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Construction of a New Gene-Fusion Expression Vector, pMONSTER

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Abstract The *Fur* (ferric uptake regulation) expression vector pMON2064 was modified to produce a Fur-fusion expression vector. A kinker site, factor Xa cleavage site, and several restriction endonuclease sites were introduced to facilitate easy cloning and isolating of the fusion protein. The resulting fusion expression vector, pMONSTER, was then used to make fusion proteins with β-galactosidase and the protease of the human immunodeficiency virus type 1 (HIV-1 PR). Strain SW4020 harboring the Fur-β-galactosidase fusion vector produced blue colonies on a 5-bromo-4-chloro-3-indolyl-β-D-galactoside plate and the resulting 133 kDa fusion protein reacted with an anti-Fur antibody. The strain harboring the Fur-HIV-1 PR fusion vector produced a 29 kDa fusion protein, which also reacted with an anti-Fur antibody. The Fur-HIV-1 PR fusion protein was purified by a single column application that was designed to isolate the Fur protein. The purified Fur-HIV-1 PR fusion protein digested with factor Xa cleaved a recombinant Gag protein to release smaller fragments, including a p24 capsid protein. The Fur-HIV-1 PR fusion protein itself did not exhibit any proteolytic activity.

Key words: Fur protein, fusion expression vector, β-galactosidase, HIV protease

Many proteins are now genetically engineered and produced for various purposes. From an industrial perspective, the protein products need to be made by inexpensive and simple methods. Researchers have therefore put a lot of effort into developing efficient expression vectors and many companies have made their goods commercially available. A good expression system generally requires several conditions: (i) a stable replication and copy number control (the replication origin); (ii) a selectable marker such as a gene conferring antibiotic resistance; (iii) a strong promoter for transcription of the gene desired to be expressed. It would be advantageous if a specific agent can induce the promoter; (iv) a ribosome binding site for efficient translation initiation; and (v) a good purification system for the protein product. Many bacterial gene expression vectors (Clonetec, Pharmacia, NEB, Stratagene, Invitrogen) have adopted P_{tac} or P_{trc} promoter, which are both hybrids, derived from the *trp* and *lac* promoters [3, 15, 1]. The hybrid promoters are made strong and inducible by isopropylthio-β-D-galactoside (IPTG) and will not express unless the inducer is introduced. Sometimes, P_l and P_r of λ phage [8] are used. The bacteriophage T7 promoter is also frequently used, simply because of its differential expression by T7 RNA polymerase, which is provided by an inducible promoter [18, 19]. Purification of the protein product is another problem. Many different proteins produced by expression vectors have different purification procedures. To avoid this problem, various gene fusion vectors have been developed. Here, the protein of interest is fused with a protein whose purification is simple and well-understood. Glutathione-S-transferase [17], IgG-binding domain of protein A [10], or maltose binding protein [9] are frequently used for fusion in a gene fusion expression vector. These fusion proteins are easily isolated by glutathione Sepharose 4B, IgG Sepharose 6FF, and amylose resin, respectively. Recently, histidine patch (Invitrogen) or calmodulin-binding peptide tag (Stratagene) have also been used for easy isolation of the expressed proteins. Cleavage site at the junction of the two proteins allows for the separation of the fusion. The thrombin cleavage site (leu-val-pro-arg-↓-gly-ser) results in an additional dipeptide, gly-ser, in the N-terminal of the target protein [4]. The blood factor Xa [5, 13] cuts the end of a tetrapeptide (ile-glu-gly-arg ↓). This protease cleavage site can produce proteins with an authentic sequence.

In the present study, we developed a new expression vector producing a Fur-fusion protein. The new vector was modified from pMON2064 [20], which was originally developed to produce Fur (Ferric uptake regulation) repressor. The pMON2064 satisfies the previous requirements for a protein expression vector. The vector carries the ColE1 replication origin, ampicillin resistance gene, *recA* promoter, and translation initiation sequence from coliphage gene 10.

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Since nalidixic acid induces the SOS system, the Fur protein is expressed via the *recA* promoter. Furthermore, the protein can be isolated in a single step using immobilized metal-ion-affinity chromatography with zinc iminodiacetate agarose [20]. The newly constructed gene fusion vector was tested by making Fur-fusion proteins with β-galactosidase and human immunodeficiency virus type 1 protease (HIV-1 PR).

