

A positive genetic selection for disrupting protein–protein interactions: Identification of CREB mutations that prevent association with the coactivator CBP

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ABSTRACT The *Escherichia coli* *tet*-repressor (TetR) operator system was used to develop a variation of the yeast two-hybrid assay in which disruptions of protein–protein interactions can be identified by a positive selection. This assay, designated the “split-hybrid system,” contains a two-component reporter. The first component contains LexA binding sites upstream of the TetR gene and the second contains TetR operator binding sites upstream of *HIS3*. Interaction of one protein fused to the LexA DNA binding domain with a second protein fused to the VP16 activation domain results in TetR expression. TetR subsequently binds to the *tet* operators, blocking the expression of *HIS3* and preventing yeast growth in media lacking histidine. The utility of the split-hybrid system was analyzed by examining the phosphorylation-dependent interaction of CREB and its coactivator CREB binding protein (CBP). CREB and CBP associate through an interaction that depends upon CREB phosphorylation at Ser-133. Mutation of this phosphorylation site prevents yeast growth in the standard two-hybrid assay but allows growth in the split-hybrid strains. The split-hybrid system was used to identify other CREB mutations that disrupt its association with CBP. These mutations localized around the site of CREB phosphorylation, indicating that only a small portion of the CREB activation domain is required for CBP interaction. The yeast split-hybrid system should be useful in identifying mutations, proteins, peptides, and drugs that disrupt protein–protein interactions.

We have developed a variation of the yeast two-hybrid system (1) that permits identification of factors that abrogate or “split” the association of two interacting proteins. This yeast genetic assay, which we have termed the “split-hybrid system,” can also be used to screen for mutations that block specific associations.

The split-hybrid system converts the disruption of a protein–protein interaction into a positive selection by using the *Escherichia coli* Tn10-encoded *tet*-repressor (TetR) operator system (2). We have engineered LexA binding sites upstream of the TetR gene and have placed TetR binding sites (*tet* operators) upstream from a nutritional reporter gene, *HIS3*. Interaction of one protein fused to LexA (which contains a DNA binding domain) with a second protein fused to the VP16 activation domain results in the expression of TetR, which binds to the *tet* operators, blocking expression of *HIS3* and preventing yeast growth in media lacking histidine. As with the conventional yeast two-hybrid system, this method allows large numbers of clones to be screened by a selection. In addition, the interaction of TetR with *tet* operators can be abolished by tetracycline (Tc). The ability to modulate the selection with Tc is useful when the protein fused to LexA has intrinsic activating

capability. Furthermore, 3-aminotriazole (3AT), an inhibitor of the histidine pathway, can be used when the interaction of two proteins is relatively weak and unable to produce sufficient TetR to block histidine production fully.

In the present study, we examined two established protein–protein interactions: the serum response factor (SRF) and human T-cell leukemia virus protein Tax and the transcription factor CREB and its binding protein (CBP). Tax activates the *c-fos* promoter through the serum response element (3). This activation results from a direct interaction between Tax and SRF, as Tax does not bind to the serum response element directly (4, 5). CBP is a coactivator of CREB that mediates the transactivation of cAMP-responsive promoters (6, 7). cAMP activates the cAMP-dependent protein kinase A (PKA), which phosphorylates CREB at Ser-133, located in the kinase-inducible domain (KID). The phosphorylation of Ser-133 allows CREB to associate with CBP (6). In this paper, we demonstrate that these interactions prevent growth in the yeast split-hybrid strains. In contrast, the expression of CBP and a CREB mutant (CREB-M1; Ser-133 → Ala), which is unable to bind CBP, prevents production of TetR and allows the yeast to grow. We used the split-hybrid system to screen for other mutations in CREB that disrupt its association with CBP and determined that most of the mutations were located within a small region immediately adjacent to the PKA phosphorylation site.

