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Genetics

A simple p53 functional assay for screening cell lines, blood, and tumors

(tumor-suppressor gene/Li–Fraumeni syndrome/transcription factor/mutation detection/*ADE2* gene)

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ABSTRACT **Mutations in the p53 gene are implicated in the pathogenesis of half of all human tumors. We have developed a simple functional assay for p53 mutation in which human p53 expressed in *Saccharomyces cerevisiae* activates transcription of the *ADE2* gene. Consequently, yeast colonies containing wild-type p53 are white and colonies containing mutant p53 are red. Since this assay tests the critical biological function of p53, it can distinguish inactivating mutations from functionally silent mutations. By combining this approach with gap repair techniques in which unpurified p53 reverse transcription–PCR products are cloned by homologous recombination *in vivo* it is possible to screen large numbers of samples and multiple clones per sample for biologically important mutations. This means that mutations can be detected in tumor specimens contaminated with large amounts of normal tissue. In addition, the assay detects temperature-sensitive mutants, which give pink colonies. We show here that this form of p53 functional assay can be used rapidly to detect germline mutations in blood samples, somatic mutations in tumors, and mutations in cell lines.**

Alteration of the p53 tumor-suppressor gene (gene symbol, *TP53*) is the most common genetic defect known to occur in human tumors (1). Current models suggest that wild-type p53 prevents genetic instability and is required for the apoptotic response to radiotherapy and chemotherapy (2). Somatic p53 status may thus be a useful prognostic marker, at least in some tumor types (for examples, see ref. 2). Germline p53 mutations are responsible for the majority of cases of the familial Li–Fraumeni cancer syndrome (3) and have also been seen in some other patients with multiple tumors or a family history of cancer (4–7). Therefore, accurate detection of p53 mutations represents an important challenge in the molecular diagnosis of cancer.

Two strategies are widely used to study p53 in clinical material: screening with anti-p53 antibodies for overexpression of mutant protein (8) and nucleic acid-based screening (9). The former approach is attractive because its technical simplicity means it can be performed within the framework of existing immunohistology services. The assumption underlying protein analysis is that high-level p53 expression is a consequence of p53 mutation; however, despite the generally good correlation between overexpression and mutation, numerous exceptions to this rule have been described and the link between mutation and overexpression is now thought to be indirect (8). One important situation where cell staining cannot be used to detect mutations is in the diagnosis of germline mutations, because normal tissue from these patients generally expresses low levels of mutant p53 protein (10). Detection of p53 mutations by DNA sequencing is expensive and labor-intensive. Simpler DNA structure-based techniques, such as single-strand conformation polymorphism, denaturing gradient gel electrophoresis, RNase mismatch cleavage, and chemical mismatch cleavage (9), are attractive alternatives to sequencing but they cannot distinguish between polymorphisms, functionally silent mutations, and inactivating mutations.

Previous reports described a conceptually different approach based on a biological assay for p53 function (11–13). The critical biochemical function of p53, which is tightly linked with its tumor-suppressor activity, is the ability to activate transcription (14). The mutations observed in tumors abolish this activity (1), and our functional assays for p53 mutation all test transcriptional competence. p53 can function as a transcription factor in yeast, and by performing the functional assay in yeast it is possible to take advantage of gap repair cloning techniques, which are highly effective with small amounts of unpurified PCR products (11, 13). We present here a simplified form of the p53 functional assay in which yeast change color according to their p53 status, and we show that this assay is a rapid, simple means to detect p53 mutations in cell lines, peripheral blood lymphocytes, and tumors.

