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Genetics

Reverse two-hybrid and one-hybrid systems to detect dissociation of protein–protein and DNA–protein interactions

(*URA3*/negative selection/5-fluoroorotic acid)

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ABSTRACT **Macromolecular interactions define many biological phenomena. Although genetic methods are available to identify novel protein–protein and DNA–protein interactions, no genetic system has thus far been described to identify molecules or mutations that dissociate known interactions. Herein, we describe genetic systems that detect such events in the yeast *Saccharomyces cerevisiae*. We have engineered yeast strains in which the interaction of two proteins expressed in the context of the two-hybrid system or the interaction between a DNA-binding protein and its binding site in the context of the one-hybrid system is deleterious to growth. Under these conditions, dissociation of the interaction provides a selective growth advantage, thereby facilitating detection. These methods referred to as the "reverse two-hybrid system" and "reverse one-hybrid system" facilitate the study of the structure–function relationships and regulation of protein–protein and DNA–protein interactions. They should also facilitate the selection of dissociator molecules that could be used as therapeutic agents.**

Many biological processes rely upon macromolecular interactions such as protein–protein and DNA–protein interactions. Traditionally, the tools available to identify and characterize molecular interactions have been limited to biochemistry. However, a genetic method, the two-hybrid system, was described that allows in a single step both the identification of potential interacting proteins and the isolation of the encoding genes (1). Two interacting proteins (X and Y) are expressed in *Saccharomyces cerevisiae* as hybrids fused to a DNA-binding domain (DB-X) or an activation domain (AD-Y), respectively. DB-X/AD-Y interaction reconstitutes a functional transcription factor that activates a reporter gene driven by a promoter containing DB binding sites. Positive growth selections using prototrophic selectable markers as reporter genes are possible (2–4) and facilitate detection of protein–protein interactions: a few growing yeast colonies expressing DB-X/AD-Y-interacting proteins can conveniently be identified among many nongrowing colonies.

A one-hybrid system was also described that allows the identification of DNA-binding proteins (DBPX) and the isolation of their encoding genes (5, 6). In that setting, DBPX is expressed as a hybrid with an activation domain (DBPX-AD). Interaction between DBPX-AD and its binding site(s) activates a reporter gene that can also be a prototrophic selectable marker (5, 7).

Subsequent to their identification, it is often crucial to characterize the structure–function relationships and the regulation of protein–protein or DNA–protein interactions. In both cases, rare events that dissociate the interaction must be recognized. (*i*) The detailed characterization of the structure–function relationships of an interaction requires the identification of dissociating mutations in the two partners. (*ii*) Important regulatory mechanisms can be unraveled by the identification of molecules that mediate their function by dissociation of particular protein–protein interactions (see below). Neither the two-hybrid system nor the one-hybrid system allows detection of such dissociation events; instead they are limited to positive selection for protein–protein association events.

In this report, we describe "reverse" two- and one-hybrid systems that detect mutations or molecules that dissociate protein–protein or DNA–protein interactions, respectively.

METHODS

Yeast Strains and Manipulations. MaV52 (*MAT***a** *ura3-52 leu2-3, 112 trp1-901 his3*Δ*200 ade2-101 gal4*Δ *gal80*Δ *can1*^{R} *cyh2*^{R} *GAL1*::*HIS3@LYS2 GAL1*::*lacZ*) is a derivative of Y153 (3) obtained by 5-fluoroorotic acid (FOA) selection to eliminate *GAL1*::*lacZ@URA3* and subsequent canavanine selection. Isogenic derivatives of MaV52 containing *SPO13*::*URA3* fusions with various numbers (*n*) of GAL4 binding sites (*SPALn*::*URA3*) are MaV95 (*SPAL5*::*URA3*), MaV96 (*SPAL7*::*URA3*), MaV97 (*SPAL8*::*URA3*), MaV99 (*SPAL10*::*URA3*), and MaV94 (*SPALX*::*URA3* for which the number of Gal4 binding sites has not been established). Strains MaV108 (*MAT***a**) and MaV103 (*MAT***a**) are segregants of a cross between MaV99 and PCY2 (8). Methods for yeast manipulations were as described (9).

Construction and Integration of *SPAL*::*URA3* Reporter Genes. We used a construct in which the *SPO13* promoter drives the expression of a protein fusion in which the first 15 aa of SPO13 are fused to URA3 (pPL128; R. Strich and R. Esposito, personal communication). The *SPO13*::*URA3* fragment was excised from pPL128 and cloned into a pBSK plasmid (Stratagene). pMV252, the resulting plasmid, contains *Eco*RI sites at positions −170 and −368 in the *SPO13* promoter. A fragment containing 5 GAL4 binding sites (10) was cloned into pMV252 in the *Eco*RI sites to yield pMV262-11 and pMV262-12 with 5 and 15 GAL4 binding sites, respectively.

