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Mutation of conserved domain II alters the sequence specificity of DNA binding by the p53 protein

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We have mutagenized human p53 expressed in yeast and selected two mutants, 121F and 123A, which activate transcription from one, rather than the normal two, copies of the consensus p53 DNA binding sequence. Both mutants have a 6-fold increase in affinity for a single copy of the sequence GGG CATG CCC. The 121F mutant has a decrease, and the 123A mutant an increase, in the affinity for the sequence GAA CATG TTC. This genetic and biochemical evidence supports the crystallographic finding that amino acid 120 contacts guanine in the major groove at the second position in the consensus. The major p53 binding site in the p21$^{WAF1/CIP1}$ promoter resembles the GAA CATG TTC form of the consensus. Compared with wild type p53, the 121F mutant has a 7-fold lower affinity for the p21$^{WAF1/CIP1}$ site *in vitro*, and the 121F mutant is defective in p21 induction *in vivo*. Mutants with subtly altered sequence specificity may facilitate dissection of downstream pathways activated by p53.

Key words: DNA binding/p53/tumour suppressor

Introduction

The p53 gene is mutated in the majority of human tumours and understanding its function is therefore central to our understanding of cancer (Hollstein *et al.*, 1991). At a biochemical level it appears that p53 is a transcription factor and that inactivation of this function is the target of tumorigenic mutations (Fields and Jang, 1990; O'Rouke *et al.*, 1990; Raycroft *et al.*, 1990; Funk *et al.*, 1992; Kern *et al.*, 1992; Scharer and Iggo, 1992). In most cases this inactivation results from loss of specific DNA binding (Kern *et al.*, 1991a,b; el Deiry *et al.*, 1992), consistent with the fact that the great majority of tumorigenic mutations lie in the DNA binding domain (Hollstein *et al.*, 1991; Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993).

At least two biological pathways are known to be activated by p53, one leading to apoptosis (Yonish *et al.*, 1991), the other leading to G_1 arrest (Kastan *et al.*, 1991). While it is possible that the outcome of p53 activation is dictated by the cellular environment, related perhaps to the status of the *Rb* gene (Lowe and Ruley, 1993) or the Bcl2/Bax ratio (Miyashita *et al.*, 1994), or reflects non-transcriptional mechanisms (Caelles *et al.*, 1994), it is also possible that the decision to arrest or apoptose is taken at the promoter level. Work from several laboratories has demonstrated that wild type p53 binds to and activates transcription from two closely spaced copies of a 10 bp DNA sequence (el Deiry *et al.*, 1992; Funk *et al.*, 1992; Halazonetis *et al.*, 1993). That sequence, RRR CWWG YYY, where R = purine, W = A or T, and Y = pyrimidine, is degenerate at eight of 10 sites. Further degeneracy derives from the fact that the two copies can be spaced by up to 13 bp, and even complete departure from the consensus is tolerated at some positions. Not surprisingly, the binding sites in genes which are thought to be activated directly by p53, p21$^{WAF1/CIP1}$ (el Deiry *et al.*, 1993), *MDM2* (Wu *et al.*, 1993) and *gadd45* (Kastan *et al.*, 1992) differ significantly. The ability to manipulate downstream pathways independently using p53 mutants with subtly altered specificity would greatly facilitate studies asking why different cell types respond differently.

Tumorigenic p53 mutants have a low affinity for DNA. We describe here p53 mutants selected in yeast which have the opposite phenotype, an increased affinity for DNA. In addition, one mutant has a subtly altered sequence specificity. Based on these studies we postulate that amino acid 121 contacts DNA at the second and third positions in the consensus. During preparation of this manuscript the crystal structure of a p53–DNA complex at 2.2 Å resolution was solved (Cho *et al.*, 1994) and it shows that amino acid 120 donates H bonds to the O^6 and N^7 of guanine at the second position in the consensus. Our genetic and biochemical data thus support and confirm the crystallographic assignment. We also show that the 121F mutant activates p21 transcription poorly in mammalian cells, and it may thus be the prototype for a class of mutants which can be used to dissect downstream response pathways *in vivo*.

Results

Identification of p53 mutants with novel DNA binding properties

Almost all genomic p53 binding sites identified to date contain one perfect copy of the p53 consensus and one imperfect one. To identify critical residues in p53 which influence DNA binding, and to identify mutants which might activate transcription more efficiently from genomic sites *in vivo*, we mutagenized p53 and looked for mutants which can activate *lacZ* expression in yeast from a single copy of the p53 consensus (AGA CATG CCT). Two such mutants were identified in plasmids rescued from 10 independent yeast colonies: amino acid 121S to F in seven clones and 123T to A in the remaining three. These mutations and other relevant structural information are shown schematically in Figure 1.

To exclude fortuitous binding to sites in the promoter

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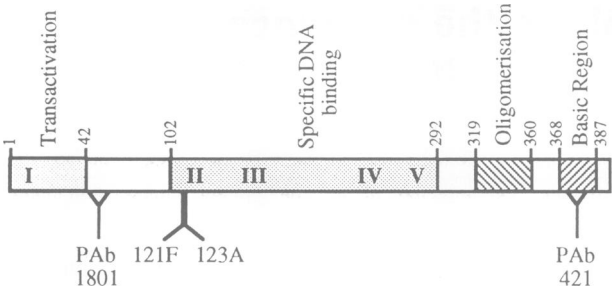

Fig. 1. Schematic diagram showing the position of the 121F and 123A mutations, the transactivation domain (Unger *et al.*, 1992), sequence specific DNA binding domain (Pavletich *et al.*, 1993), oligomerization domain (Clore *et al.*, 1994) and basic region (non-specific DNA binding, Pavletich *et al.*, 1993). I–V refer to domains conserved from man to rainbow trout (Soussi *et al.*, 1990). Tumorigenic mutations commonly lie in domains II–V (Caron de Fromentel and Soussi, 1992). The PAb421 epitope (amino acids 372–381 in human p53, Wade-Evans and Jenkins, 1985) and the PAb1801 epitope (amino acids 46–55, Y.Legros and T.Soussi, personal communication) are also shown.