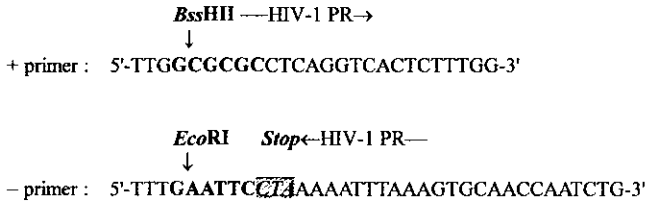

Fig. 1. Sequence of PCR primers used for cloning of the HIV-1 protease gene.
The restriction endonuclease sites *Bss*HI and *Eco*RI were introduced in the + and - primers, respectively. A termination codon TAG was also introduced in the form of *CTA* in the - primer.

MATERIALS AND METHODS

Chemicals

Restriction endonucleases were purchased from Pharmacia Biotech. Co. (Buckinghamshire, England) and the bacteriophage T4 DNA ligase was from Boehringer Mannheim Co. (Mannheim, Germany). The 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (Bistris), acrylamide, agarose, and zinc sulfate were from Sigma Chemical Co. (St. Louis, U.S.A.), Fisher Scientific (Pittsburgh, U.S.A.), and Fluka Chemika-Biochemika (Buch, Swizerland), respectively. The Sephaglass™ Bandprep kit, Sequenase version 2.0 DNA Sequencing kit, and [α-^{35}S]-dATP were purchased from Pharmacia Biotech. Co., USB Co. (Cleveland. U.S.A.), and Amersham Life Science (Buckinghamshire, England) respectively. Iminodiacetate agarose was obtained from Pierce Co. (Rockford, U.S.A.). Alkaline phosphatase conjugated anti-mouse IgG and peroxidase conjugated anti-mouse IgG were from Sigma Chemical Co. Monoclonal mouse anti-p24 IgG was purchased from Intracel Co. (Rockville, U.S.A.). The recombinant Gag protein was generously provided by the Virus Lab. in the Department of Genetic Engineering, Hallym University. All components of the bacterial medium were obtained from Difco Co. (Detroit, U.S.A.).

Strains and Plasmids

The *E. coli* strains of JRB45 (*lac*, *lon*, *ara*, *str*A, *sup*F) and SW4020 (*trp*, *his*, *arg*, *thr*, *lac*△u169, *galK*-[*fur*::Tn5] were from lab collection. The plasmid pMON2064 [20] was also from the lab collection. The plasmid mTn3-*lacZ* containing a full *lac*Z coding sequence without its own promoter was donated by the Cell Biology Lab. in the Department of Genetic Engineering, Hallym University. The plasmid pCOPIA-*lacZ* contains a complete *lacZ* sequence together with a regulatory region and was a donation from the Molecular Genetics Lab. in the Department of Genetic Engineering, Hallym University. The plasmid pSVC21 containing the wild-type protease gene of HIV-1 was a gift from the Virus Lab. in the Department of Genetic Engineering, Hallym University.

Cloning of the β-Galactosidase Gene

General molecular techniques were carried out as described by Sambrook *et al.* [16]. The plasmid mTn3-*lacZ* contained the coding sequence of the *lacZ* gene. The plasmid was digested with *Bam*H1, and the 5' protruding end was filled in with a Klenow fragment to make a blunt end. The plasmid was further digested with *Eco*RI to release a 4,019 base pair (bp) fragment containing the *lacZ* coding sequence. The fragment was isolated from 0.8% agarose gel and extracted using the Sephaglass™ Bandprep kit.

Cloning of the HIV-1 Protease Gene

The HIV-1 protease gene was amplified using a polymerase chain reaction (PCR) technique with the two primers as shown in Fig. 1. In order to clone the gene easily into the linker site in the final expression vector, the restriction sites of *Bss*HII and *Eco*RI were introduced into the + and - primers, respectively. The termination codon TAG was introduced in the form of *CTA* in the - primer since there is no natural termination codon in the protease gene [14] and the protein is processed from a polyprotein precursor. A 50 ng portion of the template DNA (pSVC21), 20 μM of each of the + and - primers, 2 mM of the four deoxyribonucleoside triphosphates, and a 10× PCR buffer were mixed in a PCR tube to a final volume of 100 μl. The reaction mixture was overlayed with 30 μl of mineral oil, heated at 98°C for 5 min, and then quickly cooled to 50°C for 1 min. The temperature was raised to 72°C and one unit of *Taq* DNA polymerase was added to the reaction mixture, which was then incubated for 2 min. A thermocycle of 94°C for 30 sec, 52°C for 1 min, and 72°C for 1 min was repeated 37 times. In the last cycle, the reaction mixture was held at 72°C for 10 min followed by 20°C for 5 min. All temperature shifts were performed in a thermocycler (Techne, Princeton, U.S.A.). The amplified DNA was finally extracted with phenol and washed with ethanol.

