Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7958–7962, September 1992
Genetics

Karyoplasmic interaction selection strategy: A general strategy to detect protein–protein interactions in mammalian cells

(Chinese hamster ovary cells/herpes simplex virus protein VP16/GAL4 protein/hygromycin resistance)

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Communicated by Vincent T. Marchesi, May 15, 1992

ABSTRACT **We describe a strategy and reagents for study of protein–protein interactions in mammalian cells, termed the karyoplasmic interaction selection strategy (KISS). With this strategy, specific protein–protein interactions are identified by reconstitution of the functional activity of the yeast transcriptional activator GAL4 and the resultant transcription of a GAL4-regulated reporter gene. Reconstitution of GAL4 function results from specific interaction between two chimeric proteins: one contains the DNA-binding domain of GAL4; the other contains a transcriptional activation domain. Transcription of the reporter gene occurs if the two chimeric proteins can form a complex that reconstitutes the DNA-binding and transcriptional activation functions of GAL4. Using the KISS system, we demonstrate specific interactions for sequences from three different pairs of proteins that complex in the cytoplasm. In addition, we demonstrate that reporter genes encoding cell surface or drug-resistance markers can be specifically activated as a result of protein–protein interactions. With these selectable markers, the KISS system can be used to screen specialized cDNA libraries to identify novel protein interactions.**

The identification and characterization of specific protein–protein interactions usually require specialized biochemical and immunological methods and reagents and can be very laborious. In addition, *in vitro* methods may fail to detect physiologically relevant, but weak, interactions that occur within the cell. Recently, several strategies have been described that present alternative methods to more rapidly identify and characterize protein–protein interactions. Successes in identifying novel protein–protein interactions have been reported using labeled proteins to screen recombinant λgt11 phage expression libraries (1–3). In addition, Fields and his colleagues (4, 5) have described a system, termed the two-hybrid system, that takes advantage of the modular nature of the yeast GAL4 transcriptional regulatory protein (6, 7). In the two-hybrid system, the two separable functional domains of GAL4—a specific DNA-binding domain and a transcriptional activation domain—are each present in different chimeric proteins. Reconstitution of GAL4 function is achieved by interaction between the chimeric proteins, with resultant activation of a GAL4-regulated reporter gene. This system has been used to detect specific protein interactions in yeast (4, 5), and a modification of this system has been used to study leucine zipper interactions of c-Myc, c-Fos, and c-Jun in mammalian cells (8). Another modification of the two-hybrid approach, termed the contingent replication assay, also has been developed (9).

We describe a strategy and reagents, based on the two-hybrid approach, that not only allow the study of known or candidate protein–protein interactions in mammalian cells but also enable the screening of specialized cDNA libraries for the detection of novel protein–protein interactions. We term this strategy the karyoplasmic interaction selection strategy (KISS). With the KISS system, the specific interaction of proteins in mammalian cells can be studied in either transiently or stably transfected cell lines. Various reporter genes can be used, including those encoding bacterial chloramphenicol acetyltransferase (CAT), cell-surface CD4, or resistance to hygromycin B. In addition, our results suggest that the system can be used to study the interaction of proteins that normally reside in the cytoplasm.

MATERIALS AND METHODS

Plasmid and Phage Constructs. Plasmids encoding chimeric GAL4 proteins, each with the 147 amino-terminal amino acid (aa) residues of GAL4, were created by subcloning cDNA in the expression vector pGALO (10). Fusion genes also were constructed with pNLVP (8), each containing a cDNA fused to DNA encoding the nuclear localization signal of the simian virus 40 large T antigen (11) and the transcriptional activation domain of herpes simplex virus protein VP16 (12). Several of the plasmids encoding chimeric GAL4 and VP16 proteins have been described (8, 10); details of other plasmids used here are available from the authors upon request. The eukaryotic expression phage vector λNLVP was constructed by ligating a 1.6-kilobase-pair (kb) *Pst* I–*Bam*HI fragment from pNLVP-B (ref. 8 and E.R.F., unpublished observations) into the *Xho* I site of λZAP (Stratagene); thus λNLVP is similar to pNLVP and contains a single *Xho* I site for cDNA cloning. The pG5E1bCAT plasmid contains five GAL4 DNA-binding sites, a minimal promoter element from the adenovirus E1B gene, and bacterial CAT coding sequence (13). The plasmid pG5E1bCD4 encodes CD4 sequence (provided by D. Littman, University of California, San Francisco) under GAL4 control; pG5E1bHygro contains hygromycin B resistance sequence (14) under GAL4 control.

