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p53-dependent growth arrest of REF52 cells containing newly amplified DNA

[(*N*-phosphonacetyl)-L-aspartate/carbamoyl-phosphate synthetase–aspartate transcarbamoylase–dihydroorotase/simian virus 40 tumor antigen/tsA58/bridge-breakage-fusion cycles)]

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ABSTRACT **The rat cell line REF52 is not permissive for gene amplification. Simian virus 40 tumor (T) antigen converts these cells to a permissive state, as do dominant negative mutants of p53, suggesting that the effect of T antigen is due mainly to its ability to bind to p53. To manipulate permissivity, we introduced a temperature-sensitive mutant of T antigen (tsA58) into REF52 cells and selected for resistance to *N*-(phosphonacetyl)-L-aspartate (PALA). Most freshly isolated PALA-resistant colonies, each of ≈200 cells, selected at a permissive temperature, arrested when shifted to a nonpermissive temperature. Growth arrest was stable, with no evidence of apoptosis, as long as T antigen was absent but was reversed when T antigen was restored. In contrast, PALA-resistant clones grown to ≈10^7 cells at a permissive temperature did not arrest when shifted to a nonpermissive temperature. All PALA-resistant clones examined had amplified carbamoyl-phosphate synthetase–aspartate transcarbamoylase–dihydroorotase (CAD) genes, present in structures consistent with a mechanism involving bridge-breakage-fusion (BBF) cycles. We propose that p53-mediated growth arrest operates only early during the complex process of gene amplification, when newly formed PALA-resistant cells contain broken DNA, generated in BBF cycles. During propagation under permissive conditions, the broken DNA ends are healed, and, even though the p53-mediated pathway is still intact at a nonpermissive temperature and the cells contain amplified DNA, they are not arrested in the absence of broken DNA. The data support the hypothesis that BBF cycles are an important mechanism of amplification and that the broken DNA generated in each cycle is a key signal that regulates permissivity for gene amplification.**

Normal mammalian cells are not permissive for gene amplification; at least three laboratories (1–4) have failed to obtain drug-resistant colonies containing amplified DNA upon selection of cells from several different species with several different drugs. By using normal human or mouse fibroblasts, Livingstone *et al.* (5) and Yin *et al.* (6) have shown that p53 is necessary to maintain the nonpermissive state: cells lacking p53 gave colonies resistant to *N*-(phosphonacetyl)-L-aspartate (PALA), an inhibitor of aspartate transcarbamylase and thus of UMP biosynthesis, and some of these colonies contained amplified carbamoyl-phosphate synthetase–aspartate transcarbamoylase–dihydroorotase (CAD) genes. Although p53 is necessary, it is not sufficient, since some cells with normal p53 function are still permissive (5, 7), probably because a regulatory step downstream of p53 has been inactivated.

Maintenance of the nonpermissive state could be based on either or both of two very different principles. The fundamental rearrangements of DNA leading to gene amplification might not occur in nonpermissive cells, or amplifications might occur in such cells but then signal the cell to arrest or to die. To address this issue, we have used the rat cell line REF52, which is very unusual in that, like normal cell strains, it is not permissive for amplification (7). REF52 cells can be made permissive by introducing simian virus 40 (SV40) tumor (T) antigen (7), which binds the p53 protein (8, 9). p53 is induced in response to DNA damage, leading to growth arrest or apoptosis (10–13). Therefore, if the DNA rearrangements that are a part of the amplification process involve broken DNA, p53-dependent regulation of amplification would be explained.

Recent understanding of gene amplification does lead to the prediction that broken DNA will be produced by some of the major mechanisms (14). Insight into amplification mechanisms in rodent cells has been provided through studies of the structure of newly amplified DNA by fluorescence *in situ* hybridization (FISH) (15, 16). We have analyzed the structures that include amplified CAD genes in PALA-resistant Syrian hamster cells. Newly amplified DNA, studied in colonies containing ≈10^5 cells, contained multiple copies of CAD, separated from each other by several megabases and present on the same chromosome arm that carries the normal single copy of CAD (17). However, when PALA-resistant Syrian hamster cells were studied much earlier, only a few cell generations after the initial event, 39% of the chromosomes carrying amplified CAD genes were found to be dicentric (18). Similarly, Toledo *et al.* (19) characterized the amplification of AMP deaminase genes in Chinese hamster cells and found evidence for both dicentric chromosomes and megabase-long inverted repeats.

