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Human p53 and *CDC2Hs* Genes Combine To Inhibit the Proliferation of *Saccharomyces cerevisiae*

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Human wild-type and mutant p53 genes were expressed under the control of a galactose-inducible promoter in *Saccharomyces cerevisiae*. The growth rate of the yeast was reduced in cells expressing wild-type p53, whereas cells transformed with mutant p53 genes derived from human tumors were less affected. Coexpression of the normal p53 protein with the human cell cycle-regulated protein kinase CDC2Hs resulted in much more pronounced growth inhibition than for p53 alone. Cells expressing p53 and CDC2Hs were partially arrested in G_1, as determined by morphological analysis and flow cytometry. p53 was phosphorylated when expressed in the yeast, but differences in phosphorylation did not explain the growth inhibition attributable to coexpression of p53 and CDC2Hs. These results suggest that wild-type p53 has a growth-inhibitory activity in *S. cerevisiae* similar to that observed in mammalian cells and suggests that this yeast may provide a useful model for defining the pathways through which p53 acts.

Nowhere are violations of cell cycle regulation more clearly illustrated than in tumor cells, where such violations can ultimately lead to destruction of the organism. A tumor cell emerges with a number of strikes against its genome, enabling it to continue through the cell cycle under circumstances in which its normal counterpart would be dormant. Mutational events mediating tumorigenesis may target components unique to regulation of cell division in a specific cell type. However, abnormalities commonly suffered by many tumor types may illuminate fundamental mechanisms governing the ability of any cell to proceed through the cell cycle.

Several lines of evidence suggest that the tumor suppressor gene p53 may negatively regulate cell division in a variety of cell types. First, inactivation of p53 is a common and perhaps rate-limiting step in the progression to malignancy for many tumor types (12). Second, the expression of wild-type, but not mutated, p53 prevents tumor cells from continuing through the cell cycle (1, 5, 21, 26, 27). Third, p53 is a nuclear protein that is a substrate for (and perhaps complexed with) CDC2Hs (3, 28, 37), a protein whose activity is critical for passage through G_1/S and G_2/M boundaries of the cell cycle (19, 34, 39).

Although the term "tumor suppressor" connotes a function attributable only to multicellular organisms, the control of cell cycle progression is critical to all eukaryotes. Genes in which recessive mutations uncouple arresting environmental cues from cell cycle progression in *Saccharomyces cerevisiae* can be considered yeast paradigms of tumor suppressors (4, 6, 25). The search for a yeast p53 homolog has so far proven unsuccessful. However, the facility of genetic analysis in yeasts prompted us to consider another possibility: mammalian p53 may still potentially interact with some yeast macromolecules which are functionally conserved. For this reason, we have examined the effects of inducing p53 gene expression in *S. cerevisiae* cells. The results, described below, demonstrate intriguing parallels between the effects of wild-type and mutant p53 expression in yeast and human cells and suggest that this approach may be useful for elucidating the pathways through which p53 acts to inhibit cell growth.

MATERIALS AND METHODS

Culture conditions. The genotype of strain YPH420, used for most experiments, was *MATa leu2 trp1-Δ63 ura3-52 prb1-1122 prp4-3 prc1-407*. Liquid media and agar contained 0.67% yeast nitrogen base and either 2% glucose or 2% raffinose for normal growth or 2% galactose for induction (35). Uracil and leucine were added to final concentrations of 20 and 30 μg/ml, respectively, for selection of appropriate plasmids. The non-protease-deficient strain YPH499 (*ura 3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) was used in some experiments, as noted.

Protein lysates. Overnight cultures grown in raffinose were diluted to an A_{600} of 0.1 in liquid media containing either raffinose or galactose, and cells were harvested after an additional 12 h of growth at 30°C. Cells were pelleted, washed once in cold phosphate-buffered saline (PBS; 40 mM K_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl), and resuspended in 100 μl of cold lysis buffer (1% Triton and 0.5% deoxycholate in PBS) plus protease and phosphatase inhibitors. Cold 0.4-mm glass beads were added, and the mixture was alternately vortexed for 30 s and put on ice a total of four times (24, 31). Lysates were separated from the beads and centrifuged for 15 min at 12,000 × *g* in a microcentrifuge. Supernatants were used for subsequent protein analysis. The protease inhibitors used were 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 10 μg of trypsin inhibitor per ml, 0.7 μg of peptstatin per ml, and 1 mM phenylmethylsulfonyl fluoride, and the phosphatase inhibitors were 1 mM $NaVO_4$ and 50 mM NaF.

Flow cytometry. Following 12 h of growth at 30°C in raffinose or galactose (see above), approximately 5 × 10^6 cells were pelleted, rinsed in TB (0.2 M Tris [pH 7.5]), resuspended in 250 μl of TB, sonicated for 10 s at a minimum level, and resuspended in TB-ethanol (30:70) for at least 1 h

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at room temperature (8, 14). The cells were pelleted, rinsed, and resuspended in 1 ml of TB. They were repelleted and resuspended in 500 μl of TB with RNase A (1 mg/ml) and incubated for 1 h at 37°C. They were then pelleted, washed, and suspended in pepsin (0.5 g of pepsin in 5.5 mM HCl) for 5 min at room temperature; they were washed once with TB and resuspended in 1 ml of propidium iodide (3 μg/ml) in TB. They were then stored at 4°C until flow cytometric analysis with an excitation wavelength of 480 nm.

Growth kinetics. Overnight cultures grown in raffinose were diluted to an A_{600} of 0.1 in medium containing either raffinose or galactose, and cells were harvested at 1- to 2-h intervals at 30°C. Increases in optical density (A_{600}) observed 5 to 10 h after dilution were used to compute doubling times. For each strain, at least two independent clones were assessed, and the difference in doubling times between clones was never more than 15%.

In vivo labeling. For all in vivo labeling experiments, overnight cultures grown in raffinose were diluted to an A_{600} of 0.1 in an appropriate medium and incubated at 30°C for 12 h. Trans Label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN) was added to 5 to 10 ml of cells at a final concentration of 100 μCi/ml and incubated at 30°C for 2 h (24, 31).

