Genetic characterization of a mammalian protein–protein interaction domain by using a yeast reverse two-hybrid system

(E2F/DP/two-step selection/negative selection/*URA3*/marked box)

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ABSTRACT **Many biological processes rely upon protein–protein interactions. Hence, detailed analysis of these interactions is critical for their understanding. Due to the complexities involved, genetic approaches are often needed. In yeast and phage, genetic characterizations of protein complexes are possible. However, in multicellular organisms, such characterizations are limited by the lack of powerful selection systems. Herein we describe genetic selections that allow single amino acid changes that disrupt protein–protein interactions to be selected from large libraries of randomly generated mutant alleles. The strategy, based on a yeast reverse two-hybrid system, involves a first-step negative selection for mutations that affect interaction, followed by a second-step positive selection for a subset of these mutations that maintain expression of full-length protein (two-step selection). We have selected such mutations in the transcription factor E2F1 that affect its ability to heterodimerize with DP1. The mutations obtained identified a putative helix in the marked box, a region conserved among E2F family members, as an important determinant for interaction. This two-step selection procedure can be used to characterize any interaction domain that can be tested in the two-hybrid system.**

The E2F transcription factor plays a key role in the temporal expression of genes required for cell proliferation (1, 2) and consists of heterodimers formed by interaction between two members of an extended family of proteins (3–13). Each of the five members of the E2F subfamily (E2F1 to E2F5) forms heterodimers with a member of the DP subfamily (DP1 or DP2) (12–15), and these heterodimers bind the promoter of their target genes (16–24). During certain stages of the cell cycle, DNA-bound E2F/DP heterodimers are found in association with the retinoblastoma gene product (pRB) or another member of the pRB family of related proteins, p107 or p130. The E2F-interacting proteins are known collectively as the pocket proteins, and the association of E2F with a pocket protein is thought to repress transcription (25–36). At specific stages of the cell cycle, when expression of the target genes is required, the pocket proteins are released and free E2F activates transcription. At least in the case of pRB, release is mediated by cell-cycle-regulated phosphorylation events (31, 37–48). Formation of E2F/DP heterodimers is critical for high-affinity binding to both DNA and pocket proteins and, therefore, is critical for temporal regulation of transcription (12, 14, 15).

Despite the functional importance of E2F/DP heterodimerization, little is known about the E2F domain(s) involved in this protein–protein interaction. Computer searches have failed to reveal obvious conservation with previously characterized dimerization motifs. *In vitro* mapping experiments using truncated E2F1 proteins have pointed to potential nonoverlapping

domains, each independently able to bind DP1. Among these, a domain corresponding to residues 206–220 of E2F1 has been shown to be important for interaction with DP1 (12, 15). However, this domain alone does not promote wild-type levels of binding since its deletion only marginally affects DP1 interaction (49).

In this paper, we describe another E2F1 domain required for interaction with DP1. This domain was identified genetically by selecting, in a reverse two-hybrid system, mutations that affect the interaction.

MATERIALS AND METHODS

General Methods and Reagents. Yeast strains and methods are described in an accompanying paper (50). Plasmids previously described are pPC97 (DB), pPC86 (AD), pPC76 (DB-Fos), and pPC79 (AD-Jun) (51), as well as pCL1 (GAL4) (52) and DB-pRB (50). The DB-DP1 and AD-E2F1 plasmids were generated by cloning PCR products corresponding to full-length DP1 or aa 159–437 of E2F1 (10) in-frame with AD in pPC86.

The β-galactosidase activities were determined as described (53, 54). Western blot analyses were as described (50). DNA sequencing was performed on one strand corresponding to aa 159–437 of E2F1. No mutations were found outside the marked box (see below).

