Genetic analysis of the Shine-Dalgarno interaction: selection of alternative functional mRNA-rRNA combinations

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Genetic analysis of the Shine–Dalgarno interaction: Selection of alternative functional mRNA–rRNA combinations

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ABSTRACT

The interaction of bacterial mRNAs with the small ribosomal subunit is strongly promoted by Watson–Crick base pairing between a purine-rich consensus ribosomal RNA-binding sequence (RBS) on mRNA and its complementary message-binding sequence (MBS) on rRNA known as the Shine–Dalgarno interaction. To identify and characterize components of the Shine–Dalgarno interaction that contribute to translation initiation, we simultaneously and randomly mutated both the MBS of the 16S rRNA gene from *Escherichia coli* and the RBS of the chloramphenicol acetyl transferase (CAT) gene and selected chloramphenicol-resistant mutant combinations. Nucleotide distribution in both mutated sequences of the survivors was nonrandom and the MBSs of the surviving clones showed a preference for purines. In addition, strong interactions between specific nucleotide pairs within each of the mutated sequences were indicated. Although the contribution of free energy of duplex formation between rRNA and mRNA was highly significant ($P < 0.001$), only 23% of the observed activity in all of the mutants could be attributed to this variable. MBSs that were lethal upon expression were also isolated. These sequences may cause overtranslation of specific messages in the cell. These data indicate that specific sequence constraints exist (primarily within the MBS) that are necessary to establish a functional threshold for translation and that only after establishment of this threshold is the level of expression significantly affected by the free energy of MBS–RBS duplex formation.

Keywords: initiation; message-binding sequence; mutation; protein synthesis; ribosome-binding sequence

INTRODUCTION

Translation is viewed conceptually as a three-step process: initiation, in which the translational machinery is assembled; elongation, in which amino acids are added to the growing peptide; and termination, in which the nascent peptide is cleaved from the final tRNA and the translational machinery is disassembled.

During initiation in bacteria, the 30S ribosomal subunit, fMet-tRNA, mRNA, three initiation factors, and GTP combine to form the 30S initiation complex. Formation of the 30S initiation complex is generally believed to be the rate-determining step in protein synthesis and is therefore the primary determinant of translational efficiency (Calogero et al., 1988; Dreyfus, 1988; Gualerzi et al., 1990; Gualerzi & Pon, 1990; McCarthy & Brimacombe, 1994). The rate of initiation, in turn, appears to be determined largely by factors affecting the mRNA–ribosome interaction (Gualerzi et al., 1990; Balakin et al., 1992; de Smit & van Duin, 1994).

Variations in mRNA sequence and structure have been shown to strongly affect expression of the encoded gene. Several elements within the message modulate the efficiency of gene expression at the level of translation by affecting formation of the 30S initiation complex. These include the start codon (Wikström et al., 1992), the second codon (Gold, 1988; Grunert & Jackson, 1994), the ribosome-binding sequence (Shine & Dalgarno, 1974; Ringquist et al., 1992), the distance between the ribosome-binding sequence and the start codon (Guillerez et al., 1991; Chen et al., 1994), the presence of putative alternative ribosome-binding sequences (Olins et al., 1988; Thanaraj & Pandit, 1989; Sprengart et al., 1990, 1996), and mRNA secondary structure (Looman et al., 1986; Lesage et al., 1992; Liebhaber et al., 1992; de Smit & van Duin, 1994).

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Ribosomal RNA has been implicated functionally in virtually every aspect of protein synthesis (Barta et al., 1984; Cundliffe, 1986; de Stasio et al., 1988; Dahlberg, 1989; Noller, 1991; Noller et al., 1992; Cunningham et al., 1993; Ofengand et al., 1993). The first direct involvement of rRNA in protein synthesis to be identified was the Shine–Dalgarno interaction (Shine & Dalgarno, 1974) in which a purine-rich region upstream from the start codon in bacterial mRNAs, the ribosomal RNA-binding sequence (RBS), base pairs with a complementary sequence, termed the message-binding sequence (MBS) found at the 3′ terminus of 16S rRNA (Eckhardt & Luhrmann, 1979; Hui & de Boer, 1987; Jacob et al., 1987; Eckert et al., 1989). This interaction plays a key role in the initiation of protein synthesis and has been shown to be a major determinant of translational efficiency in procaryotes (Hartz et al., 1991; de Smit & van Duin, 1994) and, therefore, ultimately of gene expression. However, analyses of large numbers of existing mRNA sequences have revealed only a weak correlation between the strength of base pairing (free energy of binding) of the MBS and RBS and level of expression (Ringquist et al., 1992). In addition, mutational analyses of only the RBS have produced unpredictable variations in the level of expression that do not appear to be related directly to complementarity (de Smit & van Duin, 1994). Mutations constructed in the MBS have resulted in dramatically altered cellular protein synthesis (Jacob et al., 1987) or were lethal (Hui et al., 1987; Wood & Peretti, 1991).

Poor predictability of translational efficiency based upon RBS analyses indicates that within the MBS–RBS interaction, factors other than complementarity may play a significant role in determining the efficiency with which a particular message will be translated. To identify these factors, we have randomly mutagenized both the RBS of the chloramphenicol acetyl transferase (CAT) gene and the MBS of a plasmid-encoded 16S rRNA gene and directly selected functional mutant combinations. Both functional and nonfunctional MBS–RBS combinations were then analyzed for the presence of sequence motifs that affected expression.

RESULTS

Selection of functional alternate MBS–RBS combinations

The *Escherichia coli* chromosome contains seven copies of the rRNA operon (Kiss et al., 1977). To minimize involvement of this background of wild-type ribosomes, we placed one of these operons, *rrnB*, and a selectable marker, the CAT gene, on the same plasmid and used derivatives of this construct to select alternative functional MBS and RBS combinations. The CAT gene was used because translation of the CAT message renders the cells resistant to chloramphenicol. In addition, the amount of functional CAT protein can be quantified rapidly and accurately. In our experiments, the level of chloramphenicol resistance was directly and linearly proportional to CAT activity up to 600 μg/mL (not shown).

For our initial experiments, we constructed the plasmid pRNA8, which carries the CAT gene under control of a constitutive *trp* promoter and the *rrnB* operon under control of the inducible *lacUV5* promoter. Induction of the *rrnB* operon in this construct produces cells in which plasmid-derived ribosomes constitute approximately 35–40% of the total ribosome pool as determined by primer extension (Sigmund et al., 1988) (not shown). A map of this construct is shown in Figure 1. Using PCR, we randomly mutagenized the RBS of the CAT gene and MBS of the 16S rRNA gene in the *rrnB* operon of pRNA8 and selected new MBS–RBS combinations that rendered the cells resistant to chloramphenicol upon induction with isopropyl-β-D-thiogalactoside (IPTG). This was done in two steps (Fig. 1).

First, the 5-nt *rrnB* MBS of pRNA8 was mutated so that all possible nucleotides except those found in wild-type rRNA were represented at each position of the MBS, yielding $3^5 = 243$ possible combinations. Wild-type nucleotides at each position were excluded to prevent pairing of the mutated sequences with wild-type complementary sequences in the cell. These mutations were cloned into pRNA8 and approximately 66,000 transformants were pooled and used to prepare plasmid containing the random MBS (MBS-R) for the second step.

Next, a similar scheme was used to mutate the 5 nt of the CAT RBS so that the wild-type consensus RBS (RBS-wt) was excluded and the resulting 243 different sequences were ligated into the pooled MBS-R clones and used to transform DH5 cells. To confirm the presence of all three possible mutations at each mutated position in the pool prior to selection, a portion of the transformants were selected on LB-Ap100 only, pooled, and plasmid was prepared from this pool. The plasmid pool was then added to sequencing reactions and the presence of all 3 nt at both the mutated RBS and MBS was confirmed by the presence of sequencing stops of equal intensity at each of the mutated positions (not shown).

