Mutational Alterations of Translational Coupling in the L11 Ribosomal Protein Operon of *Escherichia coli*

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Received 3 February 1987/Accepted 21 April 1987

The L11 operon in *Escherichia coli* consists of the genes coding for ribosomal proteins L11 and L1. It is known that translation of L1 does not take place unless the preceding L11 cistron is translated, that is, the two cistrons are translationally coupled, and this is the basis of coregulation of the translation of the two cistrons by a single repressor, L1. Several mutational analyses were carried out to define the region responsible for coupling L1 translation with L11 translation. First, by introducing several amber mutations into the L11 gene by a site-directed mutagenesis technique, it was shown that translation by ribosomes down to a position 21 nucleotides upstream, but not to a position 45 nucleotides upstream, from the end of the L11 cistron allowed the initiation of L1 translation. Second, deletion analysis indicated that a region located 23 to 20 nucleotides from the end of the L11 gene was involved in preventing independent initiation from L1 translation. Third, five different mutations obtained by screening for activation of the masked L1 initiation site were found to be clustered in a small region immediately upstream from the Shine-Dalgarno sequence of L1, and all of them were G-to-A transitions. These results, together with some additional experiments with oligonucleotide-directed mutagenesis, defined the region involved in the coupling and suggest that some special feature of this region, probably different from simple masking of the initiation site by base pairing, is responsible for translational coupling. The present results also suggest that there might be specific differences in the primary nucleotide sequence that distinguish independent translational initiation sites from translationally coupled (i.e., masked) initiation sites.

In many classic operons, several functionally related genes are organized into an operon and coregulated by a single repressor protein acting at a single target site, an operator. The basis for this regulation is that these genes are cotranscribed by RNA polymerase, starting from a single site near the promoter, and that interaction of the repressor with the operator inhibits the initiation of this transcription. It was recently discovered that in several ribosomal protein (r-protein) operons, coregulation of genes organized in an operon takes place in an analogous way, but at the level of translation rather than transcription (44; for reviews, see references 26 and 27). An example is the regulation of the L11 operon, which consists of the genes for L11 (*rpL*) and L1 (*rpL*). It was demonstrated that one of the gene products, the L1 protein, acts as a repressor to inhibit the synthesis of both L11 and L1 at the level of translation (13, 43) by acting at a single target site which is near the translation initiation site for L11 (2, 3, 44). The inhibition of L11 translation by L1 takes place directly, and that of L1 translation takes place only indirectly as a result of the inhibition of L11 translation; the translation of L1 does not take place unless the preceding L11 cistron is translated, that is, the two cistrons are translationally coupled. Thus, the translational coupling of adjacent r-protein cistrons is important as a basis of coregulation of several linked r-protein genes by a single r-protein repressor. Translational coupling is not unique to the r-protein operons and has been observed in several other bacterial operons (1, 12, 28, 35). Although the translational coupling in r-protein operons, such as the

L11 operon, appears to be generally tight, this does not appear to be always the case. For example, in the *galT-galK* system, the translation of *galK* was reported to be only partly coupled with that of *galT* (35). The significance of this partial coupling is not known.

The mechanism of translational coupling is not clearly understood. One major question concerns the apparent inability of distal cistrons to initiate independent translation. Another question concerns equimolar synthesis of r-proteins. Since most r-proteins (with the clear exception of L7/12) are synthesized in an equimolar ratio (27), the rate of distal cistron translation must be identical to that of proximal cistron translation in a translationally coupled system. Taking a tightly coupled system as an example, two possibilities can be considered. One is that the translation initiation site of the distal cistron is inherently defective and only a translating ribosome approaching from upstream along the mRNA, but not "free" ribosomes in the pool, can effectively initiate the act of translation at the start codon of the distal cistron. Most of the distal cistrons in the translationally coupled systems have a "good" Shine-Dalgarno sequence (SD sequence [34]), and such a sequence is required for translational coupling (12) but might not be sufficient for independent initiation because of the lack of some as yet undiscovered (hypothetical) structural features in these cistrons. According to this model, the same ribosomes continue to translate the distal cistron, and the equimolar synthesis of r-proteins can be easily explained. A second possibility is that the distal cistron has an inherent capacity for independent ribosome entry and translation initiation but is somehow masked or inhibited, e.g., by a secondary structure of mRNA. If this is the case, we would like to know how a translating ribosome approaching from upstream unmasks the downstream ribosome initiation site or

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TABLE 1. Plasmids used in this study

Plasmid	Mutation	Comments
pNO1564	None (vector)	Vector carrying the <i>lacUV5</i> promoter/operator region
pNO2625	None (parent)	Carries the L11 and L1 genes with the complete mRNA leader; see Fig. 1a
pNO2625MB1 (= pNO2626)	Amber	See Fig. 2a
pNO2625MB3 (= pNO2628)	Amber	See Fig. 2a
pNO2625MB5 (= pNO2630)	Amber	See Fig. 2a
pNO2625MB6 (= pNO2631)	GG → TT	See Fig. 2a and c
pNO2625MB8 (= pNO2773)	GG → CC	See Fig. 2a and c
pNO2731	None (parent)	Carries the L11 and L1 genes but lacks the SD sequence for L11 due to $\Delta 92$ deletion; see Fig. 1a
pNO2731 $\Delta 452$ (= pNO2733)	Deletion	See Fig. 2a
pNO2731 $\Delta 466$ (= pNO2735)	Deletion	See Fig. 2a
pNO2731 $\Delta 497$ (= pNO2736)	Deletion	See Fig. 2a
pNO2731 $\Delta 500$ (= pNO2738)	Deletion	See Fig. 2a
pNO2731 $\Delta 512$ (= pNO2739)	Deletion	See Fig. 2a
pNO2731MB5 (= pNO2741)	Amber	See Fig. 2a
pNO2731MB6 (= pNO2742)	GG → TT	See Fig. 2a and c
pNO2731NA501 (= pNO2842)	G → A	See Fig. 2b
pNO2731NA502 (= pNO2843)	G → A	See Fig. 2b
pNO2731NA508 (= pNO2844)	G → A	See Fig. 2b
pNO2731NA509 (= pNO2845)	G → A	See Fig. 2b
pNO2731NA511 (= pNO2846)	G → A	See Fig. 2b

overcomes the inhibition and how equimolar synthesis is achieved.

As a first step to answer these questions, we used mutational approaches to study the mechanism of translational coupling with the L11 operon as an experimental system. In this paper, we describe various mutations that affect translational coupling and discuss the results in connection with the questions mentioned above.

MATERIALS AND METHODS

Construction of plasmids. Two parental plasmids, pNO2625 and pNO2731, were used as references to study the effects of various mutational alterations on translational coupling (Table 1 and Fig. 1a). pNO2625 and pNO2731 are very similar to the $\Delta 1$ -6 (pNO1587) and $\Delta 2$ deletion plasmids, respectively, used in previous studies (2, 3). The construction of pNO2625 is schematically shown in Fig. 1b. The *Bam*HI site of the vector pJJS300 was first converted to a *Hind*III site (2) by digestion with *Bam*HI, filling in with the Klenow fragment of DNA polymerase I (PolIK), and blunt-end ligation with a *Hind*III linker (pNO1564). The *Eco*RI site was then eliminated by digestion with *Eco*RI, filling in with PolIK, and religation (pNO1565). The *Sal*I site was subsequently converted to a *Hind*III site by the same procedure described for the *Bam*HI site. The plasmid was then digested with *Hind*III and religated to remove the *Hind*III-*Hind*III fragment containing the *Sph*I site of the original vector. The resulting plasmid, pNO2624, was digested with *Hind*III, and a 2-kilobase (kb) *Hind*III-*Hind*III fragment from pNO1587

