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Search for the optimal sequence of the ribosome binding site by random oligonucleotide-directed mutagenesis

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ABSTRACT

Synthetic DNA duplexes corresponding to the ribosome binding site (RBS) were synthesized through the phosphite method on solid support. The synthetic RBS DNA with partial random sequences was inserted into an appropriate site between the *lpp-lac* promoter and the β-galactosidase structural gene in plasmid pMKT2. The level of β-galactosidase expression was correlated with the color intensity of the recombinant colonies on X-gal plates. The bluest colonies were isolated and characterized with respect to β-galactosidase enzyme activity and RBS sequence. There was good correlation between color intensity and the level of the enzyme activity, and this provided a reliable phenotypic screening method in the search for the optimal regulatory sequences. Novel RBS sequences obtained here show not only the unique nucleotide distribution, but also strong complementarity to the 3' end region of 16S rRNA, from which could be deduced a generalized RBS sequence, the position of the SD region, and the 16S rRNA position mediated during translation initiation.

INTRODUCTION

Translational initiation in *E.coli* is a multicomponent process requiring interactions between RNA species (mRNA, 16S rRNA and fMet-tRNA^{fMet}), initiation factors (IF-1, -2, and -3), and the ribosomal proteins (1). Shine and Dalgarno postulated that base-pairing between purine-rich sequences upstream from the AUG initiation codon in mRNA and complementary sequences near the 3' end of *E. coli* 16S rRNA might occur during translational initiation (2). Thus far, several hundred bacterial and phage mRNAs have been sequenced (3,4). Nearly all of the mRNAs include the sequences complementary to the 3' end of 16S rRNA 5 to 9 bases upstream from the AUG initiation codon. Moreover, the RBS sequence is positively mediated in the formation of the initiation complex which then affects the

regulation of protein synthesis in *E. coli* (1-3). Several essential sequential parameters influence the formation of the translational initiation complex including the length of mRNA complementary to 16S rRNA (3,4), the distance (spacer) between the Shine-Dalgarno (SD) sequence and the initiation codon (5-7), the extent to which the Shine-Dalgarno sequence and AUG are masked by the secondary structure (4,5,8,9), and the nucleotide sequences on either side of the AUG initiation codon (10,11) as well as on the spacer (12).

To date, there have been a number of studies examining the functional regulatory genes in relation to translation efficiency as influenced by RBS nucleotides (12-14). Most of the studies emphasized the significance of the individual nucleotide rather than the group of nucleotides in the region, because point mutagenesis provided only the effect of the individual nucleotide (15-17). In this study, random oligonucleotide-directed mutagenesis at a targeted region was employed to investigate the significance of the nucleotide combination as a sequence, not the effect of an individual nucleotide on translational efficiency.

We report here in that the construction of an expression vector by the replacement of an RBS sequence with synthetic random DNA and the selection of the optimal sequence allowed us a series of novel RBS sequences. A direct screening system relating β-galactosidase activity to the color intensity of colonies on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin has been developed to identify those RBS sequences which have a greater effect on β-galactosidase gene expression. Then, the plasmids obtained from these colonies were directly sequenced in the region of the RBS. The results augment the understanding of the translational role of the ribosome binding site on the basis of the primary structure. Also a variety of novel RBS sequences was explored through this approach.

MATERIALS AND METHODS

Strain, plasmids, and enzymes

E.coli strain JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, λ^-, Δ(*lac-pro*AB), F'*tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$)

was used for transformation. The $CaCl_2$ procedure and Hanahan's method were employed for the transformation of *E. coli* (18, 19). The sources of the *lpp-lac* promoter and the β-galactosidase gene were the plasmids pINIII-A1 (20) and pORF2 (21), respectively. Standard molecular cloning methods for isolation of the plasmid, digestion by restriction enzymes, kination, and ligation were employed (22).

All restriction endonucleases, T4 DNA ligase, and Klenow fragment were purchased from KOSCO enzymes (Seoul, Korea) and New England Biolabs, and the enzymatic reactions were done as directed by the manufacturer.

Oligonucleotide synthesis

Oligonucleotides were synthesized through the phosphite triester method on solid support by an automated DNA synthesizer (Beckman System Plus). Modified deprotection was adopted to simplify the procedure by employing a single treatment of conc. NH_4OH at 50°C for 12 hours. The full length DNA was purified by polyacrylamide-urea gel electrophoresis (23, 24).