Labeling with $^{32}P_i$ (100 μCi/ml) entailed growth for 12 h in a phosphate-depleted medium in which 2% galactose was the carbon source (33). The overnight cultures were pelleted and washed once in this medium before dilution to an A_{600} of 0.1. For 1 liter of medium, 150 ml of solution A (see below), 10 ml of solution B, 1 ml of solution C, 10 ml of solution D, and 4 ml of solution E were added to 825 ml of sterile H_2O containing 1 g of yeast extract and 20 g of the appropriate carbon source. Solution A was 10.21 g of potassium phthalate in 150 ml (pH 5); solution B was 20 μg of biotin, 4 mg of calcium pantothenate, 20 mg of inositol, 4 mg of niacin, 2 mg of *p*-aminobenzoic acid, 4 mg of pyridoxine HCl, 4 mg of thiamine HCl, and 2 mg of riboflavin in 100 ml; solution C was 5.72 mg of boric acid, 3.06 mg of $MnSO_4 \cdot H_2O$, 30.8 mg of $ZnSO_4 \cdot 7H_2O$, 3.9 mg of $CuSo_4 \cdot 5H_2O$, 1.84 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 17.8 mg of $FeCl_2 \cdot 4H_2O$ in 100 ml; solution D was 5 g of $MgSO_4 \cdot 7H_2O$, 20 g of $(NH_4)_2SO_4$, and 1 mg of KI in 100 ml; solution E was 3.3 g of $CaCl_2 \cdot H_2O$ and 0.5 g of NaCl in 40 ml. Solutions A, B, and C were filter sterilized, and solutions D and E were autoclaved.

Transformation. Cells were grown to an A_{600} of 1.0 in YPD (1% Bacto-Yeast extract, 2% Bacto-Peptone, 2% dextrose), pelleted, and washed once in 10 ml of LiB (0.1 M lithium acetate) (15). The cells were collected, resuspended in 10 ml of LiB, and incubated at 30°C for 1 h with shaking. Cells were pelleted and resuspended in 0.5 ml of LiB. Then 0.05 ml of cells was transformed with 1 to 6 μg of plasmid DNA. The cells and DNA were incubated at 30°C for 10 min with shaking. A 0.5-ml volume of 40% polyethylene glycol 4000 in 10 mM Tris (pH 7.5) was added, and the suspension was incubated at 30°C with shaking for 1 h. Cells were heated to 42°C for 5 min and washed twice with 1 ml of H_2O. The cells, resuspended in 100 μl of sterile H_2O, were spread onto plates containing glucose and appropriate amino acid supplements.

Western blot (immunoblot). Protein (25, 50, or 100 μg; culture conditions and lysates prepared as described above) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) at 150 V for 3 h. The gel was briefly rinsed in CB (0.039 M glycine, 0.048 M Tris, 0.0375% SDS) and was transferred, in CB, to polyvinylidene difluoride paper for 1.5 h at room temperature by using a Jannsen semidry blot apparatus. The blot was rinsed in TBS (100 mM Tris [pH 7.5], 0.9% NaCl) for 30 min and incubated in 10 ml of TTBS (TBS containing 0.1% Tween 20) with 1% nonfat dry milk for approximately 2 h; primary antibody was added at 1 μg/ml and incubated overnight at room temperature. The blot was washed three times in TTBS at room temperature, and 2.5 μCi of ^{125}I-labeled secondary antibody (goat anti-mouse or anti-rabbit immunoglobulin G) in 10 ml of TTBS with 5% nonfat dry milk was added to the blot and incubated for 1 to 2 h at room temperature. The blot was washed three times in TTBS and exposed to film.

Immunoprecipitation. Approximately 25 μg of total protein from a lysate (labeled and prepared as described above) was incubated with 1 μg of antibody in 200 μl of RIPA (10 mM Tris [pH 7.5], 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS) containing the cocktail of protease and phosphatase inhibitors described above. The mixture was incubated on ice for 2 to 8 h. Protein A-Sepharose (50 μl, suspended in RIPA at 50 mg/ml) was then added, and the mixture was rotated end over end at 4°C for 45 min. Suspensions were pelleted and washed three times in 1 ml of cold RIPA. The immunoprecipitates were separated by SDS-PAGE (10% acrylamide) and subjected to fluorography following treatment of the gel with En^{3}Hance (NEN).

Tryptic peptide mapping. ^{32}P-labeled protein (culture conditions and lysate preparation described above) was immunoprecipitated and separated by SDS-PAGE (10% acrylamide), and the 53 kDa band was excised. A single 5-h wash of this band with 25% isopropanol was followed by one overnight wash and four 2-h washes in 10% methanol. Bands were lyophilized and resuspended in 1 ml of 50 mM ammonium bicarbonate (pH 8.0) with 50 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma) per ml and rotated end over end for 24 h at 37°C. The supernatants were lyophilized, dissolved in 100 μl of distilled H_2O, and relyophilized. Samples were then dissolved in 10 μl of electrophoresis buffer (88% formic acid, glacial acetic acid, H_2O [pH 1.9]; 1:3:16), spotted on Kodak chromatogram sheets (13255 Cellulose), and subjected to electrophoresis for 40 min at 1,000 V. Plates were dried for 24 h, and second-dimension chromatography was performed in a chamber equilibrated with buffer (glacial acetic acid-pyridine-H_2O-*n*-butanol [3:10:12:15]) (20).

Two-dimensional isoelectric focusing and SDS-PAGE. Isoelectric focusing in the first dimension was performed as described previously (22) and included an equal mixture of pH 5 to 6 and pH 6 to 8 ampholytes (Pharmacia). Electrophoresis was performed at 500 V for 14 h. Tube gels were expelled and equilibrated for 1 h in Laemmli sample buffer. The gels were heated for 1 min at 80°C before loading onto an SDS-PAGE gel (10% acrylamide) for the second dimension.

Alpha-pheromone treatment. Cells previously incubated in 2% galactose for 12 h were spread onto selective plates containing 2% galactose. Discs (6 mm; cut from Schleicher & Schuell GB002 paper) were placed in the center of the plates, and 8 μl of pheromone solution (in absolute methanol) was added to the filter. The three concentrations of pheromone used were 25, 8.3, and 2.8 mg/ml. The plates were incubated at 30°C for 3 to 5 days (6).

Heat sensitivity. Cells grown in 2% galactose for 12 to 16 h at 30°C were subsequently incubated at 53°C for 25 min and then spread on selective plates containing 2% galactose. The plates were incubated at 30°C for 3 days (38).

In vitro mutagenesis. A p53 cDNA fragment was inserted into the *Bam*HI site of the pSelect vector (Promega) con-

taining a nonfunctional Amp^r gene. Two primers were added to the T4 DNA polymerase reactions: a 31-bp oligomer harboring the desired p53 mutation (5'-ACAACACCAGC TCCGATCCCCAGCCAAAGA-3') and a second primer which would restore Amp^r to the plasmid. Plasmids were selected on the basis of restored Amp^r as described in the Promega instruction manual.