(3) containing the L11 operon with the intact L11 leader sequence was inserted in the correct orientation. The resulting plasmid, pNO2625, carries the entire transcribed region of the L11 operon fused to the *lacUV5* promoter/operator. The deletion endpoint used in this fusion is at -4 with respect to the transcription start site of the original L11 operon, and this fusion is called $\Delta 4$ in this paper (Fig. 1a). pNO2731 was constructed from $\Delta 92$ (called $\Delta 2$ previously) and has a deletion endpoint at nucleotide 92 like the $\Delta 92$ plasmid [2] (Fig. 1a), but lacks the SD sequence of the *lac* operon present in the $\Delta 92$ plasmid. The $\Delta 92$ plasmid DNA was cut with *Bam*HI and digested with S1, followed by blunt-end ligation with a *Hind*III linker (Bethesda Research Laboratory). One of the plasmids constructed in this way had 12 nucleotides (including the SD sequence of the *lacZ* gene) upstream from the *Bam*HI site removed. This plasmid was cut with *Hind*III and *Sph*I, and the "intact" *Hind*III-*Sph*I fragment containing the original $\Delta 92$ deletion end was substituted for the shortened *Hind*III-*Sph*I fragment. The resulting plasmid was called pNO2731. Because the original $\Delta 92$ plasmid has a *Bam*HI site that was converted to a *Hind*III site in the process of regenerating the $\Delta 92$ deletion end, the sequence of the junction of the L11 leader with the *lac* mRNA leader in pNO2731 has some extra sequence derived from the *Bam*HI site as shown in Fig. 1a.

Deletions extending from the beginning of the L11 gene toward the beginning of the L1 gene were generated by *Bal*31 exonuclease digestion of pNO2731. At the time of religation, *Hind*III linkers were inserted. Deletions were then transferred to pNO2731 by cutting the deletion plasmids with *Hind*III and *Bgl*II and substituting each of these *Hind*III-*Bgl*II fragments for the original *Hind*III-*Bgl*II fragment in pNO2731. $\Delta 497$ was generated by cutting pNO2731 at the *Hind*III site preceding the L11 gene and at the *Nco*I site at the end of the L11 gene and filling in with PolIK, followed by blunt-end religation to delete the L11 gene up to base 497. The deletion endpoints of the constructed plasmids were analyzed by the Maxam and Gilbert sequencing method (21). Figure 2a indicates the positions of the ends of various deletions, and Fig. 3 shows the sequences at the junction of the deleted L11 genes with the *lac* mRNA leader for the various constructs. The numbering is relative to the 5' start of the intact L11 mRNA (30).

Site-directed mutagenesis. The oligodeoxynucleotides were prepared by the phosphotriester synthesis method (24) at the Biotechnology Center, University of Wisconsin, and purified by polyacrylamide gel electrophoresis, and their sequence was confirmed by the method of Maxam and Gilbert as modified by Zoller and Smith (45).

The *Eco*RI-*Sph*I fragment of pNO2625 was introduced in the homologous restriction sites of the phage M13mp18 (42). Mutagenesis was carried out essentially as described by Zoller and Smith (45). Sequence determination of the mutated phages was performed by the chain terminator dideoxy method (32). The mutated fragments were inserted back into pNO2625 or into pNO2731 (see below). The sequence of the resulting plasmids was verified by the method of Maxam and Gilbert from the *Nco*I site located at the end of the L11 gene. For the mutation (MB5) that was expected to modify the *Nco*I site, only the disappearance of the restriction site was verified.

Random generation of mutations affecting translational coupling. Plasmid pNO2731, which carries the L11 operon, was digested with *Eco*RI and *Hpa*I (Fig. 1a and 4). The 461-base-pair (bp) fragment carrying the end of the L11 gene (320 bp) and the beginning of the L1 gene (138 bp)

LL1 operon sequence

A-4

Spn I
EcoRI
NcoI
MspI
BglII

L11 L1 Pβ L10'

pLac

Amp^r

2625

b)

Diagram illustrating the cloning strategy for the L1-L1 genes:

- pJJS300** (Left): A circular plasmid with a **Plac** promoter, **BamHI**, **SphI**, and **SalI** sites. An **EcoRI** site is also indicated.
- pNO2624** (Middle): A circular plasmid with a **Plac** promoter and **HindIII** sites.
- pNO2625** (Right): A circular plasmid with a **Plac** promoter, **HindIII** sites, and the **L1-L1 genes**.

The strategy involves:

- Cloning **pJJS300** into **pNO2624** using **BamHI** and **HindIII** (indicated by (1) change BamHI to HindIII (pNO1564) and (11) remove EcoRI (pNO1565)).
- Inserting the **L1-L1 genes** into **pNO2625** using **EcoRI** and **HindIII** (indicated by (111) change SalI to HindIII and (1111) cut with HindIII and ligate (pNO2624)).

FIG. 1. Construction and structures of two parent plasmids, pNO2625 and pNO2731. (a) Structure of pNO2625 and pNO2731. The sequences of the promoter regions of the original *L11* operon (30) and of the *lac* operon are shown, with the positions of operon fusion indicated (arrows). The numbering is relative to the transcription start site (30). The Pribnow boxes in the promoter regions are over- and underlined. The SD sequences and the start codons for the structural genes are underlined, and the nucleotide sequences derived from linkers are overlined. Only some restriction endonuclease sites mentioned in the text are shown. (b) Construction of pNO2625. See Materials and Methods for details.

FIG. 2. Nucleotide sequences covering the L11-L1 intercistronic region, and the positions of various mutations. (a) Positions of deletion endpoints and base substitutions of various point mutations are shown. (b) Positions and base substitutions of nitrous acid-induced mutations are indicated. The numbers in parentheses are the number of independent mutants isolated. (c) Some inverted repeat sequences considered to participate in masking the L1 initiation site. They are indicated by three different kinds of overline. The SD sequences and the start and stop codons are underlined.

(intercistronic fragment) was purified by acrylamide gel electrophoresis. The replicative form (RF) of phage M13mp18 (43) was digested with *Sa*I, blunt-ended with PolIK, digested with *Eco*RI, and treated with bacterial alkaline phosphatase. The intercistronic fragment and the phage DNA were ligated and plated after transfection of *Escherichia coli* JM105 (42) on Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) plates containing a *lac* operon inducer, isopropyl- β -D-thiogalactopyranoside (IPTG), as described by Messing (22). This construct introduces several stop codons in the reading frame, starting from the *lacZ* gene initiation codon present upstream from the polylinker of the

phage, and the initiation of the translation of L1 is still masked by the coupling mechanism. The restriction sites used for the 3' junction produced an in-phase fusion between the beginning of the L1 gene and the *lacZ* gene α complementation fragment. The sequences of the junctions were confirmed by sequencing the single-stranded phage DNA (32). As a control, the same intercistronic fragment was prepared from a mutant plasmid (pNO2742; see Table 1 and Results) and cloned into M13mp18. The mutant fragment carries a mutation (MB6) in the distal region of the L11 gene that allows independent translation of L1.

We found that the phage carrying the wild-type intercis-

FIG. 3. Nucleotide sequences of fusion mRNAs transcribed from various deletion plasmids. The expected mRNA sequences are shown from the 5' end derived from the *lac* leader through the fusion junction for each deletion. The sequences derived from linkers are underlined, and the SD sequence and the stop and the start codons in the intercistronic region are overlined.

tronic fragment produced colorless plaques, while the phage carrying the mutant intercistronic fragment produced blue plaques. Thus, synthesis of the L1-β-galactosidase (α complementation fragment) fusion protein is evidently prevented by the translational coupling mechanism in the wild-type

phage, and we used this system to generate more mutations that disrupted the coupling.