Ligation into the Fusion Vector

Aliquots of 200 μg of the vector DNA (pMONSTER) and 200 μg of the *lacZ* DNA fragment, prepared as described above, were mixed with 50 units of T4 DNA ligase in a 20 μl reaction volume and incubated for 20 h at 16°C. Competent cells of strain SW4020 were transformed with the ligation mixture and plated on LB plates containing

300 μg/ml of ampicillin and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and the blue colonies were selected. Similarly, the 314 bp PCR product of the HIV-1 PR gene and the plasmid pMONSTER were separately digested with *Bss*HII and *Eco*RI, followed by phenol extraction and ethanol precipitation. Aliquots of 200 μg of each DNA were ligated as described above. Competent cells of SW4020 were transformed with 10 μl of the ligation mixture and spread on LB plates containing 300 μg/ml of ampicillin and those colonies producing the 3,948 bp plasmid were selected.

Production of Fur-HIV-1 PR Fusion Protein

A bacterial strain harboring the fusion vector was grown and induced essentially as described previously [20]. A cell pellet harvested from 1 l of the culture medium was resuspended in 25 ml of 20 mM Tris-Cl (pH 8.0) containing 20 mM EDTA, 5 μg/ml DNase, and 5 μg/ml RNase, and then disintegrated by French pressure cell under a pressure of 1,260 psi. The cell homogenate was centrifuged at 8,000 ×g for 30 min. Since the fusion protein was produced in the form of an inclusion body, the pellet was resuspended in 10 ml of 20 mM Tris-Cl (pH 8.0) and recentrifuged at 12,000 ×g for 30 min. The pellet was dissolved in 10 ml of 8 M urea in 20 mM Tris-Cl (pH 8.0) and dialyzed overnight against 2 l of 20 mM Tris-Cl (pH 8.0). The isolation procedure for the fusion protein was the same as for Fur protein itself [20]. In order to split the HIV protease from the fusion protein, 50 μg of isolated Fur-HIV-1 PR was dissolved in 20 mM Tris-Cl (pH 8.0) containing 100 mM NaCl and 2 mM $CaCl_2$, and then 1 μg of Factor Xa in a 50 μl reaction volume was added. The reaction mixture was incubated for 6 h at 23°C. The incubated mixture was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as well.

Preparation of Fur Antibody

After the SDS-PAGE on a 15% gel, the Fur protein band was cut and dialyzed overnight against a sufficient amount of distilled water. The protein band was homogenized in 0.7 ml of a PBS solution to make an antigen solution. A solution of 0.2 ml Fur antigen was mixed well with the same volume of complete Freund's adjuvant and injected into the abdominal cavity of a BALB/C mouse. Twenty days later, a further mixture of 0.2 ml Fur antigen and 0.2 ml of incomplete Freund's adjuvant was injected in the same way. Another 20 days later, 5 ml of pristine (2,6,10,14-tetramethylpentadecane) was injected. A 3rd Fur antigen (0.4 ml) injection was performed 5 days later. Four days thereafter a Myeloma cell was administered into the abdominal cavity of the mouse as before. After 20 days, the abdominal fluid containing Fur antibody was collected. The mouse ascite (3.5 ml) obtained from the immunized animal was centrifuged for 15 min at 20,000 ×g. An equal volume of PBS (pH 7.4) was mixed into the supernatant and ammonium sulfate was slowly added to precipitate antibody completely. The precipitate was collected by centrifugation for 20 min, dissolved in 3.5 ml of PBS (pH 7.0), and then dialyzed against the PBS (pH 7.0) for 24 h. Ten mM sodium azide was added to the dialysate, which was then stored at 4°C.

