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Development of a Unidirectional Expression Vector: in a Search of Suppressor against a Cell Death-Inducing Protein, Jpk

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Jopock (*Jpk*) has previously been ascertained that induces both bacterial and mammalian cell death. The *Escherichia coli* cells expressing Glutathion S-transferase (GST) fused Jpk showed elongated phenotype and inhibited cell growth which led eventual cell death. In an attempt to search the genetic suppressor of the lethal protein Jpk in bacterial cells, we constructed a unidirectional protein expression vector inserting *tac* promoter next to the C-terminus Jpk in pGEX-Jpk. The function of additional *tac* promoter was confirmed by substituting *lac* promoter in Plac-TOPO plasmid. The cells harboring plac-TOPO, which regulates *lacZα* gene expression under *lac* promoter, formed blue colonies in 5-bromo-4-3 indolyo-β-D-galactoside (X-gal) plate. When *lac* promoter was changed to *tac* promoter, same results were observed. Since the addition of *tac* promoter did not affect the toxic effect of *Jpk*, the pGEX-Jpk-ptac could be a useful vector for the screening of suppressor(s) for Jpk, in which GST-Jpk and a putative Jpk-suppressing protein are coexpressing from two unidirectional *tac* promoters, which response to the same inducer, isopropyl-β-D-thiogalactopyranoside (IPTG).

Key Words: Coexpression vector; *tac* promoter; Selective vector; Jpk; Hoxa7

INTRODUCTION

The *Hox* genes, which contain homeobox and act as transcription factors play key roles in development. The homeobox encodes a DNA-binding homeodomain consisting of 60-amino acid residues. A typical *Hox* gene is expressed during embryogenesis in a specific area with a sharp anterior boundary along the anteroposterior axis and during specific developmental time. A murine Hoxa-7 gene is located on the chromosome 6 and expressed during gastrulation, initially in the allantois (7.5 days p.c.) and subsequently in specific regions of the neuroectoderm and mesoderm (Krumlauf, 1994).

During the study on the Hoxa-7 gene regulation, a novel gene *Jopock* (*Jpk*) has been isolated previously as a *trans*-acting factor, which interacts with position-specific regulatory element (*PSRE*) of *Hoxa-7* gene (Cho et al., 1997). Recently, we have demonstrated that the overexpression of murine Jpk protein led F9 murine teratocarcinoma cells to the apoptotic cell death (Kim et al., 2002). Expression of *Jpk* decreased cell viability, generated reactive oxygen species (ROS), reduced mitochondrial transmembrane potential, and increased Annexin-V staining. In addition, *Jpk* seemed to influence the expression of Bcl family at the mRNA level (Kong et al., 2003). A trace amount of murine Jpk also induced in bacaterial cell death. The growth of *E. coli* harboring the Jpk expression vector was inhibited in the presence of inducer and the morphology of that bacteria gave an unusually elongated phenotype, finally leading to a cell death through a rupture at the tip of the bacteria (Park et al., 2002).

Several studies have shown that some mammalian molecules involved in apoptosis affected the viability of bacterial or yeast cells. The mammalian pro-apoptotic protein Bax, a member of the Bcl-2 family, induced both *E. coli* and yeast

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cell death (Nanbu-Wakao et al., 2000; Xu and Reed, 1998), and the death effector domain (DED) of the mammalian pro-apoptotic molecule, FADD, induced bacterial as well as mammalian cell death (Lee et al., 2000). Since the mechanism of toxic effect has shown to be similar to those of mammalian cell death, both bacterial or yeast cells have been selected as a model system for investigating the mammalian apoptosis and for the screening of the molecules related to the apoptosis.

As a part of the study on the function of Jpk, we have desired to search a molecule(s) which overcomes the *Jpk*-mediated cell death, genetically. Here we report a development of a new unidirectional vector system that can express both *Jpk* and a candidate gene, which suppresses the lethal effect of Jpk.

MATERIALS AND METHODS

1. Cloning of tac promoter

The DNA fragment encoding *tac* promoter was isolated after amplification by polymerase chain reaction (PCR) using *tac* promoter in pGEX-Jpk (previously known pGEX-c171) as a template (Park et al., 2002). The specific primers for *tac* promoter region (sense, 5'-CATAACGGTTCTGGC-AAATA and antisense, 5'-GGGACATGAATACTGTTTCC) were designed and purchased from Bioneer (Taejon, Korea). PCR was performed at 94℃ for 5 min, which was followed by 25 cycles at 94℃ 30 sec, 48℃ for 30 sec, and 72℃ for 30 sec, and then 72℃ for 10 min. The amplified products (117-bp) were subcloned into the pGEM-T Easy vector (Promega), *tac* promoter region was cleaved with *Ssp*I / *Sal*I, and then cloned into the *Sma*I / *Xho*I sites of pGEX-Jpk.

