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Permanent expression of p53 in FR 3T3 rat cells but cell cycle-dependent association with large-T antigen in simian virus 40 transformants

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The p53 oncogene is thought to play a role in the proliferation of normal and transformed cells and its expression was postulated to be cell cycle dependent. Using flow cytofluorimetry sorting of populations of exponentially growing cells, coupled to a radioimmune assay, we have investigated the accumulation of p53 along the cell cycle in normal FR 3T3 rat cells as well as in two types of SV40-transformed derivatives, one of which only expresses the large-T protein during the G2 phase of the cell cycle. p53 was accumulated at a constant level throughout the cell cycle in FR 3T3 cells. Its level and stability increased to different extents in the two types of transformants. However, the formation of complexes between p53 and large-T was modulated by the G2-restricted accumulation of large-T, thus leading to a differential increase in the levels of p53 both in exponentially growing cells and along the cell cycle. This increase in the levels of p53 appeared to be regulated at a post-transcriptional level.

Key words: cell cycle/transformation/p53 × large-T complexes/oncogene/SV40

Introduction

The protein encoded by the p53 oncogene is a short-lived (Mora *et al.*, 1982; Reich and Levine, 1984) phosphoprotein (Jay *et al.*, 1980; Crawford *et al.*, 1980) and is highly conserved in evolution (Jornvall *et al.*, 1982). It is usually present in low amounts in normal cells. When resting cells are mitogen- or serum-stimulated, an increase in the steady-state levels of p53 is observed prior to cellular DNA synthesis (Milner and Milner, 1981; Reich and Levine, 1984), suggesting a role for p53 in the progression of cells from a resting (G0 or G1) to an actively dividing state. In addition, microinjection of monoclonal antibodies directed against p53 blocks the serum-induced stimulation of cellular DNA synthesis (Mercer *et al.*, 1982, 1984). In contrast with the situation found in normal cells, p53 is generally present in elevated levels in cells transformed by a variety of agents like DNA and RNA viruses, and carcinogens (Klein, 1982; Crawford, 1983 for review). p53 levels are also elevated in variously induced primary tumors and tumor cell lines, with some exceptions.

In SV40-transformed cells, p53 is associated with the large-T protein (Lane and Crawford, 1979), in the form of non-covalent complexes (McCormick and Harlow, 1980). It appears that nearly all of p53 is complexed with T-antigen in the mouse and rat cell lines that have been analyzed (Gurney *et al.*, 1980; McCormick *et al.*, 1980; Greenspan and Carroll, 1981; Harlow *et al.*, 1981; Freed *et al.*, 1983). Association of p53 with large-T increases the half-life of the protein (Oren *et al.*, 1981; Mora *et al.*, 1982), resulting in high levels of large-T × p53 complexes, which may act in a positive way to maintain the unregulated growth of the transformed cell (Levine *et al.*, 1982). Recent studies have shown that p53 can cooperate with the *ras* oncogene in the transformation of primary rat embryo fibroblasts, and have thus suggested similar functions for p53 and c-*myc* (Eliyahu *et al.*, 1984; Parada *et al.*, 1984). However, the respective roles of p53 and large-T in the establishment and maintenance of the transformed state remain unclear (Colby and Shenk, 1982; Lane, 1984).

Infection of FR 3T3 rat cells (Seif and Cuzin, 1977) with an *A* group temperature-sensitive mutant of SV40 (SV40 *tsA30*) under controlled conditions, gave rise to two types of transformants: type N cells, which exhibit a conditionally transformed phenotype, i.e., they revert to a normal phenotype upon shift to the restrictive temperature, and type A cells, which remain transformed at this temperature (Rassoulzadegan *et al.*, 1978). We have shown previously that among these transformants, type N cells exhibit an accumulation of the large-T protein restricted to the G2 phase of the cell cycle, whereas type A cells synthesize T-antigen continuously (Imbert *et al.*, 1983a, 1984). In addition, large-T appears to be stabilized in type A cells (Imbert *et al.*, 1983b), in all likelihood through interaction with p53. By contrast, large-T is very unstable in type N cells.