Western Blot Analysis

The Fur-fusion proteins were identified by a Western blot analysis as described by Sambrook *et al.* [16]. The protein samples were loaded in duplicate on a SDS-PAGE. After the electrophoresis, one copy of the gel was stained with Coomassie Brilliant Blue and the other copy transferred onto nitrocellulose paper using a transfer kit (SemiPhor Semi-Dry Transfer Units, Hoefer Scientific, San Francisco, U.S.A.) for 45 min at 250 mA. The anti-Fur mouse antibody obtained above was then used to identify the Fur-fusion protein. For the second antibody, alkaline phosphatase-conjugated affinipure goat anti-mouse IgG (H+L) was used after a 1:10,000 dilution. In order to detect the HIV protease activity, anti-p24 monoclonal antibody was used as the first antibody and peroxidase conjugated anti-Human IgG as the second.

Activity of HIV-1 Protease

An assay of the proteolytic activity of HIV-1 PR was performed as previously described [11]. The recombinant Gag protein (p55; HIV-1 Pr55gag) was used as the HIV-1 protease substrate. A 5 μg portion of the partially purified Gag protein in 19 μl of a reaction buffer (50 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 mM disodium ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 50 mM NaCl, pH 6.5) was digested with 1 μg of either the Fur-HIV-1 PR fusion protein or the Fur-HIV-1 PR fusion protein digested with factor Xa at 37°C for 3 h.

RESULTS AND DISCUSSION

Design of Linker DNA

In order to serve as a gene fusion expression vector, the plasmid should contain several additional features. First, the fusion protein should include a cleavage site to separate the protein of interest from the Fur protein. The cleavage site of the blood factor Xa (ile-glu-gly-arg ↓) was introduced for this purpose. Second, the kinker is needed to help expose the cleavage site of the fusion protein. Three consecutive glycine codons (GGT) were provided [7] to prevent any possible folding-in of the factor Xa cleavage site. Third, a multicloning site is required for the cloning of various protein genes, therefore, the restriction sites of the endonucleases *Bss*HII, *Sma*I, *Hin*dIII, *Bsp*EI,

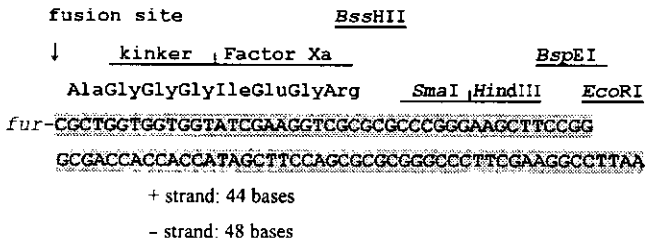

Fig. 2. Sequence of the linker DNA region.
Synthetic oligonucleotide, which contains a kinker site, blood factor Xa cleavage site, and several endonuclease recognition sites.

and *Eco*RI were inserted in the linker DNA. These restriction sites were not found elsewhere in the final expression vector. The sequence of the linker DNA is shown in Fig. 2.

Construction of pMONSTER

The plasmid pMON2064 was digested with *Nco*I and *Eco*RI (Fig. 3). Large and small fragments of the plasmid DNA were separated from the 0.8% agarose gel and harvested individually using a Sephaglass™ BandPrep Kit. The small fragment of pMON2064 was further digested with *Fsp*I, and the *Nco*I-*Fsp*I fragment was isolated as above. A 100 ng portion of the *Nco*I-*Fsp*I fragment was mixed with 100 ng of the linker DNA and 100 ng of the large fragment of the *Nco*I-*Eco*RI of pMON2064 obtained previously. To a reaction volume of 20 µl, 50 units of T4 DNA ligase were then added and the mixture was incubated at 16°C for 20 h. Competent cells of SW4020 were transformed with the ligation product and spread on an LB agar plate containing 100 µg/ml of ampicillin. Single colonies of the transformed cells were individually incubated in

