대한의생명과학회지: 제2권 제2호 1996
Korean J. Biomed. Lab. Sci.
Vol. 2, No. 2, p.231~240, December, 1996

Cloning and Expression of Human Clotting Factor 9 cDNA in *Escherichia coli*

Young-Won Lee†, Hyang-Suk Hur and Myoung-Hee Kim*

Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Taedok Science Town, P.O.Box 115, Yusong, Taejon, 305-600 Korea
**Department of Biology, Chungnam National University, Taejon, 305-764 Korea*

Astract: Human blood clotting (coagulation) factor 9 cDNA which codes for 461 amino acid has been cloned by screening human fetal liver cDNA library using PCR. This 1.4 kb cDNA spanning from the ATG initiation codon to the TAA termination codon was cloned into bacterial expression vector pGEX-2T, generating pGEX-F9 plasmid. The plasmid pGEX-F9 expresses about 73 kDa GST (Glutathione S-transferase)-Factor 9 fusion protein when introduced into *E. coli.* Western blot analysis using polyclonal antibody raised against human factor 9 confirmed this fusion protein contains factor 9 protein. The level of GST-factor 9 expression was about 20% of total protein and the purification of fusion protein was efficiently achieved by using GST agarose bead based on one step purification protocol.

Key Words: Hemophilia B; Factor 9; Glutathione S-transferase fusion protein; Expression; *Escherichia coli*

INTRODUCTION

Factor 9, a precursor of serine protease, is a plasma glycoprotein which has an essential role in the intrinsic clotting pathway[9]. A defect in clotting factor 9 causes hemophilia B, an inherited X chromosome-linked bleeding disorder which occurs in about 1 in 30,000 males. The human factor 9 gene is about 34 kb long and contains eight exons[18]. This gene is expressed in hepatocytes as a 2.8 kb-long mRNA, of which 1.4 kb is not translated (Fig. 1). Factor 9 cDNA codes for a 461 amino acid polypeptide chain, which is secreted into the bloodstream as a 57 kDa protein after post-translational modifications such as the vitamin K-dependent γ-carboxylation of 12 amino-terminal glutamic acid residues[4], the addition of several carbohydrate residues[3] and the β-hydroxylation of a single aspartic acid residue[6]. The γ-carboxylation is mediated by γ-glutamyl carboxylase, an integral membrane protein, and known to be required for the full functional activity of factor 9[7]. The propeptide consensus sequence preceding the amino terminus of the vitamin K-dependent protein has been reported as a recognition site for the carboxylase[13]. This finding was confirmed by an observation in which factor 9 with its propeptide deleted was not carboxylated[10], supporting the importance of propeptide sequence for the activity of this protein.

Currently, hemophilia B type patients have been treated by intravenous injection of factor 9 purified from normal human plasma. This

*Received Oct. 2, 1996, accepted after revision Nov. 25, 1996.

†Corresponding author

treatment is effective, but may expose patients to many side effects of virus infections such as HIV and hepatitis, and of thrombosis. Therefore, the treatment with recombinant factor 9 would be safer than plasma-derived factor 9. It has been reported that the recombinant factor 9 protein is synthesized in skin fibroblasts, myoblasts, endothelial cells and keratinocytes as well as hepatocytes[8,12,16,17]. Factor 9 proteins expressed in these cells were functional, indicating proper modifications are occurring. However, the mass production of this protein has not been accomplished and the complicated purification steps make obtaining of the recombinant factor 9 protein more difficult. In this paper we report cloning of factor 9 cDNA from human fetal liver cDNA library and an over expression of this cDNA as a GST (glutathione S-transferase) fusion protein in bacteria.