The M13 phage carrying the wild-type fragment was treated with nitrous acid to give 10 to 50% survivors as described previously (41). The phage were mixed with

FIG. 4. Construction of phage M13mp18 derivatives for the generation of mutations disrupting translational coupling of L11 and L1. The restriction sites used for cloning as well as the stop codons generated in the reading frame starting from the *lacZ* initiation codon in the vector are underlined.

exponentially growing JM105 cells and then plated on Xgal plates containing IPTG. Blue plaques were observed at a frequency of 10^{-2} to 10^{-3} , which was higher than the frequency (less than 10^{-5}) observed without mutagenesis. Mutant phage were picked from blue plaques, and DNA sequences of the insert were determined after plaque purification.

All of the five different mutations allowing blue-plaque formation were introduced back into the original pNO2731 plasmid. pNO2731 was digested with *EcoRI* and *SphI*, and the *EcoRI-SphI* fragment was replaced by the *EcoRI-SphI* fragments isolated from the RF DNA of the mutant phages (Fig. 1a). In this way five different mutant plasmids were constructed, and the synthesis of L1 from the plasmid was examined in strain GM1.

DNA-dependent in vitro protein synthesis. In vitro protein synthesis directed by the various plasmids was carried out as described previously (2, 43).

Relative synthesis rates of r-proteins. Bacterial strain GM-1 [*F'* *lacI^q* *lacZ18* *prolara* Δ (*lac pro*) *thi* (11)] carrying the various deletion plasmids was grown in AB minimal medium (10) supplemented with 0.4% glucose, thiamine (1 μ g/ml), all amino acids except lysine (40 μ g/ml each), and ampicillin (50 μ g/ml) at 37°C to a density of approximately 2×10^8 cells per ml. After the addition of 1.5 mM IPTG for 10 min, the cells were pulse-labeled with [3 H]lysine (340 pmol/ml; 87 Ci/mmol) for 1 min, followed by a 1-min chase with nonradioactive lysine (500 μ g/ml). The cells were rapidly chilled and mixed with an equal volume of control cells labeled for three to four generations with [14 C]lysine (334 mCi/mmol; 6.0 nmol/ml). Cells were then centrifuged, washed, and heated in sodium dodecyl sulfate (SDS)-lysis buffer, and selected r-proteins were precipitated with specific antisera, followed by separation by SDS-polyacrylamide gel electrophoresis. The $^3\text{H}/^{14}\text{C}$ ratios were then determined for each r-protein as described previously (3), and the $^3\text{H}/^{14}\text{C}$ ratio for each r-protein was normalized to the $^3\text{H}/^{14}\text{C}$ ratio of total protein in the sample. The values obtained in this way represent differential synthesis rates of each r-protein relative to those in ^{14}C -labeled reference cells and are given in the tables as relative differential synthesis rates. It should be noted that the values for r-proteins in the control cells were often less than 1, and this may reflect the fact that differential synthesis rates in experimental strains were determined by measuring actual synthesis rates by pulse labeling, whereas differential synthesis rates in the ^{14}C -labeled reference strain used for normalization were derived from the steady-state amounts of the respective proteins. The nonidentity of ^3H -labeled control cells with ^{14}C -labeled reference cells in this respect does not affect comparison of differential synthesis rates among various strains with different plasmids. We also note that total protein synthesis rates were practically identical for the various plasmid-carrying strains.

An example of the calculation to estimate uncoupled L1 synthesis from the plasmid operon is given here for the MB6 mutation (see Table 3). First, we assumed that translation of L11 from pNO2625 plasmid mRNA and from chromosomal mRNA was equally sensitive to L1 repression and that the rate of transcription of the L11-L1 mRNA increased 10-fold after induction of the plasmid operon (2, 3). Thus, in the strain carrying pNO2625 with the MB6 mutation, 1/10 of the total L11 synthesis is from the chromosomal operon (i.e., 0.03) and the remaining 9/10 is from the plasmid operon (0.27). Since L11 and L1 are synthesized in an equal molar ratio from the translationally coupled chromosomal operon (as confirmed by the value for pNO1564), L1 synthesis from

FIG. 5. In vitro synthesis of L11, L11 amber fragments, and L1 from various base substitution mutant plasmids (from left, pNO2625 and its derivatives and pNO2731 and its derivatives). In vitro protein synthesis reactions were carried out with the various plasmid DNAs as template at 37°C for 60 min. Protein samples were analyzed on a 12% SDS-polyacrylamide gel, and an autoradiogram of the dried gel is shown.

the plasmid operon is 2.41 (2.44 $\pm$ 0.03). Since L11 synthesis is 0.27, L1 synthesis through translational coupling is 0.27. Thus, uncoupled L1 synthesis is a total of 2.14 (2.41 $\pm$ 0.27), or 0.24 (2.14/9) per unit mRNA synthesis (corrected for the difference between plasmid and chromosomal transcriptional activity). This value corresponds to 26% (0.24/0.94) of the L1 synthesis per unit mRNA synthesis (0.94) from the chromosome operon in the control strain without the plasmid operon (pNO1564).

RESULTS

Effects of premature termination of L11 translation on the translation of L1. We used pNO2625 as the plasmid carrying a parent operon with intact translational coupling. This plasmid is very similar to pNO1587, used in our previous studies (2, 3), and translational coupling of L1 with L11 is practically complete. It carries the entire L11 and L1 structural genes as well as the entire L11 mRNA leader region, but the L11 promoter region upstream from -4 (with respect to the transcription start site) is deleted ($\Delta 4$; formerly called $\Delta 1-6$) and fused to the *lacUV5* promoter/operator (Fig. 1a). To examine how far translating ribosomes must travel along the upstream mRNA to allow L1 translation, we introduced amber stop codons at three positions by oligonucleotide-

TABLE 2. In vitro synthesis of L11 and L1 from various plasmid templates

Plasmid	Mutation	L1/L11 ratio ^a
pNO2625	None (parent)	0.73
pNO2625MB1	Amber	0.13
pNO2625MB3	Amber	0.32
pNO2625MB5	Amber	0.76
pNO2625MB6	GG $\rightarrow$ TT	2.79

^a The synthesis of L1 and L11 (or its fragments) in the experiments described in Fig. 4 was quantified by densitometric tracing of suitable autoradiograms, and the ratio of the amount of radioactive L1 synthesized to that of L11 (or its amber fragment) was calculated for each plasmid. In the case of L11 amber fragments, corrections were made for the differences in the number of methionines between the complete L11 molecule and the amber fragments, so that the values given represent molar ratios in each case.

TABLE 3. Relative differential synthesis rates of L11 and L1 after induction with IPTG; effects of point mutations on translational coupling^a

Plasmid	Mutation	Relative synthesis rate				Distribution				Uncoupled L1 synthesis from plasmid	
		Control (L3, L6)	L11	L1	L1/L11 ratio	Chromosome		Plasmid		Total	Per transcript (%)
						L11	L1	L11	L1		
pNO1564	None (vector)	0.91	0.91	0.94	1.03	0.91	0.94	— ^b	—	0.14	0.94 (100)
pNO2625	Wild type	0.90	0.83	0.97	1.17	0.08	0.08	0.75	0.89	0.14	0.015 (1.6)
pNO2625MB1	Amber	0.83	0.73	0.87	1.19	0.73	0.73	—	0.14	0.14	0.015 (1.6)
pNO2625MB5	Amber	0.88	0.53	1.01	<u>1.91</u>	0.05	0.05	0.48	0.96	0.48	0.053 (5.6)
pNO2625MB6	GG → TT	0.79	0.30	2.44	<u>8.13</u>	0.03	0.03	0.27	2.41	2.14	0.24 (25.5)
pNO2625MB8	GG → CC	0.79	0.93	0.95	1.03	0.09	0.09	0.84	0.86	0.02	<0.01 (≤1.1)

^a Relative differential synthesis rates were measured as described in Materials and Methods, and the averages of at least three independent experiments are given. The values for L3 and L6 are given as averages but was always about the same, i.e., the L3/L6 ratio was always close to 1 (average 1.04 ± 0.07 for the six plasmids shown in the table), as was the L1/L11 ratio for chromosomal synthesis in the strain with control plasmid pNO1564. (The reason that the relative synthesis rates for these control proteins are less than 1 is explained in Materials and Methods.) The L1/L11 ratio values considered significantly higher than that for the parent plasmid (pNO2625) are underlined.