Polymerase chain reaction and cloning. The polymerase chain reaction was performed as previously described (29), but reaction mixtures contained 450 ng of p53 plasmid or yeast plasmid with *GAL1* promoter, and five cycles at 95°C (30 s), 45°C (3 min), and 70°C (2 min) followed by 16 cycles at 95°C (30 s), 58°C (3 min), and 70°C (2 min) were used. The primers used to amplify the *GAL1* promoter were 5'-CTTG GTACCTGAAGTACGGATTAG-3' and 5'-CTCCTCGAGT TGATACTTTTATT-3' (16). The primers used to amplify p53 were 5'-TCATGTGCTGTGACTGCTTG-3' and 5'-GCCT CGAGGAGAAAAAACTATAATGGAGGAGCCG-3' (underlined nucleotides correspond to p53 sequences; remaining nucleotides were derived from the *Gal1* promoter). Primers contained *Kpn*I, *Xho*I, and *Eco*RI sites for efficient cloning into plasmids. The polymerase chain reaction products from these reactions were ligated sequentially and finally placed into the yeast expression vector pRS314 (36). The resultant vectors (p53, Ala-143, His-273, Asp-315) contained the *GAL1* promoter (bp 361 to 767 [16]) and an abbreviated 5' untranslated *GAL1* region joined directly to the ATG initiation codon of p53. The sequences upstream of the ATG were AGTATCAACTCGAGGAGAAAAAACTATA.

The plasmid used for regulated expression of the *CDC28* gene was YCpG1[*CDC28*] (25). This plasmid contains the *URA3* and *LEU2* selectable markers, yeast centromere and replication sequences, and the *CDC28* coding region under the control of the *GAL1* promoter. A similar plasmid was constructed for conditional expression of *CDC2Hs* by inserting a *Bam*HI fragment containing the *CDC2Hs* coding region into the unique *Bam*HI site of vector YCpG2 (34a). *Bam*HI sites were added immediately upstream of the initiation codon and downstream of the termination codon of the *CDC2Hs* cDNA (19).

RESULTS

Wild-type and human p53 cDNA sequences were inserted downstream of the *GAL1* inducible promoter on a low-copy-number, mitotically stable plasmid containing *CEN* and *ARS* sequences (36). The p53 sequence included nucleotides −133 to +1668 (relative to the translation start site) for wild-type and one mutant (Ala-143) p53, but only nucleotides −60 to +1247 for a second mutant (His-273). The constructs were transformed into an *S. cerevisiae* strain which grew well on galactose and harbored appropriate auxotrophies (YPH499). A discrete p53 transcript was produced in the presence of galactose, but p53 protein was not detectable in Western blot assays (data not shown).

In a first attempt to increase translation, constructs were introduced into a protease-deficient (YPH420) background with appropriate auxotrophies. Again, no differences in growth were observed on plates or in liquid culture; however, protein was detectable on Western blot with all three constructs. In addition, we noticed that the His-273 mutant construct, which had a shorter 5' untranslated region, expressed more p53 protein per microgram of total protein than the other two constructs did.

On the basis of this result, alterations in the sequence preceding the initiation codon of p53 were made. The final

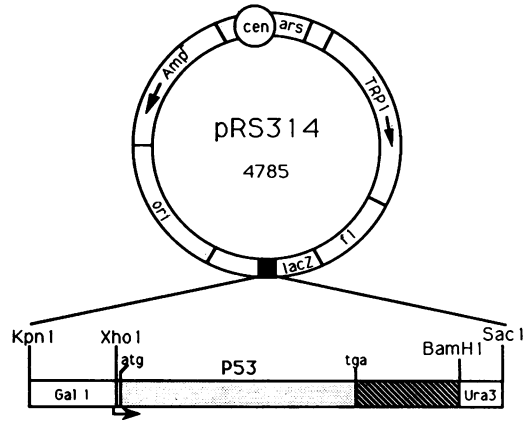

FIG. 1. Schematic diagram of the p53 expression vector. pRS314 contains *CEN/ARS* sequences and the *TRP1* gene for selection of appropriate auxotrophs. The p53 cDNA insert was ligated into the polylinker of the plasmid at *Kpn*I and *Sac*I sites (black box) and was constructed as described in Materials and Methods. Twenty-eight nucleotides derived from the *GAL1* promoter preceded the p53 coding region (hatched area). A portion of the 3' untranslated region of p53 (striped area) is followed by *URA3* promoter sequences, which contain a transcription stop site as well as a polyadenylation signal. The arrow denotes the transcription start site (40).

construct developed is shown in Fig. 1. Only 28 bp were contained within the 5' untranslated region of the predicted transcript. These 28 bp were derived totally from *S. cerevisiae* sequences and included 9 bp downstream of the *GAL1* transcription start joined to 16 bp upstream of the translation start of the *GAL1* gene (16). The additional 3 bp were derived from the *Xho*I site used to join these sequences. The sequence of the p53 protein produced in *S. cerevisiae* by this construct should be identical to that of authentic p53, with no deletions or additions of amino acids at the 5' end.

Colony growth differed between cells expressing exogenous wild-type p53, mutant p53, and no p53 (vector without insert). Colonies expressing wild-type p53 were considerably smaller than the control colonies (Fig. 2). Cells producing the Ala-143 mutant grew almost as well as the control cells, while cells expressing the His-273 mutant exhibited an intermediate colony size. Growth in liquid culture was assessed as described in Materials and Methods. Clones containing the vector alone (no p53) had a doubling time in galactose-containing medium of 4.4 h. The doubling time of cells containing the wild-type p53 was increased to 11.6 h (an increase of 250%). The doubing time of strains containing the Ala-143 mutant was 4.6 h (an increase of 5%), and the doubling time of cells containing the His-273 mutant was 6.5 h (an increase of 48%). No significant differences in doubling time were observed among the various strains when grown in raffinose-containing medium.

The growth phenotypes shown in Fig. 2 were reproducible in each of four independent transformants assessed for each construct. Differences in growth were apparent only under inducing (galactose) conditions, since all cultures grew equivalently on glucose-containing medium. We also attempted to introduce the final engineered p53 constructs (Fig. 1) into a non-protease-deficient strain (YPH499). Little difference in growth rate was observed between the strains containing either wild-type p53 or vector alone in this strain. We do not know whether this difference between strains was related to posttranslational modifications of p53 (such as

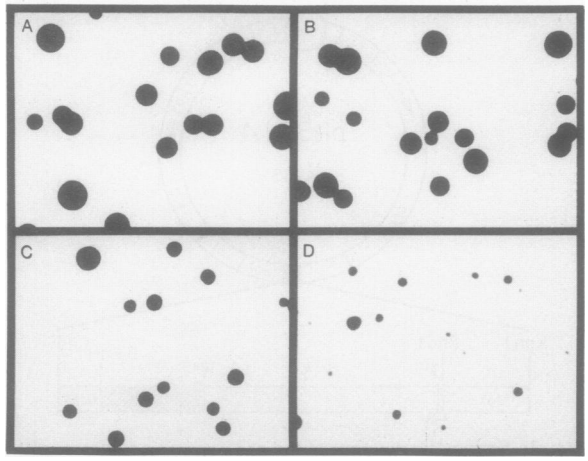

FIG. 2. Phenotype of *S. cerevisiae* expressing human p53 constructs. Cells transformed with vector (A), the Ala-143 mutant of p53 (B), the His-273 mutant of p53 (C), or wild-type p53 (D) constructs were grown to mid-log phase in liquid culture with 2% raffinose. Cells were plated onto selective plates containing 2% galactose and incubated at 30°C for 3 days. Magnification, ×6.

intracellular digestion by proteases) or to other factors, and we did not pursue this issue further.

