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Expression of Immunologically Active Porcine Recombinant TGF-β1 Precursor Protein in Baculovirus System

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In order to express recombinant porcine TGF-β1 protein in a baculovirus expression system the entire TGF-β1 gene containing extra amino acids at the N terminus was cloned into pFBa and pFBb of the Bac-To-Bac™ baculovirus expression system. One of the clones contained 106 extra amino acids and was designated pFBa-106TGF-β1, and the other had 28 extra amino acids and was designated pFBb-28TGF-β1. The orientation of the gene was identified with restriction enzyme mapping and PCR with internal TGF-β1 primers. Sf-9 cells were infected at a m.o.i. of 10 by the recombinant viruses generated from the two different plasmids. Both of the recombinant viruses produced precursor forms of TGF-β1 with expected sizes of 55 kD and 46.4 kD. These precursor forms of TGF-β1 reacted with a polyclonal antibody against human TGF-β1. No mature form of TGF-β1 protein was detected on SDS gels and an immunoblot indicated that TGF-β1 precursor is not properly processed in insect cells.

Key words: TGF-β1, baculovirus expression, recombinant protein, precursor

Transforming growth factor beta (TGF-β) is a multifunctional polypeptide whose diverse functions involve the modulation of cellular, physiological, and immunological processes. The vast majority of studies performed to date also point to its involvement in cell growth, differentiation, and regulation of extracellular matrix and various metabolic effects depending on the cell type and degree of cell differentiation (10, 14). It is now believed that TGF-β is one kind of cytokine. So far three different human isoforms of TGF-β have been reported, namely TGF-β1, TGF-β2 and TGF-β3. In addition, TGF-β4 and TGF-β5 have been identified in chicken and frog, respectively. Other proteins distantly related to TGF-β1 such as inhibins/activins, mullerian inhibitory substance and bone morphogenic proteins have also been described. Comparison of amino acid sequences among mammalian TGF-β1 has revealed a remarkably high degree of sequence conservation. Amino acid sequences of mature TGF-β1 protein in human, bovine and porcine are almost identical. One or two amino acid differences were reported in pro region (6) among mammalian TGF-β1 precursors. This sequence indentity obviously explains the lack of species specificity and implies a strong evolutionary pressure on TGF-β1. Mature transforming growth factor-β1 is a homodimeric polypetide composed of identical 112-amino acid subunits derived from carboxy termini linked by two disulfide linkages. The 25 kD TGF-β1 is the biologically active molecule and the dissociation of the dimeric structure with reducing agents results in a complete loss of its activity. Cloning and sequencing of the TGF-β1 gene from various sources have revealed that TGF-β1 propeptide is synthesized as a large molecule of 390 amino acids. This propeptide is bound to latent associated protein (LAP) until it becomes to be an active form. Latent TGF-β1 is secreted as a noncovalent complex of the 75 kD dimeric glycoprotein derived from the TGF-β1 propeptide and is eventually processed to dimeric 25 kDa of active TGF-β1(4).

Recently, clinical interest in TGF-β1 as a potential therapeutic agent and as a research tool for studying immune regulators has grown. Unfortunately, supplies of TGF-β1 from natural sources are relatively limited. Only small amounts of TGF-β1 can be extracted from platelets (1) and demineralized bone (13). As the focus of research on

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TGF-β1 protein continues to expand, expression of this protein will be essential for research and development of therapeutic agents. In most cases, recombinant TGF-β1 is expressed and purified in chinese hamster ovary (CHO) cells, which are commercially available (3).

Surprisingly, however, only a limited number of papers have been published about the production of TGF-β1 by using other expression systems. The TGF-β1 fusion protein with a collagen binding protein was successfully expressed in *E. coli* in the form of inclusion bodies, solubilized with 8M urea, and renatured to be partially active TGF-β1 (14). Porcine TGF-β1 protein was expressed in *E. coli* only in the form of inclusion bodies (5). In this study we tried to express recombinant porcine TGF-β1 protein in a baculovirus expression system. Only the precursor form of TGF-β1 was expressed in the baculovirus system but the protein had reactivity with TGF-β1 polyclonal antibody.

Materials and Methods

TGF-β1 gene

Recombinant porcine TGF-β1 gene was kindly provided by Dr. S.J. Kim NIH, USA (4, 5).

