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Expression of Partial Genes of Bacterioopsin in *Escherichia Coli*

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Bacteriorhodopshin (BR) is an intrinsic membrane protein composed of seven membrane-spanning helices (A-G). Partial genes of bacterioopsin (BOP) which encode the peptides including ABCD helices and EFG helices of bacteriorhodopsin were independently expressed in *Escherichia coli* (*E. coli*). Six inducible expression vectors were constructed, three of which contain a partial gene coding the ABCD helices of BOP (vectors pUBOAIN, pKBOAIN and pTKBOAIN), and three coding the EFG helices of BOP (vectors pUBOAIC, pKBOAIC and pTKBOAIC). The vectors pUBOAIN and pUBOAIC contain lac-promoter and the vectors pKBOAIN and pKBOAIC contain tac-promoter followed by the partial genes of BR. The vectors pTKBOAIN and pTKBOAIC contain a nucleotide fragment encoding the presequence of the manganese-stabilization protein of *Anacystis nidurans* between the lac-promoter and the BR partial gene. The expression of the resulting fusion proteins were detected by ELISA using mouse anti-BR serum. The fusion proteins prepared from *F. coli* transformed by pTKBOAIN or pTKBOAIC were estimated to comprise more than 1% of the total membrane protein in the *E. coli*.

KEYWORDS: bacteriorhodopsin, expression in *E. coli*

Introduction

Bacteriorhodopsin (BR), which is the sole protein constituent of the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump. Blaurock and Stoeckenius (1971) found that BR consists of a chromophore, retinal conjugated with a single 26 kD polypeptide bacterioopsin (BOP) with seven α helices spanning the membrane almost perpendicularly to its surface, arranged in trimers which form a two-dimensional crystalline lattice.

It has been reported by Liao, Huang and Khorana (1983) that BR molecules can be reconstituted with two chymotryptic fragments, one containing two (A and B helices) and the other five helices (C, D, E, F and G helices) of BOP. This implies that individual α-helix interacts specifically with neighboring helices and that BR is able to regenerate itself from helices in partial polypeptide fragments of BOP. Kataoka, Kahn, Tsujiuchi, Engelman and Tokunaga (1992) found that BR reconstituted from chemically synthesized peptide fragments containing helices AB and native helices CDEFG performed a normal photoreaction.

Preparations of partial peptides containing more than two helices of BR can be produced readily: helices AB and CDEFG by chymotrypsin cleavage which was found by Liao, London and Khorana (1983); and helices ABCD and EFG by $NaBH_4$ treatment which was found by Liao, Huang and Khorana (1984). The fragments of the other combinations of α-helices are difficult to obtain by such simple chemical treatment or by partial digestion of BR, so we have applied the recombinant DNA techniques to this end. Firstly, we identified four unique adequate sites for restriction enzymes in the BOP gene for taking advantage of partial genes of native BOP (Fig. 1).

Tsujiuchi, Iwasa and Tokunaga (1994) constructed the expression vector pUBO and reported the expression of BOP in *E. coli*. We designed and constructed the several expression vectors by means of recombinant DNA techniques for partial peptides of BOP. In the present paper we report on the preparation of expression vectors with pUBO and also describe the level of expression of partial genes of BOP.

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Abbreviations: BR, bacteriorhodopsin; BOP, bacterioopsin; *E. coli*, *Escherichia coli*; MSP, Manganese-stabilizing protein of *Anacystis nidurans*; IPTG, Isopropyl-β-D-thiogalactopyranoside; ELISA, Enzyme linked immuno-sorbent assay; HRPO, Horseradish peroxidase; OPD, orthophenylene diamine.

