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Reporter Gene Regulation in *Saccharomyces cerevisiae* by the Human p53 Tumor Suppressor Protein

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Abstract

This study evaluated the transcriptional regulation of four reporter genes in *Saccharomyces cerevisiae* by the human tumor suppressor protein p53. The *S. cerevisiae ADE2*, *HIS3* and *URA3* genes were used with nutritional selections and the *E. coli LacZ* gene was used to quantitate reporter gene activation. DNA elements containing binding sites for p53 were introduced upstream of several 5' truncated yeast promoters and used to express reporter genes. Human p53 cDNA was expressed at different levels by utilizing three different yeast promoters. All reporter genes were activated by p53, and in the case of nutritional selections, basal reporter gene expression could be detected in the absence of p53. A gap repair assay was evaluated and optimized for the purpose of determining whether p53 encoded in various cDNA sources was functional in transcriptional transactivation. The basal levels of reporter gene transcription in the absence of p53 could be decreased by integration of the reporter gene in the chromosome. For several expression systems, p53 appears to be limiting since higher levels of reporter gene expression were observed when the p53 cDNA was expressed from more efficient promoters. The gap repair assay can be used to determine the genotype (homozygous wild type, homozygous mutant or heterozygous) for cDNA generated from human cell lines or tissue samples. This assay can also be used to evaluate mutation rates associated with various conditions for *in vitro* PCR amplification of DNA.

Introduction

The *TP53* gene is mutant in over 50% of human cancers. The protein encoded by this tumor suppressor gene, p53, is a sequence specific DNA binding protein which is normally maintained in the cell in an inactive state due to binding by the MDM2 protein (reviewed in Oren, 1999). A variety of conditions, including DNA damage, mitotic spindle damage and various types of cellular stress, result in the dissociation of p53 from MDM2 and subsequent activation of the biological activity of p53. The tumor suppressor function appears to be due primarily to transcriptional activation by homotetramers of p53 of genes which result in cell cycle arrest or induction of apoptosis (Downehower and Bradley, 1993; Gottlieb and Oren, 1996; Haffner and Oren, 1995; Hansen and Oren, 1997; Ko and Prives, 1996; Levine, 1997; Pietenpol *et al*., 1994).

A variety of mammalian transcription factors have been shown to function as transactivators in yeast when the DNA binding site for the factor is included at an appropriate position in the 5' region of a yeast promoter (reviewed in Thukral *et al*., 1993). The entire DNA sequence of the *Saccharomyces cerevisiae* genome has been determined, and this simple microbial system is amenable to a variety of sophisticated molecular genetic techniques. Since the number of expressed genes in *S. cerevisiae* is approximately 5- to 6-fold fewer than human cells, yeast is an attractive system for functional analyses of specific human proteins since interactions with other proteins is minimized.

More than 1,700 different mutations of TP53 in human cancers and cell lines have been published, and the majority of these are missense codons which result in an amino acid substitution (http://www.iarc.fr/p53/index.html). These substitutions have been reported at 310 different residues of the 393 amino acid protein, but occur predominantly within the DNA binding domain (“core domain”; codons 100 to 300). These alterations invariably result in loss of DNA binding capacity and abolish the transcriptional activation function of p53.

In this study, we have evaluated the regulation of four different reporter genes in *S. cerevisiae* by the human TP53 gene product. A strong transcriptional activation from promoters containing a p53 DNA binding site is observed with all reporter genes. We have also formatted and characterized an assay capable of determining the transcriptional transactivation capacity of separated p53 alleles in human cellular cDNA. Since p53 mutants invariably are deficient in transcriptional transactivation, this assay can be used to determine the genetic status of cell lines used in research or human clinical specimens. In the case of human pre-cancers and cancers, knowledge of p53 status should facilitate choosing appropriate treatment options (see Discussion).

Results

Yeast Promoter Activation by Human p53

The *E. coli LacZ* gene was used as a reporter in order to quantitate promoter activation by human p53. A DNA element containing two tandem p53 binding sites (UAS_{53}; Experimental Procedures) was inserted upstream of two 5'-truncations of the yeast *TDH3* gene promoter (Figure

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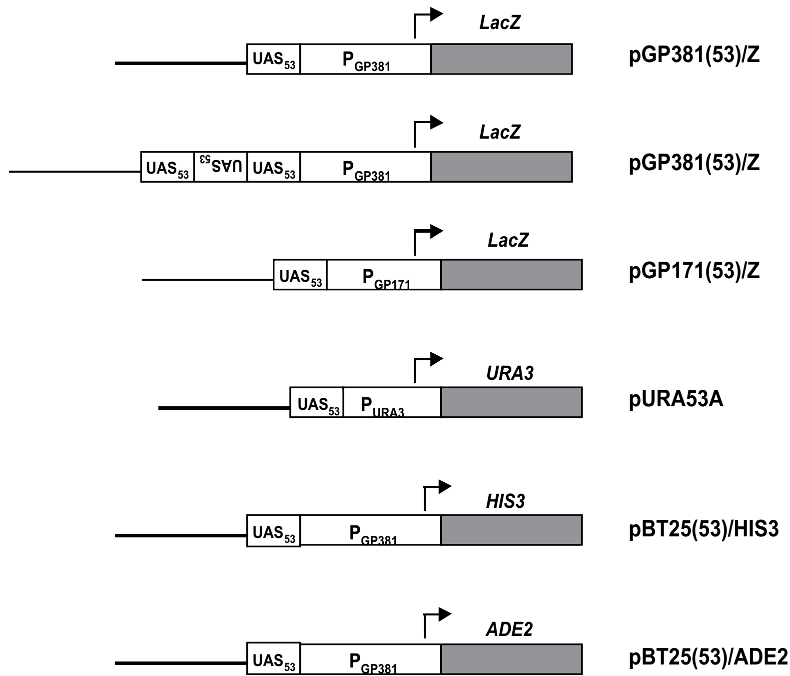
A.



B.