Cell Lines and Culture Conditions. The parental DUKXBII Chinese hamster ovary (CHO) cell line and other CHO cell lines were grown in α minimal essential medium (αMEM) with 10% fetal bovine serum, penicillin, and streptomycin. The CHO/CD4 2A5 line was generated by Lipofectin (Bethesda Research Laboratories) transfection of CHO cells with pSV2neo (15) and pG5E1bCD4 in an empirically chosen molar ratio of 1:15. Transfected cells were cloned and selected in medium with Geneticin (GIBCO) at 400 μg/ml. The L-His/Hygro 12B line was obtained by Lipofectin transfec-

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Abbreviations: KISS, karyoplasmic interaction selection strategy; CAT, chloramphenicol acetyltransferase; aa, amino acid(s).

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tion of CHO/CD4 2A5 cells with pSV2his (16) and pG5E1bHygro in a molar ratio of 1:15. Cloning and selection were carried out in medium with 5 mM L-histidinol (Sigma) and 400 μg of Geneticin per ml.

Analysis of CAT Activity, CD4 Expression, and Hygromycin B Resistance. For CAT expression analysis, DEAE-dextran transfection of CHO cells growing on 100-mm-diameter dishes at 50% confluence was performed (8, 13); CAT activity was measured 60–64 hr after transfection (17). Analysis of CD4 expression was carried out by DEAE-dextran transfection of plasmid DNAs into CHO/CD4 2A5 cells. Approximately 60–64 hr after transfection, cells were detached with 2.5 mM EDTA and analyzed for CD4 expression with a mouse anti-human CD4 monoclonal antibody (OKT4, American Type Culture Collection) at 1:20 dilution and a phycoerythrin-conjugated goat anti-mouse IgG antiserum (Southern Biotechnology Associates, Birmingham, AL) at 1:50 dilution. The relative fluorescence of the cells was measured with a FACScan (Becton Dickinson). The induction of hygromycin B resistance was analyzed by Lipofectin transfection of L-His/Hygro 12B cells. Approximately 2.0×10^6 cells were transfected with DNA in 75-mm^2 flasks, using standard protocols. Selection was initiated in hygromycin B at 400 μg/ml and Geneticin at 250 μg/ml, 60–64 hours after transfection. After 4 days of selection, the hygromycin B was reduced to 250 μg/ml. Selection was continued for a total of 12–14 days; the remaining cells were then fixed with 7% formaldehyde and stained with crystal violet.

λNLVP/Colon cDNA Library. A cDNA library was constructed with the eukaryotic expression phage vector λNLVP. cDNAs ranging in size from 0.1 to 1.6 kb were recovered with the polymerase chain reaction (PCR) (18) from a randomly primed, normal colon cDNA library that contained $>10^6$ unique clones (Clontech). cDNA inserts were isolated from agarose (19) and ligated to *Xho* I-digested λNLVP phage arms. A λNLVP/colon cDNA library of $>1.5 \times 10^6$ clones was generated and amplified. High molecular weight DNA was isolated from the amplified library (20) and was used for transfection of L-His/Hygro 12B cells.

