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A temperature-sensitive mutant of human p53

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We discovered that the human 'hot-spot' p53 mutant 143Ala is temperature sensitive for the binding of two DNA elements and for mediating the transcription of a downstream luciferase reporter gene. At 32.5°C, 143Ala possesses strong DNA binding ability. In addition, its transcriptional activities are stronger than those of wild-type p53. At 37.5°C, however, both the mutant's DNA binding and transcriptional activation functions are greatly reduced or abolished. The activity differences correlate with the presence of two conformational states of p53, which are recognized by monoclonal antibodies: at 32.5°C, 143Ala is recognized by PAb1620, which is specific for the wild-type conformation, whereas at 37.5°C, 143Ala is almost undetectable by PAb1620-mediated immunoprecipitation. Although 143Ala's binding to p53 DNA recognition elements and its activation of reporter gene transcription at 32.5°C is markedly higher than that of the wild-type p53, 143Ala inhibited proliferation less robustly than wild-type p53 and it did not increase inhibition of *ras*-induced focus formation. These results indicate a partial correlation between the events involved in transcriptional activation and events involved in the proliferation suppression of p53. 143Ala therefore will be of use in dissecting the relationship between the structure of p53 and its different cellular functions.

Key words: DNA binding/p53 mutant/temperature sensitivity/transcription

Introduction

Loss of one allele and acquisition of missense mutations in the remaining allele of *p53* are frequent in human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991). Functional loss of *p53* normal alleles leads to increased proliferation and genetic instability, which is manifested by the accumulation of genetic changes such as point mutations, chromosomal rearrangement and gene amplification (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Yin *et al.*, 1992). These accumulated genetic changes can result in cellular immortalization and tumorigenicity (Nowell, 1986; Vogelstein *et al.*, 1988; Weinberg, 1989). Introducing wild-type *p53* into the tumor cells can inhibit gene amplification (Livingstone *et al.*, 1992; Yin *et al.*, 1992), suppress cellular proliferation and tumorigenicity (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Baker *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Prosser *et al.*, 1990; Johnson *et al.*, 1991), induce apoptosis (Yonish-Rouach *et al.*, 1991) and restore radiation-induced G_1 arrest (Kastan *et al.*, 1991, 1992; Kuerbitz *et al.*, 1992). The mechanism by which p53 participates in these cellular functions has not yet been elucidated. However, the recent discovery that p53 functions as a specific transcriptional regulator (Fields and Jang, 1990; Raycroft *et al.*, 1990) suggests some clues. For example, p53 upregulates the DNA damage-inducible gene, *gadd45*, which is consistent with *p53*'s role in the mediation of DNA damage-induced G_1 arrest (Kastan *et al.*, 1991, 1992).

The p53 protein may regulate transcription either by binding to enhancer-like DNA elements directly (Kern *et al.*, 1991, 1992; El-Deiry *et al.*, 1992; Farmer *et al.*, 1992; Funk *et al.*, 1992; Kastan *et al.*, 1992; Scharer and Iggo, 1992; Zambetti *et al.*, 1992; Chen *et al.*, 1993; Zhang *et al.*, 1993a,b) or by interacting with other transcriptional regulatory factors, such as the TATA and CAAT binding proteins (Seto *et al.*, 1992; Agoff *et al.*, 1993; Liu *et al.*, 1993; Mack *et al.*, 1993). Several p53 binding elements have been identified by various techniques. One of these, the ribosomal gene cluster (RGC) element was originally isolated from human genomic DNA (Kern *et al.*, 1991). Another, the p53CON element, was isolated using a PCR-aided selection strategy called CASTing (Funk *et al.*, 1992). Both elements are found in the regulatory sequences of mouse genes and are regulated by p53 in reporter gene constructs (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992; Zauberman *et al.*, 1993). The p53CON core DNA binding element is also present in the regulatory sequence of several human genes and is regulated by p53, as demonstrated in transient transfection reporter gene assays (W.Zhang, Randhawa and A.B.Deisseroth, submitted). In addition, each *p53* mutation may have a different effect on the DNA binding and transcriptional activation functions associated with these two elements (Frebourg *et al.*, 1992; Chen *et al.*, 1993; Zhang *et al.*, 1993a). Furthermore, transcriptionally inactive p53 mutants can affect the wild-type p53 in transactivation function in either a dominant-positive or a dominant-negative manner (Zhang *et al.*, 1993b).

Wild-type and mutant p53 proteins can be associated with two different conformational states (Gannon *et al.*, 1990; Milner and Medcalf, 1991; Stephen and Lane, 1992). Structures or domains of p53 are differentially exposed in these conformational states. Monoclonal antibodies that preferentially recognize these two states are available. PAb1620 antibody immunoprecipitates the human wild-type conformation, whereas PAb240 immunoprecipitates the mutant conformation (Gannon *et al.*, 1990; Milner and

Medcalf, 1991). PAb240 also recognizes mouse mutant p53 and PAb246 recognizes the wild-type conformation of mouse p53.

The conformation of p53 appears to be important for its biological functions. For example, a mouse temperature-sensitive p53 mutant, 135Val (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991), exhibits the wild-type conformation at 32.5°C (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991), localizes in the nucleus (Ginsberg *et al.*, 1991), activates the *mdm-2* gene (Barak *et al.*, 1993) and inhibits focus formation induced by activated *ras* (Michalovitz *et al.*, 1990). At a non-permissive temperature, 37.5°C, 135Val mutant p53 protein exhibits the mutant conformation, localizes in both the nucleus and cytoplasm, fails to activate the *mdm-2* gene and fails to suppress the focus formation induced by activated *ras*.

Conformational changes of p53 also appear to be part of cell cycle activity. The conformational state of p53 changes when fresh medium is added to cells (Milner and Watson, 1990). Also, stimulation of normal lymphocytes with mitogen or acute myelogenous leukemia cells with growth factors (granulocyte-macrophage colony-stimulating factor and interleukin 3) induces a mutant-like conformation of p53 in cells, which is recognized by PAb240 (Milner, 1984; Zhang *et al.*, 1992a, 1994). These data suggest that the mutant conformation is compatible with cellular proliferation. In contrast, the wild-type p53 protein, which is elevated in *ras* and E1A-transfected cells, has the wild-type conformation, as evidenced by its pattern of immuno-precipitation using the monoclonal antibodies. This is consistent with its role in inducing apoptosis (Debbas and White, 1993; Lowe and Ruley, 1993; Lowe *et al.*, 1993).

Here we report that the human 'hot-spot' p53 mutant 143Ala (Hollstein *et al.*, 1991) was temperature sensitive for specific DNA binding, p53 binding element-mediated transcriptional activation and conformation. At 32.5°C, 143Ala functioned like wild-type p53, but at 37.5°C it acted like a mutant. Surprisingly, the marked increase observed in the transcriptional activities of 143Ala at 32.5°C did not result in a corresponding inhibition of cellular proliferation and activated *ras* gene-induced transformation. Thus, an uncoupling of the DNA binding and transcriptional activation functions of p53 from its proliferation suppression function is seen in 143Ala mutant p53 protein.

