Cell Cycle-dependent Regulation of Nuclear p53 Traffic Occurs in One Subclass of Human Tumor Cells and in Untransformed Cells

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Abstract
We have analyzed the regulation of subcellular compartmentation of mutant and wild-type (WT) p53 proteins as a function of the cell cycle using immunofluorescence microscopy and referring to different markers of position in the cell cycle in different human cells expressing either mutated (KHOS-240, A 431, and T47-D cells) or WT (WI 38 and MCF-7 cells) p53. The mutant p53 proteins present in the KHOS-240, A 431, and T47-D tumor-derived cell lines enter very rapidly in the nucleus in early postmitotic cells before the chromosomes have fully decondensed; they continue accumulating in this location without any obvious cytoplasmic retention throughout the cell cycle until prophase. Such behavior is similar to that observed for the WT p53 associating with SV40 large T antigen in human WI 38 cells transformed by SV40, but it is in contrast to the behavior of the WT p53 protein present in both the untransformed WI 38 and the tumor-derived MCF-7 cells. In these latter systems, the highest nuclear concentrations of the WT protein are always found in G1 cells that still fail to exhibit a high rate of nuclear cyclin A; past the G1-S transition, the nuclear level of WT p53 tends to decrease, possibly to the benefit of cytoplasmic expression, whereas that of cyclin A concomitantly increases, suggesting that the nuclear accumulation of WT p53 becomes restricted during the phase of DNA replication. As for Saos-2 cells stably transfected with the temperature-sensitive p53Ala-143 mutant, they become arrested before the G1-S transition with a heavy pool of nuclear p53 at 32.5°C, the temperature at which the transcripational activity of p53Ala-143 is restored. All these data are compatible with the presently acknowledged primary role for WT p53, which would be to brake transit through the G1-S border possibly by directly transactivating the p21cip1 protein.

Introduction
A very common feature of many different human tumors is the occurrence of point mutations at one p53 locus associated with a reduction of homozygosity at the second p53 allele, which causes these tumor cells to eventually express elevated levels of mutant p53 and no WT p53 (1, 2). A number of recent experimental works have converged to indicate that the WT p53 protein would act as a negative regulator of cell growth (3–7) and a suppressor of transformation and tumorigenesis (8–11). The growth-inhibitory function of WT p53 would take effect at the G1-S checkpoint, because: (a) overexpressed WT p53 either in response to X-ray or drug-induced DNA damage (12–13) or following transfection with plasmids carrying the WT p53 coding sequence (5–7) delays or inhibits transit through the G1-S checkpoint (this effect is eventually impeded by mutant p53); and (b) cells homozygous for the WT allele or heterozygous for a Li-Fraumeni syndrome-specific p53 mutant arrest in G1 when treated with N-phosphoacetyl-L-aspartic acid, an inhibitor of purine metabolism, whereas cells homozygous for mutant p53 do not arrest (14). Together, these observations have led to speculation that, first, mutations at the p53 locus are required to inactivate the inhibitory action of WT p53 on cell growth and proliferation and, second, abolishing the normal function of WT p53 is a prerequisite for tumor progression.

On the other hand, p53 has been demonstrated both to bind to DNA in a sequence-specific manner (15) and to contain a strong transcriptional activation sequence near its amino terminus (16–17). WT p53 could possibly then directly induce the expression of genes that would be involved in mediating its growth-suppressive action. A potential candidate for the role of WT p53 mediator has recently been isolated using a subtractive hybridization technique. This p53-induced gene, initially named WAF1 (18), has been shown to be identical to the CIP1 gene, the product of which, p21, binds tightly to Cdk5 and is a potent inhibitor of their activity (19). When introduced into tumor cell lines, this gene could itself act as a growth suppressor, presumably by arresting the cells at the G1-S border but not killing them (19).

Received 12/20/95; accepted 7/3/96.
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1 This study was supported by the Centre National pour la Recherche Scientifique, the Curie Institute, and the Association pour la Recherche sur le Cancer (France).
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3 The abbreviations used are: WT, wild-type; Cdk, cyclin-dependent kinase; kDa, kilodalton; DAPI, 4′,6-diamidino-2-phenylindole; ts, temperature sensitive; BrdUrd, bromodeoxyuridine; CMV, cytomegalovirus.
Yet, mutation at the p53 locus is by no means a universal and univocal marker of human cancer cells. Thus, mutant p53 has been reported in only 30% of breast cancers, and, although selecting frequently for a reduction to homozygosity at the p53 locus, most breast cancers retain the WT allele (20). In some cases, the WT p53 of tumors was shown to exhibit an unusual and heterogenous distribution pattern residing mostly inside the cytoplasm, in striking contrast to the behavior of overexpressed mutant p53 proteins, which more generally collect inside the nucleus (20–23). Interestingly, ras plus myc viral infection, which induces carcinoma in reconstituted mouse prostate, results also in a heterogeneous and peculiar WT p53 pattern of distribution, unrestricted to the nucleus (24). It is also worth noting that transfection with plasmids encoding WT p53 is powerless in inducing growth suppression of the WT p53-containing tumor cells. Considering the differential subcellular distributions of WT and mutant p53 in these two particular groups of tumors, it has been suggested that exclusion of WT p53 from the nuclear compartment might be one possible alternative used by cancer cells to bypass the WT p53 function (20). It is worthwhile here to recall, however, that in immortalized, untransformed BALB/c 3T3 and NIH 3T3 cells, the presumably WT p53 has been reported to reside only transiently in the nucleus, for about 3 h around the beginning of S-phase, accumulating in the cytoplasm for the rest of the cell cycle despite its continued synthesis (25). These latter observations are of significance, because they indicate that the subcellular compartmentation of WT p53 in untransformed cells would be tightly regulated during the cell cycle under ex vivo conditions.

