The Influence of the Nucleotide Sequences of Random Shine-Dalgarno and Spacer Region on Bovine Growth Hormone Gene Expression

Soon-Young Paik[1], Kyung Soo Ra[2], Hoon Sik Cho[3], Kwang Bon Koo[3], Hyung Suk Baik[4], Myung Chul Lee[5], Jong Won Yun[6] and Jang Won Choi[3,*]

[1]Department of Microbiology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea
[2]Department of Food and Nutrition, Daegu Technical College, Daegu 704-721, Republic of Korea
[3]Department of Bioindustry, College of Life & Environment, Daegu University, Kyungbuk 712-714, Republic of Korea
[4]Department of Microbiology, Busan National University, Busan 609-735, Republic of Korea
[5]Rice Functional Genomics Team, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon 441-707, Republic of Korea
[6]Department of Biotechnology, College of Engineering, Daegu University, Kyungbuk 712-714, Republic of Korea

To investigate the effects of the nucleotide sequences in Shine-Dalgarno (SD) and the spacer region (SD-ATG) on bovine growth hormone (bGH) gene expression, the expression vectors under the control of the T7 promoter (pT7-7 vector) were constructed using bGH derivatives (bGH1 & bGH14) which have different 5'-coding regions and were induced in E. coli BL21(DE3). Oligonucleotides containing random SD sequences and a spacer region were chemically synthesized and the distance between the SD region and the initiation codon were fixed to nine bases in length. The oligonucleotides were annealed and fused to the bGH1 and bGH14 cDNA, respectively. When the bGH gene was induced with IPTG in E. coli BL21(DE3), some clones containing only bGH14 cDNA produced considerable levels of bGH in the range of 6.9% to 8.5% of total cell proteins by SDS-PAGE and Western blot. Otherwise, the bGH was not detected in any clones with bGH1 cDNA. Accordingly, the nucleotide sequences of SD and the spacer region affect on bGH expression indicates that the sequences sufficiently destabilize the mRNA secondary structure of the bGH14 gene. When the free energy was calculated from the transcription initiation site to the +51 nucleotide of bGH cDNA using a program of nucleic acid folding and hybridization prediction, the constructs with values below -26.3 kcal/mole (toward minus direction) were not expressed. The constructs with the original sequence of bGH cDNA also did not show any expression, regardless of the free energy values. Thus, the disruption of the mRNA secondary structure may be a major factor regulating bGH expression in the translation initiation process. Accordingly, the first stem-loop among two secondary structures present in the 5'-end region of the bGH gene should be disrupted for the effective expression of bGH.

Keywords: SD sequence, spacer region, bGH expression, mRNA secondary structure

Many factors affecting expression of the heterologous proteins in E. coli are known e.g. copy number (Uhlin et al., 1979; Choi et al., 1999a), mRNA abundance (Selinger et al., 2000), codon usage and tRNA pool (Ikemura, 1985; Kanaya et al., 1999; Karlin et al., 2001), mRNA secondary structure (Wood et al., 1984; de Smit and van Duin, 1990), termination codon (Poole et al., 1995), and other nucleotide sequences (Scherer et al., 1980; Green and Inouye, 1984). The level of foreign gene expression in E.

coli, however, varies widely for different eukaryotic genes (Kim et al., 2004a; Kim et al., 2005). In prokaryotes, as the transcription and translation processes are tightly coupled, it has been widely accepted that cellular protein levels depend directly on mRNA abundance (Kim et al., 2004b; Park and Kim, 2004). It is not clear whether mRNA levels, however, can alone account for the variation in protein levels. The gene dosage and the transcripts are not the only factors in limiting a high level expression of cloned genes. Low level expression levels have been attributed to inefficient translation initiation of the mRNAs representing the heterologous gene sequences.

It has been generally observed that a Shine

* To whom correspondence should be addressed.
(Tel) 82-53-850-6756; (Fax) 82-53-850-6769
(E-mail) chjawo@daegu.ac.kr

Dalgarno sequence (Shine and Dalgarno, 1974) properly located 5' to an AUG initiation codon, is indispensable for translation (Schoner *et al.*, 1984; Ma *et al.*, 2002). The SD sequence, which is located in the ribosome binding site (RBS) of mRNA, plays an important role in formation of the initiation complex by base-pairing with the anti-SD sequence found at the 3' end of 16S rRNA. Most SD sequences could be slight variations of the consensus sequence, AGGAGG. The effectiveness of an SD sequence is determined by both its base-pairing potential with the anti-SD sequence and its spacing from the start codon (Ringquist *et al.*, 1992; de Smit and van Duin, 1994). Aligned spacing of the SD sequences generally varies from 5 to 13 bases, with optimal spacing of about 8 to 10 bases for *E. coli* genes (Hui *et al.*, 1984; Chen *et al.*, 1994). Variations of the nucleotide sequence between the SD region and the AUG initiation codon (Gold *et al.*, 1981), and sequences outside these regions (Green and Inouye, 1984) have been shown to be important in determining the efficiency of translation. Accordingly, translation initiation is commonly considered to be the rate-limiting step of translation and a major determinant of the overall expression level of a gene in bacteria (Gold, 1988; Draper, 1996). The free energy values gained by this pairing can be calculated using the computed folding program of Zuker (2003) and used as a parameter that reflects the efficiency of translation initiation (Osada *et al.*, 1999). Differences in SD binding potential to the rRNA may also explain differences in protein expression. Highly expressed genes can be predicted through the diverse prokaryotic genomes (Karin and Mrazek, 2000), in which codon usage contributes importantly to setting the level of expression of the gene.