Construction of recombinant transfer vector

The TGF-β1 gene was cloned from pPK9A that encodes porcine TGF-β1 cDNA with two point mutations resulting in amino acids changes from Cys to Ser at residues 233 and 225 in the propeptide region. The TGF-β1 gene was cut with *Bgl*II and ligated to pBlueBacIII™-TGF-β1(InVitrogen,USA). The gene was cut again with *Bam*HI and *Pst*I and ligated in frame to pFBa of the Bac-to-Bac system™ (Life Technologies, Inc., USA) to generate pFBa-106TGF-β1. pFBa-106TGF-β1 contained 106 extra amino acids including 6 His tags, spacer region and TEV protease recognition regions at the N-terminal region. Since the 106 extra amino acids at the N-terminus may have interfered with the proper processing of TGF-β1, another TGF-β1 gene was cloned by PCR with primers at the vicinity of the TGF-β1 gene resulting in 28 extra amino acids and a *Bam*HI restriction site at the N terminus, and a *Pst*I site at the other end. This DNA fragment was cloned into pFBb to generate pFBb-28TGF-β1. In order to generate two different TGF-Bacmids, pFBa-106TGF-β1 and pFBb-28TGF-β1 plasmids were transformed in DH10Bac which led to transposition of the TGF-β1 gene. White colonies were isolated in the presence of kanamycin, gentamicin, tetracycline, X-gal and IPTG. The orientation was confirmed with restriction enzyme mapping and the inserts were identified with PCR using two different internal primers : 5'-CGGGGCGA-CCTGGGCACCATCCATGAC-3', 5'-CTGCTCCACC-TTGGG CTTGCGACCCAC-3'(14).

Generation of recombinant baculovirus

Recombinant Bacmid DNA was purified as described(9) and transfected in *Spodoptera frugiperda* (Sf-9) cells by using Lipofectin™(Life Technolo- gies, Inc.USA). Briefly, 1~2 μg of plasmid DNA was mixed with 100 μl of serum free medium(SFM) and 15 μl of Lipofectin that had been premixed in the same volume of SFM. This mixture was added to Sf9 cells grown on 6 well plate (9×10^5 cells/well). The cells were allowed to be infected at 27 °C for 5 hrs. The solution was removed and 2 ml of SFM was added. Cells continued to grow for 72 hrs. Recombinant plaques were identified visually by the absence of occlusion bodies. Cells were harvested by centrifugation at 2,000 rpm for 10 min and the supernatant was saved for plaque assay. Virus titer was determined with a plaque assay and amplified up to 10^8~10^9 pfu/ml with a serial infection when necessary.

Identification of porcine TGF-β1

Monolayers of SF-9 cells were infected with recombinant virus at m.o.i of 10 in 75 cm^2 tissue culture flasks containing 9×10^6 cells/flask or in spinner cuture flask at $1\sim2\times10^6$ cells/ml. For time course analysis cells were harvested at various intervals over 120 hours. Cells (1×10^6 cells/300 μl) were lysed with lysis buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8) and the total proteins were analyzed by 15% SDS polyacrylamide gel electrophoresis (8).

Western blotting

Cell lysates were electrophoresed as described above and proteins were transferred to nitrocellulose membrane. Blots were blocked by immersion in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 2.5% BSA for 1 hr and immunological detection of TGF-β1 was carried out by incubation with TGF-polyclonal antibody (R & D Inc. USA). The TGF-β1 band was detected with both alkaline phosphatase and ECL™.

Subcellular localization of TGF-β1 in SF-9 cells

Monolayers of Sf9 cells infected for 72 hrs at m.o. i. of 10 were harvested as described above. Cell pellets were mixed with 5 volumes of nonionic detergent (1% NP40) lysis buffer per gram of cells. Cells were lysed with a 22 gauge syringe needle 3

times. Cell lysate was centrifuged at 13,000 rpm for 10 min and the supernatant was saved for cytosolic proteins. The pellet fraction was resuspended with ionic detergent lysis buffer (2% SDS, 62.5 mM Tris, pH 6.8) and saved for the nuclear fraction. Both fractions were analyzed in 15% SDS-PAGE for subcellular localization.