Mutagenic PCR. PCR mixtures (55) for aa 159–437 of E2F1 contained 100 ng of AD-E2F1 plasmid, 1 μM AD 5′ primer (5′-CGCGTTTCCAATCACTACAGGG-3′), 1 μM E2F1-specific p105 3′ primer (3), all four dNTPs (each at 50 μM), 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl$_2$, BSA (1 μg/μl), and 5 units of *Taq* DNA polymerase in 100 μl (1 min at 94°C, 1 min at 45°C, and 2 min at 72°C for 40 cycles). MnCl$_2$ (100 μM) was added after 10 cycles. To specifically mutagenize the "Marked Box," four PCRs were performed. Reaction 1 (aa 224–295) involved 5′ primer p131 (3) and 3′ primer "p15as4" (5′-CATCGATCGGGCCTTGTT-TGC-3′). Reaction 2 (aa 224–362) involved 5′ primer p131 (3) and 3′ primer "p15as6" (5′-ATCGGGACAACAGCGGT-TCT-3′). Reaction 3 (aa 261–295) involved 5′ primer p101 (3) and 3′ primer "p15as4," and reaction 4 (aa 224–362) involved 5′ primer p101 and 3′ primer "p15as4." The AD-E2F1 plasmid was digested at a unique *Bgl*II site located at nt 983 of the E2F1-encoding sequence (3).

Two-Step Selections. Approximately 10,000 Leu⁺ Trp⁺ transformants were plated on 15-cm synthetic complete plates lacking leucine and tryptophan (Sc-L-T). The first-step selection was performed by replica plating these plates to 15-cm 5-fluoroorotic acid (FOA) plates where a few hundred FOA-

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Abbreviations: FOA, 5-fluoroorotic acid; Foa^R, FOA resistant; Foa^S, FOA sensitive; 3AT, 3-aminotriazole; pRB, retinoblastoma gene product.
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resistant (Foa^R) colonies developed. These plates were then replica-plated to Sc-L-T plates for recovery and subsequently to 3-aminotriazole (3AT) plates for the second-step selection where a few colonies developed.

***In Vitro* Binding Reactions.** Proteins were produced by *in vitro* transcription/translation (TNT kit, Promega). The DP1 protein was expressed with a tag consisting of the influenza hemagglutinin epitope (HA) from plasmid pBSK-HA-DP1 (12). The wild-type and mutant E2F1 proteins were expressed using PCR products as templates. The PCR was performed with the following primers: a 5′ hybrid primer consisting of a T7 RNA polymerase promoter sequence fused to sequences annealing with the 3′ end of the GAL4 AD (5′-CCAAGCTT-CTAATACGACTCACTATAGGGAAGATGAACCC-AACAAAAAAGAGGGGTCG-3′, T7AD) and a 3′ primer annealing with sequences of E2F1 overlapping the termination codon (p105) (3). Both proteins were produced in the presence of [^{35}S]methionine. Five microliters of each *in vitro* transcription/translation reaction mixture was mixed with 200 μl of "Colrain" buffer [100 mM KCl/25 mM Hepes, pH 7.5/10 mM MgCl$_2$/10% glycerol/1 mM DTT/0.1% Nonidet P-40/leupeptin (1 μg/ml)/aprotinin (1 μg/ml)/1 mM phenylmethylsulfonyl fluoride/0.1 mM EDTA], incubated at room temperature for 30 min, diluted with 700 μl of "washing" buffer [Tris·HCl, pH 7.5/250 mM NaCl/5 mM EDTA/0.5% Nonidet P-40/1 mM DTT/leupeptin (1 μg/ml)/aprotinin (1 μg/ml)/1 mM phenylmethylsulfonyl fluoride], and incubated at room temperature for 30 min. Subsequently, the samples were separated in three tubes and various mAbs were added: anti-HA, 12CA5 (56); anti-E2F1, KH95 (12); anti-E1A, M73 (57). After a 30-min incubation at room temperature, the immunocomplexes were conjugated with protein A-Sepharose beads (Promega) (30 min at room temperature) and washed five times with 500 μl of "washing" buffer. The beads were resuspended in Laemmli buffer and boiled, and the supernatants were analyzed electrophoretically as described (58).