The *SPAL*::*URA3* alleles were integrated at the *ura3-52* locus by homologous recombination of the products of a PCR. The 5′ primer used was JB516 that contains 40 nt of the *URA3* sequence upstream of its promoter (positions −257 to −218) fused to 20 nt of the *SPO13* promoter [positions −370 to −351 (11)]: 5′-GAAGGTTAATGTGGCTGTGGTTTCA-GGGTCCATAAAGCTTGTCCTGGAAGTCT-CATGGAG-3′. The 3′ primer used was 3′URA3 [*URA3* sequence of positions +656 to +632 (12)]: 5′-TCAGGATC-

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Abbreviations: FOA, 5-fluoroorotic acid; Foa^{S}, FOA sensitive; Foa^{R}, FOA resistant; pRB, retinoblastoma gene product.

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CCTAGGTTCCTTTGTTACTTCTTCCG-3′. The PCR products were transformed (13) directly into MaV82 [MaV52 transformed with pCL1 (1)]. Integration at *ura3* was confirmed and the number of GAL4 binding sites was estimated by a PCR using genomic DNA as template, JB536 (*URA3* sequence from positions −298 to −276, 5′-GCGAGGCATATTTATGGTGAAGG-3′) as 5′ primer, and 13–5 [*SPO13* antisense sequence from positions −124 to −145, 5′-CATTTCCGTGCAAGGTACTAAC-3′ (11)] as 3′ primer.

Construction and Integration of *UAS53*::*URA3* Reporter Genes. For construction of the *1cUAS53*::*URA3* reporter gene, oligonucleotides corresponding to the p53 consensus DNA binding site [JB820, 5′-AATTTAGGCATGTCTAGGCATGTCTA-3′, and JB821, 5′-AATTTAGACATGCCTAGACATGCCTA-3′ (14)] were annealed, phosphorylated, and ligated into *Eco*RI-digested pMV252.

The *1cUAS53*::*URA3* fusion was PCR-amplified as described for the *SPAL*::*URA3* fusions and transformed into BY385 (*MAT***a** *ura3-52 his3*Δ*200 trp1*Δ*63 leu2*Δ*1 lys2*Δ*202*) containing pRB16. About 20% of the transformants selected on Sc-URA had a plasmid-dependent Ura^+ phenotype.

Plasmid Constructions. The cFos and cJun fusions (DB-cFos, pPC76; DB-Jun, pPC75; AD-Jun, pPC79) are gifts from P. Chevray and D. Nathans (8). The remaining two-hybrid fusions were generated by cloning PCR products in-frame into plasmids pPC97 (DB) (pPC97 is pPC62 containing the pPC86 polylinker) or pPC86 (AD) (8). DB-pRB, aa 302–928 of the retinoblastoma gene product (pRB); DB-pRBΔ22, aa 281–894 of mutant pRB containing a deletion of exon 22 (15); DB-p107, aa 372-1068 of p107; AD-E2F1, aa 342–437 of E2F1; AD-E2F1Y411C, aa 342–437 of mutant E2F1 containing a tyrosine to cysteine change at aa 411 (16); AD-E2F4, AA1-413 of E2F4 (15).

p2.5 was generated by inserting the *Xho*I–*Xho*I fragment of pPC86 containing the *ADH1* promoter into the *Xho*I site of pRS323 (17) and subsequently the *Sal*I–*Bam*HI fragment of pPC86 containing the polylinker and the *ADH1* terminator in *Sal*I–*Bam*HI sites of the pRS323. The p2.5 derivatives were generated by cloning PCR products into p2.5: E1A#2, aa 30–132 of E1A; E1A#4, aa 30–86 and 120–139 of E1A; E1A-CR1, aa 1–120 of E1A; pRB, aa 302–928 of pRB; E1A-CR2, aa 76–139 of E1A.

An *ADH-p53* expression vector (pRB16) was constructed by ligating the *Xho*I/*Sac*I *ADH-p53* insert of pLS76 (18) containing the *ADH1* promoter, p53, and the *CYC1* terminator into the polylinker of pRS413 (19). The plasmids for alleles R175H and R249S of p53 were constructed by ligating *Nco*I/*Stu*I fragments from pCMV-NEO-BAM (20) into pRB16.