RESULTS AND DISCUSSION

Experimental Strategy. As in the two-hybrid system (4, 5), protein–protein interactions reconstitute GAL4 function and result in reporter gene activation. Previous studies have shown that aa 1–147 of GAL4 will bind specifically to a 17-base-pair (bp) DNA sequence but will not activate transcription (6, 7). The herpes simplex virus protein VP16 does not bind specifically to DNA but does function as a transcriptional activator, and most of this activity can be localized to aa 411–455 (12, 21). As illustrated in Fig. 1, a chimeric protein containing both GAL4 DNA-binding and VP16 transcriptional activation domains will activate transcription of a gene downstream of the GAL4 DNA-binding sites and E1B minimal promoter (8, 10, 11). Similarly, if the GAL4 and VP16 domains are present in the same complex and in appropriate conformation, as a result of specific interaction between the sequences fused to each of the GAL4 and VP16 domains, then the reporter gene should be expressed. In contrast, if the sequences linked to the GAL4 and the VP16 domains do not interact with sufficient affinity to form a stable complex, or if the conformation of the complex is not functional, then the reporter gene will not be expressed. Various reporter genes might be used, including genes that allow selection of cells either by drug resistance or physical techniques, such as fluorescence-activated cell sorting. Because functional interaction of the chimeric proteins occurs in the nucleus and selectable reporter genes can be used, we term the strategy KISS.

Cytoplasmic Protein–Protein Interactions. We sought to determine whether specific interactions between pairs of cytoplasmic proteins could be detected in the KISS system, as the GAL4 and VP16 plasmids used for generation of fusion genes contain sequences that should localize each chimeric protein to the nucleus. In addition, we wished to determine whether the KISS system would permit study of proteins of varying sizes and also those that interact with endogenous CHO cellular proteins of high abundance. To this end, we determined whether chimeric proteins containing sequences from two subunits of the actin-capping protein CapZ could interact and reconstitute GAL4 function. CapZ localizes to the Z line of chicken skeletal muscle and consists of α and β subunits of 33 and 31 kDa, respectively. cDNAs for two variants of the α subunit and one β subunit have been identified (22–24). The two subunits copurify through multiple fractionation steps and are known to cap actin filaments as a dimer (25, 26). Chimeric GAL4 and VP16 proteins with nearly full-length CapZ sequences were encoded by fusion genes (Fig. 2*A*). Cotransfection of plasmids encoding GAL4/CAPZβ and NLVP/CAPZα resulted in an activation of CAT expression from the pG5E1bCAT reporter gene to >5-fold the levels seen in any control transfections (Fig. 2*B*). In two