To select transformants containing novel MBS–RBS combinations that were nonlethal and that translated CAT mRNA specifically, the doubly mutated transformants were induced for 4 h in SOC medium containing 1 mM IPTG and plated on the same medium containing either 100, 200, 300, or 350 μg/mL chloramphenicol. To identify highly functional combinations, 2.5×10^6 transformants were screened at the highest antibiotic concentration. Screening this number of recombinants provided a confidence level of greater than 99.9% that each of the $243^2 = 59{,}049$ possible combina-

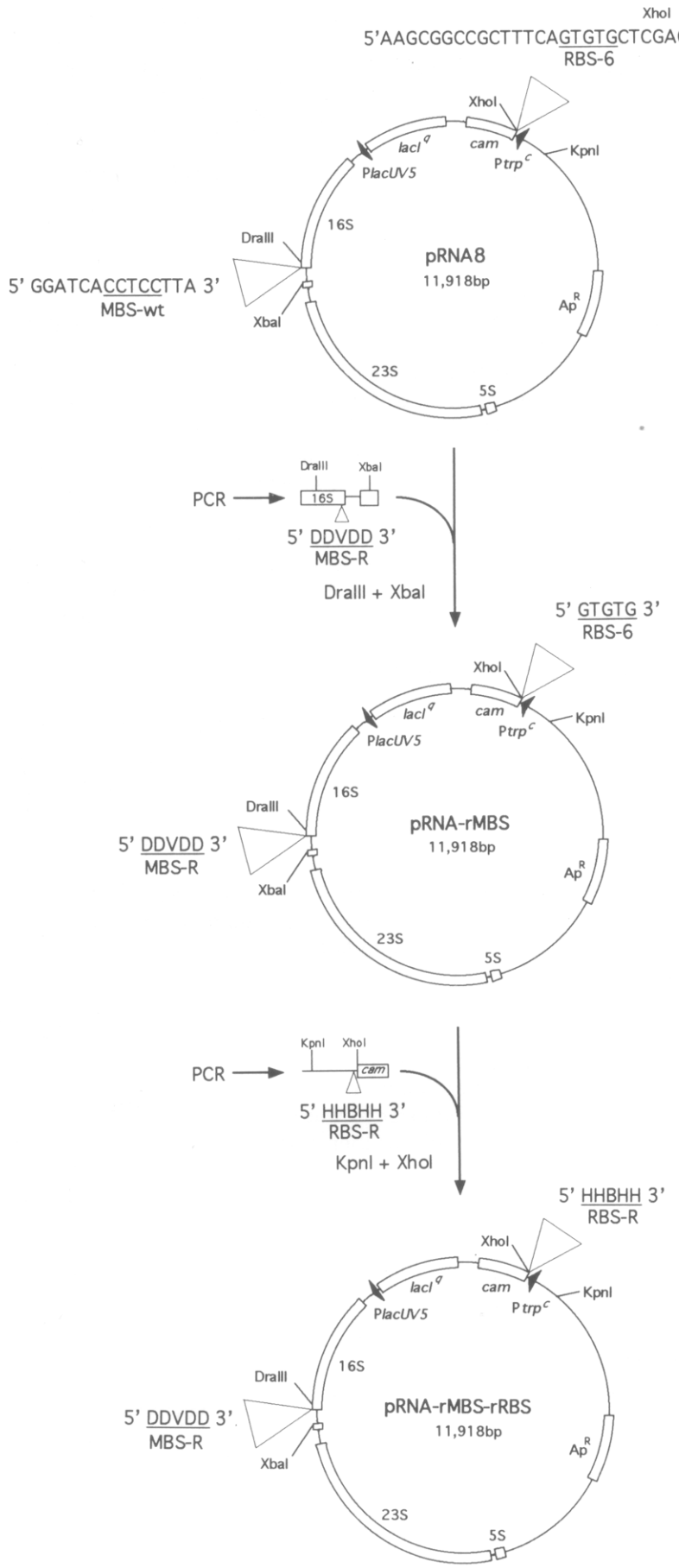

FIGURE 1. Scheme for construction of alternative MBS–RBS mutants. Ap^r, ampicillin resistance; *cam*, CAT gene; *lacI*^{q}, lactose repressor; P*lacUV5*, *lacUV5* promoter; P*trp*^{c}, constitutive *trp* promoter. The MBS and RBS mutagenic sequences are shown: B = C, G, T; D = A, G, T; H = A, C, T; V = A, C, G. Restriction sites used are indicated and details of the construction are discussed in the text.

tions was represented at least once (Clarke & Carbon, 1976).

Functional analysis of selected mutants

Levels of chloramphenicol resistance were determined for the survivors both in the presence and absence of IPTG. These data are presented in Table 1. The range of minimum inhibitory concentrations (MICs) of chloramphenicol for the isolates in the presence of inducer was from 100 to 700 μg/mL.

Chloramphenicol resistance in the selected clones may result from CAT mRNA translation by either plasmid-derived ribosomes or chromosomally derived (wild-type) ribosomes, or a combination of the two. To quantify the degree of interaction specifically between the plasmid-derived ribosomes and the CAT message, MICs were determined in both the presence and absence of inducer. The difference in chloramphenicol resistance between induced and uninduced cells (ΔMIC) represents the level of expression due only to translation by plasmid-derived ribosomes.

Sequence analysis of selected mutants

Nucleotide sequences of the MBS and RBS from 59 of the isolates were determined. Each mutated position was analyzed to determine the effects of nucleotide identity upon expression and upon selection of nucleotides at other mutated positions. Among the sequenced clones, three duplicate mutant sequences and four unprogrammed mutations were identified. Three of the unprogrammed mutants contained a wild-type nucleotide in the RBS and the other contained a single nucleotide deletion in the RBS. Unprogrammed mutations presumably occurred during amplification by Taq DNA polymerase. These mutants and four additional clones that did not show an increase in chloramphenicol resistance upon induction were excluded from statistical analyses. The remaining sequences are shown in Table 1.

Nucleotide distribution at each mutated position

To determine if selection of nucleotides in the chloramphenicol-resistant clones at each of the MBS and RBS positions was random, the distribution of nucleotides at each position was examined for goodness of fit to an even distribution among possible substitutions. Results of these analyses are presented in Figure 2. Selection of nucleotides at each of the five positions of the MBS was nonrandom with the exception of the 5′ position, M1. In positions M2–M5, a preference for purines was indicated, although, in positions M2 and M4, "G" was preferred specifically. Interestingly, in the RBS, nucleotide distribution at the 3′ (R5), 5′ (R1), and center (R3) nucleotides was random, and, although distributions at the other two positions (R2 and R4) were nonrandom, pyrimidines were not preferred, despite the preference for purines observed in the MBS. Virtually all of the selected CAT sequences were complementary to nucleotides adjacent to the mutated MBS positions. Thus, nucleotide selection in each of the RBS positions was not strictly dependent upon the sequence of the corresponding mutated MBS site (see below).

One explanation for this is that pairing with fixed adjacent sequences to the RBS (such as the downstream CUC) is responsible for the observed preferences. To test this, we removed all of the clones in which pairing with this sequence was indicated and reanalyzed the data. These analyses produced virtually identical results to those obtained from the total pool. Similar results were obtained when pairing interactions of the RBS were analyzed.