Protein Steady-State Levels by Western Blot Analysis. Yeast cells were grown to midlogarithmic phase, harvested, washed, and resuspended in 100 mM Tris·HCl, pH 8.0/20% glycerol/1 mM EDTA/0.1% Triton X-100/5 mM $MgCl_2$/10 mM 2-mercaptoethanol (2ME)/1 mM phenylmethylsulfonyl fluoride (PMSF)/leupeptin (1 μg/ml)/pepstatin (1 μg/ml) for pRB/E2F1-related experiments or 50 mM Tris·HCl, pH 7.5/1% SDS/5 mM EDTA, pH 8.0/14 mM 2ME/1 mM PMSF for the p53-related experiment at a cell density of about 10^9 cells per ml. Cells were disrupted by vortex mixing in the presence of glass beads for 10 min at 4°C. Debris was pelleted by centrifugation at 12,000 rpm in a microcentrifuge for 15 min at 4°C. Approximately 50 μg of proteins was subjected to Western blot analysis (21).

RESULTS

Yeast strains were generated in which expression of DB-X/AD-Y or DBPX hybrid proteins is toxic under particular conditions (negative selection). Under these conditions, dissociation of an interaction should provide a selective advantage thereby facilitating detection: a few growing yeast colonies in which DB-X/AD-Y (or DBPX/binding site) fail to interact should be identified among many nongrowing colonies containing interacting DB-X/AD-Y (or DBPX/binding site) (Fig. 1). This concept was demonstrated using well-characterized protein–protein and DNA–protein dissociation events.

***URA3* Counterselectable Marker.** The reverse two-hybrid and one-hybrid negative selections are based on two concepts: (*i*) the use of a reporter gene whose expression causes toxicity under specific growth conditions (counterselectable marker), and (*ii*) the construction of alleles of that gene whose expression responds very tightly to a transcription factor.

We used the counterselectable yeast gene *URA3*, which encodes an enzyme involved in uracil biosynthesis. Yeast cells that express *URA3* grow on medium lacking uracil (Ura^+ phenotype, positive selection). However, the *URA3*-encoded enzyme can also catalyze the transformation of 5-fluoroorotic acid (FOA), into a toxic compound (22). Therefore, *URA3* expression is toxic for yeast grown on medium containing both uracil and FOA [FOA-sensitive (Foa^S) phenotype, negative selection].

When fused to *URA3*, most yeast promoters described confer a Ura^+ phenotype in the absence of any specific transcription factor (23, 24). This is probably because basal levels of expression of *URA3* are sufficient to promote growth on medium lacking uracil. To reduce the basal levels of expression, we used a fusion between the *SPO13* promoter and

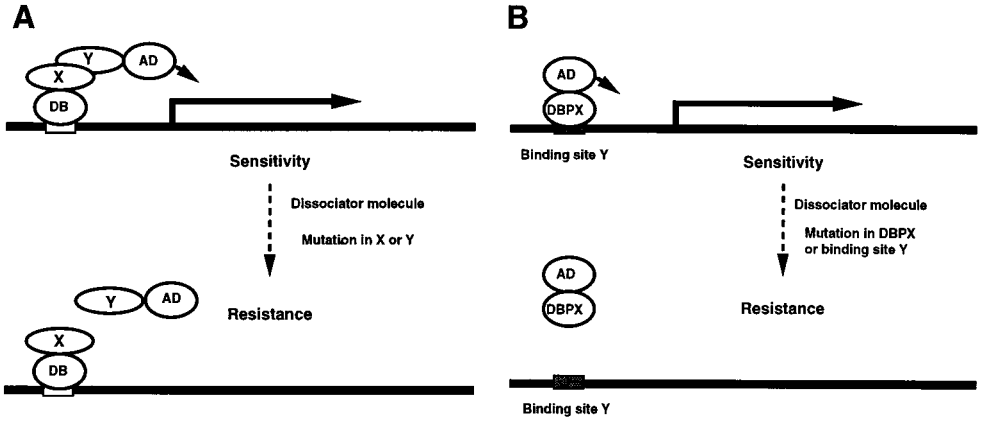

FIG. 1. Reverse two-hybrid (*A*) and one-hybrid (*B*) systems. The reconstitution of a transcription factor activates a reporter gene whose expression is lethal under particular growth conditions (Sensitivity). Expression or presence of a dissociator molecule or mutations in one of the interacting partners results in decreased expression of the reporter gene and selective advantage (Resistance).

the *URA3* open reading frame. *SPO13* is only activated under sporulation conditions (25) and tightly repressed under normal conditions by a cis-acting upstream repressing sequence (11, 26–30). Thus the fusion between the *SPO13* promoter and the wild-type *URA3* open reading frame (*SPO13*::*URA3*) confers a very tight Ura$^-$ FOA-resistant (Foa^R) phenotype under normal growth conditions (Fig. 2*B* and see Fig. 4*B*).

