

Full Length Research Paper

Construction of expression vectors carrying mouse peroxisomal protein gene (PeP) with GST and Flag labels

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The aim of this study was to construct expression vectors carrying mouse peroxisomal protein gene (*PEP*-cDNA) in prokaryotic and mammalian expression vectors in chimeric cDNA types, encompassing GST and FLAG with *PEP*-cDNA. *PEP*-cDNA was sub-cloned in pGEX6p2 prokaryotic expression vector in order to label this gene with GST to purify PEP protein for further biochemical analysis and identifying related proteins thereafter. FLAG-PEP recombinant DNA was produced and sub-cloned in pUcD3 eukaryotic expression vector to express tagged-PEP protein for transient transfection analysis and identifying intracellular localization of PEP protein in future experiments. *PEP*-cDNA was amplified in different PCR reactions using pEGFP-*PEP* vector and 2 sets of primers introducing specific restriction sites at the ends of *PEP*. PCR products with *Bam*HI/*Sal*I restriction sites were treated by restriction enzymes and inserted into the pGEX6p2, downstream of GST tag. *PEP*-cDNA containing *Bam*HI/*Apa*I restriction sites and FLAG gene (which amplified using pUcD3-FLAG-*PEX3* vector) were used as templates in secondary PCR for amplifying FLAG-*PEP* recombinant DNA. FLAG-*PEP* fragment was treated by enzymatic digestion and inserted into the pUcD3 eukaryotic expression vector. pGEX6p2-*PEP* and pUcD3-FLAG-*PEP* constructed vectors were transformed into the one shot TOP10 and JM105 bacterial competent cells, respectively. Positive colonies were selected for plasmid preparation. Results confirmed correct amplification of the expected products. *PEP*-cDNA in both PCR reactions encompasses 630 bp. FLAG fragment containing designed sites was 77 bp and FLAG-*PEP* fragment was 700 bp. Sequencing of constructed vectors confirmed that *PEP*-cDNA was tagged appropriately and inserted free of mutation and in frame with GST and FLAG.

Key word: *PEP* cDNA, pGEX6p2 vector, peroxisome, PTS1 signal.

INTRODUCTION

Peroxisomes are ubiquitous organelles in almost all eukaryotes that function to rid the cell of toxic substances. Peroxisomes were discovered by pioneer works of Belgian biologist Christian de Duve in 1966 (De Duve, 1969). They perform many biochemical functions of lipid

metabolism, including the β -oxidation of very long chain fatty acids, biosynthesis of plasmalogens and structural ether lipids abundant in the central nervous system, interconversion of cholesterol to bile acids, and glyoxylate transamination (Shimizu et al., 1999; Furuki et al., 2006). Mature peroxisomes are spherical, with diameters between 0.5 and 1.0 micrometer. Each peroxisome is delimited by a single membrane and contains a fine granular matrix (Latruffe et al., 2000).