MATERIALS AND METHODS

Plasmid Construction. For pLexA–CBD, a DNA fragment containing the CREB binding domain (CBD) of CBP (amino acids 461–682) was PCR amplified and inserted into the *EcoRI* and *BamHI* sites of pBTM116 that carries the *TRP1* gene (8). For pLexA–SRF, a DNA fragment containing SRF was digested from pCGN–SRF (gift from M. Gilman, Ariad Pharmaceuticals, Boston) with *XhoI* and *BamHI*. The *XhoI* site was blunt-ended, ligated with *BamHI* linkers, and inserted into the *BamHI* site of pBTM116. pLexA–Lamin was a gift from R. Sternglanz (The State University of New York at Stony Brook). For pVP16–CREB, pcDNA3/CREB283, containing the VP16 transactivation domain fused to the rat CREB 341 (amino acids 1–283) (a gift from R. Maurer, Oregon Health Sciences University, Portland), was digested with *XbaI*, blunt-ended, and ligated with *BamHI* linkers. The fragment encoding the pVP16–CREB fusion protein was digested with *BamHI* and *HindIII* and inserted into pVP16, which carries the *LEU2* gene (9). For pVP16–Tax, the DNA sequence encoding Tax was digested from pS6424 (10) with *BamHI* and inserted into

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Abbreviations: TetR, *tet*-repressor; Tc, tetracycline; 3AT, 3-aminotriazole; SRF, serum response factor; PKA, cAMP-dependent protein kinase A; KID, kinase-inducible domain; CBD, CREB-binding domain; CBP, CREB binding protein; β -gal, β -galactosidase.

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pVP16. For pLeu, pVP16 was digested with *Hind*III and *Bam*HI to remove the VP16 transactivation domain, blunt-ended, and religated. For pLexA-VP16, the VP16 transactivation domain was PCR amplified, digested with *Clal*, blunt-ended, and inserted into the *Sma*I site of pBTM116. For pRS306/8xLexA op/TetR, the ADH terminator sequence was digested from pBTM116 with *Sph*I and *Pst*I, blunt-ended, and cloned into the blunt-ended *Not*I site of pRS306 (11) to yield pRS306/Term. PCR was used to link the 5' promoter sequence of the yeast *HIS3* gene (nucleotides -75 to +23) to the translational start of TetR. Sequences encoding the simian virus 40 large T-antigen nuclear localization signal (NLS) were fused to the C terminus of TetR. The PCR product was digested with *Eco*RI and *Bam*HI and inserted into pRS306/Term. This construct, pRS306/*HIS3*:TetR/Term, retains the fusion of TetR to the nuclear localization signal, followed by four amino acids generated by the vector backbone (Arg-Ile-His-Asp). The LexA binding site multimer from the plasmid pSH18-34ΔSpe (gift from R. Brent, Harvard Medical School) was PCR amplified (9), digested with *Eco*RI, and subcloned into pRS306/*HIS3*:TetR/Term to produce pRS306/8xLexA op/TetR. For pRS303/2*x*tet op-*LYS2*, PCR was used to engineer one copy of the *tet* operator (created by annealing complementary oligonucleotides) into position -53 of pRS303 (11). For the second *tet* operator site, a *Mlu*I restriction site was engineered into position -22 in the *HIS3* promoter by PCR. One copy of the *tet* operator was inserted into the *Mlu*I site to yield pRS303/2*x*tet op. The *LYS2* gene was isolated from pLYS2 (9) with *Eco*RI and *Bam*HI, blunt-ended, ligated with *Sst*I linkers, and ligated into pRS303/2*x*tet op. For pVP16-CREB(*Bgl*II-*Sac*II)-LacZ, β -galactosidase (β -gal) was PCR amplified from pSV- β -gal (Promega) and inserted into the *Not*I site of pVP16 to produce pVP16-LacZ. A PCR fragment containing CREB (amino acids 1-283) was inserted into pVP16-LacZ at the *Bam*HI site. To generate a cassette vector for subcloning, PCR was used to engineer a *Bgl*III site at nucleotides 273-278 and a *Sac*II site at nucleotides 500-505 of CREB.

