Translational regulation of the L11 ribosomal protein operon of *Escherichia coli*: mutations that define the target site for repression by L1

Mark S.Thomas⁺ and Masayasu Nomura*

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. The mRNA target site for this repression is located close to the Shine-Dalgarno sequence for the first cistron, *rplK* (L11). By use of a random mutagenesis procedure we have isolated and characterized a series of point mutations in the L11 leader mRNA which eliminate or greatly diminish the regulation by L1. The mutations define a region essential for translational regulation upstream of the L11 Shine-Dalgarno sequence and identify a region of structural homology with the L1 binding site on 23S rRNA. These results are also consistent with the previously proposed model for the secondary structure of the L11 leader mRNA.

INTRODUCTION

It has now been established that the synthesis of most, if not all, ribosomal proteins (r-proteins) are feedback-regulated at the level of translation by certain key r-proteins. Each r-protein regulatory unit contains the gene for its own unique translational repressor. According to the model for feedback regulation of r-protein translation each repressor r-protein preferentially binds to specific sites on rRNA during ribosome assembly. Each repressor r-protein also recognizes a specific target site on its own message. Binding of the repressor r-protein to this site occurs when r-protein synthesis exceeds that needed for ribosome assembly and results in inhibition of translation of all the r-proteins encoded within the regulatory unit. Thus competition between the binding sites on r-protein mRNA and rRNA modulates the translation of r-protein mRNA to match the synthesis of rRNA in the cell [for reviews see (1-3)].

One system which has been fairly well characterized is the L11 r-protein operon which contains the genes for L11 and L1. Previous experiments have identified L1 as a translational repressor regulating the synthesis of both L11 and L1 (4-6). The mRNA target site for this repression has been localized to a region near, or at, the translation initiation site of the

first (L11) cistron (7,8). An understanding of the mechanism of translational feedback repression depends upon a knowledge of the mRNA sequences necessary for binding of L1. This paper describes the isolation of a number of mutations in the L11 leader mRNA which reduce or eliminate repression by L1. We discuss these results in relation to both the secondary structure model proposed for the L11 leader mRNA (K. Kearney and M. Nomura, submitted for publication) and the possible mechanism of translational repression.

MATERIALS AND METHODS

Construction of plasmids pNO2863 and pNO2864 is shown in Fig. 1. *E. coli* MC1061 [*araD139*, Δ (*ara-leu*)7697, Δ (*lacIPOZYA*)X74, *galU*, *galK*, *hsdR* *hsdM*⁺ *rpsL*] (9) was kindly provided by N. Minton. NO3607 is a spontaneous rifampicin resistant (*rpoB*) mutant of MC1061. NO3608 carries the MN2 mutation in the L11 operon leader sequence and as a result overproduces the repressor r-protein L1 as well as L11 (10), but its growth rate is about the same as NO3607. The strain NO3608 was constructed from MC1061 by P1 transduction from NO3294 (10) and selection for rifampicin resistance. The presence of the MN2 mutation was confirmed by transformation of some of the Rif^r transductants with pNO2863 and then comparing the resulting β -galactosidase activity with that produced by NO3607 containing pNO2863. The presence of the MN2 mutation in NO3608 results in a greatly repressed level of *rplK-lacZ* translation from pNO2863 (see Results).

Conditions for ultraviolet (UV) light mutagenesis were as described previously (11). Briefly, pNO2863 DNA (1 μ g in a small drop) was irradiated for 90 seconds at a distance of 70 cm from a 60W germicidal lamp and used to transform UV-irradiated NO3608 cells. β -galactosidase activity was assayed according to Miller (12) and values are expressed in Miller units. For DNA sequencing, a *HindIII* fragment containing the entire L11 leader and first 12 codons of L11 was transferred from the mutant derivatives of pNO2863 (Fig. 1) to M13mp9 (13). Sequencing reactions were carried out as described before (14).

RESULTS

Construction of system for selection of L1 target site mutations

In order to isolate mutations in the L1 target site we constructed two plasmids (pNO2863 and pNO2864) in which the *lacZ* gene was fused in phase with the first 12 codons of the L11 gene (*rplK*) (Fig. 1). pNO2863 contains the

Fig. 1 - Construction of pNO2863 and pNO2864.