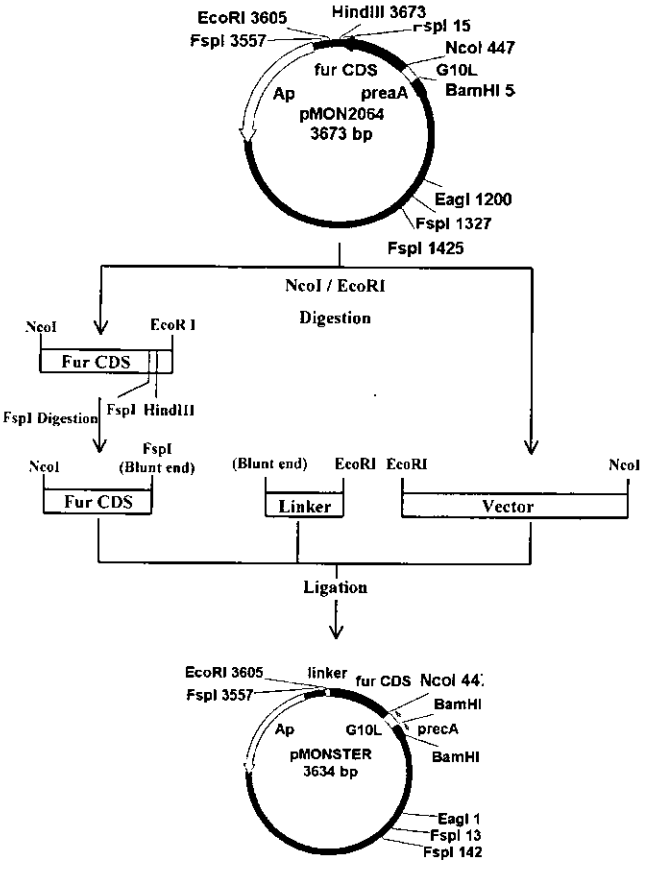

Fig. 3. Construction of gene fusion expression vector, pMONSTER.
The synthetic linker DNA was replaced at the *Fsp*I (*Fsp*I 15, near the end of the Fur coding sequence) and *Eco*RI sites of pMON2064.

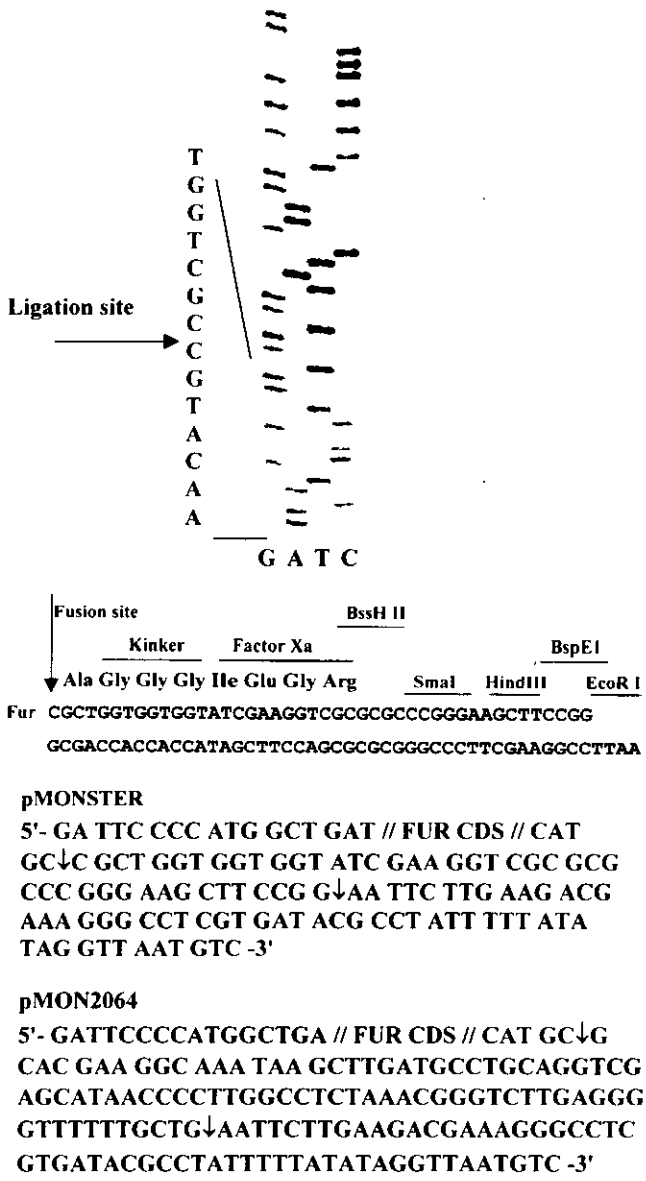

Fig. 4. DNA sequence of the linker region of pMONSTER.
The DNA sequence shows the successful ligation of the linker DNA. The arrow points in the pMONSTER and pMON2064 sequences indicate the altered regions of the plasmid. The change results in 25 additional amino acids at the end of the Fur protein, which terminates at a TAG stop codon in pMONSTER.