Bovine growth hormone (bGH) is a natural peptide that is produced in the pituitary gland of cattle (Paladini *et al.*, 1983; Secchi & Borromeo, 1997). It has been reported that bGH not only stimulates proportional growth in animals but that the peptide also regulates the metabolism of protein, carbohydrates, and fat (Morikawa *et al.*, 1982). The bGH also elicits a biological response by binding with high affinity to specific receptors on the cell membrane of target cells. Further, bGH has been shown to produce dramatic increases in the milk yield of lactating cows (Bauman *et al.*, 1988). Natural bGH exists as four biologically active variants of 190 or 191 amino acids with a molecular mass approximating 22,000 daltons. The bGH molecule has two disulfide bonds (at the 53-164 and 181-189 positions) and is synthesized as a precursor with 217 amino acids containing a hydrophobic signal peptide of 26 amino acid residues. The signal peptide is removed from the N-terminus during

synthesis and secretion from the pituitary somatotropic cells (Secchi & Borromeo, 1997). Several groups have reported on the expression of bGH in *E. coli* (Schoner *et al.*, 1984; George *et al.*, 1985; Hsiung *et al.*, 1987; Wingfield *et al.*, 1987; Choi *et al.*, 1999b). The expression of unmodified cDNA coding for bGH, however, has proven to be difficult (Wingfield *et al.*, 1987; Tomich *et al.*, 1989; Choi and Lee, 1996) regardless of the promoter strength, the SD sequence, host strains, and culture conditions. These difficulties indicate that the bGH coding sequence has some inherent properties that inhibit a high level expression in *E. coli* which may be one or more of the following: two putative secondary structures at the beginning of the coding region (Tomich *et al.*, 1989); a number of non-preferred codons present in the bGH gene (Seeburg *et al.*, 1983; George *et al.*, 1985); a basic pI value of the bGH (Saito *et al.*, 1987). Our previous studies have centered on overcoming low transcriptional levels and poor translational efficiencies. The sequences in the first 9 codons of bGH mRNA were modified to disrupt RNA secondary structure around the start codon and to neutralize the base properties of the protein, and finally the gene dosage was increased using the pUC19 plasmid with a mutated ColE1 replicon (Choi and Lee, 1996). As a result, an appropriate amount of bGH was produced in *E. coli* that harbored different recombinant plasmids.

In this study, we have examined the effects of the SD sequence and nucleotide sequences of the spacer region on translational efficiencies of two bGH derivatives which have different 5'-terminal sequences. The bGH1 gene has an original sequence of bGH and the bGH14 gene contains the modified 5' terminal sequence of bGH, in which the first amino acid of alanine was replaced with glutamic acid. We investigated and compared the mRNA secondary structures between the 5'-end sequence of bGH, the SD sequence and nucleotide sequences of the spacer region and the correlation between the expression levels and other factors.

Materials and Methods

Bacterial strains, plasmids, and culture media

E. coli HB101 (*hsd*R, *hsd*M, *leu*, *pro*, *rec*A, *sup*E) was used for transformation and the propagation of plasmids. *E. coli* BL21(DE3) (F', *hsdS*, *gal*, $\lambda CI^{ts}857$, *ind1*, *sam7*, *nim5*, *lacUV5*-T7 gene1) and *E. coli* BL21(DE3)pLysE strains were used for the expression of the T7 promoter system. These strains contain a single copy of the gene for T7 RNA polymerase in the chromosome that is under control of the inducible *lacUV5* promoter (Tabor and Richardson, 1985). *E.*

coli BL21(DE3)pLysE strain contains the T7 lyso-zyme, a T7 RNA polymerase inhibitor that prevents leaky expression in uninduced cells and is useful for the expression of toxic proteins (Studier *et al.*, 1990). The plasmid pT7-7 carrying the T7 promoter was used to construct the bGH expression plasmids, pTbGH1 and pTbGH14 series vectors. Plasmids pUBJ1 and pUBJ14 (Choi and Lee, 1996) were used as sources of bGH cDNAs. *E. coli* cells harboring plasmids were grown in Luria-Bertani (LB) broth supplemented with ampicillin (50 μg/ml).

Oligonucleotide synthesis and DNA sequence analysis

Oligonucleotides were synthesized by the phosphor-amidite method on an automatic DNA synthesizer (384 superoligo synthesizer) obtained from Bioneer Inc. (Korea). The oligonucleotide set was 5'-CTAGAT TAATTAAAATTTTGRRRRGNNNNNNAT-3' and 5'-C ATGATNNNNNNCYYYYCAAAATTTTAATTAAT-3' (R; A or G, N; A, C, G, T, Y; T or C). After phos-phorylation and annealing, the oligonucleotides were ligated to the bGH cDNA. The constructed plasmids were amplified in *E. coli* and extracted using the Wizard *Plus* SV Minipreps DNA purification kit from Promega (USA). The nucleotide sequences of the SD and spacer regions were determined with T7 sequenc-ing primer by the dye terminator sequencing method using a Basestation DNA Fragment Analyzer from Bionex, Inc. (Korea).