Yeast Strain Construction. Yeast were grown in yeast extract/peptone/dextrose or selective minimal medium using standard conditions (12, 13). Yeast strains were derived from AMR69 and AMR70 (9). YI584 was constructed from AMR69 and AMR70 as follows: (i) *Ura*⁻ derivatives were identified after 5-fluoroorotic acid selection (15); (ii) targeted integration of pRS306/8xLexA op/TetR was carried out by transforming with a plasmid linearized at a *Nco*I site in *URA3* and the resulting strains were examined by Southern blot analysis; (iii) targeted integration of pRS303/2*x*tet op-*LYS2* was carried out by transforming with the plasmid linearized at a *Hpa*I site in *LYS2* and the resulting strains were confirmed by Southern blot analysis; (iv) YI584 was constructed by mating the AMR69-derivative *MATa* strain containing the pRS303/2*x*tet op-*LYS2* with the AMR70-derivative *MATa* strain containing pRS306/8xLexA op/TetR. The genotype of YI584 is (*MATa*/*MATa*, *his3*Δ200/*his3*Δ200 *trp1*-901/*trp1*-901 *leu2*-3, 112/*leu2*-3, 112 *ade2*/*ade2* containing *URA*::(LexA operator)₈-TetR *LYS2*::(Tet operator)₂-*HIS3*).

Liquid Assay. YI584 was transformed as described (9) and plated on selective medium plates lacking tryptophan, uracil, leucine, and lysine. After three days growth at 30°C, a pool of colonies was diluted in 5 ml of selective media, vortexed, and sonicated for 10 sec. Cells were counted and seeded at 1000 cells/ml selective media at a final volume of 2 ml. Tc, 3AT, and histidine were supplemented as appropriate. Samples were incubated with shaking for two days at 30°C and were quantitated by measuring the OD₆₀₀.

β -Gal Liquid Assay. The β -gal liquid assays were performed as described (14).

PCR Mutagenesis and Creation of Mutant Library. Mutagenic PCR was a minor modification of that described (15).

The reaction mixture contained 20 ng of pVP16-CREB(*Bgl*II-*Sac*II)-LacZ, 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 6.1 mM MgCl₂, 0.5 mM MnCl₂, 6.7 μ M EDTA, 10 mM 2-mercaptoethanol, 1 mM primers, 1 mM each dGTP, dTTP, and dCTP, 400 μ M dATP, and 2.5 units of *Taq* DNA polymerase (Promega). After seven cycles of PCR (94°C for 40 sec, 50°C for 40 sec, and 72°C for 40 sec), the PCR product was reamplified with *Vent* DNA polymerase (New England Biolabs) for 25 cycles. The resultant PCR product was digested with *Bgl*II and *Sac*II and inserted into pVP16-CREB(*Bgl*II-*Sac*II)-LacZ. Ligations were transformed into DH5 α bacterial cells. Transformants were pooled and plasmid DNA was isolated by CsCl gradient centrifugation.

Library Screen. The yeast strain YI584 expressing pLexA-CBD was transformed with a library of pVP16-CREB(*Bgl*II-*Sac*II)-LacZ mutants. 4 ml of cells (OD₆₀₀ = 0.6) were transformed with 40 μ g of library and 4 mg of salmon sperm DNA and plated onto selective medium supplemented with 10 μ g/ml of Tc and 1 mM 3AT. β -Gal filter assays were performed as described (16) to identify full-length proteins. DNA from 536 growth(+), 5-bromo-4-chloro-3-indoyl β -D-galactoside(+) colonies was isolated and transformed into *E. coli* MC1066 cells. The pLexA-CBD plasmid was cured by culturing the MC1066 cells in the absence of leucine (17). The DNA from 193 VP16-CREB(*Bgl*II-*Sac*II)-LacZ mutants was isolated and rescreened in YI584 cells, as well as in L40 cells (two-hybrid system; ref. 16). Two-hybrid transformations were plated on selective medium supplemented with 40 mM 3AT. CREB mutants that did not interact with CBD in the rescreening process were sequenced using Sequenase (United States Biochemical, version 2.0).