MATERIALS AND METHODS

Polymerase chain reaction using library lysates as template

λgt11 human fetal liver cDNA library lysate (Dr. Yoo, Kemyung Univ., Taegu, Korea) was directly used as a template for the polymerase chain reaction. Reaction condition was as follows: 10μl of fetal liver cDNA library lysate ($3x10^5$ pfu) was boiled for 10 min and cooled on ice. Reaction was carried out in 20 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, 50 pmole of each primer, 250 μmole of each dNTP and 2.5 units of *Taq* DNA polymerase by denaturing for 1min at 94°C, annealing for 1min at 50°C and then polymerizing for 1.5 min at 72°C. This cycle was repeated 35 times except the last polymerization reaction at 72°C for 10 min. The amplified DNA product was analyzed on 0.8% agarose gel.

Primers synthesized to polymerize factor 9 cDNA

Oligonucleotide DNA primers were designed to contain restriction enzyme sites, such that cloning and manipulation could be easier. Oligonucleotide DNAs were synthesized in Korea Biotech. Inc (Taejon, Korea). The nucleotide number of the 5' end of each oligonucleotide was based on the cDNA sequence of Anson *et al.*[2)]

OH1:
5'nt45CATGATCATGGCAGAATCACC coding strand
BclI

OH2:
5'nt698GACTTCACTCGGGTTGTTGG coding strand
AvaI

OH3:
5'nt717CCAACAACCCGAGTGAAGTC noncoding strand
AvaI

OH4:
5'nt1434GGGAATTCCATCTTTCATTAAGTGAGC noncoding strand
EcoRI

Plasmid construction

To construct plasmids containing factor 9 cDNA human fetal liver cDNA library was screened by PCR. As shown in figure 2, 670 bp 5'-half of the factor 9 coding region designated as 13F9 was amplified using oligonucleotide OH1 and OH3 as primers. And also 730 bp 3'-half of cDNA designated as 24F9 was amplified by using oligonucleotide OH2 and OH 4. 13F9 DNA fragment was inserted into SmaI site within pTZ19U plasmid (Takara, Japan), generating pTZ-13F9 as shown in figure 3. Plasmid pTZ-24F9 was constructed by cloning 730 bp of 24F9 DNA fragment into *Sma*I site within the plasmid pTZ18U (Takara, Japan). Plasmid pTZ-F9, containing a full length of factor 9 coding region, was made by subcloning 730 bp *Ava*I-*Bam*HI 24F9 DNA fragment from pTZ24F9 into the corresponding sites in pTZ13F9 as shown in figure 3. For the expression of factor 9 protein in bacteria, pTZ-F9 plasmid harboring the factor 9 cDNA was digested with *Eco*RI, and 1.4 kb of *Eco*RI-*Eco*RI factor 9 cDNA fragment was subcloned into

the bacterial expression vector pGEX-2T, generating pGEX-F9 (Pharmacia).

All the restriction and DNA modification enzymes used were purchased from either Boehringer Mannheim or Promega company, and plasmids were propagated in *E. coli* strain DH5α.

Expression of GST-factor 9 fusion protein in bacteria

Overnight culture of cells transformed with pGEX-F9 was diluted 1:10 in fresh LB media containing ampicillin, and continued to grow for 2 hrs. IPTG was added to the culture at a final concentration of either 0.5 mM or 1 mM, and then aliquots were taken after 20 hour growth at 37°C. After centrifugation, the cell pellets were resuspended in 1X Laemmli buffer[11]. Proteins were separated by electrophoresis through 9 to 10% polyacrylamide gel and stained with Coomasie blue.

Purification of GST-factor 9 fusion protein

GST-factor 9 fusion protein was purified by following the Smith and Johnson's batch washing procedure with slight modifications.[15] Briefly, overnight cultures of *E. coli* transformed with pGEX-F9 were diluted 1:10 in 250 ml of LB media containing ampicillin and grown for 2 hrs at 30°C before adding IPTG to 0.5 mM. Cells grown overnight were pelleted and resuspended in 5 ml of PBS buffer. Cells were lysed on ice by mild sonication and subjected to centrifugation at 10,000g for 5 min at 4°C. The 100 μl of supernatant was mixed with 40 μl of 50% glutathione-agarose beads (sulphur linkage, Sigma) for 1 hr on a rotating platform and then beads were collected by brief centrifugation and washed three times with PBS. Proteins bound to beads were eluted by boiling in SDS sample buffer, and resolved on the SDS polyacrylamide gel.