In addition, pairing of the RBS to the fixed U's found downstream of the MBS might explain the preference for A's observed in the R1 and R2 positions. If this were the case, the strongest preference for A's should exist

RBS Position	R1	R2	R3	R4	R5
χ^2	5.38	11.38**	2.38	6.00*	1.00
RBS 5'-	AUC	ACU	CUG	ACU	UAC
MBS 3'-	GAU	GUA	GAC	GAU	GAU
χ^2	6.13*	13.63**	12.88**	16.63***	1.50
MBS Position	M5	M4	M3	M2	M1

FIGURE 2. Nucleotide distribution at each mutated position. Distribution of nucleotides at each of the mutated RBS and MBS positions was determined and converted to a percentage. This percentage is represented by the size of the font. Chi-squared analysis was used to test the null hypothesis that distribution at each position occurred randomly. χ^2, Chi-squared statistic; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 1. Sequence analysis of chloramphenicol resistant isolates.[a]

Clone	Alignment of CAT mRNA and 16S rRNA	MIC (μg of Cm/mL)		ΔG°_{37} (kcal/mol)
Random	5′ C A R1 R2 R3 R4 R5 C U C G 3′ CAT mRNA 3′ A U U M5 M4 M3 M2 M1 A C U 5′ 16S rRNA	no IPTG	1 mM IPTG	
wild-type	5′ C A G G A G G C U C G 3′ 3′ A U U C C U C C A C U 5′	500	500	−9.8
1	5′ C A A U C C C U C G 3′ 3′ A U U A G G G A A C U 5′	100	400	−8.3
2	5′ C A U A C C U C U C G 3′ 3′ A U U G G G U A A C U 5′	50	100	−4
3	5′ C A C A G U C C U C G 3′ 3′ A U U A G C A G A C U 5′	50	100	−1.9
4	5′ C A A A C C A C U C G 3′ 3′ A U U U A G U G A C U 5′	50	100	−4.1
5	5′ C A U A G C C C U C G 3′ 3′ A U U G G G U U A C U 5′	50	100	−7.6
6	5′ C A U C U U C C U C G 3′ 3′ A U U G G A G G A C U 5′	50	100	−7.4
7	5′ C A A U U A U C U C G 3′ 3′ A U U U U A A G A C U 5′	50	100	−3.1
8	5′ C A C A G A A C U C G 3′ 3′ A U U G A C U A A C U 5′	100	200	−3.6
9	5′ C A A A G U U C U C G 3′ 3′ A U U G A G U A A C U 5′	100	200	−0.6
10	5′ C A A U U C A C U C G 3′ 3′ A U U A A G U G A C U 5′	100	400	−7.7
11	5′ C A A C U C A C U C G 3′ 3′ A U U G U G A G A C U 5′	100	200	−7.1
12	5′ C A A C C C A C U C G 3′ 3′ A U U A G G G U A C U 5′	50	100	−6
13	5′ C A U C G U U C U C G 3′ 3′ A U U G A G A A A C U 5′	50	200	−2.2
14	5′ C A C A C C A C U C G 3′ 3′ A U U U U G G G U A C U 5′	50	100	−4.7
15	5′ C A C C C A C C U C G 3′ 3′ A U U G G G A A A C U 5′	50	200	−7
16	5′ C A U C C C A C U C G 3′ 3′ A U U G G G G A A C U 5′	50	100	−7.3
17	5′ C A A A C U C C U C G 3′ 3′ A U U A U C A U A C U 5′	50	100	0.8
18	5′ C A U A C A U C U C G 3′ 3′ A U U U G A G A A C U 5′	50	100	−2.1
19	5′ C A A C U C U C U C G 3′ 3′ A U U A G A G G A C U 5′	50	200	−5.6
20	5′ C A A A U A U C U C G 3′ 3′ A U U U A G A G A C U 5′	200	500	−6.2
21	5′ C A U A C C U C U C G 3′ 3′ A U U G G A G U A C U 5′	200	500	−7.3
22	5′ C A U A G U A C U C G 3′ 3′ A U U U A G G U A C U 5′	100	200	0.3
23	5′ C A A U C C A C U C G 3′ 3′ A U U A G G U G A C U 5′	200	400	−10.6
24	5′ C A C A G A U C U C G 3′ 3′ A U U U U C G G A C U 5′	100	200	−0.2

(continued)

TABLE 1. *Continued.*

Clone	Alignment of CAT mRNA and 16S rRNA	MIC (μg of Cm/mL)		ΔG°_{37}
Random	5′ C A R1 R2 R3 R4 R5 C U C G 3′ CAT mRNA 3′ A U U M5 M4 M3 M2 M1 A C U 5′ 16S rRNA	no IPTG	1 mM IPTG	(kcal/mol)
25	5′ C A U A G C A C U C G 3′ 3′ A U U A U C G U A C U 5′	200	400	−6.8
26	5′ C A A C U A A C U C G 3′ 3′ A U U G U G A U A C U 5′	100	200	−3.4
27	5′ C A A A U A U C U C G 3′ 3′ A U U A U G G A A C U 5′	100	400	−5.3
28	5′ C A A A U A U C U C G 3′ 3′ A U U A G A G G A C U 5′	200	400	−1.6
29	5′ C A C U C C U C U C G 3′ 3′ A U U A G G A G A C U 5′	50	100	−9.1
30	5′ C A U A U U C C U C G 3′ 3′ A U U A A G G U A C U 5′	100	400	−5.3
31	5′ C A A C C U A C U C G 3′ 3′ A U U A G A G G A C U 5′	50	200	−3.1
32	5′ C A A U C C A C U C G 3′ 3′ A U U A G A G G A C U 5′	100	400	−4.5
33	5′ C A A C C C C C U C G 3′ 3′ A U U G G G A G A C U 5′	100	400	−7.2
34	5′ C A A A C A U C U C G 3′ 3′ A U U G U A G A A C U 5′	200	400	−8
35	5′ C A U C C C A C U C G 3′ 3′ A U U A U G G G A C U 5′	50	200	−5
36	5′ C A C U G A U C U C G 3′ 3′ A U U A G G A G A C U 5′	200	500	−3.9
37	5′ C A U A U C C C U C G 3′ 3′ A U U U A G G G A C U 5′	100	500	−8.4
38	5′ C A A A C A C C U C G 3′ 3′ A U U G G A G A A C U 5′	150	500	−8.1
39	5′ C A A C G A A C U C G 3′ 3′ A U U G U G A G A C U 5′	100	400	−5.7
40	5′ C A U C U A U C U C G 3′ 3′ A U U A G A G G A C U 5′	100	400	−6.2
41	5′ C A U A C C U C U C G 3′ 3′ A U U G G A G U A C U 5′	100	500	−7.3
42	5′ C A U A U A A C U C G 3′ 3′ A U U A G A G A A C U 5′	200	500	−3.6
43	5′ C A A A U A C C U C G 3′ 3′ A U U G G A G U A C U 5′	100	500	−7.7
44	5′ C A C A U A C C U C G 3′ 3′ A U U G G A G U A C U 5′	150	600	−7.7
45	5′ C A C C G A C C U C G 3′ 3′ A U U G G A G A A C U 5′	100	500	−8.5
46	5′ C A U A U C C C U C G 3′ 3′ A U U G G G G U A C U 5′	100	700	−7.3
47	5′ C A A C U A C C U C G 3′ 3′ A U U G G A G U A C U 5′	100	500	−7.7
48	5′ C A U A U A C C U C G 3′ 3′ A U U G G A G A A C U 5′	200	600	−8

[a] The free energy of duplex formation (ΔG°_{37}, kcal/mol) was calculated according to the nearest neighbor model (Freier et al., 1986; He et al., 1991). The mutated nucleotides are underlined and potential duplex formations are boxed. MICs were performed as described in Materials and methods.

in the R1 position. Our data, however, clearly show that the strongest nucleotide preferences were found at position R2. Thus, pairing with adjacent sequences does not appear to explain the observed nucleotide preferences in either the MBS or RBS.

Does nucleotide identity affect translation?