Peroxisomes are highly responsive organelles, because

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their size, number, protein composition and biochemical functions vary dramatically depending on the organism, cell type, and environmental milieu. Peroxisomes are essential for normal human development and physiology, as demonstrated by the lethality of the peroxisome biogenesis disorders (PBD) (Wanders, 2004), a group of autosomal recessive diseases including Zellweger syndrome, rhizomelic chondrodysplasia punctata, and neonatal adrenoleukodystrophy, in which multiple peroxisomal metabolic pathways are dysfunctional because peroxisome biogenesis is compromised (Lazarow and Fujiki, 1985; van den Bosch et al., 1992; Subramani, 1998; Purdue and Lazarow, 2001). Up to now, 35 different peroxins (proteins involved in peroxisome biogenesis) have been identified, of which only 18 are present in human. Most peroxins are peroxisomal membrane proteins or interact through docking sites with the peroxisomal membrane. A complex peroxin interaction network controls biogenesis and division (Pex11p, 23p, 25p, 27p, 28p, 29p, 30 p, 31p, and 32p) and allows for the recognition of peroxisome target proteins through specific receptors (Pex5L p, 5Sp, 7p, 18p, 20p, and 21p), for membrane protein assembly (Pex3p, 15p, 16p, Pex19p, and 24p), for the docking of these receptors (Pex13p, 14p, and 17p), for receptor recycling and protein import (Pex1p, 4p, 6p, 8p, 9p, 22p, and 26p), and for the translocation of proteins to peroxisomal matrix (Pex2p, 10p, and 12p) (Subramani et al., 2000; Lazarow, 2003; Titorenko and Rachubinski, 2004). With respect to biogenesis of peroxisomes and as peroxisomes lack DNA, all peroxisomal proteins are synthesized on cytoplasmic free polysomes and are post-translationally transported to pre-existing peroxisomes (Lazarow and Fujiki, 1985). Two types of peroxisome-targeting signal (PTS) for the matrix proteins are identified: the C-terminal tripeptide-SKL and its conserved variants (PTS1) for most proteins (Baker et al., 2000; Baker and Sparkes, 2005; Gould et al., 1989; Miura et al., 1992) and PTS2, the N-terminal cleavable nonapeptide, - (R/K)(L/V/I)X₅(H/Q)(L/A)-, present in several proteins such as 3-ketoacyl-CoA thiolase (thiolase) of fatty acid β -oxidation pathway (Osumi et al., 1991; Singha et al., 2004; Swinkels et al., 1991). Membrane proteins are sorted to peroxisomes by targeting signals distinct from PTS1 or PTS2. Various peroxins have been shown to act as receptors for the different PTSs or as docking sites for these receptors (Roger et al., 2002; Heiland and Erdmann, 2005). In contrast to protein sorting to peroxisomes, much less is known about the mechanism of peroxisome proliferation and the proteins involved in this process. A few proteins (such as Per8p, Pas4p, and Pmp27) have been implicated directly in regulating this process (Tam et al., 2003; Tan et al., 1995; Crane et al., 1994; Marshall et al., 1995).

One of the peroxisomal matrix proteins, termed Peroxisomal Protein (PEP), has been cloned in mouse in 2002. *PEP* cDNA encodes the protein which its primary

structure is formed by 209 amino acids, with the C-terminal tail (SKI) closely resembling SKL, the consensus sequence for PTS1 (Ferrer-Martinez et al., 2002).

Analysis of *PEP* sequence demonstrates that the only region of *PEP* similar to a protein with a known function is an FnIII module that includes residues 31 to 114 (Ferrer-Martinez et al., 2002).

All FnIII motifs share three highly conserved residues, which correspond to Trp51, Leu92, and Tyr98 of the *PEP* amino acid sequence, and the same secondary structure of seven antiparallel-strands (Craig et al., 2004; Li et al., 2004).

Studies have shown that *PEP* expression in mouse embryo is different in various tissues, while its reason is unclear. Therefore we were interested to construct expression vectors carrying *PEP*-cDNA with GST and FLAG labels for further analyses. In the present study, *PEP*-cDNA was inserted downstream of *GST* and *FLAG* genes in pGEX6p2 prokaryotic expression vector (Figure 5A) and pUCD3 eukaryotic expression vector (Figure 5B) to used tagged-*PEP* proteins for further analyses.

MATERIALS AND METHODS

Construction of pGEX6p2-*PEP*

The coding region of *PEP* (*PEP*-cDNA) was inserted into the pGex6p2 vector (Pharmacia Biotech) in order to constructing the GST-*PEP* fusion protein prokaryotic expression vector, pGex6p2-*PEP* (Figure 1). PCR experiments were performed in an Eppendorf Mastercycler gradient thermal cycler that is described in details. Primers used during this study were ordered from Bioneer (Korea) and presented in the Table 1.

