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**Mutational analysis of the L1 binding site of 23S rRNA in *Escherichia coli***

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Doctor Said, James R.Cole\* and Masayasu Nomura<sup>†</sup>

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Department of Biological Chemistry, University of California, Irvine, CA 92717, USA

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**ABSTRACT**

The L11 ribosomal protein operon of *Escherichia coli* contains the genes for L11 and L1 and is feedback regulated by the translational repressor L1. Both the L1 binding site on 23S rRNA and the L1 repressor target site on L11 operon mRNA share similar proposed secondary structures and contain some primary sequence identity. Several site-directed mutations in the binding region of 23S rRNA were constructed and their effects on binding were examined. For *in vitro* analysis, a filter binding method was used. For *in vivo* analysis, a conditional expression system was used to overproduce a 23S rRNA fragment containing the L1 binding region, which leads to specific derepression of the synthesis of L11 and L1. Changes in the shared region of the 23S rRNA L1 binding site produced effects on L1 binding similar to those found previously in analysis of corresponding changes in the L11 operon mRNA target site. The results support the hypothesis that r-protein L1 interacts with both 23S rRNA and L11 operon mRNA by recognizing similar features on both RNAs.

**INTRODUCTION**

Synthesis of many ribosomal proteins (r-proteins) are feedback regulated translationally. The general outlines of this regulation are now well understood [for reviews see (1-4)]. Briefly, the r-protein genes are grouped in translational regulatory units, with each unit consisting of co-transcribed r-protein genes; however, all the r-protein genes in a transcriptional unit are not necessarily members of a single translational regulatory unit. Each translational regulatory unit includes a single repressor r-protein that, when synthesized in excess of the amount necessary for ribosome assembly, can interact with its own mRNA, blocking its own translation and the translation of the other r-proteins in the regulatory unit. Stimulation of degradation of the repressed mRNA may also take place as a consequence of the translational block. In the cases that have been studied, repressor r-proteins act by binding to a single target site near the translation start site of one (usually, but not always the first) cistron in

the translational regulatory unit. Most of the r-proteins identified as translational repressor proteins have also been identified as primary binding proteins in ribosome assembly and can bind directly to rRNA *in vitro* (for a review on ribosome assembly, see e.g. ref. 5). Some (but not all) of the identified mRNA target sites have considerable primary and secondary sequence similarity to the binding site of the corresponding repressor protein on rRNA (6-8). Such similarity suggests, but does not prove, that the protein may recognize the same features in both rRNA and mRNA.

One of the best studied pair of r-protein binding sites are the rRNA and mRNA binding sites for r-protein L1. The secondary structures for both regions have been examined using structure-specific nuclease analysis (9, 10). Phylogenetic studies for both rRNA (9, 11) and L11 operon mRNA (12) show evolutionary conservation of these proposed secondary structures. Both the proposed secondary structure for the L1 binding site on 23S rRNA and the proposed structure for the L1 repressor target site on L11 operon mRNA share a similar secondary structure and contain some primary sequence identity. [Similarity was first noted by Gourse *et al.* (11) and Branlant *et al.* (9), and later more fully analyzed in this laboratory (6, 10, 13,)]. Furthermore, the L1 target site on L11 operon mRNA has been extensively studied by introduction of both random and site directed mutations (6, 14). These studies verified that some regions of the proposed secondary structure are required for L1 binding to mRNA *in vivo*. In addition, these studies identified bases not paired in the secondary structure models that are important for L1 recognition. If L1 in fact recognizes the same structural features in both rRNA and mRNA as discussed above, one can predict that recognition of rRNA by L1 may also use the sequence elements corresponding to those identified as important for mRNA-L1 interaction. In this paper, we have examined this prediction by constructing several mutations in the L1 binding region of 23S rRNA and by testing the effects of these mutations on L1 binding both *in vitro* and *in vivo*. For the *in vitro* test, we used a classical nitrocellulose filter binding assay. For the *in vivo* test, we devised a novel method which involves a conditional overproduction of a fragment of 23S rRNA containing the L1 binding site with or without mutational alterations and measurements of the extent of derepression of translation of L11-L1 mRNA. Results presented in this paper have demonstrated that the same structural features are indeed recognized by L1 in the interaction with both rRNA and mRNA.

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**MATERIALS AND METHODS****Materials**

[ $\gamma$ -<sup>32</sup>P]ATP, [ $\alpha$ -<sup>32</sup>P]UTP, [ $\alpha$ -<sup>35</sup>S]dATP and a site-directed mutagenesis kit were purchased from Amersham. Oligodeoxyribonucleotides used for site-directed mutagenesis were obtained from Synthetic Genetics (San Diego, CA) and the Agouron Institute (La Jolla, CA). T7 DNA polymerase (Sequenase Kit) was provided by United States Biochemical Corp. (Cleveland, OH). Filters type HA 0.45  $\mu$ M were obtained from Millipore Corp. (Bedford, MA). Bethesda Research Laboratories Gaithersburg, MD) was the source of T7 RNA polymerase, and RNase inhibitor (RNasin) was obtained from Promega Biotec. (Madison, WI).