To assess p53 expression in transformants, we immunoprecipitated p53 from metabolically labeled lysates prepared at various times after induction with galactose (Fig. 3). Uninduced cultures did not exhibit any p53 protein expression. In cultures containing galactose, p53 protein expression increased during the 12-h incubation for both the mutant and wild-type protein species. There were no significant differences between the amount of p53 produced in the strains containing wild-type p53 or the Ala-143 or His-273 mutants (Fig. 3; data not shown). Western blots (not shown) confirmed the immunoprecipitation data, demonstrating that similar levels of p53 protein were found in strains producing the wild-type and mutant forms. Therefore, different levels of the p53 expression could not explain the differences that were observed.

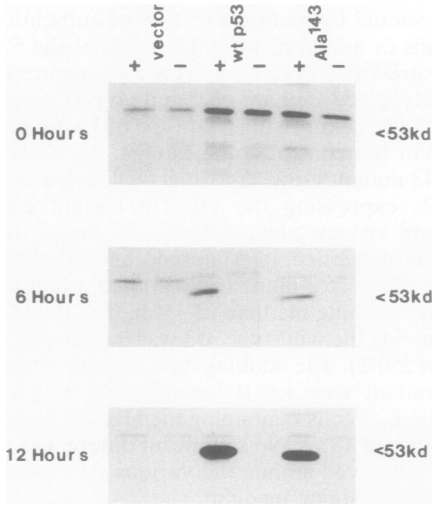

FIG. 3. Induction of p53 protein expression in liquid culture. Individual yeast colonies were grown to mid-log phase in liquid culture with 2% raffinose. Cultures were subsequently diluted to an A_{600} of 0.1 in selective medium containing 2% galactose. Cells were harvested from liquid cultures at the time points indicated after addition of galactose, and protein lysates were prepared as described in Materials and Methods. Cells contained vector only, wild-type p53 (wt p53), or the Ala-143 mutant of p53. Immunoprecipitations were performed with a monoclonal antibody to p53 (+ lanes) or mouse immunoglobulin (– lanes) as a control. The migration of the mutant p53 is slightly faster than that of the wild-type protein. A background band migrating at 58 kDa was sometimes observed.

We also tested these strains under conditions which are known to affect cell cycle progression. We reasoned that cells producing the mutant p53 might be refractory to the growth inhibition mediated by pheromone whereas cells expressing wild-type p53 might be more sensitive to the pheromone (i.e., lower concentrations causing growth arrest). In a halo assay performed under inducing conditions, cells containing wild-type p53 still grew considerably more slowly than the control cells or cells producing the Ala-143 mutant, but the zones of growth inhibition formed around filters containing different pheromone concentrations were the same for all strains (data not shown). We also tested cell viability after exposure to elevated temperature. All of the strains emerged from stationary phase in a similar manner after heat treatment (see Materials and Methods). Therefore, p53 expression did not appear to differentially affect the response to heat shock or pheromone.

A current model for regulation of p53 activity invokes phosphorylation by the CDC2Hs kinase as the critical event (3, 28, 37). To test for an in vivo interaction between these two proteins in *S. cerevisiae*, we introduced a human *CDC2Hs* gene construct into cells already containing a p53 construct. We also tested yeast genes known to regulate the cell cycle, specifically *CLN2* (10), *CLB1* (9), and *CDC28* (the yeast homolog of human *CDC2Hs*). All constructs were under the control of a galactose-inducible promoter on plasmids containing *CEN* and *ARS* functions (25). A striking reduction in colony size was observed in cells expressing both wild-type p53 and CDC2Hs (p53/CDC2Hs cells [Fig. 4]). None of the other tested genes, including *CDC28*, had any effect over and above that observed with wild-type p53 alone. Cells expressing the mutant Ala-143, when transformed with *CDC2Hs* and induced with galactose, grew at a level intermediate between that of p53/CDC2Hs and control cells (Fig. 4). In liquid cultures, the Ala-143/CDC2Hs cells exhibited a doubling time of 6.5 h (an increase of only 45% over the control cells), whereas the doubling time of the wild-type p53/CDC2Hs cells was increased by sixfold (to 26 h).

At a microscopic level, approximately 90% of p53/CDC2Hs cells appeared to be large and unbudded when grown on galactose (Fig. 5D). In contrast, only 30% of cells transformed with vector alone or with the CDC2Hs construct without p53 were unbudded. Cells expressing wild-type p53, but no CDC2Hs, were indistinguishable in morphology from the control cells (Fig. 5A). Interestingly, the buds of CDC2Hs-expressing cells were often extended and oblong (Fig. 5B). For cells expressing the Ala-143 mutant of p53 as well as CDC2Hs, 50% of the cells exhibited the morphology of the unbudded p53/CDC2Hs cells; most of the budded cells had the extended morphology observed in strains expressing CDC2Hs alone.

To determine whether these cells arrested in a specific phase of the cell cycle, we performed flow cytometry. Strains containing wild-type p53 or wild-type p53 plus either CDC2Hs or CDC28 plasmids exhibited G_1 and G_2/M peaks typical of a cycling population of cells when grown in media