2. Analysis of promoter activity

In order to confirm whether the 117-bp *tac* PCR product contains the promoter activity, plac-TOPO was constructed by digesting pCR®2.1-TOPO® (Invitrogen, Carlsbad, California, USA) with *Eco*RI and self ligation. To change, *lac* promoter in plac-TOPO to *tac* promoter, the plasmid was modified in two steps. First, the *lac* promoter region was deleted; Δplac-TOPO was constructed by ligation with *Pvu*II / *Nco*I fragment and Klenow-filled *Hind*III / *Nco*I fragment of plac-TOPO. Next, ptac-TOPO, containing *tac* instead of *lac* promoter in plac-TOPO was manufactured; 117-bp *tac* promoter cloned in pGEM-T easy was digested with *Ssp*I / *Nco*I and subcloned into *Eco*RV / *Not*I site of Δplac-TOPO. Then, the three constructs were transformed into DH5α on LB plates containing ampicillin (50 μg/ml), X-gal (80 μg/ml) and IPTG (200 μM).

3. Measurement of cell growth

One ml of *E. coli* strain BL21(DE3) harboring pGEX-Jpk or pGEX-Jpk-Ptac was cultured overnight in 2x YT media containing ampicillin (50 μg/ml). In the next morning, 1 ml of overnight culture was inoculated into a 50 ml of 2x YT media and then cells were cultured at 37℃ with vigorous shaking. When O.D.600 reached about 0.4, the culture was split into two batches. After that, IPTG (1 mM) was added into one batch of the culture and continued to grow. Every 30 min, 1 ml aliquot was taken from each culture and then O.D.600 was measured.

RESULTS AND DISCUSSION

As a tool for searching the genetic suppressor against Jpk function, we tried to construct a vector system, which coexpresses Jpk and a candidate suppressor gene under the control of *tac* promoters at the same time. The *tac* promoter consists of hybrid *trp-lac* promoter containing a tryptophan synthetase gene promoter (5' P*trp*, -35 region) fused to a *β*-galactosidase gene promoter (3' P*lac*, -10 region), and the operator (Olac) of *β*-galactosidase gene. Therefore, it is regulated by *lac* repressor and can be highly inducible in the presence of chemical inducer (IPTG), a lactose analog through binding to the *lac* repressor (Amann et al., 1983). Here, we used pGEX-4T-1 containing *Jpk* (pGEX-Jpk) as a useful *tac* promoter source, since the promoter activity had been confirmed previously by exerting cell death through expression of *Jpk* under the induced condition., i.e. addition of IPTG (Park et al., 2002).

The 117-bp *tac* promoter regions amplified through PCR using specific primers and the template, pGEX-Jpk. The amplified region includes -35 region of *trp* promoter, -10 region of *lac* promoter, *lac* operator and ribosome binding site, and *Ssp*I site for easy manipulation. The start codon (ATG) of GST was designed to be included in the PCR product (Fig. 1A). After confirming the size (Fig. 1B), the PCR product was first cloned into the pGEM-T easy vector.

In order to ascertain the promoter activity of the 117-bp

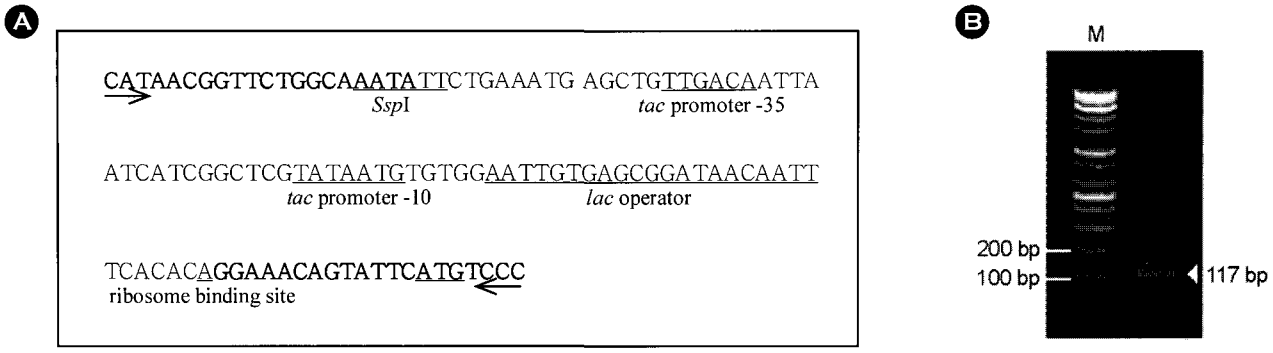

Fig. 1. Amplification of *tac* promoter. **(A)** The predicted nucleic acid sequences of *tac* promoter in pGEX-Jpk were aligned; control regions were underlined and the primers were indicated as bold writing and arrows. ATG is the start codon for GST. **(B)** PCR was performed using specific primers shown in **(A)** and the product was resolved on a 1.2% agarose gel containing 100 μg/ml EtBr. The predicted band corresponding to the ~117 bp promoter region is indicated with an arrowhead. M: 100 bp DNA ladder.