Reverse Two-Hybrid System. Since the system described herein uses the GAL4 DB (ref. 1 and see below), derivatives of the *SPO13* promoter were constructed that contain GAL4 binding sites (*SPALn*::*URA3* where *n* is the number of GAL4 binding sites, see Fig. 2*A*). The *SPALn*::*URA3* alleles were integrated into the yeast genome at *ura3*. As evidence for GAL4 inducibility, *SPAL5*::*URA3* transformants containing either wild-type full-length GAL4 or the GAL4 DB (aa 1–147) and AD (aa 768–881) expressed as two separate molecules show tight Ura$^+$ Foa^S and Ura$^-$ Foa^R phenotypes, respectively. As expected, neither GAL4 nor DB and AD have any effect in cells containing a null allele of *URA3* (*ura3-52*) (Fig. 2*B*).

GAL4-inducible *URA3* alleles confer Foa^S phenotypes in yeast cells that reconstitute mammalian protein–protein interactions in the context of the hybrid system. For example, both cFos and cJun, which associate with a relatively high affinity (32), and pRB and E2F1, which interact rather weakly in the absence of the E2F heterodimeric binding partner DP1 (33), confer a Foa^S phenotype in *SPAL8*::*URA3* strains (Fig. 2*C*). For comparison, DB-cFos/cJun and DB-pRB/AD-E2F1 interactions induce 100 units and 0.5 unit of β-galactosidase-specific units expressed from *GAL1*::*lacZ*, respectively, while GAL4 induces 3000 units under identical conditions. The Ura phenotype was usually consistent with the Foa phenotype: i.e., Ura$^+$ Foa^S or Ura$^-$ Foa^R, respectively (Fig. 2*C*). However, cells expressing DB-pRB/AD-E2F1 show a Ura$^-$ Foa^S phenotype. Intermediate levels of expression may be sufficient to produce toxic doses of the FOA metabolite but are too limiting for the synthesis of uracil.

The relative affinity of a dissociator for either one of the partners of an interaction is unknown prior to its identification.