The aim of this step is amplification of *PEP*-cDNA using pEGFP-*PEP* as a template, with primers introducing *Bam*HI and *Sal*I restriction sites at both of 5' and 3' ends, respectively. 25 μ l of PCR reactions containing 50 ng template DNA, 5 pmol (100 nM) each of primers, 0.25 μ l *Pfu* DNA polymerase (Fermentas), 0.5 μ l dNTPs at 10 mM (Fermentas), 2.5 μ l 10X buffer of *Pfu* (200 mM Tris-HCl with pH 8.8 at 25°C, 100 mM (NH₄)₂SO₄, 100 mM KCl, 1% Triton X-100, 1 mg/ml BSA and 20 mM MgSO₄) was applied for a PCR by the following conditions: 4 min of denaturation at 94°C followed by 35 cycles of amplification (94°C 1min; 65°C 1min; and 72°C 2 min.), and ended to 10 min at 72°C. The amplified product of this step with the length of 647 bp (*PEP*) was purified by QIAprep Spin Miniprep kit (Qiagen). Purified product was used for digestion with restriction enzymes. Both of pGex6p2 vector and amplified *PEP*-cDNA were cut with *Bam*HI (TaKaRa)/*Sal*I (TaKaRa) restriction enzymes and were ligated by TaKaRa ligation kit (TaKaRa). Transformation was done immediately using one shot TOP10 competent bacterial *E.coli* cells (Invitrogen). Insert check analysis on grown colonies was done the next day. Plasmid preparation from bacterial colonies was done by QIAgen plasmid miniprep kit (Qiagen). To confirm insertion of *PEP*, two strategies were applied: First, PCR on constructed vector with primers which used for *PEP* amplification. The second one was sequencing. Samples were sent for sequencing through Takapozist Company.

Construction of pUcd3-*FLAG*-*PEP*

The coding region of *PEP* (*PEP*-cDNA) was tagged with *FLAG*

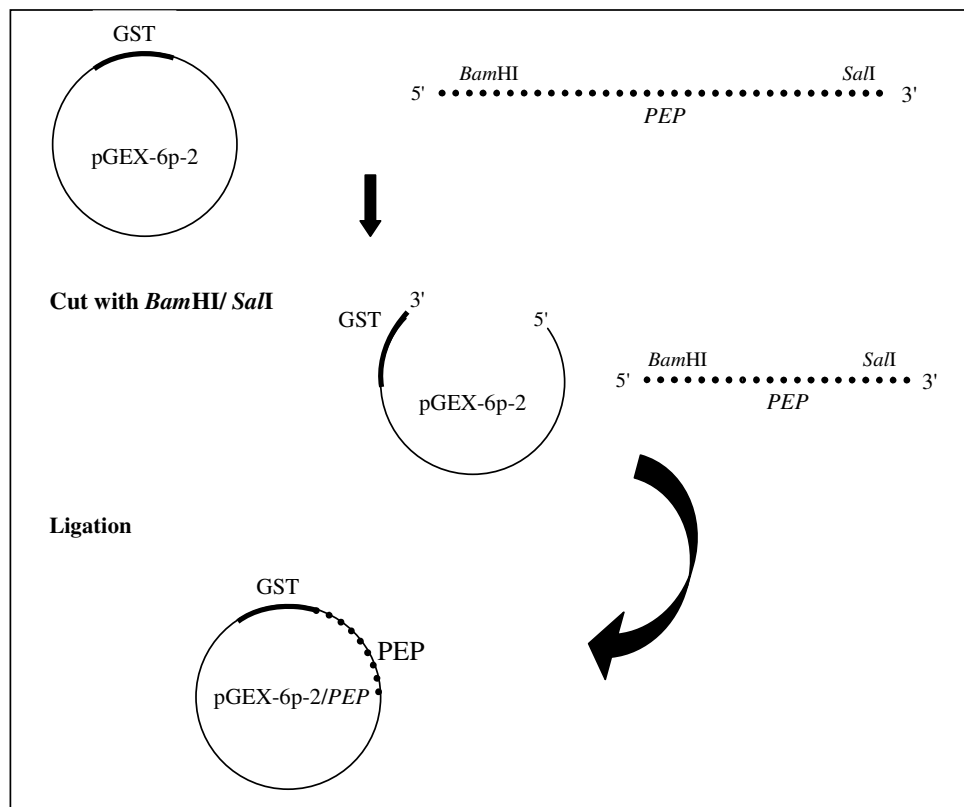


Figure 1. PCR reaction amplified *PEP*-cDNA containing *Bam*HI and *Sal*I restriction sites at ends. *PEP* fragment and vector were cut by the same enzymes and ligated with each other that led to insertion of *PEP* downstream of *GST*.