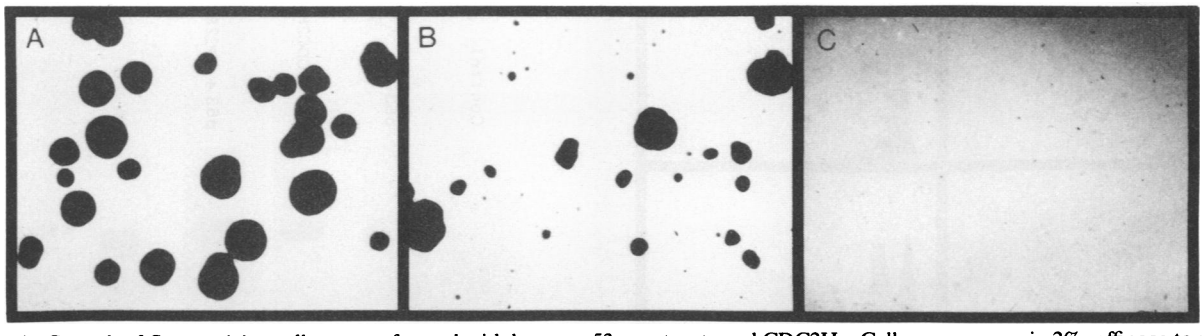

FIG. 4. Growth of *S. cerevisiae* cells cotransformed with human p53 constructs and CDC2Hs. Cells were grown in 2% raffinose to mid-log phase and spread onto selective plates containing 2% galactose. Colonies were photographed at a magnification of ×10 after 7 days of growth at 30°C. The colonies formed in panel A contained the CDC2Hs vector and were slightly scalloped, compared with the colonies shown in Fig. 2A. Cells containing both the Ala-143 mutant of p53 and the CDC2Hs vector were highly scalloped and grew slowly (B). Cells containing wild-type p53 and CDC2Hs vector (C) formed very small colonies.

containing raffinose (e.g., Fig. 6A and C). In galactose, cells containing wild-type p53 or wild-type p53 plus CDC28 were distributed in G_1 and G_2/M phases of the cell cycles as in the uninduced cultures (e.g., Fig. 6D). However, *S. cerevisiae* expressing both p53 and CDC2Hs accumulated largely in G_1 (Fig. 6, top).

We considered several possibilities to explain the reduced growth of p53/CDC2Hs cotransformants. In each case, we compared p53/CDC2Hs cells with p53/CDC28 cells, which grew at a rate comparable to that for cells transformed with p53 alone. The first possibility was that the p53 protein levels were increased in the CDC2Hs-containing strain. This was eliminated by the results of Western blot experiments: p53 expression was similar in both cases (Fig. 7). In addition, the levels of CDC2Hs and CDC28 produced in strains containing exogenous *CDC2Hs* and *CDC28* genes, respectively, appeared to be similar as assessed by Western blot assay (data not shown).

p53 is highly phosphorylated in mammalian cells (3, 23), and one of the kinases responsible for this phosphorylation may be CDC2Hs. We therefore considered the possiblity that CDC2Hs was able to phosphorylate p53 and thereby activate its suppressing capabilities. As a first test of this possiblity, we metabolically labeled *S. cerevisiae* cells with ^{32}P and immunoprecipitated p53 with monoclonal antibodies. The results shown in Fig. 8 demonstate that p53 is indeed phosphorylated when expressed in *S. cerevisiae*, whether expressed alone or with either of the two kinases under *GAL1* control. The levels of phosphorylation were equivalent in the p53/CDC2Hs and p53/CDC28 strains. However, this experiment assessed only whether p53 was phosphorylated in these yeast strains and not necessarily the

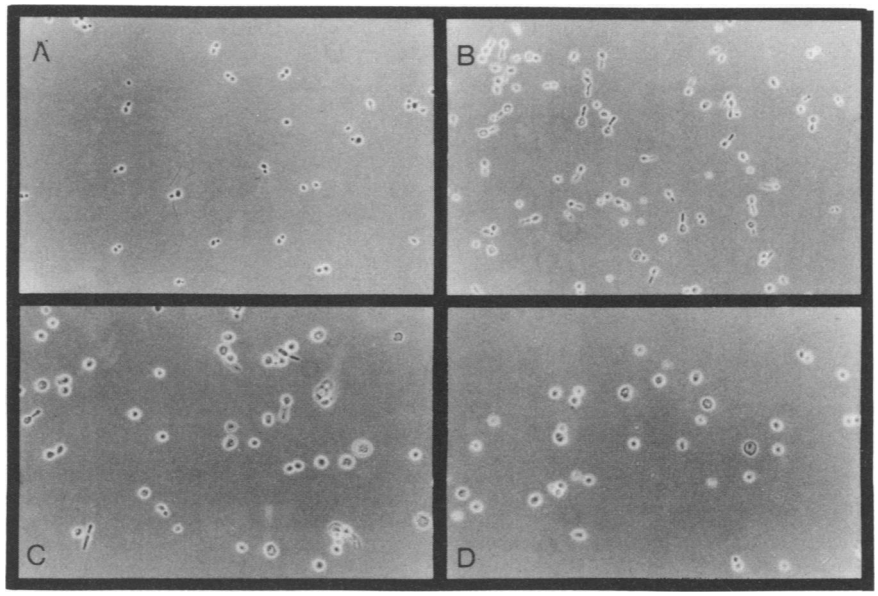

FIG. 5. Morphology of cells coexpressing human p53 constructs and CDC2Hs. Individual colonies were grown in liquid culture with raffinose to mid-log phase at 30°C, diluted to an A_{600} of 0.1 in medium containing 2% galactose, and grown for 12 h. Cells were fixed by the method described in Materials and Methods. (A) Vector plus CDC28; (B) vector plus CDC2Hs; (C) Ala-143 plus CDC2Hs; (D) wild-type p53 plus CDC2Hs. The CDC2Hs plasmid conferred a long-bud phenotype (B), whereas cells containing the CDC28 plasmid (A) had the same morphology as the parent cells grown in galactose. Cells containing p53 but not CDC2Hs were indistinguishable in morphology from those shown in panel A. Magnification, ×400.

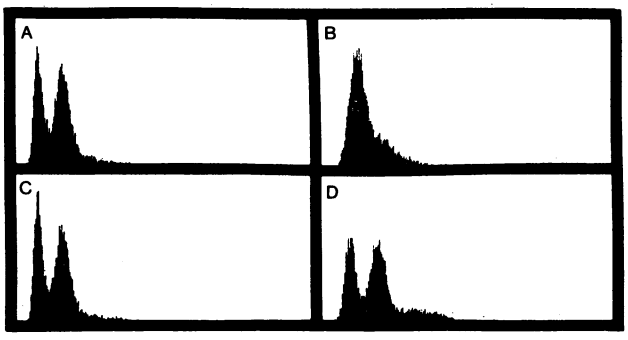

FIG. 6. Flow of cytometry of *S. cerevisiae* coexpressing human wild-type p53 and CDC2Hs (A and B) or p53 and CDC28 (C and D). Flow cytometry profiles of cells grown in 2% raffinose (A and C) and in the presence of 2% galactose (B and D) are shown. The number of cells is depicted on the vertical axis, and fluorescence emission is depicted on the horizontal axis.

steady-state level of p53 phosphorylation. To determine the extent of phosphorylation of p53 protein, we performed two-dimensional gel electrophoresis on p53 proteins labeled with [^{35}S]methionine in vivo. In previous studies, we have shown that the phosphorylated forms of p53 can be identified as isoforms with a molecular mass of 53 kDa on two-dimensional gels (17). The results of such assays performed on the proteins from *S. cerevisiae* cells are shown in Figure 9. Three major forms of p53 were identified, presumably representing three different phosphorylation states, in both p53/CDC2Hs and p53/CDC28 strains. The isoforms observed were similar to those seen from mammalian cells in analogous experiments (17).

