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p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer

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Gal4-p53 fusion constructs demonstrate that wild type p53 is a potent transactivator in human lung cancer cells with the transactivation domain for p53 residing in amino acids 1-42. Strikingly, a variety of lung cancer derived p53 mutations occurring outside this domain disrupt this activity. Temperature sensitive conformational shifts of p53 mutant proteins to the wild type form exist and, with a temperature downshift, several mutants become transcriptionally active. Wild type p53 protein is known to form oligomers with mutant p53 and cotransfection of wild type and mutant genes shows that p53 acts in a transdominant manner that is independent of the DNA binding specificity. Transcription is either increased or decreased depending on whether the wild type is more or less abundant than the mutant form. Finally, lung cancers differ in their ability to support the transactivation related functions, providing evidence of other abnormalities of the p53 system in human cancer.

Key words: activation domain/lung cancer/p53/temperature sensitivity/transactivation activity

Introduction

The mechanism(s) by which wild type p53 regulates cell growth while mutant p53 proteins have lost growth control and in some cases play an active role in tumorigenicity are not clear. p53 fused to the DNA binding domain of Gal4 is able to activate transcription (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990) and binds to sequences adjacent to the SV40 origin of replication (Bargonetti *et al.*, 1991). Therefore, it is possible that p53 regulates cell growth through these mechanisms and it would be of great interest if mutations in p53 alter these functions. While p53 specific DNA binding sequences have been reported (Bargonetti *et al.*, 1991; Kern *et al.*, 1991) and the muscle-specific creatine kinase gene enhancer contains a p53 responsive element (Weintraub *et al.*, 1991), it is not known if p53 mediates its biological functions through these target sequences. However, the finding that wild type p53 fused to the Gal4 DNA binding domain exhibits transcriptional transactivation activity (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990) provides a method to test whether the p53 mutations found in human tumors will disturb transactivating activity.

p53 mutations with corresponding loss of the wild type allele are common in human cancers, especially in human lung cancers (Takahashi *et al.*, 1989; Chiba *et al.*, 1990; Hollstein *et al.*, 1991; Levine *et al.*, 1991; D'Amico *et al.*, 1992; Mitsudomi *et al.*, 1992). Does loss of the transactivating capacity of p53 occur in the context of these human tumor mutants? From the initial reports, conducted in Chinese hamster ovary, yeast or HeLa cells, one naturally occurring mouse p53 mutant ($p53_{val135}$) appeared to lose activity while a human mutant ($p53_{his273}$) retained transactivating capacity (Fields and Jang, 1990; Raycroft *et al.*, 1990). Thus, it is important to examine wild type human p53 function in human cancer cells and to assess the corresponding activity of p53 mutations derived from human cancer in these same cells. To this end, we used several lung cancer cell lines which already had defined p53 mutations as the recipients for wild type p53 and specific, mutated p53 genes which had been isolated from lung cancer cells. Since the p53 mutations found in human tumors, such as lung cancers, are spread over the coding region resulting in various amino acid changes, we also wished to test which of these mutants might change the transactivating capacity of p53. As with mutations in the retinoblastoma gene (Kaelin *et al.*, 1991), it was possible that these mutations would localize important functional domains of p53. However, there were several other known facets of p53 biological activity that needed to be related to transcriptional activation. Since mutant p53 can cooperate with a mutated *ras* gene to transform cells, it has been postulated that mutated p53 molecules interfere with wild type p53 function in a dominant negative manner (Levine *et al.*, 1991). Thus, it was important to learn if mutant p53 proteins would disrupt wild type p53 transcriptional activity. Based on the findings that a mouse p53 mutant ($p53_{val135}$) was temperature sensitive for its ability to suppress oncogene transformation (Michalovitz *et al.*, 1990) and that some, including several lung cancer mutants, showed conformational temperature sensitivity (Milner and Medcalf, 1991; Medcalf *et al.*, 1992), we also tested whether any of these p53 mutants exhibited transactivating activity which was temperature sensitive.

Results

Human lung cancer cells support wild type p53 transactivation while p53 mutants are significantly diminished in this activity

We designed chimeric Gal4-p53 constructs that contained the DNA binding domain of Gal4 fused to the entire coding sequence of the human p53 gene containing either the wild type sequence (Gal4-$p53_{wt}$) or different mutations under the control of simian virus 40 (SV40) regulatory sequences (Figure 1). The six mutants had all been isolated from human lung cancer cells and included missense amino acid substitutions and deletions: $p53_{154}$ (Gly to Val); $p53_{179}$ (His to Glu); $p53_{247}$ (Asn to Ile); $p53_{273}$ (Arg to Leu);

$p53_{del\ ex4}$, from which the entire exon 4 (amino acids 33–125) is deleted; and $p53_{del\ 262-269}$, with eight amino acids deleted in exon 8 from codons 262–269 (Materials and methods and Figure 1). All Gal4 fusion constructs were sequenced and shown to be in the correct reading frame.

The fusion constructs were introduced into three different human non-small cell lung cancer (NSCLC) cell lines, H23, H1299 and H358. The H23 cell line has lost the normal p53 allele and has an endogenous missense mutation in the p53 gene at codon 246 (Met to Ile) (Takahashi *et al.*, 1989) which is associated with the production of large amounts of mutant protein (Bodner *et al.*, 1992). The other two cell lines, H1299 and H358, produce no endogenous wild type or mutant p53 protein (Bodner *et al.*, 1992). The H1299 cell line has a homozygous 5′ intragenic deletion of the p53 gene, while the H358 cell line has a large homozygous deletion of the entire gene (Takahashi *et al.*, 1989; Mitsudomi *et al.*, 1992).

We first tested for expression of the wild type and mutant proteins and their ability to localize in the nucleus. When H1299 cells containing a homozygous intragenic deletion for the p53 gene were transiently transfected with either wild type or mutant p53 constructs containing the Gal4 DNA binding domain (Gal4–$p53_{wt}$, Gal4–$p53_{154}$ and Gal4–$p53_{247}$) and then immunostained with an anti-p53 monoclonal antibody (pAb 421), an equivalent frequency (~1% of the transfected cells) and the same amount of nuclear staining of the p53 protein were seen with all the constructs (Figure 2A) while control transfections of H1299 with Gal4 were negative (data not shown).

