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A Mammalian Expression Vector for the Expression of GAL4 Fusion Proteins with an Epitope Tag and Histidine Tail

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Expression of newly cloned cDNAs in mammalian cell lines is an essential tool for the functional analysis of the proteins encoded by these cDNAs. In many instances, however, evaluation of the protein is difficult because of the difficulty in purification of the expressed protein and/or the lack of specific antibodies which react with the proteins on Western blots or for immunocytochemistry or immunoprecipitation. A number of gene fusion systems have been employed in which a known peptide is fused to the expression product of interest and the fusion protein is purified using affinity chromatography and identified in extracts or by immunocytochemistry using antibodies directed against the affinity handle peptide. The DNA-binding domain of the yeast transcription factor GAL4 is widely used to construct fusion proteins with putative transcription factors to evaluate potential *trans*-acting domains. Because of the lack of commercially available anti-GAL4 antibodies, the further biochemical characterization of these fusion proteins has remained difficult. We describe the construction of two mammalian expression vectors, pMFH/GAL4 and pMFH2/GAL4 (where pMFH stands for *pM*2, *F*lag, *H*istidine tail), which encode the DNA-binding domain of the yeast transcription factor GAL4 with a Flag peptide (consisting of the 11-amino-acid leader peptide of the gene *10* product from bacteriophage T7) at the NH_2-terminus and a tail of six histidines at the COOH-terminus. Unique restriction sites allow both the construction of fusion proteins with the GAL4 DNA-binding domain and the replacement of the *GAL4* fragment with another insert. Proteins encoded by this vector are biologically active, can be easily precipitated and purified by interaction of its histidine tail with immobilized Ni^{2+}, and can be visualized on Western blots with an antibody against the Flag peptide.

The expression of proteins in mammalian cell lines has become a vital tool for the characterization of eukaryotic proteins. Expression in nonmammalian systems, such as bacteria, yeast, or baculovirus, is often not ideal for a number of reasons. Bacteria will frequently produce at least partially insoluble recombinant proteins and do not process proteins in a manner characteristic of mammalian cells. While insolubility of recombinant proteins usually does not pose a severe problem in yeast or insect cells, the potential lack of proper interacting proteins in these systems can lead to anomalous protein interactions and function. Furthermore, recombinant proteins are generally expressed at lower (though probably more physiological) levels in mammalian cell lines than in bacteria, yeast, or insect cells. These facts justify the development of efficient purification, enrichment, and detection procedures for exogenous proteins expressed in mammalian cells.

An ideal antibody should immunoprecipitate the protein of interest with high efficiency and should detect it specifically on Western blots and in cytological specimens. Often an antibody fulfilling all these criteria is not available. One solution to this problem lies in the use of fusion proteins in which a chimera is formed between the recombinant protein and a short sequence of amino acids which serve as an epitope that is recognized by a generally available antibody (e.g., 1).

In this paper we describe two mammalian expression vectors, pMFH/GAL4 and pMFH2/GAL4, with several

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convenient features. The plasmids contain the SV40 origin/early promoter region, which results in extrachromosomal replication and thus high expression levels in COS cells and a wide variety of other cell lines. The fusion protein encoded by these vectors consists of the 11-amino-acid leader peptide of the gene *10* product from bacteriophage T7 (Flag), the DNA-binding domain (amino acids 1–147) of the yeast transcription factor GAL4, and the protein of interest followed by a tail of six histidines at the COOH-terminus. By employing an antibody against the Flag epitope, Western blots or immunoprecipitation can be carried out. The histidine tail permits the efficient purification and precipitation of the fusion protein even under denaturing conditions. Because of the presence of several unique restriction sites between the Flag epitope- and the GAL4-encoding sequence, the latter can be excised and fusion proteins without the GAL4-encoding sequence can be constructed containing the Flag epitope and histidine tail. *Sal*I sites outside the coding region allow the easy transfer of the entire expression cassette into other vectors.

MATERIALS AND METHODS

Antibodies

A commercial monoclonal T7·Tag antibody ($IgG_{2b,K}$) directed against the Flag epitope was obtained from Novagen (Madison, WI) and used at 1:3000 dilution. A polyclonal rabbit anti-GAL4 antibody, used at 1:1000 dilution, was a gift from Stephen A. Johnston and Karsten Melcher, University of Texas Southwestern Medical Center (Dallas, TX). Horseradish peroxidase (HRP)[3]-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) and HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) were both used as secondary antibodies at 1:3000 dilution.