Results

Transcriptional activation of p53CON-luciferase and RGC-luciferase by p53 mutant Ala143 is temperature sensitive

When the p53 binding element p53CON or RGC is placed 5′ of a luciferase reporter gene in an expression vector, wild-type p53 activates luciferase gene expression (Funk *et al.*, 1992). At 37°C, none of the five p53 mutant proteins used in this study (143Ala, 175His, 248Trp, 273His and 281Gly) stimulated RGC-mediated luciferase transcription. In contrast, two of the mutant p53 proteins (143Ala and 273His) were able to stimulate p53CON-mediated transcription (Zhang *et al.*, 1993a). We also observed that the activity of 143Ala increased greatly if the cells transfected with this mutant were cultured at room temperature as compared with 37°C. These results suggested that 143Ala may be a temperature-sensitive mutant.

To test this hypothesis, we cotransfected K562 cells with a plasmid containing a p53 cDNA and a luciferase reporter gene which contains either the RGC of p53CON element. We then cultured the transfected cells at either 37.5°C or 32.5°C. Transient luciferase expression assays showed that 143Ala's ability to activate both p53CON- and RGC-mediated transcription was temperature dependent (Figure 1). At 37.5°C, 143Ala failed to promote RGC-mediated transcription and promoted only a low level of p53CON-mediated transcription. However, at 32.5°C, the activity of 143Ala was increased more than 100-fold. Cotransfection of the luciferase reporter gene construct with plasmids containing wild-type p53 and other p53 mutants cDNA generated only 3- to 7-fold induction in luciferase activity at 32.5°C. This increase was independent of the presence of p53 since it was also observed when the CMV.neo parental vector was used alone. At 25°C, a similar pattern

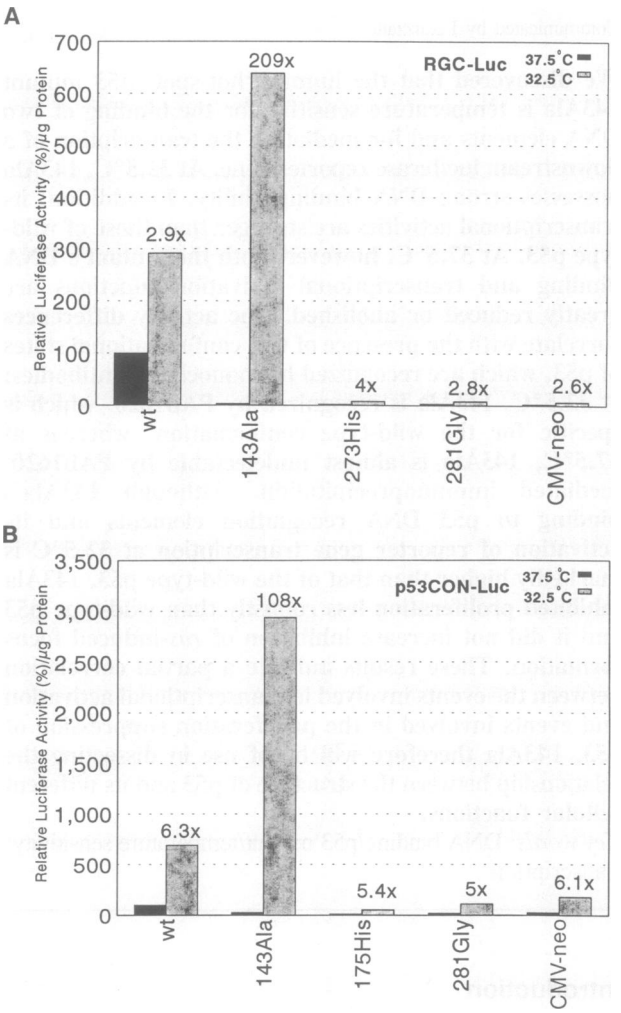

Fig. 1. The relative transcription activities of wild-type and mutant p53 at 32.5°C and 37.5°C. Exponentially growing K562 cells were transfected with either wild-type (wt) *p53*, the mutant *p53* indicated or a CMV.neo vector along with the reporter gene constructs RGC-Luc (**A**) or p53CON-Luc (**B**). Cells were incubated for 48 h. The protein was extracted, and the luciferase activities were analyzed as described in Materials and methods. Protein was also quantitated, and the luciferase activity per μg of protein was calculated and normalized against that of wild-type p53 at 37.5°C. The result is an average of three independent experiments. The magnitude of the increase in activity at 32.5°C relative to 37.5°C is indicated above the bars (×-fold).

of increased 143Ala activity was also evident but it was not quite as high as that seen at 32.5°C (data not shown).

We reasoned that the different transcriptional promoting activities of 143Ala at the different temperatures could have resulted from different levels of p53 protein expression in the cells. To test this hypothesis, we used Western blot analysis to measure the p53 protein levels in the transfected cells cultured at different temperatures. Figure 2 shows that the p53 protein levels at the two temperatures were similar, indicating that 143Ala was equally stable at the two temperatures. The data also indicated that the observed difference in transcriptional activity (Figure 1) reflected a real functional difference of 143Ala protein at the two temperatures. The slightly greater electrophoretic mobility of 143Ala seen on the Western blot reflected the presence of a polymorphic Arg at codon 72 in the cDNA (Zhang *et al.*, 1992b, 1993a). A surprising discovery from these experiments was that the activity of 143Ala at 32.5°C was consistently higher than that of wild-type p53. Therefore, at this temperature, 143Ala not only reversed its phenotype to the wild type, but actually gained additional activity.

During the course of our work, Chen *et al.* (1993) reported that the 143Ala p53 protein showed increased activity for activating p53CON-mediated transcription at 30°C, but this was not true for activating RGC-mediated transcription. In that study, the 1299 cell line, which is a p53-null lung cancer cell line, was used in transient expression experiments. We repeated these experiments in our laboratory; however, we found that 143Ala was also temperature sensitive for activating RGC-mediated transcription in 1299 cells (data not shown). Thus, we believe that temperature-sensitive transcriptional activity is an intrinsic characteristic of 143Ala.

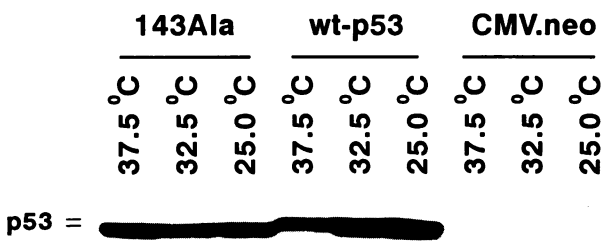

Fig. 2. The levels of p53 protein in transfected cells. Protein (40 μg) extracted from cells transfected with wild-type (wt) p53, 143Ala or CMV.neo vector was analyzed for the levels of p53 protein by Western blotting using DO-1 monoclonal antibody. 143Ala has a faster electrophoretic mobility because it has Arg at codon 72 instead of the Pro in wild-type *p53* (Zhang *et al.*, 1992b).