2 ml of LB broth containing 300 μg/ml of ampicillin for 16 h. The plasmid DNA was prepared from each culture and digested with *Bsp*EI, which has a unique restriction site in the linker of the plasmid. Those colonies harboring a plasmid containing the *Bsp*EI restriction site were then selected and stored at -70°C. In Fig. 4, the sequence of the linker region shows the details of the plasmid modification. The end (tgc ↓) of the *Fsp*1 restriction site of the Fur-containing fragment and blunt end (↓ cgc...) of the linker DNA were joined as we expected. Due to the intentional removal of the native Fur stop codon and the introduction of a linker DNA, a new stop codon, TAG, was generated at 88 bases downstream from the *Fsp*I ligation site. This resulted in the addition of 25 more amino acids (75 DNA bases) to the Fur protein in pMONSTER. The strain SW4020 harboring pMONSTER produced a 20 kDa Fur protein instead of a 17 kDa protein as with pMON2064 (see below, Fig. 6).

Fur-β-Galactosidase Fusion

The newly constructed fusion expression vector, pMONSTER, was used to create the β-galactosidase fusion. The nucleotide sequence of the resulting recombinant plasmid pMONSTER-*lacZ* showed the correct ligation of *lacZ* into the *Sma*I site of pMONSTER (data not shown). The *E. coli* strain SW4020 (pMONSTER-*lacZ*) produced an apparent 133 kDa molecular size protein compared to the 116 kDa of the native β-galactosidase (Fig. 5). The 133 kDa protein of strain SW4020 (pMONSTER-*lacZ*) showed a positive reaction with a Fur antibody in a Western blot analysis (Fig. 5), thus indicating that the protein is a Fur-β-galactosidase fusion. The fact that strain SW4020 (pMONSTER-*lacZ*) formed blue colonies on a X-gal plate (data not shown) suggests that the Fur-β-galactosidase fusion protein was enzymatically active in the cell. This result indicates the successful construction and proper functioning of the new fusion expression vector.

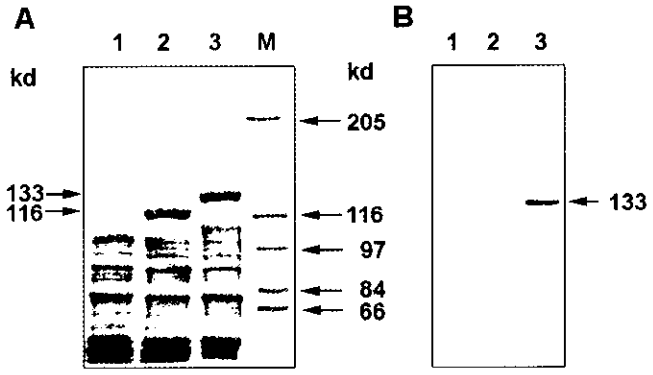

Fig. 5. Expression of Fur-β-galactosidase fusion protein.
6% SDS-PAGE (A) and Western blot (B) analysis of *E. coli* SW4020, lane 1; *E. coli* SW4020 (pCOPIA-*lacZ*), lane 2; *E. coli* SW4020 (pMONSTER-*lacZ*), lane 3; and molecular weight marker, lane M. All the samples were induced with nalidixic acid (50 g/ml) and homogenated by sonication. In the Western blot analysis, a polyclonal mouse anti-Fur antibody was used as the first antibody and alkaline phosphatase conjugated anti-mouse IgG as the second antibody. The Fur-β-galactosidase fusion protein (133 kDa) was recognized by the Fur antibody.

Fur-HIV-1 PR Fusion

The protease gene of the human immunodeficiency virus was also used to test the newly constructed fusion expression vector. A DNA fragment of the 314 bp HIV-1 protease gene was obtained by PCR (see Materials and Methods). The PCR product was digested with the restriction endonucleases *Bss*HII and *Eco*RI and then ligated with pMONSTER which had also been treated with the same enzymes. The resulting fusion vector, pMONSTER-HIV-1 PR, was sequenced and the correct ligation was confirmed (data not shown). The Fur-HIV-1 PR fusion plasmid was expressed in SW4020 after transformation. Figure 6 shows that the 29 kDa Fur-HIV-1 PR fusion protein was detected by anti-Fur antibody.