METHODS

Strains, Media, and Plasmids. The yeast expression vectors used for gap repair (pSS16) and expression of wild-type p53 (pLS76) have been described (13). The p53 transcription reporter plasmid was made by cloning the *ADE2* open reading frame downstream of a *CYC1* minimal promoter containing three copies of the RGC p53 binding site (15). The vector containing this promoter was derived from pLS37 (11). pLS37 was cut with *Bam*HI and *Tth* III 1 to release the *lacZ* gene and self-ligated to regenerate the *Bam*HI site, yielding pLS207. A *Bam*HI site was engineered before the *ADE2* start codon in pASZ11 (16) by using an oligonucleotide cloned into the *ADE2* *Xba* I site at codon 3, 5′-GGATCCATGGATTCTAGA-3′, yielding plasmid pLS196. *ADE2* was transferred from pLS196 on a 1.9-kb *Bam*HI–*Bgl* II fragment into the *Bam*HI site in pLS207, yielding pLS208. This was cut with *Eco*RI to release the 2μ plasmid origin and self-ligated, yielding pLS210.

Standard yeast manipulations were performed as described (17). pLS210 cut at the *Apa* I site in *URA3* was integrated into a strain isogenic with CWO4 (16) and selected for uracil prototrophy, giving yIG397. Integration at the *URA3* locus was confirmed by Southern blotting. The genotype is *MAT*a*ade2-1 leu2-3,112trp1-1his3-11,15can1-100ura3-1 URA3* 3×RGC::p*CYC1*::*ADE2*.

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Cell Lines and Clinical Samples. TT and TE human esophageal carcinoma cell lines were obtained from the Japanese Cancer Research Resources Cell Bank. LN malignant astrocytoma cell lines were obtained from the Neurosurgery Service, University Hospital, Lausanne, Switzerland. Lymphocytes were fractionated from whole blood and suspended in RNA lysis buffer (Quickprep micro mRNA kit; Pharmacia). Resection specimens of head and neck tumors were frozen in liquid nitrogen and stored at −70°C; biopsy specimens were placed directly in RNA lysis buffer.

RNA Extraction and Reverse Transcription (RT)–PCR. mRNA was purified with a Quickprep micro mRNA kit (Pharmacia) and eluted in 300 μl of water containing 50 units of RNase inhibitor (Boehringer). cDNA was synthesized at 46°C for 1 hr by 100 units of Superscript II (BRL) from 10.5 μl of mRNA in 20 μl of RT buffer (BRL) containing 0.4 μg of hexamers (Pharmacia). PCR was performed with primers P3 [5′-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTG-AA(S)C-3′, where (S) represents a phosphorothioate linkage] and P4 [5′-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT(S)G] for 30–35 cycles of 94°C for 30 s, 65°C for 60 s, and 78°C for 80 s. Two microliters of the cDNA reaction product was amplified in 20 μl of *Pfu* buffer (Stratagene) plus 1.25 units of *Pfu* DNA polymerase, 100 ng of primers, 10% (vol/vol) dimethyl sulfoxide, and 50 μM dNTPs. After PCR, 5 μl of the product was checked by gel electrophoresis and 5 μl was used directly for yeast transformation.

A first-strand cDNA synthesis kit (Pharmacia) was used with equivalent results for cell lines, head and neck tumors, and some blood samples. Primers which amplify the entire 1.2-kb p53 open reading frame (12) were used for PCR from astrocytoma and esophageal cell lines, head and neck tumors, and p53 plasmids. In practice the P3 and P4 primers are preferred because the product is slightly shorter (1 kb) and they contain nuclease-resistant phosphorothioate linkages at the 3′ end which protect against degradation by DNA polymerases with 3′-to-5′ exonuclease activity (18). Deep Vent polymerase (New England Biolabs) was used to amplify p53 from plasmids and esophageal cell lines. Deep Vent gives satisfactory results with abundant templates [10^6-fold amplification gives ≈10% background red colonies (19)], but the higher fidelity of *Pfu* polymerase makes it the enzyme of choice when the input template copy number is below ≈10^5.