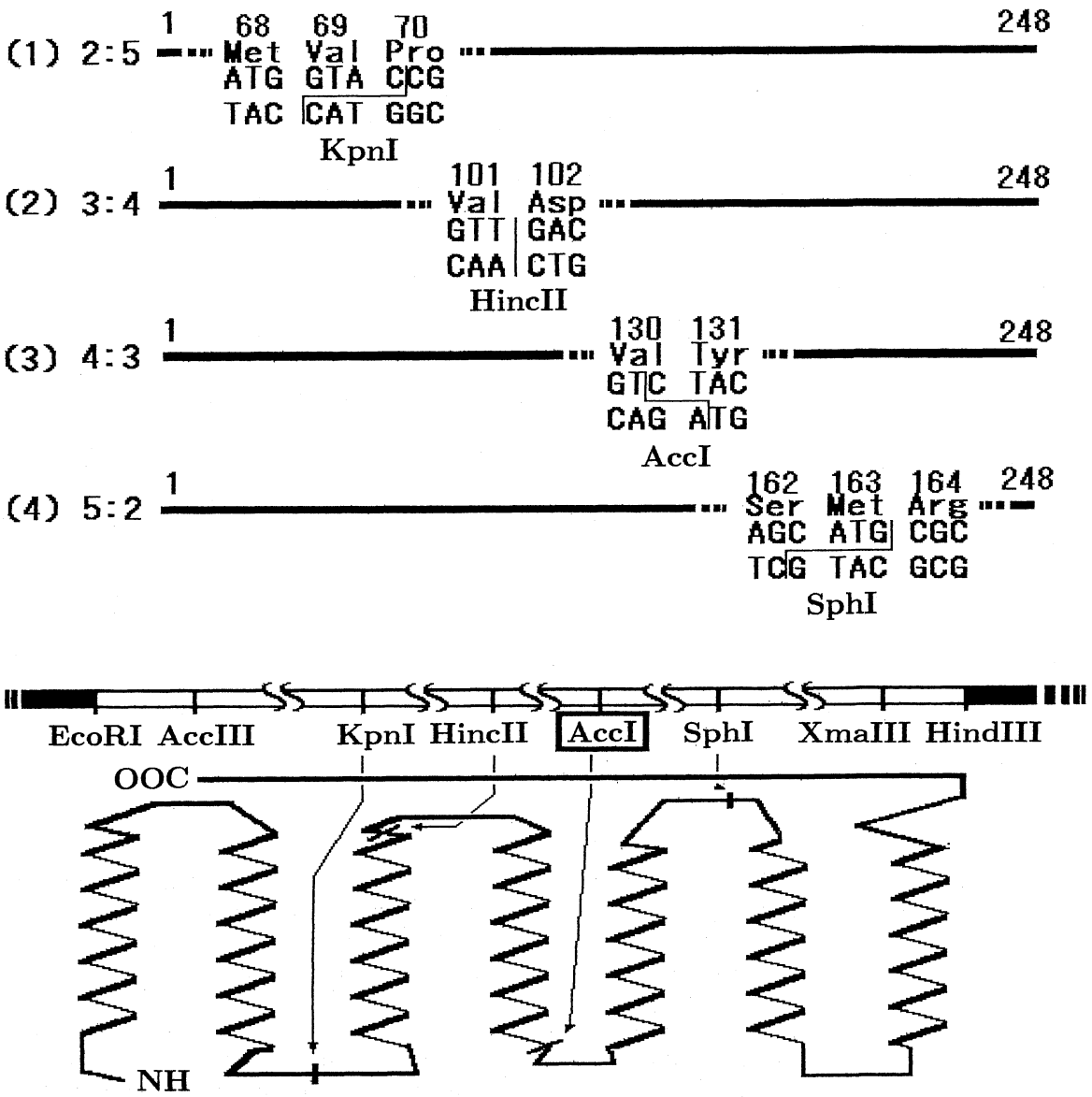

Fig. 1 Restriction sites in Bacterioopsin gene. Top: (1) (2) (3) and (4) are sites of restriction enzymes *KpnI*, *HincII*, *AccI and SphI*, respectively. The ratio beside the numbers ((1)-(4)) indicates the ratio of lengths of the expected partial peptide fragments when the DNA is digested with the indicated restriction enzyme. Center: Restriction sites represented in Bacterioopsin gene. Bottom: Restriction sites in the folding model of Bacteriorhodopsin.

Materials and Methods

Vectors The plasmid pUBO was constructed from the vectors pUC18 and pHV-18.34-Δ-E3, as reported by Tsujiuchi, Iwasa and Tokunaga (1994). Plasmid pKK223-3 was purchased from Toyobo Co., Japan, and plasmid pTK2 was kindly provided by Prof. T. Kuwabara who constructed the plasmid (1988).

Enzymes and DNA preparation Restriction enzymes, T4-DNA ligase, bacterial alkaline phosphatase and T-4 polynucleotide kinase were purchased from Toyobo and used as recommended. DNA fragments treated with restriction enzymes were isolated on low-melting agarose gels and recovered by glass powder adhesion, and separation. Synthetic oligonucleotides for linkers were synthesized with a commercial DNA synthesizer (Applied Bio Systems 381A, USA), purified by high performance liquid chromatography (Pharmacia LKB, Sweden) and were desalted by C-18 Sep-Pak columns (Pharmacia LKB, Sweden). The oligonucleotides were phosphorylated with T-4 polynucleotide kinase. Ligations were carried out with equi molar amounts of restriction fragments and synthesized oligonucleotides (0.1-1.0 pmol) at 14°C for 12 h. *E. coli* strains JM105 were transformed with the ligation mixture by the $CaCl_2$ procedure. Transformants were selected on LB plates containing 35 μg/ml ampicillin. The rapid boiling procedure was applied for small-scale plasmid preparation. Large-scale plasmid preparation was carried out by extraction with alkaline SDS and centrifugation to equilibrium in CsCl gradients

containing ethidium bromide.