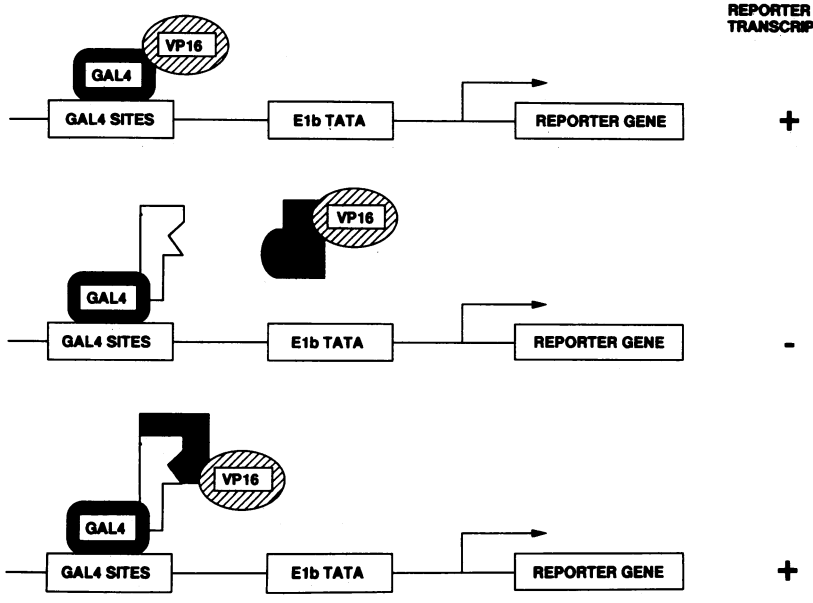

FIG. 1. Outline of KISS system. A chimeric protein consisting of the GAL4 DNA-binding domain (aa 1–147 of GAL4) and a transcriptional activation domain from the herpes simplex virus protein VP16 (either aa 411–490 or aa 411–455) can specifically activate transcription of a reporter gene located downstream of GAL4 DNA-binding sites and the E1B minimal promoter (*Top*). Similarly, two chimeric proteins, one encoding a chimeric GAL4 protein and the other encoding a chimeric VP16 protein, can activate the reporter gene, if the domains fused to the GAL4 and VP16 sequences can complex with appropriate conformation (*Bottom*). However, if the domains fused to the GAL4 and VP16 sequences do not interact specifically to form a complex that reconstitutes GAL4 function, the reporter gene cannot be activated (*Middle*).

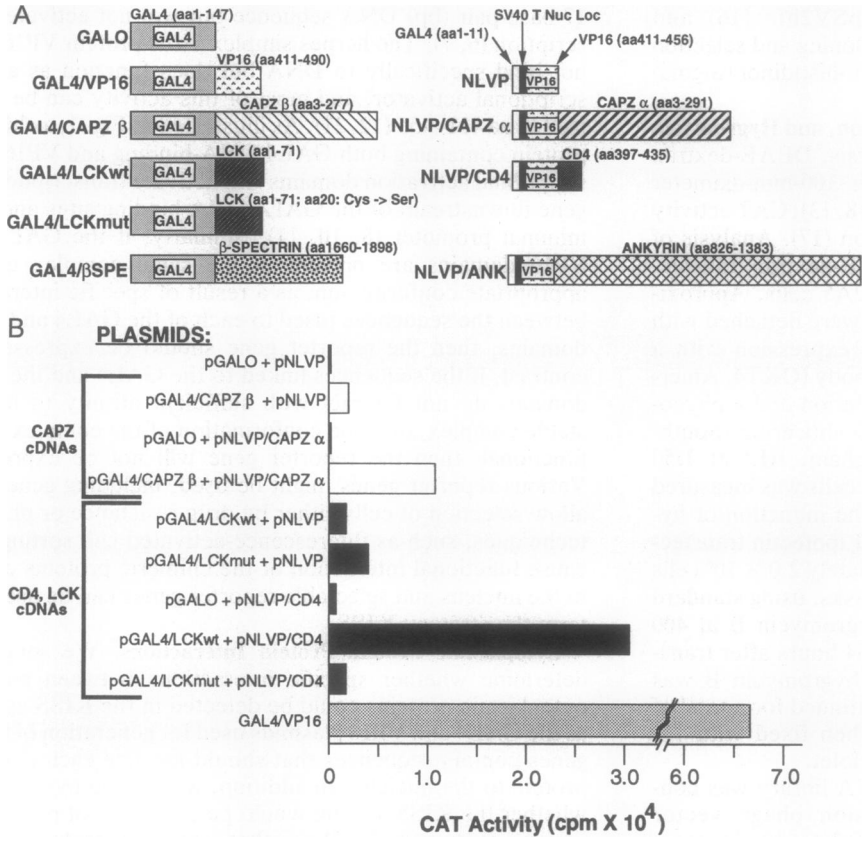

FIG. 2. Detection of protein–protein interactions for pairs of cytoplasmic proteins. (*A*) For various chimeric proteins, the sequences fused to the GAL4 and NLVP domains are indicated. They include sequences from the chicken actin-capping protein subunits CapZα_2 (22) and CapZβ (24), the murine tyrosine kinase Lck (27), the cytoplasmic domain of human CD4 (28, 29), and human β-spectrin (30, 31) and ankyrin (32). SV40 T Nuc Loc, simian virus 40 T-antigen nuclear localization signal. (*B*) Results of transient transfection analysis of CHO cells with 1 μg of the reporter-gene plasmid pG5E1bCAT and 1 μg of each fusion plasmid for the various combinations are shown. Average CAT activity measured in a single experiment performed in duplicate for all plasmid combinations is shown; results are representative of a total of three experiments, each performed in duplicate. Bar indicating CAT activity for pGAL4/VP16 is discontinuous.