Yeast Transformation. yIG397 should routinely be cultured on complete medium supplemented with adenine (200 μg/ml) to avoid selection of spontaneous suppressors of the endogenous mutant *ade2* locus. To test p53 status, yeast were cotransformed with unpurified PCR product, linearized vector, and carrier DNA by the lithium acetate procedure (13), plated on synthetic minimal medium minus leucine plus adenine (2.5–5 μg/ml), and incubated for 2–3 days at 35°C. Red colonies are clearly identifiable at this stage but the color is more intense after an additional 2 days at 4°C.

Recovery of p53 Plasmids from Yeast and DNA Sequencing. p53 expression plasmids were rescued from yeast lysed with glass beads (17). p53 cDNA was amplified with M13-tagged PCR primers and sequenced on both strands with fluorescent M13 primers on an Applied Biosystems 373A automated sequencer.

RESULTS

Assessment of p53 Status by Colony Color. Ade2$^-$ cells grown on medium containing limiting adenine turn red because of the accumulation of an intermediate in adenine metabolism (16). The strain used for assessment of p53 status (yIG397) contains an integrated plasmid with the *ADE2* open reading frame under the control of a p53-responsive promoter. When the strain is transformed with a plasmid encoding wild-type p53, the cells express *ADE2*, grow normally, and form white colonies. Cells containing mutant p53 fail to express *ADE2*. Consequently, they form red colonies which are smaller than normal because adenine is limiting for growth. The overall outline of the assay is shown in Fig. 1. An unpurified human p53 RT–PCR product is cloned into a constitutive yeast expression vector by homologous recombination *in vivo* after cotransformation of p53 and vector into yeast, followed by selection of recombinants on plates lacking leucine, as described (13). Since the p53 expression vector is linearized at codons 67 and 346, the assay tests the entire DNA-binding domain (aa 102–292; ref. 20).

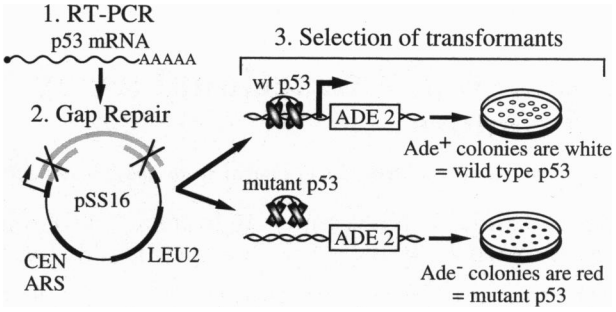

FIG. 1. Outline of the assay. Step 1: p53 mRNA is reverse transcribed, amplified by PCR, and cotransformed into yeast with a linearized expression vector carrying the 5′ and 3′ ends of the p53 open reading frame. Step 2: Gap repair of the plasmid with the PCR product results in constitutive expression of human p53 protein. Step 3: Yeast which have repaired the plasmid are selected on medium lacking leucine. The medium contains sufficient adenine for growth of Ade$^-$ cells, but they form red colonies. Hence colonies containing wild-type p53 are white (*ADE2*$^+$) and colonies containing mutant p53 are red (*ade2*$^-$).

Testing the Assay with Defined p53 Mutants. To confirm that the strain with the integrated *ADE2* reporter correctly distinguishes wild-type and mutant p53, the assay was performed with wild-type p53 cDNA and a panel of known mutants. Linear vector alone gave fewer than five colonies per transformation. These background colonies contained self-ligated vector and were therefore red. Transformation with circular plasmid encoding wild-type p53 gave only white colonies, but PCR-amplified wild-type cDNA gave a small number of red colonies because of PCR-induced mutations [3% after 10^9-fold amplification with *Pfu* polymerase (19)]. Functional assays were performed with eleven p53 mutants (143A, 156P, 175H, 245C, 248W, 249S, 252P, 258K, 273H, 281E, and 282W) by gap repair using PCR-amplified plasmid DNA. At least 200 colonies were examined for each mutant, and in every case 100% of the colonies were red. Thus the *ADE2* reporter strain scores wild-type and mutant p53 correctly.

