

Fig. 2 Autoradiography of 7.5% SDS-polyacrylamide gels of ^{35}S -methionine-labelled *Xenopus* oocyte proteins after injection of TMV RNA and various concentrations of tRNA^{Tyr} isolated from *Schizosaccharomyces pombe* and *D. melanogaster* respectively. Experimental procedures are as described in Fig. 1 legend. Synthesis of the 160K TMV readthrough protein is stimulated proportionally as a function of the concentration per oocyte of $\text{tRNA}^{\text{Tyr}}_{\text{pombe}}$ or $\text{tRNA}^{\text{Tyr}}_{\text{Drosophila}}$ (anticodons G Ψ A) isolated from *Drosophila*³, respectively, whereas $\text{tRNA}^{\text{Tyr}}_{\text{Drosophila}}$ (anticodon Q Ψ A) isolated from *Drosophila*³ is unable to induce this readthrough event. All three tRNAs were at least 95% pure^{3,5}.

might contribute to production of appreciable amounts of the 160K protein. These circumstances might also be responsible for the approximately equal efficiency in the reading of the TMV RNA stop codon by wild-type tRNA^{Tyr} and amber or ochre suppressor tRNA^{Tyr} (Fig. 3 and ref. 2). From the above we conclude that the leakiness of the TMV RNA stop codon is due to the misreading of an amber stop codon by a wild-type tRNA^{Tyr} . This is analogous to readthrough of the UGA termination codon of the coat protein of phage QB, thus producing a product indispensable for infection in *Escherichia coli*²⁷⁻²⁹.

Geller and Rich³⁰ have proposed that the readthrough phenomenon might represent a control of gene expression at the level of termination. They pose the question of whether readthrough of certain proteins occurs, for example, in certain developmental stages of an organism, or after transformation of cells. In fact, significant changes can be observed in the extent of Q base modification in tRNAs isolated from different ontogenetic stages in *D. melanogaster*^{19,31}. In transformed mammalian cells the Q base modification disappears almost completely³². Whether these changes reflect any activities, as suggested by Geller and Rich³⁰, is not known. Putative changes in the extent of Q base modification of the tRNAs in TMV-infected tobacco plants have yet to be demonstrated. Thus, gene expression could be regulated by a single modification enzyme.

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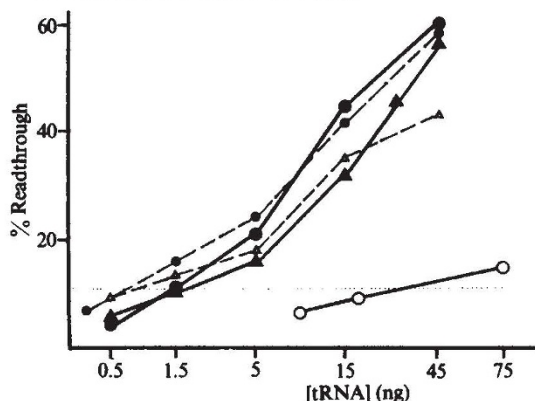


Fig. 3 Dependence of the amount of the 160K TMV readthrough protein on the concentration of various tRNAs. Experimental procedures are as described in Fig. 1 legend and by M.B. *et al.*². ●, tRNA^{Tyr} (anticodon G Ψ A) from *Drosophila*³; ▲, tRNA^{Tyr} (anticodon G Ψ A) from *S. pombe*⁴; ■, *Saccharomyces cerevisiae* amber suppressor tRNA^{Tyr} (ref. 2); △, *S. cerevisiae* ochre suppressor tRNA^{Tyr} (ref. 2); ○, tRNA^{G} .

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***E. coli* ribosomal protein L10 inhibits translation of L10 and L7/L12 mRNAs by acting at a single site**

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The genes coding for ribosomal proteins L10 and L7/L12 and for the subunits for RNA polymerase β and β' are clustered within the same transcription unit (β operon) located at the 88-min region of the *Escherichia coli* genome^{1,2}. The regulation of these genes is of particular interest because of the ratio of synthesis of the proteins (copy ratio L10:L7/L12: β : β' = 1:~4:0.2:0.2) and because synthesis of the ribosomal proteins and of β and β' is apparently subject to different regulatory signals (see ref. 3 for a review). It was previously shown that certain ribosomal proteins, including L10, can inhibit the translation of their own mRNA and of mRNA of other genes from the same transcription unit⁴⁻¹¹. This regulation is physiologically significant in coordinating the synthesis of ribosomal proteins and ribosomes. Although L10 was previously shown to inhibit its own translation *in vitro*⁵, the mechanisms for regulation of L7/L12 synthesis have remained unknown. We report here the results of *in vivo* and *in vitro* investigations demonstrating that L10 regulates L7/L12 synthesis, as well as its own synthesis, by acting near the L10 translational start site.