RESULTS

Characterization of the Split-Hybrid System. The yeast split-hybrid strain contains two integrated reporter constructs (Fig. 1A). One reporter contains the coding sequence of TetR flanked upstream by the yeast *HIS3* minimal promoter and a tandem array of LexA operators. To ensure efficient transport of TetR into the nucleus, the nuclear localization signal of simian virus 40 large T antigen was fused to the C-terminal end of TetR. The other reporter contains *tet* operator sequences inserted into the promoter region of the *HIS3* gene. The expression of TetR in this system depends on the formation of a complex between one protein fused to LexA and another protein fused to the VP16 activation domain. When the fusion proteins interact, the TetR gene is activated. The TetR protein binds to the *tet* operators and represses promoter activity of *HIS3*, which causes the yeast to be auxotrophic for histidine. Conversely, when the interaction of two fusion proteins is disrupted (Fig. 1B), no TetR protein is generated, allowing expression of the *HIS3* gene and producing yeast prototrophic for histidine.

To test the utility of the reporter constructs, we first transformed the yeast split-hybrid strain YI584 with pLexA-VP16. pLexA-VP16 encodes a fusion protein containing the VP16 activation domain and LexA and is a strong transactivator for promoters containing LexA operators. Yeast expressing LexA-VP16 generate TetR protein as detected by gel mobility shift assay (data not shown). These yeast were unable to grow in the absence of histidine, indicating that the overexpressed TetR is capable of binding to *tet* operators and preventing the expression of *HIS3* (Fig. 2A and B). These strains grew on plates containing histidine, however, indicating that TetR overexpression was not toxic. Yeast expressing pLexA-lamin and pVP16, which do not interact in a yeast two-hybrid system (Fig. 2C) and are thus not expected to lead to the production of TetR, grew in the absence of histidine (Fig. 2A and B).

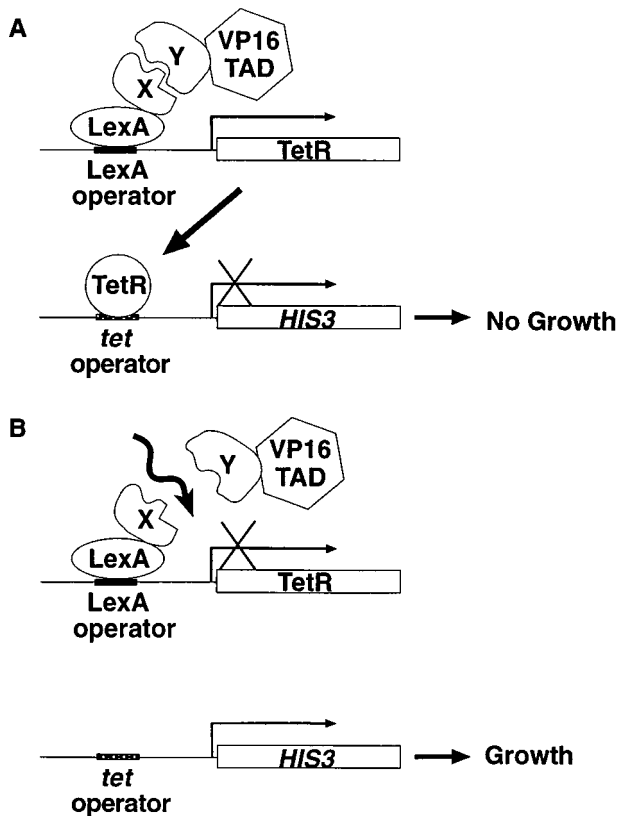


FIG. 1. The yeast split-hybrid system. (A) Interaction of protein X fused to LexA with protein Y fused to the VP16 transactivation domain (TAD) promotes the expression of TetR which, in turn, binds to *tet* operators upstream of the *HIS3* gene, preventing the growth of yeast in medium lacking histidine. (B) Disruption (depicted by wavy arrow) of the interaction between proteins X and Y prevents expression of TetR, permitting expression of *HIS3* and allowing growth of yeast in medium lacking histidine.