In the present study, we have measured the half-life of p53 in normal and SV40-transformed FR 3T3 rat cells, and have determined the levels of p53 along the cell cycle in the various cell lines. p53 was expressed at a constant level during the cell cycle in actively growing FR 3T3 cells, but the association between p53 and large-T was found to be modulated by the cell cycle-dependent expression of large-T in type N cells.

Results

Stability of p53 and large-T in normal and transformed cells

FR 3T3 cells and two SV40 transformants representative of each type, namely SV-*tsA30*-An3 and SV-*tsA30*-N1, were labelled with [^{35}S]methionine, and the label was chased in cold medium for increasing periods of time. The cell extracts were immune precipitated sequentially with anti-p53 monoclonal antibodies PAb 421 (Harlow *et al.*, 1981) and then with anti-SV40 T-antigen hamster serum. Control experiments have shown that upon a 1-h [^{35}S]methionine labelling, all of p53 and no large-T were immune precipitated with PAb 421 (data not shown), whereas upon a long (4 h) labelling period, cross-immunoprecipitation was observed: PAb 421 precipitated a small fraction of large-T, and a small fraction of p53 was precipitated with PAb 416, a monoclonal antibody directed against the SV40 large-T protein (Harlow *et al.*, 1981). This could suggest that these two proteins are only weakly associated in FR 3T3-SV40 transformants. The immune precipitated proteins were analyzed by polyacrylamide gel electrophoresis and the half-lives of the two proteins were determined graphically after scanning of the autoradio-

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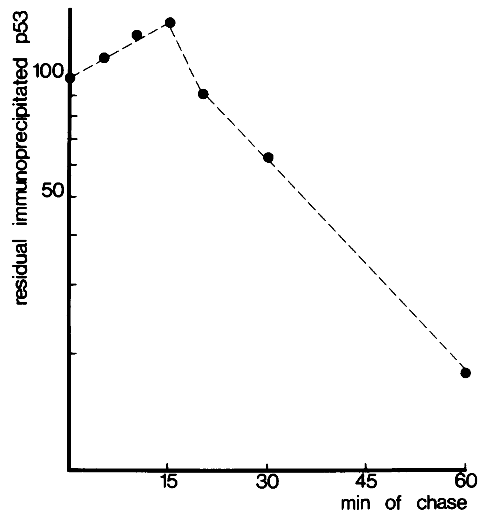

Fig. 1. Stability of p53 in normal FR 3T3 rat cells. Exponentially growing cells were labelled with [^{35}S]methionine and the label chased for increasing periods of time as described under Materials and methods. The autoradiograms were scanned, and the amounts of labelled p53 were plotted as a function of the time of chase.

Table I. Determination of the half-lives of the large-T and p53 proteins[a]

Cell line	Large-T		p53	
	33°C	40.5°C	33°C	40.5°C
SV-*tsA30*-N1	20	15	35	20
SV-*tsA30*-An3	>90	65	>90	70
FR 3T3			17	[b]

[a]Cells grown at either 33 or 40.5°C were labelled with [^{35}S]methionine, the label chased for increasing periods of time, and p53 and large-T were immune precipitated as described under Materials and methods. The half-lives are expressed in minutes.
[b]Not determined.

grams. In FR 3T3 cells (Figure 1), the half-life of p53 was <20 min; it increased to ~30 min in type N cells at the permissive (33°C) temperature (Table I). The half-life of large-T was similar to that of p53 in these cells. In contrast, in type A cells, the two proteins exhibited increased stabilities: >90 min at 33°C and ~1 h at 40.5°C (Table I). The two proteins therefore appeared to have a common fate in each type of transformant.

