presence of pBR322, again indicating reduced transcription, reduced translation or degradation of the mRNA. Plasmids pSC124 and pSC120 carry C to G transversions at positions 1577 and 1580, respectively. Both of these fusions direct a high level of β -galactosidase activity in the presence of pBR322. They also produce high levels of activity in the presence of pNF1337, which indicates a loss of feedback regulation. The feedback ratio is 1.2 for both of these mutants. Plasmid pSC115 carries a G to A transition at position 1581, the base of the 1548–1581 stem structure. This mutant produces a high level of β -galactosidase activity in the presence of pBR322, but this activity is somewhat reduced in the presence of pNF1337. The feedback ratio for this mutant is 1.7, which indicates that feedback regulation is reduced, but not completely abolished. Computer analysis of the 1548–1581 stem using the Fold program (Zuker & Stiegler, 1981) reveals a structure identical with that determined experimentally (see below). All of the single base mutations are at positions predicted to be involved in base-pair formation. Since all of the mutations abolish feedback regulation, we were led to believe that base-pairing in the 1548–1581 region is a requirement for this regulation.

(c) *Pseudorevertants restore feedback regulation*

Pairs of point mutations in the 1548–1581 stem were combined in order to test the idea that base-pairing was necessary for feedback regulation. Plasmid pSC119 carries both the 1549 G to C and 1580 C to G transversions from plasmids pSC72 and pSC120. Although the primary sequence is altered at two positions in this double mutant, there is no predicted change in the secondary structure of the 1548–1581 stem, as shown in Figure 3(b). The predicted effect of this double mutant is to restore pairing between the bases located at positions 1549 and 1580. This mutant produces a high level of β -galactosidase activity in the presence of pBR322, but this activity is reduced 7.4-fold when pNF1337 is used as the donor plasmid, indicating that feedback regulation has been restored. Plasmid pSC131 carries single base transversions at positions 1552 (G to C) and 1577 (C to G). Again, the secondary structure of the 1548–1581 stem is predicted to be restored. A high level of β -galactosidase activity is produced by this fusion in the presence of pBR322, and this level is reduced about fourfold when pNF1337 is used as the donor plasmid (i.e. the feedback ratio is 4.0). This indicates that feedback regulation has been restored to almost a wild-type level; the results are summarized in Table 1. The ability of these double mutants to restore feedback regulation leads us to conclude that base-pairing in the 1548–1581 stem is necessary for feedback regulation and that the primary sequence *per se* may be less important than structure in providing features that are required for L10-L7/L12 binding.

(d) *Secondary structure analysis of a portion of the rplJ mRNA*

Previous studies have predicted the existence of several regions of secondary structure in the untranslated leader region of the *rplJ* mRNA. One of the predicted structures was the 1548–1581 stem described above and shown in Figures 2 and 3. Results of the genetic analysis described above led us to believe that such a structure was likely to exist. The nucleotide-specific chemical modification reactions described by Peattie & Gilbert (1980) were used to examine the secondary structure of the mRNA in this region. Both DMS and DEP modify specific nucleotides at positions that are involved in secondary or tertiary interactions. When RNA is modified under denaturing conditions, most or all of these potentially reactive sites are accessible to the reagents. Under native conditions, the RNA assumes a characteristic secondary structure and the reactive sites on those nucleotides that are involved in the formation of Watson–Crick base-pairs or tertiary interactions are no longer accessible to the reagent. DMS reacts primarily at the N-7 position of guanosine (Lawley & Brooks, 1963). It also methylates efficiently the N-1 position of adenosine and the N-3 position of cytidine (Lawley & Brooks, 1963; Singer, 1976). The latter two modifications can be detected as a result of their ability to block the polymerizing activity of reverse-transcriptase (Inoue & Cech, 1985). Although the N-7 position of guanosine is the most reactive site for DMS modification, such modifications do not block reverse-transcriptase and so they are not detected using this method (Inoue & Cech, 1985; Moazed *et al.*, 1986). DEP carbethoxylates adenosine primarily at the N-7 position under the conditions used (Leonard *et al.*, 1971; Ehrenberg *et al.*, 1976). Again, this modification can be detected because it blocks reverse-transcriptase (Inoue & Cech, 1985). Although the N-7 position of adenosine is not directly involved in base-pairing, DEP is a useful reagent for structure-probing because it is sensitive to base stacking (Peattie & Gilbert, 1980).

