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Two-Cistronic Expression Plasmids for High-Level Gene Expression in *Escherichia coli* Preventing Translational Initiation Inhibition Caused by the Intramolecular Local Secondary Structure of mRNA

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Two-cistronic expression plasmids for the wild-type solubilized domain of porcine NADPH-cytochrome P450 reductase (PsCPR) gene in *Escherichia coli* were systematically constructed using a solubilized domain of porcine cytochrome b_5 gene (*Psb5* gene) or a derivative of it as the first cistron to examine their utility for second gene expression preventing the translational inhibition caused by the intramolecular local secondary structure of mRNA at the ribosome-binding site (RBS). The mRNAs from the plasmids lacking an RBS for the second cistron (SD2) accumulated very low levels of PsCPR, while those from the plasmids having an SD2 accumulated higher levels of PsCPR. The level of accumulation of PsCPR by the mRNA from plasmid pCbSD-T-CPR-3, which has an SD2 upstream of the termination codon of the first cistron, was higher than for those with an SD2 in the intercistronic region. The predicted intramolecular local secondary structures at the SD2 of mRNAs from these plasmids were stable enough to cause translational initiation inhibition. These results indicate that the use of a two-cistronic expression plasmid is an effective way to overcome translational initiation inhibition. Improved plasmids, pCP1 and pCP2P, were constructed from pCbSD-T-CPR-3. Using these plasmids, the solubilized donain of porcine NADH-cytochrome b_5 reductase was also highly accumulated on prevention of the translational initiation inhibition. These plasmids are expected to be useful tools for the comprehensive high-level expression of heterologous genes in *E. coli* cells.

Key words: mRNA, ribosome-binding site, secondary structure, Shine-Dalgarno sequence, two-cistronic expression plasmid.

Abbreviations: CBB, Coomassie Brilliant Blue; IPTG, isopropyl-β-D-thiogalactopyranoside; Pb5R, solubilized domain of porcine NADH-cytochrome b_5 reductase; Psb5, solubilized domain of porcine cytochrome b_5; PsCPR, solubilized domain of porcine NADPH-cytochrome P450 reductase; RBS, ribosome-binding site; SD, Shine-Dalgarno; SD1, SD sequence for the first cistron; SD2, SD sequence for the second cistron.

Heterologous gene expression involving the recombinant DNA technique is a basic and necessary technique for the identification, characterization, and structural study of a protein molecule that can be seldom prepared from a natural source. Although *Escherichia coli* cells do not have posttranslational modification systems such as the addition of a sugar chain, heterolologous gene expression in *E. coli* is widely used for the production of recombinant proteins. This is because heterologous gene expression in *E. coli* has the following advantages: (i) gene manipulation techniques are well established, (ii) a large amount of the protein can be synthesized in a short time, and (iii) fermentation scale up is easy in comparison with gene expression with eukaryotic cells.

The levels of expression of heterologous genes in *E. coli* depend on a number of factors at both the transcriptional and translational levels (*1*). Various plasmid vectors have been designed and widely used for the expression of heterologous genes in *E. coli* cells. Most of these expression plasmids have a high-copy number origin of replication such as ColEI, strong promoters such as *trp*, *lac*, *tac*, λP_L, and *T7* promoters, appropriate regulatory elements to synthesize large amounts of mRNAs (*2*, *3*), and a ribosome-binding site (RBS), which is known as the Shine-Dalgarno (SD) sequence and is complementarily recognized by the 3′-end region of the 16S rRNA in the 30S subunit of ribosomes in *E. coli* (*4*). However, the levels of expression of the heterologous genes depend on the individual genes, and a heterologous gene is not always highly expressed with such a conventional expression plasmid. Therefore, a universal expression plasmid that enables high-level heterologous gene expression independent of the target genes is required.

One reason for the low level of gene expression with a conventional high-level expression plasmid is the formation of unfavorable intramolecular double-stranded structures of mRNA at the RBS (*5*). The formation of a stable intramolecular double-stranded structure of mRNA at the RBS, prevents complementary recognition of the RBS by 16S rRNA, resulting in the low-level gene

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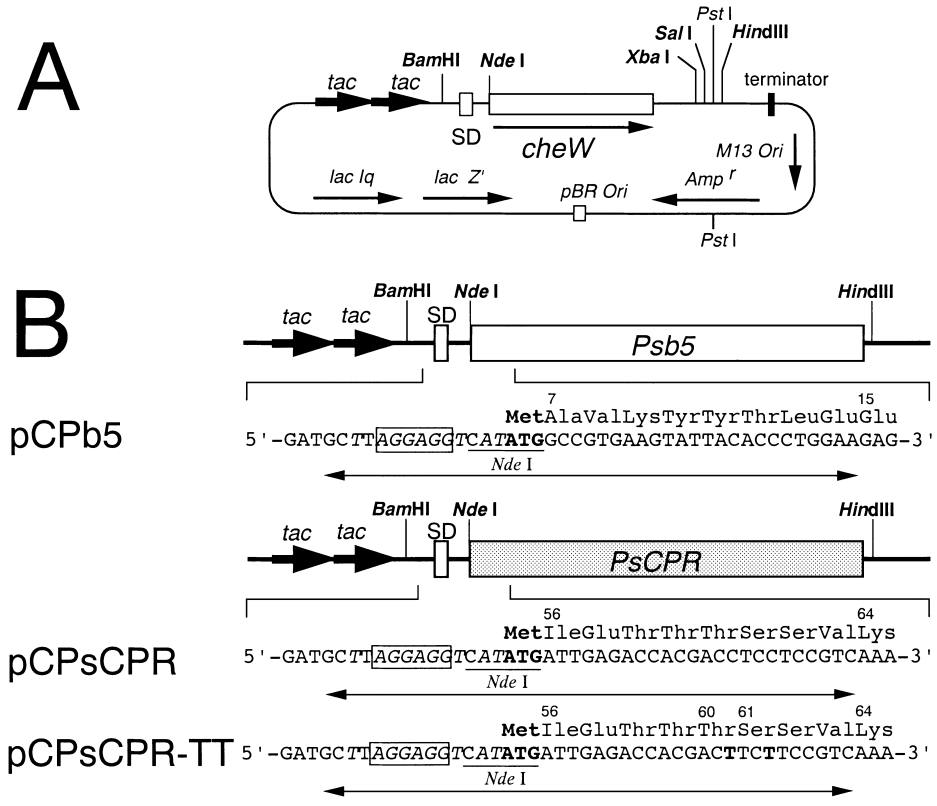

Fig. 1. **Plasmid pCW$_{ori}^{+}$, and partial structures of the expression plasmids for Psb5 and PsCPR.** A: The structure of plasmid pCW$_{ori}^{+}$. B: The partial structures and nucleotide sequences of pCPb5 (*18*), pCPsCPR (*8*), and pCPsCPR-TT (*8*), which are derivatives of pCW$_{ori}^{+}$. Plasmids pCPb5 and pCPsCPR have structures in which the *Nde*I–*Hin*dIII region containing the *cheW* gene in pCW$_{ori}^{+}$ is substituted with the *Nde*I–*Hin*dIII fragments containing the *Psb5* and *PsCPR* genes, respectively. Plasmid pCsCPR-TT is a derivative of pCsCPR, and has silent mutations in the triplets encoding Thr^{60} and Ser^{61} (bold), which dramatically increase the level of expression of the *PsCPR* gene in *E. coli* cells (*8*). In these plasmids the nucleotide sequence upstream of the *Nde*I site is identical to that of pCW$_{ori}^{+}$. The nucleotides that are complementary to the 3′-terminal region of *E. coli* 16S rRNA are shown in italics. The nucleotides encoding the hypothetical RBS are boxed. The ATG encoding initiation codon and the formylmethionine residues are shown in bold letters. The solid lines with arrows at both terminals indicate the regions of the nucleotide sequences that were used for the local secondary structure prediction of mRNA.

expression caused by the inhibition of translational initiation. We have previously elucidated that the translational initiation of the wild-type solubilized domain of porcine NADPH-cytochrome P450 reductase (PsCPR) gene (*PsCPR* gene) in *E. coli* cells, using expression plasmid pCW$_{ori}^{+}$ (*6*, *7*), is markedly inhibited by the formation of a highly-stable intramolecular double-stranded structure of mRNA at the RBS, and demonstrated that modulation of the local secondary structure of mRNA by introducing silent mutations in the section of the gene encoding the N-terminal region of the protein based on the predicted local secondary structure of mRNA at the RBS is a useful approach to control and to increase the level of expression of the heterologous gene in *E. coli* cells (*8*).