Western blot

E. coli strain DH5α either transformed or non-transformed with pGEX-F9 plasmid was induced with IPTG, and then sonicated as above. Proteins from each cell pellet and supernatant were separated on the SDS containing denaturing polyacrylamide gel, and electrophoretically transferred onto nitrocellulose membrane in the buffer consisting of 0.02 M Tris base (pH 8.3), 0.15 M glycine and 20% methanol. Membrane was blocked by incubating in PBST buffer (PBS plus 0.05% TWEEN 20) containing 3% BSA at room temperature for 2 hours, and then incubated with rabbit polyclonal antibody raised against human factor 9 protein (Sigma) for 1.5 hours at 37°C. After washing with PBST buffer twice for 15 min each at room temperature, membrane was reacted with horse radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG for 1.5 hours at room temperature. Membrane was then incubated with HRP substrate containing 4-chloro-1-naphthol and H_2O_2 until brown color appears.

RESULTS

Screening of 1.4 kb coding region of factor 9 cDNA by using polymerase chain reaction

The full genomic DNA sequence of 34 kb of factor 9 gene has been reported[18]. Its cDNA is 2.8 kb, of which 1.4 kb is the protein coding region. In order to clone coding region of factor 9 cDNA we have synthesized oligonucleotides complementary to the factor 9 cDNA based on the nucleotide sequence of Anson *et al.*[2] Oligonucleotide DNA primers (Fig. 1) were designed to contain restriction enzyme sites, such that cloning and manipulation could be simple as described in the Materials and Methods. Since factor 9 is expressed in liver cell specifically, human fetal liver cDNA library

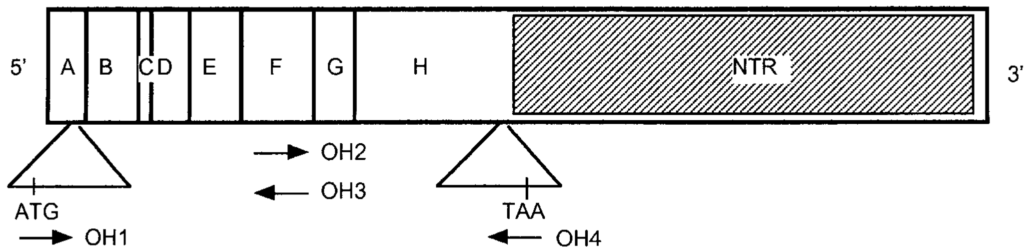

Fig. 1. Schematic diagram of factor 9 cDNA and the positions of the synthesized oligonucleotide primers (OH1, -2, -3, and -4) used to amplify cDNA by PCR method. Eight exons are denoted as capital letters (A through H) and the long 3' nontranslated region (NTR) is marked. The translation initiation (ATG) and termination (TAA) codons are also marked.

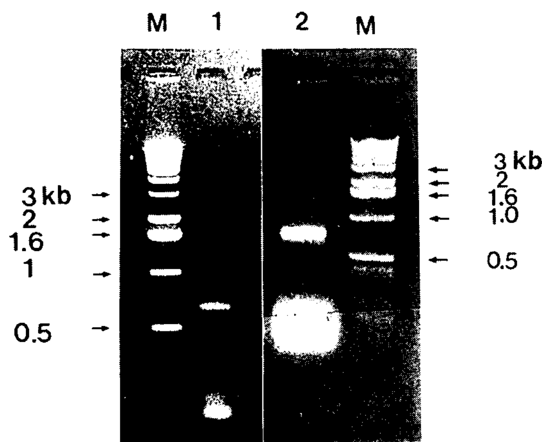

Fig. 2. Agarose gel electrophoresis of factor 9 cDNA fragment amplified by PCR. Lane 1: 670 bp 5' half of the factor 9 cDNA fragment amplified using the oligonucleotides OH1 and OH3, Lane 2: 730 bp 3' half of the factor 9 cDNA fragment amplified with OH2 and OH4 as primers; M is 1 kb DNA ladder marker.

was screened by PCR using library lysates as templates. As shown in figure 2, 670 bp 5'-half of the factor 9 coding region designated as 13F9 was amplified using oligonucleotide OH1 and OH3 as primers. And also 730 bp 3'-half of cDNA designated as 24F9 was amplified by using oligonucleotide OH2 and OH4.

