Expression and purification of recombinant proteins are fundamental techniques in molecular biology. Many different cell systems ranging from bacteria to mammalian cell lines are commonly used. One of the most widely used systems is the baculovirus expression system. An alternative for large-scale production of eukaryotic proteins are stably transfected Schneider Drosophila line 2 cells (Catalog No. CRL-1963: ATCC, Rockville, MD, USA), abbreviated SL2 or S2, derived from Drosophila melanogaster embryos (13). The generation of stable transfectants of insect cells (2) is fast, and it circumvents amplification, titration and storage of virus stocks. In addition, tightly regulated promoters, such as the metallothionein (MET) promoter (4), are available to control production of the recombinant protein.

Surveillance and purification of an expressed protein can be simplified by epitope-tagging. Widely used epitopes are the hemagglutinin (HA) (14) and the FLAG® epitopes (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) (10), which consist of nine (YPYDVPDYA) and eight (DYKDDDDK) amino acid peptides, respectively. Both epitopes can easily be detected by commercially available antibodies. The FLAG epitope also is recognized by a protease enterokinase and thus, if necessary, can be removed.

Here, we describe transfer vectors designed for the regulated expression of dual epitope-tagged proteins in SL2 cells. The recombinant proteins contain both the HA and the FLAG epitope at their N terminus. Thus, either antibodies to HA and/or FLAG can be used for detection and purification. The presence of two epitopes could be of particular advantage if antibodies to one of the epitopes cross-react with cellular proteins. In addition, SL2 cell lines were established that express the epitope-tagged transcription factors Sp1, Sp3, a mutant of Sp3 and HNF3α.

To generate the HA/FLAG expression vectors, a pair of phosphorylated complementary oligonucleotides, 5'-AATTTGGATCCTGACCACTGGATACCCCTATGATGTTCCGAATTT-ATGCCCTCGACTACAAAGGATGACGATAAAAG-3' and 5'-AATTCTTTATCGTCACTGCTTTTCTGAAGAGCATAACGACCATCATATAGGTTACCATGAGG-3', was synthesized, annealed and cloned into the EcoRI site of the plasmid pRmHa-3, which is a derivative of the plasmid pRmHa-1 (4). The plasmid pRmHa-3 contains the copper-inducible MET promoter, a multiple cloning site (MCS) and the polyadenylation signal of the Drosophila melanogaster alcohol dehydrogenase (ADH) gene. pRmHa-3 is identical to pRmHa-1 with the exception that it contains three additional single cloning sites. The 5' end of the annealed double-stranded oligonucleotide was designed such that the EcoRI site was destroyed after cloning (GAATTCG), whereas the 3' EcoRI site remained intact (GAATTCC). Plasmids containing the correct insert orientation were identified by restriction analyses and sequencing. The resulting plasmid (pMET-HA/FLAG) contains, downstream of the MET promoter, a consensus Kozak sequence (12) flanking the AUG start codon and the codons for the HA and FLAG epitopes followed by a MCS (Figure 1A).

To test the vector, four open reading frames encoding the transcription factors Sp1 (11), Sp3 (8), a mutant of Sp3 (Sp3SD) (6) lacking the inhibitory domain and HNF3α (5) were subcloned into the MCS of pMET-HA/FLAG (Figure 1B). The resulting plasmids pMET-HA/FLAG-Sp1, pMET-HA/FLAG-Sp3, pMET-HA/FLAG-Sp3SD and pMET-HA/FLAG-HNF3α were analyzed in transient transfection experiments using appropriate reporter constructs (3,9). These experiments show that the epitopes do not interfere with the activation properties of the transcription factors (data not shown).

To generate stable transfectants of SL2 cells, the recombinant transfer vectors were transfected into SL2 cells along with the plasmid pLTR-Hygro (2) at a 19:1 molar ratio using the calcium-phosphate method (7). The plasmid pLTR-Hygro contains the hygromycin-

![Figure 1. Vectors for inducible expression of epitope-tagged proteins in SL2 cells.](image-url)