Preparation of partial BOP genes The gene of native BOP contains four unique sites of restriction enzymes for preparing partial genes of BOP. These enzymes (*KpnI, HincII, AccI and SphI*) can digest the gene at the site corresponding to the peptide between α helices B and C (Met68-Val69-Pro70), C and D (Val101-Asp102), D and E (Val130-Tyr131), and E and F (Ser162-Met163-Arg164), respectively (Fig. 1).

Construction of pUBOAIN and pUBOAIC The plasmid pUBO (3.4-kb) was digested with restriction enzymes *HindIII* and *AccI* into two fragments (0.36 and 3.0 kbp). The smaller one contains nucleotides coding α-helices EFG of BOP. The larger one contains the remaining nucleotides corresponding to α-helices ABCD. We synthesized *AccI-HindIII* linker as shown in Fig. 2 (linker(1)) and ligated it to the larger fragment. *E. coli* strain JM105 was then transformed by this ligation mixture. The plasmid pUBOAIN thus obtained was identified in ampicillin-resistant transformants. The size of the insert was checked by agarose gel electrophoresis and its orientation confirmed by sequencing.

The plasmid pUBO was also digested with restriction enzymes *EcoRI* and *AccI* into 0.39-kbp and 3.0-kbp fragments. The smaller one (0.39-kbp) contains nucleotides corresponding to the α-helices ABCD of BOP. The Larger one contains the remaining nucleotides corresponding to α helices EFG. We synthesized also *EcoRI-AccI* linker as shown in Fig. 2 (linker(2)) and ligated it to the larger fragment. *E. coli* strain JM105 was transformed by this ligation mixture. The plasmid pUBOAIC was identified in an ampicillin-resistant transformant. The size of the inserts was checked by agarose gel electrophoresis and its orientation confirmed by sequencing.

Construction of pKBOAIN and pKBOAIC The smaller *EcoRI-HindIII* fragment prepared by digesting pUBOAIN or pUBOAIC with *EcoRI* and *HindIII* was inserted between the *EcoRI* and *HindIII* sites of the plasmid pKK223-3 (Fig. 3). *E. coli* strain JM105 was transformed by the ligation mixture and the plasmids pKBOAIN and pKBOAIC were identified in ampicillin-resistant transformants. The size of the insert was checked by agarose gel electrophoresis.

Construction of pTKBOAIN and pTKBOAIC The plasmid pTK2 contains the nucleotides encoding the signal peptide of manganese-stabilization protein (MSP) (Fig. 4(a)) inserted in the *XbaI-HindIII* site of pUC18. We designed the expression vector pTKBOAIN and pTKBOAIC containing lacZ promoter, the nucleotides encoding MSP signal peptide, an alanine residue, the *BglII-EcoRI* linker (Fig. 4(b)) and the partial gene corre-