These observations and others [reviewed by Stark (14)] led to the proposal that bridge-breakage-fusion (BBF) cycles, originally identified by McClintock (20), might be important in amplification. Random breakage of DNA between the centromeres of a dicentric chromatid during cell division can lead to asymmetric distribution of the target gene into the two daughter cells (amplification in one cell and deletion in the other). Continuation of this process in subsequent cell cycles can lead to further increases in copy number and to the structures that have been observed. BBF cycles might be initiated either by DNA breakage or by fusion of the telomeric regions of sister chromatids (14). In either case, formation of a dicentric chromatid in a single cell will lead to the appearance of broken DNA in all the descendants of this cell, until the dicentric chromatid is lost (selected against if it carries the amplified target gene) or when the broken ends are healed, for example, by acquiring telomeric sequences (21).

In the present study, we have used a temperature-sensitive mutant of SV40 T antigen, tsA58 (22), to manipulate permissivity in REF52 cells by shifting them from 33°C, where the

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Abbreviations: BBF, bridge breakage fusion; PALA, *N*-(phosphonacetyl)-L-aspartate; CAD, carbamoyl-phosphate synthetase–aspartate transcarbamoylase–dihydroorotase; FISH, fluorescence *in situ* hybridization; T, tumor; SV40, simian virus 40.

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mutant T antigen is functional and the cells are permissive for amplification, to 39.5°C, where T-antigen function is lost and the cells are not permissive. Both the activity of tsA58 necessary for viral replication (23, 24) and the ability to bind to p53 (25) are lost at 39.5°C. By allowing amplification events to begin at a known time at 33°C and then shifting the cells to 39.5°C at several different times thereafter, we have found that cells with newly amplified DNA are arrested reversibly and without evidence of apoptosis at the nonpermissive temperature but that the capacity to arrest is lost progressively with time of growth at 33°C. The data are consistent with the predictions of the BBF mechanism, in which broken DNA appears in the initial amplification event and is propagated through many cell generations, and with the observations cited above that dicentric chromatids, obligatory intermediates in BBF cycles, are lost upon prolonged growth of drug-resistant populations.

MATERIALS AND METHODS

Cell Lines. REF52 cells were infected with the virus LJ-tsSVLT (26), which expresses tsA58 (provided by Hartmund Land, Imperial Cancer Research Fund). Infected cells were selected with G418 (400 μg/ml) and the expression of T antigen was confirmed by Western blot analysis (data not shown). The known C-to-T mutation at codon 438 (27) was confirmed in the infected cells by use of the PCR, followed by DNA sequencing (data not shown). All cell lines were cultured at 10% CO_2/90% air in Dulbecco's modified Eagle's medium, supplemented with 10% (vol/vol) fetal bovine serum.

PALA Selection. To obtain fresh PALA-resistant colonies, groups of 10^3 cells (total 2.4×10^4 cells) were placed into individual wells and expanded to 4×10^4 cells per well at 33°C. All the cells in each well were replated into separate 9-cm plates for selection at 33°C with 33 μM PALA (3× LD_{50}). After 4 weeks, the number of PALA-resistant colonies in each plate was determined. These colonies were used immediately for temperature-shift experiments, without replating.

Detection of Proliferating Cells. Cells were grown for 3 hr in 10 μM 5-bromo-2′-deoxyuridine (BrdUrd) and then fixed with methanol for 30 min. The BrdUrd incorporated into DNA was stained with a monoclonal antibody by using a kit obtained from Amersham.