Subcloning and sequencing

The 13F9 and 24F9 DNA fragments amplified by PCR were cloned into the SmaI restriction enzyme sites in the plasmids pTZ19U and pTZ18U respectively, generating p13F9 and p24F9 as shown in figure 3. The plasmid pTZ-F9, in which two 5'-and 3'-half of the factor 9 cDNA fragments were combined, was constructed by inserting 670 bp of AvaI to *Bam*HI fragment from p24F9 into the corresponding sites of the plasmid p13F9. This plasmid construction was confirmed through the agarose gel electrophoresis after restriction endonuclease digestions as well as sequencing of the construct (data not shown). After confirming the presence of ATG translation initiation and TAA stop codons, 1.4 kb *Eco*RI fragment harboring factor 9 gene was transferred into the expression vector for protein production (Fig. 3).

Expression and purification of GST-F9 fusion proteins in bacteria

In order to overexpress human factor 9 in bacteria, glutathione S-transferase (GST) fusion vector, pGEX-2T, has been adopted. GST fusion protein can be easily purified by using glutathione-agarose bead because it binds to the bead. Furthermore, a thrombin cleavage site within the pGEX-2T vector makes the isolation of intact foreign protein possible.

The plasmid pGEX-F9, containing human factor 9 gene, directs the synthesis of factor 9 protein fused at the C terminus of Sj26, a 26 kDa glutathione S-transferase(GST) when its *tac* promoter is induced by IPTG[15]. DH5α cells transformed with the plasmid pGEX-F9 was treated with a final concentration of either 0.5 mM or 1 mM IPTG for 20 hours to induce the expression of fusion protein. When total