To investigate the feasibility of using the split-hybrid system to study protein-protein interactions, a pair of interacting proteins, SRF and Tax, was tested. pLexA-SRF was transformed into strain Y1584 along with pVP16-Tax. This transformation failed to yield any colonies in medium lacking histidine (Fig. 2A and B). In contrast, when LexA-SRF was cotransformed with a vector encoding the VP16 activation domain alone, yeast growth occurred in the absence of histidine. LexA-SRF and VP16-Tax interact relatively weakly in the yeast two-hybrid system (Fig. 2C), yet almost completely abolish growth when expressed in the split-hybrid strain. VP16-Tax is slightly toxic (unpublished observations), which may explain why LexA-CBD and VP16 activate LacZ expression more strongly than LexA-SRF and VP16-Tax in the two-hybrid strain (Fig. 2C) but are unable to fully abolish yeast growth when expressed in the split-hybrid strain (Fig. 2B).

Modulation of the Split-Hybrid System. A major advantage of using the TetR-operator system is the ability to block TetR binding with Tc. This ability to modulate TetR binding is critical when the LexA fusion protein has intrinsic activating capability, as illustrated in experiments using yeast expressing the CREB-binding domain (CBD) of CBP fused to LexA (LexA-CBD). Yeast expressing LexA-CBD and VP16 had substantially decreased growth in medium lacking histidine (Fig. 2A and B). Indeed, the levels of growth observed when yeast were cotransformed with pLexA-CBD and pVP16 or pVP16-CREB were almost indistinguishable. To distinguish growth differences between strains expressing VP16 and VP16-CREB, varying concentrations of Tc were added to liquid cultures of yeast grown in the absence of histidine. The

repression of yeast growth was relieved by Tc in a dose-dependent fashion (Fig. 3A). These results demonstrate that Tc can overcome basal transactivating capability of the LexA-CBD fusion protein.

To use the split-hybrid system most efficiently, it is important to minimize background growth that might be confused with disrupted protein-protein associations. This can be accomplished with 3AT, a competitive inhibitor of the *HIS3* gene product. For instance, in the presence of 10 $\mu\text{g/ml}$ Tc, the yeast strain transformed with pLexA-CBD and pVP16-CREB still conferred $\approx 12\%$ growth in liquid culture (Fig. 3A). To diminish this background, varying concentrations of 3AT were added to the culture media in the presence of 10 $\mu\text{g/ml}$ Tc. At 0.25 mM 3AT, the growth of the yeast strain expressing LexA-CBD and VP16-CREB was $< 5\%$, while the growth of the control strain was still maintained at 70% of control levels.

Selection of Mutants Defective in a Protein-Protein Interaction. One use of the split-hybrid system is to identify point mutants that disrupt protein-protein interactions. The binding of CREB to CBP has been shown to require the phosphorylation of CREB Ser-133, located in the KID (6, 7). Changing Ser-133 to alanine (CREB-M1) abolishes the ability of CBP to activate CREB-mediated transcription. We tested CREB-M1 in the split-hybrid system and verified that this mutation prevents the interaction with CBP (data not shown). Precisely which other residues of the KID of CREB are required for binding to CBP are unknown, however. To define these residues, the KID (amino acids 102-160) of CREB 341 was randomly mutagenized using PCR.