FISH. Cosmids containing rat CAD sequences were cloned from a library of rat genomic DNA (provided by Richard Akeson, University of Cincinnati). Hamster CAD cDNA (28) was used as a probe. One of three overlapping clones (CRC10, which contained ≈40 kb of rat DNA) was used as a probe in FISH analyses, which were carried out by standard methods (17, 18, 29). Metaphase spreads were prepared by culturing cells for 90 min in Colcemid (BRL; 50 μg/ml). Floating cells were collected by shaking, incubated in 75 mM KCl, fixed, and dropped onto slides. Chromosomal DNA was denatured in 70% (vol/vol) formamide for 5 min at 75°C. CRC10 DNA (50 ng) was labeled with biotinylated dUTP (Bionick labeling kit; BRL), denatured at 100°C for 10 min, and hybridized to metaphase spreads with 10 μg of sonicated rat genomic DNA as competitor. Hybridization signals were amplified once with fluorescein isothiocyanate-conjugated avidin and biotinylated anti-avidin (Vector Laboratories). The DNA was stained with propidium iodide (ICN) at 1 μg/ml.

Induction of waf1 mRNA by p53. PALA-resistant clones L1 and L2 (see below) were grown in PALA for seven more passages at 39.5°C, then placed into PALA-free medium for 3 days, and γ-irradiated from a cesium source. Total cellular RNA was extracted with guanidinium thiocyanate (30) and 10 μg of RNA was loaded onto a formalin/agarose gel. Mouse *waf1* cDNA (from Bert Vogelstein, Johns Hopkins University) (31) was used as a probe. Hybridization was carried out in 50% formamide/0.65 M NaCl at 42°C. Human glyceraldehyde 3-phosphate dehydrogenase cDNA (32) was used as an internal control. Quantitation was performed with a PhosphoImager (Molecular Dynamics).

RESULTS

Dependence of Permissivity on p53 in REF52 Cells. Expression of SV40 T antigen is sufficient to make REF52 cells permissive for gene amplification (7). The ability of T antigen to bind to p53 (8, 9) may be responsible for this effect, since it has been well established in other cells that the nonpermissive state depends upon the presence of functional wild-type p53 protein (5, 6). However, since SV40 T antigen binds to Rb and related proteins in addition to p53 (33), it is important to evaluate these interactions with respect to permissivity. Introduction of the adenovirus E1A protein does not allow REF52 cells to give rise to PALA-resistant colonies (7), ruling out the possibility that binding of Rb (or Rb family members such as p107) to T antigen is sufficient to make REF52 cells permissive for CAD gene amplification. Thus, the effect of SV40 T antigen on permissivity is likely to be due primarily to its ability to bind to p53.

Analyses of p53 function, including induction of a p53-dependent gene in response to γ-radiation (see below) and alteration of permissivity for gene amplification with dominant negative mutant p53 proteins, support the conclusions that p53 is wild type in REF52 cells and that its inactivation is sufficient to make REF52 cells permissive. cDNA clones for expression of mutant p53 proteins were obtained from Peter Chumakov (Engelhardt Institute of Molecular Biology, Moscow). A pool of ≈500 REF52 colonies expressing a mutant of human p53 with a C141 → Y replacement was prepared by transfection, and expression of the mutant protein was confirmed by Western blot analysis (data not shown). Selection with 33 μM PALA (3× LD_{50}) yielded 30 colonies from 9×10^5 cells, whereas selection with PALA of 6×10^5 control cells (transfected with the puromycin-resistance marker only) gave no colonies. A similar experiment was performed with a dominant negative mutant of human p53 with a R175 → H replacement. Selection with PALA at 3× LD_{50} of a pool of ≈400 transfected cells, grown to 4×10^5 cells, gave five colonies. We conclude that inactivation of wild-type p53 is sufficient to make REF52 cells permissive for amplification and that this is the primary mechanism for the effect of SV40 T antigen on permissivity.