Table 1. Primer sequences for *PEP* amplification to construct *GST-PEP*. Forward primer introduces *Bam*HI restriction site at the 5' end of *PEP* and reverse primer introduces *Sal*I restriction site at the 3' end of *PEP*.

F	5ATG'GATCCCCCCCAGGGCCGTGCGCCT 3' <i>Bam</i> HI
R	5'AAAAG' TCGACTCATATCTGCTGCGGAGGAGA 3' <i>Sal</i> I

Table 2. Primer sequences for *PEP* amplification to construct *FLAG-PEP*. Forward primer introduces *Bam*HI restriction site at the 5' end of *PEP* and reverse primer introduces *Apal* restriction site at the 3' end of *PEP*.

F	5' ATGGATCCTGCCCCCAGGGCCGTCCGCCT 3' <i>Bam</i> HI
R	5' AAAAGGGCCCTCATATCTTGCTGCGGAGGAGA 3' <i>Apal</i>

gene in its upstream, with two steps PCR reactions and then *FLAG-PEP* was inserted into the pUcD3 vector in order to constructing the pUcD3-*FLAG-PEP* under regulation of *SRA* promoter. PCR experiments were performed again in an Eppendorf

Table 3. Primer sequences for *FLAG* amplification to construct *FLAG-PEP*. Forward primer introduces *Not*I restriction site at the 5' end of *FLAG* and reverse primer contains 15 nucleotides similar to 5' end of *PEP* at the 3' end.

F	5'ATAAGAATGCGGCCGCCACCATGGATTACAAGGAC3' <i>Not</i> I
R	5'CTGGGGGCAGGATCCCAAGCTTATCGTCGTCGTC3' <i>Bam</i> HI

Mastercycler gradient thermal cycler that its condition is described in details. Primers used during this study were ordered from Bioneer (Korea) and presented in the Tables 2 and 3.

Step 1: The aim of the first step of PCR is production of *PEP* and *FLAG* fragments. *PEP*-cDNA was amplified using pEGFP-*PEP* as template, with primers introducing *Bam*HI and *Apal* restriction sites at 5' and 3' ends, respectively (Table 2). *FLAG* gene was also amplified using pUcD3-*FLAG-PEX3* (was constructed by Ghaedi et al., 2000 in Kyushu University) as template, with forward primer introducing *Not*I restriction site in its 5' end and reverse primer containing *Bam*HI restriction site and 9 sequences from 5' end of *PEP*-cDNA at its 5' site, that it was designed for next step (Table 3 and Figure 2A).

25 μ l of PCR reactions containing 50 ng template DNA, 5 pmol (100 nM) each of primers, 0.5 μ l *Pfu* DNA polymerase, 0.5 μ l dNTPs at 10 mM, 2.5 μ l 10X buffer of *Pfu* (200 mM Tris-HCl with

