Conditional Inhibition of Transformation and of Cell Proliferation by a Temperature-Sensitive Mutant of p53

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Summary

Mutant p53 can contribute to transformation, while wild-type (wt) p53 is not oncogenic and actually inhibits transformation. Furthermore, wt p53 may act as a suppressor gene in human carcinogenesis. We now describe the temperature-sensitive behavior of a particular mutant, p53val135. Like other p53 mutants, it can elicit transformation at 37.5°C. However, at 32.5°C it suppresses transformation, behaving like authentic wt p53. Moreover, the proliferation of transformed cells expressing p53val135 is dramatically inhibited at the permissive temperature. Significantly, the inhibition of both transformation and proliferation is reversible upon temperature upshift. These data demonstrate that the ability of wt p53 to suppress transformation is not due to a general lethal effect, but rather to a reversible growth arrest. p53val135 may prove instrumental for gaining insight into the cellular and molecular properties of wt p53.

Introduction

The p53 protein has frequently been suggested to play a role in cell proliferation and in neoplastic processes (reviewed in Rotter and Wolf, 1984; Oren, 1985; Jenkins and Stürzbecher, 1988). Mutant forms of mouse p53, when cotransfected into primary rodent cells together with activated *ras*, can cause the induction of transformed foci, while such apparent oncogenic activity is not manifested by wild-type (wt) mouse p53 (Finlay et al., 1988; Eliyahu et al., 1988; Hinds et al., 1989). Furthermore, wt p53 can actually inhibit focus formation when introduced into primary rat embryo fibroblasts (REFs) together with various combinations of transforming genes (Finlay et al., 1989; Eliyahu et al., 1989). The latter include *myc* + *ras*, adenovirus E1a + *ras*, and adenovirus E1a + E1b, as well as mutant p53 + *ras*. Various p53 mutants fail to exhibit such a transformation inhibitory activity and sometimes even enhance focus formation (Finlay et al., 1989; Eliyahu et al., 1989). The ability of wt p53 to inhibit focus formation in vitro is consistent with the suggestion that it may act as a tumor suppressor or anti-oncogene.

The notion that wt p53 is a tumor suppressor is strongly suggested by the demonstration that tumor-derived cells, including those from human tumor biopsies, often do not express wt p53. The responsible mechanisms involve chromosomal deletions, gene inactivations, truncations, and point mutations, the latter resulting in the production of mutant rather than wt p53 (Mowat et al., 1985; Wolf and Rotter, 1985; Masuda et al., 1987; Ahuja et al., 1989; Baker et al., 1989; Takahashi et al., 1989; Nigro et al., 1989). The loss of wt p53 expression may therefore play a crucial role in the induction or development of tumors, including major types of human cancer.

While these findings underscore the importance of understanding the mode of action of wt p53, very little progress has been made in this respect. To date, attempts to stably overexpress wt p53 in nonvirally transformed mammalian cells have not been successful, probably reflecting a pronounced selective disadvantage of constitutive wt p53 overexpression. This problem has thus far prevented the establishment of an appropriate system for studying the cellular and biochemical activities of wt p53. A potential way of overcoming this difficulty is by generating cells in which wt p53 activity can be experimentally manipulated. One conceivable option involves the use of inducible transcriptional control elements to drive the expression of wt p53. However, our attempts to produce cell lines overexpressing wt p53 under control of the metal-inducible mouse metallothionein promoter have so far failed (data not shown), probably due to the detrimental effects of the basal levels of wt p53 present in the uninduced state.

An alternative approach toward achieving manipulatable wt p53 activity could potentially rely on the use of a temperature-sensitive (ts) p53 mutant. Such a ts mutant may be expected to possess wt activity at the low temperature but behave like other mutants at elevated temperatures.

We now describe the analysis of a mouse p53 mutant that behaves according to these predictions. This mutant, carrying a substitution from alanine to valine at position 135, cooperates effectively with *ras* in the cotransformation of REFs when assayed at 37.5°C. In this respect, it behaves like other p53 mutants. However, unlike such other mutants, it efficiently suppresses REF transformation by *myc* + *ras* at 32.5°C, similarly to authentic wt p53. Cell lines harboring this mutant are reversibly growth arrested at 32.5°C, suggesting that wt p53 overexpression may inhibit cell proliferation. This mutant thus provides a very promising tool for studying the mechanism of action of wt p53.

Results

Transformation of REFs by p53val135 + *ras* Is Temperature Dependent

Mutant p53, in cooperation with *ras*, can contribute to the transformation of primary REFs while wt p53 cannot. The mutant mouse p53 containing valine instead of alanine at position 135 has been one of the most extensively studied mutants in this system (Finlay et al., 1988, 1989; Eliyahu et al., 1988, 1989; Hinds et al., 1989).