Determination of the levels of p53, large-T and p53 × large-T complexes in the various cell lines by radioimmune assay (RIA)

Among the monoclonal antibodies directed against the mouse p53, only two, PAb 122 (Gurney *et al.*, 1980) and PAb 421 (Harlow *et al.*, 1981), reacted with the rat p53. However, these two antibodies recognize determinants close to each other on the p53 molecule (Benchimol *et al.*, 1982; Wade-Evans and Jenkins, 1985), which precluded their use in a RIA. The alternative approach developed for the titration of the human p53 (Benchimol *et al.*, 1982), was therefore adopted. PAb 421 monoclonal antibodies were used in the first step, and in the second step, a polyclonal serum from breast cancer patients was added to the reaction wells. The human determinants were recognized by ^{125}I-labelled 8a4 monoclonal antibodies, directed against the Fc portion of human IgG (Lowe *et al.*, 1982). Sera from breast cancer patients have been shown, by immune precipitation, to recognize the mouse p53 (Crawford *et al.*, 1982) as well as the rat p53 (our unpublished results). The amount of serum required to set

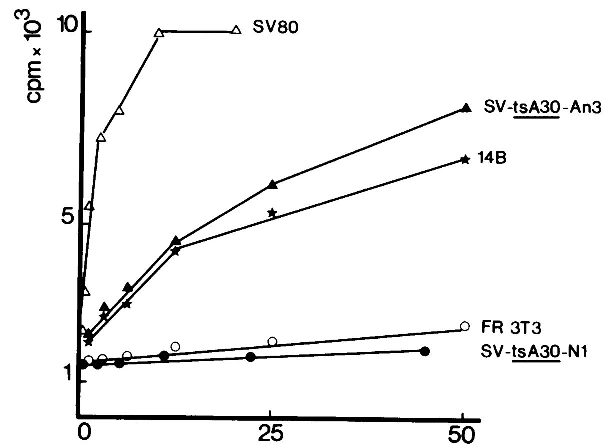

Fig. 2. Radioimmune assay for the titration of p53 in the various cell lines. The RIA was a modification of that described for the human p53 by Benchimol *et al.* (1982). The relative amounts of p53 were determined from the slopes of the curves. The figure shows the results of one experiment.

Table II. Relative levels of p53, large-T and large-T × p53 complexes during the cell cycle

Cell line		E[a]	G1	S	G2
FR 3T3	Total p53[b]	5	4	6	5
SV-*tsA30*-N1	Total p53	5	1	3	10
	Total T[b]	23	4	12	43
	T × p53[b]	10	ND[c]	5	26
SV-*tsA30*-An3	Total p53	39	34	38	48
	Total T	106	71	95	126
	T × p53	58	48	60	68

[a]E corresponds to cells in exponential growth.
[b]Amounts of p53, large-T and large-T × p53 complexes were determined by radioimmune assays as described under Materials and methods, and are expressed relative to those in the 14B cell line, taken as reference. The figures represent the mean values of several independent experiments.
[c]Non-detectable.

up a quantitative radioimmune assay for the rat p53 was determined in preliminary experiments (data not shown).

Figure 2 shows the results obtained with this combination of antibodies. The lower amount of p53 detectable in our cells was decreased to 0.6% of the amount detected in SV80 cells (SV40-transformed human cells, Todaro *et al.*, 1966), taken as a reference in these determinations (Benchimol *et al.*, 1982). By comparison, the amount of p53 in the SV40-transformed 14B rat cell line (Botchan *et al.*, 1976), was only 14% of the amount in SV80 cells. 14B was taken as an external standard in further determinations. FR 3T3 cells, as well as the type N transformant, contained a low level of p53 (Table II), whereas an 8-fold increase in the amount of p53 was noticed in the A transformant.

Another RIA involving two monoclonal antibodies directed against large-T, PAb 416 and PAb 419 (Harlow *et al.*, 1981), was used to measure the relative levels of large-T in the different transformants (Table II). SV-*tsA30*-N1 cells contained low levels of large-T, in agreement with its G2-restricted accumulation, in contrast with the high amounts present in type A cells.