Preparation of RNA and dot blot hybridization

Total RNA was isolated from *E. coli* BL21(DE3) har-boring each plasmid using the RiboPure-Bacteria RNA Isolation Kit from Ambion Inc. (USA). After 4 h induction, cell density was measured at OD_{600} and the volume corresponding to 1×10^9 cells was taken and followed the protocol recommended by the manufacturer. For the RNA dot blot study, the amount of RNA corresponding to 2×10^8 cells was applied directly to a nylon membrane and hybridization was performed as described by Sambrook *et al.* (1989). The partial fragment of bGH DNA was used as a probe.

Identification of bGH produced in E. coli

E. coli transformants were grown at 37°C overnight in LB broth supplemented with 50 μg of ampicillin. The cultures were diluted 1/200 using the same medium and incubated at 37°C. When the cell density reached $OD_{600} = 0.5$, IPTG (isopropyl-1-thio-β-galactoside) was added to a final concentration of 1 mM. After an ad-ditional incubation for 6 h, lysates were prepared from one ml of the culture. The cell pellets were sus-pended in 200 μl of sample buffer (0.05 M Tris-HCl, pH 6.8, 0.1 M DTT, 2% SDS, 10% glycerol, and 0.1% bromphenolblue) and boiled at 90°C for 5 min. The samples were fractionated on 12% SDS-PAGE as described by Laemmli (1970).

Western blot analysis

After proteins were separated on SDS-PAGE, the pro-teins were then transferred to a PVDF membrane us-ing Trans-Blot SD apparatus (BioRad, USA). The blot was blocked for 1 h in phosphate buffered saline (PBS) containing 3% (w/v) skim milk and incubated with polyclonal anti-bGH antibody (1:2,000 dilution) for 1 h. After washing unbound primary antibodies with washing buffer (PBS + 0.5% Triton X-100, v/v) three times for 10 min each, the blot was treated with alkaline phosphatase or horseradish peroxidase-con-jugated goat anti-rabbit antiserum (1:2,000 dilution) and developed with BCIP/NBT (Sigma, USA) sub-strate solution or the ECL system (Amersham Pharmacia Biotech, USA).

Prediction of the mRNA secondary structure

The mRNA SD sequence was evaluated by its ability to base-pairing with the corresponding sequence in the rRNA. Thus, the quantitative measure for an SD sequence was the free energy gain when such base pairing occurs. To determine the sequences which de-stabilize the mRNA secondary structure or yield low free energy values (to positive direction) for inducing the expression, the free energy values were calculated for the subsequences from transcription initiation site to +51 of the various bGH transcripts using mfold (version 3.1) web server by Zuker (2003).

Results and Discussion

Synthesis of oligonucleotides containing random SD sequences and the spacer region

Based on the nucleotide sequence of bGH, there are two potential stem-loop structures in the 5'-region of the mRNA which may interfere with translation (Tomich *et al.*, 1989). The first stem-loop structure is weak with a free energy of ⁻6.2 kcal/mole and results in a low level of expression of bGH because the start codon AUG is involved in hydrogen bonding. The second stem-loop structure can be formed through the codons for amino acid residues 12-25 (20 kcal/mole) and does not affect the expression of bGH, although the structure has a stronger hydrogen bond than in the first structure. Accordingly, it was thought that the first structure might be an important factor for in-creasing the bGH expression. To search for optimum sequences that destabilize the first stem loop structure, oligonucleotides (33mer in length) were designed as described in Materials and Methods. The SD sequence was designed to consist of 6 nucleotides (GRRRRG),

Table 1. Nucleotide sequences of Shine-Dalgarno and the spacer region of the pTbGH1 series expression vectors

Clone No.	SD region		Spacer region		G value[a] (Kcal/mole)	No. of base pair[b]	Expression (%)[c]
	−15	−10	−1	+1			
pTbGH1-1	TT GAAAGG		GGTGGAATC	ATG	30.0	5	ND
pTbGH1-2	TT GGAAAG		ACATATATC	ATG	29.6	6	ND
pTbGH1-3	TT GAAAGG		CATCGTATC	ATG	29.9	4	ND
pTbGH1-4	TT GAAGAG		CTGTAAATC	ATG	28.9	3	ND
pTbGH1-8	TT GGAAAG		GCCAACAATC	ATG	34.5	4	ND
pTbGH1-9	TT AGGGAG		GATGGTATC	ATG	31.5	5	ND
pTbGH1-10	TT GAGGAG		GGAAGCATC	ATG	32.5	4	ND
pTbGH1-11	TT GAGGAG		GACGGTATC	ATG	33.2	3	ND
pTbGH1-13	TT GGGGAG		AAACAAATC	ATG	29.8	5	ND
pTbGH1-14	TT GAGAGG		CGCGTAATC	ATG	30.8	5	ND
pTbGH1-16	TT AAAGAG		CGTTCTATC	ATG	29.2	6	ND
pTbGH1-17	TT GAAGAG		TATTGAATC	ATG	29.0	4	ND
pTbGH1-18	TT GGAAAG		TAGGAAATC	ATG	28.8	5	ND
pTbGH1-19	TT GAGGAG		ACACAGCATC	ATG	37.2	4	ND
pTbGH1-20	TT GGAAGG		CACACAATC	ATG	32.1	6	ND
pTbGH1-21	TT AAAGAG		CGTTCTATC	ATG	29.2	6	ND
pTbGH1-22	TT GGAAGG		CACACAATC	ATG	32.1	6	ND
pTbGH1-23	TT GGGGAG		GGTGCCATC	ATG	32.9	6	ND

a: The free energy was calculated from the transcription initiation site to +51 of the bGH cDNA using mfold web server by Zuker (2003)
b: Number of base complementary to the 3'-end sequence of 16S rRNA (5'GAUCACCUCCUUA3') in the 30S ribosomal subunit
c: Expression amount for total cell proteins
ND : not detected