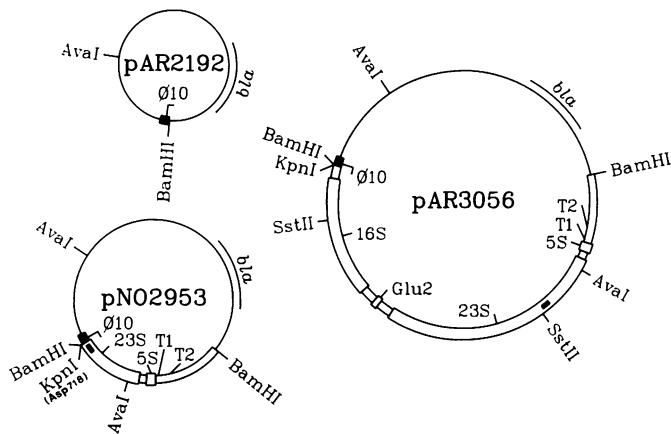
**Bacterial strains and plasmid construction**

The strain BL21(DE3) carries a copy of T7 RNA polymerase gene under *lac*-promoter control on a defective lambda prophage (15). Derivatives of this strain carrying pAR2192 or pN02953 (Fig. 1) were used for *in vivo* experiments. pAR2192 carries the  $\phi$ 10 promoter for T7 RNA polymerase and was used as a control vector. pN02953 is a derivative of pAR2192, and carries the 3' third of the 23S rRNA gene containing the L1 binding site. pN02953 was constructed as follows. Plasmid pAR3056 (16; see Fig.1) was cut with restriction enzymes *Sst*II and *Asp*718 (an isoschizomer of *Kpn*I). Both sticky ends were made blunt by simultaneously filling in the 5' overhang of the *Asp*718 sites and digesting the 3' overhang of the *Sst*II sites with T4 DNA polymerase. The resulting mixture was subsequently ligated in the presence of *Kpn*I linkers followed by transformation. Transformants were screened for the presence of plasmids of the expected size. One candidate was selected (pN02953) and the new junction sequenced after transfer to M13mp18. The sequence data indicated a loss of one of two C residues at the junction between the filled-in *Asp*718 site and the *Kpn*I linker. The reason for this loss was not investigated. Transcription from the T7 promoter on pN02953 is thus predicted to start with the sequence:

pppGGGAGACCACAACGUUUCCCUCUAGCGGGAUCCGGUACGGUACCGG<sub>2048</sub>

where G 2048 is the first rDNA residue in the transcript. The underlined C residue indicates the position where an additional C residue was lost during construction.

Various mutant derivatives of pN02953 were constructed using oligonucleotide directed mutagenesis. pN02953 was cleaved by *Bam*HI and the fragment carrying the L1 binding site was cloned into the *Bam*HI site of



**Figure 1.** Structures of pAR2192, pNO2953 and pAR3056. Locations of rRNA genes, the  $\phi 10$  promoter ( $\emptyset$ ), terminators (T1, T2) for *rrnB*, and the gene for  $\beta$ -lactamase (*bla*) are shown together with pertinent restriction enzyme sites. It should be noted that *Asp718* is an isoschizomer of *KpnI*. The L1 binding region of 23S rRNA is indicated with a small filled box.

M13mp18. Various synthetic oligonucleotides with length ranging from 15 to 21 nucleotides were used as primer to generate M13 phages carrying desired base alterations at the L1 binding site. In the case of mutant plasmids pNO2943 and pNO2974, the method described by Zoller and Smith (17) was followed. In the case of the other mutants, the Amersham oligonucleotide-directed mutagenesis kit was used as described by the manufacturer. Mutational alterations at the L1 target site were confirmed by DNA sequencing of the M13 mutant derivatives, covering at least the L1 binding site shown in Fig. 2B. *BamHI* fragments carrying the mutant sequences were then cut from replicative forms of the M13 derivatives and reinserted into the *BamHI* site of pAR2192 to yield mutant derivatives of pNO2953 (plasmids pNO2973 - pNO2980 and pNO3013). Nucleotide alterations in these plasmids are indicated in Table 1 and 2.