While investigating the biological activity of this mutant, we noted that transformation efficiencies tended to be low-
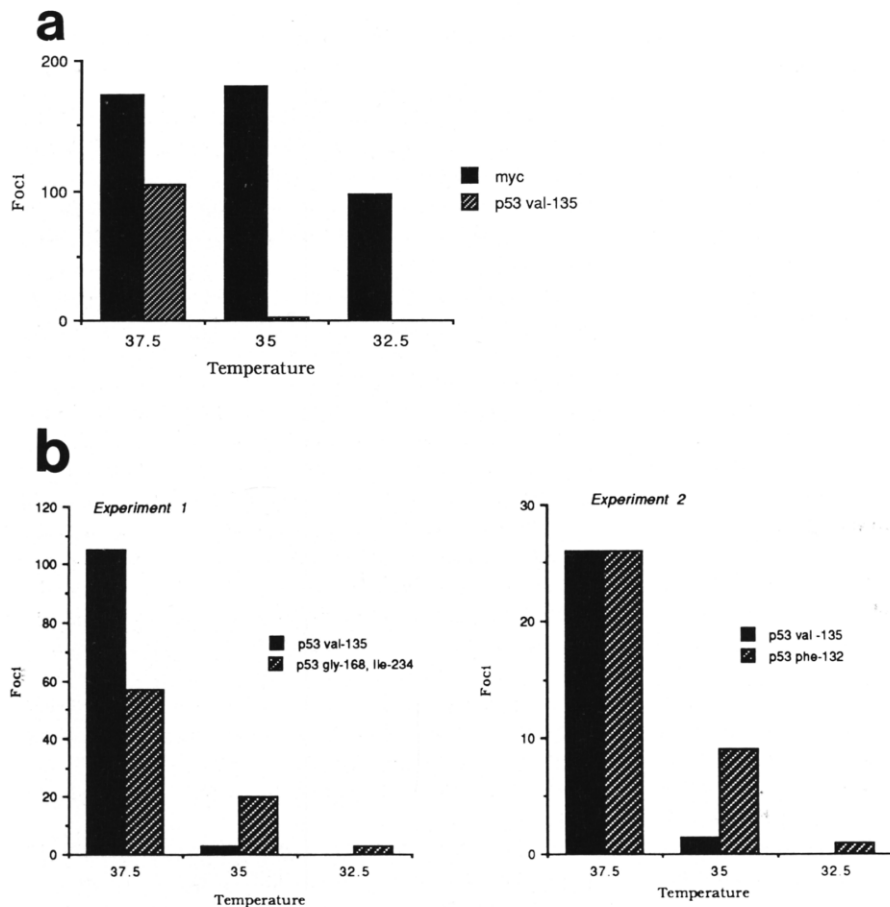
a

b

Figure 1. Effect of Temperature on *ras* + *myc*- and *ras* + p53–Mediated Focus Formation

(a) REFs were transfected at 37.5°C with plasmids encoding *myc* (pLTRmyc, 4 μg per dish) and *ras* (pEJ6.6, 4 μg) or with p53val135 (pLTRp53cGval135, 4 μg) and *ras* (4 μg). Immediately after glycerol shock (14 hr posttransfection), cultures were shifted to the indicated temperature, at which they were maintained for 10 days (*myc* experiment) or for 14 days (p53 experiment). Values on the vertical axis refer to the average number of transformed foci per 90 mm dish.

(b) REF cultures were cotransfected with 4 μg of the *ras* plasmid and 4 μg of a plasmid encoding one of the following p53 mutants: p53phe132 (pLTRp53cGphe132); p53gly168,ile234 (pLTRcgly168,ile234), or p53val135 (pLTRp53cGval135). Following glycerol shock, cells were maintained at the indicated temperature for 14 days.

er when the incubator temperature fell significantly below 37°C. To determine whether p53val135-mediated transformation is indeed temperature dependent, the experiment shown in Figure 1a was carried out. It is obvious that transformation by p53val135 + *ras* was almost completely abolished at 35°C. Furthermore, no transformed foci could be detected at all at 32.5°C. This was clearly not a general feature of *ras*-mediated REF transformation, as focus induction by *myc* + *ras* was as efficient at 35°C as it was at 37.5°C; at 32.5°C, there was only a slight reduction in the number of foci (Figure 1a).

The marked temperature dependence of p53val135-mediated focus formation could be attributed to some cellular property required for p53 but not *myc*-mediated REF transformation. Alternatively, however, it could indicate that p53val135 is a ts mutant of the protein. The latter possibility is of great interest, as a ts mutant of p53 may provide major clues toward understanding the mode of action of this protein. Hence, we compared the transforming ac-

tivity of p53val135 to that of other p53 mutants. The latter represent mutations occurring naturally in the tumor-derived mouse fibrosarcoma cell line Meth A (DeLeo et al., 1979; Bienz et al., 1984; Arai et al., 1986; Eliyahu et al., 1988; Finlay et al., 1988). One such plasmid, pLTRp53c-Gphe132, carries a single point mutation causing a substitution from cysteine to phenylalanine at position 132. This plasmid was generated by site-directed mutagenesis (see Experimental Procedures) and is otherwise identical to pLTRp53cGval135, the p53val135 expression vector used above. The other plasmid, pLTRcgly168,ile234, carries two point mutations at positions corresponding to residues 168 and 234; unlike the two other p53 plasmids, it contains only p53 cDNA and no genomic sequences (see Experimental Procedures).

As seen in Figure 1b, lower temperatures negatively affected focus induction by all three mutants. However, the temperature dependence of transformation was much more pronounced in the case of p53val135. These data
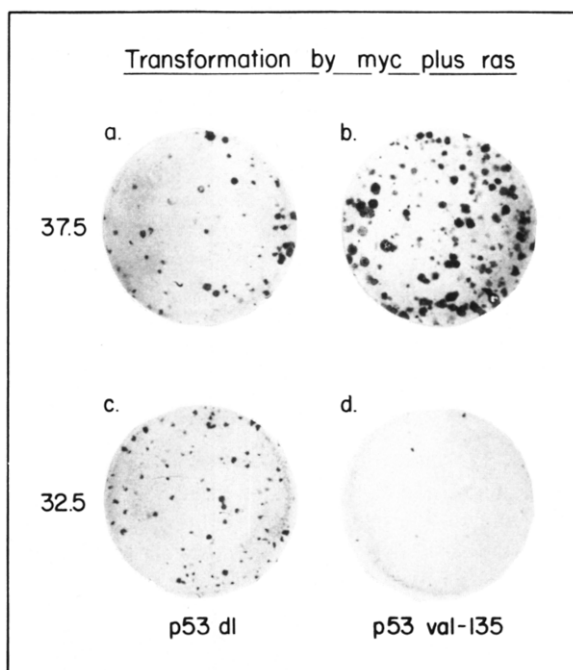
Figure 2. Effect of p53val135 on Transformation by *myc* + *ras* at Different Temperatures