The mouse temperature-sensitive p53 mutant 135Val activates the expression of the *mdm-2* gene at 32.5°C but not at 37.5°C (Barak *et al.*, 1993). To determine whether 135Val is also temperature dependent in a human cell background, we tested transactivation by 135Val in our transient functional assays. The data in Figure 3 show that 135Val was temperature sensitive in activating both p53CON- and RGC-mediated transactivation in K562 cells. Our data are consistent with previously reported characteristics of 135Val (Michalovitz *et al.*, 1990; Barak *et al.*, 1993).

Increased levels of transcriptional activation of luciferase reporter gene by 143Ala at 32.5°C correlates with its increased binding to the p53 binding DNA elements

The p53 protein must bind to p53CON and RGC elements to activate p53CON- and RGC-mediated transcription (Funk *et al.*, 1992; Kern *et al.*, 1992; Zhang *et al.*, 1993a). Therefore, we hypothesized that the different transcriptional activities of 143Ala at 32.5°C and at 37.5°C may have resulted from altered binding capability of these p53 binding elements. We used mobility shift assay to examine this possibility. In these assays, protein was extracted from transfected cells that were incubated at either 32.5°C, 37.5°C or 25°C. The protein then was mixed with radiolabeled p53CON or RGC oligonucleotide in the presence or absence of anti-p53 monoclonal antibody PAb421. Previous work (Funk *et al.*, 1992; Hupp *et al.*,

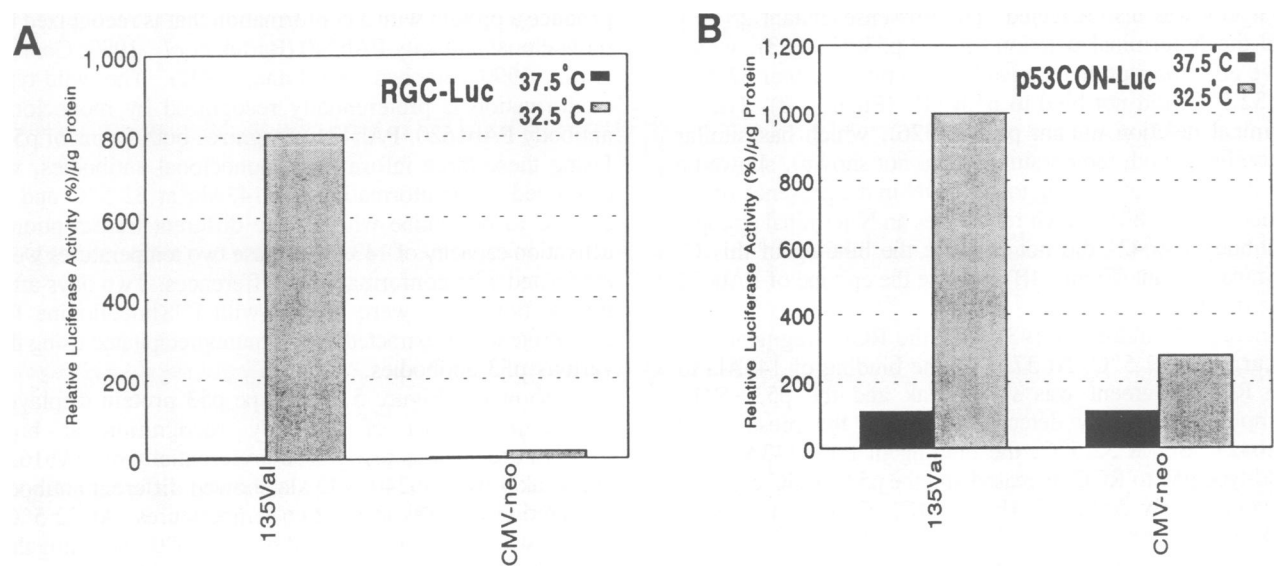

Fig. 3. The activity of mouse p53 mutant 135Val in activating RGC-mediated (**A**) or p53CON-mediated (**B**) transcription in K562 cells. The expression vector for the mouse mutant 135Val was cotransfected with the RGC-Luc or p53CON-Luc reporter gene into K562 cells. The transfected cells were incubated at either 32.5°C or 37.5°C. Protein was extracted and the luciferase activity was analyzed.