Results

Recombinant virus construction

Cloning of the baculovirus transfer vector containing the entire TGF-β1 coding gene was carried out as described in the Bac-To-Bac manual (Fig. 1). The cloning procedure of the TGF-β1 gene from pPK9A involved direct cloning and PCR amplification to generate pFBa-106TGF-β1 and pFBb-28TGF-β1 respectively. Because both clones contained *Bam*HI and *Pst*I sites, the orientation of the TGF-β1 gene in both plasmids was determined by restriction enzyme mapping (Fig. 2). The TGF-β1 gene insert from the transfer vector, bacmid and recombinant virus was also identified with PCR us-

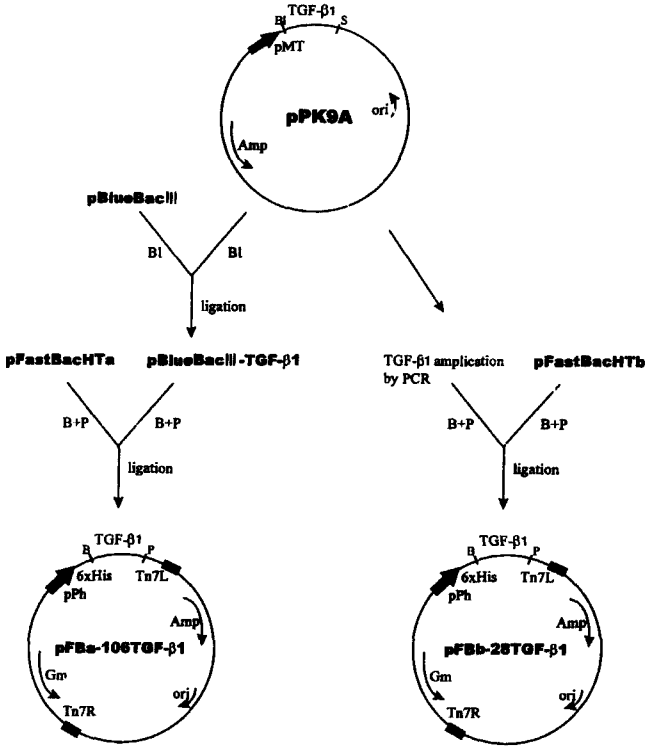

Fig. 1. Schematic diagram of the generation of two TGF-β1 transfer vectors. Recombinant porcine TGF-β1 gene from pPK9A was either subcloned in pBlueBacIII or amplified by PCR with primers to generate *Bam* HI and *Pst*I sites. TGF-β1 gene digested with *Bam* HI and *Pst*I was ligated to baculovirus transfer vector in frame pFastBacHTa and pFastBacHTb to make pFBa-106TGF-β1 and pFBb-28TGF-β1, respectively. Bl, *Bgl*II; B, *Bam* HI; P, *Pst*I; S, *Sal*I sites.

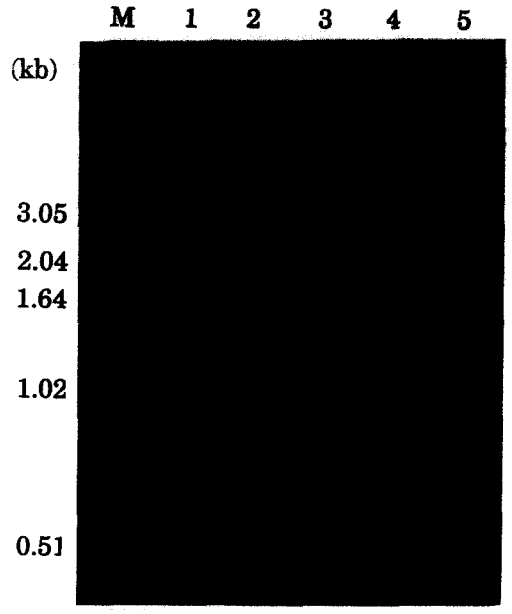

Fig. 2. The identification of TGF-β1 gene in transfer vector. The inserted TGF-β1 gene was indentified with restriction enzyme digestion. 1: pFBa transfer vector, 2: pFBa-106TGF-β1 vector, 3: pFBa-106TGF-β1 digested with *Bam* HI, 4: pFBa-106TGF-β1 digested with *Bam* HI and *Pst*I, 5: TGF-β1 gene from pBlueBacIII, M: 1 Kb DNA ladder.

Fig. 3. Identification of TGF-β1 by PCR with internal primers. The TGF-β1 gene was identified at the various steps of cloning procedures by PCR. 1: PCR product from pFBa-106TGF-β1, 2: PCR product from TGF-β1 Bacmid, 3: PCR product from recombinant virus, M: 1 Kb DNA ladder.