A combination of plasmids encoding *myc* (1 μg) and *ras* (1.5 μg) was cotransfected into REFs together with a plasmid encoding p53val135 (1.5 μg) or with a control plasmid (pLTRp53dl, 1.5 μg). Cells were maintained at 37.5°C for 9 days or at 32.5°C for 13 days, after which cultures were stained with Giemsa stain.

are consistent with the notion that p53val135 may be a ts mutant.

p53val135 Can Suppress Oncogene-Mediated Transformation at 32.5°C

According to the classic definition of a ts mutation, such mutant protein should exhibit wt activity at the low, permissive temperature, while being biochemically impaired at the high, restrictive temperature. Consequently, attempts to determine the ts nature of any particular p53 mutant

should employ a system in which wt p53 activity can be monitored. Obviously, the REF cotransformation assay employed above cannot address this issue conclusively, as wt p53 is completely devoid of any transforming activity in this system (Finlay et al., 1988; Eliyahu et al., 1988; Hinds et al., 1989). On the other hand, wt p53 has been shown to suppress efficiently the ability of various oncogene combinations to elicit foci in transfected REFs (Finlay et al., 1989; Eliyahu et al., 1989), making this a most appropriate system for assessing the temperature sensitivity of p53 mutants. Therefore, we investigated the ability of p53val135 to suppress oncogene-mediated focus formation at different temperatures. As can be seen in Figure 2, the inclusion of a p53val135 plasmid in a *myc* + *ras* transfection greatly eliminated focus formation at 32.5°C, relative to a parallel experiment employing a highly deleted version of this plasmid (p53dl). Interestingly, at 37.5°C, p53val135 markedly enhanced *myc* + *ras*–mediated transformation, as previously reported for E1a + *ras* (Finlay et al., 1989).

To determine whether the properties exhibited in Figure 2 were common to different p53 mutants or unique to p53val135, the effect of two additional mutants on *myc* + *ras* transformation was compared at 37.5°C and at 32.5°C. As seen in Table 1, only p53val135 suppressed focus formation at 32.5°C. This property of p53val135 is very similar to the behavior of wt p53 in the same system (Finlay et al., 1989; Eliyahu et al., 1989; see also Table 1). Hence, these results strongly suggested that p53val135 is indeed a ts mutant of p53, possessing wt activity at 32.5°C. This activity is presumably abolished at 37.5°C, a temperature at which this mutant may actually act in a dominant-negative fashion (Herskowitz, 1987; Stürzbecher et al., 1988; Finlay et al., 1989; Eliyahu et al., 1989).

Reversibility of the Inhibition of Focus Formation by p53val135

The ability of wt p53 to suppress focus formation could be due to a nonspecific lethal effect of wt p53 overproduction, resulting in cell death. Alternatively, it could be a manifestation of a more specific effect of wt p53 on cell proliferation. To distinguish between these possibilities, we took

Table 1. Temperature-Dependent Transformation of REFs by *myc* plus *ras* in the Presence of wt or Mutant p53

| Cotransfected Combination | Foci per Dish | | | | | |
| | Expt. 1 | | Expt. 2 | | Expt. 3 | |
	37.5°C	32.5°C	37.5°C	32.5°C	37.5°C	32.5°C
myc + *ras* + p53dl	215	103	56	18		
myc + *ras* + p53wt	1	0	0	0		
myc + *ras* + p53val135	198	8	99	1	132	19
myc + *ras* + p53cys270	92	83	165	105	50	44
myc + *ras* + p53phe132	77	55	110	63	43	90

REFs were cotransfected with a combination of plasmids encoding *myc* (pLTRmyc), *ras* (pEJ6.6), and one of the p53 variants or a highly deleted version of p53 (pLTRp53dl). Three different mutant p53 forms were employed: p53val135 (encoded by pLTRp53cGval135), p53cys270 (pLTRp53cGcys270), or p53phe132 (pLTRp53cGphe132). In all experiments, 800,000 REFs in a 90 mm dish were transfected with 1 μg of the *myc* plasmid, 1.5 μg of the *ras* plasmid, and 1.5 μg of the p53 plasmid. Transfected cultures were maintained at 37.5°C for 12–14 hr, subjected to glycerol shock, and then grown at the different temperatures (37.5°C or 32.5°C) for 12 additional days. Numerical values denote the average numbers of foci calculated from two parallel cultures in each individual experiment.
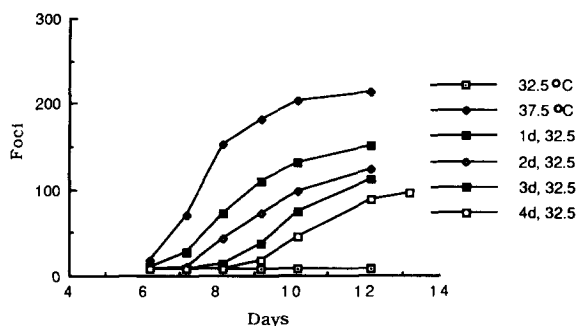
Figure 3. Focus Induction by *myc* + *ras* + p53val135 Following Different Incubation Periods at 32.5°C

