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Expression of an Antimicrobial Peptide Magainin by a Promoter Inversion System

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Abstract A method was developed for the controlled expression of an antimicrobial peptide magainin in *Escherichia coli*. A series of concatemeric magainin genes was constructed with a gene amplification vector, and fused to the 3' end of *malE* gene encoding the affinity ligand, *E. coli* maltose-binding protein (MBP). The construct directed the synthesis of the fusion protein with the magainin polypeptide fused to the C-terminus of MBP. The fusion protein was expressed in a tightly regulatable expression system which was under the control of an invertible promoter. The MBP-fused magainin monomer was expressed efficiently. However, the expression level of the MBP-fused magainin in *E. coli* decreased with the increasing size of multimers possibly because of the transcription and translation inhibition by the multimeric peptides. After purification using an amylose affinity column, the fusion protein was digested by factor Xa at a specific cleavage site between the monomers. The recombinant magainin had an antimicrobial activity identical to that of synthetic magainin. This experiment shows that a biologically active, antimicrobial peptide magainin can be produced by fusing to MBP, along with a promoter inversion vector system.

Key words: Antimicrobial peptide, expression, promoter inversion vector, *Escherichia coli*

Antimicrobial peptides have received increasing attention in recent years because of their contribution as a first line of host defense against infections of pathogenic microorganisms [6]. A large number of antimicrobial peptides have been identified from various sources, including amphibians [2], insects [10], mammalians [16], plants [3], invertebrates [19] and prokaryotes [11]. These peptides exhibit potent antimicrobial activities against a broad range of microorganisms, including bacteria, protozoa, fungi and viruses [13, 16]. The antimicrobial peptides have been shown to exert their activity by the formation of multimeric pores directly through the lipid bilayer of the cell membrane [18, 28]. Despite the activities of these peptides against microorganisms, they do not induce lysis of erythrocytes or lymphocytes at comparable concentrations [30, 31]. The mature peptides, derived by the processing of large precursors, share common structural features such as a high content of basic amino acid residues and a global distribution of hydrophobic and hydrophilic residues leading to amphipathic conformations [23].

The antimicrobial peptides have been obtained from living organisms, and this has become a limiting factor for their mass production and widespread applications. Chemical synthesis could be an alternative method. However, the chemical synthesis of peptides longer than 10 amino acids in length is not cost-effective. Therefore, a biological expression system, if developed successfully, would be one of the most cost-effective approaches for the production of large quantities of antimicrobial peptides. Recently, several groups have attempted to produce antimicrobial peptides in different biological systems. Cecropin A has been expressed in baculovirus expression systems [1, 9] and in *E. coli* [4], insect defensin from *Phormia terranovae* in yeast [22], human defensin HNP-1 and CEME [27] in various bacterial expression systems [20], magainin in the erythrocytes of transgenic mice [26], and moricin from *Bombyx mori* in *E. coli* [7]. However, most of these methods have limitations, such as the proteolysis of the fusion protein [20], or the toxic effects of the expressed peptides to the host [5]. As a new approach, we developed a bacterial expression system for the antimicrobial peptide magainin

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by a promoter inversion and gene amplification vector system. The promoter inversion vector, which could tightly control the expression of lethal genes, prevented the toxic effects of the antimicrobial peptide to the host. And the tandem linkage of multiple copies of a peptide-encoding sequence was used because the recombinant concatemer could be quantitatively converted into monomeric peptides, resulting in a high yield. In this study, we have shown that biologically active magainin could be produced using the promoter inversion vector system.

MATERIALS AND METHODS

E. coli Strains, Enzymes and Vectors

E. coli strains, XL1-Blue and D1210HP (Stratagene, La Jolla, CA, U.S.A.) were used as hosts for subcloning and gene expression, respectively. pUC18 (New England Biolabs, Beverly, MA, U.S.A.) and pBluescriptII SK(-) (Stratagene) were used as vectors for subcloning and pBBS1 [14] for amplification of a peptide gene. pMal-c (New England Biolabs) and pNH18a (Stratagene) were used as a source for the *malE* gene and a promoter inversion vector, respectively. pBEST*luc* (New England Biolabs) was used as a control DNA for the *in vitro* transcription – translation reaction. *E. coli* cells were grown in LB medium at 30°C or 37°C and 50 μg/ml of ampicillin was added for plasmid-containing strains. Restriction enzymes and other modifying enzymes were purchased from New England Biolabs and used according to the recommendations of the suppliers. A mini-scale preparation of DNA was carried out using the alkaline lysis method [17] and large quantities of vector DNA were prepared by the PEG precipitation method [24]. Other recombinant DNA techniques were exploited as described by Maniatis *et al.* [17] and Sambrook *et al.* [24].