#### **In vitro RNA transcription**

$^{32}\text{P}$ -labeled RNA containing the L1 binding site (in the wild-type or mutant forms) was prepared by transcribing *AvaI* cleaved pNO2953 and its derivatives with T7 RNA polymerase. The reaction mixture (125  $\mu\text{l}$ ) contained the following: 40 mM Tris-HCl, pH 8.0, 8 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol (DTT), 25 mM NaCl, 2 mM spermidine, 0.4 mM each ATP, CTP, GTP and 0.08 mM UTP (containing 100  $\mu\text{Ci}$  of  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ ), 80 units RNasin, about 40  $\mu\text{g}/\text{ml}$  plasmid DNA template cut with *AvaI*. Reaction was initiated by adding 200 units of T7

RNA polymerase and the mixture was incubated at 37°C for 45 min. Non-radioactive RNA transcripts were also prepared in parallel by replacing  $^{32}\text{P}$ -UTP with non-radioactive UTP (0.4 mM). After the incubation, RNase-free DNase was added to a final concentration of 5  $\mu\text{g}/\text{ml}$  followed by 10 min incubation at 37°C. Carrier glycogen (20  $\mu\text{g}$  in 1  $\mu\text{l}$ ) and 2.5 volumes cold ethanol were then added to precipitate RNA. RNA was dissolved in 20  $\mu\text{l}$  buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM DTT and 40 units RNasin) and frozen at -70°C. The integrity of the RNA transcripts was confirmed by carrying out electrophoresis on 2% agarose gel in 1x TAE buffer (10 mM Tris-acetate, pH 8.0, 1 mM EDTA) and visualizing RNA under UV lamp (for cold RNA) or visualizing RNA by autoradiography (for [ $^{32}\text{P}$ ]RNA). The expected run off transcript is 774 nucleotides long including 45 bases of non rRNA sequence (see above) and 23S rRNA sequence from base 2048 to 2776 (18; for the location of L1 target site, see Fig. 2B). For  $^{32}\text{P}$ -RNA, autoradiograms gave a single RNA band and its size estimated from size markers was consistent with the expected size.

#### Filter binding assay

L1 protein was dialyzed overnight against "dialysis buffer" (0.5 M urea, 2 mM potassium phosphate buffer, pH 6.5, 1 M KCl, 1 mM DTT). Just prior to binding assays, the dialyzed L1 preparation was incubated at 37°C for 20 min and then cooled to room temperature.  $^{32}\text{P}$ -labeled-RNA and non-radioactive RNA were suitably mixed (see below) in 20 mM Tris-HCl pH 7.4, 25 mM KCl, 10 mM  $\text{MgCl}_2$ , 0.1 mM DTT and 5% dimethyl sulfoxide (DMSO) and incubated at 65°C for 5 min and then cooled to room temperature. [ $^{32}\text{P}$ ]RNA (1  $\mu\text{l}$ ) was first mixed with 35  $\mu\text{l}$  "reconstitution buffer" [30 mM Tris-HCl, pH 7.4, 20 mM  $\text{MgCl}_2$ , 0.35 M KCl and 6 mM 2-mercaptoethanol]. Four  $\mu\text{l}$  of dialysis buffer containing various amounts of L1 protein were then added and the reaction mixture was incubated at 37°C for 20 min. Thus, the final concentration of components in the 40  $\mu\text{l}$  reaction mixture was: [ $^{32}\text{P}$ ]RNA (about 1  $\mu\text{M}$ ; 0.8 to  $1.4 \times 10^5$  cpm), L1 (about 5  $\mu\text{M}$ ), 410 mM KCl, 50 mM urea, 27 mM Tris pH 7.4, 18 mM  $\text{MgCl}_2$ , 0.12 mM DTT, 0.13% DMSO, 0.2 mM  $\text{K}_2\text{HPO}_4$  and 5.3 mM 2-mercaptoethanol. [In experiments with the wild type [ $^{32}\text{P}$ ]RNA and various concentrations of L1, the concentration of L1 (about 5  $\mu\text{M}$ ) used was shown to be near saturation; see also the legend to Table 1.] After the incubation, the reaction mixtures were chilled on ice for about 1 hr and filtered through a Millipore type HA nitrocellulose filter by gentle suction at room temperature, followed by washing with 5 ml of reconstitution buffer. Filters with the retained RNA-L1 complexes were dried and the amounts of radioactive RNA were determined. The

TABLE 1. Binding of [<sup>32</sup>P]rRNA fragment and its mutant derivatives to ribosomal protein L1 *in vitro*.

Plasmid used as template	Location of mutation	Nucleotide change	[ <sup>32</sup> P]rRNA bound (% of wild type)	(% induction from <i>in vivo</i> experiment)*
pNO2953	(wild type)	-	(100)	(100)
pNO2973	2123/24	GG→CC	7±3	(1)
pNO2976	2174/75	CC→GG	9±5	(5)
pNO2975	2123/24	GG→CC	41±5	(94)
	2174/75	CC→GG		
pNO2974	2125/26	GA→TC	3±1	(11)
pNO2977	2125	G→A	49±5	(61)
pNO2978	2125	G→C	45±3	(52)
pNO2979	2125	G→T	48±6	(84)
pNO2980	2126	A→G	17±4	(10)
pNO3013	2126	A→C	24±4	(4)

[<sup>32</sup>P]RNAs were prepared from various plasmid DNA templates indicated in the table after digestion with *Ava*I as described in Materials and Methods. The amounts of [<sup>32</sup>P]rRNA retained by nitrocellulose filters were measured and compared to the amounts obtained with transcripts from pNO2953 which contains the wild type L1 binding site. The average values from two independent experiments are shown for mutant transcripts together with standard deviations. The values obtained with pNO2953 varied from experiment to experiment and were on the average 70% (±24%) of the input values (which were 0.8 to 1.4 x 10<sup>5</sup> cpm). Background values (4±2% of the input values) obtained in the absence of L1 are subtracted.