Nonrandom distribution of nucleotides among the selected functional clones suggests that nucleotide identity within the mutated sequences affects CAT translation. To determine the effect of nucleotide identity at each of the mutated positions upon the level of CAT translation, the mean activities of all mutations at a given position were compared. Table 2 shows the results of single-factor analysis of variance of the effect of nucleotide identity on the level of expression at each mutated position for the chloramphenicol-resistant isolates. Some of the CAT RBS mutants were able to be translated by chromosome-derived, wild-type ribosomes. The extent of translation by wild-type ribosomes is indicated by the chloramphenicol MIC of the mutants in the absence of IPTG (Table 1). To exclude the contribution of CAT translation by wild-type ribosomes in the mutants, MIC values determined in the absence of inducer were subtracted from those determined in the presence of inducer and the resulting ΔMIC values were used in the analyses of MBS–RBS interaction. Within the MBS, these data indicate a significant effect of nucleotide identity upon the level of expression at position M2 ($P < 0.05$) and at position M3 ($P < 0.05$). Within the RBS, the positions that showed a significant effect of nucleotide identity upon expression were R3 ($P < 0.05$) and R5 ($P < 0.05$). Thus, it appears that, although the particular nucleotide present at RBS positions R3 and R5 was not critical in selecting functional mutants (Fig. 2), nucleotide identity at these positions did have an effect upon the amount of CAT protein produced by the selected constructs.

Covariation of nucleotides within the MBS and RBS

Because intrastrand interactions as well as interstrand interactions of the mutated sequences may also affect expression levels significantly in this system, mutant sequences were examined to determine if the presence of a particular nucleotide at one position affected nucleotide selection at other mutated positions. To identify potential interactions within each mutated sequence, the paired distribution of selected nucleotides within the same sequence was examined for goodness of fit to independence. These analyses are shown in Figure 3. Within the RBS, significant covariation is indicated only between positions R3 and R4 ($P < 0.05$). However, within the MBS, significant covariation is indicated between nucleotides M2 and M3 ($P < 0.01$), M2 and M4 ($P < 0.05$), and between M3 and M4 ($P < 0.01$). These data indicate that the MBS is highly constrained.

Alignment of the paired sequences

We aligned each of the mutated combinations (Table 1). Clearly other alignments are possible. The alignments

TABLE 2. Effects of nucleotide identity upon translation.[a]

Message Binding Sequence					Ribosome Binding Sequence				
			ΔMIC					ΔMIC	
Position	Base	Count	Mean	F	Position	Base	Count	Mean	F
M1	A	14	200	0.68	R1	A	22	210	0.27
	G	20	190			C	9	180	
	U	14	250			U	17	230	
M2	A	12	160	4.04*	R2	A	26	220	0.31
	G	29	250			C	15	190	
	U	7	120			U	7	210	
M3	A	18	270	3.54*	R3	C	19	170	4.44*
	C	5	100			G	11	170	
	G	25	190			U	18	280	
M4	A	9	200	1.85	R4	A	20	260	2.96
	G	28	240			C	20	200	
	U	11	150			U	8	120	
M5	A	19	220	1.31	R4	A	16	160	4.34*
	G	21	230			C	16	290	
	U	8	140			U	16	190	

[a] Data from Table 1 were analyzed to determine the effect of nucleotide identity upon CAT translation by analysis of variance (ANOVA). *, $P < 0.05$. Mean ΔMIC values were rounded to the nearest 10 μg/mL.

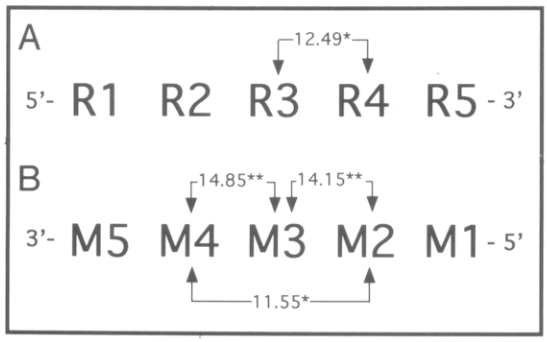

FIGURE 3. Paired distribution of selected nucleotides within the same sequence. To determine if the presence of a particular nucleotide at one position within either the MBS or RBS affected the selection of nucleotides at other position within the same sequence, we used Chi-squared analysis to test the null hypothesis that nucleotide identity at one position did not affect distribution of nucleotides at either of the other positions within the sequence. Pairs of nucleotides for which the probability of the observed distribution was less than 0.05 are connected by arrows and the Chi-squared statistic for the pair is indicated along with the probability: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

shown are those that are most energetically favorable and that do not involve noncanonical base pairs other than G·U. Most of the aligned sequences were not complementary exclusively between the mutated nucleotides. Instead, extensive pairing occurred between the mutated positions on one strand and nucleotides adjacent to the mutated positions on the opposite strand (Table 1). Pairing in this manner may affect positioning of the start codon near the anticodon of fMet-tRNA in the P site. Therefore, the effect of alignment upon translational efficiency was examined.

A well-established determinant of translational efficiency in mRNAs is the distance between the RBS and the start codon. Previous studies have shown that the paired nucleotides of RBSs are optimally located 7–9 nt from the start codon (Stormo et al., 1982; Ringquist et al., 1992; Chen et al., 1994). The distance from the 3′ paired nucleotide of the mutant RBS to the AUG start codon of CAT mRNA was calculated for each of the selected mutants (Fig. 4A, RBS–AUG). The mean RBS-AUG distance for all functional mutants was 6.8 nt, with a range of 5–11 nt. To determine the effect of RBS–AUG distance upon CAT translational efficiency, the mean activity at each distance was determined and is shown in Figure 4B. Translation of the CAT message was maximal when the RBS–AUG distance was 5 nt, although significant activity remained as this distance increased. However, no functional mutants were isolated in which the RBS–AUG distance was less than the 5-nt optimum.

Previous studies on the effect of the RBS–AUG distance on translational efficiency have been performed in systems in which the MBS was not mutated. In our study, both the MBS and RBS were mutated. Although mutagenesis did not involve insertion or deletion of nucleotides, the potential for extended pairing in the double mutants between the mutated and nonmutated residues on both molecules exists. Hence, neither the MBS nor the RBS were fixed with respect to each other. To account for such differences in alignments, a fixed position on each RNA molecule was chosen. Several nucleotides in small subunit rRNA have been implicated in P site formation (Ofengand et al., 1982; Brimacombe et al., 1990; Moazed & Noller, 1990; Mitchell et al., 1993), however, the precise location of the P site on the ribosome is unknown. We therefore chose the AUG start codon of the CAT message and the 3′-terminal nucleotide of 16S RNA and the distance between these fixed positions was calculated (Fig. 4A, 3′16S–AUG).

The optimal distance in wild-type messages between the 3′ nucleotide of 16S RNA and the first nucleotide of the start codon of mRNA is 15–17 nt (Chen et al., 1994) (see also Fig. 4A). In our selected mutants, this distance varied from 11 to 18 nt, with an overall mean distance of 13.9 nt. The effect of this distance upon translational efficiency in the selected mutants is shown in Figure 4C. Clones in which distances were longer than in the wild-type sequence all retained significant activity. However, only one clone was isolated in which this distance was shorter than 12 nt, and the ΔMIC of this clone for chloramphenicol was 100 μg/mL. None of the selected clones were able to pair so as to give a distance of less than 11 nt.

In our mutagenesis scheme, 243 different sequences are possible at both the MBS and RBS. In the region between the RBS and the AUG of the CAT message, the only sequence that could not pair with one of the possible mutated MBSs was the pentanucleotide sequence CGAGA (Fig. 4D) (3′16S–AUG = 9 nt) because the complementary sequence would have required a U at the center position of the mutant MBS and this nucleotide was excluded by the experimental design. Thus, even though distances shorter than 11 nt were possible, none were observed in the functional mutants, suggesting that these mutants were nonfunctional and therefore selected against.