REFs were cotransfected at 37.5°C with a mixture of plasmids encoding *myc*, *ras*, and p53val135 (1, 1.5, and 3 μg per 90 mm dish, respectively). Parallel cultures were maintained at either 37.5 or 32.5°C for the entire course of the experiment, or incubated for various intervals (1–4 days) at 32.5°C, following which they were shifted back to 37.5°C. The cumulative number of foci is plotted as a function of the total number of days posttransfection.

advantage of the wt-like activity of p53val135 at 32.5°C. Specifically, we tested whether REFs, transfected with *myc* + *ras* in the presence of p53val135 and maintained for various periods at 32.5°C, could still exhibit focus formation when shifted back to 37.5°C. As seen in Figure 3, while efficient inhibition of focus formation was obtained when cultures were maintained at 32.5°C, foci could develop at 37.5°C even after 4 days of incubation at the low temperature. It should be noted, though, that increased incubation periods at 32.5°C progressively decreased the number of foci eventually developing upon shifting the culture back to 37.5°C. The inhibitory effect of p53val135 at 32.5°C is thus at least partially reversible, arguing against a lethal activity of the overproduced protein.

Proliferation of Cells Transformed by *ras* + p53val135 Is Inhibited at 32.5°C

The inhibitory effect of the wt-like activity of p53val135 on focus formation could be exerted on the initiation of transformation, on transformed cell proliferation, or on both. To address this issue, the proliferation of stable cell lines derived from p53val135 + *ras* foci at 37.5°C was measured at different temperatures. For comparison, a similar analysis was performed on normal REFs and on a REF cell line transformed by *myc* + *ras*. As seen in Figure 4A, the proliferation rates of REFs and clone 51 cells, transformed by *myc* + *ras*, were only mildly affected by low temperatures. On the other hand, the proliferation of lines transformed by *ras* + p53val135 was significantly more sensitive to low temperatures. The effect of temperature was more pronounced in clone 112, which did not proliferate at all at 32.5°C and was markedly inhibited even at 35°C. In clone 2, a significant interference with cell proliferation was seen only at 32.5°C. Most importantly, the conditional growth arrest mediated by p53val135 is specific to this p53 variant as proliferation of cells transformed by p53phe132 + *ras* was not affected significantly at 32.5°C (Figure 4B).

It is noteworthy that no dramatic differences in cell morphology of clones 2 and 112 could be observed at different temperatures under our experimental conditions. However, clone 6 cells, also derived from a p53val135 + *ras* focus (Pinhasi-Kimhi et al., 1986), did exhibit marked morphological changes at 32.5°C. Under these conditions, a significant proportion of clone 6 cells became big and flattened (data not shown). The nature and the extent of this phenomenon are still under investigation.

Stable lines transformed by *myc* + *ras* in the presence of p53val135 are also growth inhibited at 32.5°C (Figure 4C). This is consistent with the effect of cotransfected p53val135 on *myc* + *ras*-mediated focus formation (Figure 2; Table 1).

Cell Cycle Distribution of p53val135 + *ras*-Transformed Cells Arrested at 32.5°C

To characterize further the nature of the growth arrest induced in p53val135 + *ras*-transformed cells at 32.5°C, we examined the cell cycle distribution of various cells at this growth-restrictive temperature. As seen in Figure 5a, the cell cycle distribution of clone 51, transformed by *myc* + *ras*, was not altered at all at 32.5°C. REFs normally multiply more slowly at lower temperatures (Figure 4A). Correspondingly, they exhibited a relative decrease in the proportion of cells in S and G2/M phases of the cell cycle at 32.5°C. The pattern of cell cycle distribution of cells arrested at 32.5°C was complex and varied among individual cell lines. Whereas some, such as clone 112 and clone 2 (Figure 5b), exhibited primarily a G0/G1 arrest with no cells detectable in S phase and a variable proportion found apparently in G2/M, other lines (e.g., clone 26) appeared to be arrested also in S. A similar distribution was also observed among lines transformed by a combination of *myc*, *ras*, and p53val135 (data not shown). Hence, overproduction of wt p53 may cause growth arrest at multiple phases of the cell cycle.

