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# Induction of Growth Arrest by a Temperature-Sensitive p53 Mutant Is Correlated with Increased Nuclear Localization and Decreased Stability of the Protein

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**A temperature-sensitive mutant of p53, $p53^{Val-135}$, was found to be able to arrest cell proliferation when overexpressed at 32.5°C. While much of the protein was cytoplasmic in cells proliferating at 37.5°C, it became predominantly nuclear at 32.5°C. Concomitantly, $p53^{Val-135}$ became destabilized, although not to the extent seen in primary fibroblasts.**

The cellular protein p53 has been implicated in the control of cell proliferation and tumor progression. In its wild-type (wt) form, it probably acts as a tumor suppressor, whereas various mutant forms exhibit oncogenic activities (for recent reviews, see references 9, 18, 20, and 21). In most cases studied so far, p53 behaves as a distinctly nuclear protein (2, 4, 13, 28), although its intracellular distribution can be altered by certain mutations (27). One system that has been especially informative in the biological characterization of p53 involves transformation of primary rat embryo fibroblasts (REFs). In this system, certain p53 mutants can elicit transformed foci when cotransfected together with *ras*; stably transformed cell lines have been established from many such foci (6, 8, 11, 17, 24). Conversely, plasmids encoding wt p53 suppress oncogene-mediated transformation (7, 10). While the precise mechanisms responsible for these grossly diverse properties of mutant and wt p53 are unclear, potential promise is carried by the recent identification of a temperature-sensitive mutant of p53, $p53^{Val-135}$ (23). At 37.5°C, $p53^{Val-135}$ cooperates efficiently with *ras* in the transformation of REFs. On the other hand, at 32.5°C it behaves like wt p53 and strongly inhibits oncogene-mediated transformation. Moreover, cells expressing $p53^{Val-135}$ become growth arrested when shifted to 32.5°C.

In an attempt to identify properties of $p53^{Val-135}$ that are altered concomitantly with the induction of its growth-inhibitory activity, we determined the intracellular localization of the protein at each temperature. Cells transformed by $p53^{Val-135}$ plus *ras* were analyzed by indirect immunofluorescence (12), using the p53-specific monoclonal antibody PAb421 (16). At 37.5°C, a substantial fraction of the protein was found in the cytoplasm (Fig. 1a). Variations among individual cells could be observed, with respect to both the overall intensity of staining and the relative intensity of the nuclear compartment. Upon the shift to 32.5°C, almost all the p53 became nuclear (Fig. 1b and c); a weakly positive signal was discernible in the cytoplasm of some of the more intensely staining cells. A predominantly nuclear localization of p53 could be detected as early as 4 h after the shift to 32.5°C (Fig. 1b). Similar results were obtained with another line transformed by $p53^{Val-135}$ plus *ras* (Fig. 1d and e).

To determine whether this behavior was a unique property of $p53^{Val-135}$, we analyzed the staining pattern of p53 in a cell line transformed by *ras* plus a different mutant, $p53^{Phe-132}$. At 37°C (Fig. 1f), the distribution of p53 in these cells was very similar to that seen in the $p53^{Val-135}$ lines. However, this pattern was not altered when the cells were incubated at 32.5°C (Fig. 1g). Cells overexpressing $p53^{Phe-132}$ do not arrest at 32.5°C (23). Thus, there was a clear correlation between the increased nuclear localization of the protein and its ability to exert a growth-inhibitory effect.

The cells used in the previous experiments exhibited a transformed phenotype at 37.5°C as a result of the additional presence of activated Ha-*ras*. To determine whether the latter fact had any relevance for the observed relocalization of $p53^{Val-135}$, a similar experiment was performed with a cell line derived by immortalization of REFs with $p53^{Val-135}$. These cells are nontransformed, and their proliferation was very efficiently inhibited at 32.5°C (Fig. 2). The results (Fig. 3a and b) were practically identical to those obtained for the corresponding transformed lines.

We have recently characterized a novel tumor-derived mouse p53 mutant. This mutant protein, $p53^{Cys-270}$, is very stable, does not possess any growth-inhibitory activity, and, unlike $p53^{Val-135}$ and $p53^{Phe-132}$, does not associate with hsc70 (15). Cells derived by transformation of REFs by a combination of $p53^{Cys-270}$ and *ras* were next subjected to analysis by immunofluorescence, following incubation at either 37.5 or 32.5°C. Unlike the two other mutants described above, $p53^{Cys-270}$ was strictly nuclear at 37.5°C, and an identical pattern was retained at 32.5°C (Fig. 3c and d).