Table 2. Nucleotide sequences of the Shine-Dalgarno and spacer regions of the pTbGH14 series expression vectors

Clone No.	SD region		Spacer region		G value[a] (Kcal/mole)	No. of base paird[b]	Expression (%)[c]
	−15	−10	−1	+1			
pTbGH14-3	TT GGAAAG		CATACCATC	ATG	28.6	5	ND
pTbGH14-8	TT GGAAAG		CGGGTAATC	ATG	27.5	5	ND
pTbGH14-9	TT GGGGGG		CCCCACATC	ATG	27.1	5	ND
pTbGH14-10	TT GGAAAG		CATACCATC	ATG	28.6	5	ND
pTbGH14-11	TT GGAAGG		GAAACAATC	ATG	26.3	7	7.4
pTbGH14-13	TT GAAAAG		GAATTCATC	ATG	23.9	5	7.2
pTbGH14-14	TT GAGGAG		CTCGCTATC	ATG	24.8	4	6.9
pTbGH14-15	TT GGGGGG		GCGTCTATC	ATG	29.2	6	ND
pTbGH14-19	TT GGAAAG		CATACCATC	ATG	28.6	5	ND
pTbGH14-22	TT GGAAAG		CATACCATC	ATG	28.6	5	ND
pTbGH14-23	TT GAGGAG		GTTGCGATC	ATG	25.6	4	8.5
pTbGH14-24	TT GGGGAG		TCAGCAATC	ATG	26.8	8	ND
pTbGH14-25	TT GAGGAG		GTTGCGATC	ATG	25.6	4	8.2
pTbGH14-26	TT GAGGGG		GCAGCTATC	ATG	29.2	6	ND
pTbGH14-29	TT GAGAAG		ACCGACATC	ATG	27.2	4	ND
pTbGH14-30	TT GGGAAG		TCAGCAATC	ATG	26.8	8	ND
pTbGH14-31	TT GAAGAG		TCATATATC	ATG	22.9	5	7.9
pTbGH14-32	TT GGAAAG		CGTTTAATC	ATG	23.9	5	7.0
pTbGH14-34	TT GAGGAG		GTTGCGATC	ATG	25.4	4	7.3

a: The free energy was calculated from the transcription initiation site to +51 of the bGH cDNA using mfold web server by Zuker (2003)
b: Number of base pairs complementary to the 3'-end sequence of 16S rRNA (5'GAUCACCUCCUUA3') in the 30S ribosomal subunit
c: Expression amount for total cell proteins in percent
ND: not detected

in which the A or G nucleotide was randomly introduced. The AT-rich block (TTAATTAAAA TTTT), which was reported to enhance expression level by enriching the sequence flanking the SD region with AT-rich nucleotide (Choi *et al.*, 1999b), was introduced in the 5'-untranslated region upstream from the SD sequence. The distance between the SD region and the AUG initiation codon (spacer region) was fixed to nine bases. Among the nine bases, six were randomly synthesized with the A, C, G, and T nucleotide, respectively. Both ends of the oligonucleotides were designed with *Xba*I and *Nco*I for easy cloning to a vector and insertion into bGH DNAs. The synthesized oligonucleotides were phosphorylated with T4 polynucleotide kinase, heated to 95°C (equal molar amounts), and allowed to anneal by slowly cooling to room temperature overnight. The annealed oligonucleotides were fused with bGH1 and bGH14, respectively and the oligonuleotide sequences were analyzed after obtaining appropriate recombinant clones as shown in Tables 1 and 2.