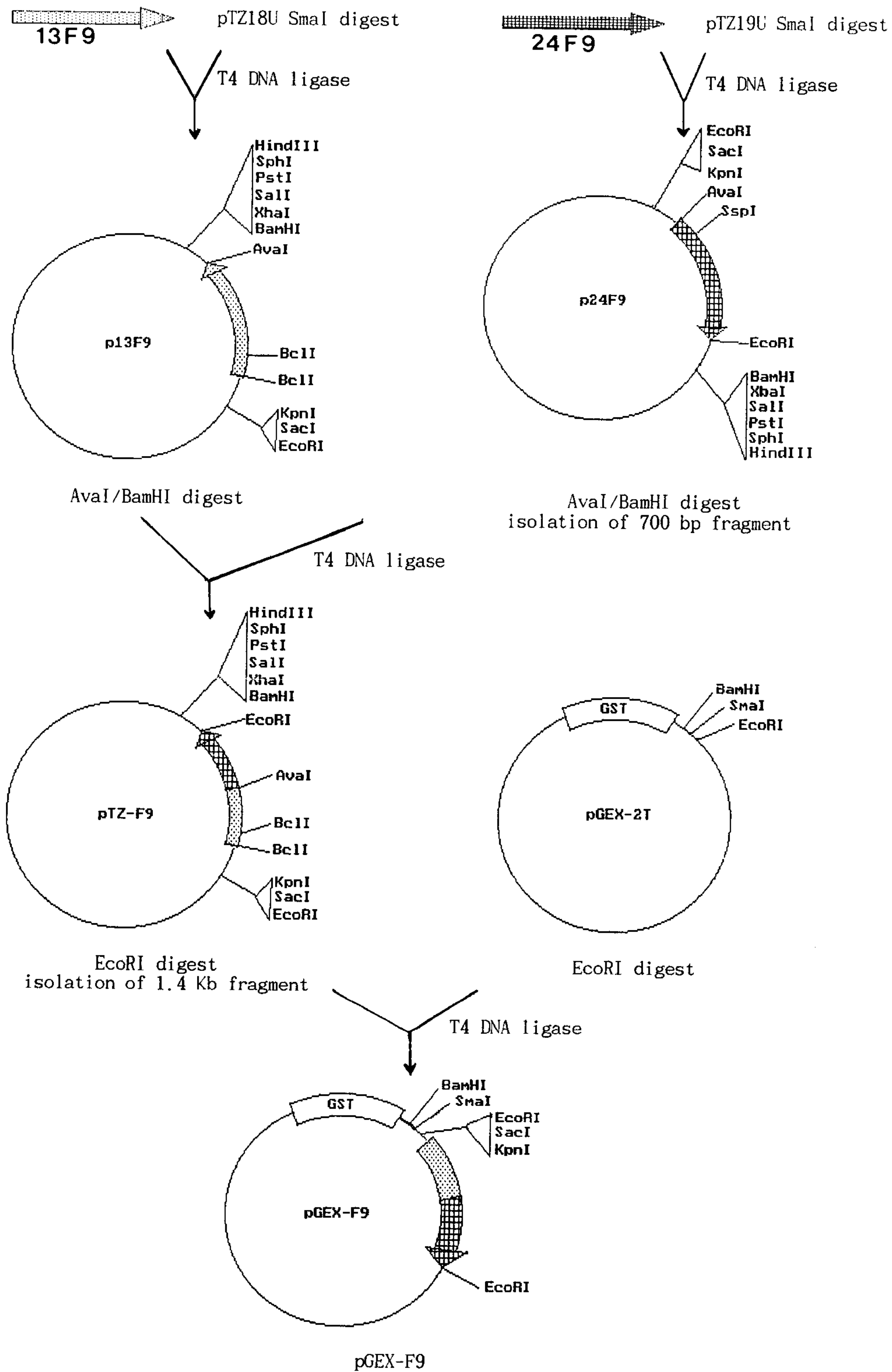

Fig. 3. Construction scheme for the recombinant plasmids pTZ-F9 and pGEX-F9. GST stands for the glutathione S-transferase. Restriction endonuclease recognition sites used for plasmid construction were also shown.