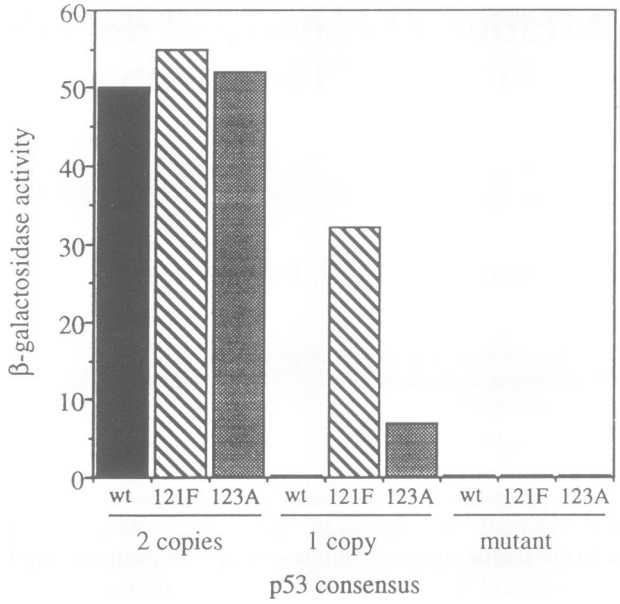

Fig. 2. Yeast β-galactosidase assays with wild type, 121F and 123A mutant p53 using reporter plasmids containing the DED1 promoter. '2 copies': AGG CATG TCT AGG CATG TCT; '1 copy': AGG CATG TCT; 'mutant': AGG AATT TCT AGG AATT TCT.

of the plasmid used for the mutagenesis screen, the mutants were tested for activation of transcription from a plasmid with a different minimal promoter (pDED1 instead of pCYC1). As before, both mutants activate transcription from a single copy of the consensus (Figure 2).

Western blotting shows that the effect is not due to changes in protein level, and immunoprecipitation with conformation-sensitive antibodies (PAb240 and PAb1620) shows that the mutants are in the wild type conformation (data not shown).

The mutants have altered DNA binding properties in vitro

To obtain high affinity DNA binding *in vitro* it has previously been shown that the C-terminus of p53 must be either deleted or bound to the antibody PAb421 (Hupp *et al.*, 1992). We have confirmed this observation, and for our *in vitro* studies we used p53 truncated at amino acid 368 ('p53 CΔ368').

Whole cell extracts containing the mutants were tested initially for *in vitro* DNA binding by electrophoretic mobility shift (bandshift) assay using probes containing the form of the consensus (AGA CATG CCT) employed in the mutagenesis screen. Mutant and wild type p53 bind strongly to probes containing two copies of the consensus (data not shown). All three forms of the protein also shift a probe containing a single copy of the consensus (Figure 3). Both mutants bind better than wild type protein, although the difference is less marked than in the transcription assays (Figure 2). The shift is p53 specific since with full length p53 it is induced by PAb421, and with truncated p53 (CΔ368) it supershifts with anti-p53 (PAb1801) but not control antibody (PAb204). It is sequence specific since it competes with specific (Figure 3, 'cons') but not non-specific (Figure 3, 'mut') oligonucleotide competitor.

Examination of published sequences from which the p53 consensus was derived reveals a bias towards certain forms of the consensus being preferred at the first and last three positions (el Deiry *et al.*, 1992; Funk *et al.*, 1992; Halazonetis *et al.*, 1993). Oligonucleotides encoding single copies of all eight possible palindromic variants of this part of the consensus were therefore tested in order

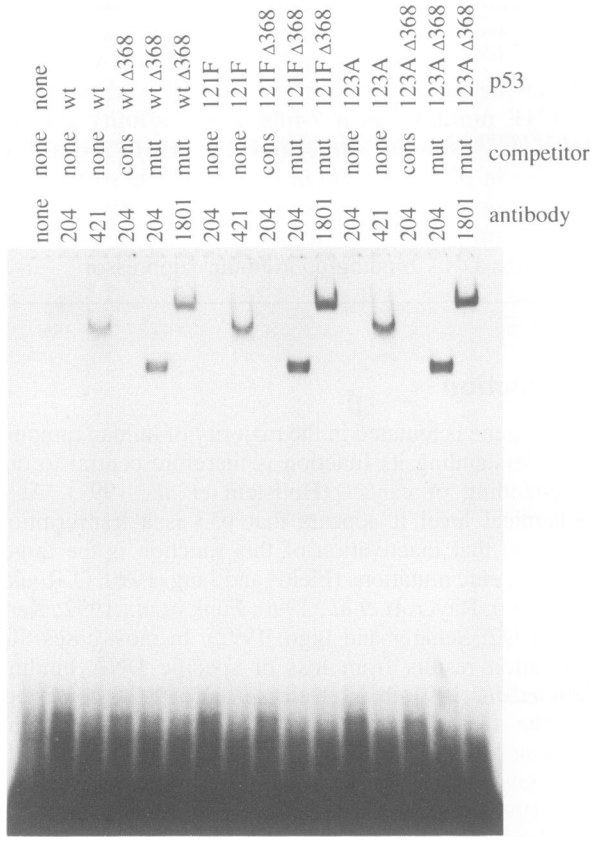

Fig. 3. Bandshift assays with full length and CΔ368 wild type, 121F and 123A mutant p53. The probe (RI.50/51) contains a single copy of the form of the consensus used for the screen: AGA CATG CCT. PAb421 and PAb1801 are anti-p53 antibodies; PAb204 is a control antibody. cons, 50-fold excess of double-stranded competitor oligonucleotide containing two copies of the consensus (Hupp *et al.*, 1992); mut, 50-fold excess of unrelated double-stranded oligonucleotide competitor (Hupp *et al.*, 1992).