Cloning of the Magainin Genes into the Promoter Inversion Vector

A DNA fragment containing the *malE* gene encoding the *E. coli* maltose-binding protein (MBP), was PCR-amplified from pMal-c vector with primers A (5'-GAAGATCTGGAATTGTGAGCGG-3') and B (5'-GTTTTCCCAGTCACGAC-3'). The PCR product was purified, *Sal*I-digested and cloned into the promoter inversion vector pNH18a which was digested with *Sma*I and *Sal*I, resulting in pNH18aMBP.

The 93-bp DNA fragments encoding magainin were tandemly multimerized using the vector pBBS1 as described in Fig. 2. Vector pBBS1-M1, which contains a magainin gene, produced monomeric DNA fragments with asymmetric cohesive ends of 5'-CCCC/5'-GGGG upon digestion with *Bbs*I. Purified monomeric fragments were tandemly multimerized by self-ligation for 2 h and cloned into the *Bbs*I-digested pBBS1. With XL1-Blue transformants selected, the number of monomers in the vector was determined by cleaving the vector with *Bam*HI and *Xba*I whose sites flank the multimer. The orientation of the individual monomers within the vector was determined by digesting the vectors with *Eco*RI which cuts a site in the vector and a site in each monomeric fragment. The vectors containing monomer, dimer, tetramer and hexamer of the magainin gene were selected and named pBBS1-M1, -M2, -M4 and -M6, respectively. To express the multimeric magainin genes with an invertible promoter [8], the *Kpn*I-*Xba*I fragments containing the multimeric magainin genes of the vectors, pBBS1-M1, -M2, -M4 and -M6, were cloned into the vector pNH18aMBP which was digested with *Kpn*I and *Xba*I, resulting in pNH18aMBP-M1, -M2, -M4 and -M6, respectively.

Int-mediated Promoter Inversion

E. coli host D1210HP lysogen was transformed with the expression vectors, pNH18aMBP, pNH18aMBP-M1, -M2, -M4, or -M6. Each transformant was inoculated into 3-ml LB medium supplemented with ampicillin to a final concentration of 50 μg/ml and grown at 30°C for 9 to 12 h. Each culture was then diluted 1 : 100 into fresh LB medium containing 50 μg/ml ampicillin and grown at 30°C. At $OD_{600} = 2.0$, the culture was heated at 42°C for 10 min. Then, the temperature of the culture was shifted to 30°C and isopropyl-β-D-thiogalactoside (IPTG) was added to a concentration of 0.6 mM. The cells were harvested 5 h after heat-induction and plasmids were isolated by the alkaline lysis method. The promoter inversion was analyzed by the *Eco*RI restriction analysis of the isolated plasmids.

Expression and Purification of Recombinant Magainin

To analyze the expression level of the multimeric peptides, each transformant was inoculated into 3-ml LB medium supplemented with ampicillin to a final concentration of 50 μg/ml and grown at 30°C for 9 to 12 h. Each culture was then diluted 1 : 100 into fresh LB medium containing 50 μg/ml ampicillin and grown at 30°C. At $OD_{600} = 2.0$, the culture was heated at 42°C for 10 min. Then the temperature of the culture was shifted to 30°C and IPTG was added to a concentration of 0.6 mM. The cells were harvested 5 h after heat-induction and whole cell lysates from the uninduced and induced cultures were analyzed by SDS-PAGE [12]. For the purification of recombinant magainin, *E. coli* cells harboring the expression vector pNH18aMBP-M4, which has four copies of the magainin gene, were cultured with 4-liter LB medium at 30°C in a 5-liter

fermenter. At OD_{600} = 2.0, the culture was heated at 42°C for 10 min, the temperature of the culture was shifted to 30°C and then IPTG was added to the cultures (4 liters) at the final concentration of 0.6 mM. The cells were harvested 5 h after heat-induction by centrifugation at 4, 300×*g* for 10 min at 4°C and resuspended in 1/10 of the culture volume with the column buffer (20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and lysed by sonication (Labsonic U, B. Braun Biotech International GmbH, Melsungen, Germany). After the lysis of cells, the supernatant was recovered by centrifugation at 13, 000×*g* for 30 min at 4°C, diluted fivefold with the column buffer and applied to an amylose-agarose affinity column equilibrated with the same buffer at 4°C. The column was washed with the column buffer to remove unbound proteins, and the bound proteins were eluted with the column buffer containing 10 mM maltose. The eluted proteins were pooled and dialyzed against 20 mM Tris/HCl buffer (pH 7.4). The fusion proteins were cleaved by incubating with factor Xa (New England Biolabs) at 4°C for 24~48 h, and then the cleavage of the fusion proteins was analyzed by SDS-PAGE [12] and tricine-SDS-PAGE [25]. After the cleavage reaction, the samples were applied to a 3.9×300 mm Delta Pak C18 column (Waters associates, Milford, MA, U.S.A.) with a linear gradient of 0% buffer A to 50% buffer A at 1 ml/min for 1 h [buffer A; acetonitrile containing 0.1% (v/v) trifluoroacetic acid]. Each peak was collected and examined for an antimicrobial activity.