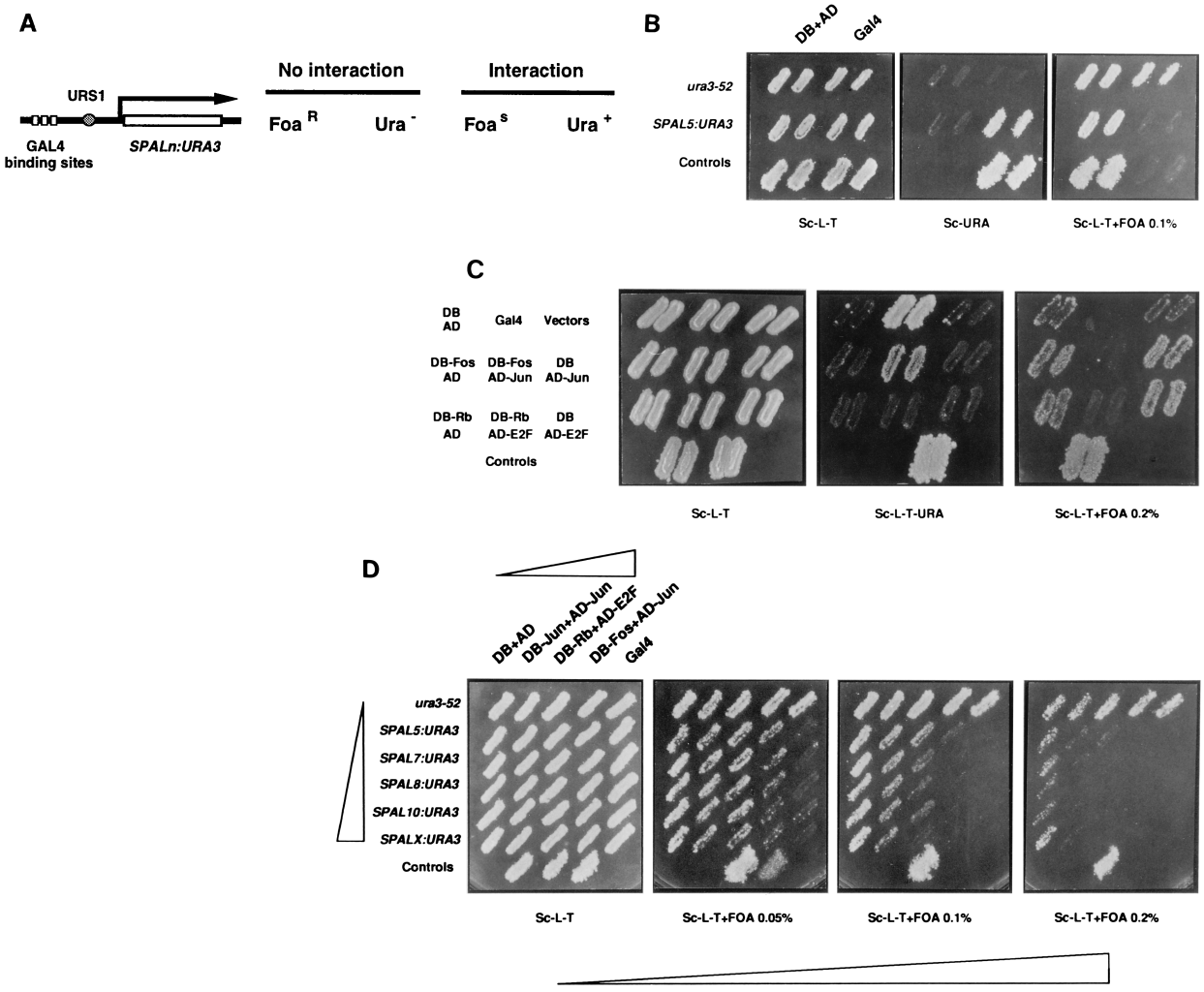

FIG. 2. Human protein–protein interactions confer Foa^S phenotype to *SPAL*::*URA3* yeast cells in a titratable manner. (*A*) *SPALn*::*URA3* contain n GAL4 binding sites and the upstream repressing sequence in the *SPO13* promoter fused to *URA3*. When a GAL4 DB is used, *SPALn*::*URA3* fusions are expected to generate Foa^S Ura$^+$ or Foa^R Ura$^-$ phenotypes in the presence or absence of interacting hybrid proteins, respectively. (*B*) Strains MaV52 (*ura3-52*) and MaV95 (*SPAL5*::*URA3*) were cotransformed with pCL1, a *LEU2* plasmid expressing GAL4 (1) and pRS314 (19) (Gal4) or with pPC97 and PC86 (8) expressing GAL4 DB and AD separately (DB+AD). Six Leu$^+$ Trp$^+$ transformants were tested; two are shown. Patches of cells growing on synthetic complete medium lacking leucine and tryptophan (31) (Sc-L-T) were replica-plated onto a Sc-URA plate and a plate containing 0.1% FOA (Sc-L-T+FOA 0.1%). The control strains are *ura3-52* (two left patches) and wild-type (two right patches). The plates were incubated at 30°C and photographed 3 days later. (*C*) Strain MaV99 (*SPAL10*::*URA3*) was cotransformed with the plasmids indicated ["vectors" are plasmids pRS314 and pRS315 (19)]. The yeast patches were manipulated as described in *B*. (*D*) Titration of the Foa^S phenotype. Derivatives of MaV52 were cotransformed with the plasmids indicated above the left panel. The yeast patches were manipulated as described in *B*.

Likewise, the extent of phenotypic effects due to particular mutations is unknown. Ideally then, conditions for identifying dissociation events should allow even small decreases in *SPAL*::*URA3* transcriptional activity to be detected phenotypically. For every particular interaction considered, it is therefore important to establish the minimal number of GAL4 binding sites in *SPALn*::*URA3* and the lowest concentration of FOA required to confer a Foa^S phenotype. We tested this notion using the two interactions described above, DB-cFos/AD-cJun (strong interaction) and DB-pRB/AD-E2F1 (weak interaction), as well as the reconstitution of cJun/cJun dimers (very weak interaction). In each case, one particular combination of conditions could be found that corresponded to a limit of growth threshold, suggesting that by titrating the conditions, a large spectrum of dissociators or mutations affecting a particular interaction could be identified (Fig. 2*D*).

The concept of a growth advantage mediated by the dissociation of a protein–protein interaction was tested in the context of interactions between the retinoblastoma family of proteins and the E2F transcription factors. In mammalian cells, pRB and p107 interact with (34–37) and thereby repress the transcriptional activation function of the DNA-binding transcription factors E2F1 and E2F4 (16, 38), respectively. Dissociation of this interaction, either by mutations in pRB or by expression of a viral oncoprotein like E1A, causes loss of proliferation control (39).

A mutation affecting the interaction domain of one interacting partner should significantly reduce the expression of the *SPALn*::*URA3* fusion, thereby resulting in a Foa^R phenotype. Deletion of exon 22 of pRB is associated with tumorigenicity and results in a pRB allele (pRBΔ22) that fails to associate with E2F1 (40). When expressed as a fusion to DB along with AD-E2F1, this form of pRB conferred a Foa^R phenotype although expression of both wild-type DB-pRB and mutant pRBΔ22 was comparable (Fig. 3 *A* and *B*).