(a) pNO2767 and pNO2698 contain the entire L11 leader sequence fused, by means of a *HindIII* linker, to the *lac* promoter. Both plasmids also carry the L11 gene and 5' part of the L1 gene. pNO2698 carries, in addition, the MN2 mutation which abolishes regulation by L1. Construction of both plasmids has previously been described (10). pNO2861 and pNO2862 were constructed from pNO2767 and pNO2698 respectively by the transfer to pBR322 of a *HindIII*-*SphI* fragment containing the entire L11 leader, L11 gene, and a 5' portion of the L1 gene. (b) pNO2863 and pNO2864 were created by replacing the *PstI* fragment in pSKS105 (15) with the *PstI* fragment containing the L11 leader from pNO2861 and pNO2862 respectively. In both plasmids, the *lacZ* gene is fused in phase with the first 12 codons of *rplK* (L11). Translation of the L11- β -galactosidase fusion protein from pNO2863 is under control of L1. Pertinent restriction enzyme sites are denoted as follows; H = *HindIII*, P = *PstI*, S = *SphI*. The stippled region represents the L11 leader.

entire L11 leader region and the L11- β -galactosidase fusion is therefore under the translational control of L1. pNO2864 carries the MN2 mutation in the L11 leader (see Table 1). This mutation, originally derived from pNO2597 (16), abolishes translational regulation by L1. These plasmids do not contain the *lac* promoter originally present in the vector pSKS105 (Fig. 1). Despite the absence of the *lac* promoter, expression of the *rplK-lacZ* fusion

TABLE 1. Effect of L11 leader mutations on β -galactosidase synthesis from pNO2863

Plasmid ^a	Location of mutation ^b	Nucleotide change ^c		<u>β-galactosidase activity (Miller Units)</u>		
				N03607 back-ground	N03608 back-ground	Repression Ratio ^d
pNO2863("parent")	-	-	(-)	481	59	8.15
pNO2864 (MN2)	49/50	GG→CC	(-)	1517	1240	1.22
pNO2877 (MN101)	18	G→A	(1)	961	352	2.73
pNO2878 (MN102)	32	A→G	(2)	738	113	6.51
pNO2880 (MN104)	35-42 ^e	U insertion	(1)	2580	1860	1.39
pNO2881 (MN105)	40-41	C insertion	(1)	1978	1844	1.07
pNO2882 (MN106)	48	C→U	(1)	3561	3307	1.08
pNO2883 (MN107)	49	G→U	(2)	1201	911	1.32
pNO2885 (MN109)	49	G→A	(2)	1255	666	1.89
pNO2887 (MN111)	50	G→C	(2)	1439	1110	1.30
pNO2889 (MN113)	50	G→A	(3)	1901	1544	1.23
pNO2894 (MN118)	51	G→A	(3)	1824	1430	1.28
pNO2896 (MN120)	52	G→U	(2)	1082	467	2.32
pNO2897 (MN121)	52	G→C	(1)	898	378	2.38
pNO2898 (MN122)	53	A→G	(1)	1104	894	1.24
pNO2899 (MN123)	53	A→U	(1)	1120	969	1.16
pNO2900 (MN124)	53	A→C	(2)	1164	947	1.23
pNO2902 (MN126)	54	G→A	(1)	945	644	1.47
pNO2903 (MN127)	74	C→A	(1)	1900	992	1.92
pNO2905 (MN129)	75	C→U	(2)	1420	422	3.37
pNO2907 (MN131)	76	C→U	(3)	1290	219	5.89
pNO2909 (MN133)	52,53	GA→AG	(1)	1229	782	1.57
pNO2910 (MN134)	52,53	GA→UU	(1)	1176	823	1.43

^aMutation designation is given in parenthesis.^bNumbers refer to the nucleotide sequence in Fig. 2 (a). For simplicity only one example of each different mutation is shown.^cThe frequency of occurrence of each type of mutation is given in parentheses.^dThe repression ratio gives a measure of the effect of each mutation on translational regulation by L1 (see Results) and is obtained by dividing the β -galactosidase activity for a particular mutant plasmid in N03607 by the activity for the same plasmid in N03608.^eThe precise location of the U insertion cannot be determined.

still occurs although at a greatly reduced level. The element responsible for directing the transcription of the *rplK-lacZ* fusion is probably the pBR322-derived P4 promoter (17). Removal of the *lac* promoter had two main advantages; (a) selection of L1 target site mutations was not hampered by a presumed high background of *lac* regulatory mutants and (b) strains containing plasmids in which the *rplK-lacZ* fusion is under control of the *lac* promoter/operator were found to be inviable on media containing inducers of the *lac* operon thus precluding the isolation of L1 target site mutations. Regarding the latter observation we believe that this is a result of overproduction of the abnormal L11- β -galactosidase fusion protein.