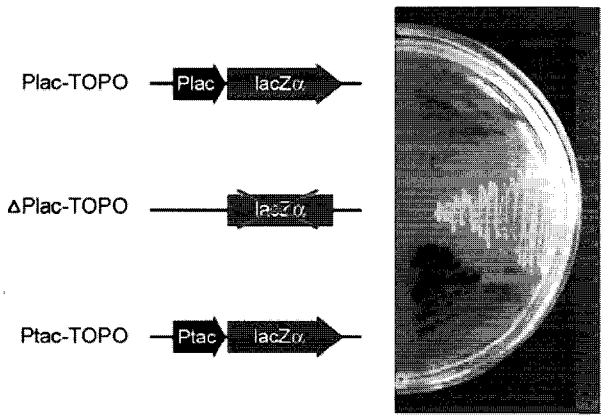

Fig. 2. Promoter analysis of 117-bp Ptac fragment. Plasmids harboring Plac-TOPO (positive control), ΔPlac-TOPO (negative control deleted the promoter region) or Ptac-TOPO replaced the Plac with Ptac promoter were transformed into *E. coli* DH5α. Transformants were streaked on LB plate containing ampicillin, X-gal and IPTG. And then a photograph was taken after overnight incubation at 37℃.

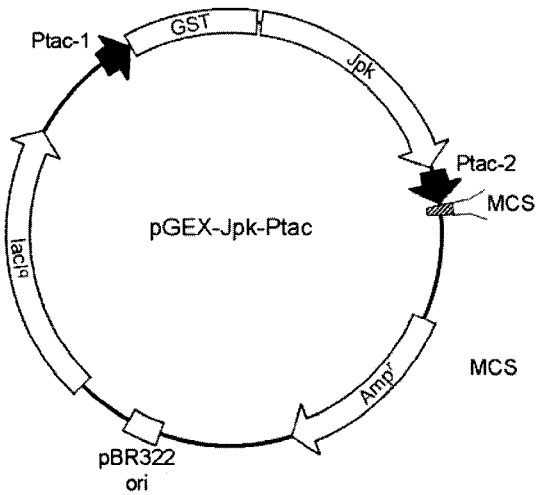

Fig. 3. Schematic drawing of the vector, pGEX-Jpk-Ptac. Ptac, *tac* promoter; GST, glutathione S-transferase; Jpk, *Jopock*; MCS, a multiple cloning site (ATCAC TAGTG AATTC GCGGC CGCCT GCAGG TCGA: *Spe*I, *Eco*RI, *Not*I/*Bst*Z1, *Pst*I); Amp^r, the ampicillin resistance gene; pBR322 *ori*, the pBR322 origin of replication; lacI^q, *lac*I gene with promoter-up mutation.

fragment we took advantage of the blue-white screening ability of pCR®2.1-TOPO® vector, originally designed for the cloning PCR products. The vector contains *lac* promoter and the coding sequence of *lacZα* fragment; hence, the bacteria cells expressing α-complementation of β-galactosidase are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-3-indolyo-β-D-galactoside (X-gal), which is converted by β-galactosidase into insoluble dense blue compound. For the experiment, pCR®2.1-TOPO® was circularization by self-ligation with two *Eco*RI sites in the multiple cloning site, and then designated as pPlac-TOPO. The cells harboring pPlac-TOPO plasmid form the blue colony in LB plate containing X-gal and IPTG, expectedly. Then, in order to replace *lac* with 117-bp *tac* promoter, we constructed pΔPlac-TOPO by deleting *lac* promoter from pPlac-TOPO. The colonies transformed with pΔPlac-TOPO colored white when incubated in LB plate containing X-gal and IPTG, since the vector did not have any promoter. When *tac* promoter was introduced to pΔPlac-TOPO, the transformants formed blue-colored colonies again as shown Fig. 2. This indicates that the 117-bp fragment has a full promoter activity.