The amount of p53 × large-T complexes was measured in the two types of transformants with a third RIA, as described under Materials and methods. In actively growing cells, p53 × large-T complexes were found to be present in 6-fold higher amounts in type A than in type N transformants (Table II). This result could be related to the G2-restricted expression of large-T in type

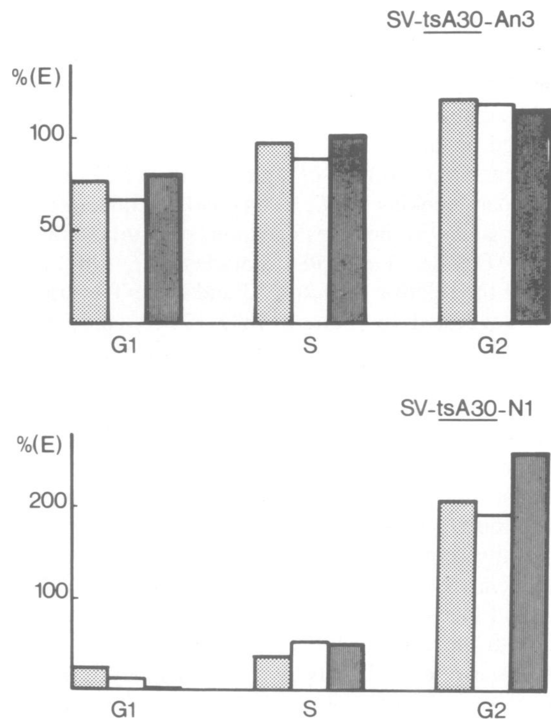

Fig. 3. Cell cycle variation of the amounts of p53, large-T and p53 × large-T complexes in the two types of transformants. The amount of p53 (dotted), large-T (white) and p53 × large-T complexes (hatched) were calculated, for each cell line, from the data listed in Table II, but were expressed as a percentage of the values determined for these parameters in the exponential phase of growth, respectively.

N transformants; to test this hypothesis, we titrated the relative levels of p53 × large-T complexes in the various phases of the cell cycle in both types of transformants.

Steady-state levels of p53, large-T and large-T × p53 complexes in the various phases of the cell cycle

Cells, labelled *in vivo*, were sorted as a function of their DNA content into G1, S and G2 fractions using a flow cytofluorimeter (Imbert *et al.*, 1984), and the amounts of p53 in the various fractions were determined by RIA. Low and not significantly different amounts of p53 were detected in the three fractions isolated from FR 3T3 cells, indicating that p53 levels remained roughly constant throughout the cell cycle (Table II). p53 was clearly more abundant in the G2 than in the G1 or S phases in type N cells (Figure 3). In contrast, high levels of p53 were found in all the phases of the cell cycle in type A cells, with a slight increase in the total amounts from G1 to G2 (Table II and Figure 3).

The variations in the steady-state levels of large-T along the cell cycle were determined (Table II). The amount of large-T increased with respect to cell size, from G1 to G2 cells in the type A transformant, whereas a significant amount of large-T was only detected in the G2 phase in the N transformant.

p53 × large-T complexes were found in all the phases of the cell cycle in type A cells (Table II and Figure 3). The relative amount of complexes in G2 as compared with G1 was 1.4, a value identical to the relative amount of total p53 in these phases, respectively. By contrast, in type N cells, complexes were not detectable in G1, but mainly found in the G2 phase, and to a much lesser extent in the S phase.