Figure 4 shows the results of DEP and DMS probing experiments done under a variety of conditions. A and C residues located in the stem-loop (C-1561, A-1562, A-1563) are modified under both native and denaturing conditions, an indication that these bases are not involved in secondary or tertiary interactions. Similarly, the A and C residues (A-1571, A-1572, C-1574, C-1575) in the bulge-loop are accessible under all the conditions tested, suggesting that these bases are in a single-stranded region (Fig. 4(a), see lanes 2 to 9 and Fig. 4(b), lanes 2 to 4). The A at position 1554 is accessible under semi-denaturing conditions, possibly due to unstable base-pairing at that position. The A residues at positions 1551, 1565, 1566 and 1567 are not accessible under native conditions, but they are modified at higher temperatures under denaturing conditions, when the secondary structure is denatured. Similarly, C

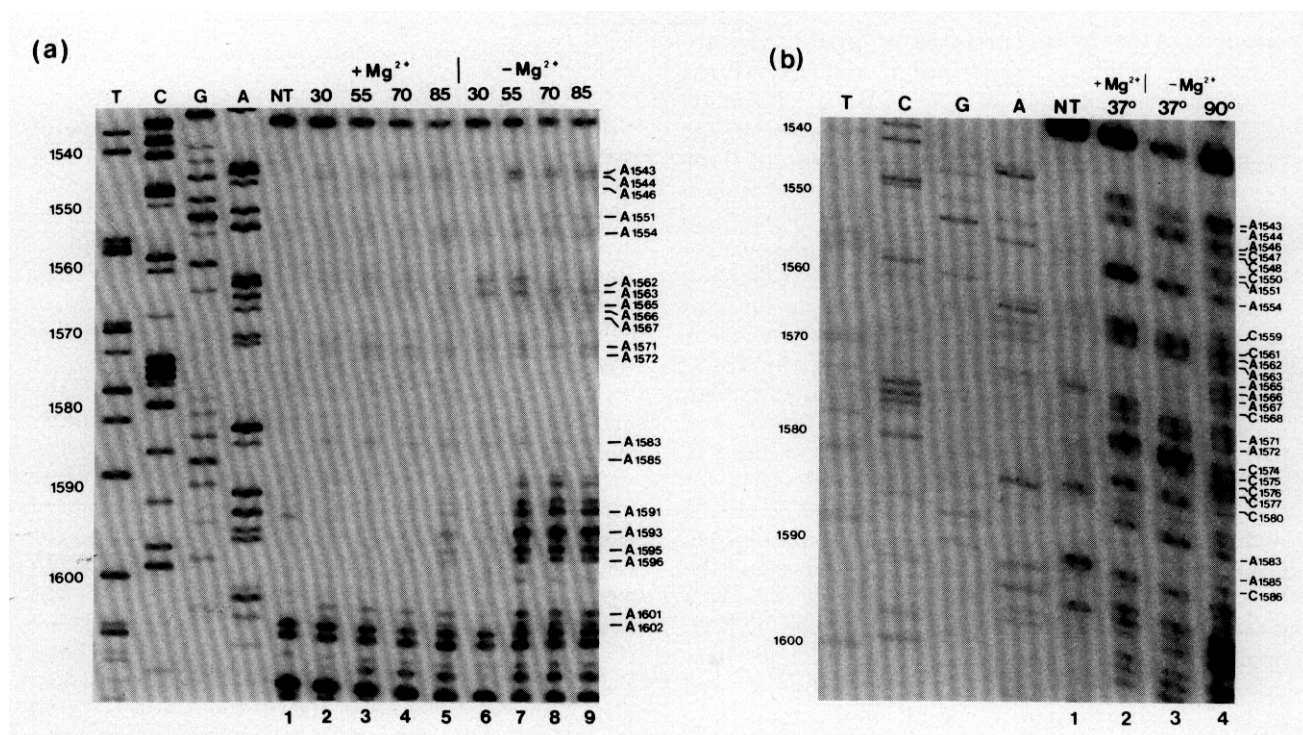


Figure 4. Sequence gel showing chemical modification of part of the leader region of the *rplJ* mRNA. (a) DEP modifications. A, G, C and T, DNA sequence markers. Lane 1, primer-extension products from untreated RNA. Lanes 2 to 5, primer-extension products from RNA modified in the presence of 10 mM-MgCl₂ at the indicated temperatures for 60 min, 8 min, 3 min and 1.5 min, respectively. Lanes 6 to 9, same as lanes 2 to 5, except that RNA was carboxymethylated in the absence of MgCl₂. (b) DMS modification. A, G, C and T, DNA sequence markers. Lane 1, primer-extension products from untreated RNA. Lane 2, primer-extension products from RNA modified at 37°C in the presence of 10 mM-MgCl₂. Lanes 3 and 4, products from RNA methylated in the absence of MgCl₂ at 37°C and 85°C, respectively.

residues at positions 1548, 1550, 1559, 1568, 1576 and 1577 are accessible only under denaturing conditions and are likely to be involved in base-pairing (compare lanes 2 and 3 to lanes 8 and 9 in Fig. 4(a), and lane 2 to lane 4 in Fig. 4(b)). The results of RNA structure-probing experiments are summarized in Figure 5. They show that the secondary structure of this part of the L10 mRNA is identical with that predicted earlier on theoretical grounds (Christensen *et al.*, 1984). The secondary structure analysis allows us to confirm that the regulatory mutations described above are likely to affect the structural features of the RNA as predicted.