The apparently very strict scheduling of p53 localization in the immortalized, untransformed mouse cell lines led us to suspect that the nuclear timing of p53 in WT p53-containing human tumor-derived cell lines could also be tightly regulated as a function of the cell cycle in the same way as it is in untransformed mouse and, perhaps, human cells. To test this hypothesis, we have performed an analysis, using the immunofluorescence technique, of subcellular distribution as a function of the cell cycle of WT p53 present in the MCF-7 cell line (derived from a human breast carcinoma), which we compared with: (a) that of WT p53 expressed in human untransformed WI 38 cells; (b) that of the WT p53 protein stably complexed to the large T antigen in WI 38 fibroblasts transformed by SV40; and (c) that of the mutant p53 proteins in three human tumor-derived cell lines (KHOS-240, A 431, and T47-D). This study clearly indicates that inactivated p53 proteins (through either mutation or complex formation with a DNA virus-transforming protein) enter the nucleus in early–mid-G1, reaching their maximal nuclear rate in fully spread cells, generally prior to entry into S-phase; they maintain elevated levels of nuclear p53 expression throughout S-phase, G2, and even prophase, exhibiting at no one stage of the cell cycle any evident cytoplasmic retention. In contrast, the WT p53 protein in human untransformed as well as tumorous MCF-7 cells, although also entering the nucleus in early–mid-G1 and reaching a maximal nuclear rate generally prior to entry into S-phase, sees its level of nuclear expression decreasing, possibly to the benefit of cytoplasmic expression, as the cells progress into S-phase and approach mitosis.

**Results**

**Immunolocalization of Mutant and WT p53 Proteins in Asynchronous Human Normal and Tumor-derived Cell Lines**

Two different mouse monoclonal anti-p53 antibodies (PAb 122 and NCL-DO7) and one rabbit polyclonal anti-p53 antibody (R1A) were used to examine the subcellular localization of p53 proteins in the different human normal and tumor-derived cell lines described in "Materials and Methods".

In asynchronous populations of the human cells carrying either a mutant p53 (KHOS-240, A 431, and T47-D cells) or a WT p53 complexed with SV40 large T antigen (SV40 and VA13-WI 38 cells), the three different antibodies exhibit almost exclusively a nuclear distribution in the majority of the cells in subconfluent or loosely packed cell cultures (shown for PAb 122 and R1A; Fig. 1) and a diffuse cytoplasmic distribution only during mitosis, being then totally excluded from the condensing chromosomes (Fig. 1, small arrows). Very distinctive features are obtained in asynchronous populations of either the untransformed WI 38 or the MCF-7 breast carcinoma cells that express a WT p53. First, the three different antibodies seem to exhibit differential affinities for nuclear WT p53; typically, the percentage of nuclear WT p53-positive cells underscored with NCL-DO7 reproducibly amounts to about 60% versus 15–20% with PAb 122. In both cases, the nuclear concentration of WT p53 is highly variable from one cell to another inside a given population, and a few cells (2–4%) in the MCF-7 line exhibit an abnormally high level of nuclear WT p53 (Fig. 2, A and C, arrowheads). Polyclonal R1A (Fig. 2A), on the other hand, generates high, unspecific, diffuse cytoplasmic staining that could prevent immunodetection of WT p53 inside the nucleus of cells in which the level of expression is low. Fig. 2, A–F, illustrates in the MCF-7 cell line this uneven reactivity of the three antibodies toward WT p53.

Western blot analysis of extracts from WI 38 and MCF-7 cells confirmed our immunofluorescence observations, because polyclonal R1A gave rise to numerous contaminating bands in addition to a ~53-kDa one, and monoclonal PAb 122 produced a barely detectable 53-kDa band (not shown), whereas monoclonal NCL-DO7 readily revealed the presence of a 53–54-kDa doublet in the absence of any significant contaminating background. Fig. 3 presents the results of immunoblotting experiments conducted with monoclonal NCL-DO7 on equivalent amounts (250 μg protein) of the various human normal and tumorous cell lines analyzed. Interestingly, whereas this anti-p53 antibody apparently reacts with a unique, broad, 53-kDa band in KHOS-240, A 431, and T47-D cells, which carry mutant p53 protein, it reveals clearly a 53–54-kDa doublet in cell lines carrying WT p53 protein, as well as in the HeLa cell line (which has been reported to contain a low p53 level; Refs. 26 and 28) and the VA13-WI 38 cell line (in which WT p53 forms a complex with SV40 large T antigen). Immunofluorescence observation has confirmed the Western blot analysis, because we have been able to detect faint, yet visible (apparently both nuclear and cytoplasmic) staining of HeLa cells with NCL-DO7 (Fig. 2, G and H), which is in agreement with a previous report (28) using a different polyclonal anti-p53 antibody, HZp53R. Also,
Fig. 1. Double labeling of asynchronous populations of human cells with anti-p53 antibodies PAb 122 (C, E, G, and I) and R1A (A) and with DAPI (B, D, F, H, and J). The cell lines examined are KHOS-240 (A–D), T47-D (E and F), A 431 (G and H), and VA13-WI 38 (I and J). Prominent nuclear staining with anti-p53 antibodies is evident in the majority of the cells, except on rare occasions (large arrowheads), and the p53-specific labeling is diffusely distributed (or absent) in mitotic cells, avoiding the condensing chromosomes (small arrows).
Fig. 2. Double labeling of asynchronous MCF-7 (A–F), HeLa (G and H), and WI 38 (I and J) cells with various anti-p53 antibodies (A, C, E, G, and I) and DAPI (B, D, F, H, and J). Anti-p53 antibodies are R1A (A), PAb 122 (C), and NCL-D07 (E, G, and I). Large arrowheads in A and C, nucleus with abnormally elevated p53 staining.
the immunofluorescence pattern revealed by NCL-D07 in the human untransformed WI 38 cell line closely resembled that observed in the tumor-derived MCF-7 cell line (Fig. 2, I and J). In consequence, all the data presented in this report regarding WT p53 were obtained using the NCL-D07 anti-p53 antibody, which appeared to be the more sensitive WT p53-specific antibody.