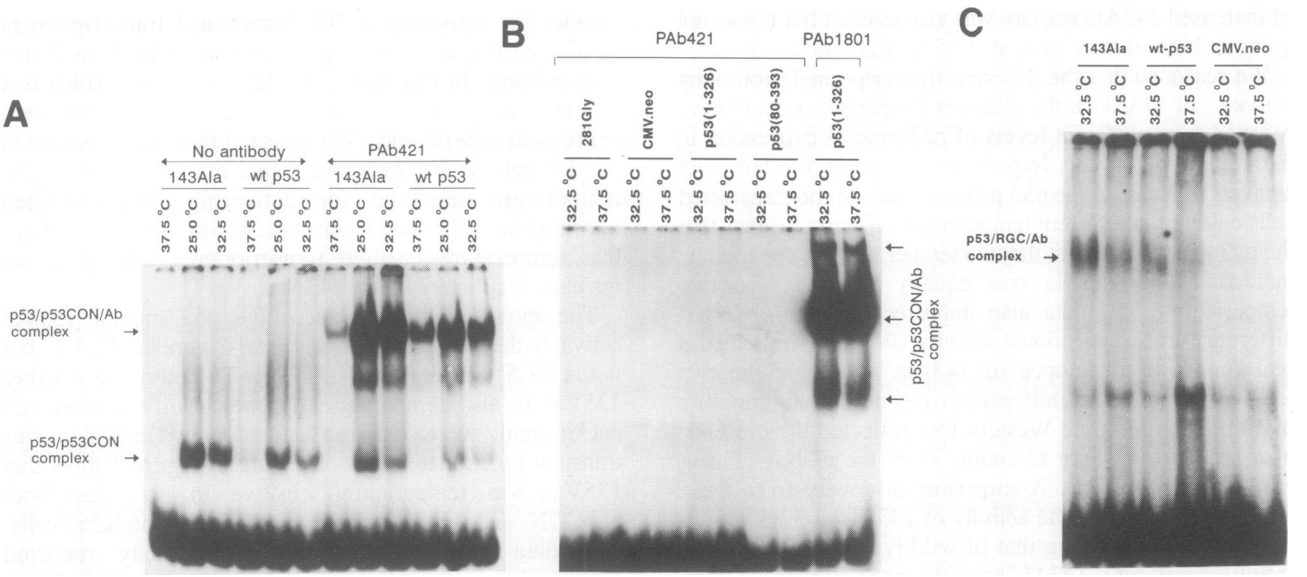

Fig. 4. Temperature-sensitive DNA binding of 143Ala. Protein was extracted from transfected cells incubated at either 37.5°C, 32.5°C or 25.0°C. Protein (10 μg) was mixed with 1 ng of radiolabeled p53CON (**A** and **B**) or RGC (**C**) oligonucleotide in either the absence or the presence of anti-p53 antibody PAb421 or PAb1801 as indicated. PAb421 was included in the binding experiments shown in (**C**). Twenty minutes after incubation at room temperature, the mixture was loaded on a 5% polyacrylamide gel and subjected to overnight electrophoresis at a constant voltage of 110 V. The gel was dried and exposed to Kodak X-AR film. p53(1−326) is a deletion mutant which is missing 67 amino acids, including the epitope of PAb421, from the C-terminus of p53. Deletion mutant p53(82−393) has 80 amino acids deleted from the N-terminus of p53.

1992; Zhang *et al.*, 1993a) reported that PAb421 can increase p53's ability to bind to the oligonucleotide.

The data in Figure 4A shows that the binding of 143Ala to p53CON increased at 25°C and 32.5°C in the mobility shift assay. At 37.5°C, the binding of 143Ala to p53CON was only detected in the presence of the anti-p53 antibody PAb421. At 32.5°C and 25°C, 143Ala binding to p53CON was greatly increased and was evident even in the absence of PAb421. Wild-type p53 also showed increased binding to p53CON oligonucleotide at 25°C and 32.5°C but to a much lower degree than was seen with 143Ala p53 protein. In the presence of PAb421, a complex of intermediate electrophoretic mobility which formed between p53 and p53CON was also detected. The missense mutant 281Gly and the N-terminal deletion mutant p53(82−393), which have no transcriptional activating function at either 37.5°C or 32.5°C, did not bind to p53CON (Figure 4B). The C-terminal deletion mutant p53(1−326), which has similar activities at both temperatures (data not shown), showed a similar degree of binding to p53CON in the presence of the antibody PAb1801, which recognizes an N-terminal epitope. Antibody PAb421 did not enhance the binding of this C-terminal mutant (Figure 4B) because the epitope of PAb421 was deleted.

Increased binding of 143Ala to the RGC fragment also occurred at 32.5°C. At 37.5°C, the binding of 143Ala to the RGC fragment was very weak and the p53−RGC complex was barely detectable even in the presence of PAb421. But at 32.5°C, the binding of both 143Ala and wild-type p53 to RGC increased and the p53−RGC complex became clearly detectable (Figure 4C). Consistent with its lack of transcriptional activity, the mutant 281Gly did not bind to RGC at either temperature.

Thus, it appears that the increased transcriptional activation capacity of 143Ala at 32.5°C correlated with its increased binding affinity for the two p53 DNA binding elements. However, an independent change unique to 143Ala must also have occurred, since wild-type p53 showed a similar increased binding to DNA but this was not accompanied by a concomitant and substantial or specific increase in transcriptional activation function at 32.5°C. By the same token, stronger binding ability did not always translate into stronger transcriptional capability.

Mutant 143Ala has different conformations at 37.5°C and 32.5°C

The conformation of p53 is important for its normal functions (Michalovitz *et al.*, 1990; Ginsberg *et al.*, 1991; Martinez *et al.*, 1991). Many missense mutations within the *p53* gene produce a protein with a conformation that is recognized by monoclonal antibody PAb240 (Bartek *et al.*, 1990; Gannon *et al.*, 1990; Stephen and Lane, 1992). The wild-type conformation is preferentially recognized by monoclonal antibody PAb1620. PAb421 recognizes both forms of p53. Using these three informative monoclonal antibodies, we examined the conformations of 143Ala at 32.5°C and at 37.5°C to determine whether the different transcriptional activation capacity of 143Ala at these two temperatures were associated with conformational differences. Two days after transfection, cells were labeled with [^{35}S]methionine for 2 h. Protein was extracted and immunoprecipitated using the various p53 antibodies.

As shown in Figure 5, wild-type p53 protein displayed the same pattern of antibody recognition at both temperatures: it was recognized preferentially by PAb1620 but weakly by PAb240. 143Ala showed different antibody recognition patterns at the two temperatures. At 32.5°C, 143Ala was immunoprecipitated by PAb1620, indicating that it had the wild-type conformation at this temperature. But at 37.5°C, 143Ala was barely immunoprecipitated by PAb1620. In spite of these differences, similar levels of 143Ala were immunoprecipitated by mutant conformation-

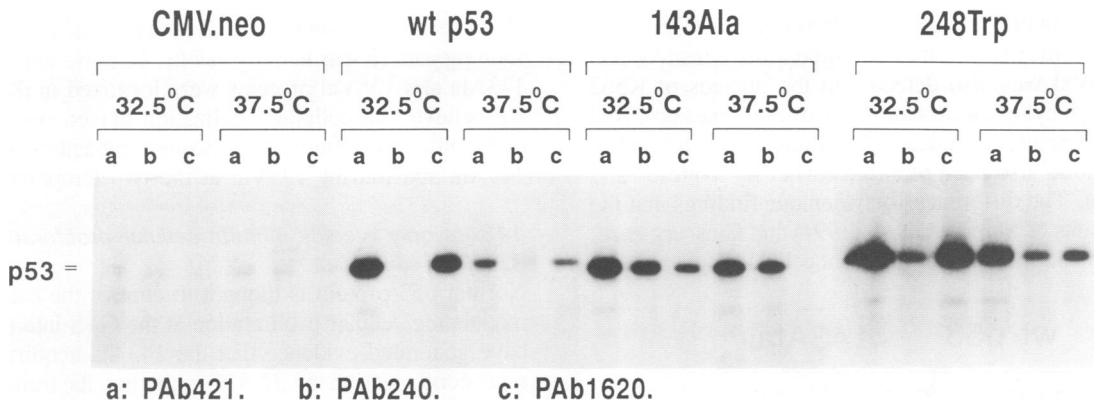

Fig. 5. Conformational states of 143Ala at 32.5°C and 37.5°C. Cells transfected with constructs indicated were labeled with [^{35}S]methionine for 2 h. Protein was extracted and immunoprecipitated with monoclonal antibody: (**a**) PAb421, which recognizes both wild-type (wt) and mutant forms of p53; (**b**) PAb240, which recognizes a mutant conformation or (**c**) PAb1620, which recognizes wt conformation.

specific antibody PAb240 at both temperatures. This result clearly indicates that 143Ala possessed different conformations at the two temperatures. This conformational change provides a simple explanation for the mutant's two transcriptional activities. It does not, however, explain the increased DNA binding of 143Ala at 32.5°C because wild-type p53 underwent no such conformational change, but also had increased DNA binding affinity at 32.5°C. Therefore, it is possible that the conformational change observed in these experiments affected only the transactivation domain localized on the N-terminus of p53 (O'Rouke *et al.*, 1990; Zhang *et al.*, submitted). Our hypothesis predicts that this kind of conformational change will not affect the transcriptional activity of p53 mutants that have no DNA binding ability. This prediction was borne out in tests with the 248Trp mutant p53 protein, which showed no transcriptional activity at either temperature. Our results indicate that the immunoprecipitation of 248Trp by PAb1620 was greater at 32.5°C than at 37.5°C (Figure 5).