Construction of the pTbGH1 and pTbGH14 series expression vectors

The pT7-7 vector was used as a starting vector to construct a series of expression plasmids for two types of bGH cDNA that differed with respect to the DNA sequence at the 5'-end of the bGH coding region. The strategy for the construction of expression plasmids with a random SD region and a spacer sequence at the 5'-untranslated region of the bGH gene was described as follows: the pUBJ1 and pUBJ14 plasmid (Choi and Lee, 1996) contain the full-length bGH cDNA except for differences at the 5'-end sequence that were digested with *Nco*I and *Hin*dIII, eluted, and ligated with the annealed oligonucleotide, respectively. The ligated products were cleaved with *Xba*I and *Hin*dIII to remove multimeric forms and then the 0.7 kb *Xba*I-*Hin*dIII fragments were obtained by elution on agarose gel. The pT7-7 vector containing the T7 promoter was also digested with the same enzymes, *Xba*I and *Hin*dIII, and fused with the 0.7 kb *Xba*I-*Hin*dIII fragments containing random SD sequences and the bGH gene. The fused products were introduced into *E. coli* HB101 and the appropriate clones were screened by size selection. After confirming the insert DNA with double digestion, the sequences of random SD and spacer region were finally analyzed by the dideoxy chain termination method (Sanger *et al.*, 1977). The resulting expression plasmids were named pTbGH1 and pTbGH14 series vectors. The correct sequences of 18 clones among the pTbGH1 series plasmids and 19 clones for pTbGH14 series plasmids were analyzed as shown in Tables 1 and 2. The sequences of SD and spacer re-
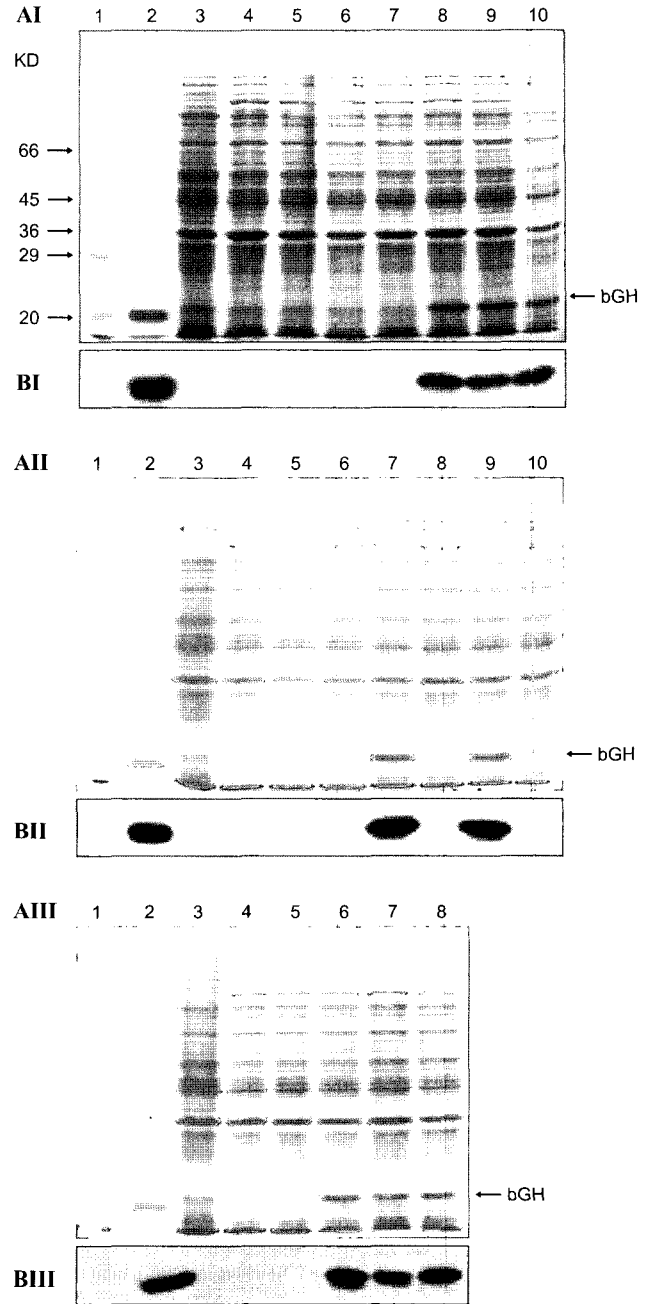

Fig. 1. SDS-PAGE and western blot analysis of *E. coli* BL21 (DE3) harboring pTbGH14 series vectors. Panels AI, AII and AIII, 12.5% SDS-PAGE; Panel BI, BII and BIII, Western blot. The arrows indicate the position of bGH. (C) indicates uninduced cells and (I) induced cells. Lanes in panel AI indicate: 1, protein marker (Sigma, M3913, USA); 2, purified bGH (5 μg); 3, pTbGH14-3 (C); 4, pTbGH14-3 (I); 5, pTbGH14-8 (I); 6, pTbGH14-9 (I); 7, pTbGH14-10 (I); 8, pTbGH14-11 (I); 9, pTbGH14-13 (I); 10, pTbGH14-14 (I). Lanes in panel AII: 1, protein marker (Sigma, M3913, USA); 2, purified bGH (5 μg); 3, pTbGH14-15 (C); 4, pTbGH14-15 (I); 5, pTbGH14-19 (I); 6, pTbGH14-22 (I); 7, pTbGH14-23 (I); 8, pTbGH14-24 (I); 9, pTbGH14-25 (I); 10, pTbGH14-26 (I). Lanes in panel AIII: 1, protein marker (Sigma, M3913, USA); 2, purified bGH (5 μg); 3, pTbGH14-29 (C); 4, pTbGH14-29 (I); 5, pTbGH14-30 (I); 6, pTbGH14-31 (I); 7, pTbGH14-32 (I); 8, pTbGH14-34 (I).

gion, free energies, and base pairing between SD and 16S rRNA are summarized in these two tables. To correlate a relationship between optimal sequences and expression levels, those clones were induced for bGH expression.