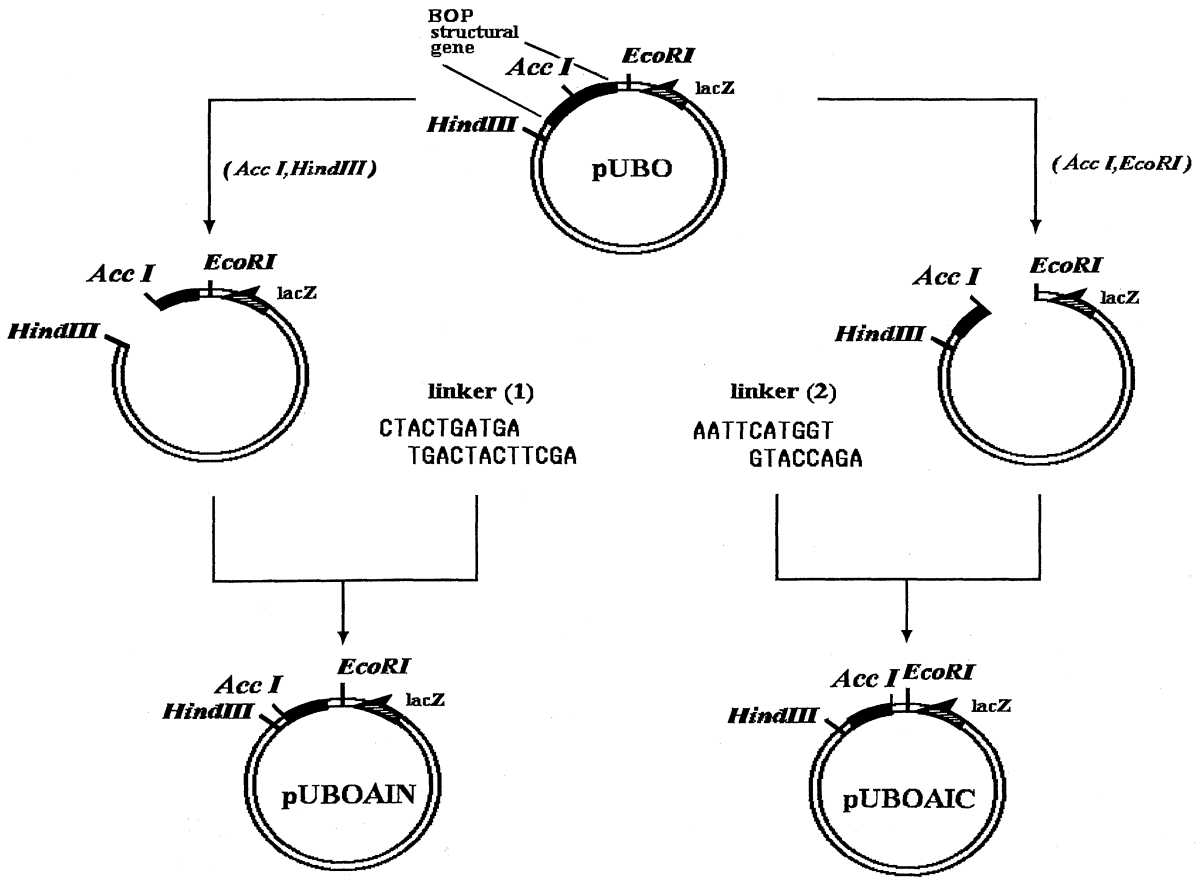

Fig. 2 The procedures for construction of pUBOAIN and pUBOAIC. Plasmid pUBOAIN has a partial gene corresponding to α helices ABCD of BOP. Plasmid pUBOAIC has that of helices EFG. A synthesized DNA linker (1) for pUBOAIN contains a stop codon (TGA) while that (2) for pUBOAIC contains a start codon (ATG).

