Novel Vectors for Co-Expression of Two Proteins in *E. coli*

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ABSTRACT

Two new vectors, pAC28 and pEGST, for the co-expression of recombinant genes in E. coli *were developed. This two-plasmid system allows for an efficient expression and purification of large amounts of protein-protein complexes formed in bacterial cells. We have utilized this new system to express and isolate a stable complex of two human proteins, hematopoietic cell tyrosine phosphatase (HePTP) and mitogen-activated proteins kinase Erk2. This approach is useful for biochemical and structural studies of protein-protein interactions.*

INTRODUCTION

Protein-protein interactions have attracted a lot of attention in recent years (1). In most studies, the proteins are separately expressed and purified, followed by complex formation in vitro. However, this technique is often unsuccessful because separately expressed proteins may not be stable enough to form a stoichiometric complex. A number of studies have reported in vivo formation of protein complexes in bacteria through cloning of both genes in a single vector (2,11). This technique has its disadvantages in the complicated cloning procedures, the large size of the resulting plasmid (3), and the inefficient isolation of the complex. A natural alternative to the above methods is to use two-plasmid co-expression systems. This was recently utilized to produce two subunits of the virion-like reverse transcriptase of human immunodeficiency virus (5) and the von Hippel-Lindau Cancer Syndrome (VHL) tumor suppressor protein together with elongin (4,11). However, a convenient pair of vectors that would allow efficient expression and rapid purification of protein complexes from bacterial cells is not yet available.

We have constructed two vectors, termed pAC28 and pEGST, specifically designed for the overproduction of co-expressed proteins in the same bacterial cell. We demonstrate their utility for the co-expression and purification of two human proteins, the mitogen-activated protein kinase Erk2 and the hematopoietic protein tyrosine phosphatase (HePTP). These two proteins form a stable complex in lymphoid cells (8–10). We believe that the two vectors described here may prove useful for investigation of other protein complexes as well.

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Enzymes

Expression plasmids pET23b and pET28a were obtained from Novagen (Madison, WI, USA), pGEX-4T-2 was from Amersham Pharmacia Biotech (Uppsala, Sweden), and pACYC177 was from New England Biolabs (Beverly, MA, USA). The NpT7-5/Erk2 plasmid DNA containing the full-length cDNA for Erk2 (6) was kindly provided by Dr. E. Goldsmith (University of Texas Southwestern Medical Center, Dallas, TX, USA). The pEF-HA/HePTP plasmid DNA encoding an N-terminally hemagglutinin epitope tagged HePTP was as described earlier (9). *E. coli* strains XL10-Gold and BL21(DE3) were supplied by Stratagene (La Jolla, CA, USA). Restriction endonucleases and T4 DNA Ligase were obtained from New England Biolabs. *Pwo* DNA polymerase was from Roche Molecular Biochemicals (Mannheim, Germany)

Construction of Expression Vectors

Standard gene cloning techniques (7) were used to obtain the two new vectors. Plasmids pET28a and pACYC177 were digested with *Fsp*I and *Cla*I. Two DNA fragments of 1723 bp (pACYC177 mixture) and 3457 bp (pET28a mixture) were purified, ligated, and verified by sequencing. The vector pEGST was obtained by cloning of a PCR fragment of the glutathione-S-transferase (GST) gene from pGEX-4T-2 into the *Nde*I-*Bam*HI sites of pET23b. The construct was verified by sequencing.

Construction of Plasmids Harboring Erk2 and HePTP cDNAs

Erk2 full-length cDNA was cloned into *Bam*HI-*Hin*dIII sites of pEGST, whereas HePTP was cloned into the *Nde*I-*Hin*dIII sites of pAC28 using the