Synthetic dsDNA-1 :

```
          XbaI EcoRI  SD           I.C. SalI  HindIII
5'-CTAGAGAATTCGAGGGTATTAATAATGCAGTCGACA
     TCTTAAGCTCCCATAATTATTACGTCAGCTGTTCGA-5'
```

Synthetic dsDNA-2 :

```
          EcoRI              SphI   SalI  HindIII
5'-AATTCGPPPPNNNNNNNGCATGCAGTCGACA
     GCYYYYNNNNNNNCGTACGTCAGCTGTTCGA-5'
                     I.C.
```

P, Y, and N stand for purine, pyrimidine, and random bases with equivalent molar ratios, respectively. I.C. stands for the initiation codon.

Synthetic primer for sequencing:

5'-GTTTTCCCAGTCACGACGTTGTA

The position of the primer is 13 bases downstream from the unique BamHI site.

Annealing

Annealing of the synthetic oligonucleotides for dsDNA (36 base long dsDNA-1, and 31 base long dsDNA-2) was performed by mixing an equimolar ratio of the corresponding oligonucleotides in an annealing buffer containing 0.1 M NaCl, 10 mM Tris-HCl pH 7.8, and 10 mM EDTA, heating to 98°C for 5 minutes, and followed

by overnight slow-cooling of the solution to room temperature.

Construction of plasmids, pMKT1, pMKT2 and pMKT2-series

After digestion of plasmids, pINIII-A1 and pORF2 with both PstI and BamHI restriction endonucleases, a small DNA fragment (about 1.1 kilo base pairs) carrying both the *lpp-lac* promoter and the RBS, and a large DNA fragment (about 5.5 kilo base pairs) carrying the entire β-galactosidase gene were obtained, respectively. Following the ligation of these two fragments with T4 DNA ligase, *E. coli* strain JM109 was transformed with the resulting plasmid pMKT1 by the $CaCl_2$ procedure.

Since pMKT1 does not have the correct reading frame for the β-galactosidase gene, the small XbaI-HindIII fragment of pMKT1 was replaced with the synthetic dsDNA-1 carrying the RBS sequence and XbaI and HindIII cohesive ends (Fig. 1). The resulting plasmid named pMKT2 was used to transform JM109 cells. The colonies were grown on LB plates containing ampicillin, X-gal and IPTG. To confirm the construction of pMKT2, plasmid DNA was purified from the blue colonies, and analyzed with several different restriction endonucleases.

For the construction of the pMKT2-series, the synthetic dsDNA-2 was phosphorylated with T4 polynucleotide kinase and joined with the large EcoRI-HindIII fragment of pMKT2 using T4 DNA ligase. The resulting plasmids possibly containing multicopies of dsDNA-2 were digested with HindIII to remove any extra copies. Then, the resulting large fragment possessing only one dsDNA-2 was electroeluted, self-joined in the presence of T4 DNA ligase, and transformed JM109 cells through Hanahan's method.

β-Galactosidase assay

For the β-galactosidase assay, *E.coli* were cultured overnight in M9 minimal media (3 mL) containing ampicillin (40 μg/mL), and subcultured in fresh minimal media for 3 hours. The expression of the β-galactosidase gene was induced by adding IPTG (1 mM), and the culture was allowed to grow at 37°C for 3 hours. A β-galactosidase assay of the samples was performed according to Putnam and Koch (25). The harvested cell fraction in Z buffer (1 mL) supplemented with chloroform (10 μL) and 0.1% SDS solution (50 μL) was reacted with *o*-nitrophenyl-β-D-