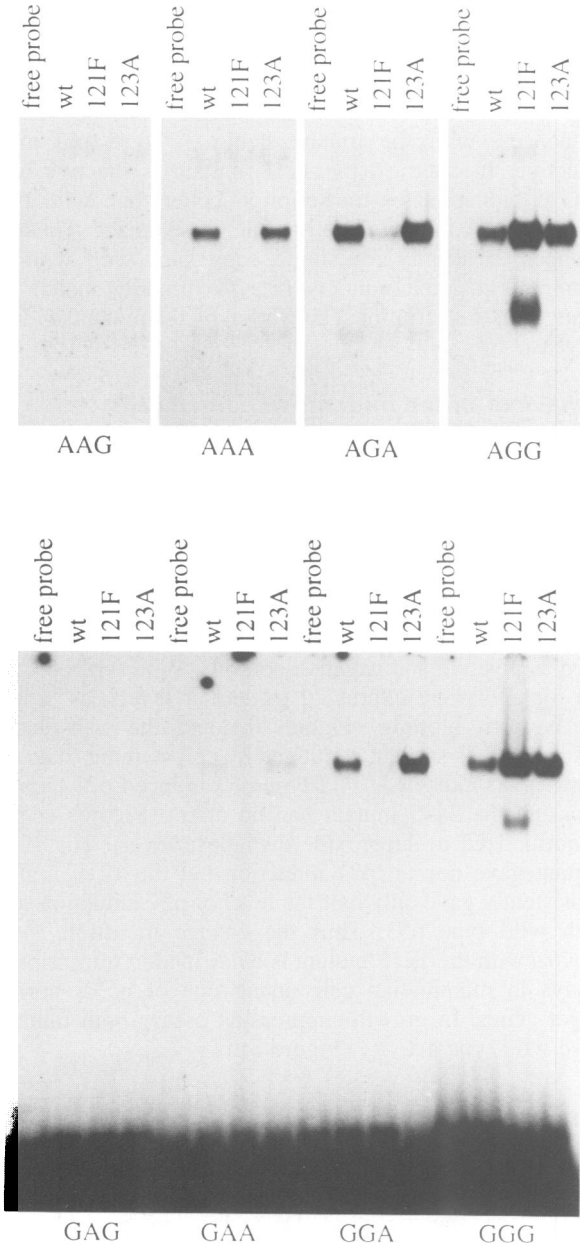

Fig. 4. Bandshift assays with CΔ368 wild type, 121F and 123A mutant p53 and palindromic probes containing single copies of the p53 consensus. 'AAG', AAG CATG CTT; 'AAA', AAA CATG TTT; 'AGA', AGA CATG TCT; 'AGG', AGG CATG CCT; 'GAG', GAG CATG CTC; 'GAA', GAA CATG TTC; 'GGA', GGA CATG TCC; 'GGG', GGG CATG CCC.

to detect subtle changes in the DNA binding properties of the mutants (Figure 4). Palindromic probes were used on the grounds that both halves of a p53 dimer (the basic DNA binding unit) would be presented with the same form of the consensus sequence. (Note that a 'single copy' of the consensus actually contains two pentamers, for example the AGA CATG CCT sequence used for the screen contains AGA CA and AGG CA.) The binding in Figure 4 is p53 specific and sequence specific, as determined by supershifting and competition experiments, and Western blotting shows that the effects are not due to changes in protein level (data not shown). The faster migrating complex seen with the 121F mutant and the

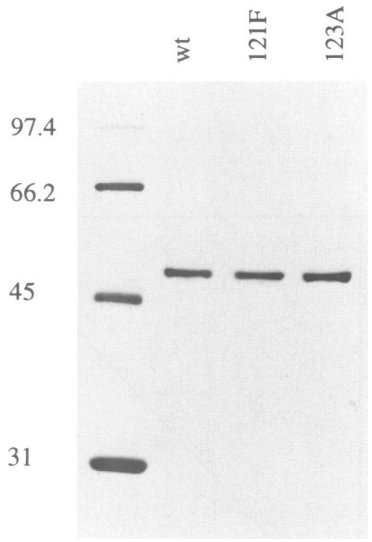

Fig. 5. Silver stained gel of purified CΔ368 p53 protein eluted from the DNA column. Markers in kDa.

'RGG' probes (Figure 4) probably contains p53 dimers (Halazonetis and Kandil, 1993; data not shown). Changes at the first position in the pentamer do not alter the specificity of binding by one or other mutant. None of the proteins tested binds to the 'RAG' probe; this is consistent with the fact that it is under-represented in genomic target sequences (el Deiry *et al.*, 1992). The 123A mutant has an increased affinity relative to wild type p53 for the 'RGA' and 'RGG' probes. Relative to wild type p53 and the 123A mutant, the 121F mutant has an increased affinity for the 'RGG' probe and a markedly reduced affinity for the 'RGA' and 'RAA' probes. Thus, the 121F mutant has an altered sequence specificity in bandshifts with crude extract *in vitro*.

Quantitative analysis of DNA binding in vitro

In order to characterize the changes in more detail we purified p53 and measured its affinity for DNA. We used CΔ368 C-terminally deleted protein in order to eliminate confounding variation in repression by the C-terminus (Hupp *et al.*, 1992). The tendency for p53 to become denatured in heterologous systems is an obstacle to quantitative analysis. To avoid this we purified active protein by DNA affinity chromatography (Figure 5), and tested it immediately after elution from the DNA column. Immunoprecipitation with conformation-sensitive antibodies (PAb1620 and PAb240) shows that the purified protein is in the wild type conformation (data not shown).