In primary cells, wt p53 exhibits an extremely short half-life ($t_{1/2}$) (25). In primary REFs, the $t_{1/2}$ of p53 is approximately 15 min (5). On the other hand, $p53^{Val-135}$ is much more stable, at least in transformed cells (11, 14, 19). In view of the observed intracellular distribution of $p53^{Val-135}$ at 37.5°C, one could propose that its extended half-life is due to the exclusion of a major fraction of the protein from the nucleus. At 32.5°C, practically the entire population of $p53^{Val-135}$ becomes nuclear. In addition, much less hsc70 is associated with $p53^{Val-135}$ at 32.5°C than at 37.5°C; such association has been shown to correlate with p53 stabilization (11). One could therefore anticipate that $p53^{Val-135}$ would be far more labile at 32.5°C. Consequently, a pulse-chase experiment was performed (Fig. 4). Clone 112 cells (see Fig. 1) were plated at a density of $2 \times 10^5$ per 90-mm dish 3 days prior to the onset of the experiment, maintained at 37.5°C or shifted to 32.5°C 14 h before addition of the radioisotope, and then pulse-labeled with [$^{35}$S]methionine

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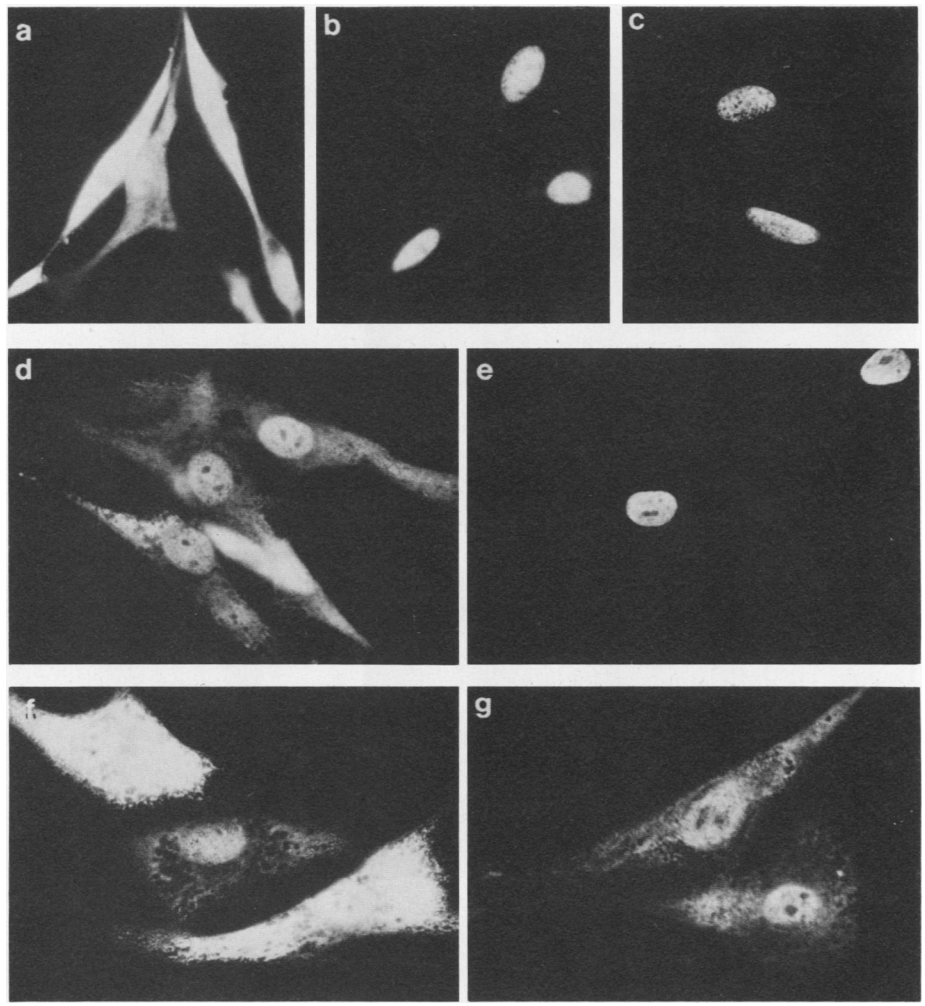


FIG. 1. Immunofluorescent staining of p53 in transformed cells. Clones 112 and 2 are independent lines derived from REFs following transformation by *ras* plus $p53^{Val-135}$. R-phe132-5 is derived from REFs transformed by *ras* plus $p53^{Phe-132}$. Cells were stained with monoclonal antibody PAb421 (see text). (a) Clone 112, grown at 37.5°C; (b) clone 112, 4 h after the shift to 32.5°C; (c) clone 112, 24 h at 32.5°C; (d) clone 2, 37.5°C; (e) clone 2, 24 h at 32.5°C; (f) R-phe132-5, 37.5°C; (g) R-phe132-5, 24 h at 32.5°C.