RESULTS

Two-Step Selections. To locate and characterize the heterodimerization domain(s) of E2F1, we used genetic selection to identify a collection of single amino acid changes in E2F1 that specifically alter its ability to bind DP1. We designed a strategy based on genetic selections in yeast to identify mutations that prevent protein–protein interaction. A useful selection procedure should allow the identification of informative mutations within a large library of randomly generated alleles, thus permitting an unbiased approach. The strategy we have developed is based on variations of the two-hybrid system developed by Fields and Song (52) and modified by others (50, 59–61). The variations described herein facilitate detection of the relevant missense mutations in two sequential selection steps. The first step is a negative selection for mutations that impair E2F1/DP1 interaction, and the second step is a positive selection for a subset of those mutations that maintain expression of full-length stable protein (Fig. 1A). The second step should prevent the isolation of relatively uninformative mutations, such as truncations, frame shifts, or any mutation that affects stability, processing, or folding.

To facilitate the first-step negative selection, we designed a "reverse" two-hybrid system in which protein–protein interactions induce expression of a toxic reporter gene (*SPAL10::URA3*; ref. 50). When tested in the context of the two-hybrid system in *SPAL10::URA3* yeast strains, protein–protein interactions confer sensitivity to FOA (Fig. 1B and ref. 50). As an indication of the biological relevance of the "reverse" two-hybrid system, it was demonstrated that physiologically relevant dissociation of a previously characterized interaction, either by mutation or by expression of a competitive dissociator molecule, results into a FOA-resistant (Foa^R) phenotype (50).

To facilitate the second-step positive selection, we introduced in *SPAL10::URA3* strains the titratable *GAL1::HIS3* reporter gene (60). *HIS3* encodes an enzymatic activity specifically inactivated by the competitive inhibitor 3-aminotriazole (3AT). Two-hybrid-dependent *GAL1::HIS3* expression levels establish the maximal 3AT concentration tolerated by yeast cells containing a given protein–protein interaction (His$^+$ phenotype). Consequently, wild-type and mutant alleles of an interaction partner can be phenotypically discriminated
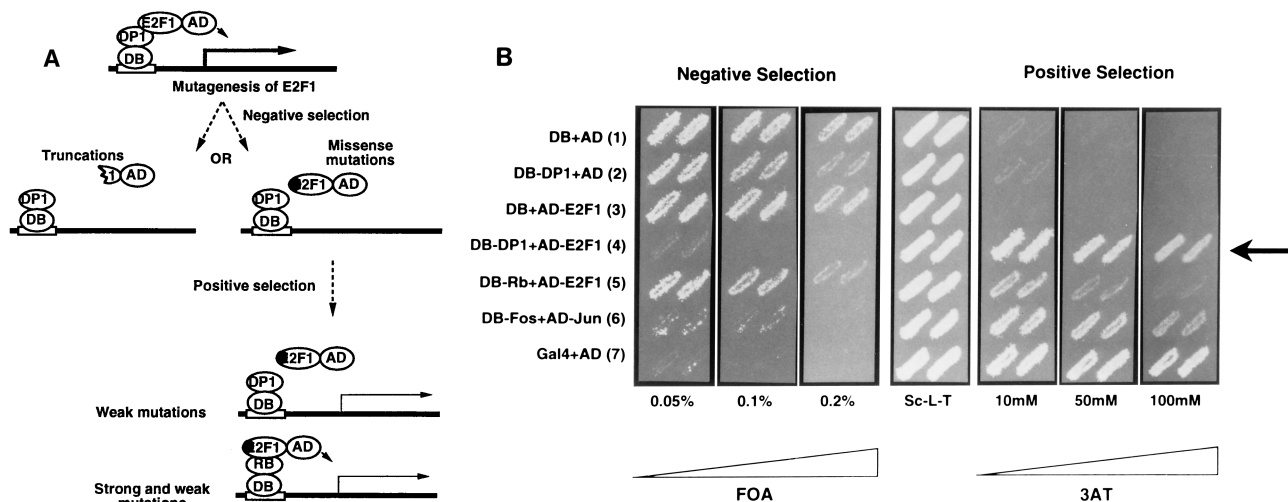