Analysis of Normal Tissue. To define more exactly the background number of red colonies when starting from clinical samples, RNA was extracted from two sources of normal tissue: tonsils removed from healthy children with recurrent tonsillitis and peripheral blood lymphocytes from healthy adults. Twelve tonsils tested with *Pfu* polymerase gave 2 ± 1% red colonies. Sixteen samples of peripheral blood lymphocytes tested with *Pfu* polymerase gave 5 ± 2% red colonies (Fig. 2*a*).

Identification of Germline p53 Mutations. The previous yeast functional assay was developed to detect p53 mutations in families with Li–Fraumeni syndrome (13). We have used the new assay to test one patient whose family history met the criteria for Li–Fraumeni syndrome. Fifty-five percent of the colonies were red (Fig. 2*b*), and sequencing of plasmids rescued from multiple colonies and from uncloned cDNA showed that the patient was heterozygous for a 273C mutation (CGT → TGT).

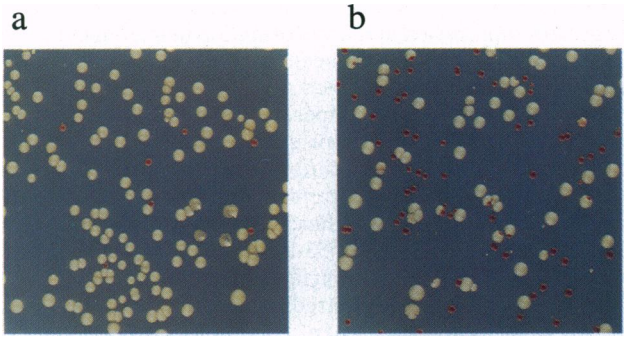

FIG. 2. Analysis of peripheral blood lymphocytes. (*a*) Normal donor (5% red colonies). (*b*) Patient with a germline 273C mutation (55% red colonies).

We have also used the new assay to study lymphocyte RNA from patients with multiple tumors of diverse types (Table 1). Ten out of 11 patients tested gave background levels of red colonies. One patient gave 58% red colonies, and sequencing of plasmids rescued from multiple red colonies, and subsequently of uncloned cDNA and genomic DNA, showed that the patient was heterozygous for a 273H mutation (CGT → CAT).

Identification of p53 Mutations in Tumors. To test the suitability of the assay for analyzing tumor samples, RNA was extracted from head and neck tumors, reverse transcribed, amplified, and transformed into yIG397 (Table 2 and Fig. 3). Eight out of 10 tumors scored unequivocally as mutant (48–100% red colonies), and the other two scored as wild type (3–10% red colonies). Clonality was tested and confirmed for two of the tumors by sequencing DNA amplified from multiple red colonies (195T and 173L; see Table 2). Thus the *ADE2* assay can be used to identify p53 mutations in tumors.

Identification of p53 Mutations in Cell Lines. Results of functional assays performed on three astrocytoma cell lines and four esophageal carcinoma cell lines are shown in Table 3. LN319, which was previously shown to be homozygous for the 175H mutation (21), gave 100% red colonies, and sequencing of p53 cDNA confirmed the presence of the 175H mutation.

Table 1. Functional assay testing lymphocytes from patients with multiple tumors

Age at first tumor, years	No. of primary tumors	Red colonies, %	Diagnoses*
16	3	4	Sarcoma, meningioma, cancer of testis
19	3	6	HD, ADCC, cancer of breast
24	3	4	Meso, cancer of testis and colon
32	2	8	NHL, BCC
34	3	58†	Sarcoma, cancer of breast (2×)
50	2	4	Melanoma, cancer of breast
51	2	3	Cancer of thyroid and prostate
51	2	3	HCC, cancer of esophagus
55	2	6	NSCLC (2×)
58	3	4	SCLC, CLL, cancer of prostate
54	5	8‡	Choroid melanoma, cancer of cervix, lung, thyroid, and breast

*HD, Hodgkin disease; ADCC, adrenocortical carcinoma; Meso, mesothelioma; NHL, non-Hodgkin lymphoma; BCC, basal cell carcinoma; HCC, hepatocellular carcinoma; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; CLL, chronic lymphatic leukemia.
†273H mutation (CAT).
‡Deep Vent polymerase; all other samples were tested with *Pfu* polymerase.