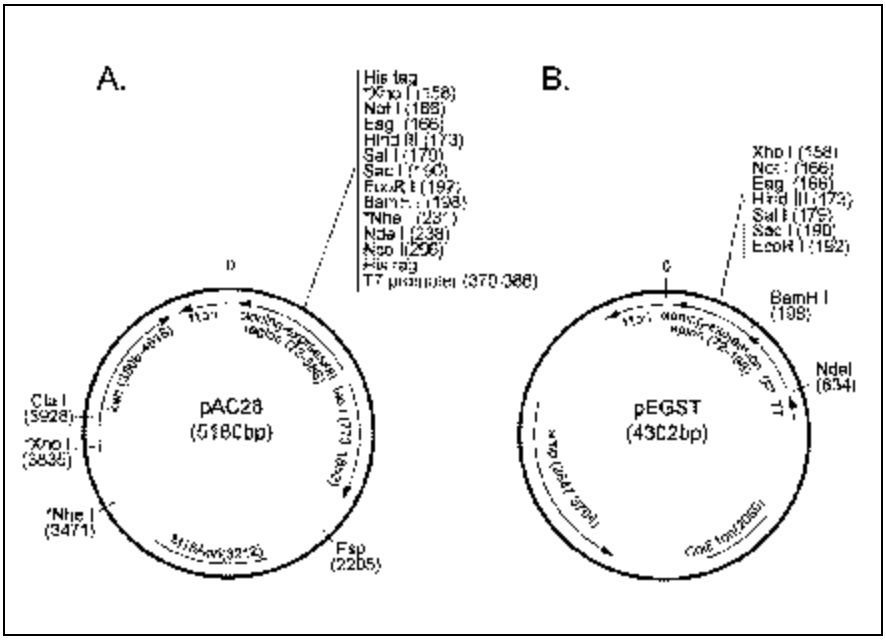

Figure 1. Map of the two new vectors, pAC28 and pEGST. Duplicate restriction sites are indicated with an asterisk.

standard PCR technique and with plasmids NpT7-5/Erk2 and pEF-HA/HePTP as templates. The resulting constructs pEGST/Erk2 and pAC28/HePTP were confirmed by sequencing.

Expression and Purification of GST-Erk2 Protein

Plasmid DNA pEGST/Erk2 was transformed into *E. coli* BL21(DE3) strain. The cells were grown at 37°C in LB medium (7) until the A_{590} = 0.4–0.5. Expression of the protein was induced with the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration 0.5 mM, and the bacterial culture was incubated at room temperature for 4 h. The cells were suspended in ice-cold sonication buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol). Lysozyme (to final concentration 0.25 mg/mL) and Triton® X-100 (to 0.2%) were added, and cells were lysed at room temperature for 20 min. Cell lysate was clarified by centrifugation at 30 000× *g* for 20 min at 4°C. Supernatant was applied to a Glutathione Sepharose™ 4B (Amersham Pharmacia Biotech). The column was washed with sonication buffer. GST-Erk2 was eluted with buffer containing 50 mM Tris-HCl, pH 8.8, 150 mM NaCl, and 25 mM reduced form of glutathione (Sigma, St. Louis, MO, USA). Proteins were analyzed by electrophoresis in 12% SDS polyacrylamide gels.

Expression and Purification of HePTP Containing 6×His

Plasmid DNA pAC28/HePTP was transformed into *E. coli* BL21(DE3) strain. The cells were grown at 37°C in LB media (7) until the A_{590} = 0.4–0.5. Then, IPTG was added to final concentration of 1 mM, and bacterial culture was incubated at room temperature for 4 h. The cells were centrifuged and lysed as described above. Cell lysate was applied to a Ni-NTA (Qiagen, Valencia, CA, USA). The column was washed with 20 mM imidazole in sonication buffer. 6×His-tagged HePTP was eluted with 100 mM imidazole in sonication buffer and analyzed by electrophoresis in 12% SDS polyacrylamide gels.

