Baculoviral Transfer Vectors for Expression of FLAG® Fusion Proteins in Insect Cells

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The baculovirus expression system has become widely used for recombinant eukaryotic protein production because of its ability to produce large quantities of biologically active eukaryotic proteins (12,13). The high expression levels are achieved by strong baculoviral promoters that exert their function in insect cells infected with a recombinant baculovirus. Insect cells provide a eukaryotic environment for posttranslational modifications and folding which, in some cases, are essential for producing biologically active proteins. The system has also proven that the insect cell itself is useful for functional studies of foreign proteins and for studies into the biogenesis of some subcellular structures (1,2,11). Efforts continue to be made to make the system easier and more versatile; these include adoption of a larval production system for large-scale production of proteins (14) and modification of the insect cell glycosylation system for the production of more authentic recombinant proteins (7).

Epitope tagging of a protein not only allows the surveillance of the protein with a specific monoclonal antibody but also simplifies the purification of the protein by using an immunoaffinity column. The FLAG® epitope (Scientific Imaging Systems, Eastman Kodak, New Haven, CT, USA) consists of a highly hydrophilic eight-amino acid peptide (DYKDDDDK), which can be easily detected by commercially available anti-FLAG® monoclonal antibodies (6). The amino acid sequence DDDDK in the FLAG peptide provides the recognition sequence for the protease enterokinase. The FLAG fusion protein can be purified in one step using an anti-FLAG immunoaffinity column. The FLAG fusion vectors (Figure 1), a pair of complementary oligonucleotides with upstream BglII and downstream Ndel and BamHI sites, 5'-GGAAGATCTATGG-ACATACAGTGAGCGATGCAAGCATAATGGATCCGCG-3' and 5'-CGCGGATCCATATGCTTGTATCCTGTCGCTCCTGTAGTCATAGATCTTCC-3' (restriction sites are underlined), were synthesized. The purified oligonucleotides were annealed, digested with BglII and BamHI and inserted into BamHI-digested pBacPAK8™ and pBacPAK9™ plasmids (CLONTECH Laboratories, Palo Alto, CA, USA). The plasmids containing the correct insert orientation were identified by DNA sequencing. The resulting plasmids, pSK277 and pSK278, are identical except for the orientation of multiple cloning sites (MCSs) and contain a FLAG epitope coding sequence upstream of the MCS, permitting the production of a fusion protein with an amino-terminal FLAG tag. The MCS contains an additional unique restriction site, Ndel (CATATG), which is useful for subcloning of genes from the ATG initiation codon.

To test these vectors, three open reading frames (ORFs) encoding yeast...
transcription factors were subcloned into either pSK277 or pSK278. The ORFs of the SUB1 (8) and TUP1 (16) genes were amplified by polymerase chain reactions (PCRs) of Saccharomyces cerevisiae chromosomal DNA with primers (5′-GGGAATTCCATGSCTGATTTACGTT and 5′-CGGGAATTCCTTCTTATTGGCGCTATTTTTTTA-3′) for SUB1 and 5′-GGGAATTCCATATGACTTCGACCCTTCCGAAATACG-3′ and 5′-CCGCTCGAGTTAATTGTGGCCGTATTTTTTTATA-3′ for TUP1 and subcloned into NdeI/EcoRI-digested pSK277 and NdeI/XhoI-digested pSK278, respectively. A 0.5-kb BamHI/XhoI fragment containing the ORF of the SRB6 gene (15) was subcloned into BamHI/XhoI-digested pSK278 after creating a BamHI site in front of the ATG initiation codon of the gene by in vitro site-directed mutagenesis using an oligonucleotide (5′-CCCTTTTTTTTTATACAGGATCCAAAATGAGACCC-3′). The recombinant transfer plasmids were cotransfected with Bsu36I-digested BacPak6 Viral DNA (CLONTECH) into Spodoptera frugiperda-derived SF21 insect cells according to the manufacturer’s instructions, and recombinant baculoviruses were isolated by plaque assay. The production of FLAG-tagged recombinant proteins was identified by Western blot analysis of extracts of SF21 cells infected with the recombinant viruses using the anti-FLAG M2 monoclonal antibody.