We examined the feedback regulatory properties of L10 *in vitro* using a DNA-directed protein synthesizing system with various DNA templates containing all or portions of the β operon (Fig. 1). When a template containing the entire β operon (λ rif^d18) was used, addition of purified L10 to *in vitro* reaction mixtures specifically inhibited synthesis of L10 and L7/L12 but did not inhibit synthesis of β and β' or of any other protein (Fig. 2, lanes 1–4; Table 1). By transcribing λ rif^d18 DNA, extracting the mRNA and then using the mRNA to direct protein synthesis, we found, as have others⁵, that the inhibition by L10 occurs at the level of mRNA translation (data not shown). When a *Hind*III DNA fragment containing only the distal portion of the L10 gene, the entire L7/L12 gene and a proximal portion of the β gene was used to direct protein synthesis, addition of L10 did not affect the synthesis of L7/L12 (Fig. 2, lanes 5, 6). We therefore conclude that L10 acts at a single site and inhibits both L10 and L7/L12 synthesis and that this site is in the promoter-proximal portion of the β -operon transcript.

When a template (pNO1525) containing the entire L10 structural gene and only a portion of the L10 leader sequence was used to direct L10 synthesis, addition of L10 caused marked inhibition of L10 synthesis (Table 1). This result indicates that the 5'-proximal 186 nucleotides of the mRNA are not essential for the repressor action of L10. Using two additional DNA templates, it was also demonstrated that the repressor action of L10 does not require the presence of the distal portion of the L10 cistron. The template λ *trp-lac*1, made up of DNA of a hybrid '*trp-lac*' λ phage carrying the β -operon promoter and the proximal portion of the L10 gene fused in phase to the distal portion of the *trpB* gene, directed synthesis of an L10-*trpB* fusion protein; the synthesis was specifically inhibited by L10 (Table 1). Similarly, specific inhibitory action of L10 was demonstrated using an *in vitro* system in which the synthesis of an L10- β -lactamase fusion protein was directed by a plasmid DNA template, pFM20, that includes the promoter and only the proximal portion of the L10 gene fused in phase to the β -lactamase gene. These results, summarized in Table 1, imply that the target site for L10 repressor action is located between a portion of the L10 leader sequences and the proximal portion of the L10 gene, as shown in Fig. 1. This region includes a possible stem-loop structure in the leader mRNA¹² containing four point mutations¹³ that prevent L10 synthesis and which were thought to be involved in the feedback regulatory mechanism for regulation of L10 synthesis. However, two such mutants are in a portion of the leader sequence (at positions 1,515 and 1,516) not required for feedback inhibition. Using a similar *in vitro* system, Brot *et al.*⁵ found that L10 inhibited translation of L10

Table 1 Relative synthesis rates of proteins in the presence of L10

Template	Protein synthesized		
	L10*	L7/L12	Control protein
λ rif ^d 18	0.25	0.38	0.92
<i>Hind</i> III(1,700 bp)	—	1.05	—
pNO1525	0.14	—	1.08
λ <i>trp-lac</i> 1†	0.29*	—	0.94
pFM20	0.53*	—	0.99

Proteins were synthesized as described in Fig. 2 legend in the presence and absence of 1.9 μ M L10. Synthesis of L10 and L7/L12 from λ rif^d18 and synthesis of L10 from pNO1525 were determined by immunoprecipitation followed by gel electrophoresis. Syntheses of all other proteins were determined directly by gel electrophoresis. Fluorograms made using preflashed film were scanned with a Joyce-Loebl densitometer. Ratios of amounts of synthesis obtained in the presence of L10 to synthesis obtained in its absence are shown. Proteins listed as 'control' proteins for each template are: λ rif^d18, β , β' ; pNO1525, β -lactamase; λ *trp-lac*1, β -galactosidase; pFM20, a 35,000-molecular weight β -lactamase-L10 fusion protein containing the N-terminus of β -lactamase and the C-terminus of L1.

* For λ *trp-lac*1 and pFM20, synthesis of the L10-*trpB* and L10- β -lactamase fusion proteins was measured. The proteins of molecular weights 57,000 and 13,000 respectively were identified using anti-L10 antisera.