Steady-state levels of p53 mRNA along the cell cycle

The steady-state levels of p53 mRNA in the various cell lines were determined by dot blot hybridization using cytoplasmic

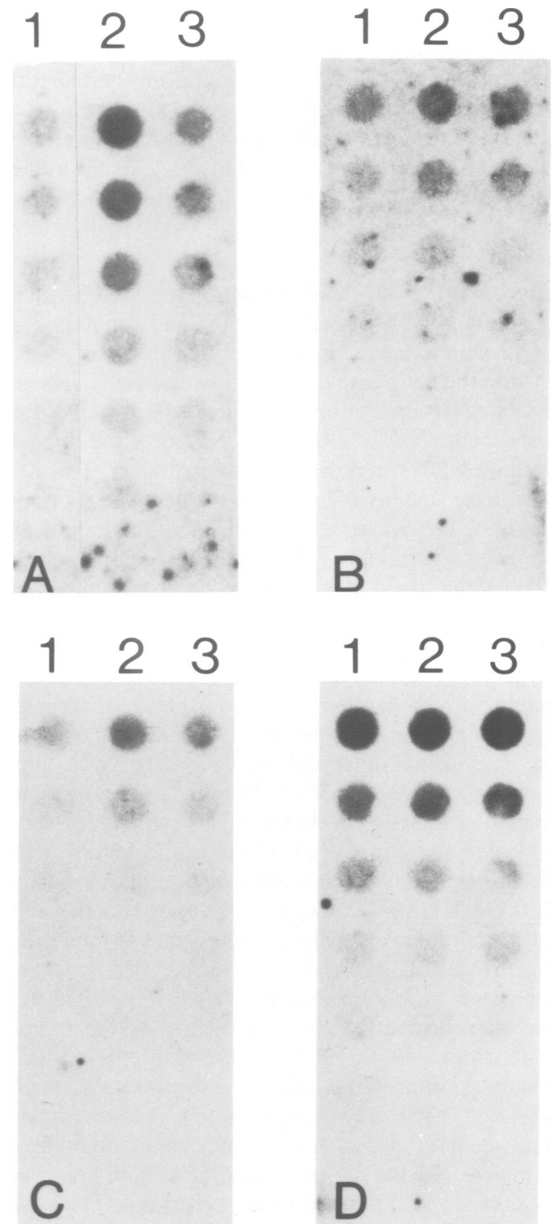

Fig. 4. Steady-state levels of p53 mRNA in the various cell lines in exponential growth (**A**) and in sorted cell fractions (**B – D**). Cytoplasmic RNA was isolated from exponentially growing cells or from sorted cell fractions, denatured, serially diluted and filtered onto nitrocellulose sheets. The dot blots were hybridized with a combination of ^{32}P-labelled pp53-271 and C1 9 cDNA clones as described under Materials and methods. (**A**) **Lanes 1 – 3** contained cytoplasmic RNA extracted from 5 × 10^6 cells of the FR 3T3, SV-*tsA30*-N1 and SV-*tsA30*-An3 cell lines, respectively. (**B – D**) Correspond to the sorted G1, S and G2 (**lanes 1 – 3**, respectively) fractions of FR 3T3 (**B**), SV-*tsA30*-N1 (**C**) and SV-*tsA30*-An3 (**D**) cell lines. For each cell line, the three fractions were standardized in number of cells. In the experiment ilustrated above, 1.15 × 10^6 FR 3T3 cells and 2 × 10^5 SV-*tsA30*-N1 and SV-*tsA30*-An3 cells in each phase of the cell cycle were used. The data shown above are from different experiments with internal controls.

RNA preparations made from actively growing cells. The specificity of hybridization of the two murine p53 cDNA clones used, pp53-271 (Oren *et al.*, 1983), and C1 9 (Benchimol *et al.*, 1984) to rat p53 mRNA was ascertained by Northern blotting: both probes revealed a single band of ~2 kb (data not shown). The amounts of p53 mRNA were similar in the normal and transformed cells in active growth (Figure 4A). Moreover, the

pattern of accumulation of p53 RNA as a function of the phase of the cell cycle was similar in all the cell lines tested (Figure 4B–D), including FR 3T3 cells. Therefore the increase in the levels of p53 in the transformed cells must result from the stabilization of p53 through its association with large-T, such an association being restricted to the G2 phase of the cell cycle in type N cells.