The p53–Gal4 fusion expression plasmids with the G5E1bCAT reporter plasmid, which contains five copies of the Gal4 DNA binding sites upstream from the adenovirus E1b promoter driving the CAT gene, were then transfected into all three lung cancer cell lines and the cells assayed for CAT activity (Figure 3). A control expression plasmid (pRSV/L) for the luciferase gene was included to monitor and standardize for transfection efficiency (see Materials and methods). Human wild type p53 exhibited potent transcriptional transactivation activity (~90-fold over background) which was even stronger than that of the native Gal4 transcription factor in these cells (60- to 70-fold over background). The three missense mutants at codons 154, 179 and 247, the exon 4 deletion and the exon 8 deletion of eight amino acids, all showed >90% loss of activity. Of interest, the lung cancer cells differed in the magnitude of transactivation activity. In fact, the exon 4 deletion mutant exhibited no activity. For several of the p53 mutants, transfection into H23 and H1299 revealed some activity. H358 cells (containing the large homozygous p53 deletion) showed significantly less Gal4 and Gal4–$p53_{wt}$ transactivation than the other two lung cancer lines and lack transactivating activity for the p53 mutants tested. In striking contrast to the other mutants, the Gal4–$p53_{273}$ construct, one of the p53 mutational 'hot spots', was even more active (~100-fold over background) than wild type in all of the lung cancer cell lines. This was particularly marked in H358 cells (Table I). All of these results, including the high activity of the wild type and the codon 273 p53 mutant and loss of activity of the other p53 mutants, were also found in transfection studies with monkey COS7 cells (data not shown).

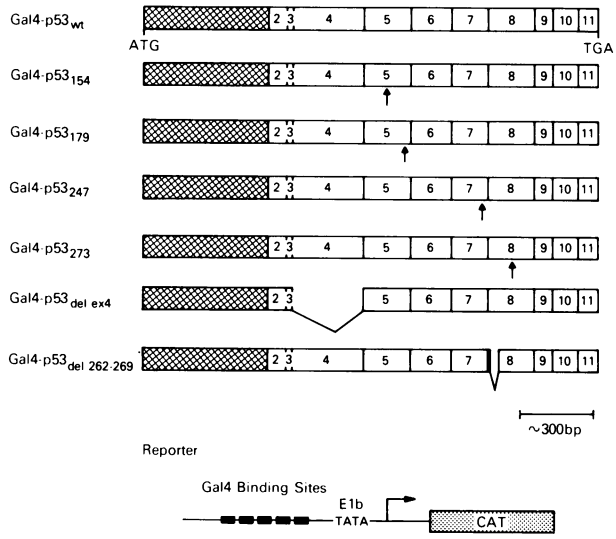

Fig. 1. Schematic representation of chimeric Gal4–p53 constructs. The Gal4 DNA binding domain (amino acids 1–147), denoted by hatched boxes, is fused to the entire coding region of either wild type or mutant p53 sequences, denoted by open boxes containing exons 2–11 with position of ATG and TGA indicated. Arrows show the approximate location of the mutations and gaps connected by lines show deletions. The codon containing the mutation or the region deleted (del) are included in the name of each chimeric construct. The reporter plasmid contains five Gal4 DNA binding sites (black boxes), the E1b TATA sequence and the CAT gene (stippled box).

Localization of the p53 domain participating in transactivation to amino acids 1–42

One of the initial reports of Gal4–p53 transactivation showed activity in a construct involving the first 73 amino acids of p53 (Fields and Jang, 1990) while another showed that the first 160 amino acids were active (O'Rourke *et al.*, 1990). The exon 4 deletion mutant which leaves intact amino acids 1–32 of the p53 protein resulted in loss of all transactivation activity in all host cells tested while the other mutants retained some activity (Figure 3). Therefore, we created two additional Gal4 fusion constructs in an attempt to further localize the activating domain. The Gal4–p53 (amino acids 1–13) construct with the sequence coding for the first 13 amino acids contains part of exon 2 while the second construct, Gal4–p53 (amino acids 1–42), contains p53 sequence including exons 2, 3 and part of exon 4 coding for the first 42 amino acids (Figure 4). These constructs were cotransfected with the G5E1bCAT reporter plasmid into the H358 cell line and compared to wild type p53 and the codon 273 p53 mutant activities (Figure 4). While the Gal4–p53 (amino acids 1–13) construct failed to transactivate, the Gal4–p53 (amino acids 1–42) construct had activity comparable to that of Gal4–$p53_{wt}$. The identical pattern of activity for these constructs was seen in the other two lung cancer lines and in COS7 cells (data not shown). Thus, the domain including the first 42 amino acids of the p53 protein, a region rich in acidic amino acids and proline residues, is sufficient for transactivation, while the Gal4–$p53_{del\ ex4}$ construct containing amino acids 1–32 lacks this activity. The other mutants, which have lost considerable activity, all contain amino acids 1–42 but have other downstream lesions. They may have some other disturbance in p53 function, possibly related to conformational changes reported for such mutants (Medcalf *et al.*, 1992).