Enzymatic Manipulations of DNA

All enzymatic manipulations were carried out according to standard protocols (2) or following the manufacturers' instructions.

Construction of pMFH/GAL4–Kid-1N

Preparation of insert. The pBXG1/Kid-1N plasmid has been described previously (3). It contains sequence encoding a fusion protein including the NH_2-terminal 147 amino acids of yeast GAL4 and the first 195 amino acids of Kid-1. The region encompassing nucleotides 4 to 441 of *GAL4* and nucleotides 312 to 878 of *Kid-1* was PCR amplified from pBXG1/Kid-1N with the forward primer 5′-ATTTGC**GAGCTC**GAAGCTACTGTCTTCT-3′ and the reverse primer 5′-ATTTGC**AAGCTT**TTTTATAGCGTTTATC-3′ (nucleotides in bold indicate the restriction sites for *Sac*I and *Hin*dIII, respectively). The PCR product was digested with *Sac*I and *Hin*dIII and subcloned into *Sac*I/*Hin*dIII-cut pET21b (Novagen), creating pET21b/GAL4–Kid-1N. The entire PCR product was sequenced to confirm that no mutations were introduced. pET21b is a prokaryotic expression vector encoding a fusion protein with the 11-amino-acid leader peptide of the gene *10* product from bacteriophage T7 (Flag) at the NH_2-terminus and a tail of six histidines at the COOH-terminus. pET21b/GAL4–Kid-1N was digested with *Xba*I and *Dra*III, releasing the PCR product together with sequence encoding the Flag epitope at the 5′-end and six histidines at the 3′-end. The insert was blunt-ended with T4 DNA polymerase, and *Sal*I linkers (5′-CGGTCGACCG-3′) were ligated onto it. After a digest with *Sal*I, the insert was gel-purified and subcloned into pM2; the resulting construct was designated pMFH/GAL4–Kid-1N (Figs. 1a and 1b).

Preparation of vector. pM2 (4) (a gift from Ivan Sadowski, University of British Columbia, Vancouver, Canada) was digested with *Bgl*II and *Xba*I, which releases the GAL4-encoding sequence. The linearized plasmid was subsequently blunt-ended with T4 DNA polymerase, and *Sal*I linkers (5′-CGGTCGACCG-3′) ligated onto it. After a digest with *Sal*I, the plasmid backbone was gel-purified and used for the ligation.

Construction of pMFH/GAL4

pMFH/GAL4–Kid-1N was digested with *Pst*I and *Hin*dIII (thus removing the Kid-1N fragment) and blunt-ended with T4 DNA polymerase. The vector fragment was gel-purified and self-ligated (Figs. 1a and 1b).

Construction of pMFH2/GAL4–Kid-1A

Preparation of insert. The pBXG1/Kid-1A plasmid contains sequence encoding a fusion protein including the NH_2-terminal 147 amino acids of yeast GAL4 and the first 54 amino acids of Kid-1 (5). The region encompassing nucleotides 4 to 441 of *GAL4* and nucleotides 312 to 473 of *Kid-1* was PCR amplified from pBXG1/Kid-1A with the forward primer 5′-ATTTC**GGATCCCGGGTACCAGCTGATCGATAAGAGCTC**GAAGCTACTGTCTTCT-3′ (nucleotides in bold indicate the restriction sites for *Bam*HI, *Sma*I, *Kpn*I, *Pvu*II, *Cla*I, *Sac*I) and the reverse primer 5′-ATTTGC**AAGCTTGATATCTAGATCT**GCCATGGAGGCCAG-3′ (nucleotides in bold indicate the restriction sites for *Hin*dIII, *Eco*RV,

[3] Abbreviations used: HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; CAT, chloramphenicol acetyltransferase.