Discussion

In one group of SV40-transformants (type N cells) derived by infection of FR 3T3 rat cells with the early mutant *tsA30*, the large-T protein, encoded by the early viral region, is expressed only during the G2 phase of the cell cycle and is very unstable. In the second group of transformants (type A cells), large-T is expressed continuously along the cell cycle and appears to be stabilized, possibly through interaction with p53. The differences observed in the stability of large-T in the two types of transformed cells could be the consequence of different interactions between large-T and p53, thought to be a cell cycle protein (Mercer *et al.*, 1982; Reich and Levine, 1984). These two potential modes of interaction between large-T and p53 were investigated by first analyzing the expression of the rat p53 along the cell cycle in actively growing normal and transformed FR 3T3 cells, and then determining its consequences on the formation of complexes between p53 and large-T in the transformants.

Pulse-chase labelling experiments have shown that p53 in FR 3T3 cells had a half-life of < 20 min, a value similar to that determined for this protein in mouse 3T3 cells (Reich and Levine, 1984). The increase in the half-life of the protein in the transformants suggested that it was stabilized through the formation of complexes with large-T, though this increase was not comparable with that reported previously for other transformed rodent cells (Oren *et al.*, 1981; Mora *et al.* 1982).

The determination of the steady-state levels of p53 in the normal and transformed cells was performed with a radioimmune assay (Benchimol *et al.*, 1982). The level of p53 in FR 3T3 cells was low and similar to that measured for mouse 3T3 cells (Benchimol *et al.*, 1982). It only apparently increased in type A cells. However the presence of low but detectable amounts of complexes in type N cells suggested that p53 could be accumulated at least during the G2 phase of the cell cycle. The variation in the levels of p53 and large-T × p53 complexes was determined in purified cell fractions. FR 3T3 cells accumulated p53 at a quite constant level throughout the cell cycle. In type N cells, the level in G1 was roughly the same as in normal cells; it increased to reach a maximum in G2. The level of complexes along the cell cycle showed a strong correlation with the level of p53: complexes were undetectable in G1, and found predominantly in G2. Type A cells exhibited high levels of both p53 and large-T × p53 complexes throughout the cell cycle. These results strongly suggest that p53 was stabilized in FR 3T3-SV40 transformants through the formation of complexes with the large-T protein; this was clearly shown by the study of the accumulation of p53 RNA. Both normal and transformed cells contained equivalent amounts of p53 RNA. In the case of FR 3T3 cells, similar amounts of p53 mRNA were detected in the G1, S and G2 phases, and this pattern was unchanged in the transformants. This suggests that the level of p53 is not controlled at a transcriptional level but rather via the formation of complexes with large-T, that is, at a post-transcriptional level, as suggested previously (Oren *et al.*, 1981). This may also hold true for p53 in the G2 phase of the cell cycle in SV-*tsA30*-N1 cells, though the determination of the half-life of the protein in the various phases of the cell cycle could not be done by cell sorting of pulse-chase-labelled cells. As regards both the stabilities of p53 and large-T as well as the interaction between these two proteins, FR 3T3-SV40 transformants appear different from the other SV40-transformed rat and mouse cells studied to date.

The recent demonstration of the capacity of p53 to extend the cell's lifespan (Jenkins *et al.*, 1984) and to cooperate with activated *ras* genes in the transformation of primary rat embryo fibroblasts (Eliyahu *et al.*, 1984; Parada *et al.*, 1984) raises the question of the relative roles of p53 and large-T in transformation. As suggested by Lane (1984), SV40 could transform primary cells by first immortalizing them through stabilization of p53: p53 and SV40 large-T would then act as a pair of cooperating oncogenes, like polyoma virus large- and middle-T (Rassoulzadegan *et al.*, 1982) and other viral and cellular oncogenes (Land *et al.*, 1983; Ruley, 1983). In the case of an SV40-transformed FR T3 cell line, this cooperation, although limited to the G2 phase of the cell cycle, appears sufficient to confer a fully transformed phenotype. One may speculate that specific events in G2 are required for progression of the cell to mitosis, and that large-T (or large-T × p53 complexes) could interact with the cell machinery at this point.