Fig. 1.   Two-step selection. (*A*) Expression of interacting proteins DP1 and E2F1 (or potentially any other protein pair) in fusion with a DNA-binding domain (DB, DB-DP1) and an activation domain (AD, AD-E2F1), respectively, reconstitutes a transcription factor (52). This in turn activates reporter genes in the same host cells whose expression can either be toxic (negative phenotype) or required for viability (positive phenotype) depending on the growth conditions. See text for details for two-step selection schemes. (*B*) DB-DP1/AD-E2F1 interaction confers titratable positive and negative phenotypes. Patches of MaV103 cells (50) transformed with the indicated plasmids and grown on synthetic complete medium lacking leucine and tryptophan (62) (Sc-L-T) were replica-plated onto plates containing FOA at the indicated concentrations or lacking histidine and containing the indicated concentrations of 3AT. The plates were incubated at 30°C for 3 days. Note that neither DB-DP1 nor AD-E2F1 expressed separately are active in this growth assay. Note also that in the yeast two-hybrid system, DB-pRB/AD-E2F1 interaction is detectable in the absence of DP1 expressed. DB-Fos/AD-Jun interaction and expression of full-length GAL4 are shown as positive controls.
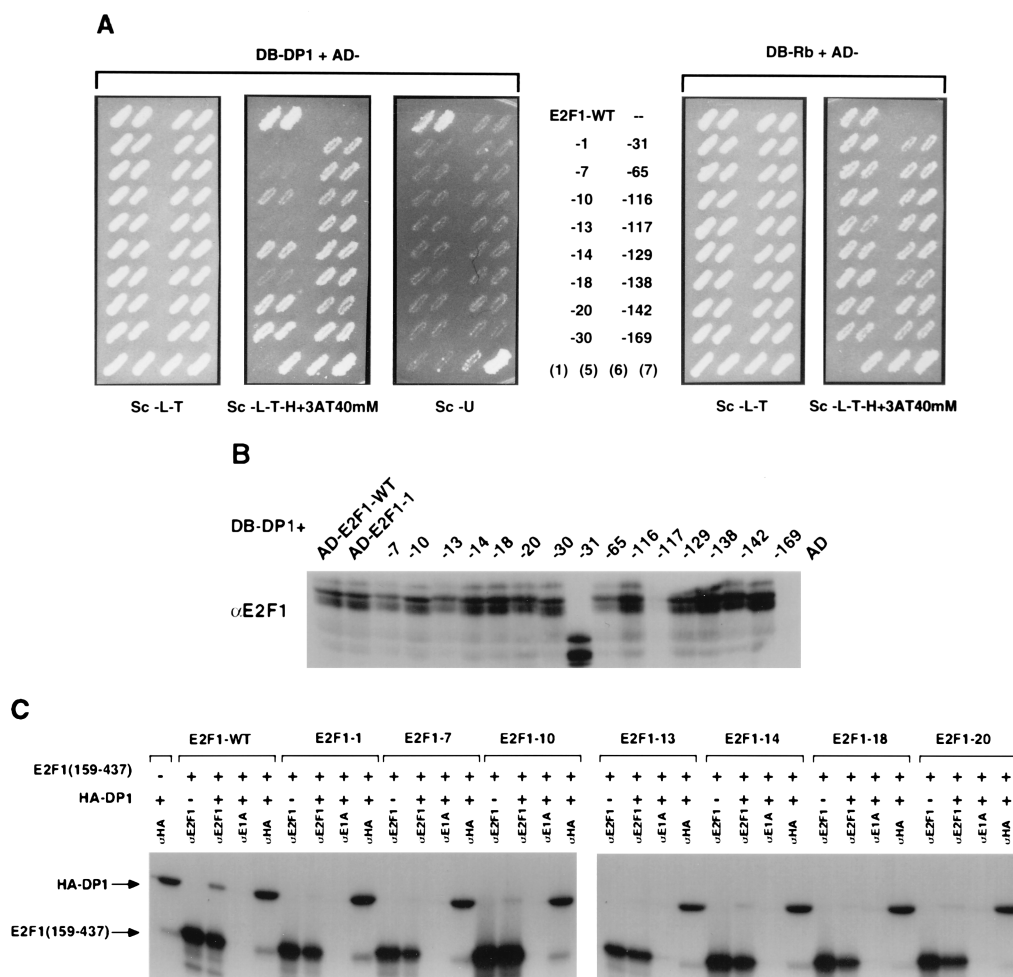
FIG. 2.   Phenotypes of 16 E2F1 mutant alleles. (*A*) Growth phenotypes. DB-DP1-containing (three panels to the left) or DB-pRB-containing (two panels to the right) containing strain MaV103 was transformed with the AD plasmids indicated: E2F1-WT, wild-type E2F1 positive control; −, no fusion negative control. The numbers below are names for the different E2F1 alleles. Six individual Leu$^+$ Trp$^+$ transformants were tested for each allele, two of which are shown. Patches of cells growing on Sc-L-T were replica-plated onto Sc-L-T-H+3AT (40 mM) and Sc-U plates. Under the conditions of our assay, the His phenotype is less stringent than the Ura phenotype and, therefore, E2F1 alleles with weak mutations promote growth on plates containing 3AT but not on plates lacking uracil (Sc-U). The four patches at the bottom represent four control strains (see Fig. 1*B*). The plates were incubated at 30°C and photographed 3 days later. (*B*) Expression levels of mutant AD-E2F1 mutant alleles. Yeast extracts were examined by protein immunoblot analysis with mAb anti-E2F1 KH129. In each case but one, the protein levels were comparable between samples. Note the size shift of AD-E2F1-31 due to an in-frame deletion (aa 285–324). (*C*) *In vitro* binding reactions. HA-DP1 and AD-E2F1 wild-type mutant alleles were translated *in vitro* in the presence of [^{35}S]methionine and, after mixing, subjected to immunoprecipitation with the indicated mAbs. DB-DP1 and AD-E2F1 hybrid proteins alone were used as negative controls. Note the presence of a HA-DP1 degradation product comigrating with AD-E2F1 in lane 1 corresponding to HA-DP1 alone. This band makes the coimmunoprecipitation with mAb 12CA5 (anti-HA) rather uninterpretable. However, mAb KH95 (anti-E2F1) unambiguously coimmunoprecipitates HA-DP1 when wild-type AD-E2F1 is mixed (see lane 3). mAb M73 (anti-E1A) is used as a negative control antibody.