Most of 143Ala protein is localized in the nucleus at both 32.5°C and 37.5°C

The cellular localization of the mouse p53 mutant 135Val also depends on the temperature (Gannon and Lane, 1991; Ginsberg *et al.*, 1991; Martinez *et al.*, 1991). 135Val is detected in the nucleus at 32.5°C, but it is detected in both the nucleus and cytoplasm at 37.5°C. Therefore, the difference in the mutant's conformation may alter its affinity for the nuclear matrix, thus changing its transcriptional activity.

To test whether a change in cellular localization or nuclear affinity was the molecular basis of the decreased transcription activation function of human 143Ala at 37.5°C, we investigated these characteristics at the two temperatures. The localization of p53 was first analyzed by immunohistochemical analysis using the anti-p53 monoclonal antibody PAb1801. Wild-type p53 and other missense p53 mutants were analyzed simultaneously as controls. As shown in Figure 6, most of the 143Ala protein, as well as most of the wild-type and other missense mutant proteins, was detected in the nucleus at both temperatures. Cellular localization of p53 then was analyzed using cellular fractionation experiments, in which both transient and stably transfected cells were separated into nuclear and cytoplasmic

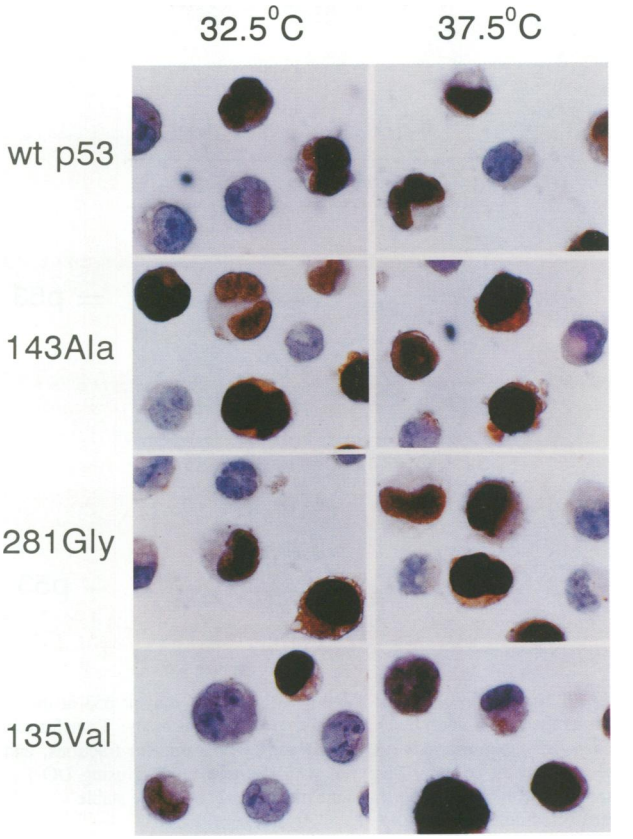

Fig. 6. Nuclear localization of wild-type (wt) and mutant p53 at 32.5°C and 37.5°C detected by immunohistochemical assay. After incubation at the two temperatures for 48 h, the transfected cells were fixed on polylysine-coated coverslips, incubated with anti-p53 monoclonal antibody PAb1801 (for human p53) or PAb421 (for mouse mutant 135Val), and detected with avidin-biotinylated peroxidase complex assay. The brown color represents a positive signal.

fractions by low-salt extraction. Under these conditions, the nuclear retinoblastoma protein (Rb) could be separated into two categories: the tightly binding underphosphorylated Rb, which remains in the nuclear fraction, and the weakly binding hyperphosphorylated Rb, which leaks into the cytoplasmic fraction. The presence of p53 in these two subcellular fractions was examined by Western blot analyses. This assay also detected 143Ala and other mutant p53 proteins

predominantly in the nucleus at both temperatures (Figure 7). Similarly, the majority of mouse temperature-sensitive p53 mutant 135Val was also detected in the nucleus of K562 cells, although cytoplasmic staining also sometimes occurred (Figure 5). Exclusively nuclear locations of this mutant in human cells has also been reported earlier by Vojtesek and Lane (1993). The differences between our findings and the previous results of Martinez *et al.* (1991) and Ginsberg *et al.* (1991) are most likely due to different cellular backgrounds.

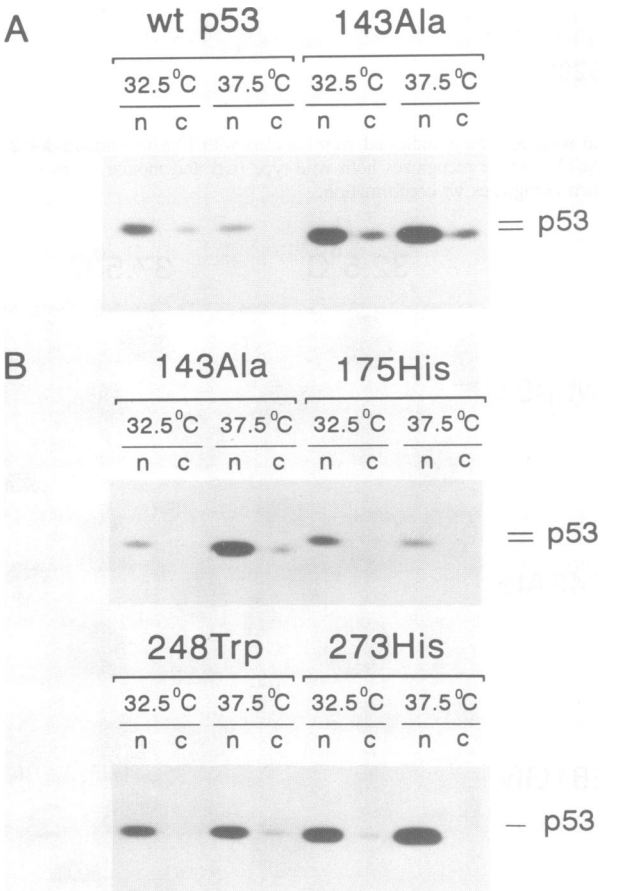

Fig. 7. Nuclear localization of wild-type (wt) and mutant p53 at the two temperatures analyzed by cellular fractionation assay. Transfected cells were separated into nuclear (n) and cytoplasmic (c) fractions, and the presence of p53 was analyzed with Western blotting using DO-1 monoclonal antibody: (**A**) transient transfected cells, (**B**) stable transfected cells.