The presence of the MN2 mutation on pNO2864 resulted in a 3-fold increase in β -galactosidase activity in strains containing this plasmid relative to strains containing pNO2863. This is in accordance with previous observations regarding the regulatory effects of the MN2 mutation (10). However, there was only a subtle color difference between colonies formed by strains containing either of these two plasmids on lactose MacConkey medium. In order to increase the difference in β -galactosidase activity between strains containing these plasmids and by that means facilitate mutant detection we used NO3608 as the host strain (see Materials and Methods). Overproduction of L1 repressor r-protein in this strain greatly represses translation of the L11- β -galactosidase fusion protein from pNO2863 without affecting significantly translation of the pNO2864-encoded fusion protein (Table 1). The low level of L11- β -galactosidase fusion protein synthesis in NO3608 (pNO2863) results in the production of white colonies on lactose MacConkey agar whereas colonies of NO3608 (pNO2864) are pink. The 20-fold difference in β -galactosidase activity between NO3608 (pNO2863) and NO3608 (pNO2864) also results in a pronounced growth rate difference on minimal medium containing lactose. The former strain forms small colonies after 4 days growth whereas only 1-2 days growth of the latter strain is necessary for the formation of visible colonies on this medium.

Isolation and characterization of L1 target site mutations.

pNO2863 DNA was irradiated with UV light and used to transform UV irradiated NO3608 cells as described in Materials and Methods. Selection for mutants giving rise to high levels of β -galactosidase activity was made on lactose MacConkey or lactose minimal plates. "Lac⁺" mutant colonies appeared at a frequency of about 0.1%. Mutations were assigned to either the chromosome or plasmid by re-transforming NO3608 with plasmid isolates from

the original mutants and screening for the appearance of the same Lac⁺ phenotype. Mutations assigned to the plasmid were further characterized by transformation of an isogenic strain which does not overproduce L1 repressor (NO3607). By comparing the β -galactosidase activity of NO3607 carrying a particular mutant plasmid with NO3608 containing the same plasmid it was possible to determine the nature of the mutation. For example, a mutation which completely abolishes regulation by L1 will result in a β -galactosidase activity which is the same in both backgrounds. Thus the ratio of β -galactosidase activity for a plasmid carrying such a mutation (i.e. MN2) in NO3607 relative to NO3608 will be approximately equal to unity (Table 1). On the other hand, mutations may be selected as a result of an increased plasmid copy number or efficiency of *rplK-lacZ* translation. Such mutations, although resulting in higher levels of β -galactosidase activity will still have a high "repression ratio" similar to that observed for the regulated plasmid pNO2863 (Table 1). Therefore, the repression ratio provides a measure of the effect of each mutation on translational regulation by L1; high ratios reflect little or no interference with regulation by L1 whereas low ratios indicate that the mutation exerts a strong negative effect on the regulation. Using this type of analysis we were able to discriminate between mutations having regulatory effects from those which resulted in an increased β -galactosidase activity due to some other reason.

Subsequent DNA sequence analysis resulted in the identification of 34 mutant derivatives of pNO2863 which had alterations in the L11 leader sequence. These mutations represent 21 different nucleotide changes [Table 1, Fig. 2(a)] most of which were transitions or transversions and include two double mutations (MN133 and MN134). Interestingly, both of the double mutations involve the same pair of purine residues. Since the double mutations affect the two adjacent residues, it is likely that each of them has derived from a single mutational event. Two insertion mutations (MN104 and MN105) were also found within a stretch of 6 consecutive uridine residues in the mRNA sequence (nucleotides 36-41). No deletion mutations were obtained. It is evident that most of the mutations affecting the regulation by L1 occur at nucleotides 48-54 and 74-76. Therefore, this region must be essential for regulation by L1. There also appears to be an important role for some of the uridine residues around nucleotide 40. These results are discussed below in relation to the secondary structure of the L11 leader mRNA and possible mechanism of translational repression by L1.

DISCUSSION

As mentioned in the introduction, the current model for translational feedback regulation can be regarded as involving a competition between repressor r-protein binding sites on rRNA and r-protein mRNA. If the same site on the repressor r-protein is involved in the interaction with both rRNA and mRNA, one might expect the presence of certain structural elements which are common to both target sites for each particular repressor r-protein. Regarding the binding site for L1 on 23S rRNA [Fig. 2 (b)] there is experimental evidence for a region of double-stranded RNA containing 3 G·C base pairs (nucleotides 2127-2129 and 2159-2161) (18) which appears to be phylogenetically conserved (19). Based on this knowledge and the results of mutational analyses it was proposed that a similar G·C stem structure is formed by part of the L11 leader mRNA (nucleotides 49-51 and 74-76) and is essential for translational regulation by L1 (8, 19). Physical evidence for the existence of this stem structure has recently been obtained using structure-specific nuclease analysis of the L11 leader mRNA (K. Kearney and M. Nomura, submitted for publication).