on plates containing increasing concentrations of 3AT (Fig. 1*B* and data not shown).

E2F1/DP1 Interaction in the Reverse Two-Hybrid System. To reconstitute DP1/E2F1 interaction in the context of the two-hybrid system, we fused these molecules to the GAL4 DNA-binding (DB; aa 1–147) and activation (AD; aa 768–881) domains, respectively. To maintain reproducible and relatively low levels of expression, the hybrid molecules were expressed from the *ADH1* promoter present on centromeric plasmids (51). Since DP1 apparently lacks any functional activation domain, we fused its full-length coding sequence to DB (DB-DP1). Since expression of full-length E2F1 fused to AD is toxic to yeast cells (data not shown), we used a C-terminal domain of E2F1 (aa 159–437) required for binding to both DP1 and pRB but with no apparent toxic effect, in fusion to AD (AD-E2F1). On the basis of Foa and His phenotypes, neither DB-DP1 nor AD-E2F1 could activate the *SPAL10::URA3* and *GAL1::HIS3* reporter genes when ex-

pressed alone. However, when coexpressed in yeast cells, these hybrid molecules mediated strong FOA-sensitive (Foa^S) and His$^+$ phenotypes.

The Foa^S phenotype was titrated to establish the minimal number of GAL4 binding sites (data not shown and ref. 50) in combination with the lowest concentration of FOA needed to confer a Foa^S phenotype (0.1%, Fig. 1*B*).