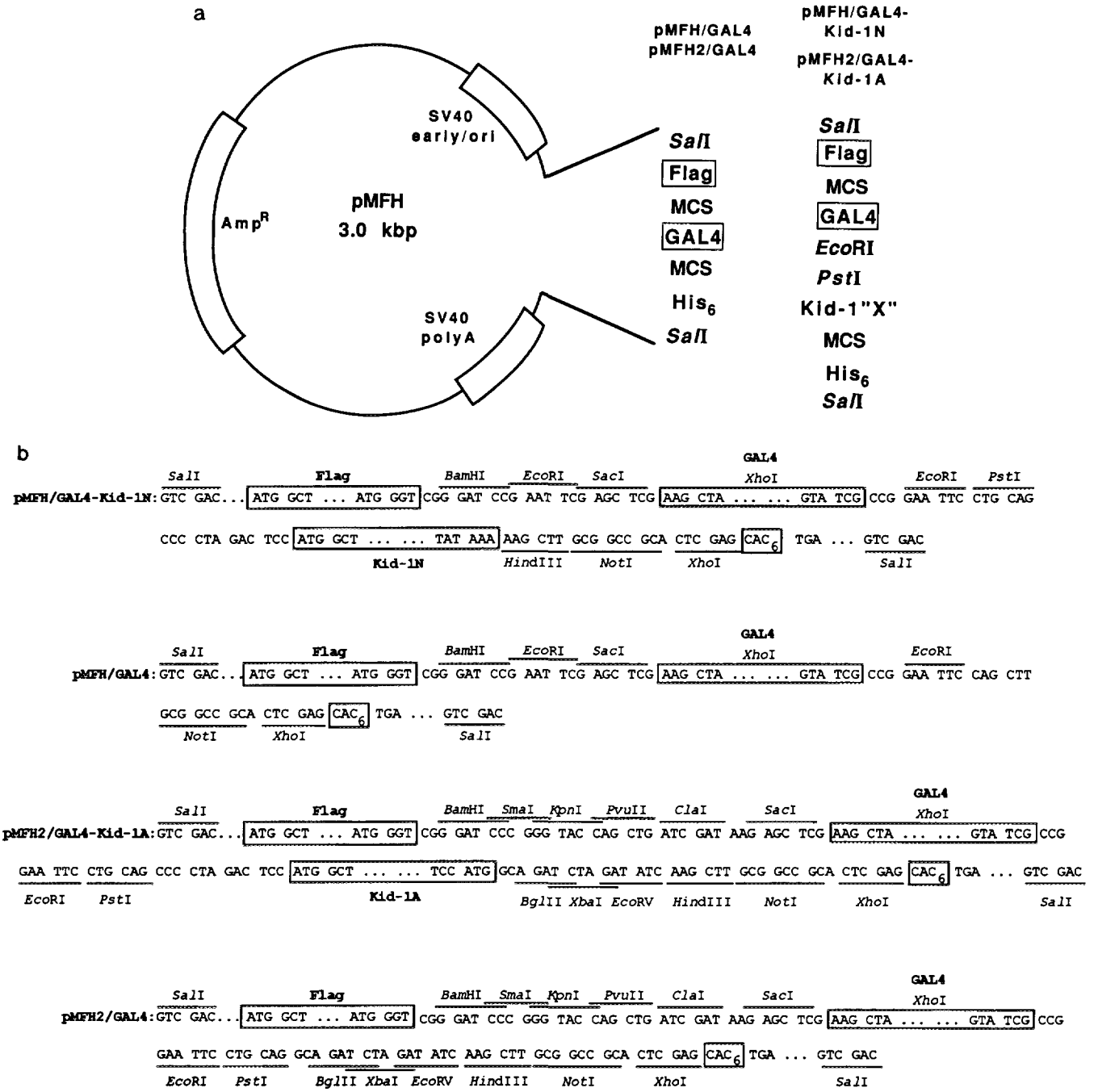

FIG. 1. (a) Plasmid maps. Maps of the expression plasmids pMFH/GAL4–Kid-1N, pMFH2/GAL4–Kid-1A, pMFH/GAL4, and pMFH2/GAL4. The backbone of the pMFH vectors is approximately 3000 bp in size and contains a gene coding for β-lactamase (Amp^R) for selection in *Escherichia coli* as well as the early promoter (including the origin of replication) and the polyadenylation signal of SV40. In all four plasmids the expression cassette is flanked by *Sal*I sites upstream of the Flag-encoding and downstream of the histidine tail-encoding region. In the case of the pMFH/GAL4 and pMFH2/GAL4 plasmids, multiple restriction sites are located immediately upstream and downstream of the sequence coding for amino acids 2 to 147 of GAL4 (compare with b). The plasmids pMFH/GAL4–Kid-1N and pMFH2/GAL4–Kid-1A (Kid-1N and Kid-1A are shown as Kid-1"X") contain additional sequence coding for various portions of the NH_2-terminus of Kid-1. (b) Partial sequence of pMFH/GAL4–Kid-1N, pMFH/GAL4, pMFH2/GAL4–Kid-1A, and pMFH2/GAL4. Sequences coding for the Flag, GAL4 (2–147), Kid-1N (1–189), Kid-1A (1–54), and the six histidines are boxed. Both the first two and last two codons of the Flag, GAL4, Kid-1N, and Kid-1A are shown. The first *Sal*I site is separated by approximately 40 nucleotides from the start codon; the second *Sal*I site lies 0.4 kbp downstream from the stop codon. All restriction sites shown are not present in the plasmid backbone.