The Arrest of p53val135 + *ras*-Transformed Cell Proliferation Is Reversible

To rule out further a nonspecific lethal effect of p53val135 at 32.5°C, and presumably also of wt p53, we tested whether the growth arrest of stable cell lines at this temperature was reversible. To that end, cells were maintained at the growth-restrictive temperature for different intervals, plated at low density, and shifted to 37.5°C. The relative plating efficiencies, normalized for cells that had not been exposed to the growth-restrictive temperature, are plotted in Figure 6. It should be noted that under our experimental conditions, only a small proportion of the plated cells gave rise to colonies, even when cells were continuously maintained at 37.5°C. Typically, the proportion of colony-forming cells in the absence of exposure to 32.5°C was about 10% and varied somewhat among cell lines. Nevertheless, in all four lines, cells could regain their proliferative capacity even after 72 hr at the growth-restrictive temperature. Thus, the arrest of cell proliferation at 32.5°C can be reversed in a significant proportion of p53val135 + *ras*-transformed cells.
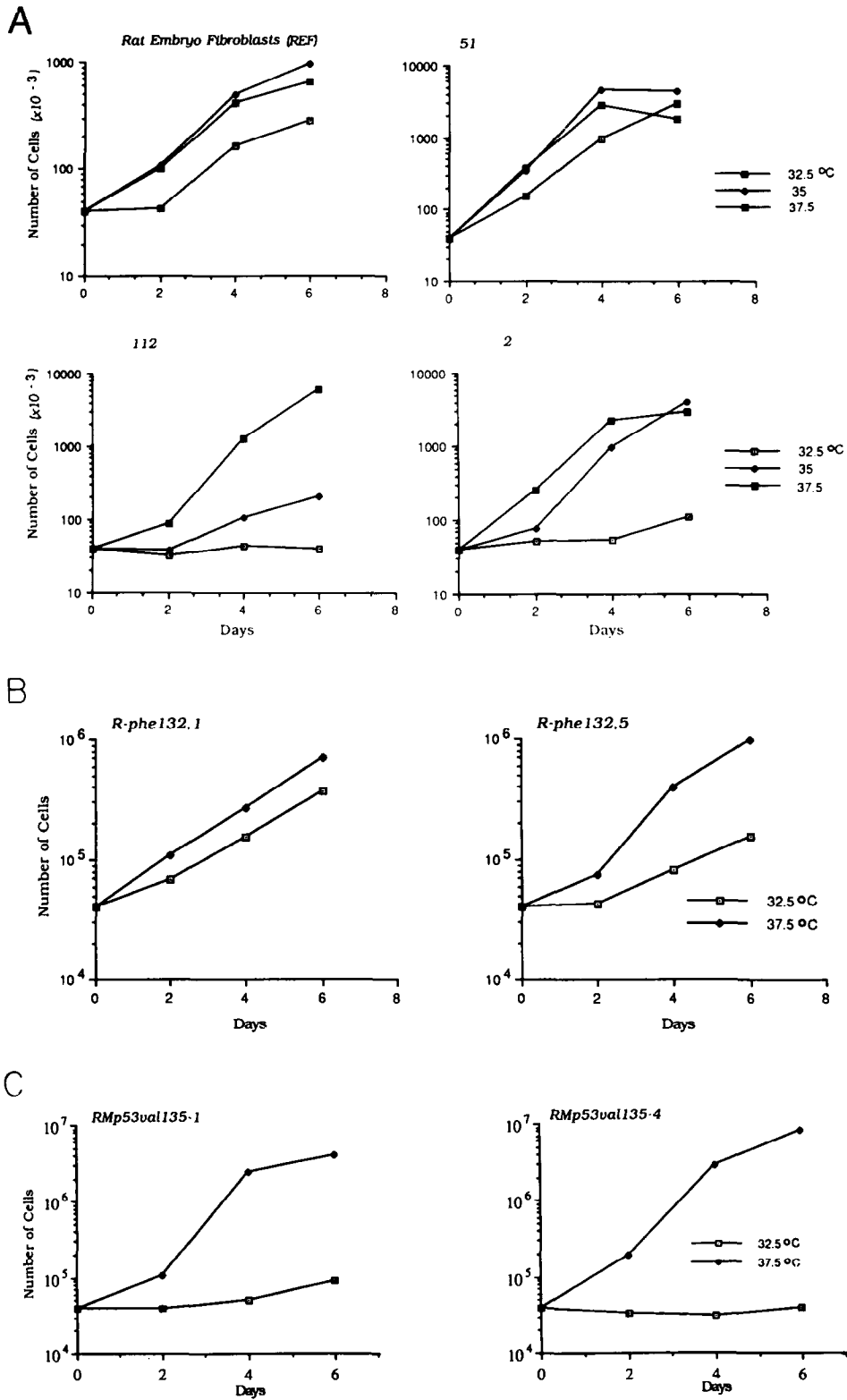
Figure 4. Growth Curves of REFs and of Transformed Cell Lines at Different Temperatures

Cells of the indicated types were plated at a density of 40,000 cells per 60 mm dish and incubated at various temperatures. Cells from parallel dishes were trypsinized and counted every few days.

(A) 51, a line derived from REFs transformed by *myc* and *ras*; 112 and 2, two independently isolated clones of cells derived from REFs that had been transformed by p53val135 + *ras*.

(B) R-phe132,1 and R-phe132,5 are two different cell lines originating in REFs that had been transformed by p53phe132 + *ras*.

(C) RMp53val135-1 and RMp53val135-4 are cell lines that originated in REFs that had been transformed by *myc* + *ras* + p53val135.

a

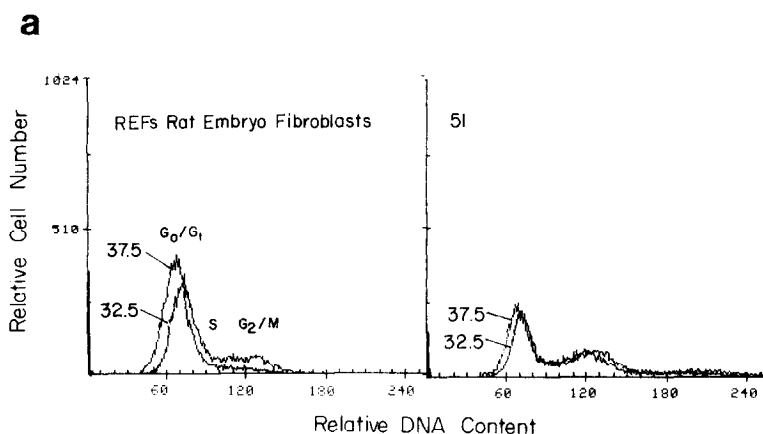**b**

Relative DNA Content

Figure 5. Cell Cycle Distribution of REFs and Transformed Cell Lines Grown at 37.5°C or 32.5°C