(A) The vector pMET-HA/FLAG contains the copper-inducible MET promoter, a consensus Kozak sequence (CCACC), an initiating ATG followed by the codons for the HA and FLAG epitopes, a MCS and the 3' untranslated region of the Drosophila melanogaster ADH gene. Unique cloning sites (EcoRI, SacI, KpnI, Smal, XbaI and SalI) are indicated. The cleavage site for the enterokinase is depicted by an arrow. (B) Expression vectors for the epitope-tagged transcription factors Sp1, Sp3, the Sp3 mutant Sp3SD and HNF3α were transfected along with the selection plasmid pLTR-Hyg (copia-Hygro) to generate stably transformed SL2 cell lines. The 3 zinc fingers of the transcription factors Sp1, Sp3 and Sp3SD are depicted by 3 black strips. The small deletion in Sp3SD is depicted by a hatched strip.
B-phosphotransferase gene under the control of the strong constitutively active copia long terminal repeat (LTR) promoter. Selection of the cells was performed with 300 µg/mL of hygromycin-B (Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Confluent hygromycin-resistant cell layers were obtained after three weeks.

Expression of the HA/FLAG-tagged transcription factors was analyzed in pooled stable transfectants after induction with 500 µM CuSO4 for 24 h. Recombinant proteins were identified by western blot analyses and electrophoretic mobility shift assays (EMSA) of nuclear extracts. All four proteins were expressed at similar levels (Figure 2). In addition, antibodies to the HA epitope (anti-HA polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the FLAG epitope (anti-FLAG® M2 monoclonal antibodies; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), both recognized their corresponding peptides in all four fusion proteins (Figure 2 and data not shown).

For large-scale growth and purification of recombinant proteins, SL2 cells were grown in suspension in the absence of hygromycin-B essentially according to the instructions of Invitrogen (www.invitrogen.com/), with the exception that Schneider medium supplemented with 10% fetal calf serum tested for growth of insect cells (both from Life Technologies GmbH, Eggenstein, Germany), 2 mM l-glutamate, antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin), 0.1% Pluronic F-68 and 10 µg/mL of heparin (both from Sigma-Aldrich Chemie GmbH) was used. Expression was induced with 500 µM CuSO4 at a cell density of 1 × 10^7 cells/mL for 24 h. EMSAs of nuclear extracts prepared from suspension culture cells also revealed regulated, high-level expression of all four proteins, which can be purified by anti-HA and/or anti-FLAG affinity chromatography.

In conclusion, the HA/FLAG-tagged transfer vectors described here are useful tools for transient expression studies of transcription factors, the generation of stable SL2 cells and the inducible expression and purification of recombinant proteins.

REFERENCES
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Figure 2. Induction of HA/FLAG-tagged mammalian transcription factors in stable transfectants. Crude nuclear extracts from non-induced (-) or Cu^{2+}-induced (+) SL2 cells were prepared by the micropreparation technique of Andrews and Faller (1) and analyzed by EMSA. Double-stranded oligonucleotides used as probes were labeled by filling in 4-bp protruding ends using [32P]dCTP (3000 Ci/nmol) and Klenow fragment (Boehringer Mannheim, Mannheim, Germany). Binding reactions contained 0.1 ng (15,000 cpm, Cerenkov counting) of [32P]-labeled GC oligonucleotide (9) for Sp1, Sp3 and Sp5SD and 0.1 ng of OEII oligonucleotide (3) for HNF3β. The binding reaction was performed as described (3) with 2.5 µg of protein extract from stable transfectants of pMET-HA/FLAG-Sp1 (lanes 1 and 2), pMET-HA/FLAG-Sp3 (lanes 3 and 4 and 9–11), pMET-HA/FLAG-Sp5SD (lanes 5 and 6) and pMET-HA/FLAG-HNF3β (lanes 7 and 8). Antibodies to the HA (αHA, lane 10) or the FLAG (αFLAG, lane 11) epitopes were included in the binding reactions as indicated at the top. Specific supershifted complexes are indicated by the right by an arrow.