To assess the effect of dissociator molecules on the Foa^S phenotypes resulting from interacting proteins, we tested the effect of overexpressing E1A in cells expressing specific pairs of DB-pRB or DB-p107 and AD-E2F hybrid molecules. Dissociation of these complexes by E1A has been well documented and functionally disrupts growth regulation *in vivo* (42, 43). To ensure proper synthesis of dissociators, a novel plasmid (p2.5) was designed with the following features: (*i*) a 2-μm sequence that permits the maintenance of the plasmid in relatively high copy, (*ii*) a different selectable marker allowing independent selection from the two-hybrid-expressing plasmids, (*iii*) the same *ADH1* promoter that was used for the expression of the two hybrids, and (*iv*) a nuclear localization signal coding sequence upstream of the polylinker such that each dissociator expressed could potentially be transported to the nucleus.

Full-length 12S E1A in *SPAL8*::*URA3* cells containing DB-pRB confers a very strong Foa^S phenotype probably due to the presence of spurious activation domains in 12S E1A (data not shown). We circumvented that problem by designing two variants of full-length 12S E1A (E1A#2 and E1A#4) with deletions that maintain the integrity of two conserved regions (CR1 and CR2) of 12S E1A and eliminate the cryptic activation domains. CR1 and CR2 domains have been previously shown to be both necessary and sufficient for pRB/E2F dissociation in mammalian cells (42).