^b —, Gene not present on plasmid or too small to be measured.

directed in vitro mutagenesis. Their positions were 111 nucleotides (MB1), 45 nucleotides (MB3), and 21 nucleotides (MB5) upstream from the end of the L11 gene (Fig. 2a).

Two experimental approaches were used to examine the effects of these mutations on L1 translation. One was the use of a plasmid DNA-directed in vitro protein-synthesizing system, and the other was in vivo measurement of the rate of r-protein synthesis after induction of the plasmid operon in the strains carrying the original or mutant plasmids.

In vitro protein synthesis was carried out with [³⁵S]methionine, and the products were separated by polyacrylamide gel electrophoresis (Fig. 5). β -Lactamase, the product of the plasmid-encoded ampicillin resistance gene, served as a control for estimation of the relative efficiency of r-protein synthesis. The premature termination of translation of L11 in MB1 and MB3 gave rise to the synthesis of shortened proteins, as expected, and strongly reduced the synthesis of L1. Densitometric scanning of suitable autoradiograms of the gel (Table 2) indicated that the decrease in L1 synthesis relative to L11 synthesis became greater with the increase in the distance between the stop codon introduced and the end of the L11 cistron. Only when a stop codon was introduced close to the natural end of L11 (21 bases in MB5) was L1 translated as efficiently as the wild-type sequence.

Differential synthesis rate of L11 and L1 were measured after induction of the plasmid-borne operon and normalized to the differential accumulation rates in a reference strain (see Materials and Methods) (Table 3). The same measurements were also carried out for r-proteins L3 and L6, which are encoded in the S10 and *spc* operons, respectively, and should not be affected by induction of the plasmid-borne operon under the experimental conditions used (Table 3).

It can be seen that the translation of L11 and L1 from the wild-type plasmid pNO2625 with the intact leader sequence was regulated as expected. It was previously shown that, after induction with IPTG, the synthesis rate of L11-L1 mRNA in strains carrying plasmid pNO1580, which is almost identical to pNO2625, increased about 10-fold, but the synthesis rate of L11 and L1 remained unchanged, indicating the presence of efficient translational feedback regulation. In the following calculation, we assumed that in the strain carrying pNO2625, the transcription rate increased to the same extent, i.e. 10-fold, and therefore the efficiency of translation (per transcription event) was decreased by a factor of about 10 relative to that in the control strain carrying the vector plasmid pNO1564.

If translational coupling is complete in this system, the ratio of L1 synthesis to L11 synthesis should be 1. Thus, this value is a sensitive measure for finding the effects of mutations on translational coupling and is included in Table 3. We note that the value (1.17) found for the wild-type plasmid pNO2625 was actually slightly higher than 1. It was not established whether this was due to experimental error or represents a very small escape of L1 synthesis from translational coupling.

Regarding the first amber mutation, MB1, the L11 synthesis analyzed did not include the L11 amber fragment, and the data indicate that no significant synthesis of L1 took place from this plasmid.

In the MB3 mutation, the L11 amber fragment from the plasmid overlapped and was only incompletely separated from the intact L11 protein in polyacrylamide gel electrophoresis, and consequently interpretation of the data was complicated. However, no definite indication of synthesis of L1 from the plasmid was obtained (data not shown).

In the strain carrying the plasmid with the MB5 mutation, measured L11 synthesis also included the mutant protein from the plasmid. In this case, we found a clearcut decrease in the synthesis of L11, and L1 synthesis remained approximately the same. This result shows that L1 synthesis took place mainly from the plasmid. In addition, the data suggest that this synthesis was probably uncoupled from the translation of L11 and escaped regulation of L1. It is unclear whether all or a part of the ribosomes that reached this amber codon actually participated in L1 synthesis. However, assuming that the mutant form of L11 was stable during the 1-min pulse period, one can conclude that L1 synthesis is actually higher than L11 synthesis and therefore that at least 50% of L1 translation from the plasmid was carried out by ribosomes from the pool. These results suggest that the L1 initiation site is perhaps inherently an independent translation initiation site but is masked in the absence of translation of the upstream cistron. However, the calculation shows that the efficiency of uncoupled translation of L1 per transcript in this case was only about 6% of that of the coupled translation of L1 in normal strains without the plasmid operon (Table 3; see Materials and Methods for the calculation; see also below for the possibility of lower estimations in the calculation based on the in vivo data).

The unmasking observed with the MB5 amber mutation was probably caused by ribosomes translating at or up-

FIG. 6. In vitro synthesis of L11 and L1 from various deletion mutant plasmids. See the legend to Fig. 5 and Materials and Methods for details.

stream from this amber site. Alternatively, the change in mRNA sequence caused by the mutational base substitution (AT → TA) might be sufficient to cause an unmasking effect in some way. To distinguish between these two possibilities, experiments were carried out with pNO2731 with and without the MB5 mutation. Plasmid pNO2731 has the genes for L11 and L1 intact, but the leader region up to nucleotide 92 (Δ92; formerly called Δ2), including the SD sequence for L11, is deleted, and the L11 and L1 genes are fused to the *lacUV5* promoter/operator through a *Hind*III linker sequence (Fig. 1b). This plasmid is very similar to pNO2615 carrying the same Δ92 deletion used in the previous study (2) and does not express the L11 gene due to the absence of the SD sequence or the L1 gene because of the tight L11-L1 translational coupling. A weak but definite synthesis of L1 was observed in vitro from the template carrying the MB5 mutation (pNO2731MB5), while no significant L1 synthesis was observed with the parent plasmid (pNO2731) (Fig. 5). The results of in vivo measurements of L1 synthesis indicated only very weak independent synthesis of L1 from the plasmid gene (2.5% of L1 synthesis from the translationally coupled chromosomal operon; Table 5). Although the in vitro results indicate that the mutational alteration (AT → TA) itself, rather than the creation of the amber codon, caused a small unmasking effect, quantitative considerations of the data suggest that the alteration of mRNA structure alone was probably not sufficient to explain the larger unmasking observed with the pNO2625MB5 plasmid, and translating ribosomes from upstream reaching the amber codon (created by the MB5 mutation) may also contribute to the unmasking.

Deletion analysis of the mRNA region responsible for masking the L1 translation initiation site. Since the results described above suggested that the L1 initiation site was somehow masked and that ribosomes translating the upstream mRNA unmasked or activated the L1 initiation site, we carried out a deletion analysis to define the upstream mRNA region responsible for masking the L1 initiation site.

Various deletion derivatives of pNO2731 were prepared as described in the Materials and Methods section. The ends of the deletions are indicated in Fig. 2a, and the sequence of the 5' end of the mRNA from some of these deletions is shown in Fig. 3. Since there was no potential translation initiation site upstream from the L1 initiation site, the synthesis of L1 from these deletion plasmids, if it takes place, means unmasking of the L1 initiation site by deletion. Again, both in vitro and in vivo experiments were carried out to test this possibility.