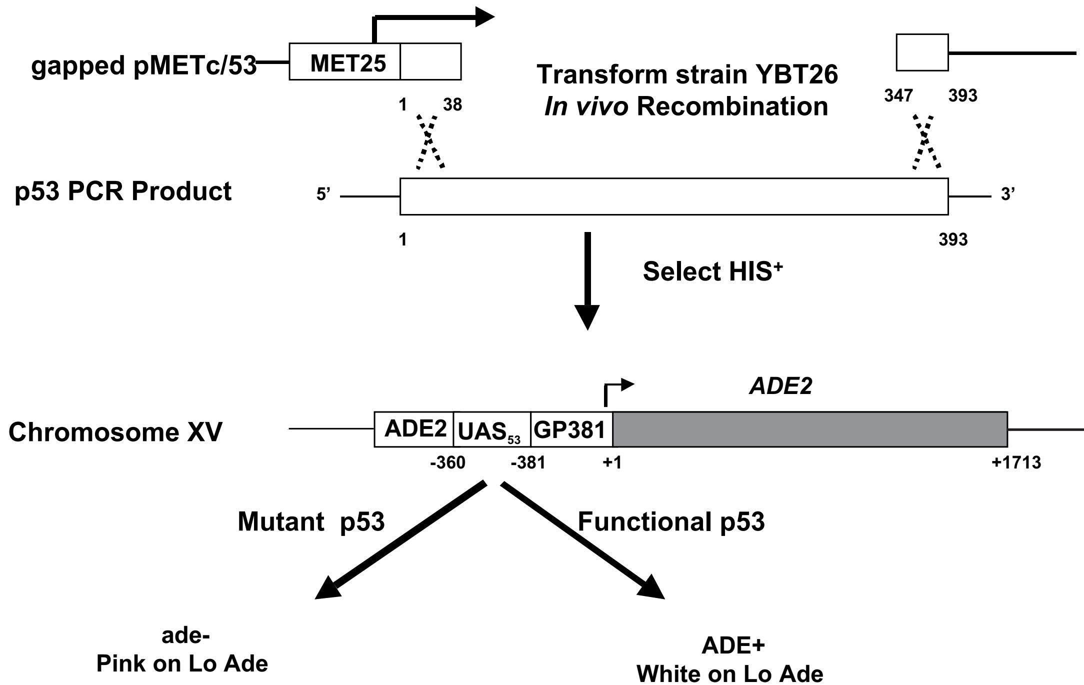


Figure 1. A. Promoters utilized to express various reporter genes under control of human p53 in yeast. The indicated plasmids were constructed as described in Experimental Procedures. Depicted are the four reporter genes, the 5'-truncated promoter elements and the orientation(s) of the p53 DNA binding site (UAS_{53}; Experimental Procdures). B. Schematic of the p53 functional assay of separated alleles. p53 DNA is PCR amplified from various sources, mixed with gapped pMETc/53 and transformed into strain YBT26, selecting for histidine prototrophs as described in Experimental Procedures. Individual yeast clones contain a single p53 cDNA allele, and its function is assessed by color on selective plates containing low adenine.

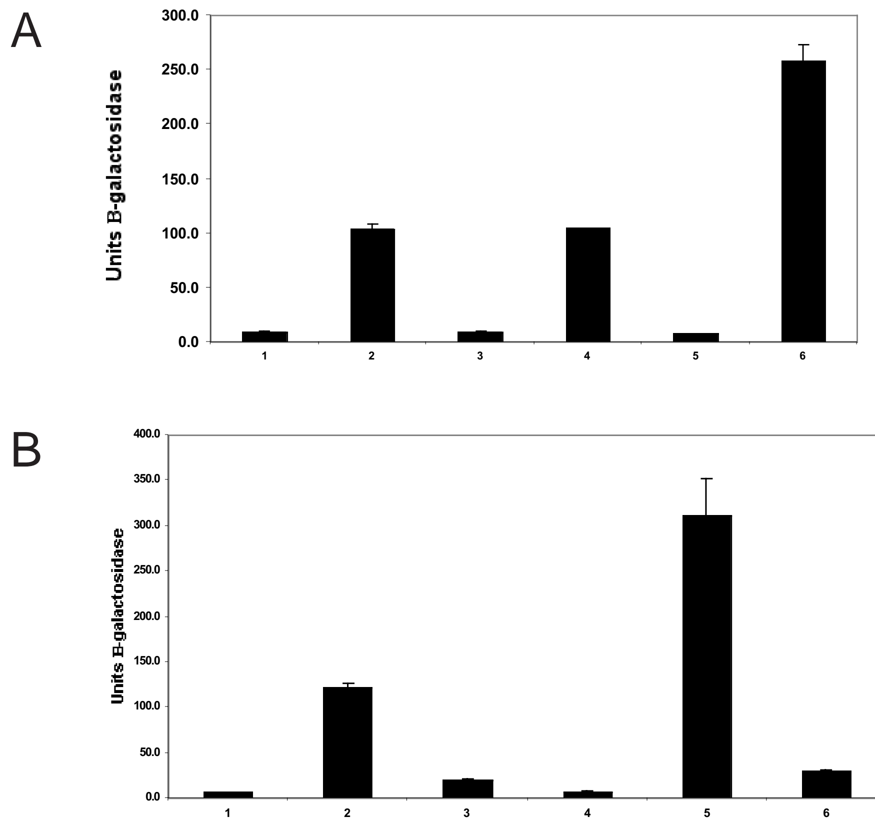


Figure 2. Regulation of *LacZ* reporter gene expression by p53. β-galactosidase was quantitated in various strains as described previously (Bitter, 1998). A. Strain YPH500 containing the following plasmids used were: (1) pGP171(53)/Z and pBT26; (2) pGP171(53)/Z and pBT26/53; (3) pGP381(53)/Z and pBT26; (4) pGP381(53)/Z and pBT26/53; (5) pGP381(53-3)/Z and pBT26; (6) pGP381(53-3)/Z and pBT26/53. B. Strain YPH500 containing the following plasmids used were: (1) pGP171(53)/Z and pBT26; (2) pGP171(53)/Z and pBT26/53; (3) pGP171(53)/Z and pBT26/53; (4) pGP171(53)/Z and pMETc; (5) pGP171(53)/Z and pMETc/53; (6) pGP171(53)/Z and pMETc/53. For culture (6), the medium was supplemented with 530 μM methionine.

1A). The GP381 promoter has a 5' endpoint at –381 while the GP171 promoter 5' endpoint is –171 relative to the ATG initiator codon (Bitter *et al.*, 1991). A promoter which included three head to tail inserts of the UAS_{53} upstream of the GP381 promoter was also constructed. These promoters were assembled immediately upstream of the *E. coli LacZ* gene and introduced into *Saccharomyces cerevisiae* strain YPH500 harboring either pBT26 or BT26/53 (Experimental Procedures). The data in Figure 2A demonstrate that expression of all three reporter genes is activated in the presence of human p53. The GP171 and GP381 promoters containing UAS_{53} produce 103 and 105 units, respectively, of β-galactosidase when human p53 is co-expressed in the cell. Thus, p53 mediated reporter gene activation is not significantly altered by changing the position of UAS_{53} 110 bp relative to the transcription initiation site. The reporter gene which contained three head-to-tail inserts of UAS_{53}, pGP381(53)/Z, was transcribed significantly more efficiently (269 units) than those containing a single UAS_{53}. The 2.5-fold higher levels of β-galactosidase expressed from this promoter demonstrate synergistic activation by multiple p53 DNA binding sites.

Effect of p53 Expression Levels on Promoter Activation

Three different yeast promoters were utilized to express p53 cDNA and the levels of β-galactosidase produced from pGP171(53)/Z measured (Figure 2B). In all cases, expression of p53 increased reporter gene activation, and an effect of p53 expression level was observed. When the efficient *TDH3* gene promoter was utilized (pBT26/53) 122 units of β-galactosidase were produced. When p53 cDNA was expressed from the truncated *TDH3* promoter (GP381) in pBT30/53, which is approximately 100-fold less efficient than the native promoter (Bitter *et al.*, 1991), only 20 units of β-galactosidase were produced which is approximately 3-fold higher than that measured in a strain not expressing p53. When the p53 cDNA was expressed from the *MET25* promoter, 311 units β-galactosidase were produced. If methionine was included (530 μM) in the culture medium for this strain, the amount of β-galactosidase produced decreased to 29 units. Methionine is known to repress the *MET25* promoter and a previous report (Mumberg *et al.*, 1994) demonstrated a 4-fold decreased protein expression level for genes expressed from this vector when greater than 500 μM methionine was included in the culture medium. All plasmids utilized in these experiments contain yeast centromeres and are therefore stably maintained at

Table 1

Strain	Media	Colonies on indicated day			
		Day 2		Day 5	
		CFU	% of Total	CFU	% of Total
YBT27; pBT26	SD, A,H,Ly,T,U	726		774	
	SD, A, H, Ly,T	0	0%	0	0%
	SD, A,H,L,Ly,T, 0.1% FOA	250[a]	34%	448[b]	58%
YBT27; pBT26/53	SD, A,H,Ly,T,U	344		390	
	SD, A, H, Ly,T	334	97%	340	87%
	SD, A,H,L,Ly,T,0.1% FOA	0	0%	0	0%

[a] Colonies are very small relative to those on SD, A,H,Ly,T,U plates.
[b] Colonies are medium sized relative to the large colonies on SD, A,H,Ly,T,U plates.

one or two copies per cell (Bitter *et al.*, 1987). Cumulatively, these results demonstrate that intracellular levels of p53 protein are limiting for promoter transactivation, and that expression of p53 cDNA from more efficient promoters yields increased reporter gene expression.