Dissociation constants were calculated by titrating protein against DNA at 25°C in the absence of competitor (the CΔ368 proteins used here lack the C-terminal single stranded DNA binding domain), and binding was quantitated by phosphorimaging of bandshift gels. Figure 6 shows a typical titration; the dissociation constant was taken as the concentration giving 50% binding (i.e. log bound/free = 0). The p53 concentration is expressed in moles of tetramer because the unbound protein has the mobility of tetramers on gel filtration (data not shown) and p53 binds DNA as a tetramer (Halazonetis and Kandil, 1993). Although the tetramerization domain was

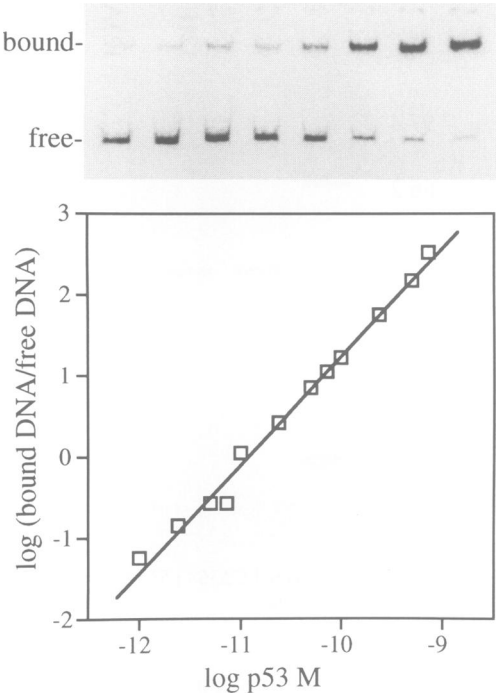

Fig. 6. Bandshift gel and Hill plot of CΔ368 123A mutant p53 binding two copies of the 'GGG' probe (RI.107: GGG CATG CCC GGG CATG CCC). p53 concentration is expressed as mol/l tetramer. The upper points were run on another gel.

previously mapped (Sturzbecher *et al.*, 1992) to the region deleted in the protein used here, subsequent work has shown that the minimal tetramerization domain lies proximal to the deletion (Pavletich *et al.*, 1993; Wang, *et al.*, 1993; Clore *et al.*, 1994).

Since p53 aggregates at concentrations above 10^{-8} M in our assay, the 'RAG' probe was not examined. The calculated dissociation constants for the other sites (Table I) confirm the results obtained with crude extract and single copy sites: the 123A mutant has a higher affinity than wild type for all three single copy sites; and the 121F mutant has a higher affinity for the 'GGG' site but a lower affinity for the other sites. The striking result is that both mutants have a 6-fold increased affinity for a single copy of the 'GGG' site.

In vitro binding to a genomic target

The affinity of wild type CΔ368 p53 for probes containing two copies of the 'GAA' site is 11-fold lower than it is for the other palindromic sites. Interestingly, the 'GAA' site most closely resembles the major p53 binding site in the p21 promoter (el Deiry *et al.*, 1993). We therefore measured dissociation constants for the p21 site itself (Table I). The results show that this site discriminates between wild type p53 and the two mutants: 121F binds much worse and 123A much better than wild type p53.

The 121F and 123A mutants have altered activity in vivo

The changes in affinity and specificity were also tested *in vivo* in yeast transcription assays using the 'GRR' single copy probes cloned upstream of the DED1 minimal promoter and tested *in vivo* in yeast (Table II). Western blotting shows that the effects are not due to changes in protein level (data not shown). As expected, single copies of the 'GAG' and 'GAA' sequences are unresponsive to p53 *in vivo*. Wild type p53 and the 123A mutant both activate transcription from the 'GGA' and the 'GGG' sequences, with the mutant being 3- to 5-fold more effective. In contrast, the 121F mutant is inactive with 'GGA' but activates transcription better than wild type and the 123A mutant from the 'GGG' sequence. Thus the changes in affinity for single copy sites seen with C-terminally deleted mutants *in vitro* broadly match the changes in transcriptional activation by the mutants *in vivo* in yeast.

Behaviour of the mutants in human cells

The biochemical data suggest that the 123A (high affinity) mutant will have wild type, or supra-wild type, activity in mammalian cells and that the 121F mutant will be defective in p21 induction. To test these predictions we transfected p53-minus Saos-2 cells with the mutants and measured p21 induction by RNase protection and growth suppression using G418-resistant colony formation assays. Wild type p53 and the tumorigenic 249S mutant were used as positive and negative controls. Wild type p53 and the mutants were expressed to similar levels, as judged by Western blotting (Figure 7b) and the transfection efficiency was similar, as judged by cell staining (data not shown). As expected, wild type p53 induced p21 expression and the 249S mutant had no effect (Figure 7a; p21 is normalized to superoxide dismutase level). The 123A mutant gave normal p21 induction but the 121F mutant consistently gave only half the level of p21 induction seen with wild type p53. Thus the change in affinity seen *in vitro* with the 121F mutant is confirmed in transcription assays in mammalian cells using one of p53's normal target genes. In growth suppression assays both mutants have wild type activity (Figure 8).

Discussion

The main conclusion from this work is that mutations in the highly conserved, central region of the protein can modify the specificity of DNA binding by p53. The p53–DNA cocrystal structure has recently been solved (Cho *et al.*, 1994), and it shows amino acid 120 binding in the major groove to the second purine in the consensus. The change in specificity of the 121F mutant identified here affects the second and third purines in the p53 consensus. Since subtle changes of this sort commonly follow mutation of DNA contact residues, our genetic data confirm and support the crystallographic assignment.