\*In the *in vivo* experiments described in Table 2, "% induction" was calculated for L1 and L11 (the values in parentheses in Table 2). Averages of the values for L1 and for L11 are given here to make comparison to the *in vitro* binding results easier.

filters used for the experiments were pre-treated with 0.5 M KOH for 20 min, washed extensively with water and then kept in 0.1 M Tris-HCl pH 7.4 at 4°C (19).

#### In vivo measurements of protein synthesis rates

Plasmid containing derivatives of BL21(DE3) were grown at 37°C in MOPS minimal media (20) supplemented with thiamine at 0.1 µg/ml, ampicillin at 100 µg/ml, and 0.4% glucose. At an OD<sub>600</sub> of 0.2 (about 2 x 10<sup>8</sup> cells/ml), isopropyl thiogalactoside (IPTG) was added to a final concentration of 1 mM

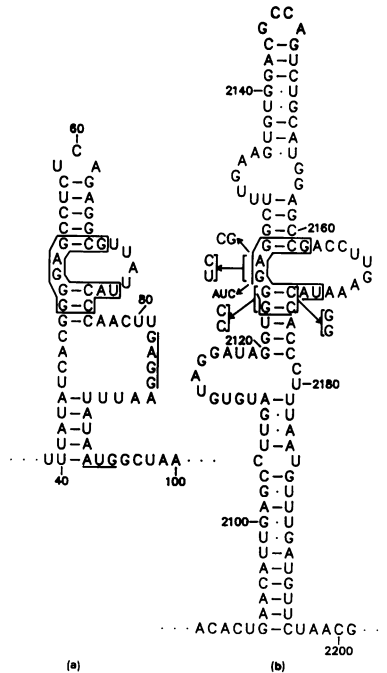
and growth continued for 20 min. After 20 min, 5 ml portions of each culture were pulse labeled for 1 min with 200  $\mu\text{Ci}$  of [ $^3\text{H}$ ]lysine (97 Ci/mmol) and chased for 1 min with non-radioactive lysine (final concentration 100  $\mu\text{g}/\text{ml}$ ). The BL21(DE3) derivative carrying plasmid pAR2192 was pulse labeled as described above both with and without IPTG addition.  $^3\text{H}$ -labeled cultures were mixed on ice with an equal volume of a long-term  $^{14}\text{C}$ -labeled reference culture of BL21(DE3) carrying plasmid pAR2192 grown for two generations in the presence of [ $^{14}\text{C}$ ]lysine (355 mCi/mmol, 4  $\mu\text{Ci}/\text{ml}$ ).

Selected r-proteins were separated by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis as described (14). The  $^3\text{H}/^{14}\text{C}$  ratio for each specific protein was divided by the  $^3\text{H}/^{14}\text{C}$  ratio for total protein in that sample. This value represents the differential synthesis rate of the specific protein normalized to the differential accumulation of the same protein in the  $^{14}\text{C}$ -labeled reference cells. The ratio of two synthesis rates defined in this way and measured under different conditions is equal to the ratio of the relative differential synthesis rates of the protein under the two conditions. The data presented in Table 2 represents the differential synthesis rates relative to those in BL21(DE3) carrying pAR2192 after induction.

It should be noted that by mixing the [ $^3\text{H}$ ]lysine-labeled experimental cells with [ $^{14}\text{C}$ ]lysine-labeled carrier cells before lysis and by expressing the final result as the relative differential synthesis rate, the errors associated with sample handling (pipetting error), variable recovery and differences in cell density all cancel. With this method the residual error is due, for the most part, to the error in quantification of the radioactivity in the final sample (counting error) and to incomplete purification of the sample proteins.