Effect of free energy upon level of expression

The selected sequences were aligned and the free energy for each interaction was calculated according to the nearest neighbor model (Freier et al., 1986; He et al., 1991). To assess the relationship between free energy and the level of expression, regression analysis was performed. The correlation coefficient between ΔG°_{37} and ΔMIC is significant at $P < 0.001$ ($F = 13.4$). However, as shown in Figure 5, only 23% ($r^2 = 0.226$) of the observed translational activity of the mutant CAT mRNAs can be explained by the strength of the MBS–RBS interaction.

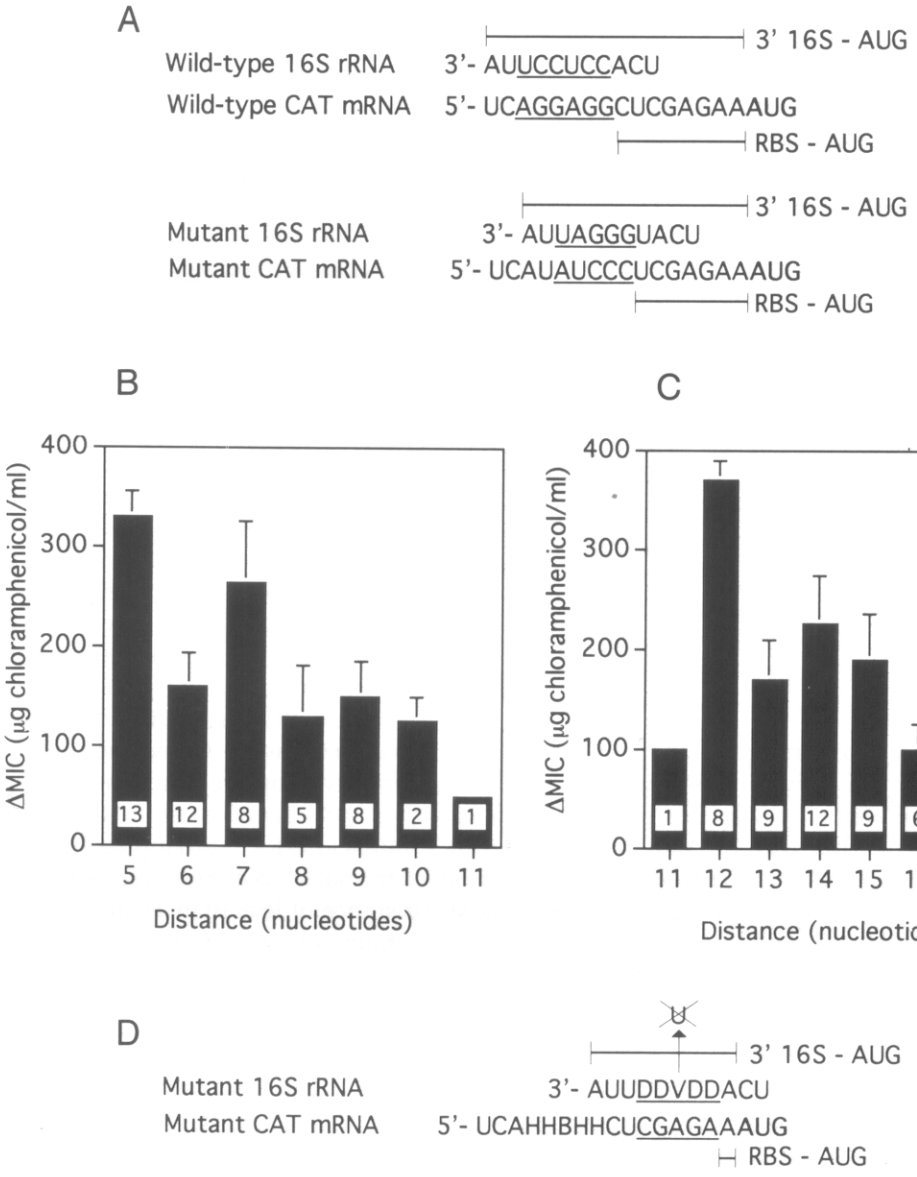

FIGURE 4. Effects of start codon spacing upon translational efficiency in the selected mutants from Table 1. MBS and RBS from functional mutants were aligned (**A**) and the effect of spacing between the CAT start codon and the 3′ paired nucleotide of the CAT message (**B**) or between the CAT start codon and the 3′ terminus of 16S RNA (**C**) was determined by averaging the CAT activities of all mutants at each distance. Error bars indicate the standard error of the mean. The number of mutants at each distance is indicated within the bars. Base pairing is prohibited by exclusion of "A" at position M3 in the MBS mutants (**D**). Alignment of mutated MBSs with all other positions between the mutated RBS and start codon is possible.

Identification and analysis of dominant lethal mutations

Other groups have reported lethal phenotypes of strains expressing altered MBS and RBS (Hui et al., 1987; Jacob et al., 1987; Wood & Peretti, 1991). To identify potentially lethal MBSs, transformants from the original MBS-R pool (Fig. 1) were plated on LB ampicillin medium and spotted onto medium containing 1 mM IPTG. Ninety-eight ampicillin-resistant transformants were screened and five clones were isolated that failed to grow when transcription of the plasmid-derived *rrnB* operon was induced. Two of these lethal plasmids contained the same MBS. In addition, the MBS and RBS described previously (Hui et al., 1988) were incorporated into our system by site-directed mutagenesis (Table 3, pRNA6). The effect of expression of the lethal sequences is shown in Figure 6. Cultures were grown to an OD_{600} of 0.1 and induced with 1 mM IPTG. Growth was strongly inhibited by induction of the mutant *rrnB* operons and the induced cultures appeared to lyse within 2–6 h. To determine if lysis was indeed oc-

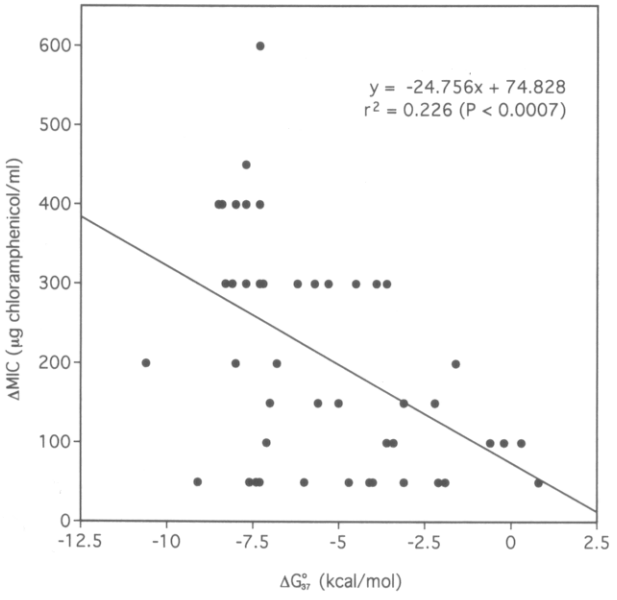

FIGURE 5. Linear regression analysis between free energy (independent variable, ΔG°_{37}) and translational efficiency (dependent variable, ΔMIC). *r*, correlation coefficient; *P*, probability that the observed relationship is due to chance alone.