Expression of the bGH gene in E. coli harboring pTbGH1 and pTbGH14 plasmids

The constructed plasmids were transferred into E. coli BL21(DE3). The resulting cultures were grown and induced as described in the Materials and Methods. After the addition of IPTG, by which the T7 RNA polymerase gene in the host chromosome is induced and the product specifically initiates transcription of the bGH gene under the T7 promoter, the total cell lysates were analyzed by SDS-PAGE and western blot. As can be seen in Fig. 1, eight clones (pTbGH14-11, 14-13, 14-14, 14-23, 14-25, 14-31, 14-32, and 14-34) among the pTbGH14 series of plasmids produced bovine growth hormone in the range of 6.9% to 8.5% of total cell protein; there were no detectable levels of bGH in any cells harboring the pTbGH1 series of plasmids (with natural sequence of bGH gene; data not shown). These findings indicate that without modification of the 5'-end of the bGH gene, there is no expression of bGH although optimal sequences of the SD and spacer regions were introduced in the 5'-untranslated region of the bGH gene. In the case of the pTbGH14 series plasmid, in which the first amino acid was changed from alanine to glutamic acid, the free energy for the coding region from the first to the sixteenth amino acid of bGH had been reduced from −11.8 Kcal/mole to −5.76 Kcal/mole (Choi and Lee, 1996), suggesting that there is a disruption of the mRNA secondary structure present in the 5'-end of the bGH gene. When the free energy was calculated from the transcription initiation site to the +51 nucleotide of bGH cDNA using a program of nucleic acid folding and hybridization prediction (Zuker, 2003), the constructs with values below −26.3 kcal/mole (toward minus direction) were not expressed (Table 2). The constructs with original sequences of bGH cDNA did not show any expression, regardless of their free energy values (Table 1). We interpret this result to mean that the disruption of the mRNA secondary structure might be an important factor for regulating bGH expression in the translational initiation process. The first stem-loop among the two secondary structures present in the 5'-end region of the bGH gene should be disrupted for effective expression of bGH, and then by the introduction of the optimal SD and spacer regions, the level of bGH expression can be enhanced. de Smit and van Duin (1990) showed that the efficiency of the ribosome binding site is reduced by one order of
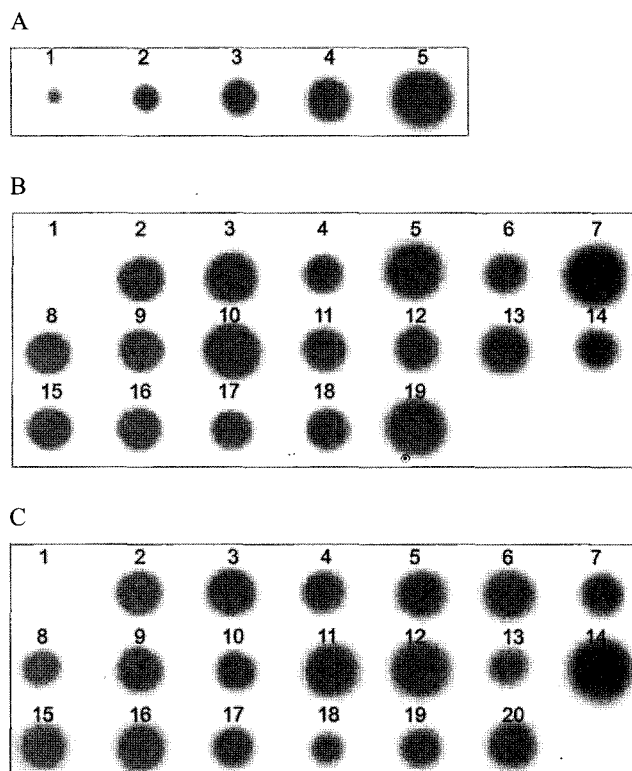

Fig. 2. RNA dot blot analysis of various pTbGH1 and pTbGH4 expression vectors containing bGH derivatives. A, Positive control (bGH): 1, 1 ng; 2, 5 ng; 3, 10 ng; 4, 25 ng; 5, 50 ng. B, RNA blot from pTbGH1 series vectors: 1, pT7-7; 2, pTbGH1-1; 3, pTbGH1-2; 4, pTbGH1-3; 5, pTbGH1-4; 6, pTbGH1-8; 7, pTbGH1-9; 8, pTbGH1-10; 9, pTbGH1-11; 10, pTbGH1-13; 11, pTbGH1-14; 12, pTbGH1-16; 13, pTbGH1-17; 14, pTbGH1-18; 15, pTbGH1-19; 16, pTbGH1-20; 17, pTbGH1-21; 18, pTbGH1-22; 19, pTbGH1-23. C, RNA blot from pTbGH14 series vectors: 1, pT7-7; 2, pTbGH14-3; 3, pTbGH14-8; 4, pTbGH14-9; 5, pTbGH14-10; 6, pTbGH14-11; 7, pTbGH14-13; 8, pTbGH14-14; 9, pTbGH14-15; 10, pTbGH14-19; 11, pTbGH14-22; 12, pTbGH14-23; 13, pTbGH14-24; 14, pTbGH14-25; 15, pTbGH14-26; 16, pTbGH14-29; 17, pTbGH14 -30; 18, pTbGH14-31; 19, pTbGH14-32; 20, pTbGH14-34.

magnitude when the stability of its secondary structure is increased by 2.3 Kcal/mole.

Correlation of transcripts, mRNA secondary structures, and expression levels

To quantitatively elucidate the correlation between the steady-state levels of bGH-specific mRNA and expression levels in E. coli BL21(DE3) harboring pTbGH1 and pTbGH14 series plasmids, dot blot analysis was carried out. As can be seen in Fig. 2, there is no difference in RNA amounts between the cells harboring pTbGH1 series plasmids and those of pTbGH14 series plasmids even though each clone in pTbGH1 (or pTbGH14) series plasmids appear a little difference of RNA amount. In case of pTbGH1 series plasmids, there was no expression of bGH in all