(a) Subconfluent cultures of REFs and clone 51 cells were maintained at 37.5°C or 32.5°C for 3 days. Cells were then trypsinized, permeabilized with Triton X-100, and subjected to nucleic acid staining with propidium iodide. Subsequently, cells were analyzed by flow cytometry. (b) Cells (1 × 10⁶) of clones 112, 2 or 26 were plated in 90 mm dishes and transferred immediately to 32.5°C for 3 days. Similarly, 200,000 cells were plated and maintained at 37.5°C. Cells were subjected to flow cytometry as described above.
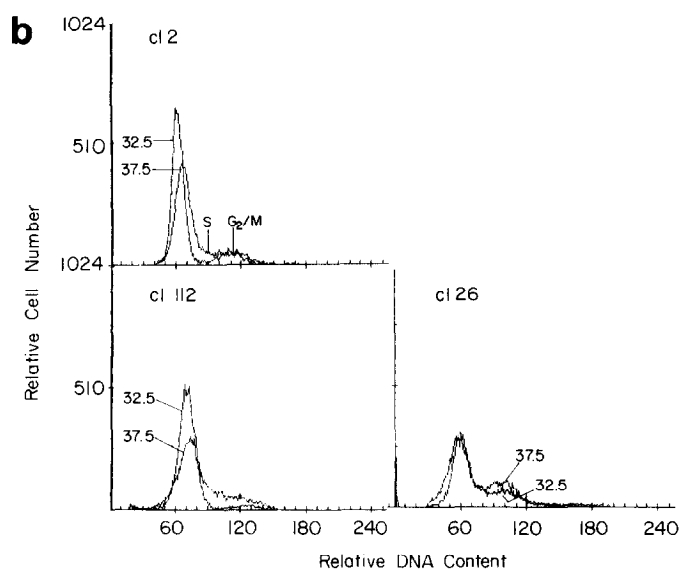
Characterization of p53 in Transformed Lines

The conclusion that p53val135 is growth inhibitory at 32.5°C rests upon the assumption that the protein is still present in significant levels at this temperature. To address this issue, cell lines transformed by a combination of ras with either p53val135 (Figure 7A) or p53phe132 (Figure 7B) were exposed to 32.5°C for increasing periods and subjected to p53 protein analysis. The results indicate that the two lines vary profoundly with respect to the properties displayed by the mutant p53 at the different temperatures. The most obvious difference concerns the formation of a p53–hsc70 complex. In the line overexpressing p53phe-132, the extent of complex formation, as measured by the hsc70/p53 ratio in the PAb421 precipitates, was practically invariant throughout the experiment (Figure 7B). On the other hand, in the line overproducing p53val135, the complex became hardly visible as early as 4 hr after the shift to 32.5°C, and was practically undetectable at later times (Figure 7A). In addition, there was a significant increase in the proportion of p53val135, which can be recognized by PAb246 in cultures exposed to the low temperature. Densitometric scanning revealed that, whereas at 37.5°C
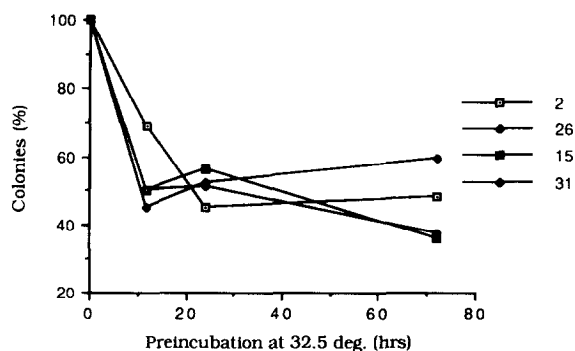

Figure 6. Colony Formation by p53val135 + ras-Transformed Cell Lines at 37.5°C Following Various Preincubation Periods at 32.5°C

Cells (40,000) of each of the four indicated cell lines were plated in 60 mm dishes. Cultures were either grown at 37.5°C for 24 hr (no preincubation) or incubated at 32.5°C for 12, 24, or 72 hr. Following these preincubation periods, cells were trypsinized and counted, and 1500 cells were plated in 90 mm dishes. Cultures were then grown for 12 additional days at 37.5°C, at which time the number of large colonies (>50 cells) was determined. Colonies were stained with Giemsa stain before being counted. The number of colonies visible in the dish that had not been subjected to preincubation at 32.5°C was taken as 100%.

Temperature	37.5°C		32.5°C							
Preincubation (hrs.)			1	1	4	4	12	12	24	24	48
Antibody 246		c	+		+		+		+		+	
421				+		+		+		+	+	+

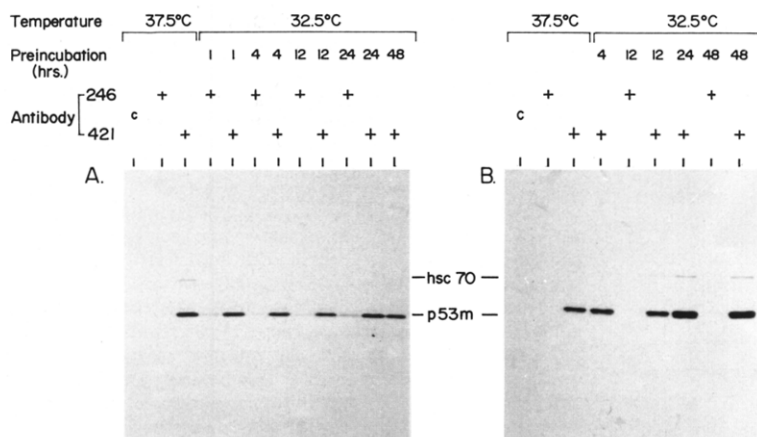| Temperature	37.5°C		32.5°C					
			4	12	12	24	48	48
		c						
			+		+		+	
				+		+	+	+