Positive Genetic Selection for Loss of p53 Function

A positive selection for cells lacking a functional p53 would have utility for selecting mutations which inactivate p53 function. Such a selection would also allow quantitation of the frequency of cells containing mutant p53 within a mixed population. For this purpose, the yeast *URA3* gene was used as a reporter. In addition to positive selection for cells expressing the *URA3* gene (growth in the absence of uracil), a positive selection for cells not expressing *URA3* exists in the form of sensitivity to 5-fluoro-orotic acid (FOA) since this compound is converted to a toxic compound by the product of the *URA3* gene (Boeke *et al.*, 1984).

For the initial experiments, yeast strains were grown in liquid culture to saturation with selection for plasmid maintenance and 10 μl loops of each culture were spread on various selective plates (data not shown). Strain YPH500 lacking any plasmid exhibited no growth on plates lacking uracil and normal growth (equivalent to that observed on SD, CAA,A,U plates) on plates containing uracil and 0.1% FOA. YPH500 is ura$^-$, FOA^R due to the *ura3-52* mutation, and growth in the presence of FOA indicates no functional Ura3p in the cells. Strain YPH500 harboring pURA3A, which contains the yeast *URA3* gene downstream from a 5' truncation of the *URA3* promoter (Experimental Procedures), exhibits a very weak URA+ phenotype taking several days longer and with significantly less growth than a strain containing a functional *URA3* gene. These results are consistent with a low level of transcription of the *URA3* gene from the 5' truncated *URA3* promoter. Consistent with this interpretation is the observation that this strain is completely FOA sensitive when selection (-trp) for the plasmid containing the *URA3* gene is maintained. The phenotype of this strain is not changed when human p53 is produced from expression vector pBT26/53 (strain YPH500; pURA3A, pBT26/53). When UAS_{53} is included upstream of the 5' truncated *URA3* gene promoter (pURA53A; Experimental Procedures), and human p53 was coexpressed in YPH500, the strain exhibited a strong URA+ and foa^S phenotype. These results demonstrate activation of the UAS_{53}-URA3 promoter by human p53 and consequent efficient transcription of the *URA3* gene.

Improved Positive Genetic Selection for Loss of p53 Function

We hypothesized that decreased basal transcription, and hence more stringent selection, might be obtained if the reporter gene was integrated into the chromosome. Strain YBT27 contains the UAS_{53}-*URA3* gene fusion, derived from pURA53A, integrated by homologous recombination at the *URA3* locus on chromosome V (Experimental Procedures), and was transformed with either pBT26 or pBT26/53. In order to accurately quantitate the uracil prototrophy and FOA resistance, these strains were grown to saturation in SD, A,H,Ly,T,U to maintain plasmid selection, serially diluted in sterile water and plated on the plates indicated in Table 1. As observed with plasmid based expression, coexpression of human p53 in the cell confers a strong URA$^+$, foa^S sensitive phenotype. In contrast to the plasmid system, however, when the UAS_{53}-*URA3* gene fusion is integrated into the chromosome and a functional p53 is not produced in the cell (YBT27; pBT26), the strain is a uracil auxotroph. No colonies were observed on plates lacking uracil, even after 5 days incubation. It appears that integration of the expression cassette into the chromosome results in a lower basal level of transcription of the *URA3* gene than when present on a plasmid. This strain also exhibits FOA resistance, although there is not 100% viability as observed for strain YPH500. The colonies grow slower than in the absence of FOA, and only approximately 60% of the plasmid containing cells (quantitated on -leu plates) were viabile after 5 days growth.

To attempt to obtain better viability of p53 negative cells on FOA plates, two concentrations of FOA which were lower than the 0.1% used in previous experiments were tested (Table 2). The results again demonstrate a strong URA+ phenotype conferred by the integrated UAS_{53}-*URA3* cassette in response to the presence of functional human p53. Strain YBT27 expressing p53 is completely sensitive to 0.05% FOA. At this concentration, the strain lacking functional p53 exhibits 45% viability on day 2, which is slightly higher than observed with 0.1% FOA (Table 1). If

Table 2

Strain	Media	Colonies on indicated day					
		Day 2			Day 5		
		CFU	Avg.	%	CFU	Avg.	%
YBT27; pBT26	SD, A,H,Ly,T,U	264[b] 184[b]	224		264[a] 184[a]	224	
	SD, A, H, Ly,T	0 0	0	0%	0 0	0	0%
	SD, A,H,L,Ly,T, 0.05% FOA	84[d] 120[d]	102	45%	100[a] 136[a]	118	53%
	SD, A,H,L,Ly,T, 0.025% FOA	276[c] 212[c]	244	100%	280[a] 220[a]	250	100%
YBT27; pBT26/53	D, A,H,Ly,T,U	204[b] 236[b]	220		208[a] 236[a]	220	
	SD, A, H, Ly,T	216[b] 304[b]	260	100%	224[a] 308[a]	266	100%
	SD, A,H,L,Ly,T, 0.05% FOA	0 0	0	0%	0 0	0	0%
	SD, A,H,L,Ly,T, 0.025% FOA	15[e] 25[e]	20	9%	200[b] 166[b]	183	83%

[a] Large size colonies
[b] Medium size colonies
[c] Small/medium size colonies
[d] Small size colonies
[e] Very tiny micro-colonies

the FOA concentration is reduced to 0.025%, the viability of YBT27 is increased to 100%. However, for YBT27 expressing p53, very tiny (barely visible) micro-colonies were apparent after 2 days incubation on plates containing 0.025% FOA. These micro-colonies represented less than 10% of the total plasmid containing cells on day 2, but increased to more than 80% after 3 additional days incubation. The size of the colonies also increased upon prolonged incubation on plates containing 0.025% FOA.

In a subsequent experiment, two intermediate concentrations of FOA were tested to define conditions where the strain lacking p53 exhibited 100% viability while the strain expressing functional p53 was completely FOA sensitive. As demonstrated by the data in Table 3, YBT27 lacking functional p53 exhibits 100% resistance to 0.03% FOA after 2 days incubation. In contrast, if human p53 is expressed in YBT27, no viable colonies appear after 2 days incubation on plates containing 0.03% FOA. After an additional 3 days incubation at 30º C, however, microcolonies begin to appear on the plates.

Positive Genetic Selections for p53 Function

The *HIS3* gene was expressed from a 5' truncated *TDH3* promoter with a UAS_{53} immediately upstream. Use of the *HIS3* gene as a reporter has the advantage that 3-amino-1,2,4-triazole (AT) stoichiometrically inhibits the His3p enzyme. Therefore, utilization of appropriate concentrations of AT can be used to decrease or eliminate the biological effects of basal levels of *HIS3* transcription. Plasmid pBT25(53)/HIS3 (Experimental Procedures) was transformed into yeast strain YPH500 containing either pBT26 or pBT26/53. The two strains were cultured to saturation in SD, A, H, Ly, U (-leu, -trp to maintain selection for both plasmids), serially diluted and plated for single colonies on SD, A, Ly, U (-leu -trp -his) plates containing various concentrations of AT. Total colonies present on the plate at various times after plating are indicated in Table 4. The strain lacking functional p53 exhibits greatly reduced viability at all concentrations of AT after two days growth. At the lower concentrations (2, 5 mM), the total number of colonies increase with time. At the highest concentration tested (20 mM), there is less than 1% viability on day two although this increases to 14% on day three, and increases to approximately 50% after 5 days incubation. Higher concentrations of AT could be used to decrease the viability of p53 negative cells even further. In contrast to cells lacking p53, coexpression of p53 in strain YPH500 containing pBT25(53)/HIS3 results in 100% viability on day two at all concentrations tested.