Assay of Antimicrobial Activity

The antimicrobial activity was examined by the ultrasensitive assay using *Bacillus subtilis* as described by Lehrer *et al.* [15]. *B. subtilis* cells were grown overnight at 37°C in 3 ml of 3% (w/v) trypticase soy broth (TSB). To obtain mid-logarithmic phase microorganisms, 50 µl of this culture was inoculated into 50 ml of fresh TSB broth and incubated for an additional 2.5 h at 37°C. *B. subtilis* cells were centrifuged at 900×*g* for 10 min at 4°C, washed once with cold 10 mM sodium phosphate buffer, pH 7.4 (NAPB), resuspended in 10 ml of cold NAPB, and their concentration was estimated by measuring the absorbance at 620 nm (A_{620}). Based on the relationship A_{620} 0.2 = 5×10^7 CFU/ml, a volume containing 1×10^6 CFU was added to 6 ml of underlay agar [10 mM sodium phosphate, pH 6.5, 1% (v/v) TSB, and 1% (w/v) agarose] and the agar was poured into a Petri dish. Samples were added directly to 3-mm-diameter wells that were made on the underlay agar. After incubation for 3 h at 37°C, the underlay agar was covered with a nutrient-rich top agar overlay and incubated overnight at 37°C to allow growth of the microorganisms. Afterwards, the gels were stained for 24 h in a solution of Coomassie Brilliant Blue R-250 (dye 2 mg; methanol, 27 ml; water, 63 ml and 37% formaldehyde, 15 ml). The spent staining solution was decanted, and replaced with an aqueous solution of 10% acetic acid and 2% dimethylsulfoxide for approximately 10 min. After this conditioning solution was poured off, the gels were dried. The presence of antimicrobial activity in the samples was indicated by the presence of a zone of bacterial clearance around the wells.

In Vitro Translation

The *in vitro* coupled transcription–translation reactions were performed with the *E. coli* S30 Extract System for Linear Templates (Promega, Madison, WI, U.S.A.) under the conditions recommended by the manufacturer. Proteins were labeled with [^{35}S]methionine and portions of *in vitro* transcription–translation reactions were analyzed by SDS-PAGE. Gels were fixed, dried and exposed to X-ray film.

RESULTS AND DISCUSSION

Principle of the Promoter Inversion Vector System

An attempt to express the antimicrobial peptide magainin in *E. coli* with an expression vector under the control of the *tac* promoter was unsuccessful because the expressed magainin was highly toxic to the host. Even the basal level expression of magainin by the *tac* promoter was harmful to the host. Most of the promoter–operator systems have their own limitations for complete repressibility because of their basal level expression. For the complete prevention of promoter leakage, an invertible promoter system [8, 21, 29] would be the most suitable. In this system, the promoter, which is flanked by two λ attachment sites, is located in a direction opposite to that of the gene to be expressed, and it can be inverted by the site-specific recombination upon induction of λ integrase as shown in Fig. 1. Therefore, the gene under the invertible promoter can be expressed upon induction of λ integrase. The DNA sequences encoding multimers of magainin can be cloned into the expression vector containing the invertible promoter, and expressed as a fusion protein of MBP, which can be purified easily using an amylose affinity column.

Cloning of Repetitive Magainin Genes into the Promoter Inversion Vector

To express magainin as a fusion protein of MBP using the promoter inversion vector, a DNA fragment containing the *malE* gene, which encodes the maltose-binding protein, was amplified from the pMal-c expression vector and cloned into the multiple cloning sites of the promoter inversion vector pNH18a, resulting in pNH18aMBP vector. The 93-bp DNA fragments encoding the magainin peptide were tandemly