However, it was pointed out (Ginsberg *et al.*, 1991) that the staining pattern was heterogeneous. Because the majority of 143Ala and 135Val proteins were localized in the nucleus, we believe that cellular localization played no part in the different transcriptional activating capacities of human 143Ala and murine 135Val at the two temperatures.

143Ala only weakly inhibits cellular proliferation at 32.5°C

Normal p53 protein is thought to control the cell cycle by modulating cellular proliferation at the G_1/S interphase. We have generated evidence that the 143Ala acquired a wild-type conformation at 32.5°C and that its transcriptional activation capacity was greater than that of wild-type p53. If the transcriptional activity is sufficient to suppress cellular proliferation, then at 32.5°C 143Ala should inhibit cellular proliferation as effectively as wild-type p53. We tested this hypothesis using methylcellulose colony plating assays in which the proliferation of transfected K562 cells at 32.5°C and 37.5°C was analyzed by colony formation.

K562 cells were transfected with the expression vector containing the neomycin-dominant selection marker and either wild-type p53; missense p53 mutant 143Ala, 175His or 248Trp; or deletion mutant p53(160–393). Twenty-four hours after transfection, 2×10^4 of the cells were mixed with 3 ml of methylcellulose containing 0.8 mg/ml G418. One set of cultures was incubated at 37.5°C and allowed to grow for 12–14 days; the other set was incubated at 32.5°C for 22–24 days (cell growth was much slower and thus required a longer incubation time). Table I shows the colony counts from these experiments. At 37.5°C, 143Ala did not inhibit the plating efficiency or the number of colonies formed. At 32.5°C it inhibited colony formation. However, it was far less effective than wild-type p53. These results provided evidence that strong transcriptional activation capacity does not necessarily correlate with strong ability to inhibit cellular proliferation.

Several colonies from the dishes incubated at 37.5°C were selected and then expanded in the presence of 1 mg/ml G418 for 3 weeks. The presence of p53 in the expanded cells was verified by Western blotting (data not shown). Most colonies transfected with wild-type p53 did not expand. One clone that did expand did not express p53 protein (data not shown). Our observation is consistent with previous findings that the presence of wild-type p53 is not compatible with continued growth of K562 cells (Johnson *et al.*, 1991). The expanded clones harboring 143Ala, 175His or 248Trp were cultured

Table I. Colony formation of transfected K562 cells on methylcellulose at 32.5°C and 37.5°C

	Experiment 1		Experiment 2		Average	
Transfectant	32.5°C	37.5°C	32.5°C	37.5°C	32.5°C	37.5°C
Wild-type p53	21	10	2	11	11	10
143Ala	79	167	14	120	46	143
175His	152	149	60	164	106	156
248Trp	161	248	ND	ND	161	248
CMV.neo	281	245	133	235	207	240
p53(160–393) + CMV.neo	336	227	194	204	265	215

For tests at 37.5°C, colonies that consisted of >50 cells were counted 2 weeks after seeding; for tests at 32.5°C, colonies that consist of >20 cells were counted 3 weeks after seeding. Different criteria of colony size were used because cell growth at 32.5°C was much slower and thus colony size was much smaller than that of 37.5°C cultures even after 5 weeks. ND, not done.

at 32.5°C and 37.5°C, and their growth was analyzed. Only 143Ala showed some growth inhibition at 32.5°C (Figure 8). These data reinforce the results of the colony formation assay.

143Ala weakly inhibits ras-induced focus formation at 32.5°C and 37.5°C

Wild-type p53 can suppress activated *ras* oncogene-induced focus formation in rat-1 cells, but the p53 mutants fail to suppress it effectively. To test whether the ability of 143Ala to suppress focus formation was temperature dependent, we co-transfected two plasmids, encoding 143Ala and activated human H-*ras* oncogene, into rat-1 fibroblasts. The transfected cells were cultured at 37.5°C for 24 h, then separated into four plates. Two were kept at 37.5°C for 14 days and the other two were incubated at 32.5°C for 21 days (a longer time was allowed to compensate for the slow cell growth at this temperature). The cells were stained and the foci were counted. The degree of suppression of focus formation by p53 was measured in terms of a percentage of foci relative to the number in control *ras*-transfected cultures (Table II). Compared with other missense mutants, 143Ala inhibited focus formation at both temperatures. However, compared with wild-type p53, 143Ala suppressed focus formation less dramatically. Similarly, other mutants studied also suppressed focus formation much more weakly than wild-type p53.

Discussion

We have identified and characterized the temperature sensitivity of the human p53 mutant 143Ala, which is a 'hot-spot' mutant frequent in human cancer (Hollstein *et al.*, 1991). Its conformation is temperature sensitive: at 32.5°C, 143Ala has a conformation recognized by PAb1620, a monoclonal antibody specific for the wild-type conformation, but at 37.5°C, the conformation of 143Ala is unrecognized by PAb1620. The mutant's different conformations correlated with its different abilities to activate transcription mediated by the p53 binding elements RGC and p53CON. At 32.5°C, 143Ala is more active in binding to DNA and promoting gene expression. At 37.5°C, 143Ala's ability to bind DNA and activate transcription was severely weakened or lost. Although 143Ala was found to possess some features of wild-type p53, the two differ in several respects. First, in transfected cells wild-type p53 was only weakly immunoprecipitated by PAb240, whereas 143Ala was strongly immunoprecipitated by the antibody. Second, at