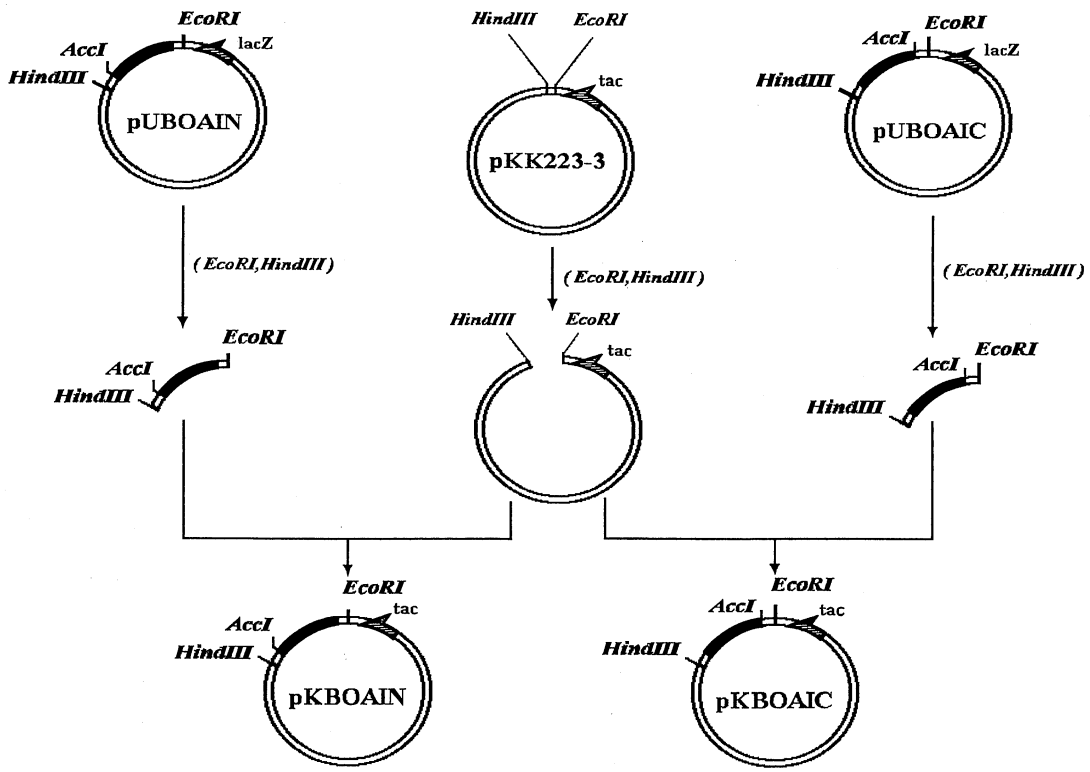

Fig. 3 Procedure for construction of pKBOAIN and pKBOAIC. The partial genes of BOP (AlNgene, 390 bp or AlCgene, 360 bp) were inserted into the *EcoRI-HindIII* site of pKK223-3 downstream of the tac promoter.

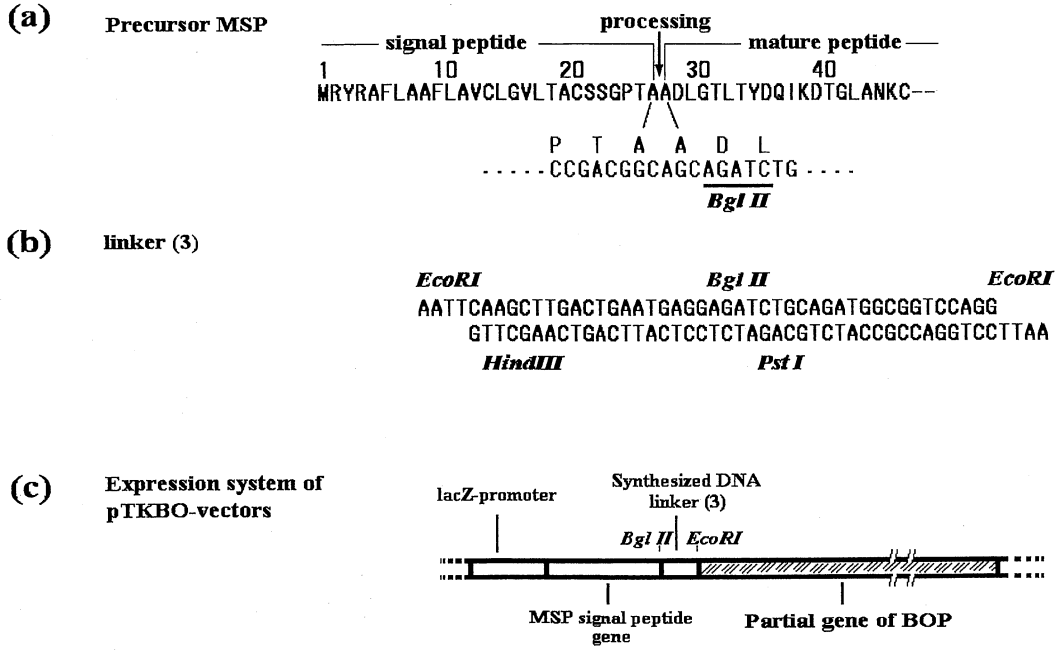

Fig. 4 Design of construction of pTKBOAIN and pTKBOAIC. (a) The amino acid sequence of precursor MSP. The signal peptide is from M1 to A27. (b) The synthesized DNA linker (3) inserted into the *EcoRI* site of pUBOAIN or pUBOAIC. (c) The design of the expression system of the pTKBO-plasmid vector. Further details are given in the text and Fig. 5.

sponding to BOP α-helices ABCD or EFG sequentially (Fig. 4(c)). To construct these two plasmids, we first constructed two plasmid vectors, pUBOAIN+L and pUBOAIC+L (Fig. 5), by inserting the synthesized DNA linker (linker (3)), as shown in Fig. 4(b), into the *EcoRI* site of pUBOAIN or pUBOAIC. The *EcoRI* site of these vectors exists uniquely upstream of the BOP partial gene. The linker contains the *BglII* site required for inserting the BOP partial gene downstream of the MSP signal peptide. *E. coli* strain JM105 was transformed by li-