**Immunolocalization of Mutant and WT p53 Proteins as a Function of the Cell Cycle in Human Normal and Tumor-derived Cells.** Untransformed WI 38 cells can readily be driven into the quiescent G0 state by serum deprivation of up to 0.5% for 36 h, in contrast to the tumor-derived cells, which, under these conditions, keep synthesizing and accumulating cyclin A (a marker of S-phase) inside their nuclei and undergo mitosis at a rate equivalent to that observed in control cell populations. Interestingly, however, the average cell cycle duration is greatly augmented in these starving conditions (from 20–22 h in 10% serum up to 32–34 h in 0.5% serum in the HeLa cell line). A thymidine-aphidicolin double block, on the other hand, very effectively prevents tumor cell progression through the G1-S boundary, because very few mitotic cells are generated during the block, but they appear at a high rate 10–15 h following release from the block. Thus, this method can be used to study more precisely cells standing at various stages of mitosis in enriched populations of mitotic cells. However, this technique should not be regarded as giving a reliable indication of the distribution of WT p53 before the transition through the G1-S checkpoint, because we and other investigators have shown that such a treatment induces an aberrant nuclear accumulation of a number of proteins, such as nonmyristoylated c-Src (29) and cyclins A and B (30). To prevent artifacts due to the use of drugs, we analyzed cells in cultures growing under standard conditions, and we determined the positions of individual cells in the cell cycle according to different markers: (a) DAPI staining, which typically becomes punctate as chromosomes begin to condense in late G2-early prophase cells (29); (b) the degree of nuclear cyclin A staining, which is quasi-null in early and mid-G1, becomes detectable but is still low in late G1, steadily augments as cells progress through S-phase and G2, and culminates in early prophase before cyclin A is degraded (31); and (c) antitubulin staining, which was used here mainly to identify postmitotic cells reentering G1, remaining at this stage still attached by pairs through their cleavage furrow (29). This analysis has been performed on several thousands of cells to give statistically valuable information.

**Study of the Cell Lines Carrying Mutant p53**

**Double Immunolabeling with Anti-p53 and Anti-Cyclin A Antibodies.** Double immunolabeling of the cells carrying mutant p53 clearly underlines the fact that high levels of nuclear expression of p53 are present in a majority of cells (>90%), whatever the degree of nuclear accumulation of cyclin A (compare in the same KHSV-240 cells the p53 and cyclin A staining in Fig. 4, A and D and B and E, respectively). This study clearly indicates that mutant p53 starts accumulating in the nucleus well ahead of cyclin A (i.e., probably in early–mid-G1) and continued to be present at a high level in this locus until late G2 and early prophase.

**Triple Labeling with Anti-p53, Antitubulin, and DAPI.** Triple labeling (Fig. 4, G–I), on the other hand, confirmed the above results and more precisely depicted the early stages of nuclear entry of p53 in postmitotic cells reentering G1. Such cells can be recognized easily by the fact that they remain attached through their cleavage furrow (Fig. 4, G–I, arrows). In addition, the relative degree of cell spreading and the sizes of the nuclei (which increase as the chromosomes keep decondensing) give reliable indications of the degree of progression through G1. Thus, a close examination of Fig. 4, G–I, which displays three pairs of postmitotic cells, would suggest that mutant p53 remains essentially excluded from the nucleus in early G1 (pair of cells on the left) but quickly starts concentrating in the nucleus as the chromosomes further decondense (Fig. 4, G–I, other two pairs of postmitotic cells). Maximal levels of nuclear p53, however, are generally encountered only in cells that have parted company and have reached their maximal spreading size [Fig. 4, G–I, two largest, unconnected cells and the early prophase cell showing typically punctate DAPI staining (large arrowheads)].

Interestingly, the relationship between anti-cyclin A (or antitubulin or DAPI) and anti-p53 labeling in the VA13-WI 38 cell line transformed by the simian sarcoma virus follows the same rules as those observed for the mutant p53 protein in the tumor-derived cell lines (not shown).