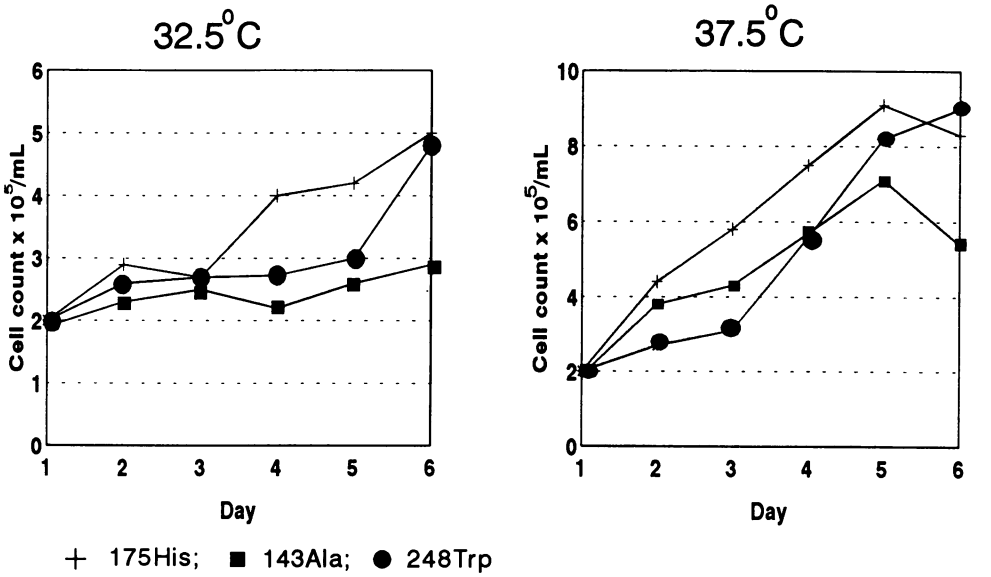

Fig. 8. Growth curve of cells stably transfected with 143Ala, 175His or 248Trp at 32.5°C or 37.5°C. Cell growth at 32.5°C was much slower than that at 37.5°C (two scales are shown).

Table II. focus formation of transfected rat-1 cells at 32.5°C and 37.5°C

	Experiment 1 (%)		Experiment 2 (%)	
Transfectant	32.5°C	37.5°C	32.5°C	37.5°C
ras	100	100	100	100
ras + wt p53	13	8.5	10	7.7
ras + 143Ala	19	16	20	18
ras + 175His	41	35	30	26
ras + 248Trp	47	35	48	28
ras + 273His	49	38	55	43
ras + 281Gly	83	65	56	46

Activated human H-*ras* gene, which has a point mutation at codon 12, was used in the experiments. For tests at 37.5°C, foci were counted 2 weeks after transfection; for tests at 32.5°C, foci were counted 3 weeks after transfection. In each experiment, two duplicate plates were counted. The focus number is normalized against control cultures using only the *ras* transfectant.

32.5°C 143Ala had a transcriptional activity higher than that of wild-type p53. Most important, wild-type p53 and 143Ala at 32.5°C had distinct differences in their abilities to suppress cellular proliferation and *ras*-induced focus formation. Wild-type p53 was not compatible with cellular growth at all, while 143Ala only slightly inhibited growth. In addition, wild-type p53 inhibited *ras*-induced focus formation more than 143Ala did.

These findings reveal an uncoupling phenomenon of DNA binding and the activation of transcription mediated by p53CON and RGC from the proliferation suppression functions of p53. At 32.5°C, 143Ala activates p53CON- and RGC-mediated transcription to a greater degree than does wild-type p53. However, 143Ala is much weaker in suppressing cellular proliferation and *ras*-induced focus formation. This lack of correlation forms a clear contrast to the apparent correlation reported to exist between the activity of p53 in regulating the *gadd45* gene and the role of p53 in arresting cells at the G_1 phase after they have been subjected to ionizing irradiation (Kastan *et al.*, 1992). Thus, transcriptional regulation does not parallel the suppression of proliferation. The transcriptional activation mediated by p53CON and RGC may be insufficient for inhibition of cellular proliferation and focus formation. This has also been observed with 273His, which activates p53CON-mediated transcription (Chen *et al.*, 1993; Zhang *et al.*, 1993a) and yet fails to inhibit cellular proliferation and focus formation. In reality, 273His may operate with a strong transforming activity since it is one of the 'hot-spot' missense mutations frequently observed in human tumors (Hollstein *et al.*, 1991).

After this manuscript was submitted, a new cellular gene, *WAF1*, was identified, that is activated by wild-type p53 (El-Deiry *et al.*, 1993). The gene product of *WAF1* or Cip1, a potent inhibitor of G_1 cyclin-dependent kinases, suppresses cellular proliferation (Harper *et al.*, 1993). This finding established a link between the transcriptional activation function of p53 and its anti-proliferative activities. Currently, we are in the process of investigating whether 143Ala mutant activates *WAF1* gene expression at 32.5°C.

Our work also showed a striking similarity between the human temperature-sensitive mutant 143Ala and the mouse temperature-sensitive mutant 135Val. The two point mutations are localized to a common area of p53. Codon 143 of human p53 corresponds to codon 137 of mouse p53, which is only two amino acids away from the mutation in 135Val. Therefore, it is reasonable to postulate that this area is a critical domain for the regulation of p53 conformation. Chen *et al.* (1993) also reported two other missense p53 mutants in which the transcriptional activity is temperature dependent. The mutations are localized at codons 173 and 247. If these two mutants also change conformation with temperature, a link between the conformation and transcriptional activity of p53 will be established further.

At least two important questions remain unanswered. Is the transcription activation function of p53 essential for its cell cycle control and its tumor suppressor functions? And if yes, what gene targets are modulated by p53? Though many have attempted to establish a link between the transcription activation and growth inhibition functions of p53, and recent discovery of p53-activated *WAF1* gene (El-Deiry *et al.*, 1993; Harper *et al.*, 1993) showed that a link may exist, the formal possibility exists that p53 may regulate proliferation through mechanisms other than DNA binding and transcriptional activation. For instance, p53 may interact with and sequester cellular proteins, thus preventing their normal effect on cellular proliferation.

Viral studies provide us with a perfect example of how this type of *trans*-dominant effect could occur. SV40 T-antigen is required for SV40 DNA replication. However, p53 binds T-antigen and prevents it from binding for replication, thus inhibiting DNA synthesis (Sturzbecher *et al.*, 1988; Wang *et al.*, 1989). p53 may regulate cellular DNA synthesis in a similar manner. This hypothesis may explain why the level of normal p53 in quiescent cells is extremely low but increases after the cells are stimulated by mitogen. p53 may exert negative feedback control over the cellular replication factors required in DNA synthesis. This function may not involve the transcriptional activity of p53. Alternatively, p53 may interact with other transcriptional factors. For example, the retinoblastoma gene product forms a complex with the transcriptional factor E2F and prevents its normal transcriptional activation function, which is required for cell cycle progression into the S-phase (Chellappan *et al.*, 1991).

However, p53's ability to transactivate through p53CON and RGC may also contribute to the p53-mediated regulation of cellular proliferation. To date, no human genes that contain functional p53CON or RGC elements in their regulatory sequences have been reported. A search for candidate genes is in progress, and the eventual demonstration of such a gene will help us to understand the biological functions of transcriptional events mediated by RGC and p53CON.