additional experiments using larger quantities of plasmid DNA, the combination of pGAL4/CAPZβ and pNLVP/CAPZα exhibited a 25- to 35-fold increase in CAT activity over that seen in control transfections (data not shown). The results suggest that GAL4 function is reconstituted as a result of interaction between the CapZ domains. This observation suggests that even if the domains to be studied are present in proteins that normally may reside in the cytoplasm, and even if these sequences might bind to abundant cellular proteins (e.g., actin), their interaction can be detected in the KISS system.

To determine whether proteins that interact at the cytoplasmic membrane could be studied in the KISS system, we sought to detect interactions between domains of the CD4 and Lck proteins. CD4, a cell-surface glycoprotein that mediates the interaction of a subset of T cells with antigen-presenting cells, contains a carboxyl-terminal cytoplasmic domain that complexes with sequences in the amino-terminal region of Lck, a tyrosine kinase with an amino-terminal myristoylation signal (33–35). We generated a plasmid encoding the GAL4 domain fused to the amino-terminal 71 amino acids of murine Lck (pGAL4/LCKwt) and a plasmid encoding the VP16 domain fused to the 38 amino acids of the cytoplasmic domain of human CD4 (pNLVP/CD4) (chimeric proteins shown in Fig. 2*A*). Analysis of CAT activity from transient transfection assays with the pG5E1bCAT reporter gene and the plasmids pGAL4/LCKwt and pNLVP/CD4 revealed CAT activities 6- to 15-fold greater than those seen in control transfections (representative results from one of three experiments are shown in Fig. 2*B*). In previous studies, a mutant form of Lck, with substitution of serine for cysteine at amino acid 20, failed to complex with the CD4 cytoplasmic domain (34, 35). In support of the specificity of CD4 and Lck interaction in the KISS system, cotransfection with a plasmid encoding sequence from this mutant fused to the GAL4 domain (pGAL4/LCKmut) and the pNLVP/CD4 plasmid resulted in no activation of CAT gene expression over control levels (Fig. 2*B*). Thus, the interaction between the cytoplasmic domain of a transmembrane protein and a (normally) myristoylated cytoplasmic protein appears to be detectable and to retain specificity in the KISS system. We did note, however, that cotransfection of a plasmid encoding the CD4 sequence fused to the GAL4 domain (pGAL4/CD4) and a second plasmid encoding the wild-type Lck sequence fused to the VP16 domain (pNLVP/LCKwt) failed to activate the CAT gene (data not shown). This result suggests that conformational considerations in the KISS system may be of importance in some cases, although differences in protein stability for the various chimeric proteins have not been ruled out.

Activation of Selectable Markers. To obtain a cell-surface marker that would be induced as a result of protein–protein interactions, we constructed a plasmid (pG5E1bCD4) with the human CD4 gene under GAL4 control. Cell lines with stable integration of pG5E1bCD4 were obtained and examined for evidence that the CD4 gene could be induced by functional GAL4 but would not be expressed constitutively. One such line, CHO/CD4 2A5, was used for further studies (Fig. 3*A*). As expected, the pNLVP/FosbZIP plasmid was not able to activate CD4 expression in transient assays (Fig. 3*B*). However, following transfection with a plasmid encoding a functional GAL4 activator (pGAL4/VP16), up to 40% of the CHO/CD4 2A5 cells expressed high levels of CD4 (Fig. 3*B*). Similarly, up to 20% of cells cotransfected with plasmids expressing Fos and Jun chimeric proteins expressed CD4. In addition, in cells transfected with a constant amount of pGAL4/FosbZIP and decreasing amounts of pNLVP/JunbZip plasmid, small populations of cells expressing CD4 could be noted with as little as 0.004 μg of input pNLVP/JunbZip DNA (Fig. 3*B* and data not shown). Thus, although not demonstrated here, use of the CHO/CD4 2A5 cell line and physical selection for CD4-expressing cells (e.g., fluorescence-activated cell sorting) should make it possible to enrich for cDNAs encoding proteins that interact with another protein of interest from a library of chimeric cDNAs.