The results of these two-dimensional gels suggested that the total level of phosphorylation of p53 was similar in the various strains, i.e., that the same number of phosphate groups was present on average in each p53 molecule. However, it was still possible that the specific sites of phosphorylation, rather than the number of sites per molecule, differed between the various strains. To determine whether the phosphorylated sites within the polypeptide differed, we performed two-dimensional tryptic peptide mapping. p53

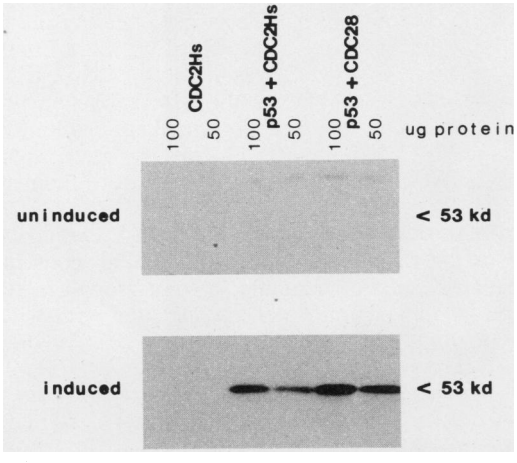

FIG. 7. Western blot analysis of cells coexpressing human wild-type p53 and exogenous CDC2Hs or CDC28. Either 100 or 50 μg of total protein per lane was loaded on SDS-PAGE gels (10% acrylamide) and transferred to polyvinylidene difluoride paper. Filters were incubated with a p53 monoclonal antibody and subsequently an ^{125}I-goat anti-mouse secondary antibody. p53 was not detectable in uninduced cultures (top panel), whereas cells grown in galactose (bottom panel) expressed similar amounts of p53 protein in the presence of either CDC2Hs or CDC28.

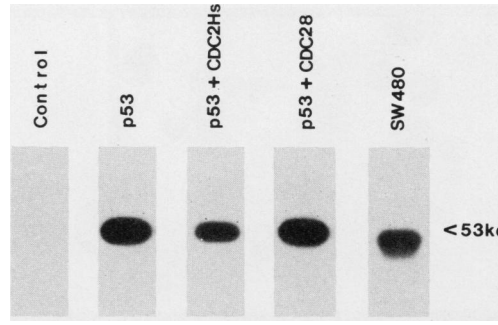

FIG. 8. Phosphorylation of human p53 in yeast cells. Individual colonies grown to mid-log phase in 2% raffinose were diluted to an A_{600} of 0.1 in phosphate-depleted medium containing 2% galactose and incubated at 30°C for 12 h. ^{32}P was added to the culture, and cells were harvested after an additional 2 h of incubation at 30°C. p53 was immunoprecipitated from protein lysates prepared from strains expressing p53 alone or with one of the two kinases. p53 was also immunoprecipitated from SW480, a human colorectal carcinoma cell line. The control lane represents immunoprecipitations performed with normal mouse immunoglobulin G. The protein from SW480 cells contined two point mutations (Arg-273 to His and Pro-309 to Ser) and migrated slightly faster than the wild-type p53 protein.

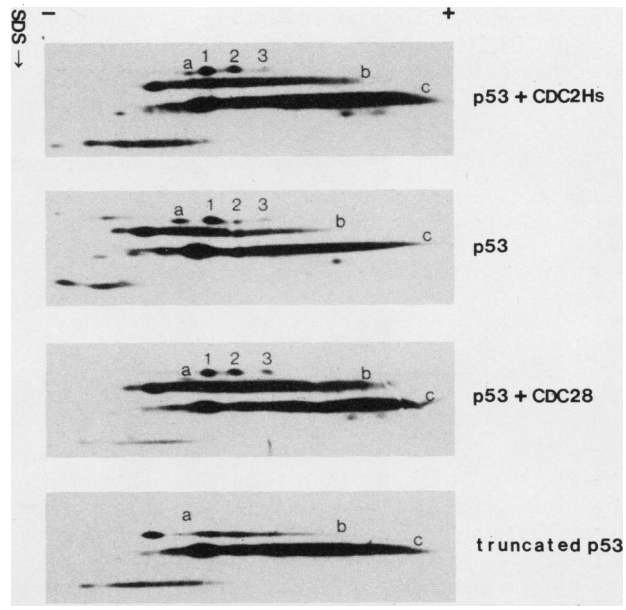

FIG. 9. Two-dimensional electrophoresis of metabolically labeled p53 in yeast cells. Proteins were labeled with $^{32}P_i$ as described in the legend to Fig. 8. Each panel represents p53 protein immunoprecipitated from cultures producing p53 alone or together with one of the two kinases. Proteins were focused at their isoelectric point in the first dimension and then run on SDS-PAGE gels (10% acrylamide) for the second dimension (the direction of electrophoresis is indicated by the arrow). A truncated p53 protein, translated in vitro, was added to each immunoprecipitate to orient the major species. Species 1, 2, and 3 were derived from the full-length p53 product produced in *S. cerevisiae*. Species a, b, and c were derived from the truncated p53 translated in vitro.