The other approach for preventing the formation of an unfavorable intramolecular double-stranded structure of mRNA at the RBS is the use of a bacterial polycistronic gene expression system, as described by Schoner *et al.* (*9–11*). It is well known that in bacterial cells, several protein genes can be co-expressed from a polycistoronic mRNA. A typical polycistronic mRNA is composed of a cistronic region and an intercistronic region. In most polycistronic mRNAs, each cistron has an RBS in the intercistronic region upstream of the initiation codon, and the translation of the genes proceeds sequentially through the cistrons. When the intercistronic region is longer than the span of the ribosome (approximately 30 bases), the ribosome binds independently at the beginning of each cistron and dissociates at the termination site. However, in some bacterial mRNAs, in which the termination codon of the first cistron is positioned near the initiation codon of the next cistron, translation between adjacent cistrons is directly linked, because the ribosome can gain access to the initiation codon of the second cistron (*12*). In such a case, since the ribosome prevents the formation of an intramolecular double-strand at the RBS for the second cistron, the second cistron can be translated independently of the formation ability of the secondary structure at the RBS. Therefore, it is considered that a more universal high-level expression plasmid that is independent of the nucleotide sequence of a heterologous gene can be constructed by aligning a highly translatable gene as the first cistron and a target gene as the second cistron appropriately to co-translate both cistrons without complete ribosome dissociation.

The genes of methionyl bovine growth hormone (Met-bGH) (*13*), methionylalanyl bovine growth hormone (Met-[Ala]-bGH) (*9*), human α_1-interferon (IFN-α_1) (*14*), human γ-interferon (IFN-γ) (*15*), protein farnesyltransferase (*16*), and spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase subunit B and its derivatives (*17*) have been expressed in *E. coli* cells using a two-cistronic expression plasmid, the levels of accumulation of the gene products being improved. Schoner described the design of a two-cistronic expression plamid that enables heterologous gene expression by preventing the formation of an unfavorable intramolecular local secondary structure of mRNA at the RBS (*11*). However, the utility of a two-cistronic expression system has not been completely established, because there is no two-cistronic plasmid for which it has been clearly elucidated that the second cistron is highly expressed through prevention of inhibition of the translational initiation caused by an

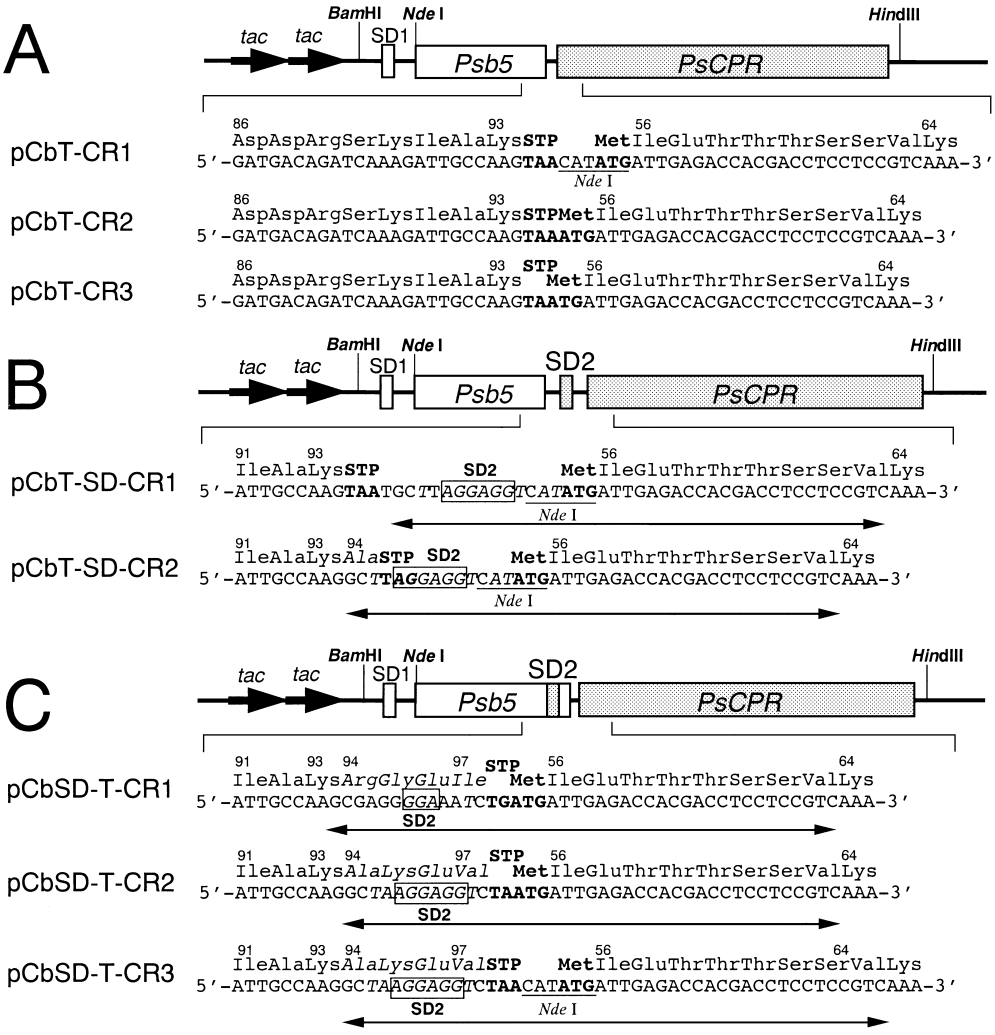

Fig. 2. **Three types of two-cistronic expression plasmids and their partial structures.** A: Partial nucleotide sequences of two-cistronic plasmids pCbT-CR1, -CR2, and -CR3, which have no SD2. B: Partial nucleotide sequences of pCbT-SD-CR1 and -SD-CR2, which have an SD2 in the intercistronic region. C: Partial nucleotide sequences of pCbSD-T-CR1, -T-CR2, and -T-CR3, which have an SD2 upstream of the 3′-terminal of the first cistron. The nucleotide sequence from CGA encoding Arg^{94} to ATG encoding the initiation codon in pCbSD-T-CR1 was designed to be similar to that in the *trp-A* gene. The nucleotide sequences from GCT encoding Ala^{94} to ATG encoding the initiation codon in pCbSD-T-CR2 and -T-CR3 are similar to the nucleotide sequence of pCW_{ori}^+ upstream of ATG encoding the initiation codon. STP in bold indicates the position of the nucleotides encoding the termination codon. The other representations of the partial nucleotide and amino acid sequences are the same as in the legend to Fig. 1.

unfavorable intramolecular secondary structure of mRNA at the RBS for the second cistron.

In this study, three types of two-cistronic expression plasmids have been constructed using the solubilized domain of porcine cytochrome b_5 (Psb5) gene (*Psb5* gene), which is highly expressed in *E. coli* cells with pCW_{ori}^+, as the first cistron (*18*), and the wild-type *PsCPR* gene as the second cistron, to determine the levels of expression of these genes. We have constructed two-cistronic plasmids pCP1 and pCP2, and have confirmed their utility for expression of heterologous genes through prevention of the formation of an unfavorable intramolecular local secondary structure of mRNAs at the RBS using the wild-type *PsCPR* gene and a mutant of the solubilized domain of porcine NADH-cytochrome b_5 reductase (Pb5R) gene (Pb5R-*Sal*I gene) (*8*). These two-cistronic plasmids are expected to be useful for comprehensive heterologous gene expression in *E. coli* cells.