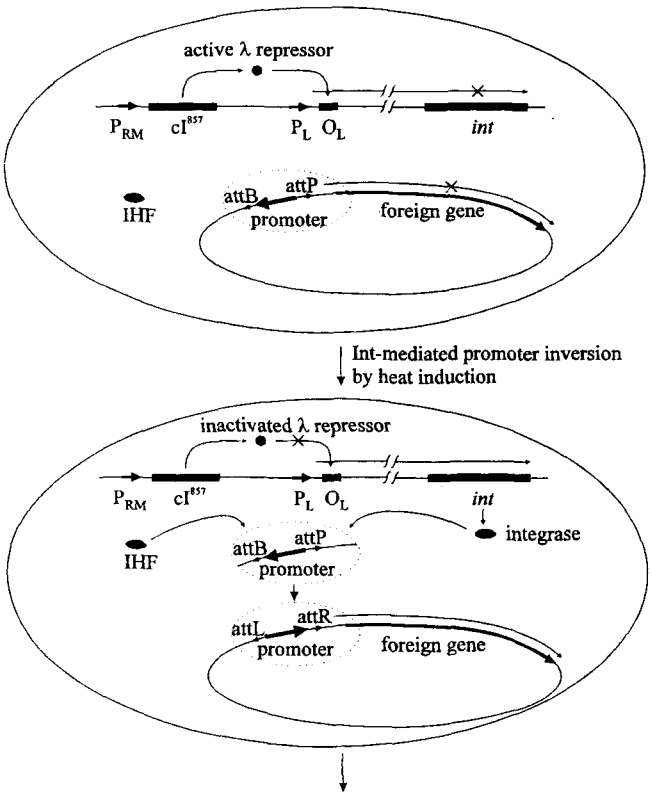

Fig. 1. Schematic representation of the promoter inversion system.

multimerized by using the gene amplification vector pBBS1 (Fig. 2). The clones containing 2 to 12 copies of the magainin monomer were selected as shown in Fig. 3. The orientation of the individual monomers within the vector was in the same direction, which was confirmed by digesting the vectors with *Eco*RI which cuts a site in the vector and a site in each monomeric fragment. Then, the fragments of DNA encoding the repetitive magainin were cloned into *Kpn*I and *Xba*I sites of the promoter inversion vector pNH18aMBP, resulting in pNH 18aMBP-M1, -M2, -M4, or -M6, which contains monomer, dimer, tetramer, or hexamer of the magainin gene, respectively (Fig. 4A). As shown in Fig. 4B, promoter inversion was analyzed by restriction analysis of the region containing the invertible module. It was shown that no promoter inversion was observed before heat shock, whereas over 80% of plasmids showed promoter inversion after heat-induction at 42°C. The expression of MBP-magainin fusion proteins was completely repressed until the culture reached the stationary phase at which the integrase was induced and the promoter was rearranged in the right orientation. This feature makes our expression system useful for the expression of toxic proteins and peptides where the

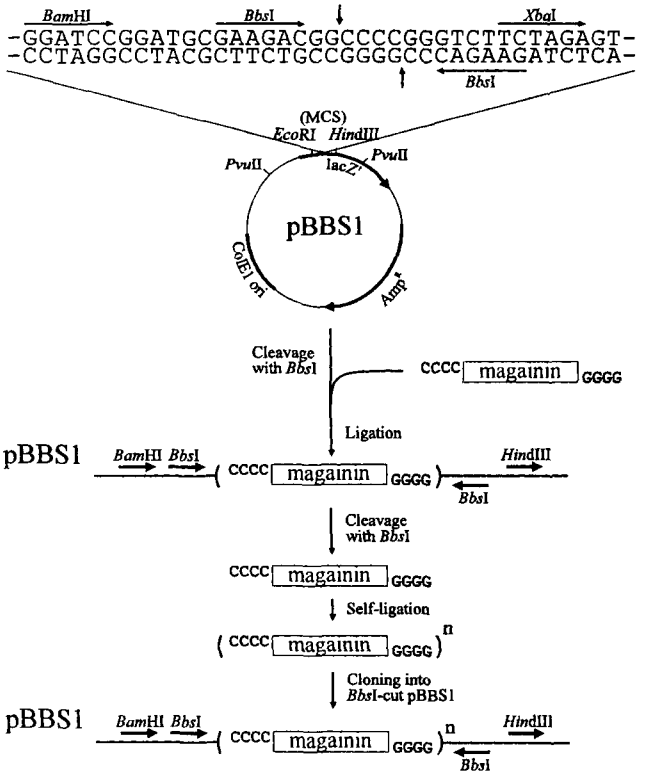

Fig. 2. Multimerization of magainin gene using the gene amplification vector pBBS1.

The gene amplification cassette contains two inversely oriented *Bbs*I sites and the same cleavage sequences. The monomeric magainin gene cloned into pBBS1 could be amplified by: (i) excision of the monomeric insert DNA by digestion with *Bbs*I; (ii) isolation of the insert DNA fragments; (iii) self-ligation of the fragments; and (iv) cloning into the original pBBS1 vector digested with *Bbs*I.

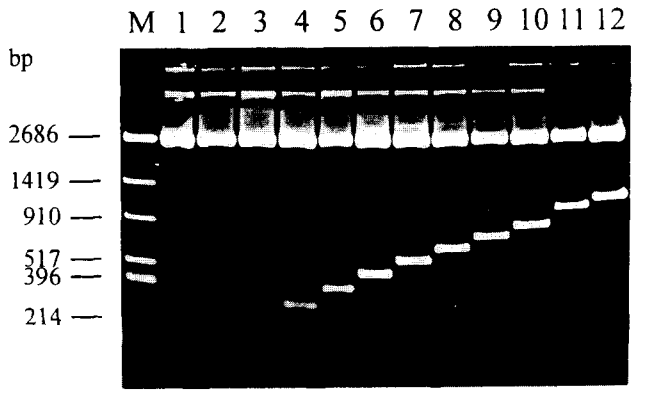

Fig. 3. Electrophoretic analysis of the multimeric magainin genes.