A.   B.

—hsc 70 —
—p53m —

Figure 7. Protein Analysis of Cells Overexpressing p53val135 or p53phe132 at Various Temperatures

Clone 2 cells (transformed by p53val135 + ras) (A) and R-phe132,4 cells (transformed by p53-phe132 + ras) (B) were either maintained continuously at 37.5°C or preincubated at 32.5°C for various periods and subsequently subjected to protein labeling. Approximately 500,000 cells per 60 mm dish were labeled at 37.5°C or at 32.5°C with 50 μCi of [^{35}S]methionine for 1 hr. p53 was immunoprecipitated from cell extracts containing equal amounts of acid insoluble radioactivity using anti-p53 monoclonal antibodies PAb246 and PAb421 (see Experimental Procedures). In parallel, control immunoprecipitation was carried out using nonimmune culture medium (c). Immunoprecipitated proteins were subjected to SDS–PAGE analysis. The positions of the transfected mouse p53 (p53m) and of the coprecipitating hsc70 are indicated.

only 3% of PAb421-reactive p53 could react with PAb246, this proportion increased to 20% after 24 hr at 32.5°C (data not shown). In the p53phe132 line the proportion of PAb246-positive protein was negligible at both temperatures. Finally, it is noteworthy that there was an apparent increase in the levels of radiolabeled p53 in the case of p53phe132 (Figure 7B), whereas no such effect was observed in the p53val135 line (Figure 7A). Whether this is due to different protein degradation rates is currently under investigation.

Discussion

The experiments described above demonstrate that p53-val135 can exert very diverse biological effects on transfected cells, depending on the temperature at which the culture is maintained. At 37.5°C it can efficiently transform REFs in concert with ras as well as enhance the transformation of REFs by myc + ras. In this respect, it behaves like other mutants described here and in earlier work (Jenkins et al., 1985; Hinds et al., 1987; Finlay et al., 1988, 1989; Eliyahu et al., 1988, 1989). However, unlike the other p53 mutants analyzed in this study, it has absolutely no transforming activity at 32.5°C. Furthermore, at this temperature it can very efficiently inhibit oncogene-mediated focus formation. Thus, it behaves like wt p53 at the low temperature. These findings strongly suggest that p53val-135 is a ts mutant. Interestingly, substitutions from alanine to valine have already been reported to result in ts proteins (Christman et al., 1989; Schmitt et al., 1988).

The interpretation that p53val135 is indeed a ts mutant gains further support from the protein analysis shown in Figure 7. It has been reported that the ability to bind hsc70 tightly is characteristic of mutant forms of p53, but not the wt protein (Finlay et al., 1988; Stürzbecher et al., 1987). In agreement with this fact, p53val135 exhibits a prominent complex with hsc70 at 37.5°C, but this interaction disappears rapidly when the cells are shifted to the growth in-

hibitory temperature, 32.5°C. Similarly, the increased reactivity of p53val135 with PAb246 at 32.5°C is also consistent with a shift to a wt protein conformation (Gannon et al., 1990).

While other p53 mutants tested here do contribute to focus formation at 32.5°C, there is still a marked reduction in numbers as compared to 37.5°C (Figure 1). This may indicate a cell-related temperature-dependent feature that facilitates p53-mediated transformation. For instance, this process may depend on rapid cell proliferation. As noted in Figure 4A, reduction of the temperature to 32.5°C significantly slows down the proliferation rate of untransfected REFs. In any event, this apparent temperature dependence is restricted to transformation by mutant p53 + ras, and it is not exhibited by myc + ras-mediated transformation (Table 1; Figure 2).

Our results indicate that the ability of p53val135 at 32.5°C and, by inference, also of wt p53, to suppress focus formation does not result from a nonspecific lethal effect of the overproduced protein. Rather, it appears to involve a reversible growth arrest, suggesting that overproduced wt p53 can negatively regulate cell proliferation. The growth arrest observed at 32.5°C is sometimes complete, as exhibited by clone 112 (Figure 4A) and additional lines (data not shown). However, in some cases the inhibitory effect may only be partial. The latter situation is evident in clone 2 after prolonged incubation at 32.5°C (Figure 4A) as well as in at least one other line analyzed (data not shown). The basis for the difference in the extent of growth inhibition between individual lines is still unknown. In many cases, the arrested cells accumulate mainly in the G0/G1 phase of the cell cycle. Hence, it is reasonable to assume that p53 plays a specific role at G1. It is noteworthy that when quiescent cultures of untransformed fibroblasts are serum stimulated, there is a marked increase in p53 in late G1 (Reich and Levine, 1984). Furthermore, experiments involving the microinjection of anti-p53 antibodies also suggest that p53 plays a role in the transition

from G0 to S (Mercer et al., 1982, 1984). Since p53 synthesis is not, in itself, modulated significantly in untransformed cells during the cell cycle (Coulier et al., 1985), it is conceivable that the G1-related effects of p53 reflect the cell cycle–regulated expression of a relevant molecular target. Alternatively, they may involve a cell cycle–regulated covalent modification of p53. Such a mechanism has recently been suggested to apply for the Rb protein, which may be active only during G1, and be inactivated by phosphorylation at other phases of the cell cycle (Cooper and Whyte, 1989, and references therein).

There are also cases where a significant proportion of the cells seem to arrest at other phases of the cell cycle in addition to G0/G1. We are currently performing a more detailed kinetic analysis of the arrest in a number of cell lines, which should assist in evaluating the significance of this observation.