Wild-type DB-DP1/AD-E2F1 interaction also induced *GAL1::HIS3* expression that allows growth at 3AT concentrations up to 100 mM (Fig. 1*B*). As expected, the AD-E2F1 fusion mediates interaction with a DB-pRB fusion, although the resulting read-outs are much weaker. The DB-pRB/AD-E2F1 interaction induced *GAL1::HIS3* expression to allow growth at 3AT concentrations limited to 20 mM (Fig. 1*B*).

Two-Step Selection for Weak Mutations. Two variations of the two-step selection were used. First, we reasoned that single amino acid changes might be found that affect heterodimerization only partially (weak mutations). This possibility was
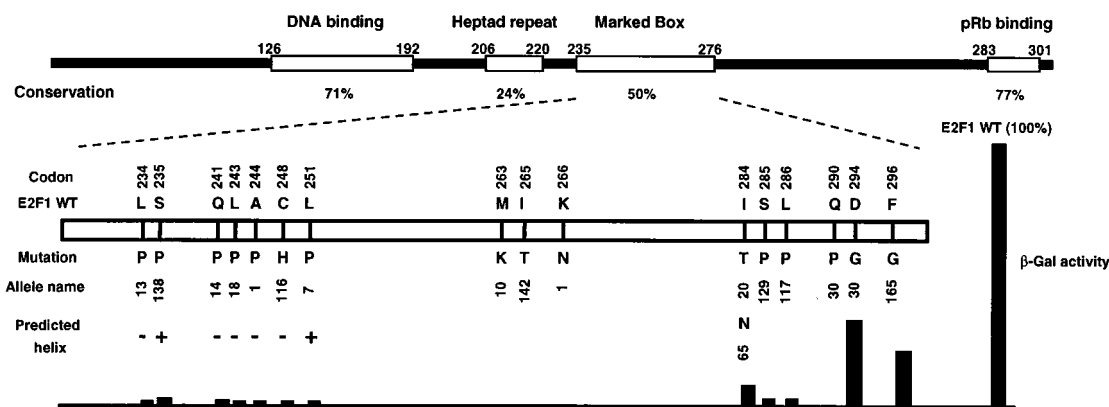
FIG. 3.   Protein sequences of mutant E2F1 alleles. A domain map of E2F1 protein is drawn, indicating either a function previously assigned (DNA binding and pRB binding) or a structural motif (Heptad repeat and marked box). The levels of conservation between E2F1 and four additional human E2Fs are indicated (percentages represent the number of positions with at least four identical amino acids) (10). The region corresponding to aa 159–437 was mutagenized. The different E2F1 alleles in the marked box are indicated with the codon number (codon), the wild-type residue (E2F1 WT), the mutant residue (mutation), and the assigned allele number (allele name). Note that two alleles contain double mutations (alleles 1 and 30). A small in-frame deletion (aa 285–324) was also obtained (E2F1-31) and is not depicted here. A putative helix is predicted by Garnier–Robson and Chou–Fasman algorithms for the wild-type E2F1 sequence around aa 231 and 248. This predicted helix is conserved (+) or disrupted (−) as indicated for the different alleles affecting the N terminus of the marked box. To quantify the defects of the different E2F1 mutant alleles, we measured their effect on expression of a *GAL1*::*lacZ* reporter gene present in the MaV103 *SPAL10*::*URA3 GAL1*::*HIS3* strain as determined by β-galactosidase activity (β-Gal activity). The AD-E2F1/DB-DP1 wild-type interaction was standardized to 100 and bar graphs representing the percent activity for AD-E2F1 alleles are shown: E2F1 WT, 100; E2F1-1, <0.5; E2F1-7, <0.5; E2F1-10, <0.5; E2F1-13, <0.5; E2F1-14, 0.7; E2F1-18, <0.5; E2F1-116, <0.5; E2F1-138, 1.5; E2F1-142, <0.5; E2F1-20, 7.5; E2F1-30, 28.8; E2F1-31, 0.7; E2F1-65, 5.4; E2F1-117, 1.1; E2F1-129, 1.2; E2F1-165, 19.0. For comparison, under identical conditions, the AD and DB plasmids gave rise to undetectable levels and DB-Fos/AD-Jun interaction or full-length GAL4 protein gave rise to 428% and 3800%, respectively.