The results presented here generally confirm the previous conclusion that this G·C stem structure is important for the regulation by L1. However, the substitution of U for C at position 75 or 76 (MN129 and MN131, respectively) still gives substantial residual repression in contrast to the substitution of G for C at the same positions which abolishes regulation more or less completely, as was found in the previous study (16). Our interpretation for this observation is that the postulated short stem structure is perhaps stabilized within the structure of the mRNA-repressor complex, and that a weakened stem structure containing one G-U base pair can still allow the complex formation to some extent leading to weak repression, whereas complete disruption of one base pair by the C to G substitution cannot. As was demonstrated in the previous study, the primary nucleotide sequence of this stem structure is not important but the stem structure they constitute is important for the mRNA-repressor interaction (16).

The importance of this region surrounding the GC base pairs mentioned above is emphasized by the occurrence of several other mutations which result in the abolition of translational regulation by L1. Nucleotide changes at positions 48, 52, 53 and 54 all have strong effects on regulation. The unpaired A residue at position 53 seems especially important in this respect and might be involved in a direct interaction with L1.

Fig. 2

(a) The proposed secondary structure of the L1 target site on *E. coli* L11 mRNA (K. Kearney and M. Nomura, submitted for publication), (b) secondary structure model of the L1 binding region on *E. coli* 23S rRNA (20), (c) secondary structure model of the L1 binding region on *Dictyostelium* 26S rRNA (19) and (d) secondary structure model of the presumed L1 binding region on *Xenopus* 28S rRNA (19). In (a) bases are numbered with respect to the first nucleotide of the L11 mRNA. The Shine-

Dalgarno sequence for L11 is indicated by a broken line and the first three codons of the *rplK* gene are overlined. The $\Delta 34$ deletion end-point (8) is also shown (see text). Mutations are indicated by arrows (to avoid confusion the double mutations MN133 and MN134 are not shown). The MN104 U insertion is shown as having occurred between nucleotides 38-39 although its exact location cannot be determined. Base changes which completely abolish regulation by L1 are shown in bold face whereas those which strongly affect regulation without completely eliminating it are shown in light face. Base changes having a very small effect on the regulation by L1 are indicated by small letters. A region of possible structural homology between these RNAs is outlined. This region of homology is more extensive when comparing only the rRNAs (broken line). It should be noted that bases 28 through 83 of *Dictyostelium* 26S rRNA, which includes the conserved stem-bulge-stem structure, are protected from nuclease digestion by r-protein L1 of *E. coli* (19). The secondary structures for (c) and (d) were redrawn from (19) and are based on the previously published secondary structure model for *E. coli* 23S rRNA (20).

Several mutations were isolated which identify other regions of the L11 leader mRNA as determinants for the regulation by L1. Two of these mutations (MN101 and MN102) are located upstream of the end-point of the $\Delta 34$ deletion [Fig. 2(a)]. It has previously been shown that the L11 leader mRNA upstream of the $\Delta 34$ deletion end-point is unnecessary for regulation by L1 (8). In agreement with this previous finding the MN102 mutation exerts only a very small, and probably negligible effect on regulation by L1. However, the MN101 mutation, which is located in a different G·C-rich stem structure (nucleotides 16-19 and 23-26), has a more pronounced effect on regulation. Since it was the only mutation found in this structure we believe that it exerts its effect on regulation indirectly. For example, disruption of this G·C-stem may result in alternative base-pairings with other G- or C-rich regions more directly involved in translational regulation.

Two other mutations (MN104 and MN105) were found downstream of the $\Delta 34$ deletion end-point but upstream of the previously identified region essential for regulation by L1. Both of these mutations are pyrimidine insertions located within a stretch of uridine residues (nucleotides 36-41) and result in complete abolition of regulation. There are several possible mechanisms which could account for the involvement of this region in the regulation by L1. One possibility is that L1 makes specific contacts with this region. We believe this to be unlikely, however, as these nucleotides are not conserved in *P. vulgaris* or *S. marcescens* even though the structure of L1 as a repressor is conserved among these two bacteria and *E. coli* (F. Sor and M. Nomura, submitted for publication). Another possibility is that this region is necessary to provide the optimum separation between the short G·C-rich hairpin (nucleotides 16-26) and the region near the L11 Shine-Dalgarno