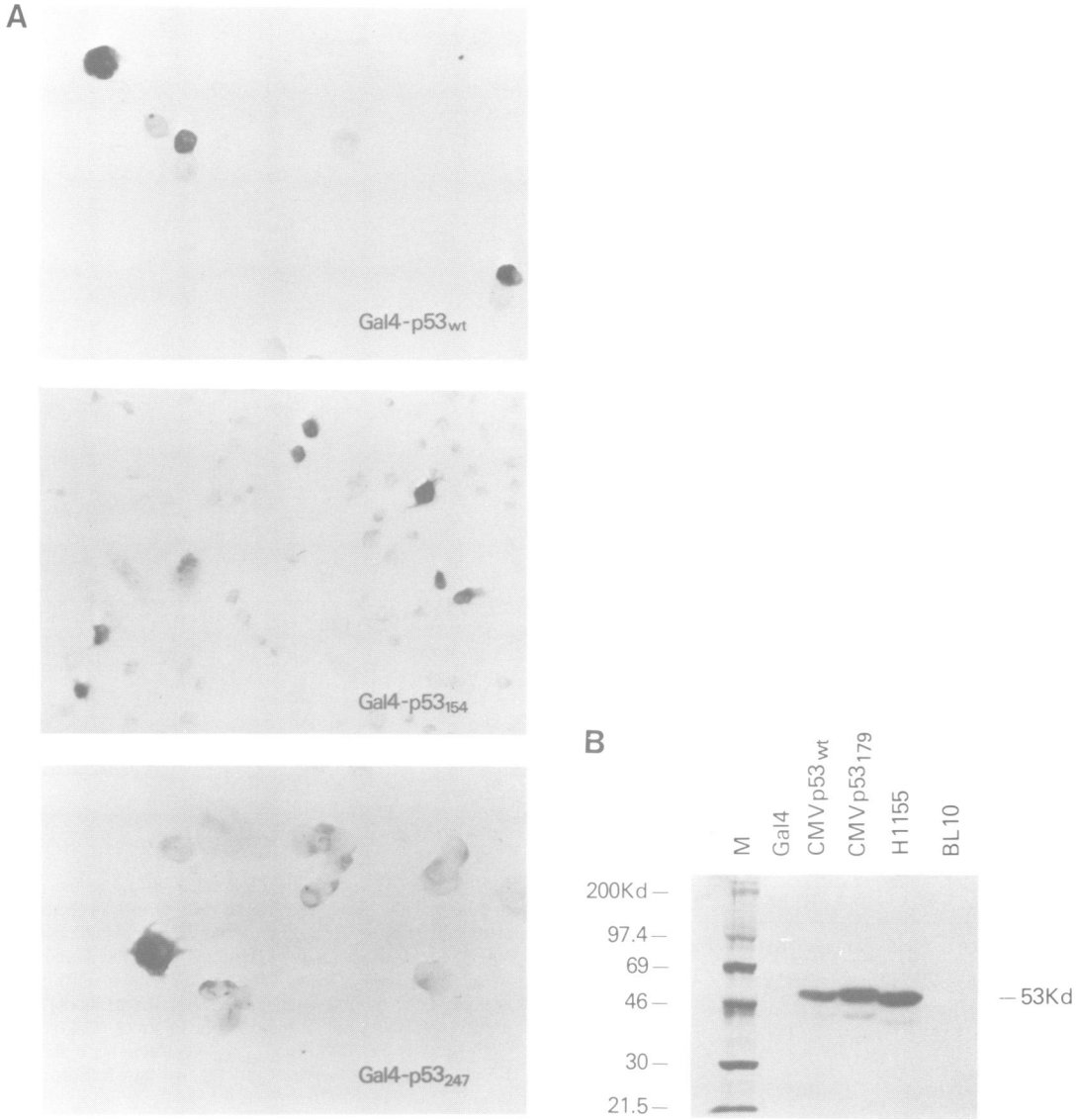

Fig. 2. Expression of wild type and mutant p53 transfected into H1299 lung cancer cells. **A.** Immunostaining. H1299 lung cells were transfected with 10 μg DNA of Gal4$-$p53$_{wt}$ (top panel), Gal4$-$p53$_{154}$ (middle panel) and Gal4$-$p53$_{247}$ (bottom panel) and immunostained with p53 antibody PAb 421 as described in Materials and methods. Top and bottom panels are at 40× and middle is at 20× magnification. **B.** Immunoblot. H1299 lung cells were transfected with 10 μg DNA of either Gal4, CMV$-$p53$_{wt}$ or CMV$-$p53$_{179}$ constructs and the lysates immunoblotted with p53 antibody, PAb 1801 (see Materials and methods). Control lysates were: NSCLC cell line, H1155, which produces abundant p53 protein (Bodner *et al.*, 1992); and a B lymphoblastoid cell line, BL10, which produces extremely low levels of p53 protein. M indicates protein marker with sizes in kDa on the left.

Many p53 mutants are temperature sensitive for transactivation

A mouse p53 mutant had been found to be temperature sensitive with respect to its ability to inhibit transformation by the combination of c-*myc* and a mutated *ras* gene (Michalovitz *et al.*, 1990). Therefore, we tested the different mutant constructs to see whether they were temperature sensitive for their transactivation activity. For this purpose, the activity was studied in the three lung cancer recipient cell lines at 30°C and 37°C. The wild type activity was either the same or decreased at 30°C compared to 37°C. The Gal4$-$p53$_{273}$ and Gal4$-$p53$_{del\ 262-269}$ mutants also showed a slight reduction in their activity at 30°C (for example 99$-$83% and 4$-$3%, respectively, in H1299), while the Gal4$-$p53$_{del\ ex4}$ remained inactive at both temperatures (data not shown). In contrast to Gal4$-$p53$_{wt}$ (Figure 5), Gal4$-$p53$_{154}$, Gal4$-$p53$_{179}$ and Gal4$-$p53$_{247}$ mutants were all temperature sensitive and significantly increased their transactivation capacity by 5- to 10-fold at 30°C, approaching 40$-$80% of wild type activity in the H23 and H1299 cell lines. Although H1299 and H358 cell lines both produce no endogenous p53 protein, they differ in their ability to support temperature sensitive transactivation. For example, in H358 cells, the construct containing the mutation at codon 179 (His to Glu) was nearly inactive at the two temperatures. In contrast, the mutation at codon 247 (Asn to Ile) in human p53 showed temperature sensitivity in all the lung cancer cells.