**Study of WT p53 Expressed in Normal, WI 38, and Tumor-derived MCF-7 Cell Lines**

**Triple Labeling with Anti-p53 and Anti-Cyclin A Antibodies and DAPI.** The observation that the intensity of the nuclear anti-p53 immunostaining detected with both anti-p53 monoclonal antibodies was highly variable from one cell to another in the MCF-7 cell line (Fig. 2) led us to suspect that such variability was not random but, rather, could reflect the fact that the level of nuclear WT p53 steadily varied as a

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* Unpublished data.
function of time during the cell cycle, achieving a maximal rate for a narrow window of the cell cycle. A close examination of Fig. 5 clearly indicates that, in subconfluent, actively growing populations of untransformed WI 38 cells (Fig. 5, J-L) and tumors MCF-7 cells (Fig. 5, A-I), the highest levels of nuclear p53 are generally encountered in cells exhibiting the lowest (or no discernible) amount of nuclear cyclin A (Fig. 5, A-F and J-L, arrowheads), and the opposite is also true (Fig. 5, A-F and J-L, arrows). In particular, WT p53 is exempt from the nucleus (in contrast to cyclin A) in late G₂-early phase cells showing punctate DAPI staining (Fig. 5, G-I, long arrows). A small percentage of cells exhibit a low nuclear level of both p53 and cyclin A (Fig. 5, A-C, stars). During mitosis until late telophase, WT p53, like cyclin A, appears to be either absent or diffusely scattered through the mitotic cells and to be excluded from the condensed sets of chromosomes (Fig. 5, A-I, short arrows). These observations strongly suggest that the nuclear concentration of WT p53 in these two cellular systems would reach a maximal rate in G₁, prior to entry into S-phase, and then would decrease as the cells progress through the S-phase and G₂.

**Triple Labeling with Anti-p53, Antitubulin, and DAPI.** In the WI 38 and MCF-7 cells, triple labeling gave results similar to those obtained for mutant p53 in postmitotic KHOS-240 cells, depicted in Fig. 4, G-I. That is, WT p53 remains essentially dispersed through the cytoplasm and excluded from the nucleus in early postmitotic cells but quickly concentrates inside this locus as the postmitotic cells (still forming pairs attached through their cleavage furrow) further expand (not shown), indicating that WT p53 starts accumulating inside the nucleus in mid-G₁ before the cells reach their full spreading size.
Fig. 5. Triple labeling of asynchronous MCF-7 (A–I) and WI 38 (J–L) cells with anti-p53 (A, D, G, and J), anti-cyclin A (B, E, H, and K), and DAPI (C, F, I, and L). One may distinguish two main cell subpopulations: (a) one with a high level of nuclear p53 and a low level of nuclear cyclin A (arrowheads in A, B, D, E, J, and K); and (b) a second population with a high level of nuclear cyclin A and a low level of nuclear p53 (arrows in A, B, D, E, J, and K). Large arrows, in G–I, late G2–early prophase cell; small arrows in A–I, mitotic and telophase cells with condensed sets of chromosomes unstained for both WT p53 and cyclin A. A few cells with fully decondensed nuclei also exhibit a low nuclear level of both p53 and cyclin A (A–C, stars).

**Immunolocalization of WT p53 as a Function of the Cell Cycle in Synchronized WI 38 Cells**

The untransformed WI 38 cells could readily be quasi-totally growth arrested by serum deprivation of up to 0.5% for 48 h. Therefore, our previous conclusions that the WT p53 nuclear concentration generally reaches a maximal rate in mid-late G1 and then tends to decrease as the cells progress through S-phase and G2 could be verified on synchronized WI 38 cell
populations. From time 0 up to 4 h after growth stimulation
induced by refeeding the cells with fresh medium supple-
mented with 20% serum, nuclear cyclin A immunostaining
appears quasi-null or very faint in the majority of the cells,
in contrast to the nuclear p53 concentration, which seems to
be already present in many cells (~60%), although at a
relatively low level (Fig. 6, A–C). Thereafter, the percentage of
nuclear cyclin A-positive cells continuously increases, reach-
ing a peak (~60%) 20–24 h following growth induction.
Strikingly, we noticed that the first cells to accumulate inter-
mediate or high levels of nuclear cyclin A (4–10 h after serum
starvation release) exhibited much lower levels (if any) of WT
p53 than their neighbors, still failing to stain for nuclear cyclin
A (Fig. 6, D–F, arrowheads and arrows, respectively). Be-
tween 24 and 32 h after stimulation (Fig. 6, G–I), the ratio of
nuclear cyclin A-positive cells remains high, and the nuclear
accumulations of cyclin A and WT p53 continue to be ap-
parently mutually exclusive in many cells, although a few
cells now exhibit a significant nuclear concentration of both
(Fig. 6, G–I, curved arrows). Past 32 h after serum starvation
release (Fig. 6, J–L), the three different patterns of nuclear
cyclin A versus nuclear p53 staining persist, although the
ratio of nuclear cyclin A-positive cells largely decreases,
because many cells have undergone mitosis and entered a
new G1. Monitoring by immunoblot the level of expression
of WT p53 in the WI 38 cells as a function of the progression
through the cell cycle showed that it reaches a maximal rate
10–15 h after stimulation and remains roughly constant up to
32 h after serum addition (not shown).