How the mutagenesis screen selected for the 121F and 123A mutants is an interesting question. Both mutants have a substantially higher affinity for 'GGG' probes containing only a single copy of the consensus and the 123A mutant also has a moderately increased affinity for 'GGA' probes. The 'RGA' pentamer is present at one end of the target sequence in the screen (AGA CA) and the 'RGG' sequence is present at the other end (AGG CA). The simplest explanation for the selection of the mutants is thus that the screen asked for an increase in affinity, and that the change in specificity of the 121F mutant was incidental.

Table I. Dissociation constants for pure p53 binding to various probes

	1× GAA	1× GGA	1× GGG	2× GAA	2× GGA	2× GGG	p21 site
Wild type	680	300	320	11	0.95	0.92	21
121F	high[a]	1100	54	14	3.8	1.6	150
123A	240	110	52	3.3	0.94	1.2	6.7

'GAA': GAA CATG TTC, 'GGA': GGA CATG TCC, 'GGG': GGG CATG CCC; '1×' means one copy, '2×' means two copies of the consensus. Expressed as $\times 10^{-11}$ M tetramer.
[a]The concentration required to achieve 50% binding induces protein aggregation ($K_D > 10^{-8}$).

Table II. Yeast β-galactosidase assays with full length wild type, 121F and 123A mutant p53 using reporter plasmids containing the DED1 promoter

p53	Reporter	Mean	SD
Wt	GAG CATG CTC	0.1	0.03
121F	GAG CATG CTC	0.2	0.02
123A	GAG CATG CTC	0.3	0.03
Wt	GAA CATG TTC	0.1	0.03
121F	GAA CATG TTC	0.3	0.03
123A	GAA CATG TTC	0.4	0.02
Wt	GGA CATG TCC	2.0	0.02
121F	GGA CATG TCC	0.2	0.02
123A	GGA CATG TCC	6.0	0.2
Wt	GGG CATG CCC	3.0	0.2
121F	GGG CATG CCC	41.0	3.0
123A	GGG CATG CCC	15.0	0.4

The most striking change in the quantitative data is the increase in affinity of the 121F and 123A mutants for the single copy 'GGG' sites. This could indicate an effect of the mutations on the oligomerization properties of the molecule, which is consistent with the appearance of a minor population of protein–DNA complexes containing p53 dimer rather than tetramer (Figure 4). However, gel filtration shows that the purified mutant proteins are tetrameric in solution. The p53 tetramer is made of two dimers (Clore *et al.*, 1994), each of which binds to a single copy of the consensus (Cho *et al.*, 1994). We interpret the presence of complexes containing dimer as follows. With a single copy probe the tetramer binds with high affinity to the consensus site and low affinity to an adjacent non-specific site, but in the gel conditions the tetramer breaks down into its constituent dimers unless binding by the 'free' dimer is stabilized by non-specific protein–DNA contacts. These non-specific contacts cannot form with short probes (particularly probes <20 bp), hence the appearance of dimer–DNA complexes in these conditions. While this satisfactorily explains most of the data, it does not explain why the increased affinity for a single copy of the 'GGG' sequence does not result in an equally large increase in the affinity for probes containing two copies of this sequence. The simplest explanation for this is that steric hindrance prevents high affinity binding to two adjacent palindromic sites. This is consistent with data suggesting that non-palindromic sites may bind wild type p53 with higher affinity than palindromic sites (Halazonetis *et al.*, 1993), and that single copies of the consensus in genomic p53 binding sites are often separated by up to 13 bp (el Deiry *et al.*, 1992).

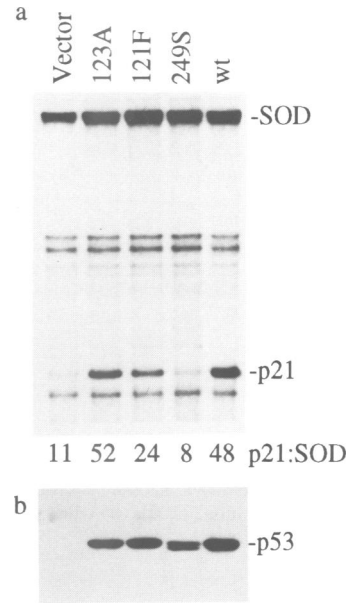

Fig. 7. (**a**) RNase protection assay using RNA extracted from cells transfected with vector alone, wild type or mutant p53. SOD was used as a loading control; the p21:SOD ratio was calculated by phosphorimaging the gel. (**b**) Western blot of cells transfected as in (a) and probed with PAb240.

Another possibility is that the mutations reduce unfavourable protein contacts with non-specific DNA, giving rise to an increased affinity for non-specific DNA. We have some evidence for this from McKay assays with the 121F mutant (data not shown), but the tendency for protein to aggregate at high concentrations precludes precise quantitation of this in the bandshift assay. Given that the 121F mutant should only bind with high affinity to one end of the probe sequence used in the screen, a change in non-specific binding is not unreasonable. Finally, an effect on allosteric regulation cannot be ruled out, although it appears from Figure 3 that the mutations do not lock the full length protein in the R state (Halazonetis and Kandil, 1993).