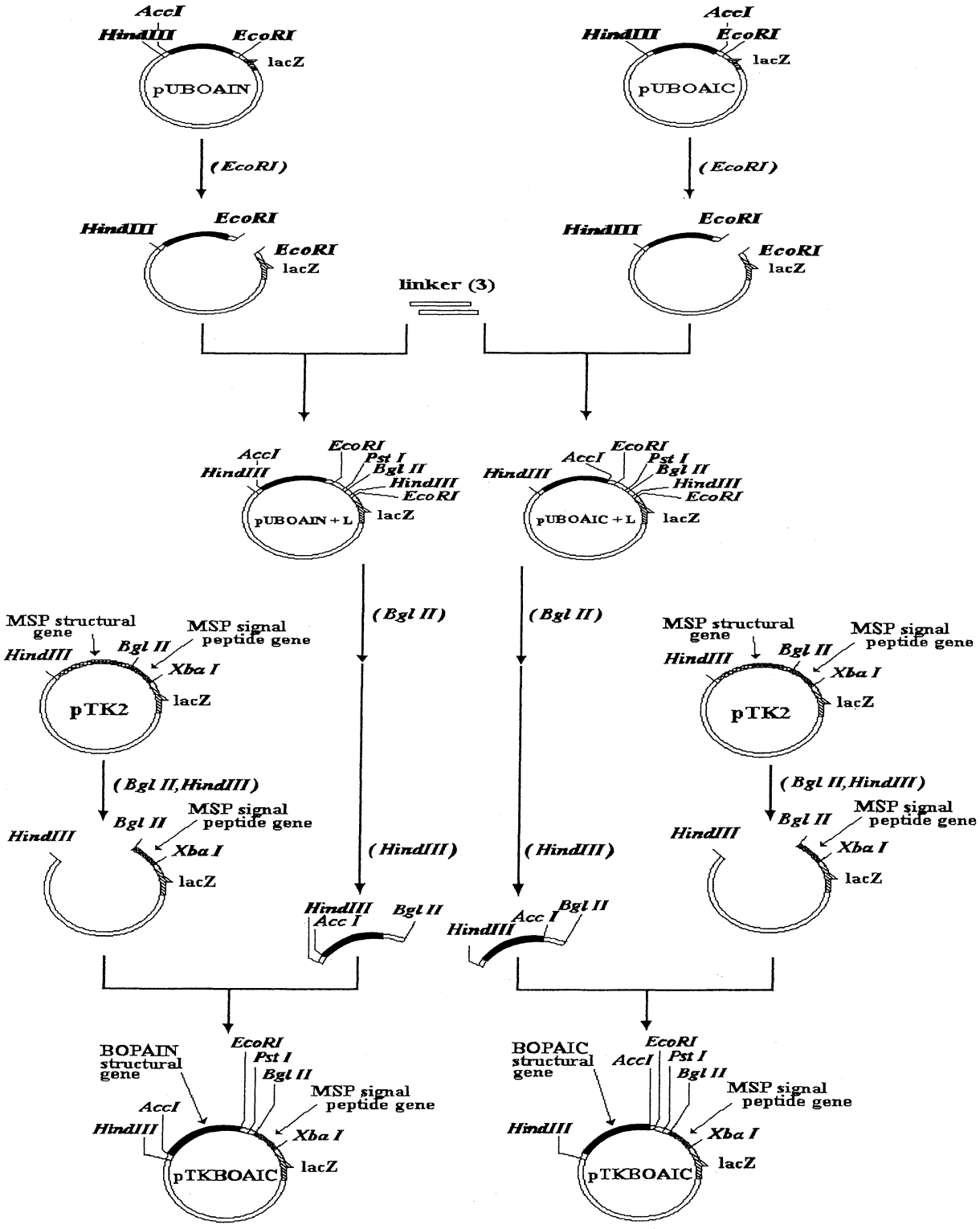

Fig. 5 Procedure for construction of pTKBOAIN and pTKBOAIC.

gation mixture involving the linker and pUBOAIN or pUBOAIC digested with *EcoRI*. The plasmids pUBOAIN+L or pUBOAIC+L were identified in ampicillin-resistant transformants and confirmed by checking the size and the nucleotide sequence around the junctions. The smaller fragments prepared from pUBOAIN+L and pUBOAIC+L digested with *BglII* and *HindIII* were ligated to the larger fragments obtained by double digestion of pTK2 with *BglII* and *HindIII*. *E. coli* strain JM105 was transformed by this ligation mixture (Fig. 5). The plasmids pTKBOAIN and pTKBOAIC were identified in ampicillin-resistant transformants. The sizes of the inserts were checked by agarose gel electrophoresis and the orientation of the inserts was confirmed by nucleotide sequences. The overall procedures for construction of pTKBOAIN and pTKBOAIC

are summarized in Fig. 5.

Preparation of Purple membrane The purple membrane fragments were purified by sucrose step gradient methods reported by Becher and Cassim (1975) for calibration of the amount of expressed BOP in the membrane fraction described below.