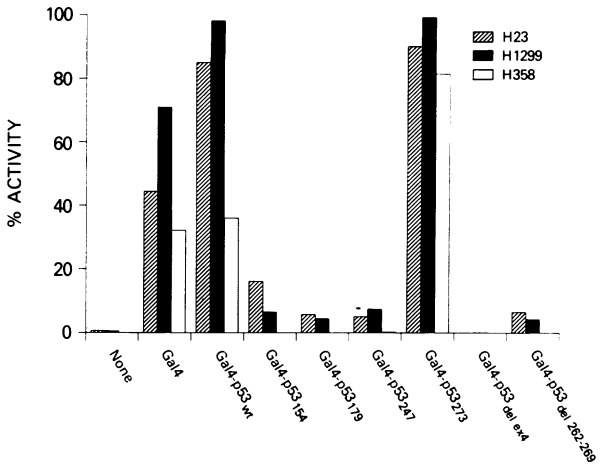

Fig. 3. Transcriptional activities of Gal4−p53 chimeric constructs in lung cells. NSCLC lung cancer cell lines, H23, H1299 and H358, were transfected simultaneously with DNAs including 5 μg reporter plasmid, 10 μg wild type or mutant p53 fusion constructs (indicated on horizontal axis) and 3 μg luciferase control plasmid (pRSV/L). Cell lysates containing equivalent amounts of luciferase activity were used for each CAT assay (see Materials and methods). Percent conversion (vertical axis) is indicated for each cell line: H23, box with diagonal lines; H1299, black box; and H358, open box. Results are representative of at least two independent transfections.

Table I. Mutant p53−Gal4 fusion proteins inhibit transactivation by the wild type p53−Gal4 fusion protein

	Host lung cancer cell lines		
	H23	H1299	H358
Construct(s) transfected[a]	% of wild type Gal4−p53 transactivation[b]		
Gal4−$p53_{wt}$	100 (97)	100 (95)	100 (28)
pBR327	0	0	0.6
vector (pSG424)	0	1	0.5
Gal4	66	51	20
Gal4−$p53_{273}$	95	103	136
Gal4−$p53_{154}$	15	5	0.4
Gal4−$p53_{del\ 262-269}$	2	2	0.6
Gal4−$p53_{179}$	2	2	0.4
Gal4−$p53_{del\ ex4}$	0.2	0	0.6
Gal4−$p53_{247}$	3	2	1
Gal4−$p53_{wt}$ +			
Gal4−$p53_{273}$	95	100	123
Gal4−$p53_{154}$	84	95	47
Gal4−$p53_{del\ 262-269}$	69	86	23
Gal4−$p53_{179}$	56	82	11
Gal4−$p53_{del\ ex4}$	40	39	2
Gal4−$p53_{247}$	37	73	8

[a]5 μg DNA of each construct was transfected. For the single constructs an additional 5 μg of pBR327 was added. All transfections included 5 μg reporter plasmid DNA and 3 μg luciferase plasmid DNA so that the total DNA transfected was 18 μg.
[b]The values in parentheses represent the actual % conversion of input [^{14}C]chloramphenicol. In all cases the amount of lysate assayed for CAT activity contained equivalent luciferase activity.

Cotransfecton of mutant and wild type p53 results in a reduction of transcriptional activity

Wild type p53 has been shown to form a complex with mutant p53 proteins, including mutants isolated from lung cancer, and this complex takes on a 'mutant' protein conformation (Eliyahu *et al.*, 1988; Rovinski and Benchimol, 1988; Finlay *et al.*, 1989; Milner and Medcalf, 1991; Medcalf *et al.*, 1992). However, whether or not mutant p53 proteins could act as dominant negative factors to disrupt normal p53 function is an important unanswered question. Thus, the effect of cotransfection of mutant and wild type p53 constructs on transcriptional activity was examined. Under similar experimental conditions, transfections of the wild type fusion construct were carried out alone or in combination with mutant p53 fusion constructs at a molar ratio of 1:1. As with previous experiments, Gal4−$p53_{wt}$ was more active than Gal4 itself, and the mutants by themselves (with the exception of the Gal4−$p53_{273}$ mutant) had greatly reduced activity in all three lung cancer lines (Table I). The combination of mutant with wild type fusion proteins (with the exception of the 273 mutant) resulted in a decrease of transactivation activity which varied depending on the mutant (Table I). As with temperature changes, the host cell lines also differed in their response to cotransfection with the mutant induced inhibition being most sensitive in H358 cells (Table I). The ability to inhibit transactivation was dependent on the relative dose of the wild type and mutant constructs. In two separate experiments when H1299 cells were cotransfected with the wild type−mutant construct (Gal4−$p53_{179}$) at molar ratios of 1:4, the transactivation activity decreased further to 25% of the wild type, while

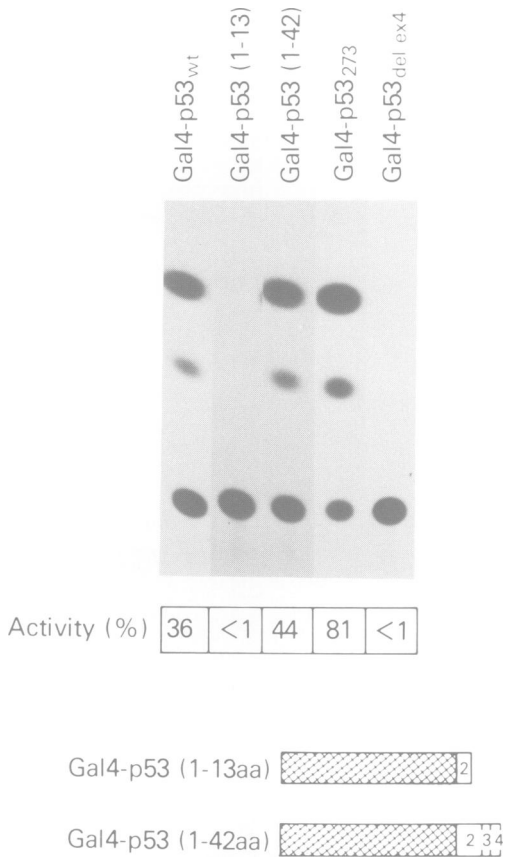

Fig. 4. The p53 transactivation domain resides in the region contained in amino acids 1−42. H358 lung cells were transfected with two artificially truncated, fused constructs, one containing p53 sequences coding for amino acids 1−13 and the other for amino acids 1−42, fused to the Gal4 DNA binding domain shown schematically at the bottom of the figure (see also Materials and methods). Amounts of transfected DNAs are as indicated in Figure 3. Gal4−$p53_{wt}$, Gal4−$p53_{273}$ and Gal4−$p53_{del\ ex4}$ constructs were used as controls. Results are representative of at least two transfections.