In the published crystal structure amino acid 120 contacts a guanine, but the consensus also allows an adenine at this position. We have measured dissociation constants for different forms of the consensus and find an 11-fold higher affinity of wild type protein for palindromic sequences with guanine rather than adenine at this position, again supporting the structural data (Table I, '2×GAA'

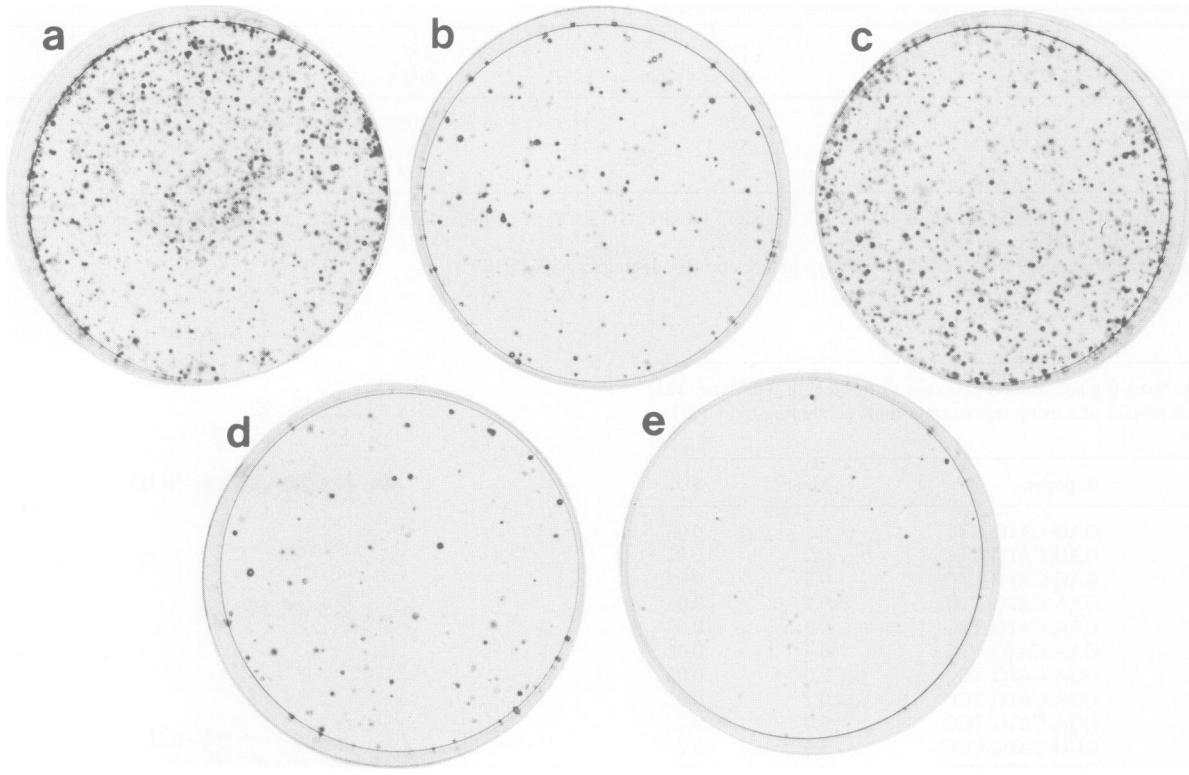

Fig. 8. Growth suppression assays. Saos-2 cells were transfected as in Figure 7 and G418 resistant colonies were selected. Plasmid (number of colonies ± SD): (**a**) vector alone (100); (**b**) wild type p53 (12±3); (**c**) 249S p53 (57±18); (**d**) 121F p53 (4±4); (**e**) 123A p53 (6±6).

versus '2×GGA' and '2×GGG'). Many genomic targets for p53 do not contain guanine at the second position in the consensus, including the proximal binding site in the p21 promoter (el Deiry *et al.*, 1993). This has the sequence c**A**A CATG T**T**g GGA CATG T**T**C, which departs from the consensus at two positions (in lower case) and has A at the second position in the basic pentamer (equivalent to T on the opposite strand) in three out of four positions. As expected, the measured affinity of wild type p53 for this site is 20-fold lower than it is for the palindromic 'GGG' site. Some tumorigenic p53 mutants activate transcription in transient transfections (Chen *et al.*, 1993; Chumakov *et al.*, 1993; Zhang *et al.*, 1993), but this activity is generally only seen at 37°C with high affinity sites. This suggests that the activity counter-selected in tumours may be transactivation of promoters containing low affinity sites (Pietenpol *et al.*, 1994).

Neither the 121F nor the 123A mutation has been found in tumours. We have carried out a preliminary characterization of these mutants in mammalian cells, from which we conclude that both retain wild type activity. The similarity of the 'GAA' probe to the p21 promoter site led us to examine p21 induction by the mutants. The 121F mutant is indeed less effective than wild type p53 at inducing p21 transcription, although it is still able to suppress colony formation. Taken at face value, this implies that p21 is not involved in suppression of colony formation (which might require induction of apoptotic genes, for example), or that transcription generally is not the critical function mediating growth suppression. In our view it would be premature to abandon the model that p53 executes its tumour suppressor functions by activating transcription, because of the excellent correlation which exists between the presence of tumorigenic mutations and loss of sequence-specific transactivation (Pietenpol *et al.*, 1994). Affinity and protein concentration together influence promoter occupancy, and we suspect that the high level of protein expressed from the CMV promoter may have masked the differences in affinity in our transient transfections in mammalian cells. Using more physiological levels of protein expressed from stable expression vectors it may be possible to activate selected target genes and thus dissect p53's downstream pathways by using carefully chosen mutants, a model we are currently testing. It is also possible that some mutants found in tumours may have rather selective defects, giving rise to a failure to induce apoptosis or failure to induce G_1 arrest.

The genetic approach described here asked for a rather general change in the DNA binding properties of p53. In future it will be possible to ask more specific questions by directing mutagenesis to the crystallographically defined DNA contact regions. Combined genetic, biochemical and physical approaches will greatly facilitate the selection of p53 mutants with subtly altered DNA binding properties. This should lead to a rapid increase in our understanding of p53's activity and accelerate the development of strategies to restore p53 function to tumours, whether by

reactivation of endogenous mutant protein or by p53 gene therapy.

Materials and methods

Strains and media

The protease deficient strain GA71 (Scharer and Iggo, 1992) was used throughout. Routine manipulation of yeast was carried out as described by Guthrie and Fink (1991).