The number of magainin genes cloned in the gene amplification vector pBBS1 was determined by cleaving the vectors with *Bam*HI+*Xba*I, whose sites flank the multimer. The digests were electrophoresed on a 2% agarose gel in TBE buffer for 2 h at 10 V/cm, and DNA bands were stained with ethidium bromide. Lane M represents size markers. Lane 1 represents the pBBS1 vector digested with *Bam*HI+*Xba*I. Lanes 2-12 represent *Bam*HI+*Xba*I-digested pBBS1-M1, -M2, -M3, -M4, -M5, -M6, -M7, -M8, -M9, -M11, and -M12, which contain one to twelve copies of the magainin monomer, respectively.

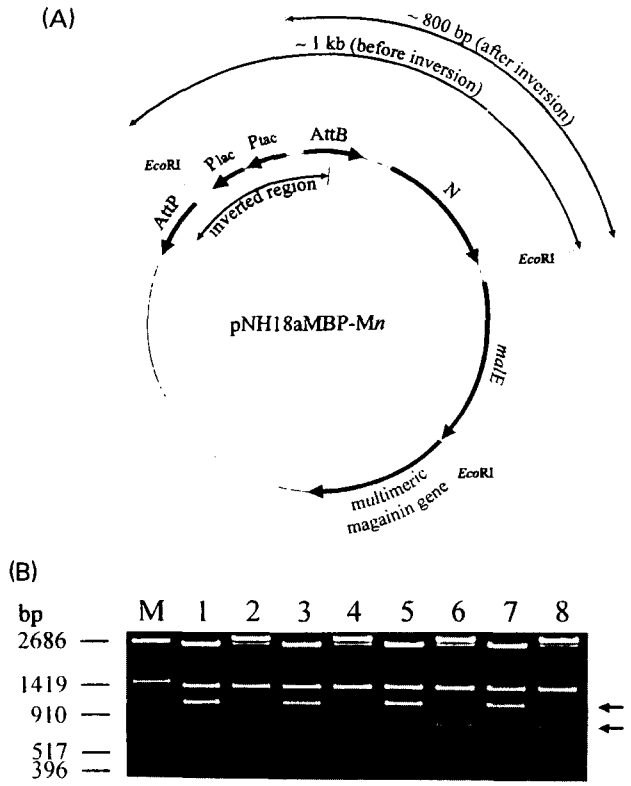

Fig. 4. Analysis of the promoter inversion.
(A) Schematic representation of promoter inversion vector pNH18aMBP-M*n*, which contains *n* copies of the magainin monomer. (B) Electrophoretic analysis of the promoter inversion. The inversion of promoter was determined by cleaving the vectors with *Eco*RI. Lane M represents size markers. Lanes 1 and 2 show D1210HP containing pNH 18aMBP-M1 before and after induction, respectively; lanes 3 and 4, D1210HP harboring pNH18aMBP-M2, lanes 5 and 6, D1210HP harboring pNH18aMBP-M4, lanes 7 and 8, D1210HP harboring pNH18aMBP-M6, respectively. A band of about 800 bp (indicated by a lower arrow) appears instead of a band of about 1 kb (indicated by an upper arrow), as the result of promoter inversion.