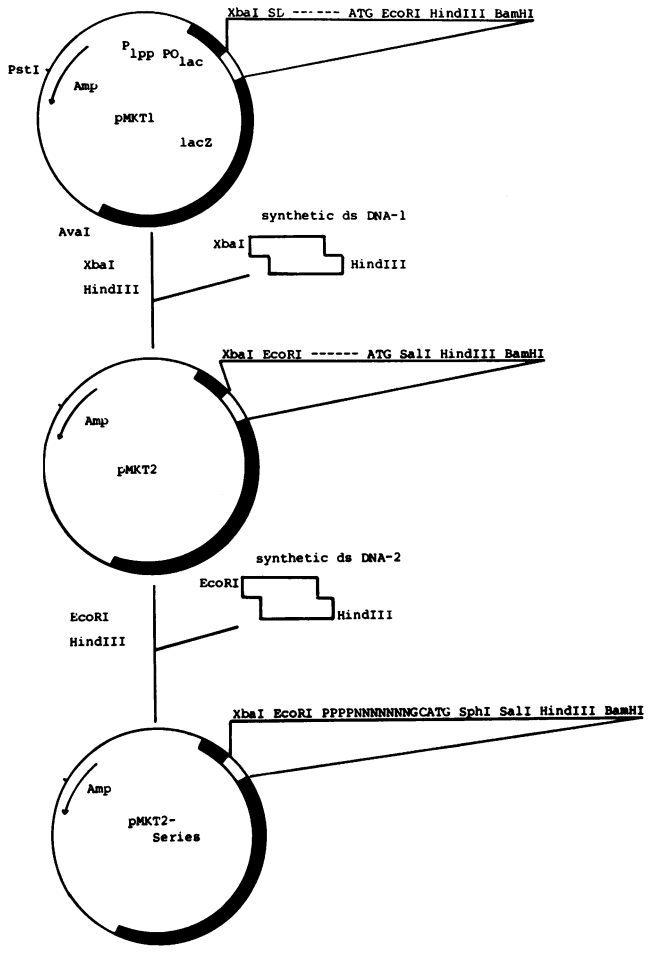


Fig. 1. Scheme for the construction of pMKT2 and the pMKT2-series. The exact sequences of dsDNA-1 and -2 are shown in the Materials and Methods. P_{lpp} and $P.O_{lac}$ mean *lpp* promoter and *lac* promoter-operator of *E.coli*. The β-lactamase and β-galactosidase genes are indicated as Amp and lacZ, respectively.

galactoside (0.2 mL, 4 mg/mL) at 28°C, and then the reaction was stopped with 1 M Na_2CO_3 solution. After the cell debris was removed by microcentrifugation, the optical density was measured at 420 nm and β-galactosidase activity was calculated, according to the following equation; β-Galactosidase activity (unit) = $OD_{420\ nm} \times 1000/(t \times V \times OD_{600})$ (t, reaction time, min ; V, assay volume, mL).

DNA sequencing

The modified dideoxynucleotide chain termination method was employed for the direct determination of RBS sequences on the double-stranded plasmid DNA. For denaturation, the plasmid DNA was incubated at room temperature for 5 minutes in the presence of 2 M NaOH and 2 mM EDTA and was ethanol-precipitated. The usual polymerization reaction was followed (26).

Computer analysis

Computer searches were performed with the Microgenie program purchased from Beckman Co.

RESULTS AND DISCUSSION

Random mutagenesis at the targeted region with synthetic DNA proved to be one of the most powerful methods for selecting meaningful DNA sequences from random nucleotide combinations. The novel RBS sequences obtained here provided new insight into the ribosome binding site in terms of sequence and position. This approach can be extended further to study and isolate useful genes from certain regulatory systems in bacteria and other organisms.

In order to search for the optimal RBS sequence through random targeted mutagenesis, a plasmid system which possesses a known regulatory and usable structural gene, convenient cloning sites, and an easy selection system was chosen. For that, we employed the *lpp-lac* promoter and β-galactosidase structural gene which provided a great deal of convenience in the proceeding experiments. Plasmid pMKT1 was constructed from pINIII-A1 (20) and pORF2 (21), which possess the *lpp-lac* regulatory sequence and the complete β-galactosidase gene, respectively (Fig. 2). Then, the synthetic dsDNA-1 was introduced not only to provide new restriction enzyme sites, EcoRI and SalI, but also to allow for the correction of the reading frame of the β-galactosidase gene in phase. The joining of the synthetic dsDNA-1 and the large XbaI-HindIII fragment of pMKT1 yielded pMKT2, in which the RBS sequence was identical to that of the *lpp* regulatory gene, the EcoRI site was shifted to just upstream from the SD region, and the new SalI site was created just following the AUG initiation codon. This facilitated the construction of pMKT2-series with the synthetic dsDNA-2.