SF21 cells were grown at 27°C to approximately 70% confluence as a monolayer in four 150-mm dishes, infected with a recombinant virus at a multiplicity of infection (MOI) of 5–10 and collected at 60 h post-infection. The cell pellet was washed with cold phosphate-buffered saline (PBS), resuspended in 5 mL of MTB-0.3 buffer (50 mM HEPES-KOH, pH 7.3, 25 mM magnesium acetate, 5 mM EGTA, 1 µM dithiothreitol [DTT], 10% glycerol, 0.01% Nonidet® P-40 [NP40], 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 2 µM pepstatin A, 0.6 µM leupeptin and 2 µg/mL of chymostatin) (the number after the hyphen indicates the molarity of the potassium acetate) (5) and then lysed by sonication. The lysate was then applied four times to 1 mL of the anti-FLAG M2 affinity gel column equilibrated with MTB-0.3 buffer according to the manufacturer’s instructions. The column was washed with MTB-0.3 buffer and equilibrated with MTB-0.1 buffer. Bound FLAG fusion protein was eluted with MTB-0.1 buffer containing 0.255 mM FLAG peptide (7-mol column equivalents). A total of 500 µg of FLAG-TUP1, 900 µg of FLAG-SUB1 and 120 µg of FLAG-SRB6 were recovered.

Coomassie® blue staining (Figure 2) of the purified FLAG fusion proteins after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) shows that the purity of the FLAG proteins is over 95%. Under these purification conditions, FLAG fusion proteins bound to and were eluted from the anti-FLAG M2 affinity column with only very low levels of contaminants. Anti-M2 monoclonal antibody showed little cross-reactivity with insect cell proteins. The electrophoretic mobility of the purified fusion proteins corresponds to the predicted molecular weight of each protein. The identity of the fusion proteins was also confirmed by Western blotting with antibodies specific to each protein as well as anti-FLAG antibody.

We have described two baculoviral transfer vectors that have a FLAG epitope coding sequence upstream of the MCS, permitting the production of a fusion protein with the FLAG tag at its amino terminus. The MCS contains an additional unique restriction site, NdeI. This site creates a second ATG start codon following the first ATG by 27 nucleotides. While neither of the start codons is part of a strong Kozak sequence (9), Western blot results show that translation occurs exclusively from the first ATG, consistent with the scanning model of translation initiation in eukaryotes (10). Further improvement in yield might be achieved by manipulating the sequence surrounding the first ATG codon. Because the FLAG sequence itself harbors the enterokinase recognition sequence, a purified FLAG fusion protein can be digested with the protease to remove the epitope tag. The FLAG fusion system in insect cells has some advantages over other fusion systems using glutathione-S-transferase (GST) or histidine tags. It can reduce degradation problems associated with large tags such as GST. Some histidine-tagged proteins are poorly purified by nickel columns, either because the affinity of these proteins to nickel is low or because of the presence of other endogenous histidine-containing proteins. In our experience, the FLAG fusion proteins are relatively stable and can be purified extensively in a single step.

In summary, the baculoviral FLAG
Benchmarks

transfer vectors and FLAG immuno-
affinity purification method described
here are especially useful tools for ex-
pression, detection and purification of
recombinant proteins expressed in in-
sect cells.

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Simplified ELISA for Detecting Antibodies to Recombinant Fusion Proteins

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Purification of recombinant proteins using affinity chromatography is a powerful technique that has widespread application. The procedure involves preparation of a plasmid encoding a fusion protein comprising the polypeptide of interest linked to a sequence with defined affinity for a solid-phase matrix. Examples of applying the principle include the binding of glutathione S-transferase (GST) fusion proteins to glutathione agarose (5,6) and the affinity of histidine-tagged proteins for nickel-nitrilotriacetic acid (Ni-NTA) resins (2,4). Important uses for the preparation of purified recombinant proteins include the raising and detection of antibodies. We describe a simple enzyme-linked immunosorbent assay (ELISA) method that exploits the binding of the recombinant protein to the solid matrix for both antigen purification and subsequent antibody detection in the sera of immunized animals. The advantage of this method over more conventional immunoassays is that procedures that may be time-consuming or prone to technical difficulties are obviated. For example, the coating of multiwell plates with antigen or the preparation of radiolabeled antigen are not necessary. In the protocol outlined here, protein X (HBx) encoded by the hepatitis B virus (HBV) was fused to a GST sequence for immunization and ELISA detection of antibody against the HBx. The principle of this method should be applicable to most recombinant proteins with an affinity tag that can be