*Xba*I, *Bgl*II). The PCR product was digested with *Bam*HI and *Hin*dIII and subcloned into *Bam*HI/*Hin*dIII-cut pMFH/GAL4-Kid-1N, creating pMFH2/GAL4-Kid-1A. The entire PCR product was sequenced to establish that no mutations were introduced.

Preparation of vector. pMFH/GAL4–Kid-1N was digested with *Bam*HI and *Hin*dIII, which releases the sequence encoding the GAL4–Kid-1N fusion protein. The linearized plasmid was dephosphorylated and the plasmid backbone was gel-purified.

Construction of pMFH2/GAL4

pMFH2/GAL4–Kid-1A was digested with *Eco*RI and *Bgl*II (thus removing the Kid-1A fragment). The vector fragment was gel-purified and ligated to an *Eco*RI/*Bgl*II adapter (5′-AATTCCTGCAGGCA-3′, 5′-GATCTGCCTGCAGG-3′) containing a *Pst*I site (Figs. 1a and 1b).

Transfections

COS cells were transiently transfected using standard protocols (2). Briefly, cells were plated the day before the transfection at a density of approximately 4×10^5 per 100-mm dish. The next day, 20 μg of DNA was added to the cells in 5 ml of DMEM–10% NuSerum–400 μg/ml DEAE dextran–0.1 mM chloroquine (unless otherwise indicated, cells were transfected with 10 μg of expression plasmid and 10 μg of pBluescript as carrier DNA). Two to three hours after the addition of DNA, the cells were shocked for 2 min at room temperature with 1× PBS–10% DMSO and new medium was added. Cells were harvested 2 to 3 days after transfection. When cell extracts were to be assayed for CAT activity, the reporter plasmid pG5SV-BCAT (3) and the internal control plasmid p_0LucSV/T1 (6) were included in the transfections. pG5SV-BCAT contains five GAL4 binding sites upstream of the SV40 promoter and the E1B TATA box, driving a CAT gene. In the case of p_0LucSV/T1 the expression of luciferase is under the control of the SV40 promoter.

Metabolic Labeling

Two or three days after the transfection, the cells were washed twice in cysteine/methionine-free DMEM (Sigma) and then incubated for 15 min in the same medium. Tran^{35}S-Label (250 μCi), an *Escherichia coli* lysate containing approximately 70% [^{35}S]methionine and 15% [^{35}S]cysteine (ICN, Irvine, CA), in 10 ml of cysteine/methionine-free DMEM/10% dialyzed fetal calf serum was added and 4 or 18 h later total cell lysates were prepared.

CAT and Luciferase Assays

CAT and luciferase assays were performed as previously described (3).

Western Blotting

Proteins were separated on a SDS–polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). Nonspecific binding sites were blocked by incubating overnight at 4°C with PBS, 0.5% Tween 20, and 5% nonfat dry milk (subsequently referred to as blocking buffer). The next morning, primary antibody was added to the membrane in fresh blocking buffer and incubated 2 h at room temperature. After two 10-min washes with PBS–0.5% Tween 20 and two 10-min washes with blocking buffer at room temperature, HRP-conjugated secondary antibody in blocking buffer was added to the membrane and incubated for 1 h at room temperature. The membrane was washed four times for 10 min with PBS–0.5% Tween 20 at room temperature and twice for 2 min each with PBS. Immune complexes were detected with the Renaissance light detection system from Du Pont (Boston, MA).

Precipitation of Histidine-Tagged Proteins with His·Bind Resin from Total Cell Lysates in a Batch

Preparation of His·Bind resin. His·Bind resin (Novagen) was washed twice in 5 vol of H_2O, resuspended in 5 vol of 50 mM $NiSO_4$, and kept for 5 min at room temperature. This $NiSO_4$-loaded His·Bind resin was then washed twice with 10 vol of H_2O and twice with 10 vol of TNU with 5 mM imidazole (TNU consists of 50 mM Tris, pH 8.0, 150 mM NaCl, and 6 M urea). This prepared resin will subsequently be referred to as "charged His·Bind resin or beads."