Table 2. Functional assay testing head and neck tumors

Age, years	No. of white colonies	No. of red colonies	Red colonies, %	Site of tumor tested*
56	11	130	92	Base of tongue†
59	160	18	10	Base of tongue†
72	10	88	90	Larynx†
51	4	300	99	Glottis
52	19	250	93	Retromolar
61	376	13	3	Base of tongue
63	17	270	94‡	Larynx
72	80	300	79	Pyriform sinus
73	0	63	100§	Pyriform sinus
74	126	116	48	Epiglottis

*All were diagnosed as squamous cell carcinoma.
†Biopsy specimen.
‡195T mutation (ACC).
§173L mutation (CTG).

TE2 and TE11 also gave 100% red colonies, and sequencing again identified clonal mutations (Table 3). LN18 was previously shown to be heterozygous for the 238S mutation (21), but 100% of the yeast colonies with LN18 cDNA were red. Direct sequencing of uncloned cDNA and restriction digestion of PCR-amplified genomic DNA (the 238S mutation destroys a *Mae* III site) confirmed that the cell line was homozygous for the mutation.

Unexpectedly, we observed pink, rather than red, colonies with the TE5 and TT4 cell lines (Table 3). This intermediate phenotype suggests that the 272L and 214R p53 mutants identified in these cell lines are partially active. Further analysis showed that these mutants were temperature sensitive, forming white colonies at 25°C and pink ones at 35°C (Fig. 4).

Functional analysis of the LN428 cell line, which contains two mutant alleles (173M and 282W; ref. 21) gave 15% white colonies (Table 3). Sequencing of plasmids rescued from nine independent red colonies showed that four contained the 282W mutation, two contained the 173M mutation, two contained both mutations, and one was wild type at these codons. This suggests that intragenic recombination can occur between different p53 alleles in yeast. To test this idea, p53 DNA from plasmids encoding 175H and 282W mutations was amplified, purified, and heteroduplexed by heating and slow cooling. Transformation of either mutant alone gave 100% red colonies, whereas transformation of the heteroduplex gave 17% white colonies. Thus, yeast can perform intragenic recombination to create wild-type p53 from two mutants. White colonies (10%) generated by intragenic recombination were also observed with the TT4 cell line (Table 3).

DISCUSSION

We have described here a simple, convenient functional assay which can selectively detect inactivating p53 mutations in periph-

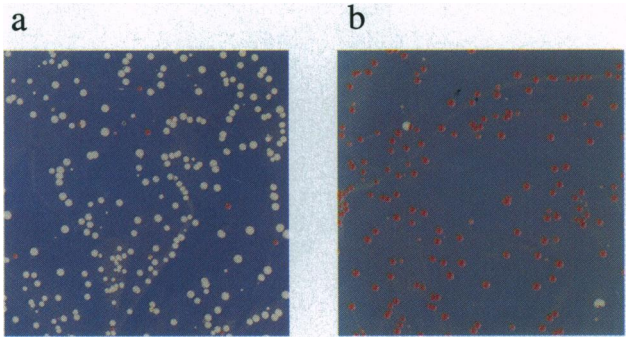

FIG. 3. Analysis of head and neck tumors. (*a*) Cancer of base of tongue (3% red colonies). (*b*) Cancer of glottis (99% red colonies).

Table 3. Functional assay testing cell lines

Cell line	No. of colonies			cDNA sequence
	Red	Pink	White	
LN18	>200	0	0	238S (TCT)
LN319	>200	0	0	175H (CAC)
LN428	165	0	30*	173M (ATG), 282W (TGG)
TE2	>200	0	0	Δ59 + frameshift†
TE5	0	>200‡	0	272L (TTG)
TE11	>200	0	0	110L (CTT)
TT4	84	100‡	20*	214R (CGT), Δ258-261§

Except where specified below, the same mutation was present in genomic DNA and cDNA.