**Immunolocalization of the ts p53^Apa^-143 Protein Transfected in Saos-2 Cells at Permissive and Restrictive Temperature**

The human p53^Apa^-143 mutant has been shown to exhibit
temperature sensitivity for its DNA-binding and transcrip-
tional activation functions following transfection in K562 leu-
kenia cells (32). This mutant p53 was stably transfected in
human osteosarcoma Saos-2 cells (which exhibit extensive
loss of p53-coding sequences; Ref. 33), as described in
"Materials and Methods." In these new host cells, the
p53^Apa^-143 protein was still ts in transcriptional activation
tests, stimulating transcription of the luciferase reporter gene
at 32.5 but not 37.5°C in the same way as in K562 leukemia
cells (not shown). The transfected human p53^His^-175 mutant,
in contrast, was ineffective at stimulating luciferase gene
transcription at both temperatures.

After immunolabeling with NCL-DO7 anti-p53 antibody,
the Saos-2 cells revealed weak background staining at 32.5
and 37.5°C (Fig. 7, a–c). In contrast, the same antibody
detected a very strong nuclear concentration of p53 antigens
in a majority of cells, whether in the p53^His^-ts-transfected
Saos-2 cells or in the p53^Apa^-143-transfected cells at 37.5 and
32.5°C (Figs. 7, d, f, h, j, and 8, a and b). Significant cyto-
plasmic immunostaining could be observed generally only in
postmitotic cells (not shown). Double immunolabeling with
anti-p53 and anti-BrdUrd clearly demonstrated that neither
p53 mutant was able to prevent entry and progression
through S-phase at 37.5°C (Fig. 7, d, e, h, and i). Double
immunolabeling with anti-p53 and anti-cyclin A, on the other
hand, confirmed that anti-p53 immunostaining was detect-
able in the nucleus at any stage of the cell cycle, i.e., in G1,
cells that expressed a low level (if any) of nuclear cyclin A, as
well as in S-phase and G2 cells, which displayed the highest
nuclear cyclin A labeling; moreover, there was no apparent
correlation between the degrees of nuclear accumulation of
cyclin A and p53 (shown in the p53^Apa^-143-transfected Saos-2 cells
at 37.5°C; Fig. 8, compare a and c).

As expected, practically no changes at all were observed
in the immunostaining patterns of the p53^His^-175-transfected
Saos-2 cells shifted at 32.5°C for 24 h and doubly labeled
with anti-p53 and anti-BrdUrd (Fig. 7, f and g). In striking
contrast, the p53^Apa^-143-transfected Saos-2 cells shifted at
32.5°C for 24 h and doubly stained with anti-p53 and anti-
BrdUrd still displayed very high levels of nuclear p53, yet the
rate of BrdUrd-positive cells dropped from ~40% at 37°C to
less than 10% at 32.5°C, indicating an important braking of
transit through the G1-S border (Fig. 7, j and k).

As for the anti-cyclin A immunolabeling, it typically varied
continuously from one cell to another in cells progressing
from late G1, through S-phase and G2, i.e., in the p53^ts-
^His^-175, transfected Saos-2 cells at 37.5 and 32.5°C and in the
p53^Apa^-143, transfected cells at 37.5°C (Fig. 8c); in the latter
cells at 32.5°C, anti-cyclin A labeling allowed detection of the
presence of two distinctive subpopulations (Fig. 8d): (a) one
representing two-thirds of the whole population exhibited a
faint fluorescence intensity typical of that generally present in
late G1 and early S-phase cells (31); and (b) the second one,
instead, accumulated a very high rate of anti-cyclin A
labeling similar to that generally found in cells standing near
the G2-M transition. Yet, very few mitotic cells were found,
but the rate of cell death was significant. However, cell death
was not negligible at 37.5°C as well, and it was impossible to
ascertain whether it increased at 32.5°C, because unhealthy
cells tend to be eliminated in the course of experimentation.

Thus, when shifted at 32.5°C, a majority of p53^Apa^-143-
transfected Saos-2 cells appeared to be arrested in late G1,
with a low level of nuclear cyclin A, as expected; presumably,
those cells that have already past the G1-S transition at the
time of the temperature shift fail to progress into S-phase, yet
they keep synthesizing and accumulating cyclin A in their
nuclei. This phenomenon is reminiscent of that occurring
following a G1-S block induced by treatment with DNA syn-
thesis inhibitors (thymidine or aphidicolin), which does not
prevent cyclins A and B1 from accumulating (30), although
it is very efficient at preventing progression through S-phase.

**Discussion**

There is a consensus of opinion arising from numerous ex-
perimental works that WT p53 exhibits a tumor suppressor
action when constitutionally overexpressed following transfe-
sion with plasmids encoding WT p53 in ex vivo culturing
systems, such as nonestablished rat embryo fibroblasts
transformed by different oncogene combinations and a num-
ber of human cultured cells derived from tumors that express

5 Unpublished observations.
Fig. 6. Triple labeling of synchronized WI 38 cells with anti-p53 (A, D, G, and J), anti-cyclin A (B, E, H, and K), and DAPI (C, F, I, and L) at various times following serum starvation release (Tm). Tm = 0–5 h (A–C), 5–15 h (D–F), 15–30 h (G–I), and more than 30 h (J–L). Arrowheads, cells that are positive for nuclear cyclin A and exhibit a low level (if any) of nuclear WT p53. Arrows, cells that are positive for nuclear WT p53 A and exhibit a low level (if any) of nuclear cyclin A. Curved arrows, cells with intermediate levels of both cyclin A and WT p53.
Fig. 7. Double immunolabeling of nontransfected (a–c), p53<sup>34−175</sup>-transfected (d–g), and p53<sup>24−143</sup>-transfected (h–k) Saos-2 cells with anti-p53 (a, d, f, h, and j), anti-BrdUrd (b, e, g, i, and k), and DAPI (c). The anti-p53 immunostaining is essentially nuclear, whatever the growth temperature, yet the Saos-2 transfected with the ts p53<sup>24−143</sup> mutant failed to undergo S-phase at 32.5°C, the temperature at which this p53 protein is transcriptionally active.