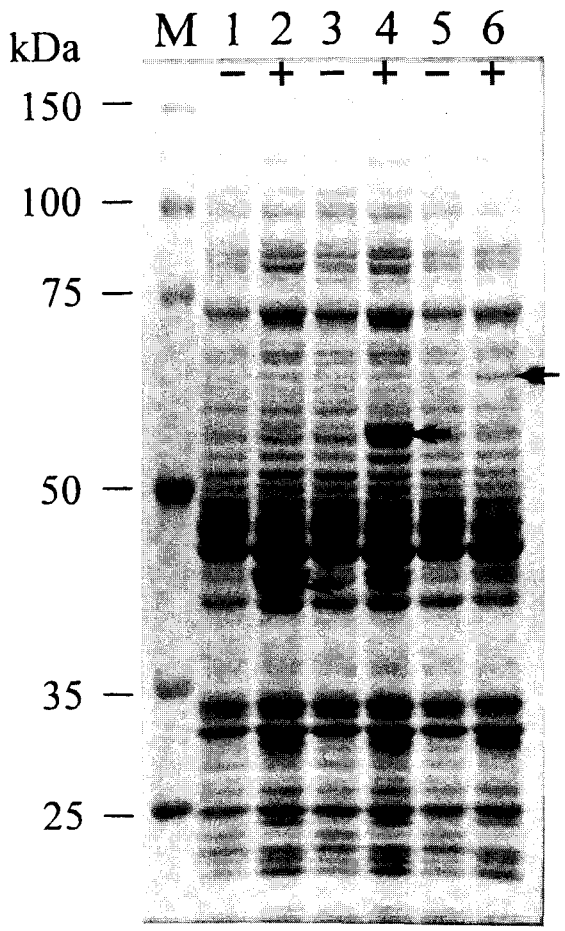

Fig. 5. Expression of multimers of magainin by a promoter inversion vector.
Whole cell lysates of cultures without (-) or with (+) induction by IPTG were analyzed by SDS-PAGE. Samples were applied to the gel after boiling for 5 min. Size markers are indicated on the left margin of the gel. Lanes 1 and 2 represent whole cell lysates from D1210HP harboring pNH18aMBP-M1; lanes 3 and 4 from D1210HP harboring pNH18aMBP-M4; lanes 5 and 6 from D1210HP harboring pNH18aMBP-M6. The positions of fusion protein bands are indicated by arrows. Samples were applied to a gel after boiling for 5 min. Proteins were stained with Coomassie Brilliant Blue.

tolerable background level of expression is very low, but a finely tunable expression system is necessary.

Expression of MBP-fused Repetitive Magainin by the Promoter Inversion Vector

Expression of the concatemeric magainin genes fused to the C-terminus of MBP, was induced in the host D1210HP by heat shock and then the addition of IPTG. Fusion proteins, which were expressed from pNH18aMBP-M1, -M4, and -M6, were analyzed by SDS-PAGE as shown in Fig. 5. The fusion protein from plasmid containing the monomeric magainin gene was successfully expressed. However, further increases in the copy number of magainin genes did not lead to higher expression. Instead, the expression level was substantially reduced as the copy number was increased.

To find the reason for the decrease in the expression level of multimers, the repetitive magainin genes cloned into the expression vector pNH18aMBP were expressed in an *in vitro* transcription-translation system in the presence of [^{35}S]Met (Fig. 6A). The *in vitro* transcription–translation experiment showed that the expression level decreased as the size of multimers was increased. It seemed that the strong positive charge of magainin multimers caused the decrease in the expression of repetitive magainin, possibly by interfering with either transcription or translation through interaction with DNAs or RNAs. When magainin was added to the reaction mixture of the *in vitro* transcription–translation, the reaction was also inhibited in proportion to the amount of magainin added (Fig. 6B). It seems that the longer the size of the multimer is, the stronger the inhibition occurs because of the increased number of positive charges.

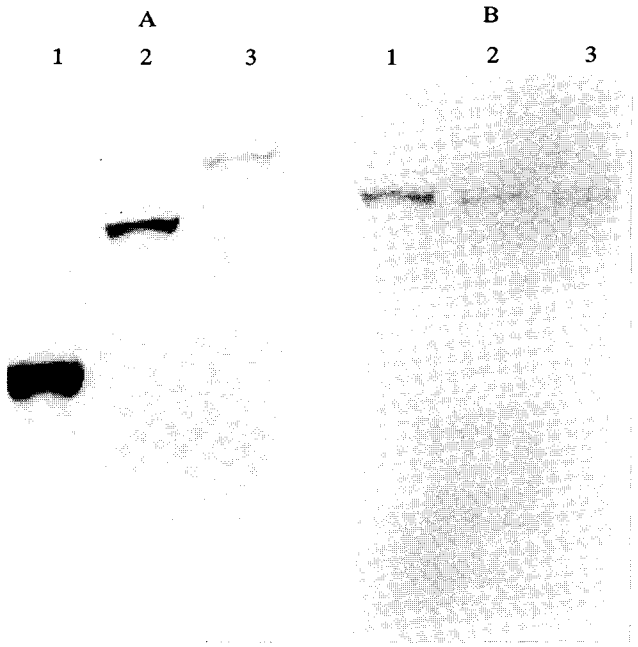

Fig. 6. *In vitro* transcription–translation of multimeric magainin genes and the effect of magainin on the *in vitro* transcription–translation.

(A) *In vitro* transcription–translation of multimeric magainin genes. The multimers, containing monomer, tetramer and hexamer, were expressed in the *in vitro* reanscription–translation system. (B) The effect of magainin on the *in vitro* transcription–translation. The molar ratio of pBEST*luc* DNA to magainin added to the *in vitro* transcription–translation reaction was 1 : 0 (lane 1), 1 : 400 (lane 2), or 1 : 4000 (lane 3), respectively.

Purification and Characterization of Recombinant Magainin

The vector containing four copies of the magainin gene was chosen for expression and purification of recombinant magainin which was expressed as a fusion protein. The fusion protein was purified by affinity chromatography on an amylose-agarose resin, in which 10 mM maltose was used to elute the fusion protein. After treatment with endoproteinase factor Xa, the recombinant magainin was purified by reverse-phase HPLC (Fig. 7A). The purified recombinant magainin was tested for its antimicrobial activity against *B. subtilis*. It was confirmed that the recombinant magainin had an antimicrobial activity almost identical to that of synthetic magainin II (Fig. 7B). Additional amino acid residues, which remained at the C-terminus of the recombinant magainin after factor Xa cleavage, seemed not to interfere with the antimicrobial activity of the recombinant magainin.