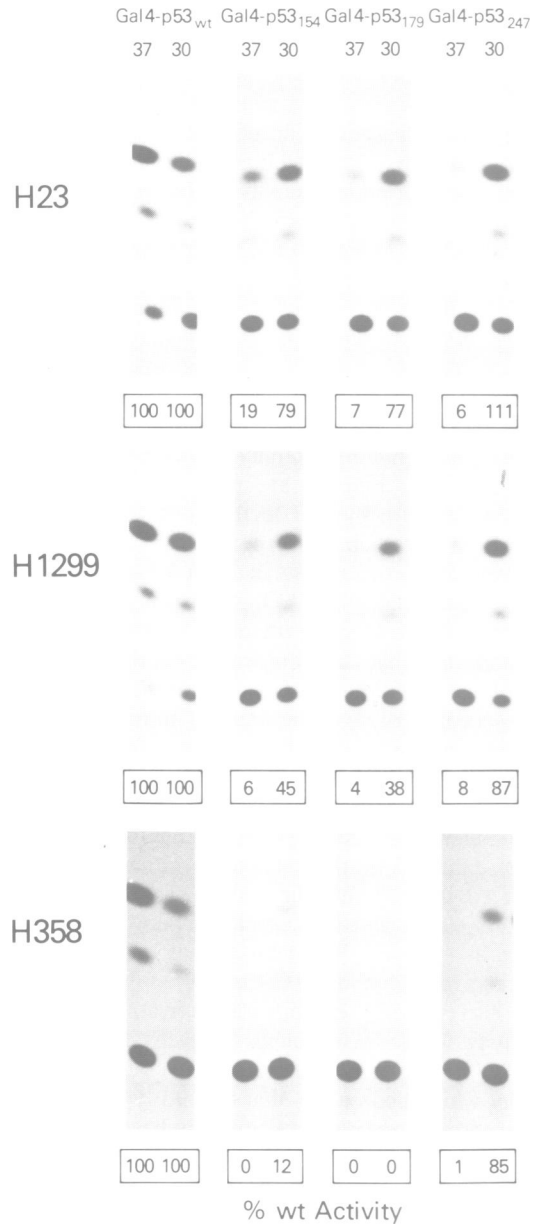

Fig. 5. Temperature sensitivity for transactivation of p53 mutants depends upon the specific mutation and the recipient lung cancer cells. Duplicate dishes of H23, H1299 and H358 lung cells were transfected at 37°C with wild type or mutant p53 fusion constructs (conditions identical to those in Figure 3) as indicated at the top of each lane. After addition of serum one set of transfected cells was incubated at 37°C and the other at 30°C for 48 h at which point CAT activity was assayed. Relative CAT activity at the two temperatures is indicated below each lane for each cell line. For each mutant, the activity is expressed relative to the wild type activity at the same temperature with the wild type p53 activity set as 100% for each temperature. Results are representative of at least three independent transfections.

it rose to 85% of the wild type activity when the wild type:mutant ratio was 4:1 (data not shown).

Mutant p53 proteins without a Gal4 DNA binding domain inhibit transactivation by the wild type p53–Gal4 fusion protein

The inhibition of transactivation when the two chimeric Gal4 fusion genes were cotransfected could be explained by competition at the level of binding to the Gal4 DNA binding sequences. Alternatively, the mutant p53 proteins could be disrupting some other facet of the function of p53. Other p53 expression vectors driven by the human cytomegalovirus (CMV) promoter/enhancer without a Gal4 DNA binding domain were tested for their expression in H1299 cells (Figure 2B). In the CMV vectors, wild type and mutant proteins were expressed at high levels, comparable with the levels of mutant p53 protein found in NSCLC cell line, H1155 (an Arg to His mutation at codon 273) (Bodner *et al.*, 1992) (Figure 2B). These are much higher levels of protein than those seen with the SV40 driven Gal4 constructs where immunoblotting could not detect transfected p53 gene expression. We then cotransfected the chimeric wild type p53–Gal4 fusion gene (Gal4–$p53_{wt}$) along with the 179 mutant without the Gal4 DNA binding domain in a CMV expression vector (CMV–$p53_{179}$) into H1299 cells. This mutant, despite the lack of the Gal4 DNA binding domain, was able to significantly decrease the transactivating activity seen with Gal4–$p53_{wt}$ (80% and 45% in two separate experiments, Table II). The same pattern of inhibition was seen when Gal4–$p53_{wt}$ was cotransfected with CMV–$p53_{179}$ into H358 cells (data not shown).

Table II. A mutant p53 protein without a Gal4 DNA binding domain inhibits transactivation by the wild type p53–Gal4 fusion protein in H1299 cells

Construct(s)	μg DNA transfected	% of wild type p53 activity[a]	
		experiment 1	experiment 2
Gal4–$p53_{wt}$	2.5	100 (42)	100 (85)
	5	143	
vector (pSG424)	5	2	1
pBR327	5	0	0
CMV–$p53_{179}$	2.5		0.3
	5	2	
Gal4–$p53_{wt}$ + CMV–$p53_{179}$	5 + 5	79	
	2.5 + 2.5		71
	2.5 + 10	19	56

[a]The values in parentheses represent the actual % conversion of input [^{14}C]chloramphenicol. In all cases the amount of lysate assayed for CAT activity contained equivalent luciferase activity. All transfections included 5 μg reporter plasmid DNA, 3 μg luciferase plasmid DNA in addition to the above stated amount of p53 fusion construct DNAs. In experiment 2, pBR327 DNA was also added to the transfections so that the total amount of DNA in each transfection was the same (20.5 μg).