#### Isolation of RNA labeled in vivo after IPTG induction

Derivatives of strain BL21(DE3) carrying pNO2953, pNO2973 or pAR2192 were grown in supplemented MOPS medium to a cell density of  $\text{OD}_{600} = 0.2$  as described in the previous section. IPTG (1 mM) was added and growth continued for 12 min. [ $^{32}\text{P}$ ]orthophosphate (60  $\mu\text{Ci}/\text{ml}$ ; 47  $\mu\text{Ci}/\mu\text{mol}$ ) was then added to each of 5 ml cultures and incubation continued for 10 min. Incorporation of  $^{32}\text{P}$  was stopped by mixing with five grams of ice. Cells were collected by centrifugation at  $4^\circ\text{C}$ , resuspended in 0.4 ml SDS lysis mixture (2% sodium acetate pH 5.2, 0.5% SDS and 1 mM EDTA), and treated with an equal volume of phenol at  $65^\circ\text{C}$  for 20 min, followed by second extraction with phenol-chloroform-isoamyl alcohol (25:24:1).  $^{32}\text{P}$ -labeled RNA was



**Figure 2.** The secondary structure models of (a) the L1 target site on L11 mRNA (10) and (b) the L1 binding region of 23S rRNA (9). The regions of primary sequence identity are boxed (see ref. 6). Mutations constructed in this work are indicated by arrows (see Table 2). Numbering of the mRNA sequence is with respect to the first nucleotide of the L11 mRNA. The Shine-Dalgarno sequence as well as the initiation codon for L11 are indicated. Numbering of the rRNA sequence is according to ref. 18.

recovered by ethanol precipitation in the presence of 30  $\mu\text{g/ml}$  tRNA, dissolved in 50  $\mu\text{l}$   $\text{H}_2\text{O}$  containing 5 mM DTT and 10 units of RNasin, and aliquots were analyzed by electrophoresis on 2% polyacrylamide-0.5% agarose gel (21).

## RESULTS

### Experimental design and construction of expression plasmid containing 23S rRNA L1 binding site or its mutant derivatives.

In order to provide high level transcription of the L1 binding site of 23S rRNA, we made use of a phage T7 polymerase - T7 promoter expression vector system (15, 16) and constructed plasmid pNO2953 (Fig. 1). This plasmid retains the 3' third of the 23S gene (with the L1 binding site) and



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the genes for 5S rRNA along with the two strong *rrnB* operon terminators, T1 and T2, under the control of T7  $\phi$ 10 promoter.

The region of L11 operon mRNA shown to be important for translational regulation by L1 is shown in Figure 2A. This region shares some sequence identity and secondary structure similarities with the L1 binding site on 23S rRNA (Figure 2B; see the regions outlined). Previous studies (6) showed that the main determinant of mRNA structure for L1 recognition is probably an internal loop surrounded by two short G-C rich stem structures, the lower G-C stem (nucleotides 49-51 and 74-76) and the upper stem (nucleotides 54-58 and 62-66). It was shown that the primary nucleotide sequence of the lower stem is not important but the stem structure is important for the mRNA-repressor interaction (14). Its function is probably to provide a structural role to maintain essential bases in the internal loop in a proper configuration; the unpaired A residue at position 53 was shown to be especially important for regulation, and G at position 52 was shown to be somewhat less important (6). Thus, to test whether similar structural features in the L1 binding region of 23S rRNA are also important for L1 binding to 23S rRNA, we constructed several derivatives of pN02953 with site-directed mutagenesis in the region of sequence identity (Figure 2 and Table 1). Two of these plasmids contain changes expected to disrupt a region of secondary structure corresponding to the lower stem of L11 mRNA mentioned above. A third plasmid carries both of these changes so that formation of the stem structure is restored. Six additional mutations were constructed to test the importance of two unpaired bases in the internal loop corresponding to A53 and G52 of mRNA mentioned above.

#### Effects of mutational base alterations on L1 binding site analyzed in vitro

<sup>32</sup>P-labeled RNA fragments carrying the L1 binding site (or mutationally altered sites) were prepared by cutting pN02953 (and mutant derivatives) with *Ava*I and transcribing it using T7 RNA polymerase. These RNA transcripts were incubated with varying amounts of purified r-protein L1. The mixtures were then passed through nitrocellulose filters. The percentage of transcripts with mutant binding sites bound to the filters were compared to the percentage of transcripts containing the wild type L1 binding site bound to the filters under the same conditions. Data from experiments using levels of L1 that were near saturating for the pN02953 transcript are presented in Table 1. [Results with lower concentrations of L1 were less reliable and no attempt was made to quantitate relative binding constants from the binding curves.] It can be seen from Table 1 (column 4) that disruption of the stem

structure (2123-24 and 2174-75) by mutational alterations abolishes the ability of the RNA fragment to bind L1 and combining the two mutations to restore a stem structure leads to a partial restoration of this ability. Alteration of A (position 2126) in the internal loop to G or C abolished the L1 binding ability nearly completely, but alteration of the adjacent G (position 2125) in the internal loop to A, C or T showed only partial effects on binding.