either a mutant (3, 34–36) or low levels of WT (10) p53. As discussed in “Introduction,” such an effect might result from an arrest at the G<sub>1</sub>-S transition induced by the WT p53-mediated overexpression of the p21<sup>Cip1</sup> protein (19). WT p53 also has been involved in mediating apoptosis (37–42), particularly following irradiation of mouse thymocytes or treatment with DNA-damaging agents. These latter findings, in addition the fact that the incidence of mutations in the p53
Fig. 8. Double immunolabeling of p53<sup>143-143</sup>-transfected Saos-2 cells at 37.5 and 32.5°C with anti-p53 (a and b), anti-cyclin A (c and d), and DAPI (e), and phase-contrast image (f). In actively growing cells at 37.5°C, cyclin A accumulates continuously from late G<sub>1</sub> through S-phase and G<sub>2</sub>; after a 24 h-temperature shift at 32.5°C, two types of cells are present: (a) G<sub>1</sub>-S-arrested cells with a low level of cyclin A; and (b) cells having past the G<sub>1</sub>-S transition at the time of the temperature shift that keep amassing cyclin A in their nuclei, although they stopped incorporating BrdUrd.
gene in human cancers is high, have led to the proposal that the normal counterpart of p53 might usually function to protect cells against neoplastic transformation. This hypothesis, however, is irrefutable with the other observation that there is another large group of tumor cells that are characterized by the expression of normal or elevated levels of endogenous WT p53 and that are not affected by transfection with the WT gene (24, 34). To remove the inconsistency, it has been speculated that, in this particular class of tumors, WT p53 is prevented from accomplishing its normal role because of an unfavorable interaction with its cellular environment. Seeing that the WT p53 present in such tumors typically exhibits a heterogeneous pattern of localization with variable degrees of nuclear concentration from one cell to another (24, 43), it has been proposed that bypassing the WT p53 function might be achieved in these types of tumors by excluding the protein from the nuclear compartment. A decisive issue was then to determine whether the variable intensity of the nuclear WT p53 immunostaining observed from one cell to another in the MCF-7 cells by previous investigators (43) and ourselves was really attributable to an abnormal phenomenon exclusive to tumor cells and not to untransformed cells. A comparison of the subcellular distribution of the WT p53 proteins expressed in the tumorous MCF-7 cell line and in untransformed WI 38 fibroblasts at different stages of the cell cycle suggests that it was not. Instead, our immunofluorescence data indicate that such variability is conditioned by the positioning of the normal and tumorous cells within the cell cycle: the intensity of nuclear WT p53 immunostaining increases very rapidly in postmitotic cells and reaches a maximal rate in late G1-early S-phase; it then tends to decrease, possibly to the benefit of cytoplasmic WT p53 staining, as the cells further progress through the S-phase and G2, and it is excluded from the condensed sets of chromosomes during mitosis. In contrast, we have shown that the nuclear accumulation of mutant p53 forms and WT p53 complexed with the large T antigen is unrestricted all along the cell cycle. Our data regarding the behavior of WT p53 in WI 38 and MCF-7 cells are similar of those previously reported for the endogenous p53 protein expressed in two untransformed mouse cell lines (BALB/c 3T3 and NIH 3T3), which has been shown to accumulate inside the nucleus for about 3 h around the G1-S transition (25). Before and after that period, the protein is essentially detected inside the cytoplasm. These data closely fit ours, except that we were not able to detect significant cytoplasmic accumulation of the WT p53 in early-mid-G1 following serum starvation release in the WI 38 cells. It is possible, however, that the rates of p53 synthesis and/or nuclear transport during G1 differ from one cellular model to the other, and, thus, depending on which rate is limiting, cytoplasmic p53 accumulation in G1 will or will not occur.