Figure 6 shows the results of the in vitro protein synthesis

experiments. Deletion of up to nucleotide 466 (Δ466), which removed most of the nucleotides coding for the L11 protein, failed to uncover L1 synthesis, and further deletion to position 488 (Δ488) gave ambiguous results, but deletion to position 497 (Δ497) gave weak but definitely positive L1 synthesis. A dramatic increase in L1 synthesis was observed with the Δ500 deletion, which removed only 3 more nucleotides than in Δ497. Although the extra sequences derived from the linkers used to construct these plasmids were different with the different deletions (Fig. 3) and hence the results are not completely unambiguous, it appears that the sequences upstream from 500, especially 498 to 500 (CAT) and thereabouts, are involved in masking the L1 initiation site. Further deletion (Δ512) reaching just to the SD sequence for the L1 cistron abolished L1 translation, as did the next deletion (Δ542). Evidently, sequences immediately upstream from the SD sequence are important for translation initiation, as already pointed out by previous workers (6, 33, 40).

The results of in vivo measurements of L1 synthesis from these deletion plasmids (Table 4) confirmed qualitatively the conclusion obtained from the in vitro experiments. A small but reproducible increase in the L1/L11 ratio for Δ466 and Δ497 may be taken as an indication of a slight escape of L1 synthesis from translational coupling. With the Δ500 plasmid, L1 translation from the plasmid was clearly demonstrated. Because L11 synthesis takes place only from the chromosome operon and L1 synthesis from the plasmid is not feedback-regulated by L1, the efficiency of L1 translation from the plasmid-derived mRNA can be calculated without much complication (Table 4). (We note that the growth rate of the strain carrying the Δ500 plasmid decreased gradually after IPTG addition, presumably due to inhibition of the synthesis of new ribosomes [13]. However, no decrease in growth rate or total protein synthesis rate was detectable at 10 min after IPTG addition when the measurements of protein synthesis rates were carried out.)

Although independent L1 synthesis from the Δ500 deletion plasmid in vivo was clearly recognized, the calculated efficiency of translation corrected for the frequency of transcription was only about 8% of that of the translationally coupled chromosome operon in the control strain without the plasmid operon. In contrast, synthesis of L1 from the Δ500 plasmid in vitro was much stronger than that from the

TABLE 4. Relative differential synthesis rates of L11 and L1 after induction with IPTG; effects of deletions on translational coupling^a

Plasmid	Mutation	Relative synthesis rate			L1 synthesis from plasmid	
		L11	L1	L1/L11 ratio	Total	Per transcript (%)
pNO1564	None (vector)	0.91	0.94	1.03	—	0.94 (100)
pNO2731	Parent (Δ92)	0.81	0.90	1.11	0.09	0.010 (≤1)
pNO2733	Δ452	0.67	0.77	1.15	0.10	0.011 (≤1)
pNO2735	Δ466	0.64	0.87	1.36	0.23	0.026 (2.8)
pNO2736	Δ497	0.68	0.88	1.29	0.20	0.022 (2.3)
pNO2737	Δ500	0.23	0.92	4.00	0.69	0.077 (8.2)
pNO2738	Δ512	0.72	0.84	1.17	0.12	0.013 (≤1)

^a Relative differential synthesis rates of L11 and L1 were measured in strains carrying the various plasmids. The values given are the averages of at least three independent experiments. Although the L1/L11 ratios for Δ466 and Δ497 (1.36 and 1.29, respectively) represent only small increases over the control value (1.03), the increases were reproducible. For other explanations, see the text and Table 3, footnotes a and b.

TABLE 5. Relative differential synthesis rates of L11 and L1 after induction with IPTG; effects of point mutations^a

Plasmid	Mutation	Relative synthesis rate			L1 synthesis from plasmid	
		L11	L1	L1/L11 ratio	Total	Per transcript (%)
pNO1564	None (vector)	0.91	0.94	1.03	—	0.94 (100)
pNO2731	Parent	0.81	0.90	1.11	0.09	0.010 (≤ 1)
pNO2731MB5	Amber	0.72	0.93	1.28	0.21	0.023 (2.5)
pNO2731MB6	GG $\rightarrow$ TT	0.12	1.94	16.17	1.82	0.202 (21.5)
pNO2731NA501	G $\rightarrow$ A	0.73	1.00	1.37	0.27	0.030 (3.2)
pNO2731NA502	G $\rightarrow$ A	0.92	1.02	1.10	0.10	0.011 (≤ 1)
pNO2731NA507	G $\rightarrow$ A	0.29	0.97	3.31	0.68	0.076 (9.1)
pNO2731NA508	G $\rightarrow$ A	0.33	1.04	3.14	0.71	0.079 (8.4)
pNO2731NA511	G $\rightarrow$ A	0.56	0.73	1.31	0.17	0.019 (2.0)

^a Relative differential synthesis rates of L11 and L1 were measured in strains carrying the various plasmids. The values given are the averages of three independent experiments. Although the L1/L11 ratios for NA501 and NA511 (1.37 and 1.31, respectively) represent only small increases over the control value (1.03), the increases were reproducible. For other explanations, see the text and Table 3, footnotes *a* and *b*.

$\Delta 4$ plasmid, that is, L1 synthesis from the coupled operon. One probable explanation is that in the absence of simultaneous rRNA synthesis to remove the L1 repressor synthesized in the *in vitro* system, L1 synthesis from the $\Delta 4$ plasmid might become repressed, leading to the observed lower efficiency of translation. The $\Delta 500$ plasmid does not carry the L1 target site, and therefore synthesis from the $\Delta 500$ plasmid was obviously free from such repression. It was previously observed that L1 (and L11) synthesis from a plasmid with a target site mutation is generally much higher than that from a plasmid with the intact target site in the present *in vitro* system (2, 3), supporting the explanation given above.

Another possible explanation is that the efficiency of L1 translation calculated from the *in vivo* data does not faithfully reflect the inherent efficiency of initiation of L1 translation on the $\Delta 500$ mRNA. Deletions eliminating upstream mRNA segments, including the L11 initiation site, may have caused increased instability of mRNA *in vivo* because of the absence of upstream translation, thereby decreasing the apparent efficiency of L1 translation. Thus, the estimates from *in vivo* experiments may represent minimum values for the efficiency of L1 translation in the various deletion plasmids. These explanations together can probably account for the apparent large difference in the degree of independent translation observed between the *in vitro* and *in vivo* experiments for the $\Delta 500$ deletion.

Point mutations affecting translational coupling constructed by oligonucleotide-directed mutagenesis. As described above, the region around nucleotide 500 of the L11-L1 mRNA appeared to be involved in masking the L1 initiation site. One possible mechanism is a sequestering of some essential sequences for initiation, such as the SD sequence or the initiation codon, by secondary (or tertiary) RNA structures as previously demonstrated, for example, for the RNA phage R17 replicase system (for a review, see reference 15). We looked for potential secondary structures in this region, and some of the inverted repeat sequences we considered are indicated by arrows over the nucleotide sequence shown in Fig. 2c.

To test whether these potential secondary structures actually participated in masking, we made several mutational alterations that would disrupt the postulated structures by oligonucleotide-directed *in vitro* mutagenesis. Among them, the only mutational alteration that had a significant effect was the MB6 mutation (GG to TT) at position 507-508. This

mutation was introduced in the pNO2625 background as well as the pNO2731 background. *In vitro* experiments demonstrated that plasmids with the MB6 mutation were superior templates for L1 synthesis (Fig. 5, Table 2). In the pNO2625 background, where the L11 cistron is intact, L1 synthesis was about three times greater than L11 synthesis. Similarly, in the pNO2731 background, the synthesis of L1 relative to the synthesis of β -lactamase was much higher than that in the pNO2625 background. Thus, the MB6 mutation clearly uncoupled L1 synthesis from L11 synthesis, making the L1 initiation site independent and apparently very efficient.