The *ADE2* gene was also expressed from a 5' truncated *TDH3* promoter with a UAS_{53} immediately upstream. Plasmid pBT25(53)/ADE2 (Experimental Procedures) was transformed into yeast strain YPH500 containing either pBT26 or pBT26/53. The two yeast strains were cultured to saturation in SD, A, H, Ly, U (-leu, -trp to maintain selection for both plasmids) and 10 μL loops were spread onto selective plates. As observed with the *URA3* and *HIS3* genes, the *ADE2* gene on a plasmid downstream of a promoter containing UAS_{53} can be used for a positive selection for functional p53. Cells expressing p53 exhibit full viability on plates lacking adenine while cells not expressing p53 exhibit only very weak growth (data not shown). The growth in the absence of p53 is presumably due to a low basal level of transcription of the *ADE2* gene. The *ADE2* reporter gene has the additional advantage that on plates containing low concentrations of adenine (2.5-4

Table 3

Strain	Media	Colonies on indicated day					
		Day 2			Day 5		
		CFU	Avg.	%	CFU	Avg.	%
YBT27; pBT26	SD, A,H,Ly,T,U	133[b]	150		139[a]	156	
		167[b]			172[a]		
	SD, A, H, Ly,T	0	0	0%	0	0	0%
		0			0		
	SD, A,H,L,Ly,T, 0.04%FOA	127[c]	120	80%	151[a]	137	89%
		114[c]			124[a]		
	SD, A,H,L,Ly,T, 0.03%FOA	142[c]	145	97%	159[a]	156	100%
		148[c]			154[a]		
YBT27; pBT26/53	D, A,H,Ly,T,U	236[b]	240		240[a]	247	
		244[b]			254[a]		
	SD, A, H, Ly,T	251[b]	267	100%	263[a]	279	100%
		284[b]			296[a]		
	SD, A,H,L,Ly,T, 0.04%FOA	0	0	0%	~50[d]	65	26%
		0			~60[d]		
	SD, A,H,L,Ly,T, 0.03%FOA	0	0	0%	~60[d]	75	30%
		0			~70[d]		

[a] Large size colonies
[b] Medium size colonies
[c] Small/medium size colonies
[d] Small size colonies
[e] Very tiny micro-colonies

μg/ml), cells which efficiently transcribe the gene exhibit normal white colonies while those in which the gene is not expressed or expressed at a low level are viable but form colonies with a pink or red color due to accumulation of an intermediate in adenine biosynthesis (Stotz and Linder, 1990).

In order to decrease the basal level of *ADE2* gene transcription in the absence of p53, strain YBT26 was constructed (Experimental Procedures) such that the UAS_{53}-GP381-ADE2 expression cassette was integrated at the *ADE2* locus on chromosome XV. This strain was transformed with either pBT26 or pBT26/53. Each strain was cultured to saturation in SD, A, H, Ly, U, serially diluted in sterile water and plated on the plates indicated in Table 5. The number of colonies which formed (CFU) after three days incubation at 30º C is presented in Table 5. In the absence of functional p53, no colonies appeared on plates lacking adenine, even after extended incubation. This presumably is due to decreased basal level transcription of the UAS_{53}-GP381-ADE2 expression cassette when it is integrated into the chromosome. When p53 is produced in the cell, the strain becomes an adenine prototroph. For the strain lacking p53, viablity is good on plates containing low concentrations of adenine but, in contrast to the strain which transcribes the *ADE2* gene at high levels, the colonies are pink . The color of colonies on plates containing low concentrations of adenine serves as the basis of a functional p53 genetic test (below).

p53 Functional Genetic Test

A functional assay of separated alleles in yeast (FASAY) was previously described for p53 (Flaman *et al.*, 1995). We have utilized the yeast strains and expression systems (above) to format and validate a FASAY of human p53. The key attributes of this system are depicted in Figure 1B. Strain YBT26 has the normal *ADE2* locus on chromosome XV replaced with the UAS_{53}-GP381 promoter-*ADE2* expression cassette (Figure 1A;

Table 4

Strain	[AT] in -his plates	Total CFU on indicated day			
		2	3	4	5
YPH500; pBT25(53)/HIS3, pBT26	0	215	226	230	234
	2 mM	9	166	205	206
	5 mM	7	145	190	199
	20 mM	2	31	120	127
YPH500; pBT25(53)/HIS3, pBT26/53	0	195	199	208	209
	2 mM	251	263	267	269
	5 mM	161	174	175	176
	20 mM	210	223	227	227

Table 5

Strain	Media	CFU	% Total	Color
YBT26; pBT26				
	SD, A, H, Ly, U	336		white
	SD, H, Ly, U	0	0%	
	SD, H, Ly, U, 3 μg/ml ade	216	65%	pink
YBT26; pBT26/53				
	SD, A, H, Ly, U	220		white
	SD, H, Ly, U	236	100%	white
	SD, H, Ly, U, 3 μg/ml ade	192	87%	white

Experimental Procedures). Vector pMETc/53 is digested with *StuI* and *AccB7I* and the linear plasmid, which has deleted codons 39 to 346 of p53, is gel purified. The "gapped" plasmid is mixed with PCR amplified p53 DNA from various sources and transformed into YBT26. The linearized vector does not transform yeast but, when co-transformed with p53 DNA, a circular plasmid can be formed *in vivo* by homologous recombination (gap repair). Histidine auxotrophs are selected to isolate clones transformed with the gap-repaired pMETc/53 vector. The p53 coding sequence in the resulting plasmid includes, at a minimum, codons 38 to 347 from the test source used for PCR amplification. The precise borders of sequences derived from the test source is determined by the recombination crossover points. The colorimetric assay for p53 transactivation (Table 5) can then be used to determine whether the test source was wild type or mutant for transactivation function.

Figure 3 presents the results of an experiment in which gapped vector and PCR amplified p53 cDNA were mixed in various ratios and transformed into strain YBT26. In the absence of p53 DNA, between 1 and 12 transformants were observed. These clones were apparently transformed by unrestricted pMETc/53 plasmid which is a trace contaminant of the gel purified gapped vector. The number of transformants was greatly increased when the gapped vector was co-transformed with p53 DNA. With the lowest mass of gapped vector (300 ng per transformation), the number of transformants was not increased by a three fold increase in p53 DNA. This indicates that the gapped vector is limiting in these transformations. Consistent with this interpretation, the number of gap-repair transformants increased when more gapped vector was used. When 600 and 1200 ng of gapped vector were used, the number of transformants was increased by increasing the mass of p53 DNA in the transformation, suggesting that p53 DNA is limiting under these conditions.