On the basis of experiments involving an analysis of either the level of p53 or of its possible function in mouse 3T3 cells arrested by serum starvation and restimulated to divide, other authors (Mercer *et al.*, 1982; Reich and Levine, 1984) have suggested on one hand, that p53 could be expressed differently along the cell cycle and, on the other hand, that it may play a role in the transition of the cell from a resting (G0) to an actively dividing (G1) state. Clearly, the analysis of pure cell fractions representative of the various phases of the cell cycle shows that p53 is continuously expressed along the cell cycle. A direct parallel may be drawn with the results obtained recently for c-*myc*: on the basis of serum starvation experiments (Kelly *et al.*, 1983; Campisi *et al.*, 1984), c-*myc* was thought to be a cell cycle protein, whereas determination of the level of c-*myc* RNA and of the protein in sorted cell fractions showed that its level does not vary during the cell cycle (Hann *et al.*, 1985; Thompson *et al.*, 1985). It was therefore postulated that c-*myc* expression could reflect the competency of a cell to enter and progress through the cell cycle, and that, in the presence of appropriate growth factors, both proliferative competency and c-*myc* expression could be maintained irrespective of the position of the cell in the cycle (Thompson *et al.*, 1985). A similar assumption can be made for the expression of p53 in normal cells; it reinforces the parallel made previously between p53 and c-*myc*, which belong to the same class of oncogenes (Parada *et al.*, 1984).

Materials and methods

Cell lines and culture conditions

The FR 3T3 cell line (Seif and Cuzin, 1977) and the SV40 *tsA30*-mutant transformed derivatives (Rassoulzadegan *et al.*, 1978), have been described. Cells were grown at 33°C in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (Gibco). The two SV40 transformants, the 14B rat cell line (Botchan *et al.*, 1976), and the SV80 human cell line (Todaro *et al.*, 1966), have been described.

In vivo stabilities of large-T and of p53

Determination of the stabilities of the two proteins at the permissive (33°C) and the restrictive (40.5°C) temperatures was performed as described previously (Imbert *et al.*, 1983b), except for the following modifications. Cells were incubated in DMEM minus methionine supplemented with glutamine and 2% serum substitute (L'Industrie Biologique Française) for 1 h and then incubated in the same medium supplemented with [^{35}S]methionine (200 μCi/90 mm dish in 2 ml; 1500 Ci/mmol, Amersham International) for 1 h. Cells were washed twice with phosphate-buffered saline (PBS) and the label was chased for increasing periods

of time by incubation in DMEM containing 10% newborn calf serum. The chase periods were 30, 60 and 90 min for type A cells, and 5, 10, 15, 20, 30 and 60 min for normal and type N cells, respectively. Cells were then lyzed, the cell extracts were first immune precipitated with PAb 421 antibodies (75 μl of tissue culture supernatant per 90 mm dish) and then with anti-SV40 T-antigen antibodies from tumor-bearing hamsters as described previously (Imbert *et al.*, 1983b, 1984). The immune-precipitated proteins were analyzed by polyacrylamide gel electrophoresis, and the dried gels were exposed to Fuji RX films with intensifying screens after fluorographic treatment.

Sorting cells as a function of their DNA content

The method has been described in detail previously (Imbert *et al.*, 1984). In each experiment, at least 2×10^6 cells in each phase of the cell cycle were sorted, and processed either for RIA or for cytoplasmic RNA preparation, as described below.

Purification of cytoplasmic RNAs, sizing and quantification of the rat p53 mRNA

Total cytoplasmic RNA was purified from normal and transformed cells as described previously (Imbert *et al.*, 1984). The size of the rat p53 mRNA was determined by electrophoresis through gels containing formaldehyde (Maniatis *et al.*, 1982), transfer to nitrocellulose and hybridization as described below. For quantification of p53 mRNA amounts, cytoplasmic RNAs were prepared from standardized fractions of exponentially growing or sorted cells, denatured, serially diluted and filtered onto nitrocellulose sheets (dot blots) using a Schleicher and Schull Manifold I apparatus, as described previously (Imbert *et al.*, 1984).