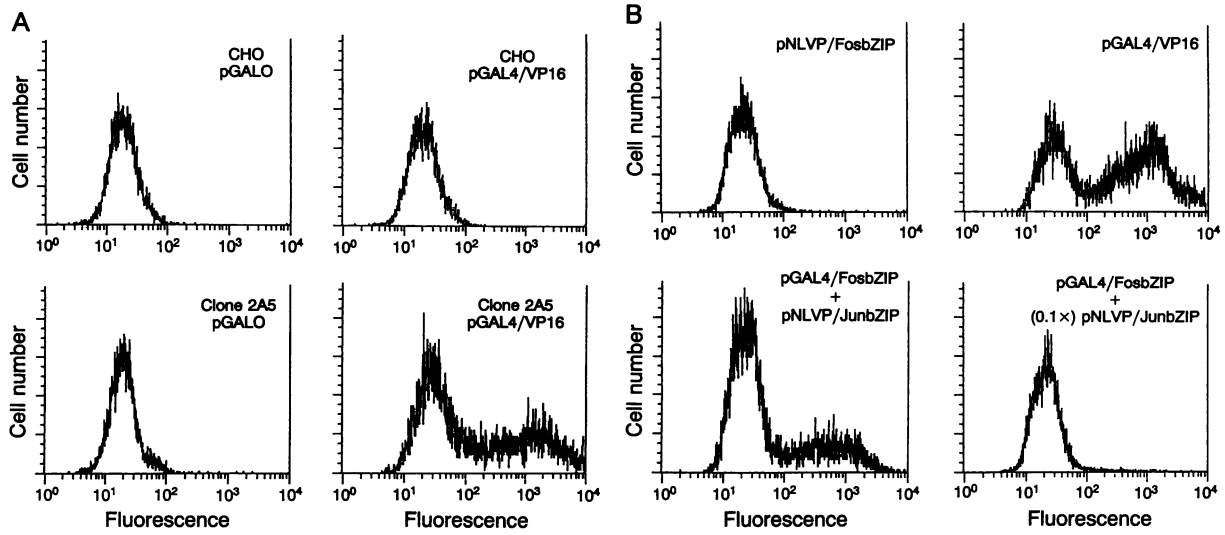

FIG. 3. Activation of cell-surface CD4 expression in a transient transfection assay. CD4 expression was analyzed 60–64 hr after transfection; the relative fluorescence of the transfected cell populations is shown. (*A*) Transfection of CHO cells and CHO/CD4 2A5 cells with either 2 μg of a plasmid encoding only the GAL4 DNA-binding domain (pGALO) or 2 μg of a plasmid encoding a chimeric GAL4 activator protein (pGAL4/VP16). CD4-expressing cells were detected following transfection of CHO/CD4 2A5 cells with pGAL4/VP16. (*B*) CD4 expression by CHO/CD4 2A5 cells following transfection with plasmids encoding GAL4 and VP16 domains. The plasmid combination encoding the chimeric Fos and Jun proteins restores GAL4 function (8). For transfection, 2 μg of each of the plasmids pNLVP/FosbZIP, pGAL4/VP16, and pGAL4/FosbZIP was used; 4 μg of pNLVP/JunbZIP or 0.4 μg of pNLVP/JunbZIP [(0.1×) pNLVP/JunbZIP] was used in the cotransfections.