*Intragenic recombination.

†Mutant intron 4 splice donor. The frameshift results from activation of a cryptic splice donor site in exon 4 (CCA-G/GT-CCA) by mutation of the normal intron 4 splice donor site (125-ACG/GTC to ACG/ATC).

‡Temperature-sensitive mutant (pink colonies). See Fig. 4.

§The in-frame deletion (Δ258–261) is caused by a mutation which creates a new splice donor site within exon 7 (256-ACA-CT/G-TAA). In the genomic DNA the mutation creates a putative in-frame stop codon (258-GAA to TAA), but this is deleted from the mature mRNA by the novel splice.

eral blood lymphocytes, tumors, and cell lines. The advantages of this assay, compared with the *HIS3* assay previously described (13), are that it does not require a replica-plating step and that it can be used to detect temperature-sensitive mutants. The absence of a replica-plating step means that large numbers of samples and many clones per sample can easily be tested. The ability to examine multiple clones is particularly useful when tumor samples are examined, because substantial contamination with normal tissue does not interfere with the assay.

Clinical material containing wild-type p53 commonly gives 5–10% red colonies under the conditions we describe, and higher values are indicative of the presence of p53 mutations. The major source of background red colonies is PCR-induced mutations. Indeed, the assay can be used to measure polymerase fidelity (19). With *Pfu* polymerase, which possesses proofreading activity, the background is around 3% red colonies after 30 doublings starting from a DNA template. The higher values with clinical samples, which use RNA as the starting material, presumably reflect the infidelity of RNA polymerase and reverse transcriptase, although other factors related to the processing of the RNA may also play a part.

Temperature-sensitive p53 mutants can readily be identified with the *ADE2* reporter strain (Table 3 and Fig. 4). The mouse 135V mutant was previously shown to be temperature sensitive for growth suppression and transformation in mammalian cells (22) and has subsequently been used in numerous studies exploiting its conditional phenotype. This mutant and the human 285K mutant are temperature sensitive for transcriptional activation of a *lacZ* reporter in yeast (11). The ability to screen rapidly for temperature-sensitive mutants with the *ADE2* assay should prove extremely useful—e.g., by facilitating the identification of cell lines containing conditional p53 mutants at the endogenous p53 locus.

The main drawback to functional testing in yeast is that high-quality p53 RNA is required if the entire mutation-prone region is to be tested in one step. This immediately disqualifies certain routinely processed pathology specimens, such as paraffin-embedded formalin-fixed tissue. We have found that the most efficient way to prevent RNA degradation is to place samples directly in RNA lysis buffer at the point of collection. This is also more convenient than using liquid nitrogen. Another disadvantage of the functional assay is that it cannot detect large deletions or mutant alleles which are poorly expressed. The latter can arise from promoter defects, splicing defects, and nonsense-mediated mRNA decay (see ref. 23 and references therein). Failure of expression of the mutant allele is relatively common with some tumor-suppressor genes, such as the familial adenomatous polyposis coli gene, that are frequently inactivated by nonsense mutations (24). In contrast, most p53 alleles contain missense mutations, which means that failure of expression of the mutant allele should give relatively few false negatives with the p53 functional assay. Furthermore, failure to detect poorly expressed alleles is offset by finding a significant number of splicing defects, which can easily be missed when only genomic DNA is tested. Detection of all mutations almost invariably requires the use of several different techniques; by screening first with the functional assay it should be possible to restrict the use of more expensive and labor-intensive techniques to those samples which do not contain straightforward missense mutations.