sequence known to be important for L1 recognition. However, this is also unlikely as the potent effects these mutations have would suggest an important regulatory role for the first hairpin which is not apparent (see above). It is also possible that there are other undetected mutations in these mutant fusion plasmids, possibly, in the *lacZ* structural gene which we did not sequence, and that the observed phenotype is a result of such undetected mutational alterations. Although we cannot exclude the possibility of such multiple mutations in the mutant plasmids, it is difficult to understand how mutations outside the L11-leader-L11-gene region could apparently abolish translational regulation and preferentially increase β -galactosidase synthesis in N03608. Thus, the significance of the region affected by the two mutations (MN104 and MN105) remains unclear.

As a result of the information generated by the present mutational analysis we have reexamined the secondary structures of the L11 leader mRNA and the L1 binding site on *E. coli* 23S rRNA. Thus we have identified a region of structural homology common to both RNAs [Fig 2(a),(b)]. We note that this region is also conserved in the L11 leader mRNAs of *P. vulgaris* and *S. marcescens* (F. Sor and M. Nomura, submitted for publication) and L1 binding sites on 23S rRNA of *P. vulgaris* and *B. stearotherophilus* (20) (except for a G $\rightarrow$ A transition at position 2162 on *P. vulgaris* 23S rRNA). The L1 binding site on 26S rRNA of *Dictyostelium* has also been determined and compared with that of *E. coli* (19). Despite divergences in primary sequence between these two L1 binding sites we find that the conserved region is still present [Fig 2 (c)]. The same structure is also conserved in the presumptive L1 binding site on *Xenopus* 28S rRNA (19), [Fig 2(d)].

As discussed earlier it was previously suggested that the G $\cdot$ C-stem (nucleotides 2127-2129 and 2159-2161) of *E. coli* 23S rRNA corresponds to the lower G $\cdot$ C-stem (nucleotides 49-51 and 74-76) of the L11 leader mRNA (8,19). According to our new suggestion this conserved G $\cdot$ C-stem in 23S rRNA represents the upper G $\cdot$ C-rich stem (nucleotides 54-56 and 64-66) of the L11 leader mRNA whereas nucleotides 2123-2124 and 2174-2175 of 23S rRNA (which are also phylogenetically conserved) correspond to the lower G $\cdot$ C-stem of the L11 leader mRNA. [It should be noted that there is strong physical evidence for the presence of the upper G $\cdot$ C rich stem of the L11 leader mRNA (K. Kearney and M. Nomura, submitted for publication).] Thus the main determinant for L1 recognition may be a bulge-loop separating two short G $\cdot$ C-rich stem structures. It is likely that L1 interacts directly with the bulge loop whereas the G $\cdot$ C-rich stems provide an essential structural role.

It should be noted that the initial selection for mutations used in the present study is based on the pseudoreversion of N03608 from *lac*⁻ to *lac*⁺. Alterations in the presumed L1 binding site which fail to give this phenotype would, of course, not be included. Thus it is possible that some other part of the L1 leader undetected by the present method is also involved in the binding of L1. For example, the L11 Shine-Dalgarno sequence might be a part of the L1 binding site, since the isolation of mutations in this region may have been precluded by their possible negative effects on translation. In this regard, we note that the base sequence CUUGA (nucleotides 2165-2169) which overlaps the L11 Shine-Dalgarno sequence, and is also present in the L1 binding region of *E. coli* 23S rRNA (nucleotides 2165-2169) was in fact previously considered to be a possible site involved in recognition by L1 (8). However, mutagenesis experiments using synthetic oligonucleotides showed that the substitution of two G residues for the CU residues at positions 79 and 80 does not affect the regulation by L1 (16).

Although the significance of the new suggestion on the structural homology between the L1 target site on mRNA and the L1 binding site on 23S rRNA discussed above is not really proven, it should be useful as a working hypothesis for further studies. For example, the new model suggests the importance of the conserved bulge-stem structure on 23S rRNA for L1 binding (Fig. 2b, see above). Such suggestions can be tested experimentally.

Finally, it should be noted that some of the mutations also result in changes in translational efficiency. This is especially true of the MN106 mutation (which results in a 2-fold stimulation of translation relative to the MN2 mutation) and the MN104 and MN105 mutations discussed above. It appears, therefore, that sequence determinants for both translational and regulatory functions overlap on the L11 leader mRNA.

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*To whom correspondence should be addressed

+Present address: Department of Molecular Genetics, Plant Breeding Institute, Maris Lane, Trumpington, Cambridge CB2 2LQ, UK

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