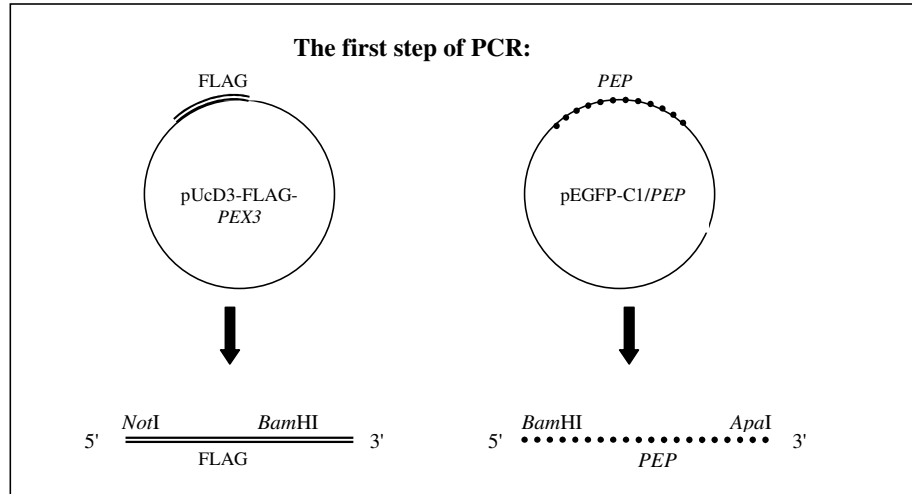


Figure 2A. First step of PCR reaction that produced FLAG and PEP fragments for the second step of PCR. Produced fragments had 15nt in common that were matched in next step of PCR.

pH 8.8 at 25°C, 100 mM (NH₄)₂SO₄, 100 mM KCl, 1% Triton X-100, 1 mg/ml BSA and 20 mM MgSO₄ was applied for a PCR by the below conditions: 5 min of denaturation at 94°C followed by 35 cycles of amplification (94°C 1 min; 65°C 1 min and 72°C 2 min), and ended to 10 min at 72°C. The amplified products of this step used as templates with the length of 647bp (PEP) and 77bp (FLAG). The products were purified by QIAprep Spin Miniprep kit (Qiagen) The amplified product was used for the step 2.

Step 2: In second step of PCR, FLAG and PEP fragments were used as templates and FLAG-PEP was amplified using FLAG forward primer and PEP reverse primer that 700bp expected product were generated (Figure 2B).

25 µl PCR reactions contained 3 µl template DNA (FLAG 2.5 µl and PEP 0.5 µl fragments), 5 pmol (100 nM) each of primers 0.5 µl Pfu DNA polymerase, 0.5 µl dNTPs at 10 mM, 2.5 ul 10X buffer of Pfu. Conditions of PCR reaction was the same as aforementioned. The product of this step was FLAG-PEP chimeric DNA. Then, both of pUcD3 vector and amplified FLAG-PEP were cut with NotI (TaKaRa) /ApaI (TaKaRa) restriction enzymes and were ligated by TaKaRa ligation kit. Transformation was done immediately using JM105 competent bacterial E. coli cells (Fermentas). Insert check analysis on grown colonies was done next day. Plasmid preparation from bacterial colonies was done by QIAGEN plasmid miniprep kit (Qiagen). To confirm insertion of PEP, two aforementioned strategies were applied again

RESULTS

Construction of pGEX6p2-PEP

PCR on pEGFP-PEP as template generated a 647 bp band (PEP), which showed amplification has been carried out correctly and designed restriction sites have been added at the ends of PEP fragment (Figure 3A). Insert check analysis on colonies which had grown one day after transformation with ligated products, showed that PEP-cDNA has been inserted into the pGEX6p2 vector (Figure 3B). Plasmid preparation was performed and constructed vectors were extracted from bacterial

The second step of PCR:

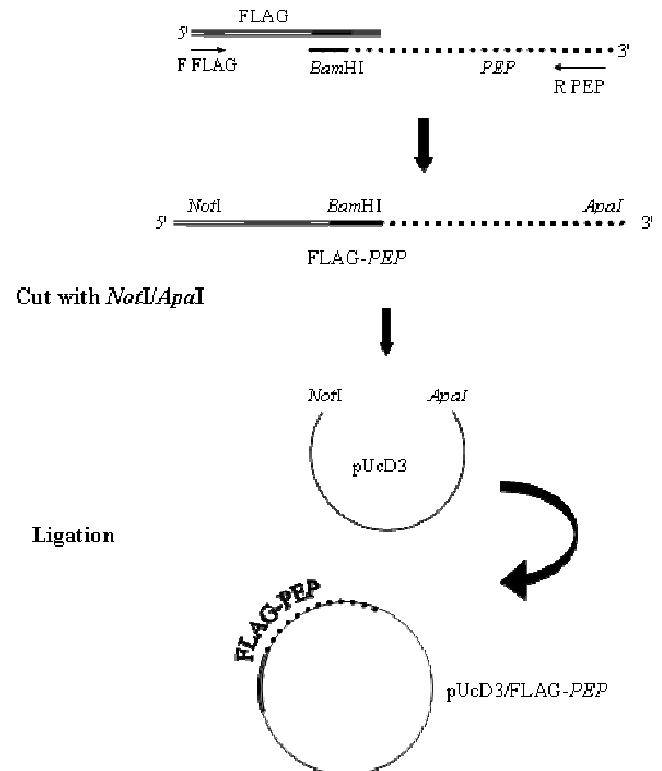


Figure 2B. Second step of PCR reaction for construction of FLAG-PEP chimeric DNA by using FLAG and PEP fragments as templates. Chimer fragment was cut and was inserted into the eukaryotic expression vector pUcD3.

colonies. To further confirm insertion of PEP into the vector, PCR were applied on constructed vector, with

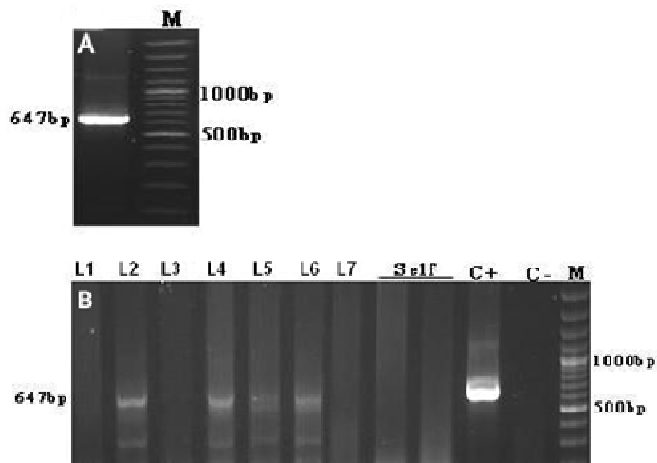


Figure 3. Construction of prokaryotic expression vector pGEX6p2-*PEP*. **A)** *PEP* amplification. **B)** Insert check analysis on grown colonies. (L2, 4, 6: positive clones containing pGEX-6p-2/*PEP*. L1, 3, 5, 7: negative clones. Self: Self ligation colonies. C+: positive control with pEGFP-C1/*PEP* as template. C-: negative control.).

primers which had been used for *PEP* amplification. This PCR generated a 647bp band as expected for *PEP* fragment (data not shown). Finally sequencing confirmed that *PEP* was inserted without any mutation downstream of GST into the vector appropriately.

Construction of pUcD3-*FLAG-PEP*

PCR on pEGFP-*PEP* and pUcD3-*FLAG-PEX3* as template in separate reaction generated a 647 bp band (*PEP*) and a 77 bp band (*FLAG*), respectively, which showed amplification, has been carried out correctly and designed restriction sites have been added at the ends of *PEP* and *FLAG* fragments (Figure 4A, B). Second step of PCR using *PEP*-cDNA and *FLAG* tandem as templates generated a 700 bp band that demonstrated amplification of *FLAG-PEP* fragment (Figure 4C). Insert check analysis on colonies which grew one day after transformation with ligated products, showed that *FLAG-PEP* has been inserted into the pUcD3 vector (Figure 4D). Then by Plasmid preparation, constructed vectors were extracted from bacterial colonies. To further confirm insertion of *FLAG-PEP* into the vector, PCR were applied on constructed vector, with primers which had been used for *FLAG-PEP* amplification. This PCR generated a 700 bp band as expected for *FLAG-PEP* fragment (data not shown). Finally sequencing confirmed that *FLAG-PEP* was inserted into the vector appropriately and it was also free from mutation.