† λ *trp-lac*1 was constructed by H. de Boer by fusing the *Hind*III-*Eco*RI fragment of λ rif^d18 containing the β promoter (nucleotides 280–2,154 in Fig. 1) to the left arm of *Hind*III-digested '*trp-lac*' phage λ RS205-7 and to the right arm of *Eco*RI-digested λ phage 459. This method has been described for other ribosomal promoters²⁷. The *Hind*III cleavage site in the *trpB* gene²⁸ is in the same reading frame as the *Hind*III site in the L10 gene.

mRNA but had "little or no effect" on L7/L12 synthesis. We obtained maximal inhibition of L7/L12 synthesis by incubating the reaction mixtures at 30 °C. At 37 °C, our results were more similar to those of Brot *et al.*, showing only 20–30% maximal specific inhibition of L7/L12 synthesis by L10 (data not shown). We believe that our results obtained *in vitro* at 30 °C reflect regulation *in vivo* because of the following results *in vivo*.

A complex containing four molecules of L7/L12 and one molecule of L10, which is formed on or off the ribosome^{14,15}, will inhibit the *in vitro* synthesis of both L10 and L7/L12 by ~50%¹⁶. We have isolated the L10-L7/L12 complex and found its *in vitro* regulatory properties identical to those of L10 alone (data not shown). Thus, the inhibition of synthesis of L10 and L7/L12 by the complex must be due to the L10 within it. The same conclusion was reached by M. Johnson, T. Christensen and N. P. Fiil (personal communication). Interaction of L10 with the target on the mRNA seems to involve the part of L10 which interacts with 23S rRNA, and not the part that interacts with the four molecules of L7/L12.

To confirm that the regulatory properties observed for L10 *in vitro* reflect its regulatory role *in vivo*, the effect of over-

Fig. 1 Genes carried by λ rif^d18 and construction of plasmids used in this study. The hatched regions of λ rif^d18 represent λ DNA and the open regions bacterial DNA. Some of the bacterial transcription units are indicated above λ rif^d18 as a P (promoter) and a horizontal arrow indicating the direction of transcription. Transcription of the β operon initiates approximately at position 1,348 (\pm 1) (ref. 12). Below λ rif^d18 is an expanded portion of the transducing phage that denotes the relevant bacterial region. Various restriction enzyme sites are indicated below the expanded region. Only the restriction enzyme sites relevant to the present study are indicated. The complete DNA sequence of this region has been determined and can be found in refs 12, 22. The numbers below the vertical arrows representing restriction enzyme sites indicate the nucleotide position of the restriction enzyme cut using the numbering system of ref. 12. Various portions of λ rif^d18 were used to construct hybrid plasmids (shown in the figure) that carry regions of the L11 and β operons. Some of the restriction sites are shown on the plasmids so that the cloned region can be compared with λ rif^d18. Plasmid pNO2020 is a derivative of pVH51 (ref. 23) and contains two copies of an *Eco*RI fragment (nucleotides 280–2,444) from λ rif^d18 and one copy of another *Eco*RI fragment from λ rif^d18 (nucleotides 2,444–3,524). For *in vitro* studies, the *Hind*III fragment that contains the L7/L12 gene was purified from pNO2020 and used to direct protein synthesis. Plasmid pRZ4006 was used as the parental plasmid for construction of pNO1525. The plasmid pRZ4006 was constructed by K. Bertrand and is a derivative of pBR322 which has the *Bam*HI-*Eco*RI fragment from pBR322 replaced with a 204-base pair (bp) *Hae*III fragment that contains the *lac* operator and promoter (personal communication). Both the *Bam*HI and *Eco*RI sites were retained in pRZ4006 and transcription originating from the *lac* promoter is directed towards the *Bam*HI site. The plasmid pNO1525 was constructed by digesting pRZ4006 with *Bam*HI, treatment with DNA polymerase I and ligation with the *Hae*III fragment containing the L10 gene purified from λ rif^d18. The resulting plasmid places the synthesis of plasmid-encoded L10 under the control of the *lac* regulatory elements. Plasmid pFM20 was constructed by ligating *Pst*I-digested pBR322 to a *Pst*I fragment (nucleotides 873–1,795) derived from λ rif^d18. Plasmid pFM20 contains the β promoter and a portion of the L10 gene fused 'in phase' to the β -lactamase gene (*bla*) contained on pBR322 (ref. 24). Plasmid pGB Δ BR2 was constructed by G. Barry and is described in ref. 17. The site for repressor action by L10 is indicated by a thick bar below the restriction enzyme site arrows.