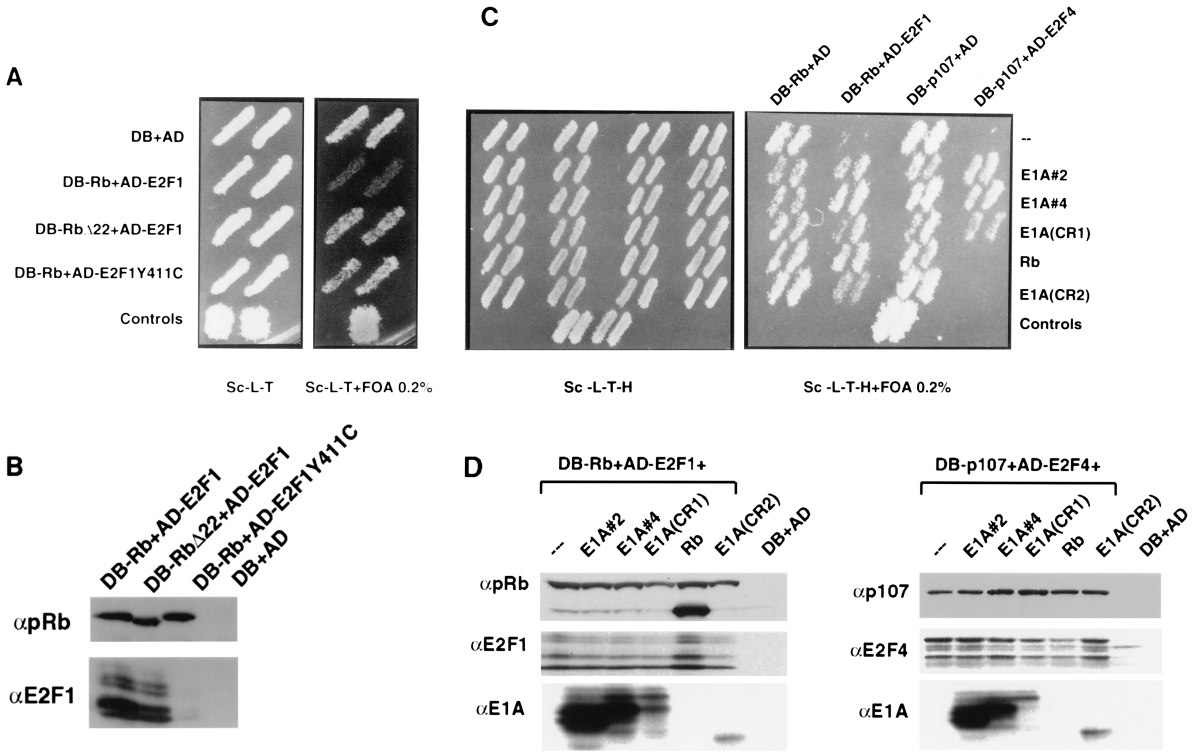

FIG. 3. Foa^S phenotype rescued by a mutation affecting an interaction domain or by a dissociator molecule. (*A*) Phenotypic effect of a mutation. Strain MaV103 (*SPAL10*::*URA3*) was cotransformed with the plasmids indicated on the left. The yeast patches were manipulated as described in Fig. 2*B*. (*B*) DB-pRB and AD-E2F1 hybrid expression levels. Extracts were examined by protein immunoblot analysis with the indicated monoclonal antibodies (mAbs): anti-pRB XZ77 (41) and anti-E2F1 KH129 (33). The E2F1Y411C allele affects the stability of the E2F1 protein and, therefore, the corresponding Foa^R phenotype (see *A*) cannot be interpreted. (*C*) Phenotypic effect of a dissociator. MaV108 (*SPAL10*::*URA3*) was cotransformed with the plasmids indicated above the right panel, and the resulting transformants were subsequently transformed with the plasmids indicated on the right. The yeast patches were manipulated as described in Fig. 2*B*. (*D*) Expression levels. Extracts were examined by protein immunoblot analysis with the indicated mAbs: anti-pRB XZ77 (41), anti-E2F1 KH129 (33), anti-E1A mixture containing LS10, LS36, LS51, and LS56 (N. Dyson, personal communication), anti-p107 SD9 (37) and rabbit anti-E2F4 polyclonal antibody C108 (Santa-Cruz). The specificity of the DB-E2F1, DB-E2F4, and E1A proteins, which run as multiple bands, can be inferred from the DB+AD negative control lane.

When introduced into cells expressing specific DB-pRB or DB-p107 and AD-E2F pairs of hybrid molecules, E1A#2- and E1A#4-encoding p2.5 plasmids rescue the Foa^S phenotype (Fig. 3*C*). Several controls demonstrated that the E1A dissociation was specific. Overexpression of E1A variants did not affect the steady-state levels of the different fusion proteins (Fig. 3*D*). E1A expression had no effect on the Foa^S phenotype resulting from the DB-DP1/AD-EF1 interaction (data not shown). Expression of CRI, was sufficient to rescue both the DB-pRB/AD-E2F1 and DB-p107/AD-E2F4 Foa^S phenotype (Fig. 3*C*). Overexpression of native pRB, in the absence of any DB sequences, rescued the Foa^S phenotype of DB-pRB/AD-E2F1 but not that of DB-p107/AD-E2F4 (Fig. 3*C*).

Reverse One-Hybrid System. The use of negative selection can be extended to study protein–DNA interactions (Fig. 4). In mammalian cells, wild-type p53 interacts with binding sites present in the promoter of its target genes and lack of binding in the context of several p53 mutant alleles can cause tumorigenicity (44, 45). To demonstrate the one-hybrid concept, we used two p53 mutations that affect its DNA-binding ability and are associated with tumorigenesis.

A p53-responsive *URA3* reporter gene (*1cUAS53*::*URA3*) was constructed by integrating a p53 consensus DNA binding site (14) in *SPO13*::*URA3* (see above) (Fig. 4*A*). The resulting constructs were integrated at the *URA3* genomic locus. Since p53 contains a native activation domain (47), it was not necessary to express it in the form of a fusion with an AD to test its DNA-binding abilities. *1cUAS53*::*URA3* cells expressing p53 exhibit Ura$^+$ and Foa^S phenotypes, while control cells containing either the p53 plasmid or the *1cUAS53*::*URA3* reporter gene alone show the opposite phenotype (Fig. 4*B*).

Mutations that affect DNA-binding should be detected by their ability to rescue the Foa^S phenotype observed with the wild-type molecule. To demonstrate this, we used two well-characterized p53 missense mutations, R175H and R249S, that are known to affect its DNA-binding ability and are associated with tumorigenesis (44, 45). Introduction of either mutation into the p53 coding region completely reversed the Ura$^+$ Foa^S phenotype (Fig. 4*B*), while wild-type and corresponding mutant proteins were expressed at similar levels (Fig. 4*C*).