4. Discussion

The *rplJL-rpoBC* operon of *E. coli* is regulated in part at the level of translation by an autogenous feedback mechanism. This regulation occurs as the result of an interaction between ribosomal proteins L10-L7/L12 and the *rplJ* mRNA leader that prevents further translation of the message. Such a mechanism is a common regulatory feature of almost all ribosomal protein operons in *E. coli*. In spite of recognition of this as an important regulatory mechanism, relatively little is known about the nature of the RNA-protein interactions, the effects of protein binding on RNA structure, or even the structural features of the RNA that are

necessary for protein binding and feedback regulation. In this study, we have partially characterized the L10-L7/L12 target site on the *rplJ* mRNA by a combination of genetic and biochemical methods.

The current results indicate that sequences in the 1548–1581 region of the *rplJ* leader region comprise an important regulatory feature of the *rplJ* mRNA. The results in Table 1 and Figure 3(a) show that deletion of the entire 1548–1581 stem structure abolished feedback regulation, but had no effect on the ability of the mRNA to be translated. This indicates that the regulatory effect of this mutation occurs at the level of translation. Deletion of the upper portion of the stem, the stem-loop, or the bulge-loop also reduced feedback regulation, suggesting that these structural features within the 1548–1581 stem might themselves be important for feedback regulation. To examine this possibility, we constructed the five point mutants described in Figure 3(b). All of these mutations are at positions that were predicted to be involved in base-pairing, and they all reduced feedback regulation. This implied that base-pairing might be necessary to allow the mRNA to assume a structure that can be recognized by L10-L7/L12 upon binding. We constructed two double mutants to test this possibility. Both of these mutants have the potential to restore base-pairing in the lower portion of the 1548–1581 stem, as shown in Figure 3(b). The double mutants showed almost

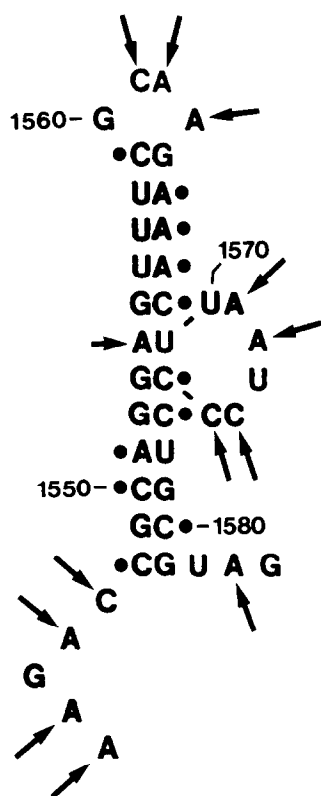


Figure 5. Summary of chemical modification results for adenosine and cytidine residues in the region of the *rplJ* mRNA that includes the 1548–1581 stem structure. Arrows indicate bases that are modified under native conditions (30°C or 37°C, 10 mM-MgCl₂). Filled circles indicate bases that are not modified under the same conditions. The small arrow at A-1554 indicates that this base is moderately reactive under these conditions. Data from several experiments are summarized in the Figure.

normal feedback regulation, a result that led us to conclude that base-pairing in the 1548–1581 stem is a requirement for this regulation. The non-feedback mutants pSC86 and pSC72 exhibited somewhat lower levels of β -galactosidase activity relative to the wild-type fusion when pBR322 was used as the donor plasmid. This could be due to reduced transcription, reduced translation, or rapid degradation of the mRNA. Further experimentation will be required to determine which of these explanations is correct.