MATERIALS AND METHODS

Materials—Plasmid pCW_{ori}^+ (*6*, *7*) was kindly provided by Dr. F. W. Dahlquist of the University of Oregon. This plasmid contains two tandem *tac* promoters and a RBS, which is highly complementary to the 3′-end region of the 16S rRNA in the 30S subunit of *E. coli* (Fig. 1A). *E. coli* strain BL21 [F^- ompT $hsdS_B$ ($r_B^-m_B^-$) *gal dcm*] was from Novagen. The enzymes for recombinant DNA technology were from TaKaRa, Toyobo, and Nippon Gene.

Construction of Two-Cistronic Plasmids—Plasmid pCbT-CR1 (Fig. 2A) was constructed as follows. An approximately 300 base pairs (bp) DNA fragment containing the *Psb5* gene was amplified from plasmid pCPb5 (*18*) (Fig. 1) by PCR using forward primer 5′-CCGGATCCATCGAATGCTTAGG-3′ (primer-1), which has the nucleotide sequence of pCW_{ori}^+ containing a *Bam*HI site (underlined) upstream of the *Nde*I site, and reverse primer 5′-*CGTGGTCTCAAT*cat**ATGTTA**CTTGGCAATC-3′ (primer-2), which has a nucleotide sequence complementary to the 3′-terminal region of the *Psb5* gene containing a termination codon (bold), an *Nde*I site (underlined), and a nucleotide sequence complementary to the 5′-terminal region of the *PsCPR* gene (italics) containing an initiation codon (lowercased). A DNA fragment of the *PsCPR* gene was amplified from plasmid pCPsCPR (*18*) (Fig. 1) using forward primer 5′-atg*ATTGAGACCACG*-3′ (primer-3), which has the nucleotide sequence of the 5′-terminal region of the wild-type *PsCPR* gene containing the nucleotides encoding the initiation codon (lowercased) and those complementary to the 5′-terminal nucleotides

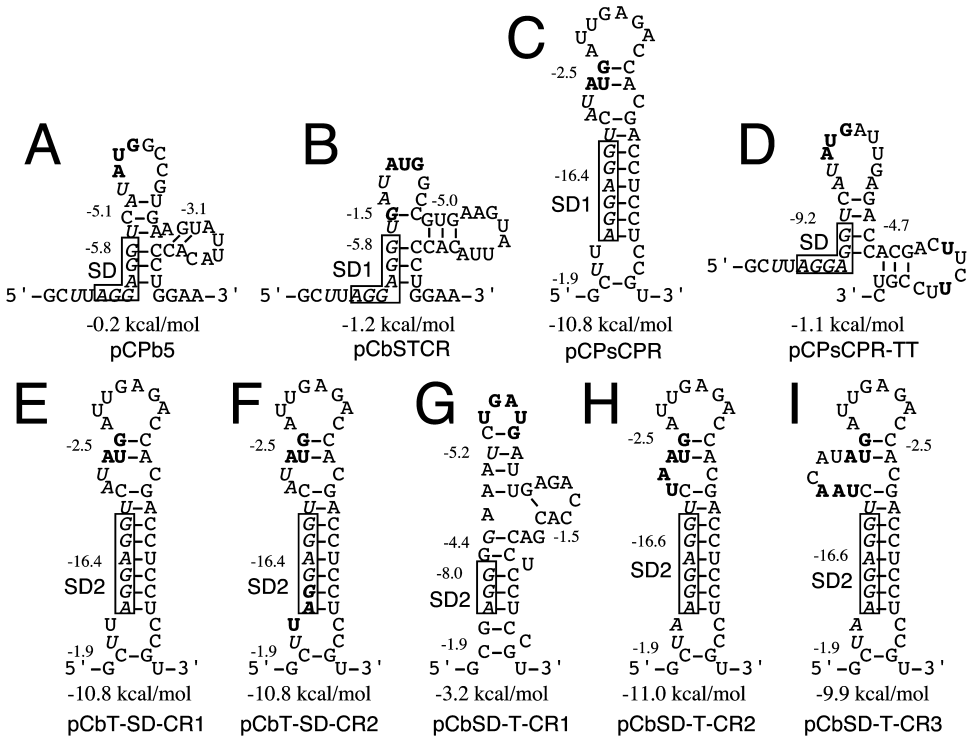

Fig. 3. **Putative partial secondary structures of mRNAs derived from the expression plasmids.** The local secondary structures of mRNAs derived from pCPb5 (A), pCbSTCR (B), pCPsCPR (C), pCPsCPR-TT (D), pCbT-SD-CR1 (E), pCbT-SD-CR2 (F), pCbSD-T-CR-1 (G), pCbSD-T-CR2 (H), and pCbSD-T-CR3 (I). These structures were predicted for the nucleotide sequences indicated in Figs. 1 and 2. The nucleotides complementary to the 3′-terminal region of *E. coli* 16S rRNA are shown in italics. The hypothetical RBS (AGGAGG or AGG) is boxed. The termination codon of the first cistron (UAA or UAG) and the initiation codon (AUG), and the silently mutated nucleotides in pCPsCPR-TT (D) are shown in bold letters. The names of the plasmids encoding the mRNAs and their predicted overall stability (ΔG) are shown under the secondary structures. The values beside the stem structures are the values of the stability of the stems (kcal/mol).

of primer-2 (italics), and reverse primer 5′-CCGCC<u>AAGCTT</u>**CTACTA**GCTCCACACACGTC-3′ (primer-4), which has a nucleotide sequence complementary to the 3′-terminal region of the *PsCPR* gene, two stop codons (bold), and a *Hin*dIII site (underlined). The two resultant PCR-products were purified, mixed, and reamplified with primer-1 and primer-4. The reamplified DNA fragment was inserted into pCW_{ori}^{+} using the *Bam*HI and *Hin*dIII sites to construct pCbT-CR1 (Fig. 2A).

Plasmids pCbT-CR2, pCbT-CR3, pCbT-SD-CR1, pCbT-SD-CR2, pCbSD-T-CR1, pCbSD-T-CR2, and pCbSD-T-CR3 (Fig. 2) were constructed by the same method as that used for the construction of pCbT-CR1 except for the use of forward primers 5′-*CGTGGTCTCAATcat***TTA**CTTGGCAATC-3′ (primer-5), 5′-*CGTGGTCTCAATCAt***TA**CTTGGCAATC-3′ (primer-6), 5′-*CGTGGTCTCAAT<u>cat</u>*<u>ATG</u>ACCTCCTAAGCA**TTA**CTTGGCAATCTTTGATC-3′ (primer-7), 5′-*CGTGGTCTCAAT<u>catATG</u>*ACCTC**CTA**AGCCTTGGCAATCTTTGATC-3′ (primer-8), 5′-*CGTGGTCTCAATCA***TCA**GATTTCCCCTCGCTTGGCAATCTTTGATC-3′ (primer-9), 5′-*CGTGGTCTCAATcat*TAGACCTCCTTAGCCTTGGCAATCTTTGATC-3′ (primer-10), and 5′-*CGTGGTCTCAAT<u>cat</u>*<u>ATG</u>TTAGACCTCCTTAGCCTTGGCAATCTTTGATC-3′ (primer-11) instead of primer-2,