We also wished to obtain a cell line containing a drug-resistance marker that could be activated by functional GAL4. The plasmid pG5E1bHygro, containing a hygromycin B resistance gene under GAL4 control, was cotransfected into the CHO/CD4 2A5 cell line with a plasmid that confers resistance to L-histidinol. L-Histidinol-resistant cells were tested for spontaneous and GAL4-inducible resistance to hygromycin B; one cell line obtained, L-His/Hygro 12B, was used for further studies. The frequency of spontaneous resistance to hygromycin in L-His/Hygro 12B cells was $<5 \times 10^{-6}$ in the selection conditions used. Lipofectin transfection of L-His/Hygro cells with no DNA or with pGALO, which encodes only the DNA-binding domain of GAL4, resulted in few, if any, hygromycin-resistant colonies (Fig. 4). In contrast, transfection of the cells with plasmid combinations that had been found to reconstitute GAL4 function in transient transfection assays resulted in stable activation of hygromycin resistance (examples in Fig. 4). Approximately $1–2 \times 10^3$ hygromycin-resistant colonies were produced by transfection of this cell line with 1 μg of pGAL4/VP16 plasmid DNA (Fig. 4 and data not shown).

In a further effort to explore the spectrum of proteins that could be studied in the KISS system, fusion genes were constructed with human ankyrin and spectrin cDNAs. These two human erythrocyte cytoskeletal proteins complex tightly with one another, with an estimated dissociation constant of 0.1 μM (36). One plasmid (pGAL4/Spe) encoded >28 kDa of β-spectrin sequence (Fig. 2*A*), encompassing the ankyrin-binding domain (30, 31), fused to the GAL4 DNA-binding domain. The other plasmid (pNLVP/Ank) encoded >65 kDa of ankyrin sequence fused to the NLVP sequence (Fig. 2*A* and ref. 32). Cotransfection of 10^6 L-His/Hygro cells with 1 μg of each of these plasmids produced about 100 hygromycin-resistant colonies (data not shown). The activation of hygromycin resistance was dependent upon the presence of intact spectrin and ankyrin sequences in the respective plasmids. The results suggest that the ankyrin and spectrin sequences interact to reconstitute GAL4 function and that interactions between protein domains as large as 28 and 65 kDa can be detected in the KISS system. In addition, the KISS system should permit definition of the minimal sequences necessary for ankyrin–spectrin interaction and may offer some advantages, since identification of the minimal regions necessary for interaction has been hampered in *in vitro* systems, due to protein insolubility (S.P.K. and J.S.M., unpublished observations).

Screening for Novel Protein–Protein Interactions. We carried out a preliminary screen to identify novel protein sequences that would interact with a chimeric GAL4 protein

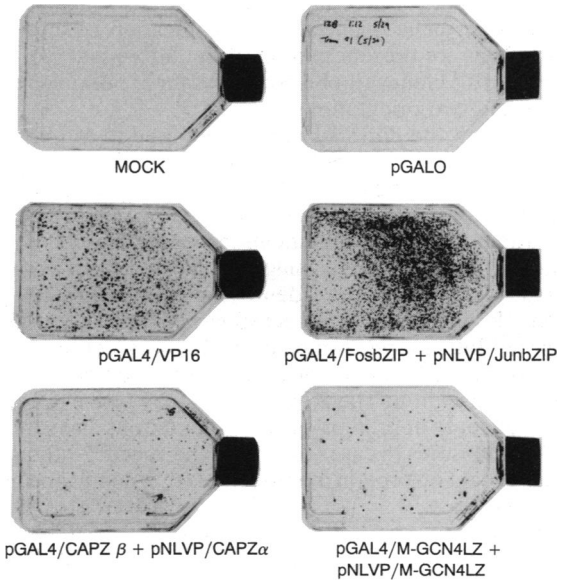

FIG. 4. Protein–protein interactions activate hygromycin B resistance. The plasmids used have been described (8) or encode chimeric proteins shown in Fig. 2*A*. Lipofectin transfection of $\approx 2 \times 10^6$ cells was carried out with no plasmid DNA (MOCK), with 0.5 μg of pGAL4/VP16 DNA, or with 2 μg of each of the other plasmid DNAs. The pairs of plasmids used have been shown to restore GAL4 function in transient transfection assays (this paper and ref. 8). The M-GCN4LZ domain contains aa 262–439 of c-Myc, except that the leucine zipper (LZ) of the yeast GCN4 protein has been substituted for the LZ of c-Myc (8). Cells remaining in the flasks following hygromycin selection were fixed and stained, and the flasks were photographed.