A DNA fragment encoding the β -gal gene was fused to the C-terminal end of VP16-CREB (Fig. 4) to allow identification of clones that contain frame-shift and nonsense mutations. Colonies positive for β -gal are presumed to contain an ORF throughout the mutated region. To facilitate subcloning of mutated sequences, we created a cassette version of the CREB cDNA that contains *Bgl*II and *Sac*II sites flanking the 5' and 3' ends of the KID, respectively. These modifications altered amino acid 168 from valine to alanine. Primers flanking the KID were used for mutagenic PCR under conditions optimized to achieve 1-3 mutations for the 177-bp region. The PCR products were introduced into pVP16-CREB(*Bgl*II-*Sac*II)-LacZ in place of wild-type sequence and a library of mutations was transformed into yeast strain Y1584 expressing LexA-CBD. Yeast transformants ($n = 27,000$) were screened, yielding about 5000 colonies capable of growing on selective media supplemented with 10 $\mu\text{g/ml}$ Tc and 1 mM 3AT. The concentration of 3AT needed to suppress background growth on plates is higher than that needed for liquid culture (Fig. 3B). Control experiments (Y1584 transformed with pLexA-CBD and pVP16-CREB(*Bgl*II-*Sac*II)-LacZ) indicated that the background colony formation probably represents 5-9% of the transformants plated under these conditions.

Two additional screening steps were performed to eliminate uninformative mutations and false positives. First, filter β -gal assays were performed on the 5000 growth(+) colonies to eliminate proteins with frame-shift and nonsense mutations. Five hundred thirty-six colonies developed a dark blue color, whereas 412 colonies remained white. The rest of the colonies developed a pale blue color. The white colonies express mutants that contain frame-shift or nonsense mutations. The pale-blue colonies may represent unstable LacZ fusion proteins, and these were not analyzed further. CREB cDNA from 193 dark blue colonies were isolated. These cDNAs were separately retransformed along with LexA-CBD into the split-hybrid strain as well as into the two-hybrid L40 strain (16). These steps eliminated false positives that arise from background colony growth (see above) and confirmed that the mutant CREB protein did not interact