The results of *in vivo* experiments with pNO2625 carrying the MB6 mutation are given in Table 3, and the results with pNO2731 carrying this mutation are given in Table 5. The calculation shows that uncoupled L1 synthesis was relatively high in both cases and corresponded to about 22 to 26% of that from the translationally coupled chromosomal operon in the control strain. It is noted that approximately the same value was obtained for the efficiency of uncoupled L1 synthesis with the MB6 mutation in both the pNO2625 and pNO2731 backgrounds, giving some confidence to the validity of the methods used for calculation. Nevertheless, because of the problem of mRNA instability in the absence of L11 translation (no L11 translation in the pNO2731 background and a large repression in the pNO2625 background), the value might be a lower estimate, as discussed above in connection with the $\Delta 500$ deletion.

We next examined the possibility that the MB6 mutation unmasked the L1 initiation site by disrupting some secondary mRNA structure, but came to the conclusion that this was probably not the case. One of the experiments that suggested this conclusion used the MB8 mutation (GG to CC rather than to TT) at the same position as MB6. If the unmasking by the MB6 mutation was the result of disruption of specific base pairing involving these GG residues, one can expect that the MB8 mutation would also disrupt the postulated secondary structure and unmask the L1 initiation site. The MB8 mutation was introduced into pNO2625, and L11 and L1 synthesis was measured *in vivo* after induction of the plasmid operon. No significant uncoupling of L1 translation was observed (Table 3). Thus, disruption of some secondary mRNA structure does not seem to be the reason for the unmasking by the MB6 mutation.

Random isolation of point mutations affecting translational coupling. We have devised a simple method to isolate mutants with defects in the translational coupling of L1 with

L11. The system uses an M13 phage derivative carrying an L1-*lacZ* fusion operon (see Materials and Methods and Fig. 4). After infection of a suitable bacterial strain, the parent phage does not produce β -galactosidase (as a hybrid polypeptide) due to masking of translation of the fusion mRNA by the presence of an upstream L11 segment. Mutations which disrupt the structure responsible for the masking effect can be isolated as phages that will give a blue rather than a colorless plaque on an Xgal plate containing a *lac* operon inducer.

We isolated 13 independent mutants in this way and identified their mutational alterations. These mutations were then reintroduced into the pNO2731 plasmid, and the efficiency of uncoupled L1 translation was measured by the *in vivo* experimental method. The mutational nucleotide alterations identified are shown in Fig. 2b, and the results of the *in vivo* experiments are described in Table 5. It was remarkable that all five different mutations in the 13 independently isolated mutants were clustered in a small segment between nucleotides 501 and 511, almost immediately upstream from the SD sequence for L1, and all of them were G-to-A transitions, even though the nitrous acid used as the mutagen was expected to cause various kinds of mutations (see Discussion). Two of the five different mutations were at positions 507 and 508, that is, at the same residues altered by the MB6 mutation described above. In fact, these two mutants showed the highest efficiency in uncoupled L1 translation, about 8 to 9% of the efficiency of the translationally coupled chromosomal operon in the control strain (Table 5). NA501 and NA511 showed weaker but probably significant uncoupling, and NA502 did not show any measurable uncoupling. The reason for the failure to detect uncoupling with NA502 is not known. As judged from the position of its mutation and its being a G-to-A transition, we suspect that NA502 is a genuine mutant with a very weak phenotype, as is also the case with NA501 and NA511, and that the detection method used for mutant isolation is perhaps very sensitive compared with the biochemical assay used for the quantitation of uncoupling in the original L11-L1 system.

The results of the analysis of the randomly isolated mutants indicate the importance of several G residues located immediately upstream of the SD sequence for L1 in connection with the masking of the L1 initiation site. Again, there is no obvious way to explain the results on the basis of any specific secondary structure of mRNA being responsible for the masking.

DISCUSSION

Identification of the mRNA region responsible for masking independent initiation of L1 translation involved in translational coupling. The results obtained in this work strongly suggest that the L1 initiation site carries all the elements required for recognition by free ribosomes in the pool and in that sense is a potentially active initiation site. First, in the plasmid with the amber mutation (MB5) which was 27 nucleotides upstream from the initiation codon, translation of the distal L1 cistron appeared to be higher than translation of the upstream L11 cistron; that is, in this case L1 was translated at least partly by ribosomes coming directly from the pool. Second, the deletion analysis indicated that deletion of upstream sequences to 29 nucleotides upstream from the AUG codon had a definite unmasking effect, and removal of a further 3 nucleotides showed a marked unmasking

effect. It should be noted that since ribosomes generally bind to the initiation site of mRNA and protect about 20 nucleotides on both sides of the AUG codon from nuclease digestion (4, 29, 38, 39), the regions of the L11-L1 mRNA affected by these deletion and amber mutations are close but probably outside the ribosome-binding site defined by nuclease digestion and are physically separated (at least 13 nucleotides) from the SD sequence, which is located 13 to 9 nucleotides upstream from the AUG codon.

In addition, various point mutations located between 501 to 511 converted the inactive L1 initiation site to an active independent site. Thus, we conclude that the lack of independent initiation of translation at the L1 initiation site is not because of the absence of some specific recognition signals for free ribosomes, but because of the presence of some mRNA regions with special features interfering with independent translation initiation by free ribosomes. The interfering mRNA features apparently involve the G residues identified by point mutations as well as the upstream regions identified by deletion analysis; the movement of translating ribosomes along the upstream mRNA (probably along the region defined by the two amber mutations MB3 and MB5) somehow overcomes this interference.

It should be noted that although various mutational alterations of the original mRNA sequence converted the masked L1 initiation site to an independent initiation site, we have not rigorously proven that the original sequence itself has an inherent ability to form a structure allowing independent initiation by free ribosomes. However, there are two known instances in which translational coupling involves masking of a clearly independent initiation site. First, in the L10 operon (also called the β operon), translation of the second cistron, L7/12, is coupled with translation of the first cistron, L10 (43), and is regulated by L10 at the target site near or upstream of the L10 initiation site (7, 14, 43). In this system, L7/12 synthesis is at least fourfold higher than L10 synthesis under normal conditions. Therefore, it is clear that translation of the upstream cistron, L10, unmasks the L7/12 initiation site, and it is mostly free ribosomes from the pool that initiate translation of L7/12 at this site, as pointed out previously (43).

Second, a somewhat analogous situation is found in the translation of r-protein cistrons in the α operon, which contains the genes for S13, S11, S4, the RNA polymerase α subunit, and L17. It is known that the synthesis of L17 from the last cistron is about equal to that of other r-proteins, but about two- to fourfold higher than the synthesis of α from the immediately upstream cistron (17; also see references in reference 20), that the synthesis of these proteins takes place from a single polycistronic mRNA, and that the translation of L17 is apparently coupled with the translation of the upstream r-proteins and not that of α (M. S. Thomas, D. M. Bedwell, and M. Nomura, *J. Mol. Biol.*, in press). Therefore, the initiation site of L17 must be inherently active, and the translational coupling appears to involve unmasking of this site by ribosomes translating the upstream r-protein cistrons.