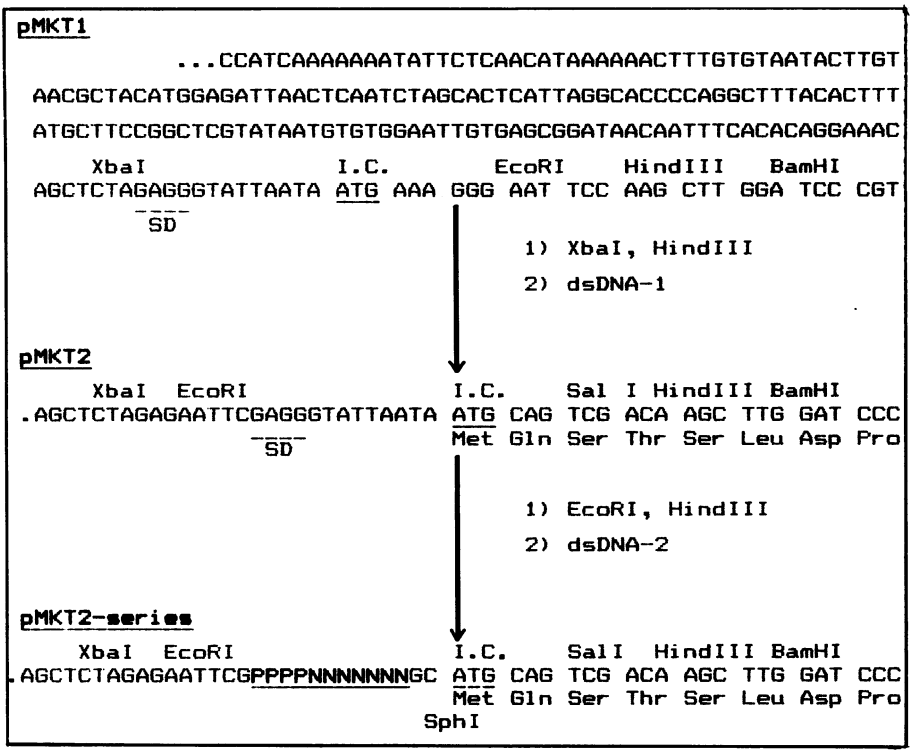


Fig. 2. Scheme for experimental strategy for mutagenesis with the synthetic dsDNA-1 and -2. I.C. is for the initiation codon. P and N stand for purine and random nucleotide, respectively.

The synthetic dsDNA-2 was designed to replace the existing *lpp* RBS DNA with random nucleotides in order to examine the effects of the sequences at the ribosome binding site on gene expression. It possesses the plausible SD region (-13 to -10) and seven base spacer (-9 to -3) in which purines (A or G) and all 4 (A, C, G, and T) nucleotides have been incorporated in an equimolar ratio, respectively. Two bases at -2 and -1 (GC) immediately preceding ATG were fixed to create the SphI site which would be an useful cloning site and provide an initiation codon for any structural gene to be expressed. The small EcoRI-HindIII fragment of pMKT2 was replaced with the synthetic DNA-2, yielding the pMKT2-series.

E.coli strain JM109 was transformed with the ligation

Table 1. Novel RBS sequences obtained from random mutagenesis and their relative β-galactosidase activities.

Mutant Number	RBS Sequences												Relative β-gal. Activity
	−15			−10				−5				+1	
1	gaauucg	A	A	G	G	A	G	C	U	U	U	gcaug	311.5
2		A	A	G	G	A	G	G	U	A	G	U	199.7
3		A	G	G	A	G	U	A	U	U	G	A	156.7
4		A	G	G	A	G	A	U	C	G	C	C	147.7
5		G	G	A	G	G	A	U	U	A	A	C	146.7
6		G	A	G	G	U	U	A	A	A	C	G	142.7
7		A	A	G	A	U	U	A	G	A	A	A	142.7
8		G	A	G	G	A	A	A	A	C	U	A g	129.9
9		A	A	G	G	A	G	G	C	U	G	A	127.7
10		A	A	G	A	G	G	C	U	U	U	U	89.7
11		A	G	A	G	U	U	A	C	U	A	A	86.9
12		A	A	G	G	A	G	G	U	A	C	U	76.9
13		G	G	G	G	G	U	G	C	U	A	G	52.0
14		A	A	G	G	C	C	A	C	A	U	G	47.0
15		A	A	G	A	G	C	A	U	G	A	G	38.9
16		G	G	A	G	G	C	G	A	G	U	C	35.8
pMKT2	gaauuc	G	A	G	G	G	U	A	U	U	A	A taaugc	100.0