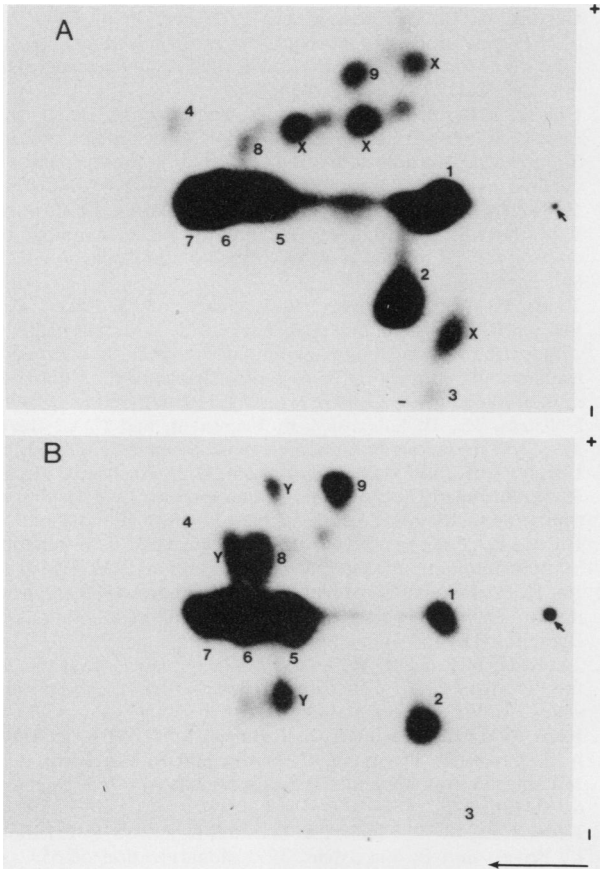

FIG. 10. Tryptic peptide maps of phosphorylated p53 in yeast and SW480 cells. p53 was immunoprecipitated from labeled lysates as described in the legend to Fig. 8 and subsequently digested with trypsin as described in Materials and Methods. The migration of peptide fragments from the origin (indicated by the small arrow) toward the electrodes is indicated by + and −. (A) Migration of phosphorylated p53 tryptic peptides expressed in yeast cells containing the p53 and CDC2Hs plasmids. (B) p53 protein immunoprecipitated from the human tumor cell line SW480. Phosphorylated peptides which migrated to similar positions are numbered 1 through 9. Peptides labeled X in panel A had no obvious counterparts in panel B, and peptides labeled Y in panel B were not represented in panel A. The large arrow indicates direction of chromatography.

was immunoprecipitated, digested with trypsin, and subjected to electrophoresis followed by ascending chromatography. The results are shown in Fig. 10, where the phosphorylated p53 peptides from yeast (Fig. 10A) or human (Fig. 10B) cells are illustrated. Nine peptides migrated to similar positions, but there were also several differences between the phosphorylation patterns observed between yeast and human cells (see the legend to Fig. 10). However, identical patterns were observed in cells expressing p53 alone and in cells expressing p53 plus CDC2Hs or CDC28, with 14 major comigrating spots apparent in each case (an example is given in Fig. 10A).

Finally, stimulated by the idea that CDC2Hs may be potentially phosphorylating p53 at a specific site, we altered the major site of p53 phosphorylation by CDC2Hs (Ser-315) (3). Assuming that phosphorylation of p53 at this site was causing the growth arrest, we attempted to create a mutant which might mimic phosphorylation at this residue (13) and thus replaced serine with aspartic acid. We guessed that if posttranslational modification occurring at this site regulated the activity of p53, the Asp-315 form of p53 might be in a permanent state of activation and, even without CDC2Hs, would be able to cause a severe growth arrest phenotype. However, when the Asp-315 form of p53 was introduced into cells alone or with CDC2Hs, the same degree of growth inhibition was observed as described above with the wild-type p53 constructs.

DISCUSSION

We have previously tried to demonstrate the existence of a p53 homolog by immunologic and recombinant DNA approaches. These included the polymerase chain reaction and cDNA library screenings with degenerate oligonucleotides corresponding to the most highly conserved regions of the p53 gene product. These experiments have all proved unsuccessful (30). The lack of a demonstrable p53 homolog in *S. cerevisiae*, however, did not discourage us from using this organism to explore potential functions. Instead, we thought that human p53 might function directly or indirectly through interacting macromolecules as a result of functional conservation between mammals and yeasts and therefore might elicit informative phenotypes. This hypothesis is now supported by the observations made here. *S. cerevisiae* haploid **a** cells, expressing wild-type p53 under the control of a modified *GAL1* inducible promoter, grew more slowly than those producing a mutant p53. Experiments in *Schizosaccharomyces pombe* with human p53 (2) or with portions of p53 fused to a DNA-binding sequence and expressed in *S. cerevisiae* (7) support the idea that p53 can alter the growth of lower eukaryotic cells. We also observed that coexpression of p53 with the human cell cycle-regulated kinase CDC2Hs, but not the yeast homolog CDC28, severely inhibited cell growth. The p53/CDC2Hs cells appeared to be at least partially arrested in G_1, since such cells were largely unbudded and contained unreplicated DNA as assessed by flow cytometry. p53 was phosphorylated in *S. cerevisiae*, but differences in phosphorylation did not account for the apparent growth rate observed in the various strains containing p53 and CDC2Hs.

The specificity of the p53 effect on *S. cerevisiae* growth was clearly demonstrated with the p53 mutant constructs. The growth of *S. cerevisiae* was affected much less dramatically by the Ala-143 mutant than by wild-type p53; the His-273 mutant behaved in an intermediate fashion. In cells expressing CDC2Hs, the Ala-143 mutant had a significant effect on growth, but to a much lesser extent than the wild type did.

Some of the effects on growth seen with *S. cerevisiae* were analogous to those observed with similar constructs in mammalian cells. For example, colony growth is severely inhibited in human tumor cells transfected with wild-type, but not mutant, p53 genes (1, 5, 21, 26, 27). Furthermore, mammalian cells expressing the wild-type p53 failed to progress to the G_1/S border whereas those transfected with mutants continued to proliferate (1, 5, 21, 26). The effects of different mutants also are distinguishable in mammalian cells. The His-273 mutant, for example, forms fewer foci than the Ala-143 mutant does when transfected with an activated *RAS* gene into primary rat embryo fibroblasts (11). Additionally, the His-273 mutant does not bind heat shock 70 proteins (11), unlike the Ala-143 mutant.

Differential phosphorylation of p53 does not appear to be the mechanism contributing to the severe growth phenotype observed in CDC2Hs compared with CDC28 cotransfor-

mants as determined by two-dimensional tryptic peptide mapping. Nevertheless, the simple fact that p53 was phosphorylated in *S. cerevisiae* proved than an endogenous yeast protein(s) is capable of interacting with p53. In the absence of phosphorylation differences, other possibilities must be considered to explain the growth inhibition mediated by CDC2Hs but not CDC28 in p53-expressing cells. p53 not only may be a substrate for CDC2Hs, but also may modulate the activity of CDC2Hs. p53 could act directly, by binding to CDC2Hs (28, 37), or indirectly, by affecting one of the many gene products already known to modulate CDC2Hs activity. We attempted to demonstrate a direct interaction of p53 with CDC2Hs by using immunoprecipitation assays in yeast cells coexpressing the two, but we could find no evidence for such interaction (30). We therefore consider an indirect effect more likely. p53 is known to bind to DNA (17, 18) and to have the potential to serve as a transcriptional activator (7, 32). Both of these biochemical activities are altered by in vivo-derived mutations such as those studied here. Moreover, it is known that the transcriptional activation function is active in *S. cerevisiae* (7). Through such activation, p53 might promote transcription of proteins mediating inactivation of CDC2Hs or could inhibit transcription of factors necessary for positive activation of the kinase. Alternatively, the overexpression of CDC2Hs could affect the expression or posttranslational modifications of genes which mediate the effects of p53 in *S. cerevisiae*, thereby accentuating them.