Even though the promoter inversion vector could be successfully used to produce antimicrobial peptides in this experiment, further increases in the production level of the antimicrobial peptide is needed for mass production. Concatemeric expression of the antimicrobial

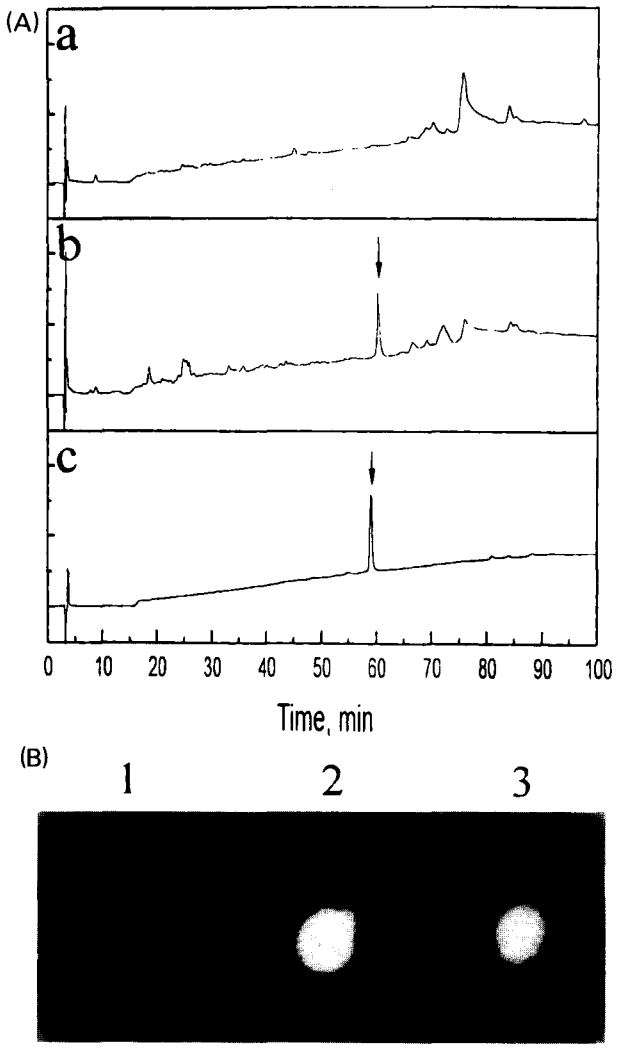

Fig. 7. Purification and antimicrobial activity assay of recombinant magainin from D1210HP containing pNH18aMBP-M4.

(A) Purification of recombinant magainin by reverse-phase HPLC. (a) Fusion protein containing dimeric magainin. (b) Factor Xa-digested fusion protein. (c) Magainin II. The arrows show the magainin peaks. (B) Assay of the antimicrobial activities of recombinant magainin II. Lanes 1, 2 and 3 represent control, recombinant magainin II and synthetic magainin II, respectiuely.

peptide would be one of the most attractive approaches for high yield production because concatemeric peptide multimers can be quantitatively converted into monomeric peptides, resulting in a high yield. However, most of the antimicrobial peptides are highly basic, which means that if their multimer gets longer, the positive charge increases, and thus results in stronger interaction with DNAs or RNAs. To overcome the interaction between antimicrobial peptide multimers and nucleic acids, an acidic peptide, which might effectively neutralize the positive charges of a basic antimicrobial peptide, can be fused to the antimicrobial peptide before multimerization. The

neutralization of a strongly basic antimicrobial peptide by an acidic peptide might also reduce the interaction of the basic peptide with nucleic acids, resulting in an increase in the expression of the antimicrobial peptide. This experiment is currently under study.