Precipitation of proteins. Cells were washed twice with PBS and scraped into a microcentrifuge tube. The cell pellet was resuspended in 200 μl of TNU–5 mM imidazole and sonicated for 15 pulses with a microtip. After centrifuging for 60 s to remove insoluble material, the supernatant was transferred into a tube with 25 μl of charged His·Bind resin. Following an incubation of 30 min at room temperature on a rotating platform, the beads were washed three times with 10 vol of TNU–5 mM imidazole. After the final wash, the beads were resuspended in 150 μl of sample buffer (125 mM Tris, pH 6.7–2.5% SDS–10% glycerol–2.5% 2-mercaptoethanol–0.01% bromophenol blue–10 mM EDTA, pH 8.0–100 mM imidazole) and boiled for 5 min before loading on a SDS–polyacrylamide gel.

Purification of Histidine-Tagged Proteins with His·Bind Resin from Total Cell Lysates over a Column

Preparation of a His·Bind resin column. A minicolumn of 100 μl of His·Bind resin was prepared in a 1-ml pipette tip plugged with siliconized glass wool. The resin was washed with 1 ml each of H_2O, 50 mM $NiSO_4$, H_2O,

and TNU-5 or 20 mM imidazole, after which the column was ready for use.

Purification of protein. Cells were washed twice with 1× PBS and scraped into a microcentrifuge tube. The cell pellet was resuspended in 200 μl of TNU-5 or 20 mM imidazole (see text) and either sonicated for 15 pulses with a microtip or, in the case of metabolically labeled cells, left on ice for 10 min. After centrifuging for 60 s to remove insoluble material, the supernatant was transferred onto the charged column. The column was washed with 20 ml of TNU-5 or 20 mM imidazole. Bound protein was eluted in 100-μl fractions in TNU-imidazole (concentrations of imidazole were as described under Results).

Fluorography

Gels either were stained first in 0.25% Coomassie blue R-250-50% methanol-10% acetic acid and destained in 50% methanol-10% acetic acid or were immediately fixed in destaining solution for 30 min. The gel was then rinsed briefly in DMSO and equilibrated twice for 45 min in DMSO. Subsequently, the gel was incubated for 45 min at room temperature in 22% (w/v) PPO (diphenyloxazole) in DMSO. After repeated rinses with H_2O, the gel was dried and exposed to X-ray film at −80°C.

RESULTS

Biological Activity of an Epitope-Tagged, Histidine-Tailed Fusion Protein

We have previously reported that the NH_2-terminus of the rat zinc finger protein Kid-1, when fused to the DNA-binding domain of yeast GAL4, confers strong transcriptional repressor activity on a chloramphenicol acetyltransferase (CAT) reporter plasmid containing GAL4 binding sites (3). To demonstrate that the addition of the leader peptide from the T7 bacteriophage gene *10* product (Flag) to the amino terminus and six histidines to the carboxy terminus did not alter this repressor activity, we performed transient transfections with the expression plasmids pBXG1/Kid-1N in the sense orientation (pBXG1/Kid-1N) or antisense orientation (pBXG1/Kid-1N,as) and pMFH/GAL4-Kid-1N and the reporter plasmid pG5SV-BCAT. Activity is reported as percentage of CAT activity obtained with the negative control pBXG1/Kid-1N,as. When either the pBXG1/Kid-1N or the pMFH/GAL4-Kid-1N plasmid was included in the transfection, CAT activity dropped considerably in both cases to comparable levels (Fig. 2).

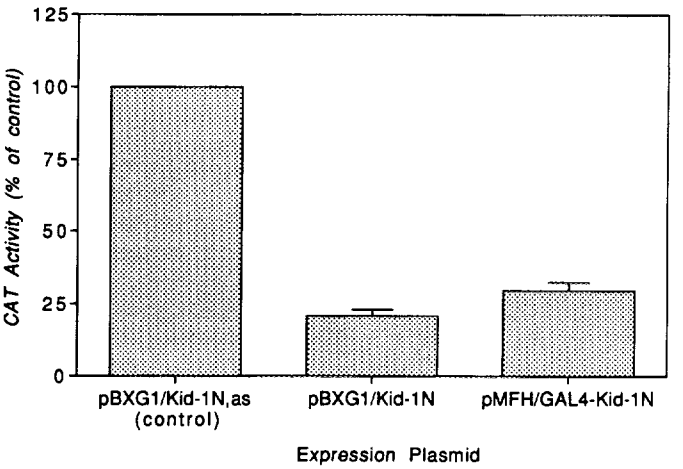

FIG. 2. An epitope-tagged, histidine-tailed GAL4-Kid-1N fusion protein is biologically active. COS cells were transiently transfected with a total of 20 μg of DNA containing 10 μg of expression plasmid (pBXG1/Kid-1N,s, pBXG1/Kid-1N,as, pMFH/GAL4-Kid-1N), 3 or 6 μg of the CAT reporter plasmid pG5SV-BCAT, 1 μg of p_0LucSV/T1 (a plasmid coding for luciferase), and Bluescript as carrier DNA. Transfection efficiencies were determined by analyzing luciferase activities. Cell extracts corresponding to equal amounts of luciferase activity were used in the CAT assay.