The RBS sequence from pMKT2 is identical to that of the *lpp* signal sequence. The initiation and termination codons preceding the actual initiation codons are underlined. The relative β-galactosidase activity is an average value from three individual experiments. The actual value of β-galactosidase activity of mutant −1 (pMKT2-1) was 16905 units.

mixture which contained all the mutations. About 1000 blue colonies of varying intensity appeared on the plate. We assumed that color intensity is linearly proportional to the expression level of the β-galactosidase gene. To confirm this assumption, we selected three hundred colonies exhibiting a variety of intensities, and assayed for β-galactosidase activity. The results indicated that the color intensities proportionally reflected the activities of β-galactosidase in the range of six hundred-fold (data not shown). Among the colonies examined, sixteen intensely colored colonies were characterized with respect to β-galactosidase activity and sequence.

Table 2. Statistical data of nucleotide distribution at each position of the RBS sequences.

Nucleotide	Position										
	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3
Group A											
A	6	6	1	3	4	3	4	2	4	2	4
G	3	3	8	6	3	3	2	1	1	3	1
C					-	-	1	2	1	2	2
T					2	3	2	4	3	2	2
Group B											
A	5	4	2	2	1	-	3	1	2	3	1
G	2	3	5	5	4	2	3	-	2	-	3
C					1	3	1	3	-	1	1
T					1	2	-	3	3	3	2

This data was obtained from sixteen RBS sequences described in Table 1. Group A and B correspond to the mutant -1 to -9, and -10 to -16, respectively. The former exhibited greater enzyme activity than that of pMKT2. Adenosine (A) of the initiation codon was numbered as +1. Positions -10 to -13 and positions -3 to -9 were random-mutagenized with dsDNA-2 (See Materials and Methods).

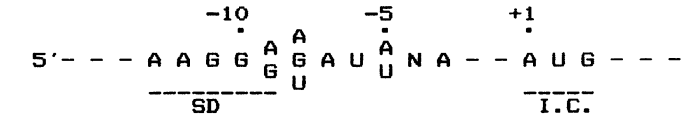
Each plasmid (pMKT2-1 through pMKT2-16) was isolated and its RBS region was sequenced from the double-stranded plasmid DNA by the modified dideoxy chain termination method (Table 1).

The sequential result shows that the nucleotide sequence itself at the spacer substantially affects the translation level. For example, both groups of mutants -1, -2, -9, and -12, and mutants -5 and -16 have identical SD sequences at the same position, but their expression levels vary in the range of 4 fold (Table 1). This indicates that the spacer sequence also appears to be important with respect to translational efficiency.

The resulting RBS sequences can be classified into two groups : Group A consists of 9 mutants with greater expression than pMKT2, and Group B of mutants with diminished expression. Both groups possess a purine rich region which has strong complementarity to the 3' end of 16S rRNA, and an AT-rich spacer (27, 28). In Table 2, Group A which exhibited greater β-galactosidase activity has an overall purine rich combination

especially A-rich. On the other hand, Group B has Gs prevalent on the RBS sequence, and Ts are dominant to As in the AT-rich spacer.

Insight into the sequential context provides an interesting possibility with respect to positional nucleotides. At positions -13 to -10 mutagenized with purine nucleotides, As are predominant at positions -13 and -12, while Gs are in preference to As at positions -11 and -10. But the possibility of other nucleotide combinations can not be ruled out if all four different nucleotides were randomly introduced. Nucleotides at positions -9 to -3 corresponding to the spacer region are purely determined by the phenotypical selection from the random DNA sequences. Positions -9 and -7 strongly prefer purine to pyrimidine nucleotides. The rest of the positions, -8, and -6 to -3, show a strong preference for A and T nucleotides. Accordingly, from the statistical data of nucleotide distribution in Group A (Table 2) the sequential context of the ribosome binding site could be postulated to be the following:



We felt that our sequential data ought to be defined in terms of the SD sequence and its position, because the data originated from the selection by random targeted mutagenesis. In order to deduce the positional information, first, a computer search of the sequential data was made to find the complementarity to the 3' end of *E. coli* 16S rRNA, which was known to be mediated through interaction with mRNA during translation initiation (13, 28). Interestingly, all of the RBS sequences exhibited good complementarity, which again confirmed the necessity of base pairing between 16s rRNA and the RBS sequences, even though it was difficult to find a direct correlation between the degree of the complementarity and the translation efficiency (Table 3).