The data in Figure 4 demonstrate utilization of the *ADE2* colorimetric assay (above) to quantitate wild type and mutant p53. An uncharacterized mutant p53 DNA, which did not transactivate the *LacZ* reporter gene, was isolated as described in Experimental Procedures. p53 DNA was PCR amplified from either this clone (Mut) or the wild type clone pBT26/53 (WT). The PCR products, either alone or in combination, were co-transformed with gapped vector and histidine prototrophs selected on plates containing low concentrations of adenine. The number of white and pink colonies from each transformation are depicted in Figure 4. In the transformations which utilized 100% p53 DNA amplified from the WT clone, 3.5% of the transformants were pink. These clones represent PCR introduced mutations which inactivate the transactivation function of the encoded p53. For the transformation which utilized 100% p53 DNA amplified from the Mut clone, only one white colony was observed in over 4,400 pink CFU counted. This white colony could derive from either a cell transformed by contaminating intact pMETc/53 or from a revertant of the uncharacterized p53 mutation. When the gapped vector was co-transformed with approximately equal amounts of WT and Mut amplified p53 DNA,

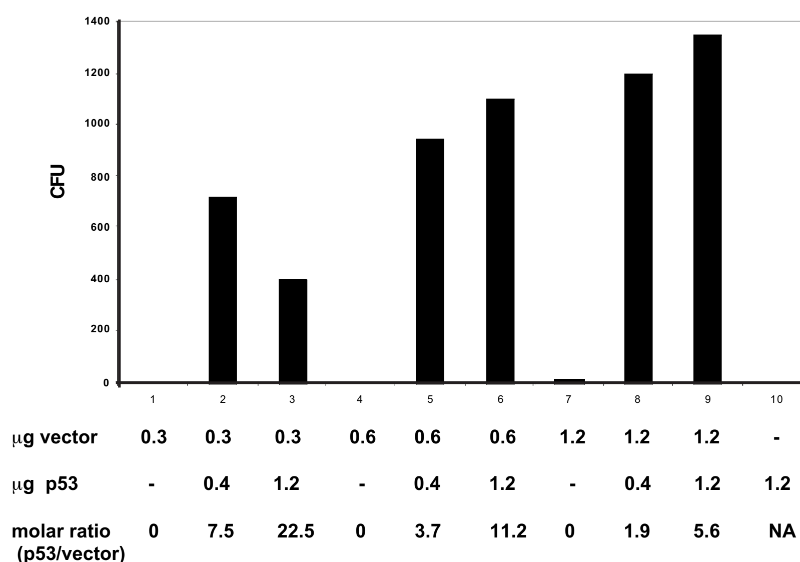


Figure 3. Optimization of the p53 functional assay. The indicated masses of gapped pMETc/53 were mixed with the indicated masses of PCR amplified p53 DNA and transformed into strain YBT26 selecting fro histidine prototrophs. The molar ration of p53 DNA to gapped vector is indicated for each transformation. The bars represent the total number of colony forming units (CFU) obtained in each transformation.

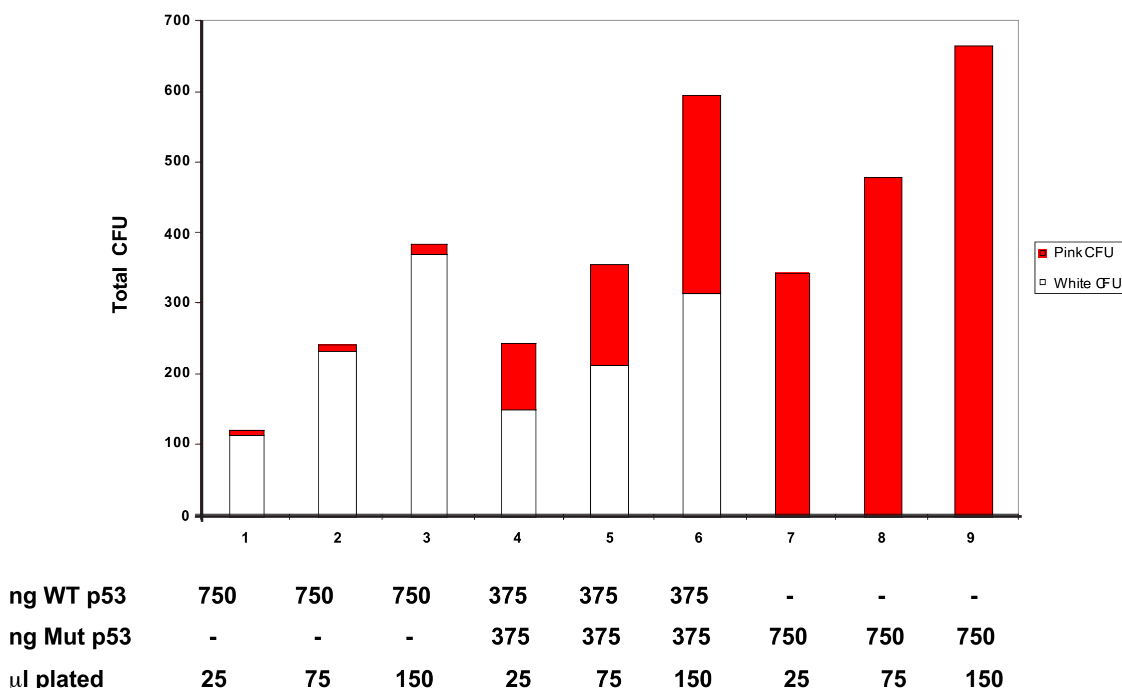


Figure 4. Detection of separated alleles in the p53 functional assay. p53 DNA was PCR amplified from either wild type or mutant plasmid as described in Experimental Procedures. The indicated masses of each PCR product were mixed with gapped pMETc/53 and transformed into strain YBT26 and 25, 75 or 150 μl of each transformation mixture plated selecting for histidine prototrophs. The bottom portion of each bar (unshaded) depicts the number of white colonies while the top portion of each bar (shaded) depicts the number of pink colonies.

approximately 42% of the total CFU were pink. The results presented in Figure 4 demonstrate that this assay can be used to distinguish between p53 cDNA samples which are homozygous WT, heterozygous WT/Mut and homozygous Mut.

Amplification of p53 DNA with *Taq* DNA polymerase under the conditions utilized (Experimental Procedures) results in base misincorporations which inactivate the transactivation function of approximately 3.5% of the *in vitro* product. In order to determine the frequency of *in vitro* generated mutations when analyzing a clinical sample, total RNA was isolated from peripheral blood lymphocytes, reverse transcribed with M-MLV reverse transcriptase and PCR amplified as described in Experimental Procedures. Only 1.1% pink CFU were observed under these conditions (Figure 5). The p53 functional assay was also used to investigate the effects of various PCR amplification conditions on fidelity of the *in vitro* product. When Mg^{++} was present at 1.5 mM and Qiagen Q solution was included at 10%, approximately 2% pink mutants were observed. If Qiagen Q solution was omitted and DMSO was included at 5%, the mutation frequency increased to 5%. If the Mg^{++} concentration was increased to 2.5 mM, the mutant frequency increased to 16%, and this was increased to 40% by inclusion of 5% DMSO. When the latter conditions were used but the mass of target DNA was increased two-fold, the mutant frequency decreased to 27%. It should be noted that these mutation frequencies are an underestimate of the misincorporation rate, since not all base substitutions will inactivate the transactivation function of p53.

It is possible that the mRNAs from the two *TP53* alleles in clinical samples may be expressed at different levels. Therefore, the sensitivity of our assay for differentially expressed alleles was measured. The wild type and mutant p53 cDNAs were mixed in the molar ratios indicated and PCR amplified as described in the legend to Figure 6. A gap-repair transformation was performed and the percentage pink colonies observed with each amplified DNA sample is presented in Figure 6. In this experiment, the reaction with 100% WT target DNA yielded 10% mutant clones after gap repair. For the reaction with 100% Mut target DNA, 99.7% mutant clones were obtained after gap-repair transformation, and the five white colonies observed out of 1,909 CFU could be due to trace contamination by un-restricted pMETc/53. With 100:1 WT:Mut target DNA, the % pink was significantly different than the 100% WT target DNA (17% vs. 10%). When the target DNA was 1:100 and 1:30 WT:Mut, the mutant frequencies were 99.4% and 97.8%, respectively. These titrations indicate that the presence of a wild type or mutant allele in a heterozygous individual can be determined in this assay even when one allele is overexpressed 30-fold relative to the other allele.