DISCUSSION

We have sub-cloned *PEP*-cDNA in prokaryotic and eukaryotic expression vectors to tag it with GST and FLAG

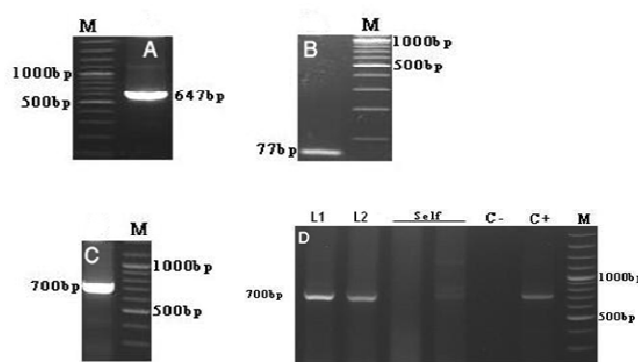


Figure 4. Construction of eukaryotic expression vector pUcD3/*FLAG-PeP*. **A)** *PEP* amplification. **B)** *FLAG* amplification. **C)** Amplification of *FLAG-PEP*. **D)** Insert check analysis on grown colonies. (L1, 2: positive colonies containing of pUcD3/*FLAG-PEP*. Self: Self ligation colonies. C+: positive control with *FLAG-PEP* as template. C-: negative control of PCR).

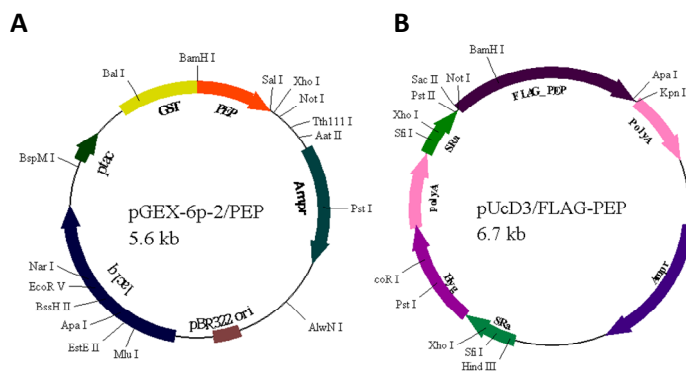


Figure 5: Map of **A)** pGEX-6p-2/*PEP* prokaryotic expression vector and **B)** pUcD3/*FLAG-PEP* eukaryotic expression vector .

tandems. Previous studies have indicated that *PEP* protein is a peroxisomal protein (Ferrer-Martinez et al., 2002). The primary structure of *PEP* protein comprised 209 amino acids, containing the C-terminal tail (SKI) closely resembling SKL, the consensus sequence for PTS1, one of the two peroxisomal targeting signals described to date. PTS1 has been found in approximately half of the peroxisomal proteins, and it directs peroxisomal proteins import from the cytosol to the organelle matrix (Subramani, 1998; Sacksteder and Gould, 2000). PTS1 was first identified in firefly luciferase and a few other proteins with the C-terminal tail sequence S/A/C-K/R/ H-L/M (Sacksteder and Gould, 2000). *PEP*-cDNA was cloned in pEGFP-C1 vector in our laboratory and transient transfection of CHO cells with pEGFP-C1-*PEP* clearly demonstrated punctuated pattern as expected for peroxisomes (Tanhaei et al., 2008). The only region of *PEP* similar to a protein with a known function is an FnIII module that includes residues 31 to 114. In order to see the possible role of this domain in molecular structure of *PEP*, proteomic analysis of *PEP*

using Flag-PEP in mammalian cell lysates and purification of PeP protein for biochemical analysis and identifying related proteins seems to be necessary. Thus our constructions can be used for the further analyses of the function of this protein.

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