Expression of partial peptides of BOP To express the partial peptides of BOP *in vivo*, cells were grown in LB broth (10 g Bacto-trypton, 5 g Bacto-yeast extract, 10 g NaCl/l) containing 35 μg/ml ampicillin. At a cell density of 0.1 optical density unit at 650 nm, 1 mM IPTG was added to the culture and the cells were cultured for additional 3 h. Cells were harvested, washed, and suspended in MF buffer (50 ml of Tris-HCl, pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). DNaseI (20 μg/ml) and RNase (20 μg/ml) were added to the cell suspension, and the mixture was sonicated. Unbroken cells were removed by centrifugation at 10,000 g for 10 min. A crude membrane fraction was collected by centrifugation at 100,000 g for 1 h. The membrane pellet was suspended in 50 mM Na-phosphate buffer (pH 7.0), and the protein concentration was determined by the Lowry method. The partial peptides of BOP were detected with mouse anti-BR serum. The amount of expressed peptides in the membrane fraction was estimated by ELISA using the mouse anti-BR serum. HRPO-conjugated anti-mouse goat IgG was used as the second antibody. To calibrate the amount of expressed BOP in the membrane fraction, three samples was prepared with the native purple membrane fragments at different concentration with the *E. coli* membrane fraction prepared from the cells transformed with pUC18. Absorbance at 492 nm (due to the decomposition product of OPD reacting with HRPO) was measured and used as an indicator of the amount of partial peptide.

Results

The amounts of partial peptides of BOP in membrane fractions of E. coli The expression plasmid vectors described above were used to transform *E. coli* strain JM105 and the expression of BOP partial peptides was induced by adding IPTG to the cultures. The *E. coli* membrane fraction from each culture was prepared and the amount of BOP partial peptides measured by ELISA. The results are shown in Fig. 6.

As the membrane fraction is usually turbid, a reference series for the absorbance measurements was made from known amounts of native purple membrane (Fig. 6, column (a)-(c)). The samples columns (a), (b) and (c) in Fig. 6 contained 4, 0.4 and 0.04% of native purple membrane in the membrane fraction of the reference culture, respectively. The samples (d)-(i) are the membrane fractions prepared from the cells transformed with the vectors, pUBOAIN, pUBOAIC, pKBOAIN, pKBOAIC, pTKBOAIN and pTKBOAIC, respectively. The absorbances were normalized against the sample containing 4% native purple membrane (column (a) in Fig. 6). The absorbance of 0.35 correspond the base value which contains little amount of partial peptides of BOP in the membrane fraction. Fig. 6 show that the partial genes were expressed only in *E. coli* cultures with an induction by IPTG.

Compared with the reference values, the membrane fraction from cells transformed with pUBOAIN or pKBOAIC contained BOP partial peptides corresponding to approximately 0.4% of the total protein (columns (d) and (g) in Fig. 6); and that of the cells transformed with pUBOAIC or pKBOAIN (column (e) and (f) in Fig. 6) contained little; and that of the cells transformed with pTKBOAIN or pTKBOAIC contained partial peptides

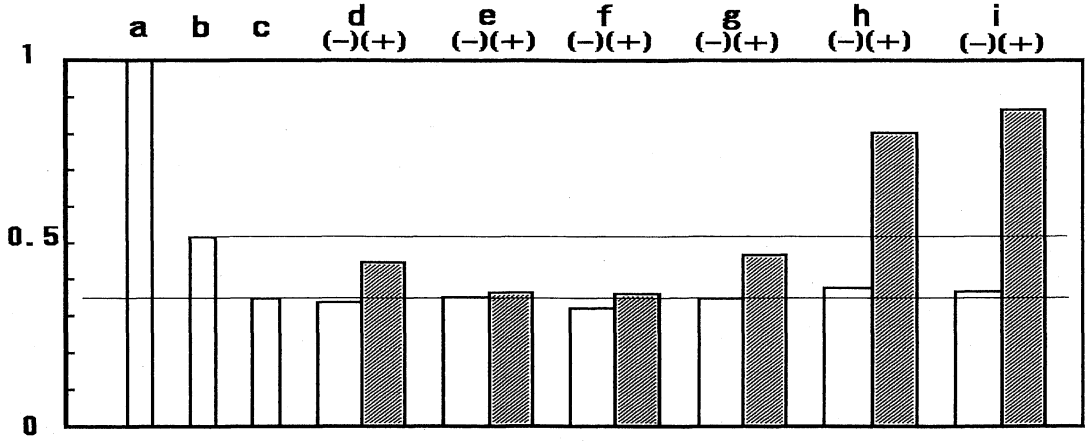

Fig. 6 Relative expression of partial genes of BOP. The ordinate is the absorbance at 492 nm observed by ELISA. All data are normalized against measurement (a). Samples (a), (b) and (c) contain the intrinsic BOP at 4, 0.4 and 0.04% of the total protein of the membrane fractions of the cells transformed with pUC18, respectively. (d), pUBOAIN; (e), pUBOAIC; (f), pKBOAIN; (g), pKBOAIC; (h), pTKBOAIN; (i), pTKBOAIC; (−), without IPTG; (+), with IPTG.