Since the 117-bp fragment (Ptac-2) amplified by PCR operated properly as a *tac* promoter as designed (Fig. 2), it was designed to be inserted at the C-terminus of Jpk cloned

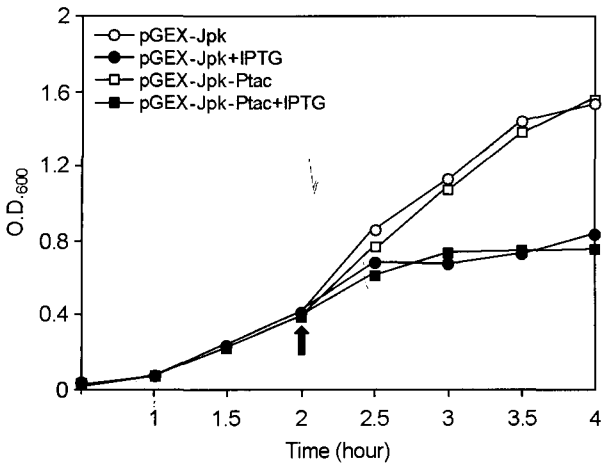

Fig. 4. Growth curve of *E. coil* BL21 (DE3) cells harboring pGEX-Jpk and pGEX-Jpk-Ptac. *E. coil* BL21 (DE3) harboring each plasmid was cultured in 2 X YT media containing ampicillin. Expression of Jpk was induced by addition of 0.1 mM IPTG; arrow indicates the time when IPTG was added. Cell growth was monitored by measuring O.D.600 every 30 min.

in pGEX-4T-1 (pGEX-Jpk-Ptac), through which Jpk and a candidate suppressor gene(s) is supposed to be expressed under the control of two *tac* promoters (Ptac-1 and -2) in the presence of inducer, IPTG (Fig. 3). The vector pGEX-4T-1 is a typical expression vector harboring an internal $lacI^q$ gene, a mutant *lac*I that synthesizes approximately 10-fold more repressor than wild type, thereby regulating *tac* promoter more tightly and probably sufficient to repress two *tac* promoters in the absence of inducer.

To see whether pGEX-Jpk-Ptac induces bacterial cell death like that of pGEX-Jpk, the growth curves of bacterial cells harboring pGEX-Jpk or pGEX-Jpk-Ptac were analyzed in the presence or absence of inducer, IPTG. As shown in Fig. 4, the growth of both cells harboring pGEX-Jpk and pGEX-Jpk-Ptac was inhibited within 30 min after induction, indicating that the additional *tac* promoter at the C-terminus of Jpk did not affect the toxic effect of Jpk.

Several genes have been identified in yeast or bacterial cells as suppressors of mammalian pro-apoptotic proteins through functional screening. For example, BI-1 was isolated from HepG2 cDNA expression library by its ability to suppress Bax-induced yeast cell death. Although identified by the yeast-based functional cloning strategy, BI-1actually inhibited Bax-induced apoptosis in mammalian cells (Xu and Reed, 1998). A mammalian pro-apoptotic protein FADD consists of the N-terminal death effector domain (DED) and the C-terminal death domain (DD). The DD recruits FAS that is oligomerized by Fas ligand and the DED domain is then associated with its homologous domain of procaspase-8, triggering the downstream pro-apoptotic cascade. The N-terminal DED of FADD induced bacterial cell death, and the genes selected from the *E.coli* genomic library to restore the viability of the bacteria harboring DED turned out to be involved in oxidation and reduction (Lee et al., 2000).

Here, we developed a vector, pGEX-Jpk-Ptac, which is useful to select genetic suppressor(s) of Jpk. It contains two *tac* promoters, which are silent in the absence of IPTG as inducer. Ptac-1 controls the expression of Jpk and Ptac-2 controls the expression of the gene of interest. Since Ptac-2 includes the ribosome binding site and an initiation codon, any cloned fragment can produce protein if the ORF is inserted in frame (Fig. 1, and 4). Following Ptac-2, multiple cloning sites (MCS; *Spe*I, *Eco*RI, *Not*I, *Bst*ZI, and *Pst*I) were included since the Ptac-2 containing fragment was isolated from the subclone in pGEM-T Easy vector, which makes the cloning simple. The *E. coli* chromosomal DNA digested with proper restriction enzyme can be introduced into this MCS of pGEX-Jpk-Ptac. If the inserted gene is a suppressor of Jpk, the transformants form colony on IPTG-containing LB plate, otherwise not. This vector might successfully offer the useful tool for searching the genetic suppressor of Jpk. We are now under try to search the genetic suppressor of Jpk using this expression vector system. In addition, pGEX-Jpk-Ptac has several advantages: 1) it can be used in any *E. coli* strain; 2) dual-expression of genes are possible in a single vector; 3) it can be used to search genetic regulator by exchanging Jpk with other interesting genes.

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