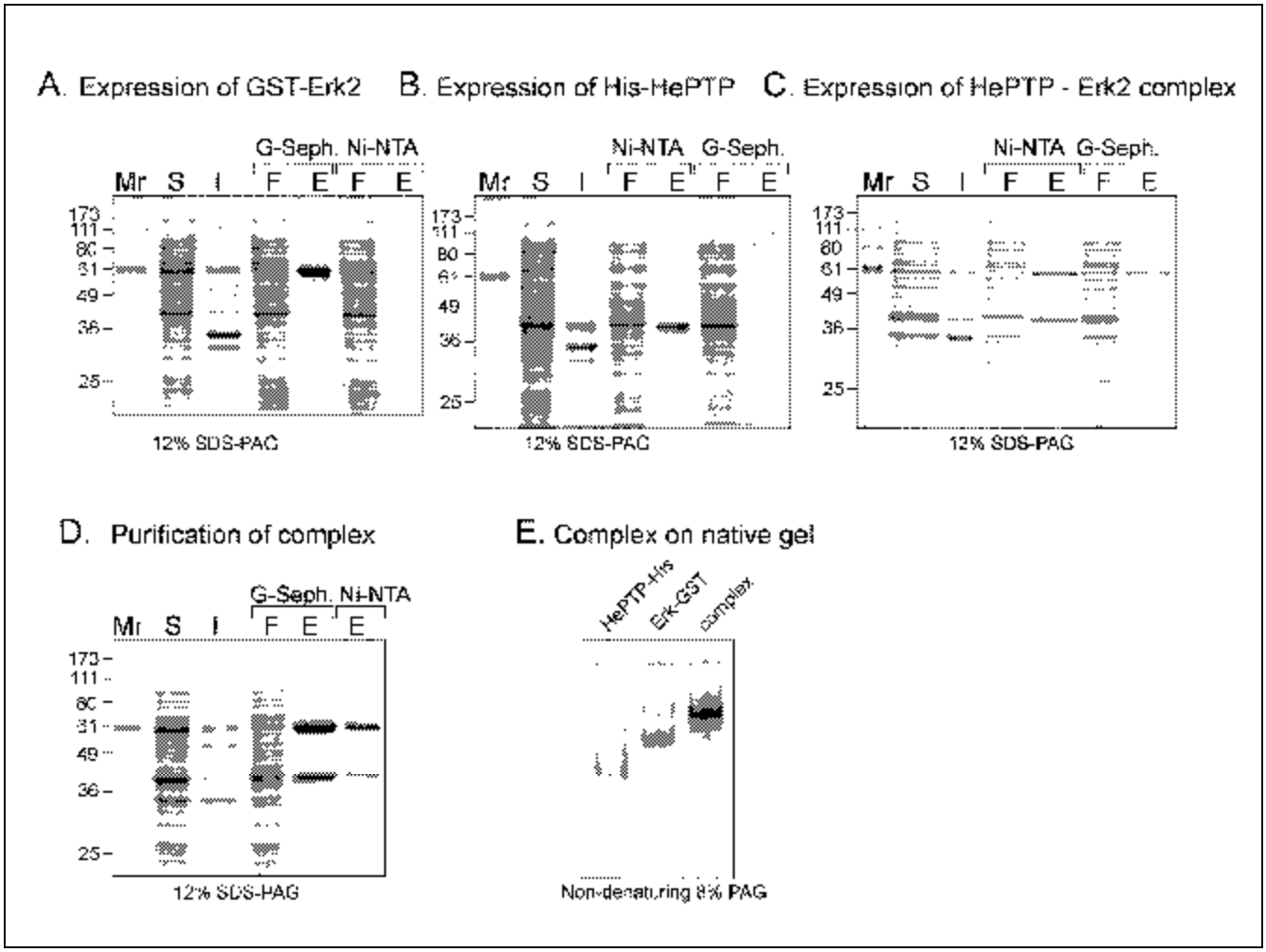

Figure 2. Expression of Erk2, HePTP, and their complex. (A) Expression of GST-Erk2. (B) Expression of His-HePTP. (C) Expression of HePTP-Erk2 complex. (D) Purification of the complex. (E) Analysis of the complex in a non-denaturing gel. The lanes are: Mr, Bench Mark™ Prestained Protein Ladder (Life Technologies, Rockville, MD, USA); S, supernatant; I, inclusion bodies; F, flowthrough; and E, eluate from the indicated resin. In panel D, the two eluate lanes represent consecutive affinity chromatography on the indicated resins.

Co-Expression of HePTP and Erk2 and Purification of the Resulting Complex

Two plasmid DNAs, pEGST/Erk2 and pAC28/HePTP, were transformed together into *E. coli* BL21(DE3). The cells were grown as above with both ampicillin and kanamycin. IPTG was added to a final concentration of 1.5 mM, and the bacterial culture was incubated at room temperature for 4 h. The cells were centrifuged and lysed as described above. The cell lysate was applied to either Glutathione Sepharose 4B or Ni-NTA agarose or both columns in sequence. Elutions were performed as above, and the protein complex was analyzed by electrophoresis in 12% SDS polyacrylamide gels or in non-denaturing 8% polyacrylamide gels using Tris/Gly buffer, pH 8.8.

RESULTS AND DISCUSSION

Construction of Novel Expression Vectors pAC28 and pEGST

The development of a co-expression system requires the construction of two plasmids containing compatible replication origins that would yield approximately equal copy numbers of both vectors in the bacterial cells. Furthermore, these plasmids should possess different selection markers, have strong inducible promoters, and encode distinct tags for rapid purification via affinity chromatography. Two currently used replication origins are known to provide approximately the same number of copies, ColE1 (15–20 copies per cell) and M15 (10–15 copies). Of these two, ColE1 is most widely used in various expression vectors (e.g., pET, pGEX, and pQE series), while M15 is sometimes used for cloning (e.g., in the pACYC series of vectors).