Isolation of Fur-HIV-1 PR Fusion Protein

The *E. coli* SW4020 (pMONSTER-HIV-1 PR) cells after induction (see Materials and Methods) were harvested and

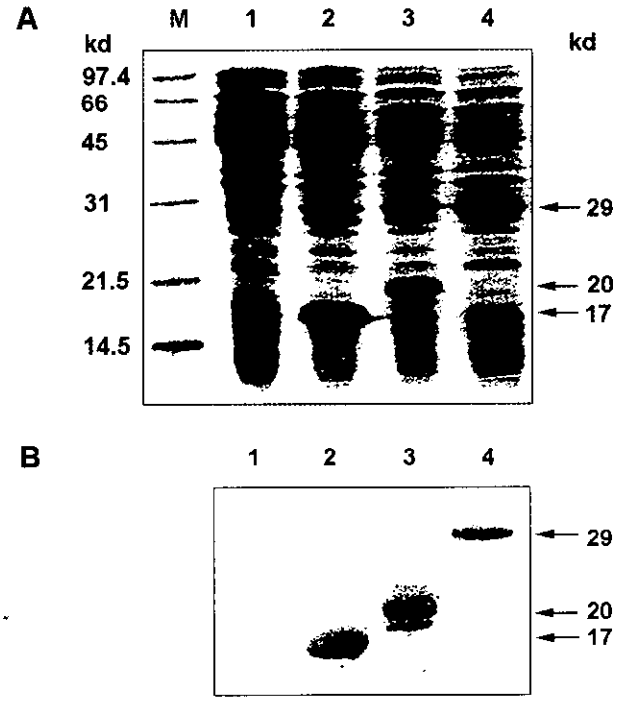

Fig. 6. Expression of Fur-HIV 1 PR fusion protein.
15% SDS-PAGE (A) and Western blot (B) analysis of *E. coli* SW4020, lane 1; *E. coli* SW4020 (pMON2064, 17 kDa Fur), lane 2; *E. coli* SW4020 (pMONSTER, 20 kDa Fur due to the deletion of native termination codon), lane 3; *E. coli* SW4020 (pMONSTER-HIV-1 PR, 29 kDa Fur-HIV-1 PR fusion), lane 4; and molecular weight marker, lane M. All the samples were treated described as in Fig. 5. A polyclonal mouse anti-Fur antibody was used as the first antibody and alkaline phosphatase conjugated anti-mouse IgG as the second antibody.

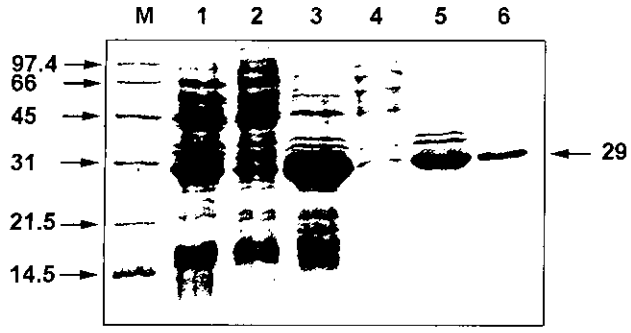

Fig. 7. Isolation of Fur-HIV-1 PR fusion protein.
15% SDS-PAGE analysis. Molecular weight marker, lane M; whole cell homogenate, lane 1; centrifugation supernatant of the cell homogenate, lane 2; resuspended pellet, lane 3; washes of the pellet, lane 4; washed pellet solubilized with 8 M urea and dialyzed, lane 5; purified Fur-HIV-1 PR by Zn-IDA agarose affinity chromatography, lane 6.

disintegrated by a French pressure cell. An SDS-PAGE analysis showed that the majority of the fusion protein was in the pellet after centrifugation of the cell homogenate, suggesting that the protein was produced in the form of an inclusion body (Fig. 7). This inclusion body seemed to be quite pure since the resuspended pellet dissolved in 8 M urea did not contain any significant amount of contamination (Fig. 7, Lane 5). More than 95% of the inclusion body was solubilized. Many proteins produced as an inclusion body have difficulties in renaturation. To avoid overexpression of the target gene, the promoter must be tightly regulated. The promoter P_{BAD} of the arabinose operon was chosen for this purpose since the expression of this promoter can be easily controlled by L-arabinose concentration [3, 6]. In contrast, thioredoxin fusion was created to prevent the formation of an inclusion body [8]. The purified Fur-HIV-1 PR fusion protein (see