clones regardless of RNA amount. Otherwise, clones with a free energy value above -26.3 Kcal/mole (toward plus direction) in the pTbGH14 series plasmids produced bGH at considerable levels. In the cases of pTbGH14-23 and pTbGH14-25 (with the higher amounts of hybridized mRNA), the expression levels were estimated to be 8.5% and 8.2% of total cell proteins respectively, while pTbGH14-11, pTbGH14-13, pTbGH14-14, pTbGH14-31 pTbGH14-32, and pTbGH 14-34 with lesser amounts of mRNA were found to express bGH amounting to 7.4%, 7.2%, 6.9%, 7.9%, 7.0%, and 7.3%, respectively (Table 2 and Fig. 2). It seems that the expression level of bGH may be proportional to the RNA amount, however, compared with the data of the pTbGH1 series plasmids, different expressions between plasmids are independent of mRNA amounts and might be due to the efficiency of translatability of these mRNAs related to the mRNA secondary structure. The amount of mRNA might not be an important factor for regulating the bGH expression although appropriate amounts of mRNA are needed to overcome some inhibitory threshold. In the case of the pTbGH1 series plasmid which did not produce the bGH, mRNA secondary structure between the SD and the 5'-end of the bGH gene may not be favorable for protein synthesis within the intracellular environment. That is, the above region of the mRNA is likely to be in a stem-loop or in a structure such that the initiation codon is not prominently presented to the environment.

It is interesting that plasmids with the more negative ΔG value (less than -26.3 Kcal/mole) in the 5'-end sequence of bGH did not produce a detectable level of bGH which suggests that ribosome accessibility to mRNA will be enhanced when the SD sequence and the initiation codon are located in a context free of local secondary structure. It is also to be noted that translational efficiencies cannot be explained solely by the primary structure of the 5'-untranslated region, but rather depends on the interaction between the 5'-untranslated region and the downstream coding region. Similar results have been reported by Warburton *et al.* (1983), in which the phenotypic expression of two point mutations located between the SD region and the AUG codon depended upon the coding information.

In conclusion, these data suggest that the mRNA secondary structure, by modification of the 5'-coding region of the bGH cDNA, should be destabilized before considering other factors and that the optimal sequence of SD and spacer regions may play important roles for the efficient expression of bGH.

Acknowledgment

References

Baumann, D.E., C.J. Peel, W.D. Steinhour, P.J. Reynolds, H.F. Tyrrell, A.C.G. Brown, and G.L. Haaland. 1988. Effect of bovine somatotropin on metabolism of lactating dairy cow. *J. Nutr.* 118, 1031-1040.

Chen, H., M. Bjerknes, R. Kumar, and E. Jay. 1994. Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. *Nucleic Acids Res.* 22, 4953-4957.

Choi, J.W. and S.Y. Lee. 1996. Optimization of bovine growth hormone gene expression in *E. coli. Mol. Cells.* 6, 712-718.

Choi, J.W., K.S. Ra, and Y.S. Lee. 1999a. Enhancement of bovine growth hormone gene expression by increasing the plasmid copy number. *Biotechnology Letters.* 21, 1-5.

Choi, J.W., H.S. Park, J.W. Yun, and Y.S. Lee. 1999b. The artificial AT-rich block enhanced the production of bovine growth hormone in *Escherichia coli. Bioprocess Engineering.* 21, 293-298.

de Smit, M.H. and J. van Duin. 1990. Control of prokaryotic translational initiation by mRNA secondary structure. *Prog. Nucleic Acids Res. Mol. Biol.* 38, 1-35.

de Smit, M.H. and J. van Duin. 1994. Translational initiation on structured messengers. Another role for the Shine-Dalgarno interaction. *J. Mol. Biol.* 235, 173-184.

Draper, D.E. 1996. Translational initiation, p. 902-908. In Neidhardt, F.C., R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger. (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.

George, H.J., J.J. L'Italien, W.P. Pilacinski, D.L. Glassman, and R.A. Krzyzek. 1985. High level expression in *E. coli* of biologically active bovine growth hormone. *DNA.* 4, 273-281.

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.

Gold, L. 1988. Posttranslational regulatory mechanisms in *Escherichia coli. Annu. Rev. Biochem.* 57, 199-233.

Green, P.J. and M. Inouye. 1984. Roles of the 5' leader region of the *ompA* mRNA. *J. Mol. Biol.* 176, 431-442.

Hsiung, H.M. and W.C. Mackellar. 1987. Expression of bovine growth hormone derivatives in *E. coli* and the use of the derivatives to produce natural sequence growth hormone by cathepsin C cleavage. *Methods Enzymol.* 153, 390-401.

Hui, A., J. Hayflick, K. Dinkelspiel, and H.A. de Boer. 1984. Mutagenesis of the three base pair preceding the start codon of the β-galactosidase mRNA and its effect on translation in *E. coli. EMBO J.* 3, 623-629.

Ikemura, T. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.* 2(1),

13-34.

Kanaya, S., Y. Yamada, Y. Kudo, and T. Ikemura. 1999. Studies of codon usage and tRNA genes of 18 unicellular organisms and quantification of *Bacillus subtilis* tRNAs: Gene expression level and species-specific diversity of codon usage based on multivariate analysis. *Gene* 238, 143-155.

Karlin, S. and J. Mrazek. 2000. Predicted highly expressed genes of diverse prokaryotic genomes. *J. Bacteriol.* 182(18), 5238-5250.

Karlin, S., J. Mrazek, A. Campbell, and D. Kaiser. 2001. Characterization of highly expressed genes of four fast-growing bacteria. *J. Bacteriol.* 183, 5025-5040.

Kim, E.Y., M.S. Shin, J.H. Rhee, and H.E. Choy. 2004a. Factors influencing preferential utilization of RNA polymerase containing sigma-38 in stationary-phase gene expression in *Escherichia coli. J. Microbiol.* 42, 103-110.