Translational coupling and equimolar synthesis of r-proteins. Although we have isolated various mutants in which L1 translation takes place independently, the efficiency of translation in these instances was relatively low compared with L1 synthesis by translational coupling. Thus, translational coupling as used by the normal operon is certainly an efficient way to translate the distal cistron. It should be noted that the mRNA regions most important for masking the L1 initiation site were almost immediately

upstream from the SD sequence for the L1 cistron, and this suggests that ribosomes translating the upstream cistron are very close to the L1 site when the L1 site is unmasked. Thus, it is highly likely that the L1 cistron is translated by the same ribosomes (or some of the same ribosomal components, for example, 30S subunits) that translate the upstream cistron under normal conditions, ensuring equimolar synthesis of both proteins. Short distances between two translationally coupled cistrons (3 nucleotides in the present system) or an overlap of stop and start codons (as in the case of the *trpED* system [28]) may undoubtedly help tight coupling in this connection. However, where translational coupling and equimolar synthesis of proteins have been demonstrated, intercistronic regions in r-protein operons vary widely; e.g., the distance is quite often 3 nucleotides as in the present L11-L1 system, but it is 41 nucleotides between S14 and S8, and yet the latter are translationally coupled along with all the other r-protein cistrons distal to L5 in the *spc* operon (L. Matthews, D. Cerretti, and M. Nomura, unpublished experiments). On the other hand, the translational coupling of L7/12 with L10 takes place with an intercistronic distance of 67 nucleotides, but equimolar synthesis of the two proteins does not take place, as mentioned above. We have previously distinguished these two classes and called the former class, i.e., translational coupling with equimolar synthesis, sequential translation (44).

In the case of translational coupling of L17 synthesis with the synthesis of the proximal α operon r-proteins mentioned above, the available published data indicate that the synthesis of all of these proteins is equimolar. If this is in fact true, the mechanism ensuring equimolar synthesis in this system would be different from that we discussed for the coupling of L11 and L1 and deserves future investigation.

Mechanism involved in masking the initiation site for L1 translation. Quite a few studies have demonstrated that the presence of some secondary RNA structures involving the SD sequence or the initiation codon reduces the efficiency of initiation of translation and that their disruption stimulates translation (5, 8, 16, 25, 31, 37; also see reference 15 for a review). More specifically, translational coupling of the replicase cistron of MS2 RNA phage with the upstream coat cistron was proposed to involve a long-range RNA-RNA base-pairing which would mask the replicase initiation site (23), and the results of deletion analysis supporting this model have been reported (5). In addition, some earlier studies also showed that the replicase initiation site is normally not accessible to ribosomes in vitro and denaturation of MS2 RNA by formaldehyde treatment converts it to an independent translation initiation site (19). However, as discussed above, we failed to identify any specific base-pairing structure responsible for the masking of the L1 initiation site in the present system. In particular, unmasking by the MB6 mutation but not by the MB8 mutation of the L1 initiation site is difficult to explain on the basis of a simple RNA-RNA base-pairing structure masking the initiation site. (It is possible, however, that the MB8 mutation forms an alternative base-pairing that also masks the initiation site, and this might be the reason for the inability of MB8 to unmask L1 initiation.) In addition, we tested the effects of formaldehyde treatment of in vitro-synthesized mRNA on L1 translation with negative results, suggesting that destruction of the secondary structure does not unmask the L1 initiation site (unpublished data).

Another possible mechanism we have considered is the (hypothetical) presence of an antisense RNA covering the intercistronic region, including the sites identified by the

mutations. According to this model, the MB6 mutation weakens the interaction of antisense RNA with mRNA, but the MB8 mutation does not. However, in preliminary experiments we have demonstrated that an L11-L1 mRNA synthesized from a phage SP6 promoter in vitro, i.e., mRNA without any such hypothetical antisense RNA, showed the same behavior, rendering such an explanation unlikely. Thus, although we have identified mRNA regions and some specific nucleotide residues involved in the masking of the L1 initiation site, we cannot offer any specific molecular model at the moment. It is possible that some unknown RNA structure is responsible for masking without utilizing ordinary base-pairing. Alternatively, some additional factor(s), e.g., ribosomes themselves, which would interact with the pertinent nucleotide residues identified, may be involved in the masking of the L1 initiation site. The (hypothetical) interaction with the ribosomes might be with the 3' end of 16S rRNA, representing competition with the true SD sequence (see discussion in reference 40) or with some other sites, inhibiting initiation by a simple physical block, and translating ribosomes from upstream would overcome such blocks.

The results of our mutant isolation studies are of particular interest for further studies on the mechanism of masking. Mutants were isolated after nitrous acid mutagenesis, which has been shown to induce transitions in both directions, AT to GC as well as GC to AT, and can also induce transversions and deletions (9, 36). Yet all the mutations found to affect the translational coupling were G-to-A transitions, and they were clustered in a region (-25 to -15 with respect to the L1 start codon) upstream from the SD sequence. We note that statistical analyses of nucleotide sequences of many translation start sites of *E. coli* and coliphage genes were previously carried out, and a paucity of G's outside the SD sequence was in fact noted in this region by previous workers (33, 40). However, these analyses included both independent initiation sites and translationally coupled (i.e., masked) initiation sites. Although the locations of the mRNA regions responsible for masking translationally coupled initiation sites surely may be different depending on the system used, we have made a preliminary inspection of this region (-25 to -15 with respect to the translation start site) from known translationally coupled r-protein cistrons (L1, L3, L4, L6, L7/12, L15, L17, L18, L30, S4, S5, S8, S11, and S14; translational coupling supported by at least some experimental evidence) and compared it with the same region from independently initiating r-protein cistrons (L5, L10, L11, L13, L14, L28, L34, S1, S2, S6, S7, S9, S10, S12, S13, S15, S16, S20, and S21; independent initiation either demonstrated experimentally or assumed because of their being the first cistron in a transcription unit). Although we failed to find any consensus sequences that might distinguish the two classes, we found that the average ratio of A to G in the former class was 1.3, whereas it was 5.2 in the latter class in this region. This might reflect a general presence of G residues responsible for masking, as suggested by the mutational analysis of the L1 initiation site mentioned above.

In conclusion, we have identified mRNA regions as well as specific nucleotide residues responsible for masking the L1 initiation site. The regions are almost immediately upstream of the SD sequence for the L1 cistron, and this suggests that ribosomes translating the upstream cistron are very close to the L1 site when the L1 site is unmasked, ensuring equimolar synthesis of both proteins, presumably by the same ribosomes. Since the translational coupling found in several r-protein operons is the basis of equimolar synthesis of

r-proteins and their coregulation by a repressor r-protein, further studies are warranted to elucidate the molecular mechanisms involved in this important process.

ACKNOWLEDGMENTS

We thank Gail Baughman for her help in some of the initial experiments in this project and J. R. Cole for useful discussion and reading the manuscript.

This work was supported by Public Health Service grant 7-RO1-GM35949-02 from the National Institutes of Health and by grant DMB-85-43776 from the National Science Foundation. Monique Bolotin-Fukuhara acknowledges fellowship support from the North Atlantic Treaty Organization during this research.