of BOP comprising more than one percent of the total protein of the membrane fraction ((h) and (i) in Fig. 6). To sum up these data, the vectors containing lac promoter or tac promoter showed low levels of expression, but vectors containing the presequence of the manganese-stabilizing protein of *Anacystis nidurans* between the lac promoter and the BOP partial gene induced expression at levels higher than 1% of total protein in the membrane fraction. This suggests that presequence of MSP increased the amount of expressed partial peptides of BOP in *E. coli* cells.

Discussion

The above results indicate that it is possible to express partial genes of native BOP which correspond to the α helices ABCD and EFG individually in *E. coli*. The plasmid vectors pUBOAIN, pUBOAIC, pKBOAIN, pKBOAIC expressed low amounts of BOP peptides: 0.4% at most. The reason for the differences in expression level, in spite of using the same promoters (lac or tac) may be that joining the short segment of structural genes of lacZ to the peptides has some influence on gene transcription.

Tsujiuchi, Iwasa and Tokunaga (1994) reported that the expression of almost the whole BOP gene was approximately 0.1% of the total protein of the membrane fraction. BR originally consists of 248 amino acid residues but the BOP expressed previously by Tsujiuchi, Iwasa and Tokunaga (1994) lacked 7 of the native amino acid residues. The ratio of the number of amino acid residues of BOP, helices-ABCD and helices-EFG is 241:131:120. The molar ratio of the numbers of antibodies bound to them is about 2:1:1. Comparing 0.1% with 0.4% in view of the reaction of anti-BR serum, the amount of partial peptides ABCD and EFG synthesized in *E. coli* is in fact four to eight times as that of BOP. If the half-life of the mRNA and the peptide, and the rates of transcription and translation are all the same for BOP, helices ABCD and helices EFG, the molar ratio of antibodies bound to partial BOPs (helices ABCD or helices EFG) and BOP itself might be four to eight. It is speculative but should be possible to titrate. Therefore it is reasonable that the expressed partial peptides were about 0.4%.

The expression vectors containing the nucleotide sequence encoding the signal peptide of *Anacystis* MSP produced much higher levels of expression than the others (more than 1% of total membrane proteins of *E. coli*). This apparently enhanced level of expression with pUBOAIN and pUBOAIC may be an effect of reduced proteolytic degradation of BOP peptides as a result of the presence of the signal peptide of *Anacistis* MSP, since this was the only difference between pTKBO and pUBO-vectors.

Acknowledgments

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REFERENCES

[1] Becher, B. and J. Y. Cassim (1975) Improved isolation procedures for the purple membrane of *Halobacterium halobium. Prep. Biochem.* 5: 161–178.

[2] Blaurock, A. E. and W. Stoeckenius. (1971) Structure of the purple membrane. *Nature New Biol.* 233: 152–155.

[3] Kataoka, M., T. W. Kahn, Y. Tsujiuchi, D. M. Engelman and F. Tokunaga. (1992) Bacteriorhodopsin reconstituted from two individual helices and the complementary five helix fragment is photoactive. *Photochem. Photobiol.* 56: 895–901.

[4] Kuwabara, T. (1988) Structure and function of the Mn-stabilizing Protein of Photosystem II. *Indo-US Workshop on Application of Molecular Biology in Bioenergetics of Photosynthesis. New Delhi. January 4–8.*

[5] Liao, M. J., E. London and H. G. Khorana. (1983) Regeneration of the Native Bacteriorhodopsin Structure from Two Chymotryptic Fragments *J. Biol. Chem.* 258: 9949–9955.

[6] Liao, M.-J., K.-S. Huang and H. G. Khorana. (1984) Regenaration of Native Bacteriorhodopsin Structure from Fragments. *J. Biol. Chem.* 259: 4200–4204.

[7] Tsujiuchi, Y., T. Iwasa and F. Tokunaga. (1994) Expession of Bacterioopsin Genes in *Escherichia coli. The Science Reports of The Tohoku University.* Series 8. Vol. 14, Nos. 2/3.