Table III. Wild type p53 protein without a Gal4 DNA binding domain increases the transactivating capacity of a Gal4–p53 mutant protein in H1299 cells

Construct(s)	μg DNA transfected	% of wild type p53 activity	
		experiment 1	experiment 2
Gal4–$p53_{wt}$	2.5	100 (92)	100 (85)
Gal4–$p53_{179}$	2.5	9	2
CMV–$p53_{wt}$	2.5	0	0.2
CMV–$p53_{wt}$ + Gal4–$p53_{179}$	2.5+2.5	65	26
	10 + 2.5	62	36

In both experiments pBR327 DNA was added to the transfections so that the total DNA transfected was 20.5 μg. See also the legend to Table II.

Wild type p53 without a Gal4 DNA binding domain is able to cooperate with a mutant p53–Gal4 fusion protein to increase transactivation

Since the previous experiments suggested that wild type and mutant p53 proteins were interacting through a domain other than those binding to DNA, we cotransfected a wild type p53 without the Gal4 DNA binding domain ($CMV-p53_{wt}$) with a mutant containing this domain ($Gal4-p53_{179}$) into H1299 cells. In this case, the wild type possesses no ability to transactivate by itself. However, $CMV-p53_{wt}$ appeared able to cooperate with the inactive mutant p53 to increase transactivating activity 7- to 12-fold (Table III). The same pattern of stimulation was seen when $Gal4-p53_{179}$ was cotransfected with $CMV-p53_{wt}$ into H358 cells (data not shown).

Discussion

While transactivation activities of various p53 constructs fused to the Gal4 DNA binding domain were reported initially (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990), these results raised a number of questions. These studies were performed in yeast, Chinese hamster ovary and HeLa cells (the latter bearing human papilloma virus oncoproteins) (Fields and Jang, 1990; Raycroft *et al.*, 1990). In one study, transactivation analyses were performed with mouse p53 constructs that did not include the C-terminal 47 amino acids (Raycroft *et al.*, 1990) while the other study (Fields and Jang, 1990) showed that deleting such C-terminal sequences actually increased activity. In fact, the region containing the first 73 amino acids alone was fully active in transactivation (Fields and Jang, 1990). Some of the mutants were artificially created (Raycroft *et al.*, 1990) and the single human mutant ($p53_{his273}$) studied, actually retained transactivating activity (Fields and Jang, 1990).

If these initial observations were relevant to the functions of human p53, it was important to analyze human cancer derived p53 mutations in human cancer cells. A multitude of different mutant p53 proteins are produced in human cancers; this is particularly true for lung cancer where the mutations are spread widely over the p53 coding region and include missense, nonsense, large and small deletions, and splicing errors resulting from intronic mutations, and occur in evolutionarily conserved as well as non-conserved regions (Takahashi *et al.*, 1989; Chiba *et al.*, 1990; D'Amico *et al.*, 1992; Mitsudomi *et al.*, 1992). We found three of the four missense mutants and the eight amino acid deletion in exon 8 lost 80–90% of the wild type transcriptional activity. In contrast to the other mutants the exon 4 deletion mutant (loss of amino acids 33–125) resulted in complete loss of transactivation activity in all the three recipient lung cancer cell lines, suggesting that a significant part of the activating domain for transactivation resides in these sequences. An artificial construct containing the first 13 amino acids of p53 was inactive, while a 1–42 amino acid construct transactivated efficiently in all the lung cancer cell lines. Thus, the activating domain can be localized to the first 42 amino acids, a region where the high negative charge and abundance of proline residues are conserved through evolution (Soussi *et al.*, 1990a,b). Acidic sequences and proline residues are characteristic features of the activating domains of viral (Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988) and cellular transcription factors (Mitchell and Tjian, 1989). Thus, it is likely that the wild type p53 protein stimulates transcription by protein–protein interaction between its activating domain and other components of the transcription apparatus. The p53 genes used in this study were fused to the Gal4 DNA binding domain, a method used to identify transcriptional activity for Fos (Lech *et al.*, 1988), the herpes simplex virus protein 1, VP16 (Sadowski *et al.*, 1988) and Myc (Kato *et al.*, 1990). However, the genes transcriptionally regulated by p53 are unknown and it remains to be determined whether p53 functions directly by binding to specific DNA sites (Kern *et al.*, 1991) or only indirectly through a protein–protein interaction.

Several of the constructs with missense mutations far downstream of the activation region have also lost 90% of their transactivating activity. Since DNA binding and oligomerization domains are provided by Gal4 and an activating domain by p53 in the construct, an additional explanation for their loss of activity must be invoked. Wild type p53 protein exists in a 'suppressor' conformation (Milner and Medcalf, 1990) while mutant p53 proteins exist in a different conformation ('mutant or promoter') as determined by reaction with two different types of monoclonal antibodies (Gannon *et al.*, 1990; Milner and Medcalf, 1991). The murine mutation $p53_{val135}$ is temperature sensitive for the ability to suppress oncogene transformation (Michalovitz *et al.*, 1990) and temperature sensitive for conformation (Milner and Medcalf, 1991) while other p53 mutations (including those from lung cancer) exhibit conformational temperature sensitivity when translated *in vitro* (Milner and Medcalf, 1990; Medcalf *et al.*, 1992) suggesting a correlation between biological and structural changes. We have shown that three missense mutations [at codons 154 (Gly to Val), 179 (His to Glu) and 247 (Asn to Ile)] exhibit transactivational temperature sensitivity. While these mutants transactivate poorly at 37°C, at 30°C their activity is increased several-fold reaching an activity which approaches that of wild type p53. Thus, we find a correlation in several mutants between temperature induced conformational changes and transactivation. The most straightforward model is that these mutations confer a conformational change on the p53 protein disrupting its interaction with other factors necessary for transcriptional activity. Similarly, with temperature downshift such mutant proteins assume a wild type conformation and we see an increase in transcriptional activity.

We could not detect loss of transactivation, temperature sensitivity or competition with wild type p53 for transactivation with the p53 codon 273 mutation (Arg to Leu). Although this mutation has been shown to have mutant conformation and conformational temperature sensitivity (Medcalf *et al.*, 1992), there is no difference in the transactivation activity of this mutation at either 37° or 30°C. A different mutation at codon 273 (Arg to His) also retained transactivation ability (Fields and Jang, 1990). The most likely explanation for the retention of activity by the $p53_{273}$ mutants is that they have lost a function provided by the Gal4 DNA binding domain.