The Protein Encoded by pMFH/GAL4-Kid-1N Can be Detected by Antibodies against GAL4 or the Flag Epitope

Total cell lysates were prepared from COS cells transiently transfected with pBXG1/Kid-1N, pMFH/GAL4, or pMFH/GAL4-Kid-1N by lysing the cells in TNU-5 mM imidazole. The lysates were incubated with Ni^{2+}-charged His·Bind resin and the captured proteins were run on a 13% SDS-polyacrylamide gel. Proteins were transferred onto a PVDF membrane and the blots were probed with an antibody against GAL4 or against the Flag epitope. When cells were transfected with any of the three expression vectors, the anti-GAL4 antibody recognized a protein of the appropriate size, indicating that all three constructs were expressed (Fig. 3). When the anti-Flag antibody was used, a protein was detected only in cells transfected with the pMFH/GAL4 or pMFH/GAL4-Kid-1N plasmids, the two plasmids encoding the Flag antigen. Protein was precipitated by the charged His·Bind resin only when it contained the histidine tail as shown by the absence of a band in the resin-bound fraction of pBXG1-Kid-1N-transfected cells.

Optimization of the Purification Protocol for Histidine-Tailed Proteins

COS cells were transiently transfected with pMFH/GAL4. Two to three days after the transfection, cells were lysed in TNU-5 mM imidazole to make total cell lysate. The lysates were loaded onto a Ni^{2+}-charged His·Bind column, the column was washed at a concentration of 5 mM imidazole, and proteins were eluted in 100-μl fractions at imidazole concentrations of 20 mM, 60 mM, and 1 M. The eluate fractions were subjected to Western blot analysis with the anti-Flag antibody. A weak signal was