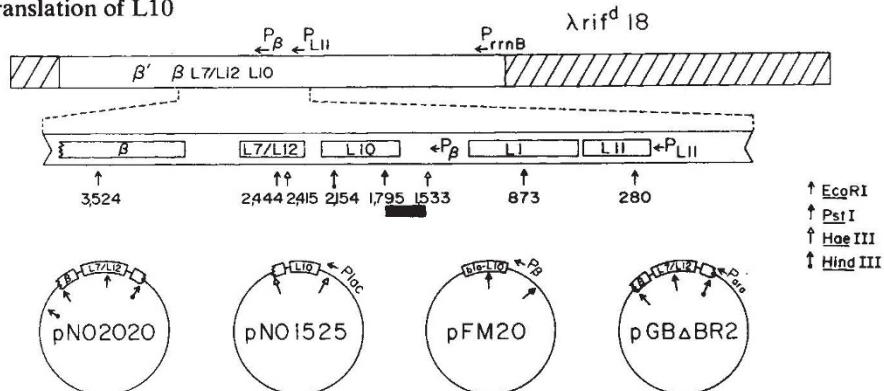
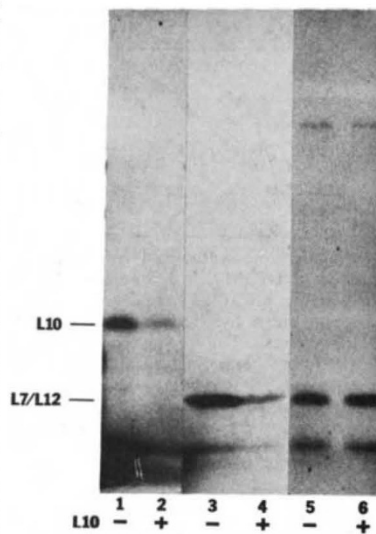


Fig. 2 Inhibition of *in vitro* synthesis of L10 and L7/L12 by addition of purified L10. L10 was purified by phosphocellulose chromatography²⁵, starting with a 1M NH₄Cl/50% EtOH wash of 70S ribosomes depleted of L7/L12 (ref. 26). L10 was stored in 6M urea, 50 mM methylamine phosphate pH 6.5 at -70°C. For use in *in vitro* experiments an aliquot of L10 (0.72 µg ml⁻¹) was supplemented with bovine serum albumin (BSA; 1.0 mg ml⁻¹) and dialysed first against 0.5 M KCl/4 M urea/2 mM potassium phosphate pH 6.5/1 mM dithiothreitol and then against KUP buffer (1.0 M KCl/1 M urea/2 mM potassium phosphate pH 6.5/1 mM dithiothreitol). *In vitro* reaction mixtures (40 µl) received either 2 µl of the resulting L10 solution (lanes 2, 4, 6) or 2 µl of KUP buffer containing 1 mg ml⁻¹ BSA (lanes 1, 3, 5). Proteins were synthesized in the presence of ³⁵S-methionine using 0.0017 µM *λ*rif^d18 DNA (lanes 1-4) or 0.009 µM *Hind*III 1,700-bp fragment (lanes 5, 6) as template, as described previously¹⁹ except that incubation was for 2 h at 30°C. The *Hind*III 1,700-bp fragment was prepared from pNO2020 (see Fig. 1). After incubations were completed, L10 was added to samples 1, 3 and 5. Reaction mixtures were analysed either directly by gel electrophoresis on a 13% polyacrylamide-SDS gel (lanes 5, 6) or by immunoprecipitation using rabbit anti-L10 antisera (lanes 1, 2) or anti-L7/L12 antisera (lanes 3, 4) followed by gel electrophoresis and fluorography¹⁹. A fluorogram is shown.



production of L10 *in vivo* was examined. The *Hae*III restriction enzyme fragment that contains the entire L10 gene and a small portion of the L7/L12 gene was inserted into a plasmid vector containing the *lac* operator and promoter so that synthesis of L10 was controlled by *lac* regulatory elements (pNO1525; see Fig. 1). The synthesis of L10 was stimulated by the addition of isopropyl thio-β-galactoside, an inducer of the *lac* operon, in a strain which harbours the recombinant plasmid, and the synthesis rates of other individual proteins were then analysed.