(40 μCi, 30 min) and chased at the respective temperatures. Protein analysis with the aid of the anti-p53 monoclonal antibody PAb421 was performed as described before (22). $p53^{Val-135}$ did possess a shorter $t_{1/2}$ at 32.5°C than at 37.5°C (1 h versus 3 h; Fig. 4). In repeated experiments, $p53^{Val-135}$ was 1.5- to 3-fold more labile at the growth-inhibitory temperature (data not shown). Nevertheless, even under those conditions it was significantly more stable than the wt p53 of primary REFs.

The findings presented above extend the recent observation, based on the study of F9 embryonal carcinoma cells, that p53 in the wt conformation is preferentially located in the nucleus (13). However, unlike F9, in this case the enhanced nuclear localization of p53 is tightly correlated with the ability of the protein to effect growth arrest. These results can be taken as further evidence for the temperature-sensitive nature of $p53^{Val-135}$ and for the conclusion that it possesses wt p53-like properties at 32.5°C.

The mechanism responsible for the alterations in the distribution of $p53^{Val-135}$ is unknown. At 37.5°C, a large fraction of the protein is complexed with hsc70, and the location of such complexed p53 molecules may be dictated

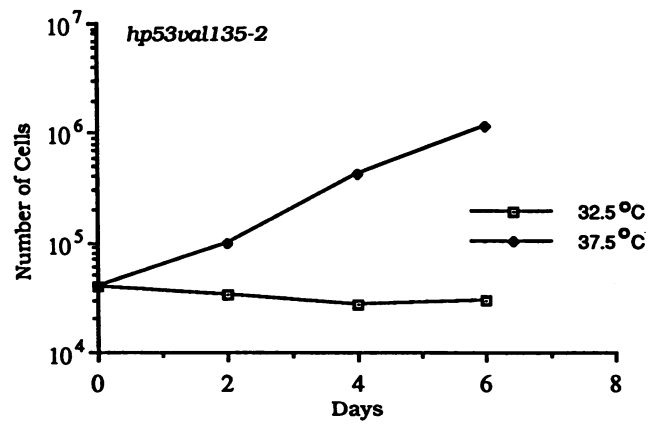


FIG. 2. Growth at different temperatures of REFs immortalized by $p53^{Val-135}$. Line hp53val135-2 was generated by cotransfecting REFs with $p53^{Val-135}$ plus plasmid pSV2hph, which confers resistance to hygromycin B, and selecting drug-resistant immortalized clones. Cells were plated at a density of $4 \times 10^4$ per 60-mm dish, and cultures were maintained at 32.5 or 37.5°C for the indicated number of days before trypsinization and counting.