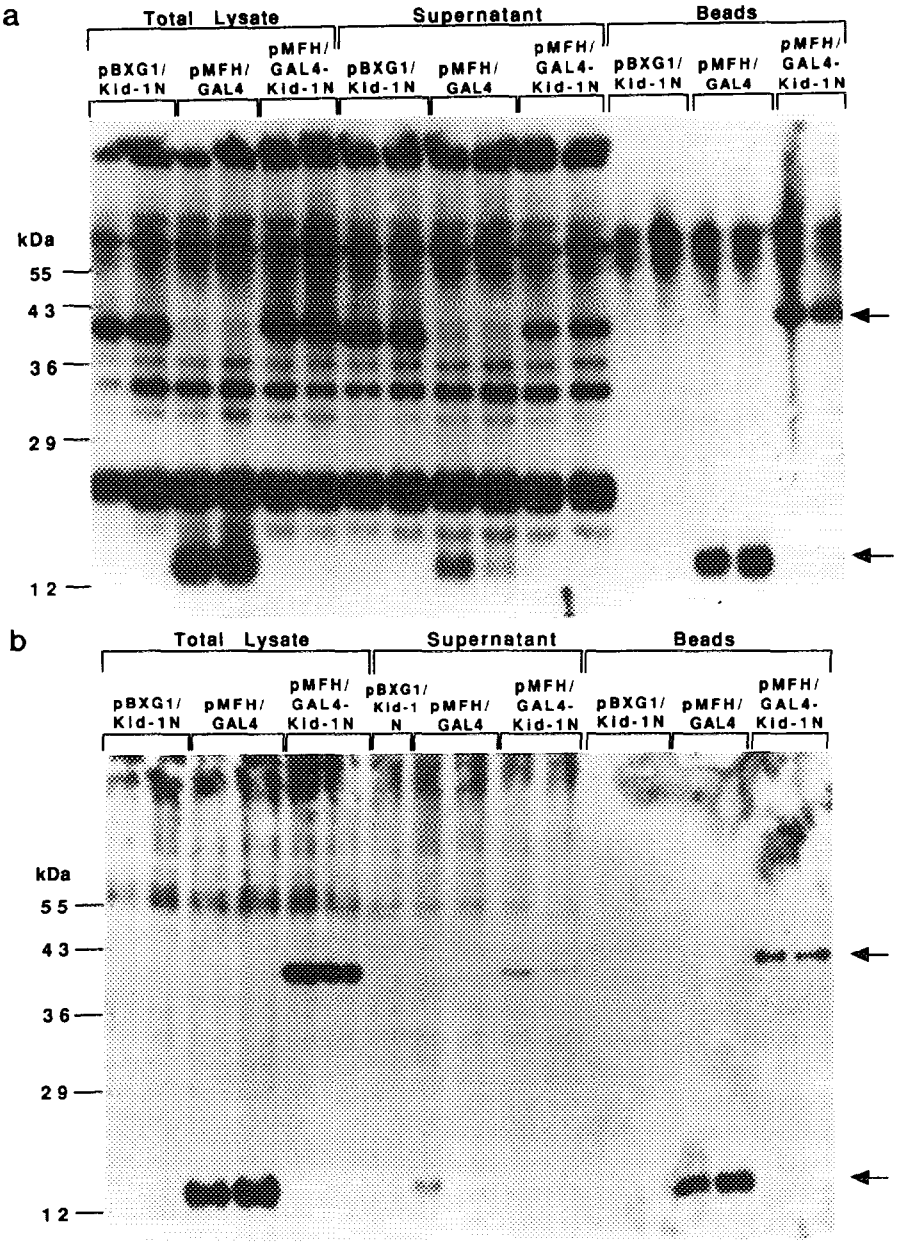

FIG. 3. An anti-Flag antibody specifically detects epitope-tagged proteins on a Western blot. Transfected COS cells were harvested and lysed in TNU–5 mM imidazole. Equal portions of (i) lysates before incubation with Ni^{2+} beads ("total lysate"), (ii) supernatants from lysates treated with Ni^{2+} beads ("supernatant"), and (iii) proteins adherent to Ni^{2+} beads ("beads") were run on a 13% polyacrylamide gel under denaturing, reducing conditions and subsequently transferred to PVDF membranes. Membranes were probed with either an anti-GAL4 antibody (a) or the anti-Flag antibody (b). Arrows point to the GAL4–Kid-1N fusion proteins or the GAL4 protein. Although some of the histidine-tagged protein appears in the supernatant after incubation with Ni^{2+} beads, the major portion adheres to the Ni^{2+} beads.

detected in the flow-through fraction, but no signal in the eluates was obtained with 20 mM imidazole. A wash solution containing 60 mM imidazole eluted a considerable amount of the GAL4 protein from the column (Fig. 4). Therefore a concentration of 20 mM imidazole was chosen for the lysis buffer and the wash buffer.

Purification from Metabolically Labeled Cells

Transiently transfected COS cells were labeled 2 days after the transfection for 4 or 18 h with [^{35}S]methionine and [^{35}S]cysteine. Cells were lysed in TNU–20 mM imidazole and the lysate was purified over a Ni^{2+}-charged His · Bind column. Bound protein was eluted with TNU–

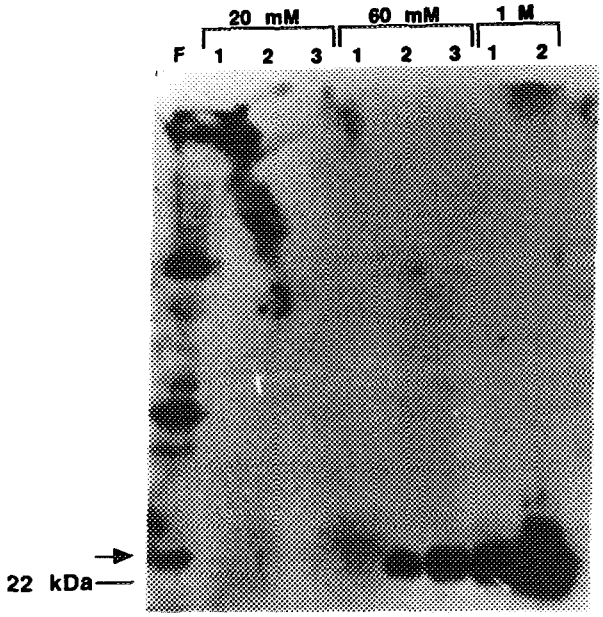

FIG. 4. Optimization of column purification conditions. Total cell lysates were prepared from COS cells transfected with pMFH/GAL4 and run on a column of charged His·Bind resin. Aliquots from the flow-through ("F"), the first three fractions of the 20 and 60 mM imidazole eluates, and the first two fractions of the 1 M imidazole eluate were run on a gel and transferred to PVDF membranes. The membranes were probed with the anti-Flag antibody.