LITERATURE CITED

1. Aksoy, S., C. L. Squires, and C. Squires. 1984. Translational coupling of the *trpB* and *trpA* genes in the *Escherichia coli* tryptophan operon. *J. Bacteriol.* **157**:363–367.
2. Baughman, G., and M. Nomura. 1983. Localization of the target site for translational regulation of the L11 operon and direct evidence for translational coupling in *Escherichia coli*. *Cell* **34**:979–988.
3. Baughman, G., and M. Nomura. 1984. Translational regulation of the L11 ribosomal protein operon of *Escherichia coli*: analysis of the mRNA target site using oligonucleotide-directed mutagenesis. *Proc. Natl. Acad. Sci. USA* **81**:5389–5393.
4. Belin, D., J. Hedgpeth, G. B. Selzer, and R. H. Epstein. 1979. Temperature-sensitive mutation in the initiation codon of the *rIIB* gene of bacteriophage T4. *Proc. Natl. Acad. Sci. USA* **76**:700–704.
5. Berkhout, B., and J. van Duin. 1985. Mechanism of translational coupling between coat protein and replicase genes of RNA bacteriophage MS2. *Nucleic Acids Res.* **13**:6955–6967.
6. Boyen, A., J. Piette, R. Cunin, and N. Glansdorff. 1982. Enhancement of translation efficiency in *Escherichia coli* by mutation in a proximal domain of messenger RNA. *J. Mol. Biol.* **162**:715–720.
7. Brot, N., D. Caldwell, and H. Weissbach. 1980. Autogenous control of *Escherichia coli* ribosomal protein L10 synthesis *in vitro*. *Proc. Natl. Acad. Sci. USA* **77**:2592–2595.
8. Cannistraro, V. J., and D. Kennel. 1979. *Escherichia coli* lac operator mRNA affects translation initiation of β -galactosidase mRNA. *Nature (London)* **277**:407–409.
9. Carlton, B. C., and B. Brown. 1981. Gene mutation, p. 222–242. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
10. Clarke, D. J., and O. Maaløe. 1967. DNA replication and the cell division cycle in *Escherichia coli*. *J. Mol. Biol.* **23**:99–112.
11. Coulondre, C., and J. H. Miller. 1977. Genetic studies of the lac repressor. III. Additional correlation of mutational sites with specific amino acid residues. *J. Mol. Biol.* **117**:525–575.
12. Das, A., and C. Yanofsky. 1984. A ribosome binding site sequence is necessary for efficient expression of the distal gene of a translationally-coupled gene pair. *Nucleic Acids Res.* **12**:4757–4768.
13. Dean, D., and M. Nomura. 1980. Feedback regulation of ribosomal protein gene expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **77**:3590–3594.
14. Fukuda, R. 1980. Autogenous regulation of the synthesis of ribosomal proteins, L10 and L7/L12, in *Escherichia coli*. *Mol. Gen. Genet.* **178**:483–486.
15. Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. S. Singer, and G. Stormo. 1981. Translational initiation in prokaryotes. *Annu. Rev. Microbiol.* **35**:365–403.
16. Iserentant, D., and W. Fiers. 1980. Secondary structure of mRNA and efficiency of translation initiation. *Gene* **9**:1–9.
17. Iwakura, Y., K. Ito, and A. Ishihama. 1974. Biosynthesis of RNA polymerase in *Escherichia coli*. I. Control of RNA polymerase content at various growth rates. *Mol. Gen. Genet.* **133**:1–23.
18. Kastelein, R. A., B. Berkhout, G. P. Overbeek, and J. van Duin. 1983. Effect of the sequence upstream from the ribosome-binding site on the yield of protein from the cloned gene for the phage MS2 coat protein. *Gene* **23**:245–254.
19. Lodish, H. F. 1970. Secondary structure of bacteriophage F2 ribonucleic acid and the initiation of *in vitro* protein biosynthesis. *J. Mol. Biol.* **50**:689–702.
20. Matzura, H. 1980. Regulation of biosynthesis of the DNA-dependent RNA polymerase in *Escherichia coli*. *Curr. Top. Cell. Regul.* **17**:89–136.
21. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
22. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20–78.
23. Min Jou, W., G. Haegeman, M. Ysebaert, and W. Fiers. 1972. Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. *Nature (London)* **237**:82–88.
24. Miyoshi, K., T. Huang, and K. Itakura. 1980. Solid phase synthesis of polynucleotides. III. Synthesis of polynucleotides with defined sequences by the block coupling phosphotriester method. *Nucleic Acids Res.* **8**:5491–5505.
25. Munson, L. M., G. D. Stormo, R. L. Niece, and W. S. Reznikoff. 1984. LacZ translation initiation mutations. *J. Mol. Biol.* **177**:663–683.
26. Nomura, M. 1986. Regulation of the synthesis of ribosomes and ribosomal components in *Escherichia coli*: translational regulation and feedback loops, p. 199–220. In I. Booth and C. Higgins (ed.), *Symposium of the Society for General Microbiology: Regulation of Gene Expression*. Cambridge University Press, Cambridge.
27. Nomura, M., R. Gourse, and G. Baughman. 1984. Regulation of the synthesis of ribosomes and ribosomal components. *Annu. Rev. Biochem.* **53**:75–117.
28. Oppenheim, D. S., and C. Yanofsky. 1980. Translational coupling during expression of the tryptophan operon of *Escherichia coli*. *Genetics* **95**:785–795.
29. Platt, T., and C. Yanofsky. 1975. An intercistronic region and ribosome-binding site in bacterial messenger RNA. *Proc. Natl. Acad. Sci. USA* **72**:2399–2403.
30. Post, L. E., G. D. Strycharz, N. Nomura, H. Lewis, and P. P. Dennis. 1979. Nucleotide sequence of the ribosomal protein gene cluster adjacent to the gene for RNA polymerase subunit β in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **76**:1697–1701.
31. Queen, C., and M. Rosenberg. 1981. Differential translation efficiency explains discoordinate expression of the galactose operon. *Cell* **25**:241–249.
32. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
33. Scherer, G. F. E., M. D. Walkinshaw, S. Arnott, and J. D. Moore. 1980. The ribosome binding sites recognized by *E. coli* ribosomes have signal character in both the leader and protein coding segments. *Nucleic Acids Res.* **8**:3895–3907.
34. Shine, J., and L. Dalgarno. 1974. The 3' terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to the nonsense triplet and ribosome binding sites. *Proc. Natl. Acad. Sci. USA* **71**:1342–1346.
35. Shumperli, D., K. McKenney, D. A. Sobieski, and M. Rosenberg. 1982. Translational coupling at an intercistronic boundary of the *Escherichia coli* galactose operon. *Cell* **30**:865–871.
36. Singer, B., and J. T. Kusmierek. 1982. Chemical mutagenesis. *Annu. Rev. Biochem.* **52**:655–693.
37. Stanssens, P., E. Remaut, and W. Fiers. 1985. Alterations upstream from the Shine-Dalgarno region and their effect on bacterial gene expression. *Gene* **36**:211–223.
38. Steitz, J. A. 1969. Nucleotide sequences of ribosomal binding sites of bacteriophage R17 RNA. *Cold Spring Harbor Symp. Quant. Biol.* **34**:621–633.
39. Steitz, J. A., and R. A. Bryan. 1977. Two ribosome binding sites from the gene 0-3 messenger RNA of bacteriophage T7. *J. Mol. Biol.* **114**:527–543.

40. Stormo, G., T. D. Schneider, and L. M. Gold. 1982. Characterization of translational initiation sites in *E. coli*. *Nucleic Acids Res.* **10**:2971-2996.
41. Tessman, I., R. K. Poddar, and S. Kumar. 1964. Identification of the altered bases in mutated single-stranded DNA. I. *In vitro* mutagenesis by hydroxylamine, ethyl methanesulfonate and nitrous acid. *J. Mol. Biol.* **9**:352-363.
42. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.
43. Yates, Y. L., A. E. Arfsten, and M. Nomura. *In vitro* expression of *Escherichia coli* ribosomal protein genes: autogenous inhibition of translation. *Proc. Natl. Acad. Sci. USA* **77**:1837-1841.
44. Yates, J. L., and M. Nomura. 1981. Feedback regulation of ribosomal protein synthesis in *E. coli*: localization of the mRNA target sites for repressor action of ribosomal protein L1. *Cell* **24**:243-249.
45. Zoller, M., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* **100**:468-500.
46. Zuker, M., and P. Stiegler. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* **9**:133-148.