Since mutant p53 genes can immortalize and cooperate with mutated *ras* oncogenes to transform primary cells in the presence of endogenous wild type p53 protein, there was an implied ability of mutant p53 protein to disrupt the function of wild type protein (Levine *et al.*, 1991).

Also, human mutant p53 proteins, including those from lung cancer, are able to form oligomers with themselves and with wild type p53 proteins and are able to change the conformation of the wild type−mutant complex into the 'mutant' phenotype as recognized by monoclonal antibodies (Milner and Medcalf, 1991; Medcalf *et al.*, 1992). The number of molecules participating in oligomer formation is unknown. Cotransfection of mutant and wild type genes with or without the Gal4 DNA binding domain in our experiments resulted in an inhibition of transactivation as predicted for a dominant negative mutation. This inhibition increased when the level of expression of the mutant protein was greater. This effect could have occurred via wild type and mutant p53 oligomerization or by the competition of mutant p53 for other cellular factors essential for transcription. However, we also found that overexpression of wild type p53 without the Gal4 DNA binding domain in the presence of mutant p53 with the Gal4 domain, resulted in reactivation of transcription by the mutant protein providing functional evidence for the transdominant nature of the two forms of the proteins in transactivation.

Although both H1299 and H358 cell lines produce no endogenous p53 protein, their ability to support p53 transactivation differs. Despite the absence of endogenous p53 expression in H1299 cells and the high level of mutant p53 produced by H23 cells, the H1299 cell line is similar to H23 in that it supports a low level of transactivation with the different mutant constructs. In contrast, cell line H358 lacks transactivating activity for most of the p53 mutant constructs and shows more dramatic inhibition of transactivation in mutant/wild type competition experiments. In addition, H358 only supports the temperature downshift reactivation of the codon 247 mutant but not the other p53 mutants. Likewise, COS cells also differ from H23 and H1299 lung cancer cells in their lack of ability to support temperature sensitive p53 transactivation (data not shown). These observations indicate that one or more factors necessary for p53 transactivation activity are present in H23 and H1299 but are not functional in H358 or COS cells and provide clues to find other components of the p53 transcriptional pathway.

Materials and methods

Plasmids and plasmid constructions

Seven fusion plasmids were constructed by ligating various p53 sequences into the *Eco*RI site of the plasmid, pSG424 (a gift from Dr Mark Ptashne), which contains the Gal4 DNA binding domain (amino acids 1−147) under the control of SV40 regulatory sequences (Sadowski *et al.*, 1988). Mutant p53 sequences were dervied from primary NSCLC tumor cDNAs with missense mutations at the following codons: 154, Gly to Val (T1490); 247, Asn to Ile (T863); 273, Arg to Leu (T861); and an eight amino acid deletion including codons 262−269 (T829) (Chiba *et al.*, 1990). Those from small cell lung cancer (SCLC) include a tumor cDNA with a mutation at codon 179, His to Glu (H1436) (Takahashi *et al.*, 1989) and a SCLC cell line cDNA (H526) with an exon 4 deletion (codons 33−125) (Takahashi *et al.*, 1990). The wild type human p53 sequence was derived from an individual primary NSCLC tumor cDNA clone (T1170) which contained no mutant p53 sequence (Chiba *et al.*, 1990). All of the cDNAs which encompassed the entire p53 coding region and adjacent untranslated 5′ and 3′ regions have been fully sequenced and contain only the reported mutations (see above references). In addition, the junction of the fused constructs was sequenced to ensure that the sequence of the fused construct was in frame. Two additional truncated fusion plasmids containing sequence coding for either the first 13 or 42 N-terminal amino acids of wild type p53 fused to the Gal4 DNA binding domain were constructed using the products generated by PCR amplification. Two separate PCR amplifications were carried out using wild type human p53 cDNA sequences as the template DNA and two pairs of primers. All primers had extraneous nucleotides comprising *Eco*RI sites at their 5′ ends. The sense primer located in the 5′ untranslated region was identical for each reaction and included the first ATG of p53: 5′-ATGCGAATTCACTGCCTTCCGGGTCACTGCCATG-3′. The two antisense primers were located in exon 2, 5′-ATGGAATTCAGGGGGCTCGACGCTAGG-3′ or in exon 4, 5′-ATGCGAATTCATCATCCATTGCTTGGGACGGCAA-3′ for the amino acids 1−13 and 1−42 constructs, respectively. The products were digested with *Eco*RI, purified as previously described (Takahashi *et al.*, 1990) and ligated into the *Eco*RI site of pSG424. The resultant constructs were sequenced to ensure that no mistakes were introduced during PCR amplification and that the p53 sequence remained in frame.

One mutant p53 expression plasmid, CMV−$p53_{179}$, under the control of the CMV promoter/enhancer contained sequences derived from a SCLC cDNA having a missense mutation, His to Glu, at codon 179 (H1436) (Takahashi *et al.*, 1990) and was first cloned into the *Eco*RI site of pcDNAI (Invitrogen) and transferred as the *Hin*dIII−*Xba*I fragment into pRC/CMV (Invitrogen) (T.Takahashi, unpublished data). The wild type p53 expression plasmid, CMV−$p53_{wt}$, contained the *Xba*I−*Xba*I fragment of php53c1 (Zakut-Houri *et al.*, 1985) also cloned into pRC/CMV (T.Takahashi, unpublished data). All constructs were confirmed by sequencing not to have changed during the cloning procedure. The construction of these plasmids was all performed by Dr T.Takahashi.

The reporter plasmid, pG5E1bCAT, contained five Gal4 DNA binding sites upstreams of adenovirus E1b promoter sequences followed by the CAT gene (Lillie and Green, 1989). Two plasmids, pSG4 encoding the entire Gal4 protein (amino acids 1−881) and pSGVP encoding the Gal4 DNA binding domain fused to the 78 C-terminal amino acids of the herpes simplex virus protein VP16 (Sadowski *et al.*, 1988), were used as positive activators. These three plasmids were gifts from Mark Ptashne's laboratory.