raised from the observation that nonoverlapping E2F1 domains might participate in DP1 binding (12). We predicted that these alleles could be recovered from two sequential selections, an initial negative selection based on the reverse two-hybrid assay by conferring a Foa^R phenotype at the concentration established in the titration experiment (0.1%, Fig. 2) and a subsequent positive selection for residual protein–protein interaction using a relatively low concentration of 3AT. Since wild-type DB-DP1/AD-E2F1 interaction allows growth at 3AT concentrations up to 100 mM, we predicted that weak E2F1 mutations could be selected that conferred a His⁺ phenotype at 10 mM 3AT.

The E2F1 coding sequence corresponding to residues 159–437 was mutagenized by the use of a PCR favoring single misincorporations. The PCR products were subsequently introduced into DB-DP1-containing yeast cells by gap-repair transformation with a linearized AD-E2F1 plasmid (55). The transformants were replica-plated to 0.1% FOA-containing plates where approximately 5% of them survived. After recovery on permissive plates, these colonies were then replica-plated to 10 mM 3AT-containing plates on which approximately 2% of the FOA-surviving colonies conferred a His⁺ phenotype. The observed Foa^R His⁺ phenotypes resulted from the expression of mutated AD-E2F1 alleles and not from a compensating genomic mutation, since reintroduction of the corresponding AD-E2F1 plasmids, after isolation and amplification in *Escherichia coli*, into fresh DB-DP1-containing yeast cells conferred identical phenotypes (Fig. 2*A Left*, E2F1 alleles 20, 30, 31, and 65).

As a control that the E2F1 alleles were specifically affected in the binding of DP1, DB-pRB/AD-E2F1-mutant interactions were tested; the pRB-binding domain of E2F1 maps to 18 residues located at the C-terminal and does not require a functional DP1-binding domain (3). Approximately two-thirds of the alleles conferred wild-type interaction with pRB and were chosen for further characterization (Fig. 2*A Right*, E2F1 alleles 20, 30, 31, and 65). The steady-state levels of the corresponding mutant AD-E2F1 hybrids were not altered, as shown by protein immunoblot analysis (Fig. 2*B*). Sequence analysis of the alleles revealed mutations in a domain identi-

fied by sequence conservation between different E2Fs and called the marked box (6). The conservation in the marked box extends from the five human E2Fs to *Drosophila* E2F (dE2F) (50% identity among human E2Fs and 35% identity between E2F1 and dE2F) (10, 63).

To characterize the possible involvement of the marked box in DP1 heterodimerization, we centered a second mutagenesis around its encoding sequence (residues 231–300) in a PCR using appropriate primers. Using the two-step selection procedure described above, we isolated six additional weak mutations in E2F1 that affected heterodimerization with DP1 but not its binding to pRB (Fig. 2*B*, E2F1 alleles 116 to 169).

Two-Step Selection for Strong and Weak Mutations. To identify important regions of the marked box, we used a second variation of the two-step selection scheme. We sought to identify single amino acid changes that strongly disrupt DP1 heterodimerization (strong mutations). In this procedure, we directly selected the AD-E2F1 mutant alleles that retained the ability to bind pRB from the colonies obtained on FOA in the first selection. In this case, the two-step selection was aimed at eliminating truncations of E2F1. Thus, the pRB-binding domain of E2F1 is located at the very C terminus of the protein and pRB binding can be used as an indication for full-length E2F1 alleles. This procedure was expected to give rise to both weak and strong mutations.

Approximately 400 Foa^R colonies obtained after the first-step selection for mutations corresponding to residues 231–300 (see above) were cured of the DB-DP1 plasmid and mated with lawns of cells of the opposite mating type containing DB-pRB. Among diploid colonies resulting from the mating, six colonies showed a His⁺ phenotype, indicative of a wild-type pRB-binding domain. These phenotypes were identical after reintroduction of the corresponding plasmids into fresh DB-DP1-containing cells and were not due to changes of the steady-state levels of the fusion proteins (Fig. 2 *A* and *B*, E2F1 alleles 1 to 18).