containing the carboxyl-terminal region (aa 262–439) of human c-Myc (GAL4/M262–439). A λNLVP/colon cDNA expression library encoding chimeric VP16 proteins was created (see *Materials and Methods*). Screening was carried out by transfection of thirty 75-mm^2 flasks of L-His/Hygro cells, each with 1 μg of pGAL4/M262–439 DNA and 1 μg of λNLVP/colon cDNA library DNA. A single hygromycin-resistant colony was obtained and expanded into a cell line. Library sequences were recovered from the DNA of the resistant cells by PCR with pairs of oligonucleotide primers flanking the cDNA cloning site in λNLVP. Seven unique cDNAs ranging in size from 104 to 280 bp were identified, subcloned, and sequenced. Those cDNAs containing an open reading frame in the context of the VP16 fusion protein sequence were subcloned into the *Xho* I site of pNLVP and tested for their ability to induce hygromycin resistance in L-His/Hygro 12B cells either by themselves or in collaboration with the GAL4/M262–439 chimeric protein.

After transfection, sequences encoded by one insert of 119 bp, known as Frag2, when present in a VP16 chimeric protein, cooperated with the GAL4/M262–439 chimeric protein to activate hygromycin resistance. Transfection of plasmids encoding either the GAL4/c-Myc or NLVP/Frag2 chimeric proteins alone did not activate hygromycin resistance. The pNLVP/Frag2 plasmid, however, was able to activate hygromycin resistance when cotransfected with pGALO. Thus, the Frag2 insert does not encode a protein that interacts specifically with c-Myc. Rather, the NLVP/Frag2 chimeric protein may cooperate with the GAL4 DNA-binding domain to activate hygromycin resistance by binding to the GAL4 domain. The predicted sequence of the Frag2 cDNA fragment, in the context of the reading frame of the VP16 chimeric protein is GRGGPVLGWAYPGPGSASPALSRSPSEGTGTPPAAG (in single-letter amino acid code). The frequency of the Frag2 insert in the λNLVP/colon cDNA library was about 1 in 10^4 clones. Thus, a preliminary estimate is that transfection of about thirty 75-mm^2 flasks of L-His/Hygro cells might allow identification of interacting cDNAs that are present in a library at a frequency of about 1 in 6.0×10^4 clones (if one considers three possible reading frames and two orientations).

At present, the KISS system can be used to establish that protein interactions identified by other methods can be observed in mammalian cells. The system also might be useful for studying the intracellular effects of small molecules and various secondary modifications on protein interactions. While preliminary studies suggest that the yeast two-hybrid system may be useful for identifying protein–protein interactions (5), the utility of the KISS system for such studies is suggested but not yet established. The interaction of some proteins may depend on proper secondary modification, such as phosphorylation. The KISS system may prove to be most useful for study of protein–protein interactions and screening of cDNA libraries in cases for which the specific interactions are dependent on protein modifications that do not occur with fidelity in yeast.

We thank the following for plasmids: Drs. J. Lillie, M. Green, I. Sadowski, M. Ptashne, R. Mulligan, B. Vogelstein, D. Littman, A. Shaw, J. Rose, O. Platt, and S. Lux. We thank Drs. D. Pardoll and H. Levitsky for help with analysis of CD4 expression; J. Barrett and A. Laudolfi for technical assistance; and Drs. P. Goldschmidt, B. Vogelstein, and J. Rose for comments on the manuscript. This work was supported in part by grants from the National Institutes of Health and the American Cancer Society.

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