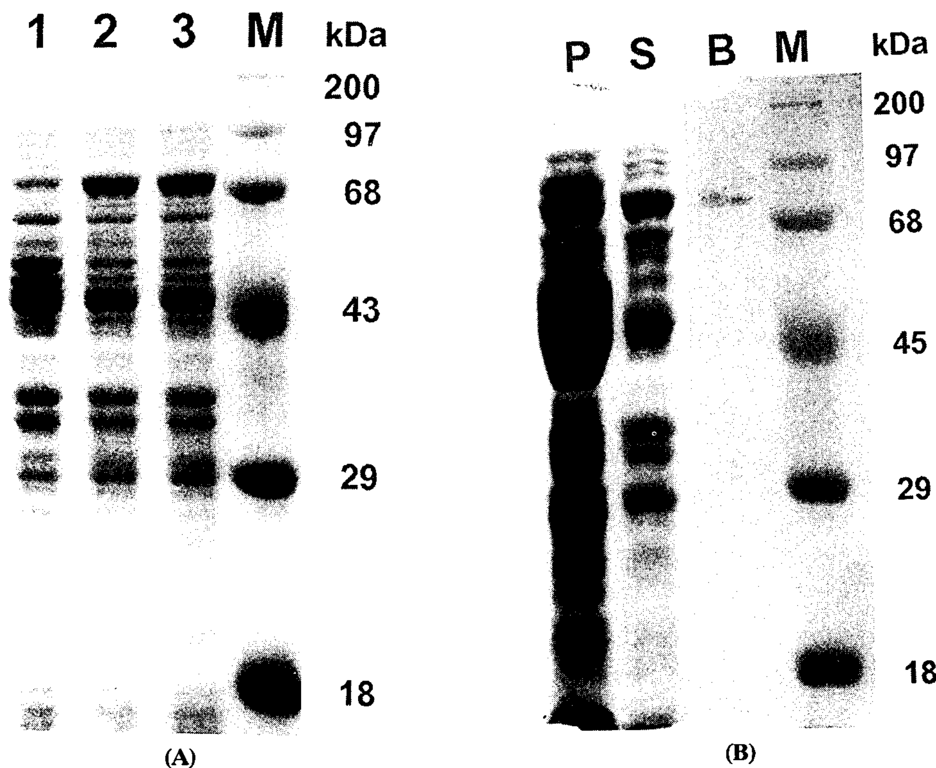

Fig. 4. (A) Expression of GST-F9 fusion protein after induction. Total proteins were resolved on a 9% polyacrylamide gel and stained with Coomassie blue. Lane 1: non-induced, Lane 2: induced with 0.5mM IPTG, Lane 3: induced with 1mM IPTG. The position and the sizes (kDa) of molecular weight marker are indicated. (B) Purification of GST-F9 fusion protein. Samples of insoluble pellet (P) and supernatant (S) and the purified protein by incubating supernatant with glutathione-agarose beads (B). The positions and the sizes (kDa) of the molecular weight marker are indicated.

crude proteins from non-induced and induced cells were resolved on 9% SDS polyacrylamide gel, about 73 kDa GST-F9 fusion proteins were detected mainly on the IPTG-induced lane (Fig. 4A). Figure 4 shows, however, the inducibility does not depend on the concentration of inducer, but does on the induction time (data not shown). Thus the induction time has been optimized to about 20 hours. The size of factor 9 protein expressed in bacteria was about 47 kDa, which is the expected size of factor 9 protein without addition of carbohydrate residues[3)]. To express GST-F9 fusion protein as a soluble form in *E. coli*, pGEX-F9 transformed cells were optimized to grow at low temperature: when it is grown at 30°C, almost 50 % of the fusion protein exists as a soluble form (Fig. 4B). Figure 4B shows the 73 kDa fusion protein present in both pellets and supernatant after sonication and centrifugation. Using the glutathione-agarose bead, GST-factor 9 fusion proteins were partially purified by batch washing procedures as described in the Materials and Methods, and 73 kDa fusion protein was recovered (Fig. 4B, lane B).

To confirm that the 73 kDa fusion protein contains factor 9 protein, Western blot analysis has been applied. Total proteins from *E. coli* transformed or non-transformed with pGEX-F9 plasmid, were prepared, and then reacted with polyclonal antibody against human factor 9. As shown in Figure 5, the amount of 73 kDa fusion protein has been increased in both pellets and supernatant upon induction with IPTG.

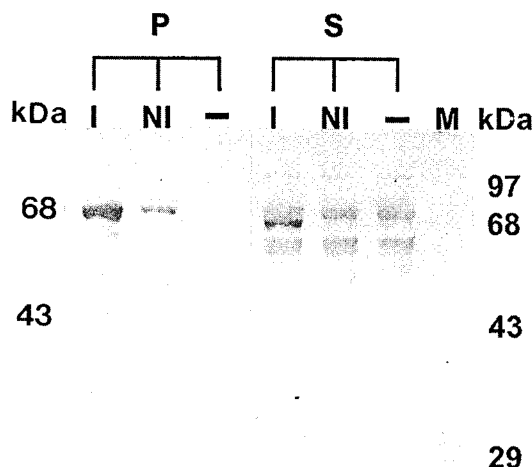

Fig. 5. Western blot analysis of human factor 9 expressed in pGEX-F9 transformed *E. coli.* S and P designate the supernatant and the pellet, respectively. Minus (-) indicates proteins from non-transformed cells, NI from transformed but not induced cells, and I from transformed and induced cells. The sizes of the molecular weight marker (kDa) are indicated. The arrow indicates GST-F9 fusion protein.

This result indicates that factor 9 is produced as a GST-F9 fusion protein of which the level is highly increased upon induc-tion. This fusion protein is present both in the supernatant as a soluble form and in the pellet as an inclusion body (Figure 5).