Cell lines

Three NSCLC cell lines established at the NCI (Brower *et al.*, 1986) were used for transfection studies: H23 (an adenocarcinoma) has lost the normal allele, bears an endogenous missense p53 mutation at codon 246 changing Met to Ile (Takahashi *et al.*, 1989) and produces high levels of mutant protein (Bodner *et al.*, 1992); H1299 (a large cell lung carcinoma) has a homozygous 5′ intragenic deletion of the p53 gene (Mitsudomi *et al.*, 1992); and H358 (a bronchioloalveolar carcinoma) has a homozygous deletion of both p53 alleles (Takahashi *et al.*, 1989). The latter two cell lines express no p53 protein (Bodner *et al.*, 1992). Both H23 and H358 have a K-*ras* mutation at codon 12 while H1299 bars an N-*ras* mutation at codon 61 (Mitsudomi *et al.*, 1992).

Transfections and CAT assays

Lung cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO or Hyclone) containing 100 μg/ml penicillin and streptomycin (Pc-Strep) each. Approximately 1×10^6 cells were plated on 100 mm dishes 1 day prior to transfection and allowed to reach 80−90% confluence. Cells were washed twice, the culture medium replaced with serum-free RPMI 1640 and Pc-Strep, and transfected using 50 μg Lipofectin according to the manufacturer's instructions (Bethesda Research Laboratories) (Felgner *et al.*, 1987) with 5 μg per dish of pG5E1bCAT, 5 μg of the various CsCl purified Gal4−p53 fusion and/or CMV−p53 expression constructs and 3 μg of pRSV/L, a plasmid containing the luciferase gene under the control of the Rous sarcoma virus LTR promoter (deWet *et al.*, 1987). The latter was used to monitor transfection and standardize the amounts of extract used for the CAT assay. Following 12 h of incubation, serum was added to the medium and 48 h later cells were harvested. One-fifth of the transfected cells were pelleted and analyzed for luciferase activity (J.Battey, unpublished method). Cell extracts were prepared from the remainder (Sambrook *et al.*, 1989) and equivalent amounts based on the luciferase activity were assayed for CAT activity (Gorman *et al.*, 1982) with [^{14}C]chloramphenicol (Amersham) using TLC (Sambrook *et al.*, 1989). The CAT activity was quantified by counting the acetylated chloramphenicol forms in either a Betascope 603 Blot Analyzer (Betagen) or a 400E Phosphoimaginer (Molecular Dynamics). CAT activity was expressed as the percentage conversion of [^{14}C]chloramphenicol into the acetylated form.

The exact amounts of the reporter and activator plasmids used in each experiment are described in the figure legends and tables. When < 12.5 μg of specific p53 plasmid DNAs were used in competition transfection experiments, pBR327 DNA was cotransfected to yield equal amounts of DNA per transfection experiment. All transfections were carried out at least in duplicate.

Luciferase activity assay

Luciferase activity was determined (J.Battey, unpublished method) as follows. Each cell pellet was resuspended in 300 μl extraction buffer [1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM dithiothreitol (DTT)] and incubated at 0°C for 5 min. Cell debris was pelleted at 13 000 r.p.m. for 5 min at 4°C. 100 μl of extract were added to 360 μl of assay buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM $KHPO_4$, pH 7.8, 1 mM DTT and 2 mM ATP). The reagents were placed in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and the reaction was initiated by the injection of 200 μl of 0.2 mM D-luciferin (Sigma) in extraction buffer containing 2 mM DTT minus Triton X-100. The light output was measured for 20 s at room temperature. The light units are directly proportional to the amount of luciferase activity in the sample being assayed.

Temperature sensitivity assay

Duplicate dishes of lung cancer cells were grown, transfected and assayed as above except that after the addition of serum, one was maintained at 37°C and the other at 30°C for the post-transfection 48 h period. From this point on the cells were processed as above.

Immunostaining

H1299 cells were transfected with 10 μg wild type and mutant p53 fusion construct DNAs (Gal4−$p53_{wt}$, Gal4−$p53_{154}$ and Gal4−$p53_{247}$) and with the Gal4 plasmid (pSG4) as a negative control (see above). Cells were trypsinized 12 h after serum addition, plated on coverslips and allowed to recover for an additional 48 h. The transfected cells were washed once with phosphate-buffered saline (PBS) and fixed with 100% acetone for 10 min at −20°C. The staining was carried out using the avidin−biotin−peroxidase technique (Vectastain ABC staining kit, Vector Laboratories) according to the manufacturer's instructions with modifications as described (Linnoila *et al.*, 1988) and using as the primary antibody a 1:10 dilution of PAb 1 (Oncogene Science) (PAb 421) (Gannon *et al.*, 1990). Cells were photographed using a Zeiss microscope.

Immunoblotting

H1299 lung cancer cells were grown and transfected with 10 μg of Gal4 or various CMV−p53 construct DNAs as described above except that the pG5E1bCAT reported plasmid and the luciferase plasmid were not cotransfected. After transfection and recovery, cells were washed twice in PBS, lysed in 1 ml of 250 mM NaCl, 50 mM Tris−HCl, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM PMSF (Sigma), 50 μM leupeptin (Boehringer Mannheim) and 50 μg/ml aprotinin (Sigma) (Laemmli, 1970), scraped into tubes and vortexed three times for 10 s every 10 min at 4°C. The cell lysates were cleared by centrifugation at 13 000 r.p.m. for 15 min at 4°C and protein measured using the Bio-Rad Microassay (Bio-Rad). Cell lysates which contained ~300 μg of protein were denatured at 95°C for 5 min in sample buffer (0.625 mM Tris−HCl, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol) and were fractionated in a reduced 10% SDS−PAGE system. Fractionated proteins were transferred to nitrocellulose membranes by electroblotting and probed as previously described (Cuttitta *et al.*, 1988) except that the primary antibody was PAb 2 (Oncogene Science; PAb 1801) (Banks *et al.*, 1986), the secondary antibody was affinity purified rabbit anti-mouse IgG1 (Pharmingen) and the detector consisted of 2.5 × 10^6 c.p.m. [^{125}I]Protein A (specific activity 33 μCi/μg).

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