Discussion

Using the *E. coli LacZ* gene as a reporter, it was possible to quantitate transcriptional activation in yeast by human p53. Reporter gene activation was not altered if the position

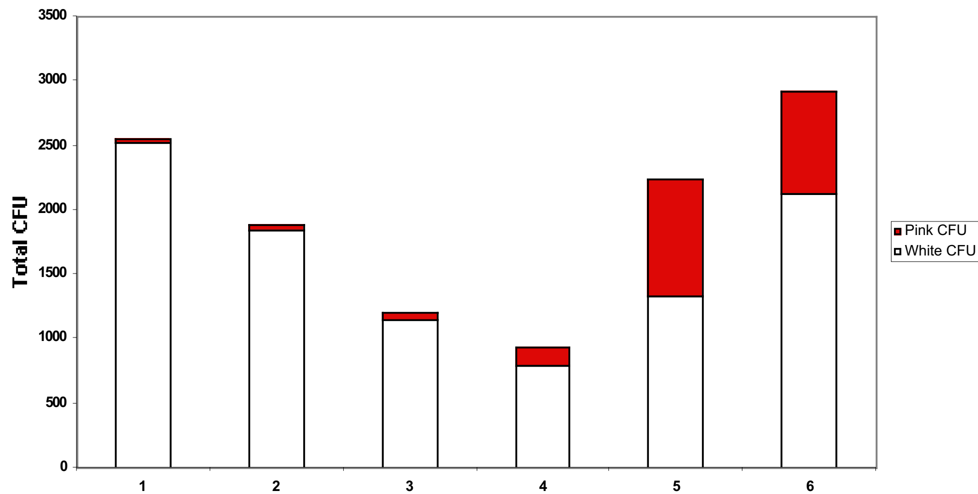


Figure 5. Functional analysis of p53 alleles from peripheral blood lymphocytes and optimization of conditions for high fidelity PCR amplification. Isolation of RNA, RT-PCR amplification of p53 alleles and gap repair in yeast was carried out as described Experimental Procedures. Bars show the total number of colonies obtained after gap repair of the p53 gene in yeast; the bottom portion (unshaded) depicts the number of white colonies while the top portion (shaded) depicts the number of pink colonies. Bar (1) represents colony forming units (CFU) obtained with RT-PCR amplified DNA from peripheral blood lymphocytes while all other bars represent CFU obtained with DNA PCR amplified from the plasmid template (Experimental Procedures). PCR conditions described Experimental Procedures were altered as follows: (1) unaltered, (2) 10% Q solution (Qiagen) included, (3) 5% DMSO included, (4) $MgCl_2$ increased to 2.5 mM, (5) 5% DMSO included and $MgCl_2$ increased to 2.5 mM , and (6) 5% DMSO included, $MgCl_2$ increased to 2.5 mM and p53 target mass increased 2-fold.

of the UAS_{53} was varied 110 bp relative to the transcription start site. However, the presence of three copies of UAS_{53} in the promoter resulted in greatly increased (2.5-fold) reporter gene expression. Since all reporter genes are expressed from centromere containing plasmids which are maintained at approximately one copy per cell (Bitter *et al*., 1987), it was somewhat surprising that reporter gene expression could be increased by expressing p53 from more efficient promoters. Apparently, when expressed from weak promoters, the concentration of p53 protein is limiting due to inefficient nuclear import, weak binding to other sites in nuclear DNA or kinetic factors associated with multimerization and DNA binding. It was demonstrated that multiple copies of UAS_{53} in the promoter increased reporter gene expression when p53 is limiting (expressed from pBT26/53; Figure 2A). This result indicates that the apparent limitation of p53 is due to the weak binding of the protein to other sites in nuclear DNA.

Several yeast genes were evaluated as reporter genes using nutritional selections. When UAS_{53} was present in

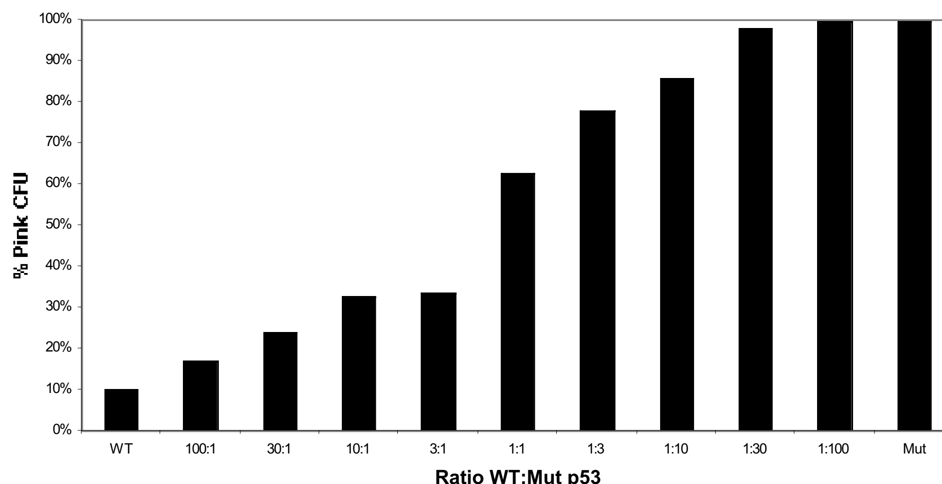


Figure 6. Sensitivity of functional p53 assay for wild-type and mutant p53 genes present in differing ratios. Plasmid wild-type and mutant p53 molecules were mixed in the indicated molar ratios, amplified by PCR and functional testing of p53 by gap repair in yeast was carried out as described in the Experimental Procedures. Following gap repair transformation of the p53 gene, over 600 histidine prototrophs (colonies) were obtained for each DNA mixture and these were scored for color; white indicating the presence of wild-type p53 and pink indicating the presence of mutant p53. Bars on the graph represent the percentage of pink CFU obtained with the particular mixture of wild-type and mutant plasmids. WT, target DNA contained all wild-type p53 plasmids, while Mut target DNA contained only mutant p53 plasmids.

the promoter expressing the *URA3*, *HIS3* or *ADE2* genes, a strong uracil, histidine or adenine prototrophy, respectively, was conferred by co-expression of p53. For the *URA3* reporter, furthermore, the strain was foa^s in the presence of p53. In all cases, however, a very weak prototrophy for the selected reporter gene was observed in the absence of p53. This is presumably due to a low basal level of transcription of the gene in the absence of p53. Apparently, only low level expression of the *URA3*, *HIS3* or *ADE2* genes is required to allow growth under the respective selective conditions. In the case of the *HIS3* gene, the phenotypic effect (histidine prototrophy) of basal level transcription could be eliminated by inclusion of appropriate concentrations of 3-amino-1,2,4-triazole in the selective plates. The basal level expression of both the *URA3* and *ADE2* genes could be decreased by integration of the reporter gene cassette at the corresponding chromosomal locus. This was evident by a lack of uracil or adenine prototrophy, respectively, if p53 was not co-expressed in the cell. The reason for decreased basal level transcription when the reporter gene is integrated into the chromosome is unclear, but may be related to differences in chromatin structure between the gene present on a plasmid and the chromosomal integrant.