Regardless of the mechanism, the studies described above provide a powerful genetic approach to defining the biochemical pathways involved in p53 gene action in eukaryotic cells. For example, it should be possible to identify factors that interact with p53 as dosage suppressors of the slow growth phenotype in the p53/CDC2Hs cells. Although it is too early to predict whether the factors which mediate p53 action will be similar in mammalian and yeast cells, the analogous effects on growth inhibition observed with wild-type and mutant forms of p53 give us hope that continuation of these studies will prove revealing.

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REFERENCES

1. **Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249:**912–915.
2. **Bischoff, J. R., and D. Beach.** Unpublished data.
3. **Bischoff, J. R., P. N. Friedman, D. R. Marshak, C. Prives, and D. Beach.** 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. USA **87:**4766–4770.
4. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a Gl cyclin, CLN2. Cell **63:**999–1011.
5. **Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend.** 1990. p53 functions as a cell-cycle control gene in osteosarcomas. Mol. Cell. Biol. **10:**5772–5781.
6. **Elion, E. A., P. L. Grisafi, and G.R. Fink.** 1990. *FUS3* encodes a cdc2+/CDC28-related kinase required for the transition from mitosis into conjugation. Cell **60:**649–664.
7. **Fields, S., and Jang, S. K.** 1990. Presence of a potent transcription activating sequence in the p53 protein. Science **249:**1046–1049.
8. **Gerring, S. L., F. Spencer, and P. Hieter.** 1990. The *CHL1* (*CTF1*) gene product of *Saccharomyces cerevisiae* is important for chromosome transmission and normal cell cycle progression in G_2/M. EMBO J. **9:**4347–4358.
9. **Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed.** 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell **65:**163–174.
10. **Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. de Barros Lopes, and S. I. Reed.** 1989. A family of cyclin homologs that control the G1 phase in yeast. Proc. Natl. Acad. Sci. USA **86:**6255–6259.
11. **Hinds, P. W., C. A. Finlay, R. S. Quartin, S. J. Baker, E. R. Fearon, B. Vogelstein, and A. J. Levine.** 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. Cell Growth Differ. **1:**571–580.
12. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
13. **Hurley, J. H., A. M. Dean, J. L. Sohl, D. E. Koshland, Jr., and R. M. Stroud.** 1990. Regulation of an enzyme by phosphorylation at the active site. Science **249:**1012–1016.
14. **Hutter, K.-J., and H. E. Eipel.** 1979. Microbial determinations by flow cytometry. J. Gen. Microbiol. **113:**369–375.
15. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
16. **Johnston, M., and R. W. Davis.** 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**1440–1448.
17. **Kern, S. E., K. W. Kinzler, S. J. Baker, J. M. Nigro, V. Rotter, A. J. Levine, P. Friedman, C. Prives, and B. Vogelstein.** 1991. Mutant p53 proteins bind DNA abnormally *in vitro*. Oncogene **6:**131–136.
18. **Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein.** 1991. Identification of p53 as a sequence-specific DNA-binding protein. Science **252:**1708–1711.
19. **Lee, M. G., and P. Nurse.** 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. Nature (London) **327:**31–35.
20. **Luna, E. J., G. H. Kidd, and D. Branton.** 1979. Identification by peptide analysis of the spectrin-binding protein in human erythrocytes. J. Biol. Chem. **254:**2526–2532.
21. **Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine.** 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. **5:**151–159.
22. **Maurides, P. A., G. R. Akkaraju, and R. Jagus.** 1989. Evaluation of protein phosphorylation state by a combination of vertical slab gel isoelectric focusing and immunoblotting. Anal. Biochem. **183:**144–151.
23. **Meek, D. W., and W. Eckhart.** 1988. Phosphorylation of p53 in normal and simian virus 40-transformed NIH 3T3 cells. Mol. Cell. Biol. **8:**461–465.
24. **Mendenhall, M. D., C. A. Jones, and S. I. Reed.** 1987. Dual regulation of the yeast CDC28-p40 protein kinase complex: cell cycle, pheromone, and nutrient limitation effects. Cell **50:**927–935.
25. **Mendenhall, M. D., H. E. Richardson, and S. I. Reed.** 1988. Dominant negative protein kinase mutations that confer a G_1 arrest phenotype. Proc. Natl. Acad. Sci. USA **85:**4426–4430.
26. **Mercer, W. E., M. T. Shields, M. Amin, G. J. Sauve, E. Appella, J. W. Romano, and S. J. Ullrich.** 1990. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA **87:**6166–6170.
27. **Michalovitz, D., O. Halevy, and M. Oren.** 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell **62:**671–680.
28. **Milner, J., A. Cook, and J. Mason.** 1990. p53 is associated with $p34^{cdc2}$ in transformed cells. EMBO J. **9:**2885–2889.
29. **Nigro, J. M., K. R. Cho, E. R. Fearon, S. E. Kern, J. M. Ruppert, J. D. Oliner, K. W. Kinzler, and B. Vogelstein.** 1991.

Scrambled exons. Cell **64**:607–613.
30. **Nigro, J. M., and B. Vogelstein.** Unpublished data.
31. **Powers, S., T. Kataoka, O. Fasano, M. Goldfarb, J. Strathern, J. Broach, and M. Wigler.** 1984. Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian ras proteins. Cell **36**:607–612.
32. **Raycroft, L., H. Wu, and G. Lozano.** 1990. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science **249**:1049–1051.
33. **Reed, S. I., J. A. Hadwiger, and A. T. Lorincz.** 1985. Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28. Proc. Natl. Acad. Sci. USA **82**: 4055–4059.
34. **Riabowol, K., G. Draetta, L. Brizuela, D. Vandre, and D. Beach.** 1989. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. Cell **57**:393–401.
34a. **Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59**:1127–1133.
35. **Rose, M. D., F. Winston, and P. Hieter.** 1988. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sikorski, R. S., and P. A. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**:19–27.
37. **Struzbecher, H. W., T. Maimets, P. Chumakov, R. Brain, C. Addison, V. Simanis, K. Rudge, R. Philip, M. Grimaldi, W. Court, and J. R. Jenkins.** 1990. p53 interacts with $p34^{cdc2}$ in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene **5**:795–801.
38. **Tanaka, K., K. Matsumoto, and A. Toh-e.** 1989. *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:757–768.
39. **Wittenberg, C., and S. I. Reed.** 1989. Conservation of function and regulation within the CDC28/*cdc2* protein kinase family: characterization of the human Cdc2Hs protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:4064–4068.
40. **Yarger, J. G., G. Armilei, and M. C. Gorman.** 1986. Transcription terminator-like element within a *Saccharomyces cerevisiae* promoter region. Mol. Cell. Biol. **6**:1095–1101.