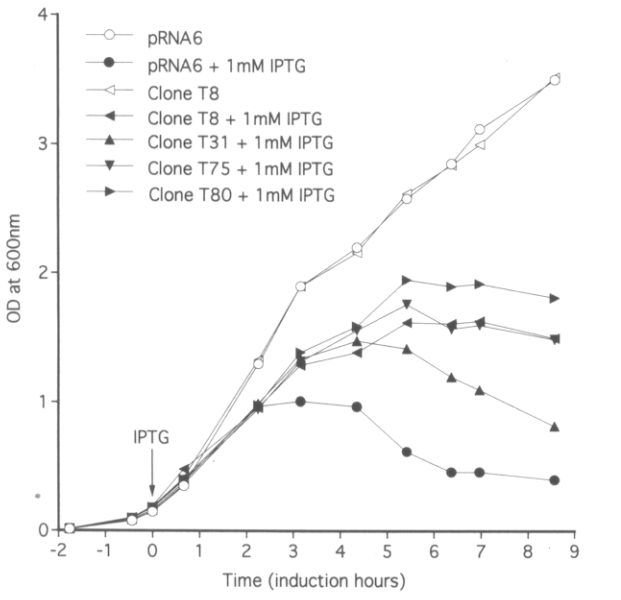

FIGURE 6. Growth characteristics of clones containing lethal sequences. Cultures of clones containing sequences presumptively identified as lethal were grown in LB-Ap100 medium and monitored at 600 nm. At $OD_{600} = 0.1$, IPTG was added and growth was monitored for the times indicated. pRNA6; RBS = GUGUG, MBS = CACAC: T8; RBS = GUGUG, MBS = UGCUU: T31; RBS = GUGUG, MBS = AGCAG: T75; RBS = GUGUG, MBS = UACAG: T80; RBS = GUGUG, MBS = GUCAG.

curring in cultures expressing the lethal constructs, one of the lethal clones isolated in this study (T8) and pRNA6 were tested for the presence of CAT activity in the culture medium. Cultures containing the lethal sequences were grown in the presence or absence of IPTG and the cells were pelleted by centrifugation. Separate CAT assays were then performed on the cell pellets and supernatants from each culture. Analysis of the growth medium revealed that CAT activity was present in the supernatant of cultures only after induction (data not shown).

MBS in lethal clones is responsible for cell lysis

Because both the MBS and RBS were mutated in these clones, lethality could be due to either of these sequences or to specific MBS–RBS combinations. To determine which sequences were responsible for lethality, derivatives of pRNA9 were constructed containing various combinations of the mutant and wild-type MBS and RBS. The mutant MBS and RBS in these constructs are from pRNA6 (Table 3). Eleven of the selected, nonlethal chloramphenicol-resistant mutants were also tested.

Cells were grown in LB-Ap100 medium either in the presence or absence of 1 mM IPTG. The growth characteristics of these cultures are shown in Figure 7. These data demonstrate that expression of 16S RNA with the MBS 5′-CACAC (MBS-6 from pRNA6) in our system causes an initial decrease in growth rate followed by a decrease in the OD_{600} of the culture within approximately 3 h. Uninduced cells or cells expressing the wild-type MBS, 5′-CCUCC (MBS-wt), all grew at the same rate and appeared to be unaffected by induction of plasmid-derived rRNA synthesis. Cell growth was also unaffected by the presence of the RBS 5′-GUGUG (RBS-6 from pRNA6) or by the presence or absence of complementarity between the MBS and RBS. Cultures containing selected nonlethal mutant MBSs did not show the lethal phenotype, but grew more slowly than those containing wild-type sequences. Samples of each culture were removed at intervals following induction, centrifuged, and the cell pellet and supernatant were assayed separately for CAT activity (data not shown). In the absence of inducer, CAT activity occurred almost exclusively in the cell pellet. However, following

TABLE 3. Plasmids used in this study.[a]

Plasmid	Marker	MBS (*rrnB*)	RBS (*cam*)
pBR322	Ap^R, Tet^R	NA	NA
pRNA6	Ap^R	5′-CACAC-3′	5′-GUGUG-3′
pRNA7	Ap^R	5′-CACAC-3′	5′-GGAGG-3′
pRNA8	Ap^R	5′-CCUCC-3′	5′-GUGUG-3′
pRNA9	Ap^R	5′-CCUCC-3′	5′-GGAGG-3′
pRNA-rMBS	Ap^R	5′-DDVDD-3′	5′-GUGUG-3′
pRNA-rMBS-rRBS	Ap^R	5′-DDVDD-3′	5′-HHBHH-3′

[a] Abbreviations: B = C, G, and U; D = A, G, and U; H = A, C, and U; V = A, C, and G.

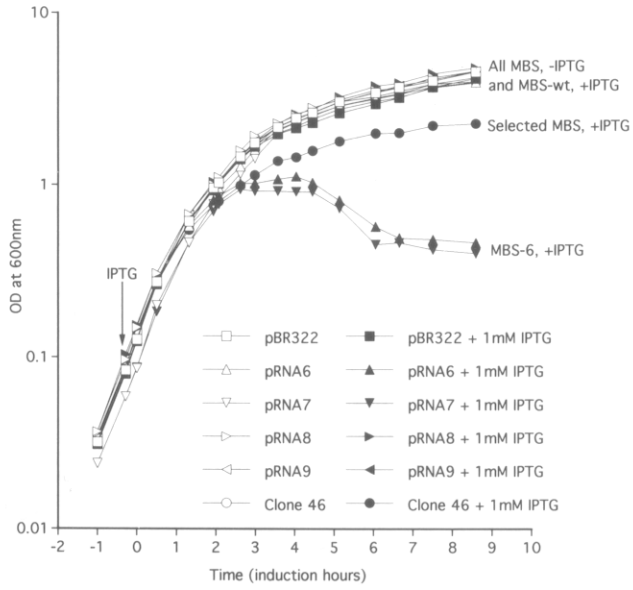

FIGURE 7. Effect of message-binding sequences on growth. Duplicate cultures of DH5 containing pRNA9 derivatives were grown in LB-Ap100 medium and monitored at 600 nm. At $OD_{600} = 0.1$, IPTG was added to the cultures and growth was monitored for the times indicated. pBR322; vector: pRNA6; RBS = GUGUG, MBS = CACAC: pRNA7; RBS = GGAGG (wt), MBS = CACAC: pRNA8; RBS = GUGUG, MBS = CCUCC (wt): pRNA9; RBS = GGAGG (wt), MBS = CCUCC (wt): Clone 46; RBS = AUCCC, MBS = GGGAU.

induction, cultures expressing the MBS-6 sequence contained significant CAT activity in the supernatant, whereas cultures expressing the MBS-wt sequence or one of the selected nonlethal sequences (selected-MBS) contained CAT activity only in the cell pellet. Thus, the observed decrease in cell density in the lethal isolates was due to cell lysis and this lysis was in turn due to expression of the mutant MBS in these clones.

The MBS of each lytic clone was sequenced and these sequences are shown in Table 4. The only characteristic shared by all of the lethal sequences, including that found in pRNA6, is the presence of a cytosine residue in the center (M3) of the MBS. Also, three of our four sequences and pRNA6 contain an adenosine residue in position M4.

DISCUSSION

Random mutations were constructed in the 5 nt comprising the MBS of a cloned copy of the 16S rRNA gene and simultaneously at the complementary 5 nt of the consensus RBS on the CAT message. From this random pool, functional interactions between the mutated RNAs were identified by selecting recombinants that produced active CAT and were therefore resistant to chloramphenicol. These sequences were analyzed for sequence identity, complementarity, and the effect of specific nucleotides upon translational efficiency. In addition, several lethal sequences were identified and analyzed.