### Effects of mutational base alterations on L1 binding analyzed in vivo

Transcription of rRNA genes from a T7 promoter by T7 RNA polymerase was first demonstrated by Steen *et al.* (16) using a derivative of *E. coli* strain BL21(DE3) carrying plasmid pAR3056. Strain BL21(DE3) carries a copy of T7 RNA polymerase gene under *lac* promoter control on a defective lambda prophage. Induction of the T7 polymerase synthesis in plasmid carrying derivatives of BL21(DE3) leads to a high rate of transcription from plasmid born T7 promoters. In fact, it was reported that the synthesis rate of rRNA from plasmid pAR3056 is roughly equal to the normal synthesis rate of rRNA (16). We used this T7 promoter/polymerase system and constructed pNO2953 and its mutant derivatives, as described above, to overproduce RNA fragments containing L1 binding site *in vivo*. We reasoned that, since the synthesis of L11 and L1 is normally repressed by a factor of 2 to 4 (22, 23), overproduction of the 23S rRNA fragment with the L1 binding site *in vivo* might compete with mRNA, removing repressor L1 to form a L1-rRNA fragment complex, and lead to derepression of the synthesis of L11 and L1. Preliminary experiments of this nature were carried out with a similar plasmid earlier and derepression of L11 and L1 synthesis was in fact observed after overproduction of rRNA fragments containing the L1 binding site (J.R. Cole, Ph.D. thesis, University of Wisconsin-Madison, 1987). Thus, we wished to examine the effects of various mutations on the ability of the rRNA fragment to bind L1 repressor *in vivo* using this system.

Derivatives of strain BL21(DE3) carrying pNO2953 or its mutant derivatives were grown in a synthetic medium and the synthesis rates of L11 and L1, and two other control r-proteins, L3 and L6, were determined at 20 min after induction of the synthesis of T7 RNA polymerase by IPTG. The results are shown in Table 2. It can be seen that the synthesis rates of r-proteins L3 and L6 remained fairly constant for all plasmids tested. However, the synthesis rates of r-proteins L1 and L11 were elevated about 2.8-fold in the strain carrying pNO2953, as expected, indicating that rRNA fragments containing the L1 target site transcribed from the plasmid is able

TABLE 2. Relative differential synthesis rates of selected proteins after overproduction of 23S rRNA fragment containing L1 target site *in vivo*

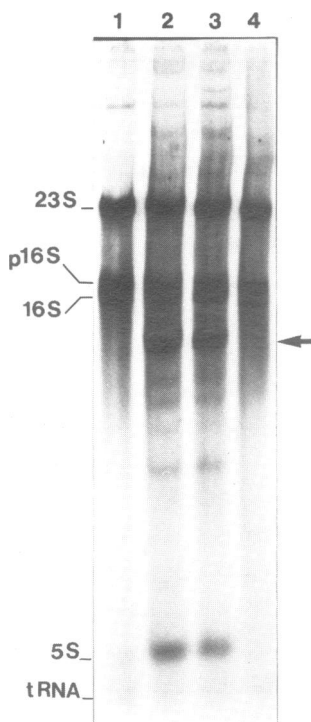
Plasmid	Location of mutation	Nucleotide change	Protein			
			L1	L11	L3	L6
pNO2953	(wild type)	-	2.85(100)*	2.83(100)*	1.07	0.93
pNO2973	2123/24	GG→CC	1.02 (1)	1.01 (1)	1.13	0.93
pNO2976	2174/75	CC→GG	1.09 (5)	1.07 (4)	1.18	0.96
pNO2975	2123/24	GG→CC	2.78 (96)	2.68 (92)	1.10	0.90
	2174/75	CC→GG				
pNO2974	2125/26	GA→TC	1.20 (11)	1.17 (10)	1.29	0.99
pNO2977	2125	G→A	2.12 (61)	2.09 (60)	1.17	0.94
pNO2978	2125	G→C	1.99 (53)	1.91 (50)	1.18	0.95
pNO2979	2125	G→T	2.55 (84)	2.51 (83)	1.04	0.91
pNO2980	2126	A→G	1.17 (9)	1.18 (10)	1.28	0.99
pNO3013	2126	A→C	1.07 (4)	1.05 (3)	1.19	0.99

Derivatives of BL21(DE3) carrying pNO2953 or its mutant derivatives (indicated in the table) or pAR2192 (a control vector plasmid) were grown in a synthetic medium and relative differential synthesis rates of r-protein L1, L11, L6 and L3 were determined, as described in Materials and Methods, at 20 min after the addition of IPTG to induce the synthesis of 23S rRNA fragment from the plasmids. The values obtained with experimental strains were normalized to the corresponding values obtained with the control strain carrying pAR2192. The normalized values from three independent experiments were very similar (standard deviations less than 10% in most of the cases), and their averages are shown in the Table.

\* For L1 and L11, "% induction" was calculated according to the equation shown below and the values obtained are given in parenthesis in the Table.