***In Vitro* Binding of Wild-Type and Mutant E2F Alleles.** To demonstrate the relevance of the marked box alleles obtained by two-step selections, we assayed the DP1 heterodimerization function of the corresponding proteins in an *in vitro* binding

reaction. Wild-type DP1 and E2F1 (residues 159–437) or E2F1 mutant proteins were produced by *in vitro* transcription/translation, mixed, and subsequently coimmunoprecipitated with an anti-E2F1 mAb. A substantial amount of DP1 protein was retained in immunocomplexes with wild-type E2F1. In contrast, a significantly reduced amount of DP1 protein was bound to each of the mutant E2F1 proteins containing weak mutations and no interaction was detected with the strong mutations (Fig. 2*C*). The variation among different alleles tested in this assay correlated with the degree of 3AT resistance observed in the two-hybrid assay (compare Fig. 2 *A* and *C*).

Quantitation of the E2F1 Mutant Alleles Defect. To quantify the defects of the different E2F1 mutant alleles, we measured their effect on expression of a *GAL1::lacZ* reporter gene present in the *SPAL10::URA3 GAL1::HIS3* strain (50) as determined by β-galactosidase activity (Fig. 3). The differences between wild-type and mutant interactions varied from 3- to more than 200-fold. With the exception of E2F1-14, all strong mutations gave rise to undetectable levels of β-galactosidase activity.

DISCUSSION

We have described genetic selections that allow single amino acid changes that disrupt protein–protein interactions to be selected from large libraries of randomly generated mutant alleles. We have selected such mutations in the transcription factor E2F1 that affect its ability to heterodimerize with DP1. The isolated mutations may either affect a residue that directly participates in the interaction or disrupt a local structural element that helps support the actual contact domains. Examination of the amino acid changes in the 16 E2F1 mutant alleles described herein suggests an interesting structural aspect of the marked box (Fig. 3). Six out of seven mutations located in the N-terminal region of the marked box result in changes to proline. As suggested by this high frequency of prolines, secondary structure predictions (64, 65) suggest the presence of an α-helix in the N-terminal region of the wild-type marked box; most of the mutations isolated in that region decrease the helix formation probability (Fig. 3). Since these proline changes confer strong phenotypes, it is likely that this putative helix in the marked box is an essential determinant of DP1 heterodimerization. A small deletion of the C-terminal part of the marked box decreases the β-galactosidase activity by 20-fold, suggesting that this region is also important for DP1 heterodimerization.

In a similar study, dominant negative mutations in p53 were selected using a third variation of the two-step selection. The selected mutations correspond to most of the residues previously shown to directly contact DNA and zinc atoms (66). These observations underline the potential structural significance of the E2F1 alleles described herein.

Residues 206–220 of E2F1 have been identified as a likely DP1-interacting domain. However, this domain was ruled out as the sole determinant of binding since its deletion only marginally affected interaction with DP1 (12, 15, 49). Thus, the mutations obtained in the two-step selections described herein suggest that both a helical structure in the marked box and the region of residues 206–220 of E2F1 cooperate to form the DP1 heterodimerization domain.

We envision that the two-step selection procedure could be used to characterize any interaction domain that can be tested in the two-hybrid system. The two-step selections for strong mutations could be performed by fusing the E2F1 18-amino acid pRb-binding domain to the C terminus of the protein of interest. In preliminary experiments, we have shown that the two-step selections can also be used to isolate conditional alleles by performing the first-step negative selection under defined restrictive conditions and the second-step positive

selection under permissive conditions (data not shown). We also anticipate that the isolation and characterization of compensatory mutations in the interacting partner of weak, strong, and conditional mutations could unravel important features of protein–protein interactions as previously described for phage and yeast proteins (67–69). In addition to facilitating the functional characterization of interaction domains, specialized alleles such as weak, strong, conditional, or dominant negative alleles generated by two-step selections could be reintroduced *in vivo* and be used to study function.

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