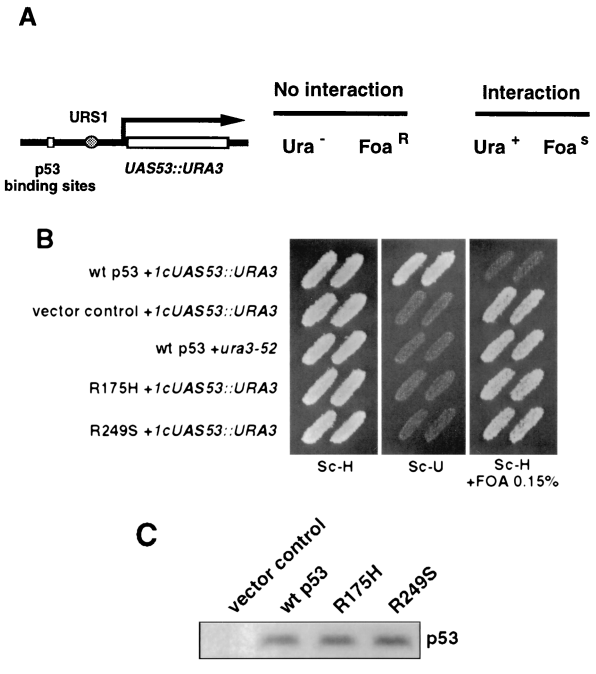

FIG. 4. Reverse one-hybrid system with p53 and its binding site. (*A*) The *1cUAS53*::*URA3* reporter gene. A fusion containing a p53 binding site and an upstream repressing sequence in *SPO13*::*URA3* is expected to generate Foa^S Ura$^+$ or Foa^R Ura$^-$ phenotypes in the presence or absence of functional p53, respectively. (*B*) Yeast growth phenotypes. Isogenic strains RBy33 (*1cUAS53*::*URA3*) and BY385 (*ura3-52*) were transformed with the plasmids indicated at the left: vector control, pRS413 (19); wild-type p53, pRB16; R175H, pRB212; R249S, pRB214. The yeast patches were manipulated as described in Fig. 2*B*. (*C*) Extracts were examined by protein immunoblot analysis with anti-p53 PAb1801 (46).

DISCUSSION

The two-hybrid and one-hybrid systems represent extremely powerful tools for the discovery of genes encoding interaction partners of proteins and DNA sequences. This type of analysis has now been extended a step further to allow characterization of these interactions using both positive and negative genetic selections enabled by the selectable and counterselectable reporter gene *URA3*.

One of the critical aspects of the negative selection is that the basal level of expression of the counterselectable marker, in the absence of interacting proteins or DNA–protein interaction should be minimal. Here, a very strong upstream repressing sequence was used to obtain such low basal level of *URA3* expression. An alternative method to eliminate the Ura$^+$ phenotype conferred by *URA3* basal levels of expression uses medium containing 6-azauracil, a pyrimidine biosynthetic inhibitor (48). However, 6-azauracil is a relatively nonspecific inhibitor mediating some of its effects on subunits of RNA polymerase II (49).

The reverse two-hybrid and one-hybrid systems can be used to efficiently analyze mutations in interaction partners as well as for the identification of additional trans-acting factors that dissociate macromolecular interactions. Our observations demonstrate that previously characterized events leading to the dissociation of protein–protein (and DNA–protein) interactions can be reconstituted in the reverse two-hybrid (or one-hybrid) system. They also suggest that previously uncharacterized dissociation events such as dissociating mutations could be specifically selected from large libraries using these genetic systems (50).

Current techniques to study the structure–function relationships of protein–protein interactions are limited to the determination of domains necessary and sufficient for interactions. Combinations of the negative selection presented herein as well as previously described positive selections (2–4) could lead to the selection of discrete single amino acid or nucleotide changes that disrupt a studied interaction in different ways. For example, we have characterized a protein–protein interaction domain of the transcription factor E2F1 by selecting for E2F1 missense mutations that abrogate E2F1 binding either weakly or strongly (50). We have also selected a large collection of dominant negative mutations in p53 that abrogate its DNA-binding affinity (51). Mutations that conditionally affect interactions also could be selected by a combination of positive/negative selections and alternate conditions of incubation (such as different temperatures). Second-site compensatory suppressor mutations of these weak, strong, dominant negative, or conditional mutations could be selected in the interaction partners. Such pairs of compensatory mutations should provide refined structural analyses on the roles of particular residues involved in an interaction. Functions of protein–protein interactions could further be studied by reintroducing the various alleles selected in the yeast reverse two-hybrid (or one-hybrid) system into suitable *in vivo* systems.

Recent studies on the regulation of particular protein–protein interactions have identified dissociator proteins (52, 53). To our knowledge, no genetic system has been described to genetically identify dissociators. They could be identified from cDNA libraries constructed in the p2.5 plasmid and transformed into *SPAL*::*URA* cells containing a DB-X/AD-Y (or a DBPX/binding site) interaction of interest.

Conceptually, in all diseases attributed to particular protein–protein or DNA–protein interactions, specific dissociation can be viewed as a potential therapeutic strategy. These target interactions include associations between proteins of a parasite and its host, unregulated associations between proteins, and also interactions responsible for the function of the downstream event of a regulatory pathway frequently mutated in a particular disease. In all cases, peptides or small compound molecules able to dissociate abnormal interactions could be critical therapeutic reagents. Identification of such molecules could be facilitated by direct selection using the reverse two-hybrid (or one-hybrid) system.

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