% Induction =  $100 \times \frac{[\text{Relative differential synthesis rate in mutant} - 1]}{[\text{Relative differential synthesis rate in wild type} - 1]}$

to compete effectively with L11 mRNA for L1 binding. The synthesis rates of L1 and L11, however, were not significantly elevated after induction of the synthesis of some of the mutant RNAs with altered binding sites, indicating that these mutations abolish the ability of the fragment to bind L1 effectively to win competition with mRNA. These mutations include the two mutations that disrupt the stem structure in question (see pNO2973 and pNO2976). Combining these two mutations and restoring a stem structure restored the ability of the RNA transcript to bind L1 to win competition (see



**Figure 3.** Polyacrylamide-agarose gel electrophoresis of RNA isolated from cells carrying various plasmids before (lane 1) and after (lane 2-4) induction of the transcription from the  $\phi 10$  T7 promoter. Strains carried the following plasmids: pNO2953 (wild type plasmid; lane 1 and 2), pNO2973 (a mutant plasmid; lane 3) and pAR2192 (a vector plasmid; lane 4).  $^{32}\text{P}$ -labeled RNAs were prepared as described in Materials and Methods. For each RNA, a sample containing  $2.5 \times 10^4$  cpm was analyzed. An autoradiogram of the dried gel is shown. The position of the overproduced 23S rRNA fragment containing the L1 target site is indicated by an arrow.

pNO2975). Mutations altering A (position 2126) in the internal loop also abolished the ability of the fragment to compete with mRNA for L1 binding (see pNO2974, pNO2980 and pNO3013). In contrast, mutations altering G (position 2125) adjacent to the A in the internal loop gave a large increase in L1 and L11 synthesis, although not as great as with the wild-type binding site, indicating that these mutations did not strongly affect the ability of the plasmid transcript to compete for L1 (see pNO2977, pNO2978 and pNO2979).

The relative degree of stimulation of L11 and L1 synthesis by overproduction of the rRNA fragment in the experiments shown in Table 2 was also expressed as % induction (see definition of % induction described in the

legend to Table 2). Averages of % induction for L11 and L1 for various mutant plasmids are shown in the last column of Table 1 to make comparison to the *in vitro* binding results easier. It can be seen that, although there are some quantitative differences between the *in vitro* and *in vivo* results expressed in this way, it is evident that both results agree by and large, as already described above (compare column 4 and 5 in Table 1).

Analysis of plasmid-encoded RNA synthesized after induction of the synthesis of T7 RNA polymerase

As described in the previous section, overproduction of the 23S rRNA fragment with the L1 binding site *in vivo* led to derepression of the synthesis of L11 and L1, and this derepression did not take place when synthesis of the fragments was induced from some mutant plasmids encoding altered L1 binding sites. We examined RNAs synthesized under these conditions. Strains carrying the wild-type plasmid (pNO2953), a mutant plasmid (pNO2973, which did not show any derepression of L11-L1 synthesis upon induction with IPTG; see above) or the vector plasmid (pAR2192) were labeled with [<sup>32</sup>P]orthophosphate between 12 and 22 min after induction with IPTG. The strain carrying pNO2953 without induction was also labeled with <sup>32</sup>P as control. RNA synthesized during this 10 min labeling period was analyzed by electrophoresis on a polyacrylamide-agarose gel, followed by autoradiography. The results are shown in Fig. 3.

It can be seen first that induction of transcription of vector plasmid DNA by T7 RNA polymerase caused some decrease in the synthesis of rRNA (23S rRNA, 16S rRNA and a precursor form of 16S rRNA) and appearance of heterogenous RNA transcripts including very large molecular weight RNA (compare lane 4 with lane 1), confirming the previous results obtained by Studier and Moffat (15). In the case of induction of transcription of pNO2953 (lane 2) and pNO2973 (lane 3), a new major radioactive RNA band can be clearly seen, at the position which corresponds roughly to that expected for RNA transcript starting from the T7 promoter and processed between the 23S rRNA gene and the 5S RNA gene (about 900 nucleotides long). Judging from the relative intensity of bands (and their relative size), molar amounts of this transcript were at least comparable to those of 16S and 23S rRNA transcribed from the chromosome. In addition, overproduction of 5S RNA (and/or its precursor) can also be recognized with these two RNA samples. From inspection of the autoradiograms, it is evident that there is no significant difference in the pattern of radioactive RNAs between the two. Thus, we conclude that, although transcription of the mutant plasmid (pNO2973) did not cause derepression of L11 and L1 synthesis, the failure is

not because of failure of the synthesis of the mutant RNA, nor because of instability of this RNA.

### DISCUSSION

Earlier studies on the L1 binding to 23S rRNA have shown that L1 protects a 169 base fragment of 23S rRNA starting at base 2067 from nuclease digestion (9). In addition, *E. coli* L1 was shown to bind to 23S rRNA from other bacterial species (9) and 26S rRNA from *Dictyostelium discoideum* (11). L1 binding regions of these rRNA were also studied by protection with L1 from nuclease digestion. However, no experimental studies have been reported to identify specific base residues involved in the interaction with L1. In the present studies, we have experimentally demonstrated involvement of specific base residues and a stem structure in L1 binding. The importance of these residues and the structure was originally inferred from the results of previous mutagenesis studies on the L1 target site of L11 mRNA (6). As in the case of the L1 target site on mRNA, unpaired nucleotide residues, especially A at position 2126, in the internal loop that is surrounded by GC-rich stems are important for L1 binding. In addition, the lower stem examined was also shown to be essential. In the latter case, the stem appears to provide an essential structural role rather than a role as L1 recognition sequence, whereas the unpaired A-2126 appears to be involved in direct interaction with L1. It should be noted that we did not study other nucleotide residues in the L1 binding region, and therefore, it is possible that L1 binding to 23S rRNA also depends on additional nucleotide residues not analyzed in this work.