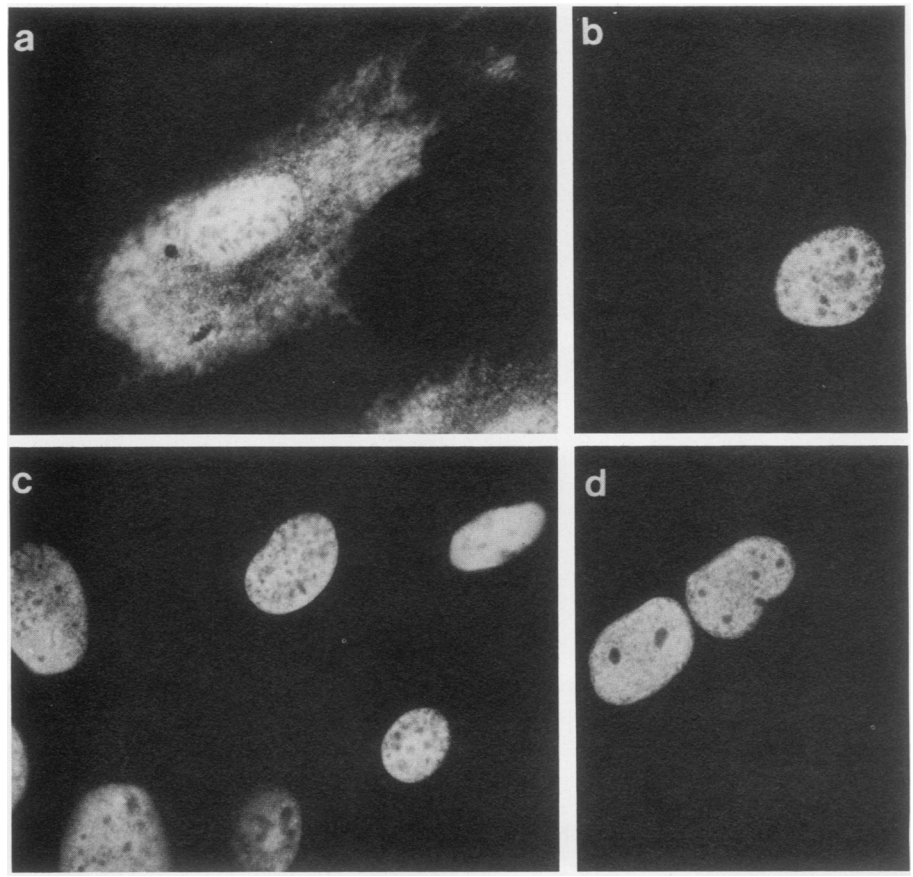


FIG. 3. Immunofluorescent staining of p53 in immortalized cells and in transformed cells. Line hp53val135-2 is described in the legend to Fig. 2. Line Rp53cys270-2 is derived from REFs transformed by *ras* plus $p53^{Cys-270}$. (a) hp53val135-2, 37.5°C; (b) hp53val135-2 kept at 32.5°C for 24 h; (c) Rp53cys270-2, 37.5°C; (d) Rp53cys270-2, 24 h at 32.5°C.

by the distribution of hsc70 (26). Upon the shift to 32.5°C, there is a marked reduction in the proportion of hsc70-bound p53 (23), which may now release free p53 for transport into the nucleus. Alternatively, the conformational change induced at 32.5°C may restore an intrinsic property of p53, which causes it to localize efficiently in the nucleus. p53 is phosphorylated in vitro, and probably also in vivo, by the Cdc2 kinase, and it has been suggested that such phosphorylation might affect the subcellular localization of p53 (1, 3). It would be of interest to test whether the interaction of Cdc2 with $p53^{Val-135}$ differs at the two temperatures.

The relocalization of p53 is an early event, already visible 4 h after temperature downshift. Therefore, it may play a key role in initiating the growth arrest. One could thus conclude that the molecular target(s) upon which wt p53 has to impinge in order to exert its antiproliferative effect is probably located in the nucleus. Nevertheless, nuclear localization per se is not sufficient for growth arrest, as is evident from the behavior of cells harboring $p53^{Cys-270}$. This finding implies that a discrete nuclear activity of p53, rather than its mere presence in the nucleus, is required to affect cell growth. One possibility is that $p53^{Cys-270}$, while retaining functional nuclear transport signals (1), fails to interact properly with a pertinent molecular target. It is noteworthy that $p53^{Cys-270}$ is deficient in its ability to bind the simian virus 40 large T antigen (15). Hence, this viral protein and the putative cellular target may recognize similar structural aspects of p53.

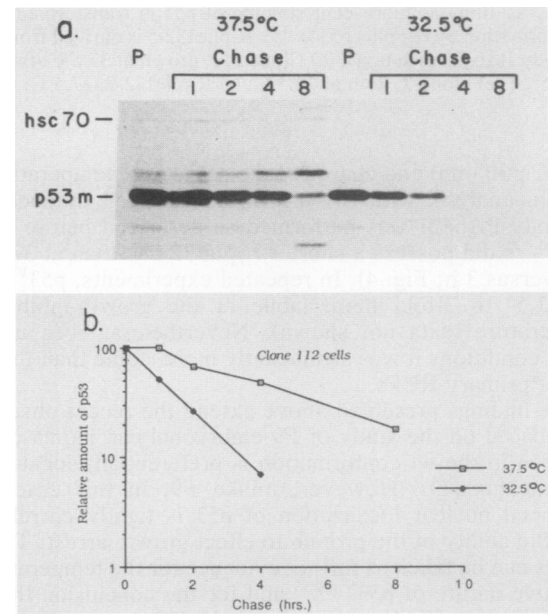


FIG. 4. Pulse-chase analysis of p53 in clone 112 at different temperatures. See text for experimental details. (a) Autoradiogram of the protein gel. Numbers on top indicate chase periods (in hours). p53m, Mouse p53. (b) Graphic representation of the data, obtained by densitometric scanning of the autoradiogram.

Finally, we show that nuclear localization of a wt-like p53, while correlated with a decreased $t_{1/2}$, is not enough to ensure very rapid degradation. The cells used here greatly overexpress the protein. Hence, at 32.5°C the proteolytic machinery may be saturated and become rate limiting. Alternatively, we would like to suggest that rapid degradation is coupled with actual activity; i.e., p53 is degraded while, or as a result of, interacting with a relevant target. Possibly, while most of the $p53^{Val-135}$ may be in the wt conformation at 32.5°C, only part of it participates in such interactions (e.g., due to limiting targets) and is therefore still degraded slowly. The extremely long $t_{1/2}$ of $p53^{Cys-270}$ (13, 15) appears to support this possibility.

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