Plasmids

*Sma*I–*Xho*I fragments of Bluescript plasmids (el Deiry *et al.*, 1992) were cloned into pLGΔ312 (Guarente and Ptashne, 1981) to give plasmids with the following p53 binding sites: pSS11 AGGCATGTCT AGGCATGTCT, pSS14 AGGCATGTCT and pSS15 AGGAATTTCT AGGAATTTCT. To give *lacZ* under the control of the DED1 minimal promoter, a *Xho*I–*Bgl*II fragment was transferred from pGM8 (Ciaramella *et al.*, 1988) into the *Xho*I–*Bam*HI sites of pLGΔ178 (Guarente and Ptashne, 1981), giving pLS168, and into pSS11-15. Additional reporters were made by cloning p53 consensus oligonucleotides (RI.39, 42, 44, 45, 105, 106, 107 and 116) into the *Xho*I site of pLS168.

Wild type human p53 was expressed from the GAL1 promoter using pLS89 (Scharer and Iggo, 1992). The CΔ368 mutant (pLS185) was obtained by *Exo*III deletion (Erase-a-base, Promega). The C-terminal nucleotide sequence is CAC TCC AGC CCT TAA, which encodes 365-HSSP-stop (the proline is a cloning artefact).

p53 was mutagenized between codons 67 and 346 using gap repair as described by Scharer and Iggo (1992). Colonies containing pSS14 and the repaired plasmid were grown on 3MM filters which were dipped in liquid nitrogen and placed in Z buffer containing X-gal to screen for suppressors (Miller, 1972). *Sgr*AI–*Nco*I fragments encoding only the suppressor mutations, TCT to TTT (121S to F) and ACT to GCT (123T to A), were cloned back into wild type vectors (pLS89 and 185) giving constructs pLS157 and 155 (121F); and pLS158 and 156 (123A), respectively.

p53 was purified from strains containing pLGΔ178-derived plasmids with a hybrid GAL1/CYC1 promoter driving p53 expression. The promoter contains two copies of the *Ear*I–*Rsa*I fragment of the GAL1 UAS cloned blunt into the *Xho*I site of pLGΔ178 (called pLS222). A *Bam*HI–*Sac*I fragment from a human wild type pGAL1 vector (Scharer and Iggo, 1992) carrying the p53 open reading frame and the CYC1 terminator was cloned into the *Ase*I–*Sac*I sites in pLS222 to give pLS228, in which translation starts at p53's own initiator methionine. To make CΔ368 truncated protein, *Sgr*A1–*Eag*I fragments from pLS185, 155 and 156 were cloned into the same sites in pLS228 to give pLS232, pLS230 and pLS231, respectively.

CMV promoter vectors were used for mammalian transfections: pC53-SN3 (Hinds *et al.*, 1990) was supplied by A.Levine; pC53-SN3 containing the 249S mutation (Frebourg *et al.*, 1992) was supplied by T.Frebourg; the parental vector was obtained by self-ligating pC53-SN3 cut with *Bam*HI; and the 121F and 123A mutations were cloned into pC53-SN3 on *Sgr*AI–*Stu*I fragments. The p21 cDNA used for RNase protection was amplified with Pfu (Stratagene) from normal tonsil cDNA by PCR with RI.91/92 and cloned blunt into the *Sal*I–*Bam*HI sites in Bluescript II (Stratagene), giving pLS291. The plasmid (pLS296) containing an *Alu*I–*Taq*I fragment of human SOD (Sherman *et al.*, 1983) in the *Sma*I–*Acc*I sites of pSP65 (Promega) was supplied by P.Amstad.

β-galactosidase assays

Strains were grown for 8 h in 2% raffinose and 12 h in 2% galactose to induce p53 expression. Protein extracts were prepared in buffer A (20 mM Tris pH 7.2, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% NP40, 2 mM DTT, 1 mM PMSF) as described by Scharer and Iggo (1992) and assayed by incubation of 10 μg (Figure 2) or 20 μg (Table I) of S100 with ONPG for 5 min at 28°C in Z buffer (Miller, 1972). β-galactosidase activity is expressed as $1000 \times A_{420}$ per μg per min. Each value is the mean of three assays ± standard deviation.

Antibodies

PAb240 (Gannon *et al.*, 1990), PAb1620 (Ball *et al.*, 1984; Hebel *et al.*, 1986; Milner *et al.*, 1987), PAb1801 (Banks *et al.*, 1986) and PAb421 (Harlow *et al.*, 1981) are antibodies against p53. PAb204 (Lane and Hoeffler, 1980) is an antibody against SV40 T antigen. For bandshift assays, antibodies were purified from hybridoma supernatant by protein A affinity chromatography as described by Harlow and Lane (1988).

Bandshift assays with yeast extract

Protein extracts at 5 mg/ml were prepared in buffer A as described above, frozen in liquid nitrogen and stored at −70°C. Probes were made as follows: RI.50/51 was labelled with ^{32}P using T4 polynucleotide kinase; RI.39-46 were labelled by incorporation of [α-^{32}P]dATP during second strand synthesis primed with a hexamer complementary to the first six residues; and RI.58 was self-annealed and extended with Klenow in the presence of [α-^{32}P]dATP.

20 μg of protein were mixed with 2.5 μg of sonicated, boiled human placental DNA, 250 ng dIdC and 100 ng monoclonal antibody or competitor as specified and incubated for 10 min at room temperature. 1–10 ng of probe were then added and the incubation was continued for 15 min. Samples were run on 4.5% polyacrylamide gels at 6 V/cm in 0.5× TBE buffer at 25°C.

Purification of wild type and mutant CΔ368 protein

Oligonucleotides were synthesised on an ABI 392 DNA synthesizer. Amino-linked RI.64 was conjugated with biotinamidocaproate *N*-hydroxysuccinimide ester, gel purified, annealed to RI.89, ligated to an equal amount of annealed RI.87/RI.88, and captured on streptavidin beads (Magnesphere, Promega).