Kim, H.G., T.N. Phan, T.S. Jang, M.J. Koh, and S.W. Kim. 2005. Characterization of *Methylophaga* sp. Strain SK1 cytochrome c_L expressed in *Escherichia coli. J. Microbiol.* 43, 499-502.

Kim, Y.M., K. Park, S.H. Jung, J.H. Choi, W.C. Kim, G.J. Joo, and I.G. Rhee. 2004b. Chlorothalonil-biotransformation by glutathione s-transferase of *Escherichia coli. J. Microbiol.* 42, 42-46.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Ma, J., A. Campbell, and S. Karlin. 2002. Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. *J. Bacteriol.* 184(20), 5733-5745.

Morikawa, M., T. Nixon, and H. Green. 1982. Growth hormone and adipose conversion of 3T3 cells. *Cell* 29, 783-789.

Osada, Y., R. Saito, and M. Tomita. 1999. Analysis of base-pairing potentials between 16S rRNA and 5'UTR for translation initiation in various prokaryotes. *Bioinformatics* 15, 578-581.

Paladini, A.C., C. Pena, and E. Poskus. 1983. Molecular biology of growth hormone. *CRC Crit. Rev. Biochem.* 15, 25-56.

Park, S.H. and J.I. Kim. 2004. Characterization of recombinant *Drosophila melanogaster* Myo-inositol-1-phosphate synthase expressed in *Escherichia coli. J. Microbiol.* 42, 20-24.

Poole, E.S., C.M. Brown, and W.P Tate. 1995. The identity of the base following the stop codon determines the efficiency of *in vivo* translational termination in *E. coli. EMBO J.* 14, 151-158.

Ringquist, S., S. Shinedling, D. Barrick, L. Green, J. Binkley, G.D. Stormo, and L. Gold. 1992. Translation initiation in *Escherichia coli*: sequences within the ribosome binding site. *Mol. Microbiol.* 6, 1219-1229.

Saito, Y., Y. Ishii, S. Koyama, K. Tsuji, H. Yamada, T. Terai, M. Kobayashi, T. Ono, M. Niwa, I. Ueda, and H. Kikuchi. 1987. Bacterial synthesis of recombinant alpha-human atrial natriuretic polypeptide. *J. Biochem.* 102, 11-122.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning : A laboratory Manual, Cold Spring Harbor Lab. New York.

Sanger, F., S. Niklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74, 5463-5467.

Scherer, G.E.F., M.D. Walkinshaw, S. Arnott, and D.J. Morre. 1980. The ribosome binding sites recognized by *E. coli* ribosomes have regions with signal character in both the leader and protein coding segments. *Nucleic Acids Res.* 8, 3895-3906.

Schoner, B.E., H.M. Hsiung, R.M. Belagaje, N.G. Mayne, and R.G. Schoner. 1984. Role of mRNA translational efficiency in bovine growth hormone expression in *E. coli. Proc. Natl. Acad. Sci. USA.* 81, 5403-5407.

Secchi, C. and V. Borromeo. 1997. Bovine growth hormone as an experimental model for studies of protein-protein interactions. *J. Chromatogr. Biomed. Sci.* 688(2), 161-177.

Seeburg, P.H., S. Sias, J. Adelman, H.A. de Boer, J. Hayflick, P. Jhurani, D.V. Goeddel, and H.L. Heyneker. 1983. Efficient bacterial expression of bovine and porcine growth hormones. *DNA* 2, 37-45.

Selinger, D.W., K.J. Cheung, R. Mei, E.M. Johansson, C.S. Richmond, F.R. Blattner, D.J. Lockhart, and G.M. Church. 2000. RNA expression analysis using a 30 base pair resolution *Escherichia coli* genome array. *Nat. Biotechnol.* 18, 1262-1268.

Shine, J. and L. Dalgarno. 1974. The 3'-terminal sequence logos: prediction of major groove binding by information theory. *Methods Enzymol.* 274, 445-455.

Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60-89.

Tabor, S. and C.C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA.* 82, 1074-1078.

Tomich, C.S.C., E.R. Olsen, M.K. Olsen, P.S. Kaytes, S.K. Rockenbach, and N.T. Hazenbuhler. 1989. Effect of the nucleotide sequence directly downstream from the AUG on the expression of bovine somatotropin in *E. coli. Nucleic Acids Res.* 17, 3179-3197.

Uhlin, B.E., S. Molin, P. Gustafsson, and K. Nordstrom. 1979. Plasmids with temperature-dependent copy number for amplification of cloned genes and their products. *Gene* 6, 91-106.

Warburton, N., P.G. Bosely, and A.G. Porter. 1983. Increased expression of a cloned gene by local mutagenesis of its promoter and ribosome binding site. *Nucleic Acids Res.* 11, 5837-5854.

Wingfield, P.T., P. Graber, G. Buell, K. Rose, M. Simona, and B.D. Burleigh. 1987. Preparation and characterization of bovine growth hormones produced in recombinant *E. coli. Biochem. J.* 243, 829-839.

Wood, C.R., M.A. Boss, T.P. Patel, and J.S. Emtage. 1984. The influence of messenger RNA secondary structure on expression of an immunoglobulin heavy chain in *Escherichia coli. Nucleic Acids Res.* 12, 3937-3950.

Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31, 3406-3415.