Materials and methods

Cells and plasmids

K562 chronic myelogenous leukemia cells and NCI-H1299 non-small cell lung cancer cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum in a 37.5°C incubator containing 5% CO_2. Northern and Western blot analyses showed that *p53* mRNA and its protein were absent in both cell lines (Koeffler *et al.*, 1986; Mitsudomi *et al.*, 1992; Zhang *et al.*, 1993a). Luciferase reporter plasmids p53CON-Luc [containing basic heat shock protein 70 (HSP70) promoter and one copy of the p53CON element] and RGC-Luc (containing basic HSP70 promoter and one copy of the RGC element) were described previously (Funk *et al.*, 1992). Human *p53* expression vectors were generously provided by Dr B.Vogelstein (Johns Hopkins University School of Medicine). Each mutant is named according to the position of the mutated codon and the mutant amino acid. All the human *p53* expression vectors are under the control of cytomegalovirus enhancer and contain a *neo* gene, conferring on them resistance to the drug G418. The deletion mutant p53(82−393) has 80 amino acids deleted from the N-terminus of p53, and deletion mutant p53(1−326) has 67 amino acids deleted at the C-terminus. Both deletion mutants were gifts from Dr Y.Shiio (The University of Tokyo, see Shiio *et al.*, 1992). The expression vector for the mouse temperature-sensitive mutant 135Val was provided by Dr G.Lozano (The University of Texas M.D.Anderson Cancer Center).

Electroporation and luciferase assay

Ten million exponentially growing K562 cells were mixed with 15 μg of *p53* expression vector (wild-type or mutant) plus 20 μg of luciferase reporter plasmids in phosphate-buffered saline (PBS) at room temperature for 10 min (the total amount of DNA transfected was normalized by the addition of sonicated salmon sperm DNA) and then were pulsed (600 μF, 420 V) using a BTX600 transfector. Transfection of 1299 cells was carried out under the conditions described earlier (Chen *et al.*, 1993). After a 10 min incubation, the cells were transferred to 10 ml of RPMI-1640 plus 10% fetal calf serum. The cells were then incubated at 37.5°C with 5% CO_2 for 48 h; 5 ml of fresh medium were added after 24 h. During the 48 h of incubation following transfection, no differences in cell growth or viability were detected on growth curves or in trypan blue exclusion assays. The

transfected cells were collected, washed with PBS and lysed in the lysis buffer provided with the luciferase kit (Promega Inc., Madison, WI). Transcriptional activity was measured using a luminometer (Pharmacia LKB Nuclear, Gaithersburg, MD). Protein concentration was quantitated using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Mobility shift assay

Ten million transfected cells were washed with PBS. Total cellular protein was isolated according to Lassar *et al.* (1991) and quantitated by the Bio-Rad method. After quantitation, 10 μg of total protein was mixed with 1 ng of ^{32}P-end-labeled p53CON or RGC for 20 min at room temperature and analyzed as described previously (Funk *et al.*, 1992; Zhang *et al.*, 1993a). When indicated, 100 ng of anti-p53 antibody PAb421 or PAb1801, or control antibody PAb419 specific for SV40 T-antigen (Oncogene Science Inc., Manhasset, NY), were added to each mixture. All oligonucleotides were synthesized with a Cruachem PS240 automated DNA synthesizer. Equal amounts of sense and antisense were mixed in 0.1 M NaCl solution, heated to 85°C for 10 min, and then gradually cooled to room temperature to allow annealing before use. End-labeling of the oligonucleotides was done according to the standard procedure.

Western blotting

Forty micrograms of each protein extract from transfected cells were boiled in sample buffer (125 mM Tris−HCl pH 6.8, 1% SDS, 2% β-mecaptoethanol, 0.01% bromophenol blue) for 5 min and then loaded onto a 10% SDS−polyacrylamide gel. After overnight electrophoresis at 45 V, the protein was transferred to an Immobilon membrane (Millipore Corp., Bedford, MA), blocked with a blocking solution (50 mM Tris−HCl pH 7.5, 0.9% NaCl, 3% non-fat milk, 0.2% bovine albumin, 0.05% Tween-20) for 4 h and then incubated overnight with anti-p53 antibody DO-1 (Oncogene Science Inc., Vojtesek *et al.*, 1992) in blocking solution (0.5 μg/ml). The levels of p53 were analyzed using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Immunoprecipitation

Cells were washed twice with PBS 48 h after transfection, and then metabolically labeled for 2 h in the following solution: 150 μCi/ml [^{35}S]methionine, 10% dialyzed fetal calf serum and 1% glutamine in methionine-free Dulbecco's modified Eagle's medium (DMEM) at 32.5 or 37.5°C in a 5% CO_2 incubator. The cells then were washed three times with PBS, lysed, precleared and immunoprecipitated with anti-p53 antibody PAb421, PAb240 or PAb1620 as described previously (Zhang *et al.*, 1992a) except that the salt concentration was maintained at 100 mM.

In situ immunohistochemistry

The method of immunohistochemical staining to detect p53 protein in cells was described previously (Xu *et al.*, 1991). The cells were allowed to adhere briefly to polylysine-precoated coverslips and then were fixed, permeabilized, blocked with 3% horse serum in phosphate-buffered saline and incubated overnight with p53-specific antibody PAb1801 (Oncogene Science, Inc.) for human p53 and with PAb421 for the mouse temperature-sensitive mutant 135Val. The antigen−antibody complex was analyzed by the avidin-biotinylated peroxidase complex method (Vector Laboratories).

Cellular fractionation

The cells were separated into nuclear and cytoplasmic fractions by low-speed centrifugation after being disrupted by a Dounce homogenizer in a low-salt buffer as previously described (Templeton, 1992).

Methylcellulose assay

After electroporation, the cells were allowed to recover for 16−24 h. Viable cells (2×10^4) were mixed with 3 ml of liquid methylcellulose (Terry Fox Laboratory, Vancouver, Canada) containing 0.8 mg/ml G418 (Gibco-BRL). The cells were cultured in a humidified 5% CO_2 incubator at either 32.5°C or 37.5°C. After 2 weeks (cultures at 37.5°C) or 3−4 weeks (culture at 32.5°C), colonies that consist of >50 cells (cultures at 37.5°C) or >20 cells (cultures at 32.5°C) were counted. Different criteria of colony size were used because cell growth at 32.5°C was much slower than that at 37.5°C and thus colonies were markedly smaller.

Focus formation assay

Rat-1 cells (generously provided by Dr M.Weil at The University of Texas M.D.Anderson Cancer Center) were seeded in 100 mm dishes with DMEM supplemented with 10% fetal calf serum. When the cells reached 60−80% confluency, 5 μg of activated *ras* expression vector (generously provided by Dr M.Tainsky at The University of Texas M.D.Anderson Cancer Center) or 5 μg of activated *ras* vector plus 5 μg of either wild-type *p53* or missense *p53* mutant expression vector were transfected using the Lipofectin kit (BRL) according to the manufacturer's instructions. The medium was replaced every 5 days for the cultures at 37.5°C and every 7 days for the cultures at 32.5°C. After 2 weeks (cultures at 37.5°C) or 3 weeks (cultures at 32.5°C), the cells were stained for 30−60 s with 1% crystal violet in 20% ethanol and were rinsed with water. The foci then were counted.

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