GA71 containing pLS230, 231 or 232 was grown for 8 h in 2% raffinose and 16 h in 2% galactose to induce p53 expression. Cells were lysed by vortexing with glass beads in buffer A, and S100 extracts were prepared as described by Scharer and Iggo (1992).

All subsequent procedures were carried out at 4°C unless otherwise stated. p53 activity was followed through the purification by bandshift assay. 40 ml S100 at 5 mg/ml were loaded at 30 ml/h on a 30 ml heparin–Sepharose column (Pharmacia). The column was washed with 150 ml of buffer A and eluted with a linear 0.1–1 M NaCl gradient in 300 ml buffer A. Peak fractions were pooled, adjusted to 100 mM NaCl, and loaded at 30 ml/h on a MonoQ HR 5/5 column (Pharmacia). The column was washed with 5 ml buffer A and eluted with a linear 0.1–1 M NaCl gradient in 10 ml buffer A. Peak fractions were exchanged into buffer B (50 μM HEPES pH 7.2, 100 mM KCl, 1 mM EDTA, 10% glycerol, 50 mM $ZnCl_2$, 5 mM DTT, 1 mM PMSF) using a Sephadex G-25 column (Pharmacia), adjusted to 50% glycerol, frozen in liquid nitrogen and stored at −70°C. Immediately before use, 100 μl aliquots were diluted to 10% glycerol in buffer C (buffer B minus EDTA), applied to the DNA affinity column pre-equilibrated with buffer C and incubated with gentle shaking for 20 min. The column was washed with 1 ml 300 mM KCl, p53 was eluted with 40 μl 500 mM KCl, and the column was regenerated with 1 M KCl, all in buffer C. The p53 concentration relative to BSA standards was determined by densitometry of Coomassie blue stained SDS–PAGE gels with an Elscript 400 scanner (Hirschmann). The oligomeric state of the protein was determined by gel filtration on a Superose 6 column (Pharmacia).

A 2 l yeast culture at an OD of 1.0 gave ~200 mg of S100, 20 mg of heparin–Sepharose fraction and 2.5 mg of MonoQ fraction. 25 μg of this material applied to a 60 μg DNA column yielded 5 μg pure p53.

Quantitative analysis of DNA binding

Probes with a specific activity of 10^9 c.p.m./μg (Cerenkov) were made by PCR amplification of pLS168-derived plasmids (above) with primers RI.94 and 11200 in the presence of [α-^{32}P]dATP (6000 Ci/mmol, Amersham). 20 μl binding reactions containing 10^{-13} M probe, 1 mg/ml acetylated BSA (New England Biolabs) and specified concentrations of p53 were incubated in buffer C at 25°C for 30 min and run on 5% polyacrylamide minigels cooled to 10°C for 30 min at 20 V/cm. The gels were dried, exposed to phosphor screens and binding was quantitated with a Molecular Dynamics PhosphorImager.

p53 expression in mammalian cells

Saos-2 cells (ATCC HTB 85) were transfected with 20 μg control vector, wild type p53 and 121F, 123A and 249S mutant p53 by lipofection (GibcoBRL) for 10 h. 24 h later total RNA was extracted with guanidinium and purified through a CsCl step gradient (Sambrook *et al.*, 1989). RNase protection was performed as described by Promega (Methods and Applications Guide, 1991) using probes transcribed with T3 and SP6 polymerase from pLS291 cut with *Rsa*I and pLS296 cut with *Ear*I, respectively. Protected bands were quantitated by phosphorimaging. For growth suppression assays, cells were transfected as above, split one in seven on day 3, 1.2 mg/ml G418 (GibcoBRL) were added on day 4, and colonies were stained with methylene blue on day 24.

The RNase protection and growth suppression assays were repeated three times. Western blots were probed with PAb240 and rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase (Dako), and developed with ECL (Amersham).

Oligonucleotides

The relevant part of the consensus is shown in bold type: **RRR** CWWG **YYY**.

RI.39 AGTGT **GGA** CATG **TCC** ACACT
RI.40 AGTGT **AGG** CATG **CCT** ACACT
RI.41 AGTGT **AAG** CATG **CTT** ACACT
RI.42 AGTGT **GAG** CATG **CTC** ACACT
RI.43 AGTGT **AAA** CATG **TTT** ACACT
RI.44 AGTGT **GAA** CATG **TTC** ACACT
RI.45 AGTGT **GGG** CATG **CCC** ACACT
RI.46 AGTGT **AGA** CATG **TCT** ACACT
RI.50 TCGAT **AGG** CATG **TCT** ATCAAACTTA
RI.51 TCGATAAGTTTGAT **AGA** CATG **CCT** A
RI.58 GAGT **GGG** CATG **CC**
RI.64 AG **TCT AGA** CATG **CCT AGA** CATG **CCT AGA** CATG **CCT**
RI.87 **GGG** CTTG **CCC GGG** CTTG **CCT**
RI.88 AG **CCC GGG** CAAG **CCC AGG** CA
RI.89 TG **TCT AGG** CATG **TCT AGG** CATG **TCT AGA** CT
RI.91 CGGGCTTCCTCTTGGAGAAGA
RI.92 CCATGTCAGAACCGGCTGGGGAT
RI.94 AACTTTTTCCCGTTCCTCCATCTC
RI.105 TCGAA **GAA** CATG **TTC GAA** CATG **TTC** T
RI.106 TCGAA **GGA** CATG **TCC GGA** CATG **TCC** T
RI.107 TCGAA **GGG** CATG **CCC GGG** CATG **CCC** T
RI.116 A **CAA** CATG **TTG GGA** CATG **TTC** TTCGAGGGGTAA-TAACTGA
11200 GGTAGAGGGTGAACGTTACAG

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