The *ADE2* reporter has the advantage that, in addition to nutritional selections for p53 function, a colorimetric phenotype can be used to distinguish yeast cells expressing a wild type p53 from those producing mutant p53 under conditions where both strains are viable. Our system allows determination of the genotype of cell lines or clinical samples with regard to p53 function: wild type, heterozygous mutant or homozygous mutant. The assay allows determination of the genotype even when transcripts of the two alleles are expressed at up to 30-fold different levels. Many p53 variants are known to be dominant negative mutants (Brachmann *et al.*, 1996). The system described here can also be used to identify such dominant negative mutations. An allele identified in a gap repair assay, which is carried on a plasmid with a *HIS3* marker, can be recovered and reintroduced into a strain with a reporter gene and also harboring pBT26/53 (*LEU2* marker). Loss of p53-mediated activation of the reporter gene indicates the allele on the *HIS3* plasmid is a dominant negative mutant.

These investigations confirm and extend previous studies in which human p53 was used as the basis for a transactivation system in yeast (Scharer and Iggo, 1992; Ishioka *et al.*, 1993). Using the *URA3* gene as a reporter Brachmann *et al.* (Brachmann *et al.*, 1996; Brachmann *et al.*, 1998) have described systems to select p53 genes with dominant-negative and suppressor alterations of common cancer mutations. In another study the *ADE2* reporter gene was used in a FASAY to test p53 function (Flaman *et al.*, 1995). The gap repair system described here tests an additional 28 codons in the 5' end of the gene, and can thus detect mutations not scored in the assay of Flaman *et al.* (1995). The variety of reporter genes and selections described in this study could be utilized in any combination to allow for quantitation of gene expression (*LacZ*), color selection (*LacZ*, *ADE2*) and nutritional selection (*ADE2*, *HIS3*, *URA3*). Taken together the p53 transactivation systems described here offer maximum flexibility and utility for further investigations of p53 function in either research or clinical settings.

Functional testing of separated p53 alleles in yeast has several advantages over physical detection techniques such as denaturing gradient gel electrophoresis (DGGE) (Fodde and Losekoot, 1994), single-strand conformation polymorphism (SSCP) (Iavarone *et al.*, 1992), heteroduplex analysis (Huber *et al.*, 1993) and sequence-specific nuclease cleavage techniques (Goldrick, 2001) which rely on expensive equipment, extensive optimization of experimental conditions and in some cases specialized enzymes and reagents The yeast-based functional assay can directly determine the transactivation potential of p53 rapidly, inexpensively, with high-throughput and without prior knowledge about the mutational spectra of p53. Moreover, the physical mutation detection techniques identify the presence of nucleotide variations in fragments of the p53 DNA sequence which must then be defined by DNA sequencing or array-based methods. In the absence of previous functional data or extensive clinical analysis, however, an observed alteration can not unambiguously be classified as either a mutation or silent polymorphism.

p53 is often dysfunctional in tumors and p53 function is known to modulate cellular sensitivity to ionizing radiation and some anticancer drugs (Hawkins *et al.*, 1996; Lowe *et al.*, 1993; O'Conner *et al.*, 1993). Moreover, in recent years, several new experimental therapies have begun to be developed which are critically based on the function or non-function of p53 in the target tumor. Knowledge of the p53 status of a tumor, therefore, will be useful in prescribing appropriate therapies. The availability of a rapid and inexpensive methodology of determining p53 status, such as the functional assay described in this report, should play an increasingly important role in implementation of effective cancer therapies.

Experimental Procedures

Expression Vectors and Genes

The expression vector pBT26 incorporates the constitutive and efficient yeast *TDH3* gene promoter, and was constructed as follows. The yeast *LEU2* gene was PCR amplified from plasmid pRS405 (Sikorski and Hieter, 1989) using the primers

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        5'-CGCGTCTAGACCACATACCTAATATTATTGCC
and     5'-CGCGATGCATAGCTACGTCGTAAGGCCG.
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The PCR product was digested with *XbaI* and *NsiI* and ligated into expression vector pBT6 (Bitter, 1998) which had also been digested with *XbaI* and *NsiI* . A clone in which the yeast *URA3* gene coding region was replaced with the yeast *LEU2* gene was identified and designated pBT26.

Expression vector pBT25 was constructed from pGP381 (Bitter *et al.*, 1991) by ligating the synthetic oligonucleotide duplex

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5'-GATCTCGAATAAACACACATAAATAAACAAACTAGTATCTCGAGTAG
       AGCTTATTTGTGTGTATTTATTTGTTTGATCATAGAGCTCATCCTAG-5'
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into the *BamHI* site and selecting a clone which regenerates the *BamHI* site adjacent to the *PGK* terminator region. This vector includes a 5' truncation of the yeast *TDH3* gene promoter (GP381 promoter) which is approximately 100-fold less efficient than the native *TDH3* gene promoter (Bitter *et al.*, 1991).

The yeast expression vector pMETc employs the yeast *MET25* gene reporter (Mumberg *et al.*, 1994). All of the yeast expression vectors utilized in this study included centromere (CEN) elements, and are therefore stably maintained in yeast at low copy number.

The coding region of the human p53 cDNA was PCR amplified from plasmid pHp53b (ATCC #57254) using the primers

5'-GCGCACTAGTGCCTTCCGGGTCACTGC
and 5'-GCGCGGATCCGTGGGGAACAAGAAGTGGAG.

The approximately 1224 bp PCR product was digested with *SpeI* and *BamHI* and ligated into *SpeI* and *BamHI* digested pBT26 to generate pBT26/53. The digested PCR product was also cloned into *SpeI* and *BamHI* digested pMETc to generate pMETc/53.

An expression vector containing the yeast URA3 gene with a 5' truncated promoter was constructed as follows. The yeast URA3 gene coding region plus 102 bp of 5' flanking DNA was PCR amplified from plasmid pYES2 (Stratagene) using the primers

5'-GCGCGTCGACTGGTATATATACGCATATGTGG
5'-GCGCGGATCCACATGCATTTACTTATAATACAG.

The approximately 931 bp product was digested with *SalI* and *BamHI* and ligated into *SalI* and *BamHI* digested pBT25. A clone in which the GP381 promoter of pBT25 was replaced with the *URA3* gene coding region and 5' truncated promoter was isolated and designated pURA3A. An expression vector containing this *URA3* gene and the 102 bp 5' truncated promoter flanked by a DNA binding site (UAS_{53}) for the human p53 transcription factor was also constructed. The synthetic DNA duplex containing two tandem binding sites for human p53

5'-CAGGCATGCCTAGGCATGCCTGT
CATGGTCCGTACGGATCCGTACGGACAGCT-5'

was ligated into *KpnI* and *SalI* digested pURA3A to generate pURA53A.

The yeast *HIS3* gene was cloned into an expression vector utilizing the weak GP381 promoter as follows. The *HIS3* gene was PCR amplified from plasmid pRS403 (Sikorski and Hieter, 1989) using the primers

5'-GCGCACTAGTGCAAGATAAACGAAGGC
and 5'-GCGCGGATCCGCAGCTTTAAATAATCGG.

The approximately 704 bp PCR product was digested with *SpeI* and *BamHI* and ligated into *SpeI* and *BamHI* digested pBT25 to generate the vector pBT25/HIS3. A vector in which this *HIS3* gene was expressed from a GP381 promoter containing a binding site for the human p53 transcription factor was also constructed. The synthetic DNA duplex containing two tandem binding sites for human p53

5'-CAGGCATGCCTAGGCATGCCTGT
CATGGTCCGTACGGATCCGTACGGACAGCT-5'

was ligated into *KpnI* and *SalI* digested pBT25/HIS3 to generate pBT25(53)/HIS3.