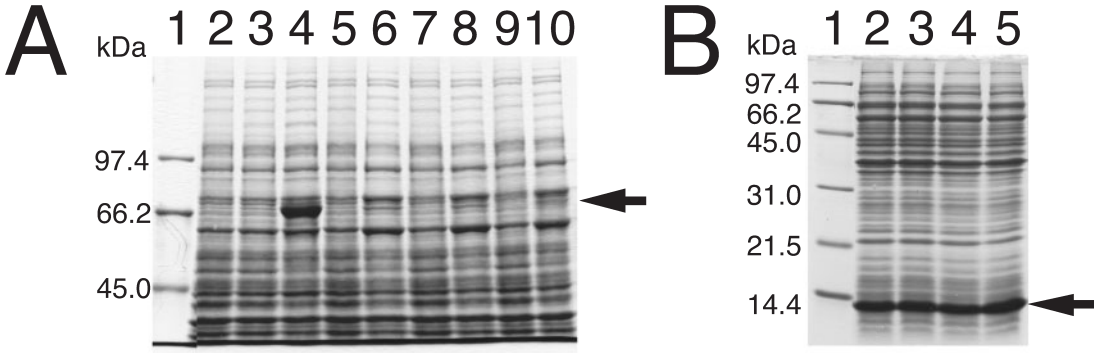

Fig. 4. **SDS-PAGE of *E. coli* whole cell extracts.** A: A 7.5% polyacrylamide gel stained with CBB R-250. Whole cell extracts of *E. coli* BL21 cells containing pCPsCPR (lanes 2 and 3), pCPsCPR-TT (lane 4), pCbT-CR1 (lanes 5 and 6), pCbT-CR2 (lanes 7 and 8), and pCbT-CR3 (lanes 9 and 10), which were cultivated in the absence (lanes 2, 5, 7, and 9) or presence (lanes 3, 4, 6, 8, and 10) of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The arrow indicates the position of the expressed PsCPR. B: A 15% polyacrylamide gel stained with CBB R-250. Whole cell extracts of *E. coli* BL21 cells containing pCbT-CR1 (lane 2), pCbT-CR2 (lane 3), pCbT-CR3 (lane 4), and pCPb5 (lane 5), which were cultivated in the presence of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The arrow indicates the position of Psb5. In both A and B, a whole cell extract from 25 μl culture fluid was analyzed. Bio-Rad SDS-PAGE Molecular Weight Standards including rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), bovine pancreatic trypsin inhibitor (21.5 kDa), and hen egg lysozyme (14.4 kDa) were used as marker proteins.

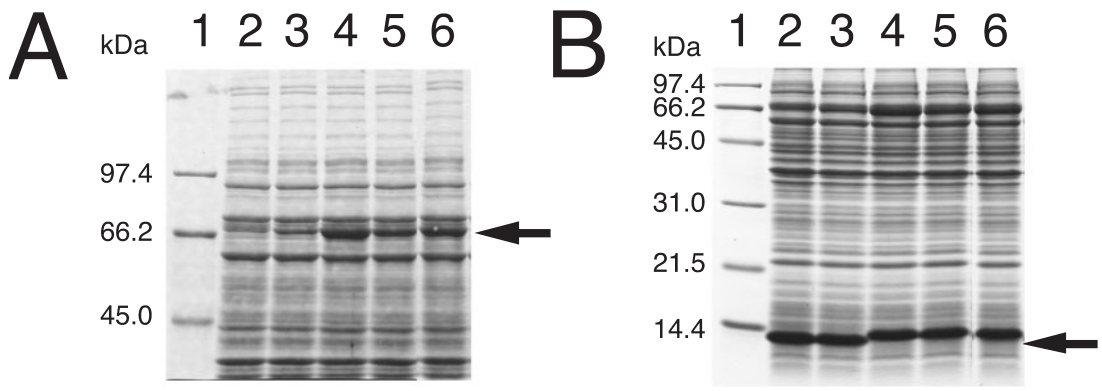

Fig. 5. **SDS-PAGE of *E. coli* whole cell extracts containing plasmids which have nucleotides encoding an SD2. 7.5% (A) and 15% (B) polyacrylamide gels stained with CBB R-250 are shown.** In both A and B, whole cell extracts of *E. coli* BL21 cells containing pCbT-SD-CR1 (lane 2), pCbT-SD-CR2 (lane 3), pCbSD-T-CR1 (lane 4), pCbSD-T-CR2 (lane 5), and pCbSD-T-CR3 (lane 6), which were cultivated in the presence of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The arrows in A and B indicate the positions of the expressed PsCPR and wild-type Psb5, respectively. The marker proteins and the amounts of the whole cell extracts, which were subjected to electrophoresis, were the same as those in the legend to Fig. 4.

respectively. In these nucleotide sequences, the nucleotides complementary to primer-3, the initiation codon, and the *Nde*I site are indicated by italics, dots, and underlining, respectively.

Pasmid pCbSTCR (Fig. 6), in which the *Nde*I site (CATATG) in pCbSD-T-CR3 was deleted by a single nucleotide change of the first C to G in this site, was constructed as follows. At first, two 5′-phosphated oligonucleotides, 5′-GATCCATCGATGCTTAGGAGGTGA-3′ and 5′-TATCACCTCCTAAGCATCGATG-3′, were annealed and then ligated with the *Nde*I–*Bam*HI fragment of pCPsCPR to construct pCb5(Δ*Nde*I). Then, a DNA fragment containing the *Psb5* gene was amplified from pCb5(Δ*Nde*I) using primer-1 and reverse primer 5′-*CGTGGTCTCAATcat*-ATG**TTA**GACCTCCTTAGCCTTGGCAATCTTTGATC-3′ (primer-12), which has a nucleotide sequence complementary to the 3′-terminal region of the *Psb5* gene, a termination codon (bold), an *Nde*I site (underlined) including an initiation codon (lowercased), and the 5′-terminal region of the *PsCPR* gene (italics). The resultant fragment was inserted into pCb-SD-T-CR3 using the *Bam*HI and *Nde*I sites to construct pCbSTCR.

Plasmids pCbSTCR-1, pCbSTCR-2, pCbSTCR-3, and pCbSTCR-4 (Fig. 6) were constructed as follows. DNA fragments, from which the 3′-terminal region of the *Psb5* gene was deleted, were amplified from pCbSTCR using primer-1 and reverse primers 5′-**TA**GACCTCCTTAG-CATGCAGCTCCCCAATG-3′ (primer-13) and 5′-*GGTC-TCAATcat*ATG**TTA**GACCTCCTTAGC-3′ (primer-14). The resultant fragment was inserted into pCPsCPR using the *Bam*HI and *Nde*I sites to construct pCbSTCR-1. Plasmids pCbSTCR-2, pCbSTCR-3, and pCbSTCR-4 were con-

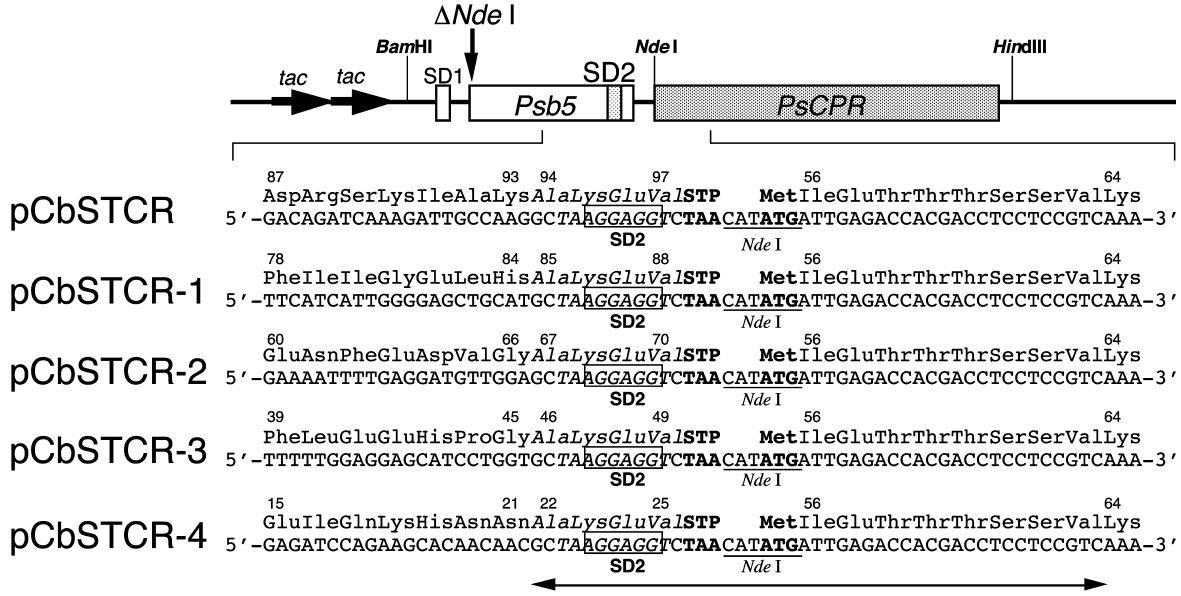

Fig. 6. **Partial structures of derivatives of pCbSD-T-CR3.** The partial structures of plasmids pCbSTCR, pCbSTCR-1, pCbSTCR-2, pCbSTCR-3, and pCbSTCR-4, which are derivatives of pCbSD-T-CR3, are shown. The representations of the partial nucleotide and amino acid sequences are the same as those in the legend to Fig. 2.