1 M imidazole in 100-μl fractions and aliquots of those eluates were subjected to polyacrylamide gel electrophoresis and fluorography. In lysates from cells transfected with pMFH/GAL4 or pMFH/GAL4–Kid-1N a band of the predicted molecular weight could be detected (Fig. 5).

DISCUSSION

Transcription factors can be divided into two major domains: the DNA-binding and *trans*-acting domains (7). Whereas the DNA-binding domain determines the recognition of a specific binding site in the promoter or enhancer region of a certain gene, the *trans*-acting domain confers a regulatory influence on transcription in a positive or negative fashion. It is possible to make chimeric proteins including the DNA-binding domain of one transcription factor and the *trans*-acting domain of another without losing or altering the activity of either domain. This technique has proven to be very useful, for example, when the binding site for a transcription factor is unknown. A commonly employed DNA-binding domain for such studies is the amino-terminal 147 amino acids of the yeast transcription factor GAL4 (recent references are 8–11), for which several mammalian expression vectors exist (see, for example, Refs. 4, 12). Further biochemical analysis of the expressed fusion proteins, however, is often difficult due to the lack of antibodies for Western blot analysis, immunoprecipitation, or immunocytochemistry and to difficulty in purification of the chimeric protein.

In this paper we describe two mammalian expression vectors, pMFH/GAL4 and the more versatile pMFH2/GAL4 (Fig. 1), which permit the production of GAL4 fusion proteins with an epitope tag at the NH_2-terminus and a tail of six histidines at the COOH-terminus. The addition of the epitope tag and histidine tail does not significantly alter the biological activity of a chimera, as demonstrated by a fusion protein between the GAL4 DNA-binding domain and the *trans*-acting domain of Kid-1 (Fig. 2). A commercially available antibody against the epitope permits the immunological detection of the fusion protein on Western blots. Because of the presence of the histidine tail at its COOH-terminus, the fusion protein can be precipitated under physiological conditions (data not shown) and even in the presence of 6 M urea. Unique restriction sites between the Flag epitope tag- and the GAL4-encoding sequence make it possible to remove the GAL4 portion and replace it with another DNA sequence which encodes another protein of interest. *Sal*I sites upstream of the Flag-encoding sequence and downstream of the sequence coding for the histidine tail allow the convenient transfer of the entire

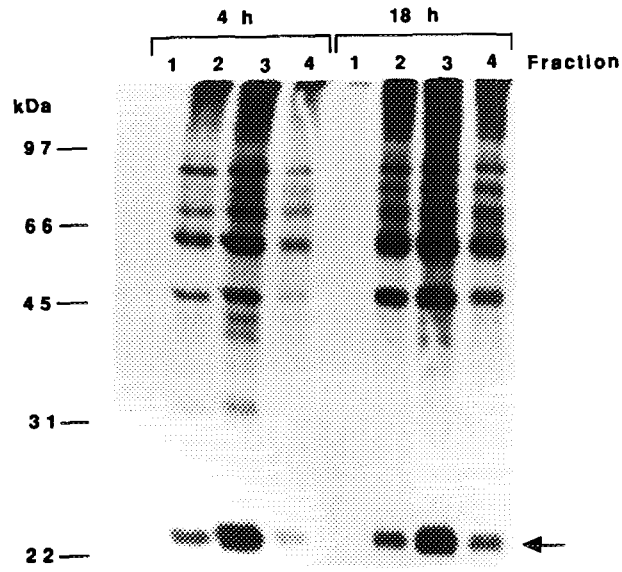

FIG. 5. Purification of metabolically labeled protein. COS cells were transfected with pMFH/GAL4. Two days after the transfection, cells were metabolically labeled for 4 or 18 h with [^{35}S]methionine and [^{35}S]cysteine. Total cell lysates were prepared in TNU–20 mM imidazole and run over a Ni^{2+} column. Proteins were eluted with four 100-μl fractions of TNU–1 M imidazole and subjected to gel electrophoresis and fluorography for two days. The arrow points to a band corresponding to the Flag–GAL4–His_6 fusion protein.

cassette into other expression vectors. We believe that because of these features the pMFH plasmids will be valuable tools for the biochemical characterization of transcription factors, as well as a variety of other proteins.

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