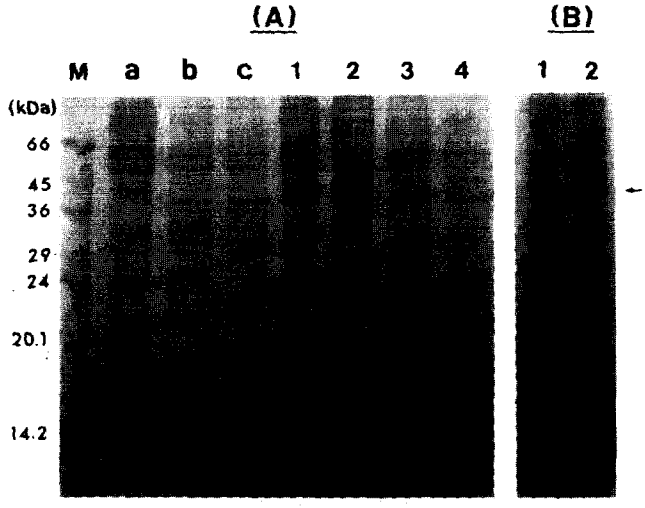

Fig. 4. Time course expression of TGF-β1 precursor protein in Baculovirus expression system. SF-9 cells were infected with recombinant virus at high m.o.i. Total protein was analyzed on 15% PAGE at various period after infection. (A): Time course expression of 106TGF-β1 precursor protein, a: SF-9 insect cells, b: SF-9 cells infected with wild type baculovirus at 72 hours post-infection, c: SF-9 cells infected with wild type baculovirus at 96 hours post-infection, 1: Cells infected with recombinant baculovirus at 48 hours post-infection, 2: Cells infected with recombinant baculovirus at 72 hours post-infection, 3: Cells infected with recombinant baculovirus at 96 hours post-infection, 4: Cells infected with recombinant baculovirus at 120 hours post-infection, (B): Expression of two TGF-β1 precursor proteins at 72 hours post-infection, 1: 106TGF-β1 precursor protein, 2: 28TGF-β1 precursor protein, Arrow indicates TGF-β1 precursor protein.

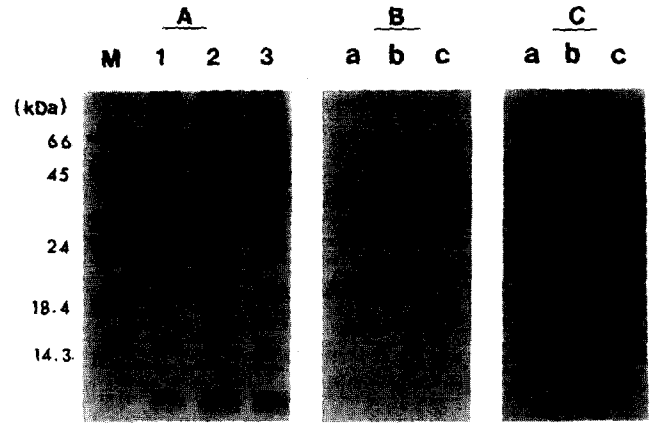

Fig. 5. Immunoblot of TGF-β1 precursor protein with polyclonal antibody. Total proteins of cells infected with two recombinant viruses at 72 hours post-infection were analyzed on 15% PAGE and stained with Coomassie Blue (A). Proteins were transferred to nitrocellulose membrane and reacted with TGF-polyclonal antibody. Colorization reaction was carried out with alkaline phosphatase (B) and ECL (C). 1) a: Cell lysates infected with wildtype virus, 2) b: 106TGF-β1 3, c: 28TGF-β1.

ing internal TGF-β1 primers (Fig. 3). The expected PCR products were detected in all samples indicating that the TGF-β1 gene was successfully cloned in recombinant baculovirus.