One important observation, which is relevant mainly to the analysis of cell lines, is the finding that the functional assay sometimes gives nonstoichiometric numbers of white colonies. This can arise for several interesting reasons. (*i*) The starting material may not be clonal, and one potential use of the assay is to follow evolution of cell lines in culture. (*ii*) There may be promoter, splicing, or polyadenylylation defects affecting the level of expression of the allele giving the white colonies, and in samples known to be heterozygous at the DNA level the functional assay thus provides a sensitive means to detect such

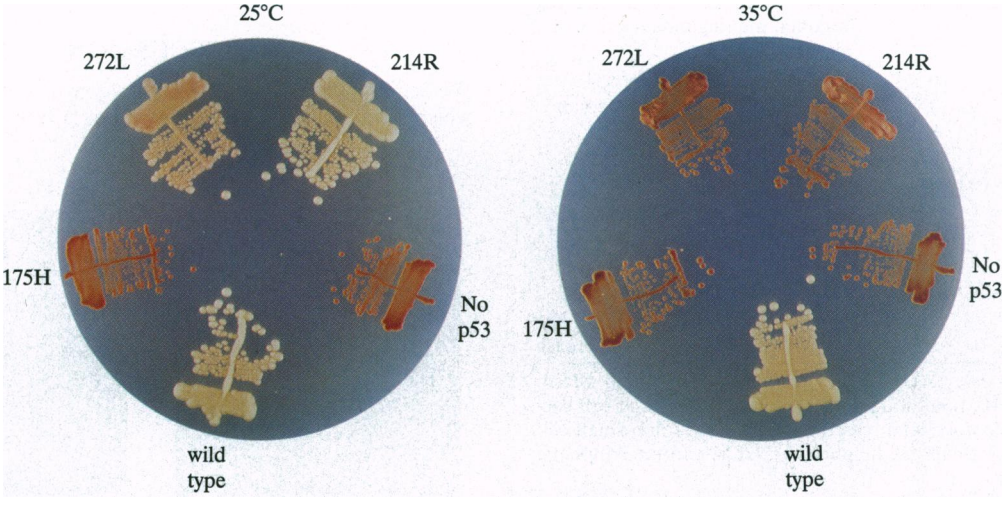

FIG. 4. Analysis of temperature-sensitive p53 mutants. Yeast expressing the specified p53 cDNAs were incubated at 25°C for 3 days (*Left*) or at 35°C for 2 days (*Right*).

defects. (*iii*) Some temperature-sensitive mutants can give a mixture of pink, white, and sectored colonies, presumably due to small differences in growth rate and plasmid copy number. (*iv*) The cell line may be heterozygous for two mutant alleles which undergo intragenic recombination to recreate the wild-type sequence. The recombination mechanism is not clear from our data, but PCR generates heteroduplex molecules, and we have shown that heteroduplex DNA can recombine in the conditions used for the assay. (*v*) Gene conversion or suppression of the endogenous mutant *ade2* locus could give white colonies, although this appears not to be a problem in the conditions we describe.

One technical point which deserves comment is that gap repair in yeast is an exceptionally efficient PCR cloning method. As little as 10 ng of unpurified PCR product can routinely be cloned with little or no vector background. The fact that potentially interesting mutants are incidentally cloned into plasmids which can easily be recovered from yeast is an important advantage of the p53 functional assay over techniques such as direct sequencing, denaturing gradient gel electrophoresis, and single-strand conformation polymorphism analysis.

Missense mutations are among the most common documented molecular alterations responsible for human genetic disease. In genes sensitive to mutation at multiple sites the distinction of a silent mutation from an inactivating mutation requires either the analysis of large pedigrees, which may not be available, or the development of a biological assay. The difficulty of establishing cosegregation of the mutant allele in complex genetic diseases, such as familial cancer, suggests that the need for functional assays will increase as more disease genes are cloned. The ease of yeast manipulation, the efficiency and convenience of cloning by gap repair, the conservation of biological pathways, and the ability of human genes of medical interest to complement defective yeast genes (25–27) make yeast a powerful tool for the development of biological assays for human genetic diseases.

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