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REFERENCES

1. Andersons, D., A. Engstrom, S. Josephson, L. Hansson, and H. Steiner. 1991. Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the antibody-binding part of protein A. *Biochem. J.* **280**: 219–224.
2. Barra, D. and M. Simmaco. 1995. Amphibian skin: a promising resource for antimicrobial peptides. *Trends Biotechnol.* **13**: 205–209.
3. Broekaert, W. F., F. R. Terras, B. P. Cammue, and R. W. Osborn. 1995. Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol.* **108**: 1353–1358.
4. Callaway, J. E., J. Lai, B. Haselbeck, M. Baltaian, S. P. Bonnesen, J. Weickmann, G. Wilcox, and S. P. Lei. 1993. Modification of the C terminus of cecropin is essential for broad-spectrum antimicrobial activity. *Antimicrob. Agents Chemother.* **37**: 1614–1619.
5. Chan, R. Y. K., R. G. E. Palfree, L. F. Congote, and S. Solomon. 1994. Development of a novel type of cloning vector for suicide selection of recombinants. *DNA Cell Biol.* **13**: 311–319.
6. Gabay, J. E. 1994. Ubiquitous natural antibiotics. *Science* **264**: 373–374.
7. Hara, S. and M. Yamakawa. 1996. Production in *Escherichia coli* of moricin, a novel type antibacterial peptide from the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **220**: 664–669.
8. Hasan, N. and W. Szybalski. 1987. Control of cloned gene expression by promoter inversion *in vivo*: construction of improved vectors with a multiple cloning site and the Ptac promoter. *Gene* **56**: 145–151.
9. Hellers, M., H. Gunne, and H. Steiner. 1991. Expression and post-translational processing of preprocecropin A using a baculovirus vector. *Eur. J. Biochem.* **199**: 435–439.
10. Hoffmann, J. A. 1995. Innate immunity of insects. *Curr. Opin. Immunol.* **7**: 4–10.
11. Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* **59**: 171–200.
12. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
13. Lee, D. G., S. Y. Shin, S. G. Lee, K. L. Kim, M. K. Lee, and K. S. Hahm. 1997. Antifungal activities of magainin-2 hybrid peptides against *Trichosporon beigelii*. *J. Microbiol. Biotechnol.* **7**: 49–51.
14. Lee, J. H., P. M. Skowron, S. M. Rutkowska, S. S. Hong, and S. C. Kim. 1996. Sequential amplification of cloned DNA as tandem multimers using Class-IIS restriction enzymes. *Genet. Anal. Biomol. Eng.* **13**: 139–145.
15. Lehrer, R. I., M. Rosenman, S. S. S. L. Harwig, R. Jackson, and P. Eisenhauer. 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* **137**: 167–173.
16. Lehrer, R. I., A. K. Lichtenstein, and T. Ganz. 1993. Defensins: Antimicrobial and cytotoxic peptides of mammalian cells. *Ann. Rev. Immunol.* **11**: 105–128.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, A laboratory manual.* Cold Spring Harbor, NY.
18. Matsuzaki, K., O. Murase, N. Fujii, and K. Miyajima. 1995. Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. *Biochemistry* **34**: 6521–6526.
19. Nakamura, T., H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, and S. Iwanaga. 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the Horseshoe Crab (*Tachypleus tridentatus*). *J. Biol. Chem.* **263**: 16709–16713.
20. Piers, K. L., M. H. Brown, and R. E. W. Hancock. 1993. Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. *Gene* **134**: 7–13.
21. Podhajska, A. J., N. Hasan, and W. Szybalski. 1985. Control of cloned gene expression by promoter inversion *in vivo*: construction of the heat-pulse-activated att-nutL-p-att-N module. *Gene* **40**: 163–168.
22. Reichhart, J. M., I. Petit, M. Legrain, J. L. Dimarcq, E. Keppi, J. P. Lecocq, J. A. Hoffmann, and T. Achstetter. 1992. Expression and secretion in yeast of active insect defensin, an inducible antimicrobial peptide from the fleshfly *Phormia terranovae*. *Invert. Reprod. Develop.* **21**: 15–24.
23. Saberwal, G. and R. Nagaraj. 1994. Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing abilities. *Biochim. Biophys. Acta* **1197**: 109–131.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning, A laboratory manual*, 2nd ed. Cold Spring Harbor, NY.
25. Schägger, H. and G. von Jägow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368–379.

26. Sharma, A., A. M. Khoury-Christianson, S. P. White, N. K. Dhanjal, W. Huang, C. Paulhiac, E. J. Friedman, B. N. Manjula, and R. Kumar. 1994. High-efficiency synthesis of human α-endorphin and magainin in the erythrocytes of transgenic mice: a production system for therapeutic peptides. *Proc. Natl. Acad. Sci. USA* **91**: 9337–9341.
27. Wade, D., D. Andrew, S. A. Mitchell, A. M. V. Silveira, A. Boman, H. G. Boman, and R. B. Merrifield. 1992. Antimicrobial peptides designed as analogs or hybrids of cecropins and melittin. *Int. J. Pept. Prot. Res.* **40**: 429–436.
28. Wimley, W. C., M. E. Selsted, and S. H. White. 1994. Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. *Protein Sci.* **3**: 1362–1373.
29. Wülfing, C. and A. Plückthun. 1993. A versatile and highly repressible *Escherichia coli* expression system based on invertible promoters: expression of a gene encoding a toxic product. *Gene* **136**: 199–203.
30. Zasloff, M. 1987. Magainin, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA* **84**: 5449–5453.
31. Zasloff, M. 1992. Antibiotic peptides as mediator of innate immunity. *Cur. Opin. Immunol.* **4**: 3–7.