Nucleotide preferences at each mutated position

One explanation for the observed nonrandom distribution of nucleotides in the selected sequences is that, because the wild-type MBS, CCUCC, was excluded from each of the mutated MBS residues, compensatory mutations favoring the complementary sequence (GGAGG) might be expected at each position to maintain a free energy similar to that found in the wild-type interaction. Two observations, however, are inconsistent with this explanation. First, only one fourth of translational efficiency can be explained by free energy of association between selected sequences (Fig. 5). This relationship is even less significant for wild-type RBS sequences (Schurr et al., 1993). Secondly, no preference for cytosine within the RBS was observed at any of the mutated positions. Because nucleotide preference was most significant in the MBS, it is possible that the sequences that were selected against were nonfunctional either because they caused unbalanced translation of wild-type mRNAs or because they permitted the formation of secondary structures within 16S rRNA that interfered with translation initiation.

Effect of nucleotide identity upon translational efficiency

The set of mutated nucleotides that affected CAT production and those that showed nonrandom distribution were significantly different. One explanation for this may be that an effect of nucleotide identity on activity exists, but that it is dependent upon intermolecular or intramolecular interactions.

We looked for possible interactions within each of the mutated sequences by determining if the presence of a particular nucleotide at one position affected nucleotide selection at other mutated positions. Only one such interaction was identified within the RBS and this was between R3 and R4 (Fig. 3). It should be noted that, although R3 and R4 showed significant sequence covariation, no nucleotide preferences were apparent

TABLE 4. Sequence analysis of lethal isolates.[a]

Clone	16S rRNA sequence
wild type	3′ A U U C C U C C A C 5′
pRNA6	3′ A U U C A C A C A C 5′
T8	3′ A U U U U C G U A C 5′
T31	3′ A U U G A C G A A C 5′
T75	3′ A U U G A C A U A C 5′
T80	3′ A U U G A C U G A C 5′

[a] Mutated MBS is underlined.

at position R3. This may indicate that the more critical nucleotide for function is R4.

In contrast, nucleotide identity at three positions (M2–M4) within the MBS was shown to affect nucleotide selection at other MBS positions significantly. Nucleotide identity at positions M2 and M4 each strongly affected selection of nucleotides at two other positions. Sequence analysis of covarying pairs revealed no selection for or against complementarity, indicating that MBS secondary structure was probably not responsible for the observed covariation. One explanation for these data is that the relationships observed within the selected MBSs are due to interaction of this sequence with either other loci within the 16S RNA or with other RNAs in the cell. To determine if the observed covariation might be due to selection against previously identified lethal MBSs (Table 4), the nucleotide pairs that were selected against were compared with the nucleotides found in the corresponding positions of lethal MBSs. These analyses revealed no similarity between the excluded covarying pairs and those found in the lethal sequences with the exception of positions M3 and M4. Cytosine at position M3 and both adenine and uracil at position M4 appear to have been strongly selected against in the functional isolates. Interestingly, these nucleotides were present in each lethal sequence we characterized. This observation is discussed below.

Alignment of the paired sequences

Alignment of the start codon of the message with the P site of the ribosome is believed to be a critical parameter affecting the rate of initiation and consequently translational efficiency (McCarthy & Brimacombe, 1994). A prediction of this model is that factors that inhibit proper alignment of these sites will also inhibit translational efficiency. One such factor is clearly the distance between the site of MBS–RBS pairing and the ribosomal P site. Thus, translational efficiency should be highest when the distance between the MBS–RBS paired nucleotides and the start codon is approximately the same as the distance between the MBS–RBS paired nucleotides and the P site. Conversely, little or no translation will occur at distances less than the minimum necessary for the start codon to reach the P site. However, because mRNA is reasonably flexible, distances greater than the minimum required for optimal alignment should still allow translation, albeit at reduced efficiency because this should decrease the local concentration of the AUG with respect to the P site.

In the random mutants, the 3′16S–AUG distance ranged from 11 to 18, with an optimum of 12 nt. Mutants in which the distance was greater than the optimum retained significant activity, presumably due to the flexibility of the mRNA. Mutants in which the distance was less than the optimum, however, showed a sharp decrease in activity that presumably represents the distance beyond which the start codon could not reach the P site. In our studies, this distance was 11 nt. Chen et al. (1994) performed a similar analysis by varying the distance between the start codon of a cloned CAT construct and the site of MBS–RBS pairing. In their study, however, only the CAT construct was mutated. These authors also distinguish between the distance from the 3′ paired nucleotide of the RBS and the first nucleotide of the CAT start codon, which they term "spacing," and the distance from a reference nucleotide in the MBS and the aligned distance to the CAT start codon, which they term "aligned spacing." Because in our studies the MBS was also mutated, the 3′ nucleotide of 16S RNA was used as a reference point instead of a nucleotide in the MBS. If the data of Chen et al. are converted to our format, they indicate an optimal *spacing* of 5 nt, which agrees with our finding of 5 nt for the RBS–AUG distance (Fig. 4A,B, RBS–AUG) in our most highly expressed constructs. The optimal *aligned spacing* (Fig. 4A,C, 3′16S–AUG) reported by these authors was 14 nt, however, significant activity (80%) was retained in constructs in which the aligned spacing was from 12 to 16 nt. In our mutants, the mean 3′16S–AUG distance was 13.9 nt in the expressed mutants and maximal activity was observed in constructs with a 3′16S–AUG distance of 12 nt. In our mutants, the 12-nt optimum also represented a minimum below which activity dropped precipitously. However, constructs with greater spacing retained significant activity. Chen et al. (1994) also found a minimum of 12 nt, and their constructs also retained significant activity as the distance increased. Thus, our data are in agreement with these authors' findings and it appears that the distance from the 3′ end of 16S RNA to the ribosomal P site in *E. coli* corresponds to a distance of 12 nt in the message.

Dominant lethal mutations

Because only five lethal mutants were analyzed, speculation regarding the cause of lethality must be regarded with caution. However, it is interesting to note that all of the sequences that were lethal contained a cytosine residue in the center position (M3) of the mutated MBS of 16S RNA. The MBS used in the specialized ribosome system of Hui and de Boer (1987) also contains a cytosine in this position and, as reported here and by others (Wood & Peretti, 1991), this sequence also causes lysis following induction. Mutants that were selected for resistance to chloramphenicol did not lyse, but grew more slowly than the wild-type when induced. This may have been due to the additional metabolic load placed upon the cells during synthesis of mutant ribosomes that were not involved in translation of wild-type messages. In addition, only 5 of the 48 functional mutants contained a C at position

M3 (Table 1), indicating that a C in this position was strongly selected against (see also Fig. 2).

One explanation for the observed lethal phenotype among the MBS mutants containing a cytosine in the M3 position is that the resulting sequence permits toxic overexpression of one or more host genes. Jacob et al. (1987) showed that a single C1538 to U mutation at position M4 slowed cell growth strongly and caused severe disruption of translational balance as indicated by 2D PAGE analysis of total *E. coli* proteins. We examined manually the upstream regions of those *E. coli* genes for which a sequence was available to identify sequences that were complementary to all of the lethal MBS sequences. This analysis revealed four genes containing sequences that might serve as RBSs with the lethal MBSs. The sequences are *sdhA*, *fhuD*, *cynT*, and *rimK*. All of these genes encode membrane proteins with the exception of *rimK*, which encodes the ribosomal protein S6 modification enzyme. Of particular note is the *sdhA* (succinate dehydrogenase flavoprotein subunit) gene (5′. . . GUGUGGGGUGUGUGAUG . . . 3′, start codon underlined), which shows complementarity to all of the mutants, but was most complementary with the most lytic MBS, MBS-6.

CONCLUSIONS

We mutated both the RBS of the CAT message and the MBS of 16S RNA and selected functional alternative pairing interactions. Analysis of these sequences indicates that a threshold for minimal expression exists and is determined primarily by motifs within the nucleotides of the 3′16S rRNA sequence.

From our data, the primary factors affecting the functional threshold are alignment of the start codon with the ribosomal P site and the existence of deleterious MBSs.