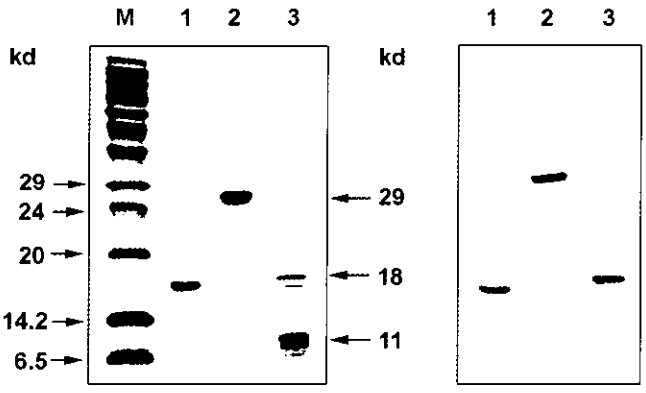

Fig. 8. Digestion of purified Fur-HIV-1 PR fusion protein with factor Xa.
15% SDS-PAGE (left panel) and Western blot (right panel) analysis of Fur-HIV-1 PR fusion protein. 5 μg of purified *fur* protein, lane 1; 10 μg of purified Fur-HIV-1 PR, lane 2; 10 μg of purified Fur-HIV-1 PR digested with 0.2 μg of Factor Xa, lane 3; molecular weight marker, lane M. The Western blot procedure was the same described as in Fig. 6.

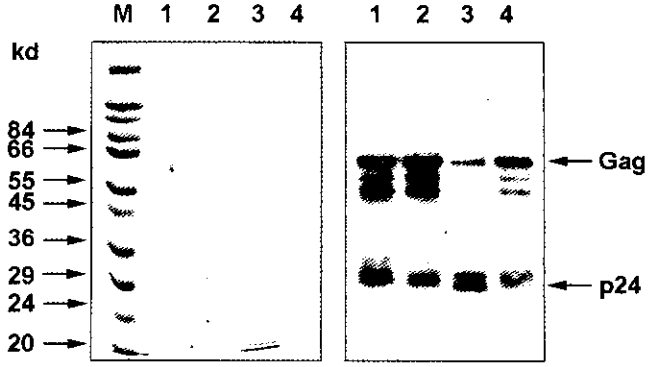

Fig. 9. Proteolytic activity of Fur-HIV-1 PR and HIV-1 PR produced by pMONSTER.
A 12% SDS-PAGE (left panel) and Western blot (right panel) analysis were performed after the incubation of the recombinant Gag protein with the Fur-HIV-1 PR fusion protein and the Fur-HIV-1 PR digested with factor Xa. Untreated 58.7 kDa recombinant Gag protein, lane 1; Gag protein incubated with Fur-HIV-1 PR, lane 2; Gag protein incubated with Fur-HIV-1 PR treated with factor Xa, lane 3; Gag protein digested with Factor Xa, lane 4. Monoclonal mouse anti-p24 IgG was used as the first antibody and peroxidase conjugated anti-mouse IgG as the second antibody.

Materials and Methods) of 29 kDa was digested with Factor Xa. SDS-PAGE and a Western blot analysis showed cleavage products of 18 kDa Fur and 11 kDa HIV-1 PR from the fusion protein (Fig. 8). Since the cleavage site of the fusion protein left four more amino acids on the Fur side (Fig. 4), the calculated molecular mass of the Fur protein appeared to be 18 kDa. Also, due to the introduction of the *Bss*HI restriction site to the HIV protease gene, the GCG codon preceded the authentic HIV-1 PR gene, which resulted in an alanine residue in the N-terminus of the protein.

Activity of HIV-1 Protease

The polyprotein of 55 kDa Gag (p55; HIV-1 Pr55gag) was processed by HIV-1 PR to produce a 17 kDa matrix protein (MA, p17), 24 kDa capsid protein (CA, p24), and 15 kDa (6+9) nucleocapsid protein (NC) [12]. A recombinant Gag protein of 58.7 (3.7+55) kDa was used as the substrate for the protease activity of the fusion protein (Fig. 9). While the fusion protein (Fur-HIV-1 PR, Fig. 9, lane 2) and factor Xa alone (lane 4) did not produce a p24 protein, the factor Xa digested-Fur-HIV-1 PR (lane 3) clearly showed a p24 (CA: 24 kDa) protein. This suggests that the HIV-1 PR separated from the Fur protein was enzymatically active while the Fur-HIV-1 PR fusion protein was not. However, the substrate Gag protein in our hand was not pure enough, as it contained anti-p24 reacting bands with various sizes. In contrast, the fusion protein treated with factor Xa had a decreased major substrate band (58.7 kDa), and produced 24 kDa and smaller protein bands along with some intermediate bands (Fig. 8, lane 3, right panel).

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