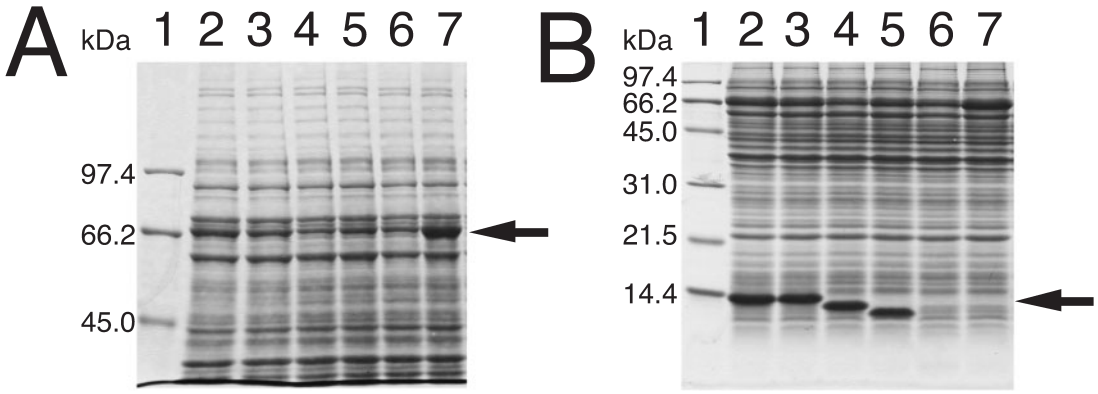

Fig. 7. **SDS-PAGE of *E. coli* whole cell extracts containing derivatives of pCbSD-T-CR3.** 7.5% (A) and 15% (B) polyacrylamide gels stained with CBB R-250 are shown. In both A and B, whole cell extracts of *E. coli* BL21 cells containing pCbTSD-T-CR3 (lane 2), pCbSTCR (lane 3), pCbSTCR-1 (lane 4), pCbSTCR-2 (lane 5), pCbSTCR-3 (lane 6), and pCbSTCR-4 (lane 7), which were cultivated in the presence of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The arrows in A and B indicate the positions of the expressed PsCPR and wild-type Psb5, respectively. The marker proteins and the amounts of the whole cell extracts, which were subjected to electrophoresis, were the same as those in the legend to Fig. 4.

structed by the same method as that for the construction of pCbSTCR-1 except for the use of reverse primers 5′-**TA**GACCTCCTTAGCTCCAACATCCTCAAAA-3′ (primer-15), 5′-**TA**GACCTCCTTAGCACCAGGATGCTCCTCC-3′ (primer-16), and 5′-**TA**GACCTCCTTAGCGTTGTTGTG-CTTCTGG-3′ (primer-17) instead of primer-13, respectively. In the nucleotide sequences of primer-13 to primer-17, the nucleotides complementary to the *PsCPR* gene, initiation codon, and *Nde*I site are indicated by italics, dots, and underlining, respectively.

Plasmids pCP1 and pCP2 (Fig. 8) were constructed by replacing the *Nde*I–*Hin*dIII fragments of pCbSTCR and pCbSTCR-4 with that of pCW_{ori}^{+}, respectively.

Plasmids pCP1-Pb5R-*Sal*I and pCP2-Pb5R-*Sal*I (Fig. 9) were constructed by replacing the *Nde*I–*Hin*dIII fragments of pCP1 and pCP2 with that of pCPb5R-*Sal*I (*8*), respectively.

The entire nucleotide sequences of the manipulated parts in the expression plasmids were confirmed by nucleotide sequencing with an ABI PRISM 310 Genetic Analizer.

Expression and Analysis of Protein Accumulation—The heterologous genes in the expression plasmids were expressed in *E. coli* BL21 cells. The cells containing an expression plasmid were cultivated in 2× YT medium containing 50 μg/ml ampicillin with shaking at 37°C overnight, and then inoculated into LB medium containing 50 μg/ml ampicillin in a ratio of 1/200 (v/v). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM for induction of gene expression after shaking at 37°C for 1.5 h, and then cultivation was continued with shaking at 37°C for 20 h.

Protein accumulation in the cells was analyzed by SDS-PAGE and Western blot analysis of whole cell extracts by the same methods as previously described (*8*). Proteins on the gel were stained with Coomassie Brilliant Blue (CBB) R-250 (Fulka).

Secondary Structure Prediction of mRNA—The local secondary structure of mRNA was predicted with a program, GeneBee (http://genebee.msu.su), on the Web (*19*, *20*), as previously described (*8*).

RESULTS

Two-Cistronic Gene Expression without SD2—Plasmids pCbT-CR1, -CR2, and -CR3 (Fig. 2A), were constructed to determine whether or not the Shine-Dalgarno sequence for the second cistron (SD2) is necessary for two-cistronic expression of the second cistron. In the nucleotide sequences of the mRNAs from these plasmids, there is no possible SD2 within 26 bases upstream from the initiation codon of the second cistron. The distance between the termination codon of the first cistron (UAA) and the initiation codon (AUG) in the mRNAs from pCbT-CR1 and -CR2 was 3 and 0 bases, respectively. In the mRNA from pCbT-CR3, the third nucleotide of the termination codon and the first nucleotide overlapped.

All of the *E. coli* BL21 cells containing these plasmids (BL21/pCbT-CR1, -CR2, and -CR3) similarly gave thin bands at the same position as the band of PsCPR on the gel only after cultivation in the presence of IPTG (Fig. 4A, lanes 6, 8, and 10). These thin bands were specifically immunostained using anti-rat CPR antibodies after Western blotting (data not shown). All of these cells gave thick bands at the same position as Psb5 after cultivation with IPTG, indicating that the *PsCPR* gene was coexpressed with the *Psb5* gene polycistronically (Fig. 4B, lanes 2–5). These results indicate that the second cistron can be coexpressed with the first cistron without an SD2, but level of expression of the second cistron was very low.

Two-Cistronic Gene Expression with an SD2—Two groups of plasmids, which produce two-cistronic mRNAs having an SD2, were constructed. One group comprised pCbT-SD-CR1 and -SD-CR2 (Fig. 2B), and the other group comprised pCbSD-T-CR1, -T-CR2, and -T-CR3 (Fig. 2C). The former plasmids encode mRNAs that have an SD2 in the region from the termination codon of the first cistron (*Psb5* gene) to the initiation codon of the second cistron (*PsCPR* gene), and the latter plasmids encode mRNAs that have an SD2 upstream of the termination codon of the first cistron. In pCbSD-T-CR1, the nucleotide sequence encoding from Arg^{94} in Psb5 to the initiation codon of the *PsCPR* gene (5′-CGAGGGGAAATCTGATG-3′) is identical to the sequence of the flanking region of