Expression of TGF-β1 protein in infected insect cells

In order to determine TGF-β1 protein synthesis in recombinant virus infected cells, Sf-9 cells were infected at a high m.o.i. with parental AcNPV or recombinant virus and were harvested at various times post-infection. Infected cell lysates were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 4). Since both recombinant viruses had very similar expression patterns only SDS-PAGE of pFBa-106TGF-β1 was used for expression data. Similar to the result from kinetic study with wild type virus where polyhedrin proteins were optimally expressed 48~72 hr after infection, recombinant virus infected cells show a protein band, evident 48 to 72 hours post-infection, corresponding in size to the 55 kD precursor form of TGF-β1. Densitometry of the stained gel indicated that the amount of TGF-β1 precursor protein was appoximately 5~10% of total proteins (data not shown). In order to identify immunological cross-reactivity, western blot was carried out with TGF-polyclonal antibody. Immunoblot data confirmed that both of the precursor forms of TGF-β1 reacted with polyclonal antibody detected with alkaline phosphatase as well as with ECL™ (Fig. 5).

In summation, recombinant TGF-β1 expressed in insect cells was not processed properly but it had immunological activity with TGF-polyclonal antibody. Both of the clones revealed that TGF-β1 precursor protein cross reacts with polyclonal antidody.

Subcellular localization of TGF-β1 protein

In order to determine the intracellular distribution of TGF-β1 in SF-9 cells, cytoplasmic and nuclear fractions were analyzed for TGF-β1 with SDS-PAGE. Most of the TGF-β1 was detected in the nuclear fraction (Fig. 6). That no soluble forms of TGF-β1 precursor form were detected in the cytosolic fraction may indicate that such localization is associated with the improper processing of TGF-β1.

Discussion

TGF-β1 is a multifuctional immune modulator in mammalian cells. Various systems have been used to express biologically active TGF-β1 in cells, including *E. coli*, yeast and mammalian cells. In most of cases, however, TGF-β1 is produced in the

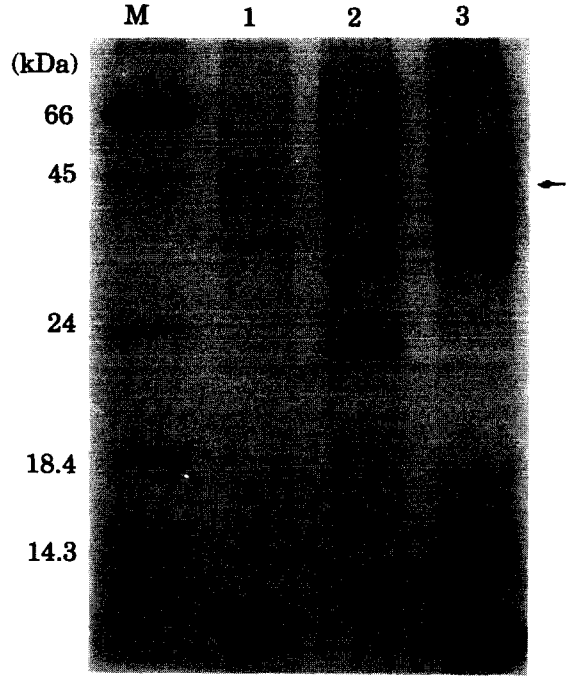

Fig. 6. Localization of 28TGF-β1 precursor protein in insect cells. Monolayers of Sf9 cells infected for 72 hours at m.o.i. of 10 were harvested and cell pellet was mixed with 5 volumes of nonionic detergent lysis buffer per gram of cells. Cell lysate was centrifuged at 13,000 rpm for 10 min and supernatant was saved for cytosolic proteins. Pellet fraction was resuspended with ionic detergent lysis buffer saved for nuclear fraction. Both fractions were analyzed in 15% SDS-PAGE, 1: Total proteins of cells at 72 hours post-infection, 2: Total proteins of cytosolic fraction, 3: Total proteins of nuclear fraction, Arrow indicates TGF-β1 precursor protein.

form of inclusion bodies and latent and precursor forms. None of these products have been used for further research. Biologically active TGF-β1 is, however, successfully expressed in chinese hamster ovary cells with different promoters (3). Usually the amount of TGF-β1 is too small to be isolated and used for other research purposes.