Benchmarks

FLAG and anti-FLAG are registered trademarks of Immunex Corporation. Dr. A. Bernard kindly provided us with the plasmid pLTR-Hygro and Prof. L.S. Goldstein provided the plasmid pRmHa-3. Drs. M. Kaliff-Suske and J. Klug are gratefuly acknowledged for critically reading the manuscript. This work was supported by Grants from the Deutsche Forschungsgemeinschaft and the Stiftung PE Kempekes. Address correspondence to Dr. Guntram Suske, Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany. Internet: suske@imt.uni-marburg.de
The methylotrophic yeast, *Pichia pastoris*, has been successfully used for the expression of a wide variety of recombinant proteins (2,3). The expression level of many of these proteins is influenced by the number of copies of the gene of interest introduced into the *P. pastoris* genome (1). In the past few years, a system for the screening of multiple-copy inserts has become commercially available (Invitrogen, Carlsbad, CA, USA). This uses expression vectors (pPIC9K for secreted protein expression and pPIC3.5K for intracellular expression) that contain the bacterial kanamycin-resistance gene, *Tn903kan*<sup>+</sup>, which confers resistance to G418 in *P. pastoris*. These vectors can either be purchased separately or as part of the Multi-Copy Pichia Expression Kit (Invitrogen). Increasing the number of copies of this gene also increases the resistance of *P. pastoris* to G418 and, therefore, this can be used to select organisms with multicopy inserts (5).

The process of screening for multiple integrated copies of foreign genes, as recommended by Invitrogen, involves transformation by either speroplasting or electroporation and then selection on plates lacking histidine. Once His<sup>+</sup> transformants are visible (usually after 2 days), they are screened for G418 resistance by scraping off all colonies and replating on G418 plates at low density (5). The density of cells plated onto G418 plates is critical, as too high a density results in large numbers of false positives. A second method recommended by the manufacturer involves the growing of (typically) hundreds of individual colonies in microplates, passing until they are all at the same density and then spotting them onto G418 plates. This is supposed to reduce the number of false positives, although it takes approximately one week longer, is very labor-intensive and, in practice, many fewer colonies can be screened. Direct selection on G418 immediately following transformation does not work, as some time is necessary for the resistance factor to be expressed. Therefore, this screening procedure can only be used subsequent to selection of transformants. Typically, resistance to 0.25–0.5 mg/L G418 suggests a single-copy insert, while resistance of 4 mg/mL can indicate 7–12 copies (5). Some other methods for identifying high-expressing *P. pastoris* clones have also been described; however, these require the availability of antibodies to the gene of interest (4,6).

We have developed a very simple method, which greatly improves this screening process. However, this method will only work following transformation by electroporation. It involves growing transformants on top of nylon membranes during the selection process for His<sup>+</sup> transformants. After 24 h, the membrane can be lifted off one plate and placed directly onto G418-containing plates. Using this method, it is not necessary to wait until colonies appear following transformation, as 24 h appears to be sufficient time for G418 resistance to accumulate. Furthermore, this approach completely eliminates the need to scrape off colonies, do cell counts and replate at appropriate densities. The other critical advantage of this protocol is that it is possible to transfer the colonies as a whole to G418-containing plates. Therefore, each colony represents one original yeast transformant and is an independent clone. Aside from the additional time involved, the problem with removing His<sup>+</sup> transformants from one set of plates and then replating onto G418 plates, is that each original colony forming unit (cfu) on the first plate becomes many colonies on a G418 plate. Therefore, G418-resistant colonies can be replicates of one original transformed colony. This hugely expands the size and cost of the screening procedure. Also, every possible cfu might not be screened due to the very large dilution necessary when replating His<sup>+</sup> cells onto G418 plates. These problems are entirely avoided by maintaining the integrity of the colony as it advances through the screening procedure.

We are now routinely using the modified screening procedure described above. An illustrative example is presented below. Preparation of electrocompetent *P. pastoris* (GS115 strain) and electroporation was performed essentially according to the Invitrogen guidelines. Eighty microliters of cells were pulsed with 10 µg of SacI-digest-

**Figure 1. Western blot analysis comparing expression levels of a FLAG-tagged protein (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) secreted from two G418-selected colonies.** Colonies, selected from a plate containing 4 mg/mL G418, were grown overnight in BMGY (1% yeast extract, 2% peptone, 100 mM phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 1% glycerol) then induced at the same OD 600 for 48 h in BMMY (1% yeast extract, 2% peptone, 100 mM phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 0.5% methanol) containing 1% (vol/vol) methanol. After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins in the cell supernatant were transferred to a nitrocellulose membrane. The anti-FLAG<sup>M2</sup> antibody (Scientific Imaging Systems [Eastman Kodak]) was then used to detect the tagged protein in the supernatant. The arrow (→) indicates the FLAG-tagged protein of interest, and the numbers indicate the molecular weights of standards.