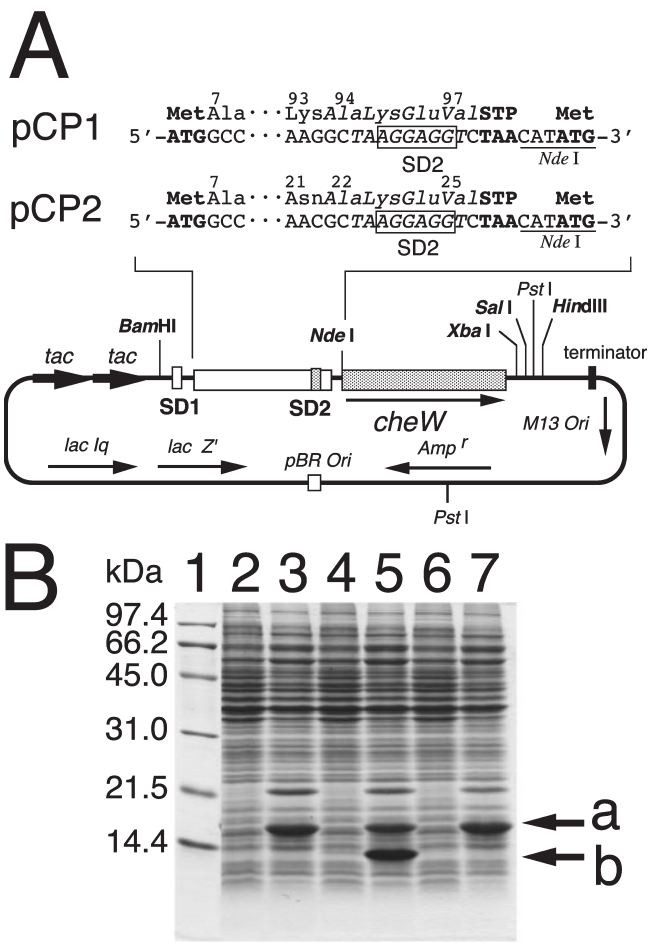

Fig. 8. **Plasmid pCP1 and pCP2, and SDS-PAGE of *E. coli* whole cell extracts containing these plasmids.** A: Structures of plasmids pCP1 and pCP2. The representations of the partial nucleotide sequences are the same as those in the legend to Fig. 2. B: A 15% polyacrylamide gel stained with CBB R-250. Whole cell extracts of *E. coli* BL21 cells containing pCW_{ori}^{+} (lanes 2 and 3), pCP1 (lanes 4 and 5), and pCP2 (lanes 6 and 7), which were cultivated in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The marker proteins and the amounts of the whole cell extracts, which were subjected to electrophoresis, were the same as those in the legend to Fig. 4. Arrows a and b indicate the positions of the bands of CheW and Psb5, respectively.

the *trpA* gene at the junction of the two-cistronic *trpB–trpA* gene pair of the tryptophan operon of *E. coli* (*21*).

The level of accumulation of PsCPR depends on both the overall stability (ΔG) of the predicted intramolecular local secondary structure of mRNA and the stability of the continuous double strand structure at the RBS. We have previously demonstrated that the predicted local secondary structure of mRNA, of which the overall ΔG value is not greater than –6.1 kcal/mol, and the 3′-portion of GAGG in the hypothetical RBS (AGGAGG), which are involved in a continuous double strand with a ΔG value of not more than –13.2 kcal/mol, greatly decreased the level of accumulation of PsCPR (*8*). The predicted local secondary structures of mRNAs derived from pCbT-SD-CR1, -SD-CR2, pCbSD-T-CR2, and -T-CR3 are similar to in the case of pCPsCPR (Fig. 3). For these secondary structures, the overall ΔG values are not more than –9.9 kcal/mol, and the whole of the hypothetical RBS is involved in the continuous double strand with ΔG values of not more than –16.4 kcal/mol, suggesting that these secondary structures have enough potential to cause translational initiation inhibition of the *PsCPR* gene (Fig. 3E, F, H and I). For the predicted secondary structure of mRNA from pCbSD-T-CR1, the overall ΔG value was –3.2 kcal/mol, and the ΔG value of the double strand involving the hypothetical RBS was –8.0 kcal/mol, suggesting that the potential of the mRNA to cause translational initiation inhibition was low (Fig. 3G).

E. coli BL21/pCbT-SD-CR1 and -SD-CR2 gave obvious bands of PsCPR after cultivation in the presence of IPTG (Fig. 5A, lanes 2 and 3). These bands were thicker than those shown by BL21/pCbT-CR1, -CR2 and -CR3 in Figure 4A, but thinner than those shown by BL21/pCbSD-T-CR1, -T-CR2, and -T-CR3 (Fig. 5A, lanes 4–6). The bands of PsCPR shown by BL21/pCbSD-T-CR2 and -T-CR3 (Fig. 5A, lanes 5 and 6) were thinner than that shown by BL21/pCbSD-T-CR1 (Fig. 5A, lane 4). The thickness of the band of PsCPR shown by BL21/pCbSD-T-CR1 was roughly similar to that shown by BL21/pCPsCPR-TT (Fig. 4A, lane 4). Plasmid pCPsCPR-TT is a mono-cistronic high-level expression plasmid for PsCPR with silent mutations at nucleotides in the PsCPR gene, which destabilize the local secondary structure of mRNA (Figs. 1B and 3D). These results suggest that the SD2s that are positioned upstream of the termination codon of the first cistron are more effective for two-cistronic high-level expression of the PsCPR gene than the SD2s that are positioned in the region from the termination codon in the first cistron to the initiation codon of the second cistron.

E. coli BL21/pCbT-SD-CR1 and -SD-CR2 gave thick bands at almost an identical position to Psb5 on a 15% polyacrylamide gel (Fig. 5B, lanes 2 and 3). *E. coli* BL21/pCbSD-T-CR1, -T-CR2, and -T-CR3 also gave similarly thick bands, but the mobilities of the bands were slightly lower than that of Psb5 (Fig. 5B, lanes 4–6), consistent with the C-terminal elongation of Psb5. The densities of these bands were similar to those of Psb5 shown by BL21/pCbT-CR1, -CR2, -CR3, and BL21/pCPb5 in Figure 4B, indicating that the existence of the second cistron minimally affected the level of accumulation of the first cistron product.

Improvement of pCbSD-T-CR3—As described above, plasmids pCbSD-T-CR2 and -T-CR3 enabled the two-cistroronic expression of PsCPR, although the predicted intramolecular local secondary structure of mRNA at the SD2 was identical to that from pCPsCPR. This finding clearly indicated that these two-cistronic plasmids were useful for heterologous gene expression in *E. coli* cells by preventing the formation of an unfavorable intramolecular secondary structure of mRNA at the RBS, which disturbs translational initiation. However, the levels of accumulation of PsCPR with these plasmids were lower than that with pCPsCPR-TT. In order to improve the expression level of the *PsCPR* gene, plasmids pCbSTCR, pCbSTCR-1, -2, -3, and -4 (Fig. 6) were constructed from pCbSD-T-CR3. In pCbSTCR, an *Nde*I site at the beginning of the first cistron in pCbSD-T-CR3 was deleted by means of a single nucleotide mutation of C to