The new vectors described in this work were constructed in the following manner: pAC28 was obtained by ligating fragments of the M15-containing vector pACYC177 and a popular expression vector, pET28a. The recombinant vector, termed pAC28 (5180 bp), carries an M15 replication origin, the kanamycin resistance gene, and the cloning expression region of pET28a (Figure 1A). It should be noted that two restrictases, *Xho*I and *Nhe*I, cannot be used for gene cloning in this vector because of extra sites outside the multiple cloning site. As constructed, this vector can be used for co-expression together with any commercially available ColE1-containing vector with a resistance selection marker other than kanamycin. To ensure efficient isolation of the expressed protein, the new pAC28 vector also encodes a 6xHis tag providing affinity to Ni-NTA. There-

fore, the other co-expression vector should encode a different tag (e.g., GST). A number of available vectors already have GST, but these usually have promoters that are weaker than the T7 phage promoter (e.g., pGEX series), or they have a kanamycin resistance gene (pET41 and pET42). To overcome these limitations, we generated a new vector by inserting the GST gene from pGEX-4T-2 into the pET23b vector (see Materials and Methods), which contains an ampicillin resistance gene and the T7 promoter (Figure 1B). The resulting vector, termed pEGST (4302 bp), was ideal for co-expression with pAC28.

Expression and Purification of HePTP, Erk2, and Their Complex

It is our long-term objective to study the structure and interaction of two human proteins, HePTP and Erk2. We therefore cloned the full-length cDNA of HePTP into pAC28 and Erk2 into pEGST and analyzed the expression of the proteins separately (Figure 2, A and B). Both proteins were expressed with a yield of about 20% of total cell proteins. The proteins also were soluble and had the correct apparent molecular weights and affinity properties.

Next, we expressed the two proteins together. Figure 2C shows the results of GST-Erk2 and His-HePTP co-expression. Judging from many independent experiments, co-expression resulted in no more than 50% reduction in each protein compared to expression alone. The two proteins bound to and were eluted from either Ni-NTA or G-Sepharose (Figure 2C, lanes 5 and 7, respectively), indicating that they formed a stable complex that can be isolated by affinity chromatography using either tag. To further show this, we purified the co-expressed protein complex by consecutive chromatography first on G-Sepharose and then on Ni-NTA (Figure 2D). Both proteins were still present in the second eluate (lane 6). An aliquot of this preparation was loaded on a non-denaturing gel (Figure 2E), which revealed a single band (lane 3) with a different mobility compared to both Erk-GST or His-HePTP alone. This band represents the complex of the two proteins. This co-expression and two-step purification procedure can be used for the effective isolation of large amounts of protein complexes formed in bacterial cells. We have also successfully expressed and purified another pair, His-Erk2 and GST-HePTP, with similar yield and complex formation.

We anticipate that the two new vectors will be useful for a number of applications. They should assist in the production and purification of protein complexes consisting of individually labile subunits and in the analysis of alternative subunits. The vectors can be

used for the screening of deletion mutants or point-mutated proteins for interactions. Finally, our system will enable the large-scale production of modified proteins by co-expression of a substrate protein with an enzyme that will modify it (e.g., a protein kinase, a protease, a methylase, etc.). This system could also be used as a rapid and selective screening method for mapping such modifications without the interference of other enzymes that tend to complicate experiments in eukaryotic cells. For example, proteins normally phosphorylated by multiple kinases could be studied using one kinase at a time.

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Elimination of Transcriptional Interference between Tandem Genes in Plant Cells

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ABSTRACT

Plant cells are commonly transformed with two or more tandemly arranged genes, but how orientation affects their expression is not well understood. We investigated the amount of transcriptional interference occurring between two adjacent genes by cloning luciferase and green fluorescent protein (GFP) genes (promoter - coding sequence - terminator) in all possible orientations and expressing the genes in tobacco protoplasts. When two genes are oriented head-to-tail (→ →), the expression of the downstream gene was reduced 80% by the upstream gene. When two genes are oriented tail-to-tail (→ ←), the expression of the upstream gene was reduced 53% by the expression of the downstream gene. There was no interference when the orientation was head-to-head (← →). Using a chemically inducible gene expression system, we showed that the downstream gene expression was reduced 71% by the induction of an upstream gene. Inserting a mammalian transcription blocker sequence eliminated the interference between the genes in tail-to-tail orientation. The interference in the head-to-tail orientation was eliminated by inserting a 2322-bp λ phage DNA fragment. The terminators in gene constructs did not prevent the transcriptional interference, and the interference was eliminated by designing the orientation of genes and by placing a transcription blocker or a λ phage sequence between genes.

INTRODUCTION

Transcriptional interference refers to the phenomenon of reduced expression of a gene caused by the transcription from an upstream or downstream promoter. It can occur when different