A striking feature that distinguishes WT p53 proteins from mutant forms and from WT p53 complexed with SV40 large T antigen under naturally occurring conditions is that the nuclear accumulation of the first ones, and not the latter ones, becomes restricted after the G1-S transition. Consequently, after the onset of DNA synthesis, the WT p53 protein should be either relocated into the cytoplasm or degraded inside the nucleus, and its further nuclear transport should be prohibited, which incites speculation that its presence within the nucleus is no longer required, and possibly unadvisable, past the G1-S transition. Intriguingly, however, the cell cycle dependency of WT p53 localization is apparently lost following its ectopic overexpression in the human tumor-derived Saos-2 (44) and HeLa (42) cells, because under these conditions, a strictly nuclear location is observed. Similarly, we found that the p53Ala-143 protein (the DNA binding and transcriptional activation functions of which are suppressed at 37.5°C and reactivated at 32.5°C; Ref. 32 and the present work) is temperature insensitive for its location and concentrates essentially in the nucleus when stably transfected in Saos-2 cells. Yet, the p53Ala-143-transfected Saos-2 cells fail to enter and progress through S-phase at 32.5°C (as judged by the fact that they fail to incorporate the thymidine analogue BrdUrd). It is interesting here to recall that Vojtesek and Lane (43) previously demonstrated that the murine p53Val-135 protein (which is temperature sensitive for its nuclear location in primary rat embryo fibroblasts; Refs. 7 and 45) is capable of adopting the temperature-insensitive localization pattern of the endogenous p53 proteins when introduced by transfection assay in different tumor-derived host cells. What clearly emerges from this collection of data is that the p53 proteins can be exposed to two drastically different cellular environments: (a) an environment such as in untransformed WI-38, BALB/c 3T3, NIH 3T3 cells (25), and a large class of breast cancer cells (20), represented here by the MCF-7 cell line, in which the intracellular distribution of the protein is temporally and spatially regulated as a function of the cell cycle, its nuclear timing being restricted to a narrow window of the cell cycle encompassing the G1-S transition; (b) an environment such as in a more widespread variety of cancer cells or cells transformed by DNA tumor viruses, in which the protein has escaped from the regulation of its compartmentation during the cell cycle and segregates preferentially inside the nucleus at any stage of the cell cycle (except in postmitotic cells). It should be noted that the Saos-2 cells (which exhibit extensive loss of p53-coding sequences; Ref. 33) and the HeLa cells (in which the WT p53 expression level is kept low, due to its degradation by the E6 oncoprotein encoded by human papillomavirus 16; Ref. 46) favor an almost quasi-exclusive nuclear location of WT p53 in transient transfection assays, behaving with respect to the control of p53 localization during the cell cycle in the same way as tumor cell lines carrying a mutant, inactivated p53 gene.

Human tumor cells can be subdivided into two main classes distinguishable by the fact that one sustains a strict regulation of WT p53 nuclear compartmentation as a function of the cell cycle (behaving in this respect like untransformed cells), which permits normal progression through S-phase; in contrast, the second major class has lost the cell cycle-dependent regulation of WT p53 nuclear expression, and this would be responsible for the tumor-suppressive action observed following reintroduction of a WT p53 gene. It has been acknowledged previously that nuclear localization is essential for the biological activity of p53 proteins (whether WT or mutant; Ref. 47) and, more recently, that a primary role for WT p53 would be to break
transit from late G1 to S-phase, possibly by directly transactivating the WAF1/CIP1 gene (18, 19). Because overexpression of WT p53 is unable to suppress cell growth in the first class of tumors (34), we would conclude that the level (and not only the timing) of nuclear p53 expression is probably also regulated in such cells. Interestingly, the p21^{169p} protein is not only a tight-binding inhibitor of G1, Cdk's, but it can also act as an inhibitor of the cyclin A-Cdk2 complex (19), the activity of which is needed for progression through the S-phase (31). It would then be expected that the WT p53-mediated transactivation of WAF1/CIP1 decreases and eventually vanishes after passage in S-phase under permissive growth conditions. This could be achieved, as in normal human and mouse cells and some WT p53-expressing tumor cells, through delocalizing WT p53 from its nuclear site and restraining its further nuclear accumulation during the process of DNA replication. Conceivably, however, exposure to DNA-damaging agents or radiation could result in activating the inhibitory WT p53-mediated regulatory pathway through relieving the break in nuclear transport and accumulation of the protein past the G1-S transition. It is intriguing indeed that WT p53-mediated apoptosis induced following DNA damage is accompanied by an intranuclear accumulation of the protein (12, 41, 48), and this appears to be part of the lethal signal (40).

It is worthy of note that homozygous null p53 thymocytes, although resisting induction of apoptosis by radiation and DNA-denaturing agents, retain a WT p53-independent apoptotic pathway on stimuli that mimics physiological cell deletion signals, namely, glucocorticoid, calcium-associated activation, and ageing in vitro, indicating that the WT p53-mediated apoptotic pathway would be activated only in response to DNA strand breakage (39, 40). These latter experimental data are of significance because they underline the possibility that certain genetic alterations such as those involved in neoplastic transformation or cell ageing need not be sensed by the WT p53-associated control machinery leading to apoptosis. On the basis of these data, and given the occurrence of a large group of tumors that express a functional WT p53, the nuclear timing and level as a function of the cell cycle of which are regulated in the same way as in normal cells, it can be inferred that tumor initiation and progression do not necessarily require bypassing the WT p53 function. It can also be predicted that tumors that do need to bypass the WT p53 function are those that have been subjected to an injury, having engendered a loss in the control of p53 nuclear traffic during the cell cycle. It is noteworthy indeed that, under naturally occurring conditions, mutant p53 proteins (as well as WT p53 complexed to SV40 large T antigen) that are inefficient in suppressing tumor growth and acting as transcriptional activators (17, 44, 46, 49) all exhibit almost exclusive nuclear compartmenting throughout the cell cycle.

**Materials and Methods**

**Plasmids and Cell Lines.** pCMVneoBAM is an expression vector containing the CMV promoter and the neomycin resistance gene under the control of SV40 promoter. This vector has been used to construct pC53-CX3 by insertion of a human p53 cDNA sequence with an alanine-to-valine substitution at codon 143. The pC53-CX3 plasmid was provided by Dr. Bert Vogelstein. Plasmid pCMVneoH175 has been constructed by insertion of a human p53 cDNA sequence with an arginine-to-histidine substitution at codon 175 in the pCMVneoBAM vector (44).