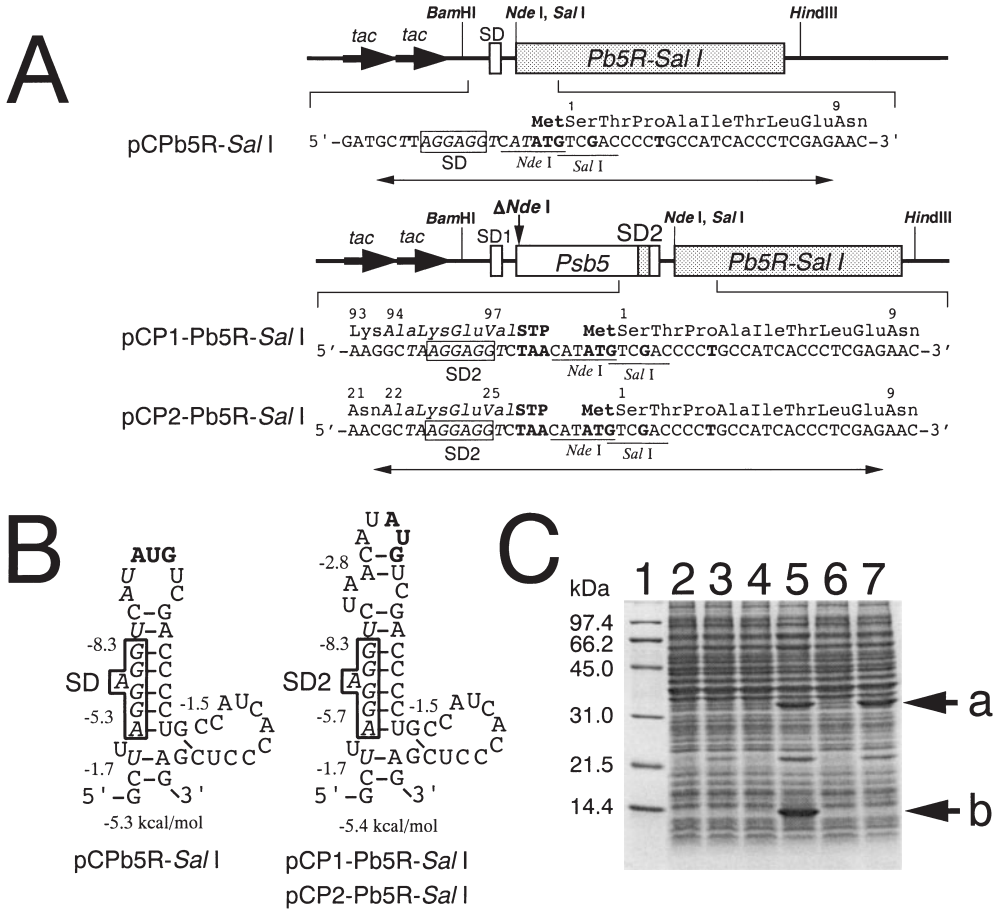

Fig. 9. **Expression of the *Pb5R-SalI* gene using pCP1 and pCP2.** A: Partial structures of plasmids pCPb5R-*Sal*I, pCP1-Pb5R-*Sal*I, and pCP2-Pb5R-*Sal*I. The representations of the partial nucleotide and amino acid sequences are the same as those in the legend to Fig. 2. B: The predicted local secondary structures of mRNAs derived from pCPb5R-*Sal*I, pCP1-Pb5R-*Sal*I, and pCP2-Pb5R-*Sal*I. The representations of the secondary structures are the same as those in the legend to Fig. 3. C: A 15% polyacrylamide gel stained with CBB R-250. Whole cell extracts of *E. coli* BL21 cells containing pCPb5R-*Sal*I (lanes 2 and 3), pCP1-Pb5R-*Sal*I (lanes 4 and 5), and pCP2-Pb5R-*Sal*I (lanes 6 and 7), which were cultivated in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of 0.2 mM IPTG, and marker proteins (lane 1) were subjected to electrophoresis. The marker proteins and the amounts of the whole cell extracts, which were subjected to electrophoresis, were the same as those in the legend to Fig. 4. Arrows a and b indicate the positions of the bands of Pb5R and Psb5, respectively.

G at the *Nde*I site. The predicted local secondary structure of mRNA at the SD1 from pCbSTCR was similar to that from pCPb5, suggesting that this mutation minimally changes the local secondary structure at the RBS for the *Psb5* gene (Fig. 3, A and B). In pCbSTCR-1, -2, -3, and -4, the nucleotides encoding Pro^{85}–Lys^{93}, His^{67}–Lys^{93}, Gly^{46}–Lys^{93}, and Ser^{22}–Lys^{93} in the Psb5 mutant were deleted in addition to deletion of the *Nde*I site at the beginning of the first cistron, respectively. The local secondary structures of mRNA in the regions corresponding to Ala^{94} in Psb5 mutants and to Val^{63} in PsCPR from the plasmids shown in Figure 6 were identical to that from pCbSD-T-CR3 (Fig. 3I).

The densities of the bands of both PsCPR and the Psb5 mutant shown by BL21/pCbSTCR were almost the same as those by BL21/pCbSD-T-CR3, indicating that the deletion of the *Nde*I site at the beginning of the first cistron minimally affected the levels of accumulation of PsCPR and the Psb5 mutant (Fig. 7, A and B, lanes 2 and 3). The band of PsCPR shown by BL21/pCbSTCR-4 (Fig. 7A, lane 7) was obviously thicker than that by BL21/pCbSTCR (Fig. 7A, lane 3), and similar to those by BL21/pCPsCPR-TT (Fig. 4A, lane 4) and BL21/pCbSD-T-CR3 (Fig. 5A, lane 4). However, the bands of PsCPR shown by BL21/pCbSTCR-1, -2, and -3 were thinner than that by BL21/pCbSTCR (Fig. 7A, lanes 3–6). The densities of the bands of PsCPR shown by the cells exhibited the following order; BL21/pCbSTCR-1 < BL21/pCbSTCR-3 < BL21/pCbSTCR-2 < BL21/pCbSTCR < BL21/pCbSTCR-4. These results indicate that a plasmid in which the first cistron is shorter does not always accumulate a higher amount of PsCPR, although the level of accumulation of PsCPR increased with pCbSTCR-4, in which the length of the first cistron was shortened to 63 bases. *E. coli* BL21/pCbSTCR-1 and -2 gave bands at positions corresponding to lower molecular weights than that of Psb5 on the 1.5% polyacrylamide gel (Fig. 7B, lanes 4 and 5). The positions of these bands of BL21/pCbSTCR-1 and -2 were consistent with the lengths of the expected first cistron products (82 and 64 amino aid residues, respectively). No obvious bands of the first cistron products derived from BL21/pCbSTCR-3 and -4 were visible on the gel (Fig. 7B, lanes 6 and 7). The synthesized first cistron products derived from pCbSTCR-3 (43 amino acid residues) and -3 (19 amino acid residues) were too short to detect on the SDS-PAGE gel under the experimental conditions used.

Construction of pCP1 and pCP2—The *Nde*I–*Hin*dIII fragments of pCbSTCR and pCbSTCR-4 were replaced with the *Nde*I–*Hin*dIII fragment of pCW$_{ori}^{+}$, which contained the *cheW* gene, and following *Xba*I, *Sal*I, and *Pst*I sites, to construct pCP1 and pCP2, respectively (Fig. 8A). These additional unique *Xba*I and *Sal*I sites are useful for replacing the second-cistron. After cultivation with IPTG, *E. coli* BL21/pCP1 and BL21/pCP2 gave thick bands at the position of the CheW protein (Fig. 8B). The band at this position shown by BL21/pCP2 (Fig. 8B, lane 7) was thicker than that by BL21/pCP1 (Fig. 8B, lane 5), and similar to that by BL21/pCW$_{ori}^{+}$ (Fig. 8B, lane 3). *E.*

coli BL21/pCP1 gave a thick band at the position of Psb5 on the gel, while BL21/pCP2 did not.

Expression of the Pb5R-SalI Gene Using pCP1 and pCP2—The translational initiation of the *Pb5R-SalI* gene was inhibited by the formation of an intramolecular stable secondary structure at the RBS of the mRNA from plasmid pCPb5R-*Sal*I (*8*). In order to determine the utility of pCP1 and pCP2 for high-level expression of the *Pb5R-SalI* gene, plasmids pCP1-Pb5R-*Sal*I and pCP2-Pb5R-*Sal*I were constructed (Fig. 9A). The predicted local secondary structures of mRNAs from pCP1- and pCP2-Pb5R-*Sal*I (Fig. 9B) showed a 7 bp long stem comprising base-paired AGG and GG, and unpaired A in the RBS, which was identical to that from pCPb5R-*Sal*I. The ΔG values of the overall local secondary structures of mRNAs from these plasmids were almost identical to that from pCPb5R-*Sal*I. *E. coli* BL21/pCP1- and pCP2-Pb5R-*Sal*I gave thick bands at the position of Psb5R on the SDS-PAGE gel after cultivation in the presence of IPTG (Fig. 9C, lanes 5 and 7), while *E. coli* BL21/pCPb5R-*Sal*I did not (Fig. 9C, lane 3). *E. coli* BL21/pCP1-Pb5R-*Sal*I gave a thick band at the position of Psb5 (Fig. 9C, lane 5), while BL21/pCP2-Pb5R-*Sal*I did not (Fig. 9C, lane 7). These results suggest that pCP1 and pCP2 are useful for high-level expression of the Pb5R-*Sal*I gene by preventing inhibition of the translational initiation caused by the formation of the intramolecular secondary structure of mRNA at the RBS.