The yeast *ADE2* gene was cloned into an expression vector utilizing the weak GP381 promoter as follows. The *ADE2* gene was PCR amplified from plasmid *S.cerevisiae* S288C DNA using the primers

5'-GCGCACTAGTAATCGGACAAAACAATCAAG
and 5'-GCGCGGATCCTAATTATTTGCTGTACAAGTATATC.

The approximately 1776 bp PCR product was digested with *SpeI* and *BamHI* and ligated into *SpeI* and *BamHI* digested pBT25 to generate the vector pBT25/ADE2. A vector in which the yeast *ADE2* gene was expressed from the GP381 promoter containing a binding site for the human p53 transcription factor was also constructed. The synthetic DNA duplex containing two tandem binding sites for human p53

5'-CAGGCATGCCTAGGCATGCCTGT
CATGGTCCGTACGGATCCGTACGGACAGCT-5'

was ligated into *KpnI* and *SalI* digested pBT25/ADE2 to generate pBT25(53)/ADE2.

Two vectors in which the *E. coli LacZ* gene was expressed from 5' truncated yeast *TDH3* gene promoters containing a binding site for the human p53 transcription factor were constructed. The synthetic DNA duplex containing two tandem binding sites for human p53

5'-CAGGCATGCCTAGGCATGCCTGT
CATGGTCCGTACGGATCCGTACGGACAGCT-5'

was ligated into *KpnI* and *SalI* digested pGP381/Z or pGP171/Z (Bitter *et al*., 1991) to generate pGP171(53)/Z and pGP171(53)/Z, respectively. The *KpnI* and *SalI* digested pGP381/Z was also treated with calf intestinal phosphatase and ligated to p53 synthetic DNA duplex which had been phosphorylated with T4 polynucleotide kinase. A clone containing three head-to-tail copies of the p53 binding site was identified and designated pGP381(53-3)/Z.

Yeast Strains and Culture Medium

A yeast strain which contained the *URA3* gene coding region downstream a 5' truncated *URA3* promoter with an adjacent p53 binding site was constructed as follows. The DNA fragment including the UAS_{53}-*URA3* gene fusion was PCR amplified from pURA53A (above) using the primers

5'-GAAGGTTAATGTGGCTGTGGTTTCAGGGTCCATAAAGCTTAAACTACCGCATTAAAGC
and 5'-GCGCGGATCCACATGCATTTACTTATAATACAG.

The approximately 930 bp PCR product contains the *URA3* gene coding sequence at one end and DNA homologous to -256 to -216 (relative the the translation start codon) of the yeast *URA3* 5' flanking DNA. This DNA was transformed (Ito *et al*., 1983) into *S. cerevisiae* strain YPH500 (Sikorski and Hieter, 1989) containing vector pBT26/53, and uracil prototrophs selected. The integration and replacement by homologous recombination of the *ura3-52* locus on chromosmome V by the *URA3*-UAS_{53}-*URA3* fragment was confirmed by PCR analysis of chromosomal DNA. This strain was cured of vector pBT26/53 by serially culturing in YPDA medium, plating for single colonies and identifying a clone which was a leucine auxotroph and had therefore lost the pBT26/53 plasmid. This strain, YBT27, has the genotype:

matα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 URA3(Δ-216/-101)::(UAS_{53})::URA3.

Strain YBT27 was transformed with either pBT26 or pBT26/53 to generate, respectively, YBT27; pBT26 and YBT27; pBT26/53.

A yeast strain which contained the *ADE2* gene coding region downstream of the GP381 promoter with a binding site for human p53 was constructed as follows. The DNA fragment including the UAS_{53}-GP381-*ADE2* gene fusion was PCR amplified from pBT25(53)/ADE2 using the primers

5'-AAGGTTAATGTGGCTGTGGTTTCAGGGTCCATAAAGCTTAAACTACCGCATTAAAGC
and 5'-GCGCGGATCCTAATTATTTGCTGTACAAGTATATC.

The approximately 2220 bp PCR product contains the *ADE2* gene coding sequence at one end and DNA homologous to -399 to -360 (relative the the translation start codon) of the yeast *ADE2* 5' flanking DNA on the other end. This DNA was transformed (Ito *et al*., 1983) into *S. cerevisiae* strain YPH500 (Sikorski and Hieter, 1989) containing vector pBT26/53 and adenine prototrophs selected. The integration and replacement by homologous recombination of the *ade2-101* locus on chromosmome XV by the *ADE2*-UAS_{53}-*ADE2* fragment was confirmed by PCR analysis of chromosomal DNA. This strain was cured of vector pBT26/53 by serially culturing in YPDA medium, plating for single colonies and identifying a clone which was a leucine auxotroph and had therefore lost the pBT26/53 plasmid. This strain, YBT26, has the genotype:

matα ADE2::UAS_{53}-GP381::ADE2 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63.

Strain YBT26 was transformed with either pBT26 or pBT26/53 to generate, respectively, YBT26; pBT26 and YBT26; pBT26/53.

The following media was used for culturing yeast. YPDA is 2% Yeast Extract, 2% Peptone, 2% Dextrose, 42 μg/ml Adenine. SD is 0.67% Yeast Nitrogen Base without amino acids, 2% dextrose. SD, CAA is SD containing 0.5% Casamino acids. Where indicated, media was supplemented with the following: A, 42 μg/ml adenine; H, 20 μg/ml histidine; L, 60 μg/ml leucine; Ly, 30 μg/ml lysine; T, 40 μg/ml tryptophan; U, 20 μg/ml uracil; FOA, 1 mg/ml 5-fluoro-orotic acid (Toronto Research Chemicals). Low adenine plates (Lo Ade) were supplemented with 2.5 μg/ml adenine.

***In vivo* Gap Repair Assay**

Plasmid pMETc/53 (above) was restricted with *StuI* and *AccB7I* and the approximately 5400 bp gapped vector purified by agarose gel electrophoresis using Qiaex II reagents (Qiagen) . The wild type human p53 cDNA was amplified from plasmid pHp53b (ATCC #57254) using the primers

5'-GCGCACTAGTGCCTTCCGGGTCACTGC
and 5'-GCGCGGATCCGTGGGGAACAAGAAGTGGAG.

The vector pBT26/53-4 contains a PCR amplified p53 cDNA constructed as described above for pBT26/53. pBT26/53-4 contains, however, an uncharacterized PCR-introduced mutation which results in synthesis of a protein not capable of transactivating the pGP381(53)/Z reporter (data not shown). This plasmid was used as the source of mutant p53 cDNA (Results, Figures 4 and 6). The gapped vector and various PCR amplified p53 DNA samples were mixed in ratios described in the text and transformed (Ito *et al.*, 1983) into yeast strain YBT26 containing pGP381(53)/Z, selecting for histidine prototrophs.

Total RNA was isolated from peripheral human blood lymphocytes using the QIAmp RNA Blood kit (Qiagen). First-strand cDNA was synthesized from approximately 1 μg RNA using murine Moloney leukemia virus reverse transcriptase (M-MLV-RT; 0.2 units) and 0.5 μg oligo $(dT)_{15}$ primers in a 20 μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 μM dNTP, and 0.05 unit Rnasin (Promega). The mixture was incubated at 23º C/10 min, 37º C/45 min, 95º C/5 min, 18º C/1 min and stored at -80º C. cDNA was PCR amplified using *Taq* DNA polymerase in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 under the following cycling conditions: 94º C/2 min; 45 cycles of 94º C/36 sec, 50º C/1 min, 72º C/2.5 min; 72º C/10 min. The 1.2 Kb PCR product was purified with the Wizard PCR Prep DNA Purification System (Promega).

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