DISCUSSION

A 1.4 kb factor 9 cDNA has been cloned by screening λgt11 human fetal liver cDNA library by using PCR. This cDNA clone has been designed to contain its own ATG translation initiation and TAA termination codons, and cloned into the pGEX-2T vector which makes the expression and purification of factor 9 simple. To over-express GST-F9 fusion protein as a soluble form in bacteria, pGEX-F9 has been optimized to grow at low temperature. When it is grown at 30°C, almost 50% of the expressed fusion protein exists as a soluble form, which can be easily purified by using glutathione-agarose bead. Furthermore, the factor 9 protein can be isolated from the GST-F9 fusion protein after cleaving with a protease, thrombin, whose recognition site is located between the GST and factor 9 in the vector.

Factor 10, which is an another coagulation factor playing an important role in the intrinsic blood clotting pathway[4], has a similar feature; that is, it is a vitamin K-dependent plasma glycoprotein and also post-translationally modified (removal of tripeptide, hydroxylation as well as γ-carboxylation). Especially γ-carboxylation, which is carried out by a vitamin K-dependent γ-glutamyl carboxylase, is known to be critical to its functional activity like the case of factor 9. Since bacterially expressed factor 9 is not modified properly, we have cloned a full length of human γ-glutamyl carboxylase cDNA by screening Alexander liver cell line and human liver cDNA library (our unpublished data). This clone is under expression in bacteria and then the effect of the carboxylation on the clotting activity of factor 9 will be tested by carboxylating bacterially expressed factor 9 *in vitro.* Since the expressed factor 9 protein reported here contains propeptide region which is required for the recognition by γ-carboxylase, the proper carboxylation is expected *in vitro.* Recently, however, it has been reported that the γ-carboxylation is not the limiting factor for the proper function of factor 9, particularly in CHO cells[14]. It is rather concluded that the removal of pre-and pro-sequence is necessary for the activity of factor 9 protein in CHO cells. Since factor 9 cloned in this paper includes pre-and pro-regions, it will be interesting to find out the effect of these sequences precisely. We are now under development of the system through which the bacterially expressed coagulation factors can be modified properly and finally contribute to produce the functional

recombinant factor 9 protein in the future.

Acknowledgements

We would like to thank Dr. H.-H. Jang for helping to prepare the figures. This work has been supported in part by Grant G70860 from the Ministry of Science and Technology of Korea.

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= 국문초록 =

인체 혈액응고 9인자 **cDNA cloning** 및 ***Escherichia coli*** 에서의 발현

생명공학연구소 유전자원센타 게놈사업실, *충남대학교 생물학과

이영원 · 허향숙* · 김명희†

인체 혈액 응고 9인자는 간에서 생성되며 461개의 아미노산으로 구성된 당단백질이다. 따라서 인체 혈액응고 9인자 cDNA를 찾기 위해 태아의 간(fetal liver) cDNA library를 PCR(Polymerase Chain reaction) 방법으로 screening 하였으며, 그 결과 ATG개시 코돈으로부터 TAA 종료 코돈까지 포함하는 1.4 kb의 9인자 cDNA를 찾았다. 또한 클론된 9인자 cDNA를 박테리아에서 발현시키기 위해 박테리아 발현 벡터인 pGEX-2T 플라스미드에 클로닝하므로써 pGEX-F9 플라스미드를 제조하였다. pGEX-F9로 형질전환된 *E. coli*에서 pGEX-F9의 발현을 유도하면 73 kDa 크기의 GST-factor9 융합 단백질이 다량 생성되며, 이 단백질이 혈액 응고 9인자 단백질을 함유하는 융합 단잭질임을 혈액 응고 9인자 항체를 이용한 Western blot으로 입증하였다. *E. coli*에서 발현된 GST-factor 9 융합단백질은 전체 단백질의 약 20%를 차지하며, GST agarose bead를 이용한 one step purificarion 방법을 통해 GST-factor9 융합 단백질을 쉽게 분리할 수 있다.

[대한의생명과학회지 2(2): 231-240, 1996년 12월]

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†별책요청 저자