The untranslated leader region of the *rplJ* mRNA was predicted to contain several regions of potential secondary structure (Fiil *et al.*, 1979; Friesen *et al.*, 1983; Christensen *et al.*, 1984). In all of these cases, the region around position 1548–1581 was predicted to form a stable stem structure with a four base stem-loop and a six base bulge-loop, as shown in Figures 2, 3 and 5. We examined the structure of the RNA in this region using the structure-specific chemical modifications described by Peattie & Gilbert (1980) in combination with primer-extension analysis as described by Inoue & Cech (1985), Lempereur *et al.* (1985) and Moazed *et al.*

(1986). The major difference between our strategy and those described previously is that we used total cellular RNA rather than the message transcribed or purified *in vitro*. We found that the patterns of chemical modification in these experiments were identical with those obtained using *rplJ* mRNA that was transcribed *in vitro* using RNA polymerase from bacteriophage T7 (data not shown). The results in Figures 4 and 5 show that the secondary structure of the *rplJ* message is indistinguishable from that predicted earlier on theoretical grounds. This result confirms that the single base mutations that abolish feedback regulation are normally involved in base-pair formation. This result also lends support to the idea that feedback regulation occurs in the double mutants, because base-pairing is restored in the 1548–1581 stem.

All of the mutations that affect feedback regulation are predicted to affect the overall structure of the 1548–1581 stem. The double mutants, however, are predicted to have no effect on secondary structure. It is interesting to note that all of the mutations do not affect feedback to the same extent. The loss of feedback regulation may reflect a decreased affinity of the RNA for protein binding. It is possible that partially feedback-defective mutants such as pSC115 still retain the ability to bind L10-L7/L12, although with a reduced affinity. This is in contrast to deletion mutants pSC65 and pSC82, in which feedback has been abolished completely. Whether these mutants affect protein binding remains to be determined experimentally. The observation that the double mutants pSC119 and pSC131 show feedback regulation leads us to suspect that the primary sequence of the lower portion of the 1548–1581 stem *per se* may not be important for protein binding. It seems likely that simple base-pairing in this region provides one of the structural features that is necessary for feedback regulation. The role of the base-paired regions may simply be to maintain other structural features in the correct position relative to one another. This idea is supported by the observation that ribosomal protein L1 from *E. coli* can bind specifically to a eukaryotic ribosomal RNA (Gourse *et al.*, 1981). The L1 binding site is observed to be highly conserved in overall secondary structure; it is composed of sequences that are only partially conserved in primary sequence, but highly conserved in secondary structure due to compensatory base changes in the stem-forming regions. In addition to this, Baughman & Nomura (1984) have shown that base-pairing in the *rplK* (L11) mRNA target site is required for feedback regulation of the L11 operon.

If base-pairing is required only to maintain other regulatory features in the correct position relative to one another, what then are the structural features required for protein binding? Peattie *et al.* (1981) have suggested that bulged nucleotides within regions of secondary structure occur commonly at RNA-protein contact sites. They

propose that an unstacked base projecting out from a helix could help distinguish that helix from similar helices in cellular RNA. Garrett *et al.* (1984) have reviewed several other examples of RNA-protein interactions that are involved in ribosome structure and function. They have classified two types of interactions based on the structural features of the protein binding sites on the RNA. Type I interactions are characterized by binding sites that contain one or two bulged bases. Type I sites are exemplified by rRNA helices that bind ribosomal proteins S8, S15 and L18. Type II sites contain unusual base-paired regions that include G·U and A·G pairs, as found in the L25 binding site on 5S RNA. Several putative type I and type II r-protein binding sites were also identified within the secondary structure models of 16S and 23S RNA, although no protein binding has yet been demonstrated at these locations (Garrett *et al.*, 1984). So, ribosomal proteins appear to bind primarily in highly structured regions of ribosomal RNA, and specific non-base-paired nucleotides are often required for the specificity of the interaction. This has also been shown to be the case for the R17 phage coat protein-RNA interaction studied by Uhlenbeck and co-workers (Carey *et al.*, 1983). Although regions of RNA secondary structure occur commonly at protein binding sites, such features are not universal. For example, the gene 32 protein of bacteriophage T4 binds to unstructured regions of its mRNA to act as a translational repressor (von Hippel *et al.*, 1982). We find that deletion of the six nucleotide bulge-loop at position 1570–1575 significantly reduces feedback regulation, suggesting that this structure might be required for protein binding. Also, preliminary results suggest that nucleotides in this region are protected from chemical modification by L10-L7/L12 binding (unpublished results). This result is interesting in light of the proposed structural homology of this region with the L10 binding site on 23S rRNA described by Draper (1987).

Previous work (Fiil *et al.*, 1979; Friesen *et al.*, 1983; Johnsen *et al.*, 1982) has shown the complexity of feedback regulation of the L10 operon, and that additional regions of the mRNA apart from those examined in this study are necessary for protein binding and post-transcriptional regulation. Further genetic and biochemical studies should allow us to identify more precisely those features of the mRNA target site that are necessary for protein binding and to determine the effects of protein binding on the secondary structure of the mRNA.

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