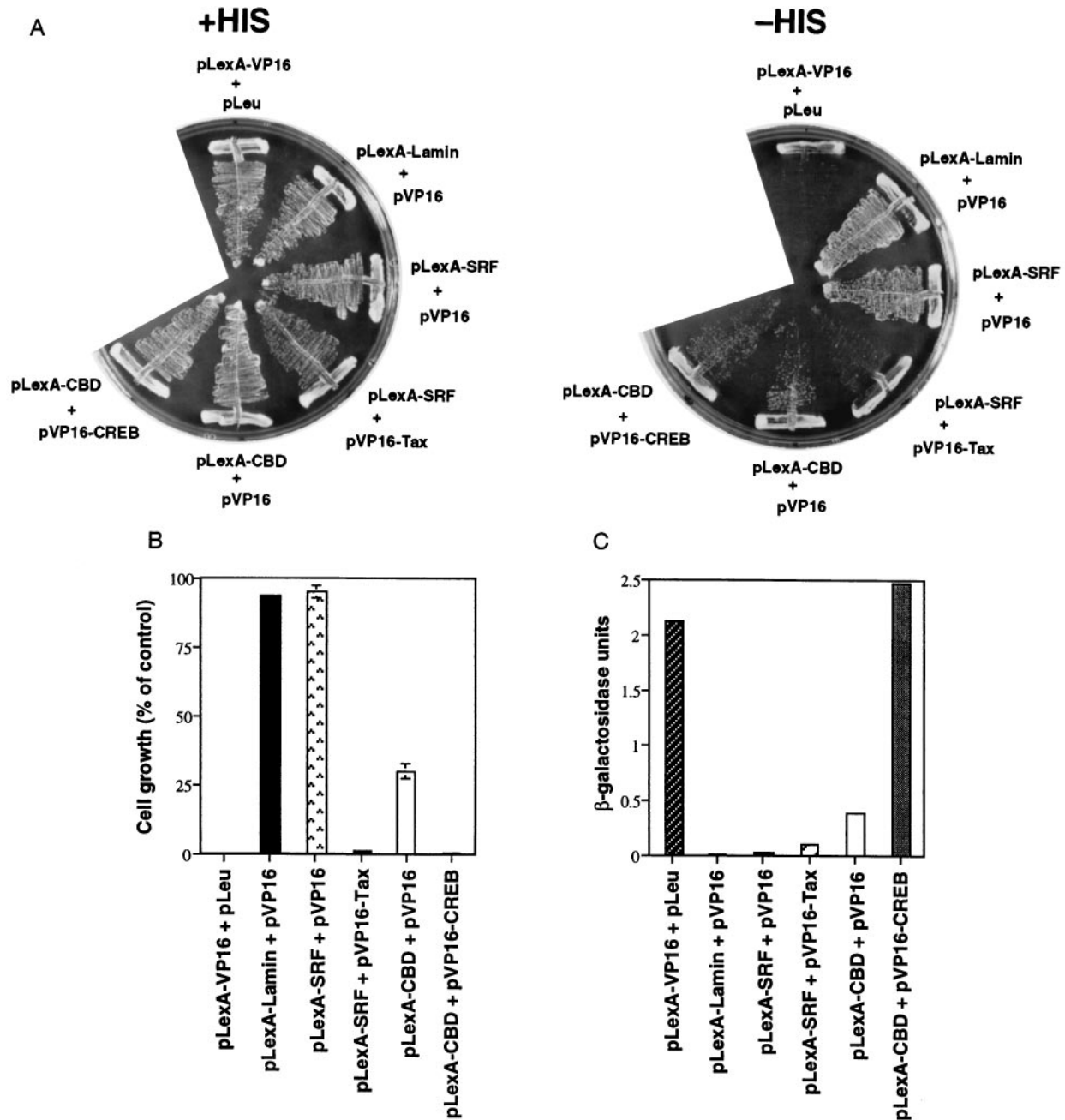


FIG. 2. Characterization of the yeast split-hybrid system. The yeast split-hybrid strain Y1584 (*A* and *B*) are the yeast two-hybrid strain L40 (*C*) were transformed with the indicated plasmid pairs. Yeast transformants were plated on selective medium plates (lacking tryptophan, uracil, leucine, and lysine) supplemented with histidine and incubated at 30°C for three days. (*A*) A pool of colonies for each interacting pair of proteins was streaked onto selective medium supplemented with histidine (*Left*) and onto selective medium lacking histidine (*Right*). The plates were incubated at 30°C for 3 days. (*B*) A pool of colonies was seeded in selective media lacking histidine. After incubation of cultures at 30°C for 2 days, growth was measured at OD₆₀₀. Data represent the mean \pm SD of two or three experiments and is presented as a percentage of the OD₆₀₀ of yeast grown in selective media supplemented with histidine. (*C*) A pool of colonies was collected and prepared for β -gal liquid assay. Data represent the mean \pm SD of two experiments. A β -gal unit = 1000 \times OD₄₂₀/time \times protein, where the time is in minutes and the protein is in mg/ml.

with CBP. Among the 193 cDNAs rescreened, 152 did not interact with CBP in the yeast two-hybrid system, 15 interacted weakly, and 26 interacted like wild-type CREB.

Seventy of the 152 CREB mutants were found to contain single amino acid changes, 64 contained two amino acid residue mutations, and 13 contained more than two mutations. Mutants containing more than one amino acid alteration were not analyzed further. The expression levels of mutant proteins with one amino acid change were determined by Western analysis to be identical to that of wild-type VP16-CREB(*BgIII-SacII*)-*LacZ*. Immunofluorescence assays dem-

onstrated that the CREB mutants were localized in the nucleus (data not shown).

The distribution of CREB mutations identified in the split-hybrid screen centered around the phosphorylation site at Ser-133 (Fig. 5). No disrupting mutations occurred outside of the region between amino acids 130–141. Most of the mutations abrogated the PKA phosphorylation motif, but others were found at Ile-137, Leu-138, and Leu-141. The ability of the split-hybrid system to detect only a limited number of CREB mutants, many of which have been proposed previously to disrupt CREB association with CBP (18), indicates the specificity of the split-hybrid system.