In lines such as 112 and 2, the initial transformation event depended on the oncogenic activity of p53val135. Therefore, one could argue that the growth arrest of these lines does not necessarily reflect wt-like inhibitory activity at 32.5°C, but rather simply the loss of a dominant oncogenic function. This argument, however, does not apply to lines such as RMp53val135-1 and RMp53val135-4 (Figure 4C). These findings indicate that the inhibitory effect of p53val135 at 32.5°C is also exerted in cases where focus induction involved the action of myc + ras. Hence, p53-mediated growth arrest seems to override the positive signals provided by these dominant oncogenes. Further work is needed to determine whether p53 and myc, both of which are nuclear, short-lived proteins, may participate in the same regulatory pathway.

Our studies also reveal that, in addition to p53val135 at 37.5°C, two other p53 mutants also fail to suppress oncogene-mediated transformation. Both of these mutants originate in tumor-derived cell lines, further confirming the notion that loss of this "suppressor" activity is positively selected for during tumor development. It would be interesting to find out whether this is a common feature of all p53 mutants found in a growing number of human and rodent tumors.

The mechanism underlying the tumor suppressor activity of wt p53 is practically unknown. We believe that the findings reported here provide a powerful experimental system for investigating this important issue. Another obvious question is whether the loss of "suppressor" activity is always coupled with the ability to cotransform REFs. In principle, these two features may reflect different properties of the mutant p53 molecule, which are induced simultaneously by only some, but not all, mutations. Hence, there may be mutants that have lost "suppression," yet do not elicit focus formation. In vivo selection of such mutants may be a late event in tumor development, requiring the prior loss of the other nonmutated p53 allele. On the other hand, mutants that exhibit transforming activity in REF cells, expressing the endogenous wt rat p53, may be of selective advantage even in tumors that still contain the normal allele. Further studies are needed to clarify this issue.

Experimental Procedures

Plasmids

pLTRp53cGval135 contains a chimera of mouse p53 cDNA and genomic DNA, including introns 2–9, under the transcriptional control of a Harvey sarcoma virus long terminal repeat. This plasmid has been previously referred to as pLTRp53cG (Eliyahu et al., 1985; Kaczmarek et al., 1986). It encodes a mutant protein with a substitution from alanine to valine at position 135 (Finlay et al., 1988; Eliyahu et al., 1988). pLTRp53cGwt, which encodes wt mouse p53, contains alanine at position 135 and is otherwise identical to pLTRp53cGval135. It was constructed from the latter by site-directed mutagenesis, employing the gapped duplex method (Kramer et al., 1984). pLTRp53cGphe132 and pLTRp53cGcys270 were derived from pLTRp53cGwt by site-directed mutagenesis as above. In each case, only one base pair substitution was introduced. These plasmids encode p53 with phenylalanine instead of cysteine at position 132 and cysteine instead of arginine at position 270, respectively. pLTRcgly168,ile234 has been previously described as pLTRp53m (Eliyahu et al., 1989) or pLTRc5 (Eliyahu et al., 1988). This plasmid contains p53 cDNA derived from Meth A fibrosarcoma cells. It contains two point mutations that alter residues 168 and 234 of the protein (Bienz et al., 1984; Eliyahu et al., 1988).

pLTRp53dl is identical to the previously described pLTRcGXK (Kaczmarek et al., 1986). It is a deleted derivative of pLTRp53cGval135 missing the bulk of the protein-coding region. Plasmid pEJ6.6 encodes activated human ras (Shih and Weinberg, 1982). pLTRmyc and pLSVmyc, which encode mouse c-myc, have been described before (Moav et al., 1986; Eliyahu et al., 1984).

Cells and Transfections

Clone 51 is a transformed cell line originating from REFs that had been transfected with myc (plasmid pLSVmyc) and ras (pEJ6.6). Clones 2, 6, 15, 26, 31, and 112 originated from REFs transformed by p53val135 and ras. In the cases of clones 26, 31, and 112, p53val135 is encoded by plasmid pMSVGp53 (Eliyahu et al., 1984), whereas clones 2, 6, and 15 were obtained using plasmid pLTRp53cGval135. R-phe132,1, R-phe-132,4, and R-phe132,5 are lines derived from REFs transformed by p53phe132 and ras (encoded by pLTRp53cGphe132 and pEJ6.6, respectively). RMp53val135-1 and RMp53val135-4 are lines obtained from REFs transformed by myc (pLTRmyc), ras (pEJ6.6), and p53val135 (pLTRp53cGval135).

Low-passage REFs and all cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were incubated at various temperatures in a 5%–6% CO_2 atmosphere. REFs were prepared and transfected, using the calcium phosphate coprecipitation method as described before (Eliyahu et al., 1984).

Cell Cycle Analysis

Cells were trypsinized, permeabilized with Triton X-100 (0.1% final concentration), and stained with propidium iodide (50 μg/ml final concentration) just prior to flow cytometry analysis in the FACS-440 machine (Becton-Dickinson).

Immunoprecipitation and Protein Analysis

Cells were labeled with [^{35}S]methionine, and cell extracts were prepared as described (Maltzman et al., 1981). Equal amounts of trichloroacetic acid–insoluble radioactivity were subjected to immunoprecipitation using the anti-p53 monoclonal antibodies PAb421 (Harlow et al., 1981) and PAb246 (Yewdell et al., 1986). Immunoprecipitated proteins were separated by SDS–PAGE on 12.5% gels (Maltzman et al., 1981). Gels were fluorographed with 2,5-diphenyloxazole-dimethyl sulfoxide, dried, and exposed to Agfa Curix X-ray film.

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Note Added in Proof

Longer exposures of the autoradiogram in Figure 7, as well as additional experiments, show that incubation at 32.5°C causes a several-fold decrease, rather than absolute disappearance, of coprecipitated hsc70 in p53val135 lines.