DISCUSSION

The levels of expression of heterologous genes in *E. coli* cells largely depend on the local secondary structure of mRNA, and modulation of the local secondary structure is one of the important strategies for controling and improving the expression level (*8*, *22–27*). We previously elucidated that the very low level of *PsCPR* gene expression in *E. coli* cells with pCPsCPR is due to the formation of a stable intramolecular secondary structure of the mRNA at the RBS, and demonstrated that destruction of the secondary structure of mRNA on introducing silent mutations based on the secondary structure prediction is a useful approach for controling and increasing the level of expression of the *PsCPR* gene (*8*). This approach was also useful for improvement of the expression levels for human inducible nitric oxide synthase (iNOS) reductase domains (Yamamoto *et al*., personal communication). However, it is hard to design an effective silent mutation of the gene, of which the nucleotide sequence is unknown. In addition, it is not realistic to introduce appropriate silent mutations into each of so many genes at the same time, particularly when comprehensive gene expression is needed to analyze the functions of the gene products. Therefore, another approach, with which one can destroy the unfavorable intramolecular secondary structure of mRNA independent of the nucleotide sequence of the target gene, is required. We considered that the wild-type *PsCPR* gene is a good reporter gene for analyzing the potential of two-cistronic expression plasmids to prevent the formation of an unfavorable intramolecular secondary structure of mRNA at the RBS. In addition, the wild-type *Psb5* gene, which is a highly expressed short gene using pCW_{ori}^{+} (*18*), is considered to be a useful gene as the first cistron in a pCW_{ori}^{+}-based two-cistronic high-level expression plasmid. In this study, we systematically constructed three types of pCW_{ori}^{+}-based two-cistronic expression plasmids, which have the *Psb5* gene as the first cistron and the *PsCPR* gene as the second cistron, to determine the level of accumulation of PsCPR in *E. coli* BL21 cells.

At first, pCbT-CR1, -CR2, and -CR3 were constructed to determine the requirement of an SD2 for the high-level expression of the second cistron (Fig. 2A). The SD2 was not necessary for two-cistrinic expression of the second cistron, but the expression levels for the second cistron were very low. This finding is consistent with the observation that the *trpA* gene in the *trpB-trpA* gene pair (*28*) and the IFN-α_1 gene in the two-cistronic expression system with the coat protein gene of bacteriophage MS2 (*14*) can be expressed in the absence of an SD2.

Then, two types of plasmids were constructed to improve the level of accumulation of the second cistron by use of an SD2 (Fig. 2, B and C). The SD2 was needed for two-cistronic high-level expression of the second cistron, and the predicted local secondary structures of mRNA from all the plasmids except pCbSD-T-CR1 suggested that the inhibition of the translational initiation on the formation of an unfavorable intramolecular secondary structure of mRNA at the SD2 was overcome by these two-cistronic plasmids. In the case of the mRNA from pCbSD-T-CR1, the predicted local secondary structure at the RBS was different and less stable than that from pCPsCPR (Fig. 3G). Therefore, direct single-cistronic expression of the *PsCPR* gene might occur in addition to two-cistronic expression, resulting in the accumulation of a very high level of the PsCPR protein.

Two-cistronic expression plasmids pCbSD-T-CR1, -T-CR2, and -T-CR3 (Fig. 2B), which encode mRNA having an SD2 upstream of the termination codon in the 3′-terminal region of the first cistron, were more effective for high-level expression of the second cistron than pCbT-SD-CR1 and -SD-CR2 (Fig. 2C), which encode mRNA having an SD2 in the region from the termination codon of the first cistron to the initiation codon of the second cistron. The enhancement of the expression level by the SD2 positioned upstream of the termination codon of the first cistron was also observed for two-cistronic expression of the Met-[Ala]-bGH (*9*) and the IFN-α_1 genes (*14*). In the tertiary structure of the 70S ribosome from *Thermus thermophilus* (*29*, *30*), the E-site, P-site, and A-site are positioned in this order in the direction from the 5′-terminal to the 3′-terminal of mRNA, and the 3″-terminal region of 16S rRNA, which recognizes the RBS in mRNA, is located near the E-site. This steric arrangement is probably conserved in the *E. coli* ribosome. At the end of translation of the first cistron, the termination codon is recognized at the A-site in the ribosome. At that time, the SD2, which is positioned upstream of the termination codon of the first cistron at an appropriate distance from the termination codon, can be positioned close to the 3′-terminal region of 16S rRNA, resulting in effective translational coupling. The level of accumulation of PsCPR by pCbSD-T-CR3 is higher than that by pCbSD-T-CR2, although the levels of accumulation of Pb5 by the two plasmids are almost identical (Fig. 5). In the mRNAs from these plasmids, the nucleotide sequences from the

SD2 (AGGAGG) to the termination codon (UAA) are identical, but the relative positioning of the termination codon and initiation codon (AUG) is different (Fig. 2C). These observations suggest that not only the distance between the SD2 and the termination codon, but also the relative positioning of the termination codon and initiation codon for the second cistron affects the efficiency of the translational coupling.

In order to improve the level of accumulation of PsCPR, the length of the first cistron in pCbSD-T-CR3 was systematically shortened (Fig. 6). Schoner (*11*) described that the length of the first cistron should be kept to a minimum to avoid unnecessary expenditure of energy and precursors for the synthesis of the peptide it encodes. Indeed, the level of accumulation of PsCPR was increased in pCbSTCR-4, which has the shortest first cistron prepared here. However, in the case of pCbSTCR-1, -2, and -3, the level of accumulation did not increase but decrease, although the first cistrons in these plasmids were shorter than those in pCbSD-T-CR3 and pCbSTCR (Fig. 7). The levels of accumulation of the first cistron products were not decreased by the use of at least pCbSTCR-1 and -2 (Fig. 7B). These results indicate that a shorter length of the first cistron is not always better for increasing the expression level of the second cistron. Further analysis is needed to determine why the level of expression of PsCPR was not increased by pCbSTCR-1, -2, and -3.

The first cistron products from pCbSTCR-3 and -4 were not detected on an SDS-PAGE gel, because the lengths of the products were too short (Fig. 7). However, the *PsCPR* genes in these plasmids were probably co-expressed with the first cistron. Since the predicted intramolecular local secondary structures of mRNAs at the SD2 from these plasmids were similar to that from pCPsCPR and stable enough to cause translational inhibition of the *PsCPR* gene, it is very improbable that the *PsCPR* genes in these plasmids were expressed at such high levels as shown in Fig. 7 independent of the first cistron.

Two-cistronic plasmids pCbSTCR and pCbSTCR-4 were improved for expression of other genes as the second cistron to construct pCP1 and pCP2, respectively (Fig. 8). It is clear that the *PsCPR* gene is highly expressed with pCP1 and pCP2, since replacement of the *Nde*I–*Hin*dIII fragment containing the *cheW* gene in pCP1 and pCP2 with the *Nde*I–*Hin*dIII fragment of pCPsCPR containing the *PsCPR* gene resulted in pCbSTCR and pCbSTCR-4, respectively. The *Pb5R-SalI* gene was highly expressed using pCP1 and pCP2 (Fig. 9), indicating that not only the *PsCPR* gene but also the *Pb5R-SalI* gene is highly expressed with pCP1 and pCP2 through prevention of the translational initiation caused by the formation of an unfavorable secondary structure of mRNA at the RBS. These plasmids are considered to be useful tools not only for high-level expression of other heterologous genes, which were unsuccessfully highly expressed using conventional other expression plasmids, through prevention of inhibition of the translational initiation, but also for comprehensive gene expression analyses. In addition, polycistronic plasmids, which will be obtained from these plasmids by increasing the number of cistrons, are expected to be useful for the direct synthesis of complex proteins that are composed of plural different polypeptide chains, and a set of proteins such as electron transfer proteins that physiologically act together. However, it should be noted that the use of a two-cistronic expression plasmid does not always allow high-level production of a heterologous protein in *E. coli* cells, because the level of production of the heterologous protein does not only depend on the efficiency of translational initiation.

In conclusion, direct evidence that a two-cistronic expression system is useful for the expression of heterologous genes in *E. coli* cells through prevention of the inhibition of the translational initiation caused by the intramolecular local secondary structure of mRNA at the RBS was provided using the *PsCPR* and *Pb5R-SalI* genes. Two-cistronic expression plasmids pCP1 and pCP2 were constructed. These plasmids are expected to be useful for comprehemsive high-level expression of heterologous genes in *E. coli* cells. We hope that the usefulness and generality of these plasmids will be confirmed by the two-cistronic expression of a number of heterologous genes other than the *PsCPR* and *Pb5R-SalI* genes using these plasmids.

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