The positional frequencies of the complementarity at each position of the RBS sequences with 16S rRNA were examined to

Table 3. Comparison of the complementarity of the RBS sequences to the 3′ end sequence of *E. coli* 16S rRNA.

Mutant	Complementarity to the 3′ end of 16S rRNA	number of base matched
	3′ A U U C C U C C A C U A G G U U G G 5′ (16S rRNA)	
	8 4 8 13 15 11 6 9 6 5 9 1 5 (number of	
	14 13 10 13 11 complement.)	
1	5′ G A A G G A G C U U U U G C A U G C 3′	11
2	G A A G G A G G U A G U G C A U G C	12
3	C G A G G A GUA U U G A G C A U G C	9
4	C G A G G A G A UCG C C G C A U G C	10
5	U U C G G G A G G A U U A A C G C A U G	9
6	U U C G G A G G U U A A A C G G C A U G	10
7	U U C G A A G A U U A G A A A G C A U G	8
8	U U C G G A G G A A A A C U A G C A U G	10
9	G A A G G A G GCU G A G C A U G C A	12
10	G G A A G A G GCU U U U G C A U G C	10
11	U U C G A G A G U U A C U A A G C A U G	9
12	G A A G G A G G U A C U G C A U G C	12
13	U U C G G G G G G U G C U A G G C A U G	9
14	U U C G A A G G C C A C A U G G C A U G	7
15	C G A A G A GCA U G A G G C A U G C	10
16	U C G G G A G G C G AGU CGC A U G C	13
pMKT2	U U C G A G G G U A U U A A U A A U G	7

The dots above the letters indicate the complementarity to the 3′ end sequence of 16S rRNA. The position for the random mutagensis and the initiation codon are underlined.

Table 4. The positional frequency of complementarity at each position of the novel RBS sequences with the 3' end of *E.coli* 16S rRNA.

Postion	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
Freq.	7	13	14	14	14	14	6	11	9	4	6	9	0	16
%	44	81	88	88	88	88	38	69	56	25	38	56	0	100

This data is derived from Table 3. The G and C residues at the positions -2 and -1 of every RBS sequence here have 0 % and 100 % complementarity, respectively, to a certain nucleotide of the 3' end of 16S rRNA.

determine if the position of the sequence could be precisely defined with this data. The results were surprising; positions -13 to -9 of every RBS sequence obtained presented nearly 90% complementarity (Table 4), in which position -9 was randomly synthesized with all four nucleotides. Hence, it can be stated that the SD sequence is located in positions -13 to -9 of the RBS sequences. Furthermore, this finding could be extended to define the SD sequence as $5'AAGG\begin{matrix}A\\G\end{matrix}$, and its position. Conversely, the data of the positional complementarity of 16S rRNA with the RBS sequences (Table 3) could pinpoint the binding position and sequence of 16S rRNS with mRNA. Consequently, the mediation sequence of 16S rRNA appeared to be 3'CCUCCA. Such analyses could infer again that the 3' end of 16S rRNA has a certain role in positioning the mRNA on the ribosome in a flexible manner during translation initiation.

The influence of the mRNA secondary structure on translation efficiency was investigated with a computer program described by Tinoco et al.(33) Although all of the possible secondary structures near and in the RBS sequences were collected (data not shown), no stable secondary structure was found. The analysis, however, indicated that there was some degree of decreasing tendency at the expression level, as the stem structures were more stablized. However, the implication of the secondary structure of mRNA relating to translation level remains to be evaluated.

It has been discussed in other reports that the initiation codon preceding the actual initiation codon of mRNA may greatly

affect translation efficiency (13). Such codons were also found in the RBS sequences of mutants-14 and -15 with different reading frames from the actual initiation codon, and their relatively low β-galactosidase activity could partly be explained by it (Table 1).

We also observed several examples which demonstrated the importance of individual nucleotides. At mutants -1 and -10, the exchange of nucleotides at positions -10 and -9 from GA to AG decreases the expression level three-fold. A single nucleotide change from G to C at position -4 in the mutants -2 and -12 lowered the expression level nearly 3 times (Table 1).

In conclusion, a direct screening system using β-galactosidase activity has successfully selected the meaningful genes by random oligonucleotide-directed mutagenesis at the targeted region. The novel RBS sequences obtained have superb functional efficiencies, as compared with those of the known functional regulatory genes. This approach could be further extended to study the variety of gene functions through change of clone construction and the introduction of a different set of random DNA.

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