The cell lines KHOS-240, T47-D, A 431, MCF-7, Hela, SV40, and VA13-W1 38 were kindly provided by Evelyne May. The untransformed WI 38 fibroblasts were obtained from the American Type Culture Collection. The Saos-2 osteosarcoma human cell line was kindly provided by Jean Feunteun. Cells were all grown in DMEM supplemented with 10% fetal bovine serum and antibiotics and maintained in a 10% CO2 humidified atmosphere. The status of the p53 gene in the various human transformed cell lines is presented in Table 1. The entire sequence of the p53-specific cDNA of all of the cell lines analyzed has been controlled by PCR sequencing, as described previously (52).

To obtain CX5.11 and MH175 cell lines, long-term transfections were performed using, respectively, pC53CX3 or pCMVneoH175, in Saos-2 cells; 5 × 10^5 cells were plated in 10-cm Petri dishes and cotransfected on the next day by 5 μg plasmid using the calcium phosphate procedure (53). Two days after transfection, Petri dishes were duplicated in culture medium containing 0.6 mg/ml geneticin (G418; Life Technologies, Inc.). After selection, individualized clones for CX5 clone or a mix of several clones for H175 clone were picked up and checked for protein expression by immunocytochemistry as described previously (54). Positive cell lines were maintained in complete DMEM containing 0.6 mg/ml geneticin.

**Cell Culture and Synchronization and Immunohistochemical and Staining Reagents: Procedure for Immunofluorescence.** Cells were cultured in 35-mm dishes on 22-mm² coverslips in DMEM containing 5% fetal bovine serum and antibiotics in 10% CO2 at 37°C for at least 2 days before immunofluorescence observation. The average cell density in standard experiments was 10^4 cells/cm². When cultures enriched in premitotic and mitotic cells were to be examined, synchronization at the G1-S boundary was first effected by a thymidine-aphidicolin double block; cells were grown in normal medium for 2 days then sequentially incubated with 2.5 μM thymidine (16 h), normal medium (8 h), and 5 μg/ml aphidicolin (16 h). Immunofluorescence with antibrulin antibody and DAPI showed that mitotic cells were most prevalent 6–9 h after release from the double block in HeLa cells and 9–11 h after release in the KHOS-240, A 431, and MCF-7 cell lines.

The immunofluorescence localization of WT and mutant p53 was performed using three different anti-p53 antibodies: the mouse monoclonal antibodies NCL-D07 (Tebu, France) and PAb 122 (hybridoma cell line obtained from the American Type Culture Collection) and a rabbit polyclonal antibody, R1A (a generous gift of John Jenkins). Visualization of the microtubules either in their interphasic organization state, in the spindle apparatus, or in the cleavage furrow was achieved using a rat monoclonal antibody (Bioysis, France). Anti-cyclin A polyclonal rabbit antiserum was a generous gift from Tony Hunter. DNA was counterstained with 1 μg/ml DAPI fluorescent dye (Sigma Chemical Co., France). Secondary antibodies were goat IgG Fab fragments from antibodies directed against either mouse, rat, or rabbit IgG (the antirat IgG was preabsorbed against a
mouse IgG column to eliminate cross-reactivity; a reciprocal preabsorption was used for the antimalouse goat IgG). These fragments were conjugated either with FITC (Interchem or Institut Pasteur, France) or tetramethyl rhodamine isothiocyanate (Interchrom). These secondary antibodies gave essentially undetectable background fluorescence.

Single- or double-fluorescent cell labeling was performed as described previously (55) following fixation with 3% formaldehyde and subsequent permeabilization by treatment with 0.1% Triton X-100 at room temperature. Alternatively, fixation was carried out by treatment with methanol at −20°C for 6 min. The two different fixation procedures generated quite similar final immunofluorescence patterns. For containing with BrdUrd, the cells had been pulsed with BrdUrd (10 μM) for 15 min before fixation and immunolabeled first for p53. At the end, the cells were fixed again with paraformaldehyde and then incubated for 8 min in 4× HCl. After extensive washes in PBS, cells were exposed for 45 min to rat monoclonal anti-BrdUrd antibody followed by fluorescein- or rhodamine-conjugated goat antirat IgG for 30 min. Fluorescent microscopy was performed with a Leitz microscope equipped with fluorescein, rhodamine, and DAPI filters using a ×40 oil objective. Photographs were taken with Kodak Tmax 400 film.

Immunoblotting. The cells were lysed in radioimmunoprecipitation assay buffer containing 10 mM Tris (pH 7.2), 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 100 mM potassium inactivator aprotinin, and the lysates were clarified by centrifugation at 10,000 × g for 20 min at 4°C. The separation of the proteins present in the cellular extracts was performed on 8.5% SDS-polyacrylamide gels after boiling the samples for 3 min in sample buffer (in standard experiments, the samples deposited on the gel each contained 250 μg of proteins). The fractionated proteins were then transferred to nitrocellulose overnight at 20 V and, after blocking, probed for the presence of the p53 antigen with the appropriate antibody solution following overnight incubation at 4°C. After several washes, the filters were incubated with horseradish peroxidase-conjugated antibody, and detection of the immune complex was performed using the enhanced chemiluminescence Western blotting system (Amersham, France).

Acknowledgments

We are indebted to Drs. Tony Hunter and John Jenkins for their generous gift of anti-cyclin A and anti-p53 antibodies and Drs. Bert Vogelstein and Jean Feunteun for providing pC53-CX3 plasmid and the Saos-2 cell line. We also thank Irène Gaspart, Colette Pouget, and Didier Aufray for their expert artwork and Françoise Amoill and Agnès Leray for typing the manuscript.

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