The overproduction of plasmid-encoded L10 caused a marked inhibition of L7/L12 synthesis, and presumably chromosome-directed L10 synthesis, but had no effect on β and

β' synthesis (Table 2). The inhibition of L7/L12 synthesis was due only to L10 overproduction because no such inhibition was observed when the strain carried a deletion within the plasmid-encoded L10 gene (data not shown). To test for a possible *in vivo* regulatory role of L7/L12, we used plasmid pGBΔBR2 which has L7/L12 gene expression under control of *ara* regulatory elements¹⁷. Addition of L-arabinose to a strain harbouring the hybrid plasmid caused an eight- to ninefold increase in the rate of L7/L12 synthesis but did not affect that of L10 or β and β' (Table 2). (We and others⁵, observed that in the *in vitro* system, purified L7/L12 does not inhibit synthesis of L10 or of L7/L12. We therefore conclude that L10 and L7/L12 are in a translational unit regulated by L10.)

How might L7/L12 synthesis be repressed by L10 acting at the L10 translational start site? One possible explanation is that efficient translation of L7/L12 mRNA requires the translation of the preceding L10 mRNA and that the inhibition of L7/L12 synthesis by L10 is a consequence of the inhibition of L10 synthesis by L10. Such 'translational coupling' has been observed in both the *trp* operon¹⁸ and the L11 ribosomal protein operon¹⁹. Ribosomal proteins seem to be synthesized from other operons in units of translational regulation with the feedback repressors acting at single mRNA target sites. We have previously suggested a mechanism of sequential mRNA translation to account for the co-regulation and equimolar synthesis of ribosomal proteins in these regulatory units¹⁹. L7/L12 differs from other ribosomal proteins in that its synthesis is at least four times faster. We suggest that translation of L10 mRNA by a ribosome 'opens' the structure of L7/L12 mRNA initiation site, allowing other ribosomes to initiate the translation of L7/L12 at least four times as efficiently as that of L10 mRNA. We might expect that, without independent feedback regulation of L7/L12 synthesis by itself, more than the four copies of L7/L12 needed per ribosome might always be synthesized. In fact there are reports that, unlike other ribosomal proteins, significant amounts of L7/L12 exist in cells as free proteins^{20,21}. Regardless of the exact stoichiometry of the rates of L7/L12 synthesis relative to L10, the present work suggests a mechanism for the co-regulation of the synthesis of L7/L12 and L10.

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Table 2 Relative synthesis rates of proteins after induction by isopropyl thio-β-galactoside or L-arabinose

Protein	Strains	
	N02396 (carries pN01525)	N02455 (carries pGBΔBR2)
S3	1.05	0.91
S4	1.00	1.05
S10	0.81	1.15
L1	1.05	1.05
L2	0.96	0.90
L4	1.00	1.17
L7/L12	0.26*	8.9*
L10	1.80*	0.97
β + β'	0.85	0.98

For labelling experiments, cells were grown in a synthetic minimal medium²⁹ supplemented with 0.4% glycerol, all amino acids (except methionine and lysine) at 50 µg ml⁻¹ and thiamine at 2 µg ml⁻¹. Cells were grown at 37°C to 2 × 10⁸ per ml. Samples of cells were pulse-labelled with ³H-lysine (646 pmol ml⁻¹; 80.5 Ci mmol⁻¹) for 1 min followed by a 1-min 'chase' with excess nonradioactive lysine. Cells were labelled before (control cells) and 10 min after addition of isopropyl thio-β-galactoside to 1 mM or 0.2% L-arabinose, depending on the strain used (experimental cells). Cells were rapidly chilled and then mixed with a suitable amount of ¹⁴C-lysine-labelled carrier cells. ¹⁴C-labelled cells were prepared by growing cells in the presence of ¹⁴C-lysine for several generations. Ribosomal proteins were extracted and separated by two-dimensional gel electrophoresis³⁰. The β and β' subunits were resolved from other proteins by SDS slab gel electrophoresis³¹. After staining the gels, the spots corresponding to the indicated ribosomal proteins were extracted, the gels oxidized with a Packard sample oxidizer and ³H and ¹⁴C measured separately. The ³H/¹⁴C ratio in each protein in the experimental cells was then compared with that in control cells using the expression:

$$\frac{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in experimental cells}}{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in control cells}}$$

The ratios obtained were then normalized to the ratio obtained for total cellular protein. Values given are the average of two independent experiments.

* Values significantly different from 1.0.