Regarding the suggested structural role of the lower GC rich stem, it should be noted that combination of the two mutations restoring a base-paired structure did not lead to complete restoration of L1 binding activity assayed *in vitro*, whereas it showed L1 binding activity comparable to the wild type when  $\kappa$  induction was used as its measure *in vivo* (see pNO2975 in Table 1 and 2). It is possible that RNA containing the two mutations transcribed from pNO2975 has in fact binding activity which is weaker than the wild type RNA, but that the difference in binding activity between the two RNAs is not manifested in the *in vivo* assay method, because the degree of derepression by the overproduction of the wild type RNA is limited by some other factors, e.g., by the synthesis rate of r-protein mRNA. The same consideration could also be applied for the apparent differences between the *in vitro* and the *in vivo* results regarding the effects of some of the mutational alterations of G

at position 2125. It is also possible that the discrepancies are in part due to some limitations in the simple *in vitro* filter binding method used in the present work. Regardless of such uncertainty regarding quantitative aspects of the effects of base alterations of rRNA on L1 binding, it is clear that L1 recognizes the same structural features in both rRNA and mRNA. Therefore, it appears likely that L1 has a single RNA binding domain which interacts with both rRNA and mRNA target sites.

Alterations of some r-protein binding sites on rRNA by *in vitro* mutagenesis were carried out previously, but their effects on r-protein binding ability *in vivo* were assessed only indirectly by analyzing over-all assembly of ribosomes (for example, ref. 24) or by following alterations of growth rate of strains harboring plasmids which carry mutant rRNA operons (25). The present method involves the ability of an overproduced RNA fragment containing the L1 binding site to derepress the translation of L11-L1 mRNA, and hence, interpretation of the results is considerably simpler compared to the previous methods, even though it still has some complications, such as those discussed above in connection with discrepancy from the *in vitro* results. In this connection, we note that the RNA fragment overproduced in this study is most likely larger than the minimum size required for L1 binding. In the present study, we examined the synthesis of L3 and L6 as control proteins and found no stimulation of their synthesis. The synthesis of L6 is regulated by S8 (26) and hence its stimulation is certainly not expected. L3 synthesis is regulated by L4, and L4 binding site is probably not present on the RNA fragment used in this system (see 27, 28), and hence, derepression of synthesis of S10 operon proteins is also not expected. However, we did not analyze other r-proteins. It is possible that there are some undiscovered (hypothetical) repressor r-proteins with a binding site on this RNA fragment. If this is the case, such r-proteins (and other r-proteins including L11) might affect the interaction of L1 with the pertinent RNA fragment or the stability of a ribonucleoprotein particle containing L1, and hence, might affect indirectly the degree of derepression of L1 and L11 synthesis. We have not characterized the state of L1 containing particles (e.g. the possibility of the presence of other r-proteins) or their stability under the experimental conditions used for the analysis of L11 and L1 synthesis. However, we found that the amount of mutant RNA fragments (synthesized from pNO2973), which did not derepress L11 and L1 synthesis, was about the same as that of the wild-type RNA fragments synthesized under the same conditions, indicating that the presence or

absence of L1 binding did not apparently affect the stability of this RNA fragment in any gross way. Since the repression of mRNA-directed synthesis of L11 and L1 *in vitro* can be reversed by the addition of 23S rRNA (in the absence of other r-proteins) (29), rRNA fragments with the L1 binding site may be able to compete with mRNA for L1 binding *in vivo* in the absence of other r-protein binding. It may be interesting to carry out deletion analysis to define the minimum "L1 binding region" necessary for derepression of L11 mRNA translation *in vivo*.

Finally, the present experiments support the previous conclusion that mRNAs for r-proteins (at least L11 and alpha operon mRNAs) are usually synthesized in excess of the amount needed for production of r-proteins (22, 23, 30). The degree of repression under normally growing cells was previously estimated to be 2 to 4-fold depending on growth conditions (22). The value estimated under the present experimental conditions (2.8-fold repression) is consistent with the previous results. Thus, translational repression plays a major role in the regulation of r-protein synthesis, at least in the case of L11 operon expression.

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\*Present Address: Department of Crop and Soil Sciences, Michigan State University, East Lansing, Michigan 48824, USA

†To whom correspondence should be addressed.

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