Northern and dot blots were hybridized with equal amounts of two ^{32}P-labelled p53 cDNA clones, pp53-271 (Oren *et al.*, 1983) and C1 9 (Benchimol *et al.*, 1984), which correspond to different portions of the murine p53 mRNA molecule. The total plasmid was labelled *in vitro* by nick-translation with DNA polymerase I (Rigby *et al.*, 1977). Blots were hybridized at 68°C for 72 h in 40 ml of 6 × standard saline citrate (SSC) containing 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (Sigma), 0.1% SDS, 50 μg/ml of denatured salmon sperm DNA (Sigma) and 10^6 c.p.m./ml of each denatured probe. After repeated washes in 2 × SSC containing 0.1% SDS at 68°C, the nitrocellulose sheets were exposed to Fuji RX films and Dupont Lightning Plus intensifying screens at −80°C. The amounts of p53 mRNA in the dot blots were quantified after scanning the autoradiograms with an ELISA reader (Titertek). Biohazards associated with the experiments described in this publication have been examined previously by the French National Control Committee.

Preparation of the cell extracts for RIA

For each cell line, four standardized fractions of sorted G1, S and G2 cells were prepared together with a control of exponentially growing cells. Cells were resuspended at a concentration of 5×10^7 cells/ml in lysis buffer (50 mM Tris-HCl pH 8.0, containing 1% NP40 and 0.15 M NaCl) (Benchimol *et al.*, 1982). Cell extracts were stored at −80°C before analysis.

Production and iodination of the antibodies

The hybridoma cell lines PAb 416, PAb 419 and PAb 421 and their culture conditions have been described (Harlow *et al.*, 1981). Antibodies were purified and labelled with ^{125}I (CEA, France) as described previously (Benchimol *et al.*, 1982). The labelled proteins were separated from the unreacted iodine on a PD 10 prepacked column (Pharmacia) equilibrated with NET/gel buffer (50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 0.04% NP40, 0.02% sodium azide and 0.25% gelatin) (Benchimol *et al.*, 1982).

Radioimmune assays

The RIA set up for the rat p53 was similar to that developed previously for human p53 (Benchimol *et al.*, 1982). Purified PAb 421 antibodies were passively bound to PVC microtitration wells by adding 50 μl of a 20 μg/ml antibody solution in 10 mM phosphate buffer pH 7.0 to each well and incubating overnight at room temperature in a humid chamber. The wells were then washed with the NET/gel buffer described above to remove the unbound antibodies and to saturate the binding capacity of the plastic. Various concentrations of cell extracts (either standardized in number of cells or for total protein concentration, according to the experiment, see Results), diluted in NET/gel buffer were added to the wells under a volume of 20 μl, and incubated for 3 h at room temperature. Wells were washed with NET/gel buffer. 20 μl of a 1:200 dilution of a pool (named PSK) of polyclonal sera from breast cancer patients were then added to the wells, and incubated for a further 3 h at room temperature. After washing, the wells were exposed to ^{125}I-labelled mouse 8a4 monoclonal antibodies (Lowe *et al.*, 1982), and incubated overnight. Wells were washed extensively with NET/gel buffer, dried and counted in a gamma counter.

The amounts of large-T were determined by another RIA using two monoclonal antibodies directed against large-T, PAb 416 bound to the PVC plates, and ^{125}I-labelled PAb 419. Amounts of p53 × large-T complexes were determined using PAb 416 antibodies bound to the PVC plates, and ^{125}I-labelled PAb 421, as well as the reverse combination (PAb 421 bound to the wells, and ^{125}I-labelled PAb 416), to exclude the possibility of non-specific aggregate formation.

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