Another indication of MBS constraints is the occurrence of highly significant nucleotide covariation within the MBS. At three positions within the MBS, certain nucleotide pairs are preferred, whereas others appear to be selected against. Although the reasons for this are unclear, it does not appear to be due to pairing interactions within the MBS, pairing with fixed sequences adjacent to the RBS, or to pairing with lethal sequences. Pairing between rRNA and mRNA is presumably strongly affected by the structures of both the RBS and MBS. Thus, one explanation for the observed covariation between specific nucleotides within the same sequence is that they permit formation of specific structures that facilitate or are required for pairing with the complementary sequence on the opposite molecule. Evaluation of this possibility will require fine structure studies of highly functional mutant MBS–RBS combinations, such as those performed by Schindelin et al. (1995) on the wild-type sequences.

Other studies have found that the strength of the MBS–RBS interaction correlates poorly with the level of translation (Melançon et al., 1990). Schurr et al. (1993) reported a mean ΔG°_{37} of −4.9 kcal/mol for all known *E. coli* genes and concluded that average free energy values from highly or poorly expressed genes did not deviate significantly from this mean. From our data, it appears that mRNA–rRNA affinity at the mutated sites is most significant in messages that are highly expressed and that the overall effect of free energy between the mRNA and rRNA, although significant, is not substantial.

The data presented here demonstrate that significant constraints exist in the formation of an efficient Shine–Dalgarno interaction, but that stable and efficient alternatives to the wild-type interaction are possible. These data also provide a functional framework for future analyses of the structural constraints upon the Shine–Dalgarno interaction and its role in the initiation of protein synthesis in procaryotes.

MATERIALS AND METHODS

Reagents

Restriction enzymes, ligase, and calf intestine alkaline phosphatase were from New England Biolabs and from Gibco-BRL. Sequenase-modified DNA polymerase, nucleotides, and sequencing buffers were from USB/Amersham. Oligonucleotides were either purchased from Midland Certified Reagent Company (Midland, Texas) or synthesized on-site using a Beckman Oligo 1000 DNA synthesizer. Amplitaq DNA polymerase and PCR reagents were from Perkin-Elmer-Cetus. [^{3}H]-Chloramphenicol (30.1 Ci/mmol) was from Amersham and [α^{35}S]-dATP (1,000 Ci/mmol) was from New England Nuclear. Other chemicals were from Sigma.

Plasmids

Plasmids used in this study are described in Table 3. A plasmid map of pRNA8 (accession U72488) and its derivatives is shown in Figure 1. The key features of this construct are: (1) it contains a copy of the *rrnB* operon from pKK3535 (Brosius et al., 1981) under transcriptional regulation of the *lacUV5* promoter; (2) it contains a copy of the lactose repressor allele *lacI^q* (Calos, 1978); (3) the CAT gene (*cam*) is present and transcribed constitutively from a mutant tryptophan promoter, *trp^c* (de Boer et al., 1983; Hui et al., 1987); (4) the RBS of the CAT message has been changed from the wild-type, 5′-GGAGG to 5′-GUGUG (mutations underlined) (Hui & de Boer, 1987), and the MBS of the 16S rRNA gene remains wild-type (5′-CCUCC); (5) the β-lactamase gene is also present to allow maintenance of plasmids in the host strain.

Bacterial strains and media

All plasmids were maintained and expressed in *E. coli* DH5 (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*) (Hanahan, 1983). Cultures were maintained in LB medium (Luria & Bur-

rous, 1957) or LB medium containing 100 μg/mL ampicillin (LB-Ap100). To induce synthesis of plasmid derived rRNA from the *lacUV5* promoter, IPTG was added to a final concentration of 1 mM at the times indicated in each experiment. Strains were transformed by electroporation (Dower et al., 1988) using a Gibco-BRL cell porator. Unless otherwise indicated, transformants were grown in SOC medium (Hanahan, 1983) for 1 h prior to plating on selective medium to allow expression of plasmid-derived genes.

Mutagenesis

Random mutagenesis of the MBS and RBS in pRNA8 was performed essentially by the method of Higuchi (1989). The overall procedure is shown in Figure 1. For each set of mutations, four primers were used: Two "outside" primers and two "inside" primers.

To mutate the MBS, two outside primers were designed to anneal to either side of the *Dra* III and *Xba* I restriction sites in pRNA8 (Fig. 1). These primers were ASD-B, 5′-GGCGAC TTTCACTCACAAAC-3′ and UNK#3, 5′-GTAATCGTGGAT CAGAATGC-3′. Inside primers for the MBS were ASD-R1, 5′-GTTGGATCADDVDDTTACCTTAAAGAA-3′ and ASD-R2, 5′-TAAGGTAAHHBHHTGATCCAACCGCAGGT-3′ (mutated residues underlined; D = A + G + T; V = A + C + G; H = A + C + T; B = C + G + T). The MBS of pRNA8 was mutated using these primers and the mutated PCR product was gel purified, digested with *Dra* III and *Xba* I, and ligated into pRNA8 that had been digested with the same enzymes. A total of 66,000 transformants was pooled and used to prepare plasmid (pRNA–rMBS, Fig. 1) for the next step.

To mutate the RBS, two outside primers were designed to anneal to either side of the *Kpn* I and *Xho* I restriction sites in pRNA8 (Fig. 1). These primers were OR2, 5′-AAATCGTC GTGGTATTCACT-3′ and SD-R2, 5′-TTCTCCTTACGCATCT GTGC-3′. Inside primers for the RBS were SD-R1, 5′-TCCATT TCTCGAGDDVDDTGAAAGCGGCCGCTTC-3′ (mutated residues underlined) and SD-R3, 5′-CTCGAGAAATGGAGA AAAAA-3′. The RBS of the CAT gene in pRNA8 was mutated using these primers and the mutated PCR product was gel purified, digested with *Kpn* I and *Xho* I, and ligated into the pRNA–rMBS pooled plasmid that had been digested with the same enzymes.

Transformants were incubated in SOC medium containing 1 mM IPTG for 4 h to induce rRNA synthesis and the induced transformants were plated on a series of LB agar plates containing chloramphenicol at varying concentrations.

CAT assays

CAT activity was determined essentially as described (Nielsen et al., 1989). Cultures for CAT assays were grown in LB-Ap100. Briefly, 0.5-mL aliquots of mid-log cultures (unless otherwise indicated) were added to an equal volume of 500 mM Tris-HCl, pH 8, and lysed using 0.01% SDS and chloroform (Miller, 1992). The resulting lysate was either used directly or diluted in assay buffer prior to use. Assay mixtures contained cell extract (5 μL or 10 μL), 250 mM Tris, pH 8, 214 μM butyryl-coenzyme A (Bu-CoA), and ^{3}H-chloramphenicol in a 125-μL volume. Two concentrations of lysate were assayed for 1 h at 37 °C to ensure that the signal was proportional to protein concentrations. The product, butyryl-^{3}H-chloramphenicol was extracted into 2,6,10,14-tetra-methyl-pentadecane (TMPD)-xylenes (2:1) and measured directly in a Beckman LS-3801 liquid scintillation counter. Blanks were prepared exactly as described except that uninoculated LB medium was used instead of culture.

MICs

MICs were determined by standard methods in microtiter plates. Overnight cultures grown in LB-Ap100 were diluted and induced in the same medium containing 1 mM IPTG for 2–3 h. Approximately 10^4 induced cells were then added to wells containing LB-Ap100 + IPTG (1 mM) and chloramphenicol at 50, 100, 200, 300, 400, 500, 600, 700, and 800 μg/mL for induced (1 mM IPTG) cultures and 50, 100, 150, 200, 300, and 400 μg/mL for uninduced (no IPTG) cultures unless otherwise indicated. Cultures were grown 24 h and the lowest concentration of chloramphenicol that completely inhibited growth was designated as the MIC.

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