In order to produce large amounts of active porcine TGF-β1, we used a recombinant porcine TGF-β1 gene in which Cys-223 and Cys-225 were changed to Ser residues by site directed mutagenesis. This amino acids substitution in the pro-region resulted in the expression of only monomeric precursor protein. When TGF-β1 is expressed in CHO system, the CHO cells release bioactive TGF-β1 that does not require activation process (4). We tried to use a baculovirus expression system because of its easy manipulation and high expression level. Two different forms of the TGF-β1 gene as described above were cloned and expressed in Sf-9 cells. In both cases, the precursor form of TGF-β1 was expressed at high levels. However, both of these TGF-β1 precursor forms were not processed properly. Usually, proteins expressed baculovirus system are properly processed and have biological activities (2). Improper processing of TGF-β1 expressed in baculovirus system is not clearly understood. Other efforts to make biologically active TGF-β1 in baculovirus systems, such as direct expression of monomeric TGF-β1 and use of different baculovirus expression vectors are in progress. These precusor forms, however, show reactivity with polyclonal anti-TGF-β1 antibodies. The production of monoclonal antibodies against TGF-β1 is very important in the characterization of TGF-β1 action *in vitro* and *in vivo*. The production of monoclonal antibodies requires relatively large amounts of TGF-β1. Since these two precursor forms have immunological cross reactivity these proteins may be good sources for producing monoclonal antibodies against TGF-β1.

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References

1. **Assoian, R.K., A. Komoriya, C.A. Meyers, D.M. Smith, and M.B. Sporn.** 1983. Transforming growth factor beta in human platelets: Identification of a major storage site, purification and characterization. *J. Biol. Chem.* **258**, 7155-7160.
2. **Barber, G.N., J.C.S. Clegg, and G. Lloyd.** 1990. Expression of the Lassa virus nucleocapsid protein in insect cells infected with a recombinant baculovirus: application to diagnostic assays for Lassa virus infection. *J. Gen. Virol.* **71**, 19-28.
3. **Bourdrel, L., C.-H. Lin, S.L. Lauren, R.H. Elmore, B. J. Sugarman, S. Hu, and K.R. Westcott.** 1993. Recombinant human transforming growth factor-β1: Expression by chinese hamster ovary cells, isolation, and characterization. *Protein Expression and Purification* **4**, 130-140.
4. **Brunner, A.M., H. Marquatrdt, A.R. Malacko, M.N. Lioubin, and A.F. Purchio.** 1989. Site-directed mutagenesis of cysteine residues in the pro region of the trasforming growth factor beta1 precursor. Expression and characterization of mutant proteins. *J. Biol. Chem.* **264**, 13660-4.
5. **Choi, E.Y., P.H. Kim, and W-H. Byeon.** 1997. Expression of immunologically detectable epitope having porcine transforming growth factor-β1 (TGF-β1) in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **25**, 137-143.
6. **Derynck, R., J.A. Jarrett, E.Y. Che, and D.V. Goeddel.** 1986. The murine transforming growth factor-β precursor. *J. Biol. Chem.* **261**, 4377-4379.

7. **Kondaiah, P., E.V. Obberghen-Schilling, R.L. Ludwig, R. Dhar, M.B. Sporn, and A.B. Roberts.** 1988. cDNA cloning of porcine transforming growth factor-1 mRNAs. *J. Biol. Chem.* **263**, 18313-18317.
8. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680-685.
9. **Luckow, V.A., S.C. Lee, G.F. Barry, and P.O. Olins.** 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* **67**, 4566-4579.
10. **McCartney-Francis, N.L. and S.M. Wahl.** 1994. Transforming growth factor beta: a matter of life and death. *J. Leukocyte Biology* **55**, 401-409.
11. **Miyazono, K., A. Olofsson, P. Colosetti, and C.-H. Heldin.** 1991. A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. The *EMBO J.* **10**, 1091-1101.
12. **Rhee, K.J., S.-U. Nham, J.-S. Yoo, S.W. Yie, G-T. Chun, E.Y. Choi, J.-H. Park, and P.-H. Kim.** 1996. Characterization of mutated transforming growth factor-β1 transformants and its modulatory effect on IgA isotype synthesis by murime B lymphocytes. *Mol. Cells* **6**, 746-752.
13. **Seyedin, S.M., T.C. Thomas, A.Y. Thompson, D.M. Rosen, and K.A. Picz.** 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc. Natl. Acad. Sci. USA* **82**, 267-2271.
14. **Tuan, T.L., D.T. Cheung, L.T. Wu. A. Yee, S. Gabriel, B. Han, L. Morton, M.E. Nimni, and F.L. Hall.** 1996. Engineering, expression and renaturation of targeted TGF-beta fusion proteins. *Connet. Tissue. Res.* **34**, 1-9.