Regulation of Permissivity by Temperature-Sensitive T Antigen. Independent clones of REF52 cells expressing tsA58 were established at 39.5°C and assayed for permissivity at 39.5°C and 33°C by selection with 33 μM PALA. All three clones tested, including clone 23, gave PALA-resistant colonies at 33°C but not at 39.5°C in a parallel selection. Clone 23 was chosen for detailed analysis. To establish PALA-resistant colonies representing new and independent amplification events, 48 aliquots, each of 10^3 cells, of clone 23 grown at 37°C were replated at 33°C, allowed to expand to 4×10^4 cells without selection, dispersed, and selected with 33 μM PALA. Twenty-seven plates had no colonies, 17 had one colony, 1 had 2 colonies, 1 had 3 colonies, and 2 had 4 colonies (30 colonies in total). This result shows that the amplification events giving rise to PALA resistance arose late during the period of unselected growth or during the time of exposure to PALA. If a cell with a preexisting amplification had been present in one of the original aliquots, it would have given rise to ≈40 PALA-resistant colonies due to expansion during the period of unselected growth.

Effects of Shifts to a Nonpermissive Temperature. When the 30 PALA-resistant colonies described above had grown to ≈200 cells per colony, the temperature was changed to 39.5°C and the cells were allowed to remain in medium with PALA for 3 days (8 colonies) or 13 days (22 colonies) more. The colonies did not grow appreciably during this period. Most

contained no or very few cells that were labeled with BrdUrd. However, three colonies (10%) did contain a substantial fraction of cells that were so labeled (Table 1). We conclude that most REF52 cells containing newly amplified CAD DNA are rapidly arrested when shifted to a condition that is not permissive for amplification.

A second experiment was performed in which colonies of intermediate age were examined. Cultures were divided and selected with PALA at 39.5°C or 33°C. No colonies were observed at 39.5°C. Four colonies (E1–E4) obtained at 33°C were picked, and each colony was expanded to ≈1000 cells at 33°C and divided into two portions 60 days after the start of selection. These were maintained for 3 days in the absence of PALA, either at 39.5°C or at 33°C. Incorporation of BrdUrd was observed with all four samples maintained at 33°C but not with three of the four samples shifted to 39.5°C (Table 2, experiment A). Further growth at 33°C of clone E2 for 10 days or of clone E3 for 26 days before a shift to 39.5°C for 3 days led to different results: now substantial incorporation of BrdUrd into the cells that had been shifted to 39.5°C was seen (Table 2, experiment B). Therefore, the properties of clones E2 and E3 changed during the additional period of growth. These results show that the failure of permissive cells carrying newly amplified DNA to grow when shifted to a nonpermissive temperature is transient and is lost upon prolonged growth under permissive conditions. To confirm this, a third experiment was performed in which each of the four PALA-resistant clones (L1–L4) was expanded at 33°C to 10^7 cells in the presence of 33 μM PALA and then shifted to 39.5°C. The cells in all four clones incorporated BrdUrd readily and continued to grow at the high temperature (Table 2, experiment C).

Properties of PALA-Resistant Cells. Five PALA-resistant clones (L1–L4 and E2) were analyzed for CAD gene amplification by the FISH method. All showed four to eight discrete CAD signals on the same chromosome (Fig. 1 and data not shown). Thus, as expected for rodent cells, PALA resistance is due to CAD gene amplification.

The morphology of PALA-resistant cells that had been arrested by temperature shift was examined after 3 or 16 days at 39.5°C (Fig. 2). The cells showed enlargement of the cytoplasm and a streaked appearance and, in general, were similar to PALA-sensitive REF52 cells soon after PALA-induced growth arrest. It is noteworthy that, even after 16 days, the arrested cells were largely intact and that nuclear condensation and cytoplasmic fragmentation, hallmarks of apoptosis (34), were not observed.

By using clone E3, we examined the reversibility of arrest. In parallel with Table 2, experiment A, a third portion of E3 cells was shifted to 39.5°C for 3 days and then shifted back to 33°C. After 3 days more, the BrdUrd assay revealed that 6 of 117 cells were positive, similar to the 9 of 119 BrdUrd-positive cells found in the population that was maintained at 33°C (Table 2). Therefore, the growth arrest observed at 39.5°C is reversible when the cells are returned to the permissive temperature.

Table 1. Incorporation of BrdUrd into new PALA-resistant colonies shifted to a nonpermissive temperature

	No. of colonies				
Exp.	Total	0% B^+	0.5–1.5% B^+	2–5% B^+	>5% B^+
A	22	13	7	0	2
B	8	6	0	1	1

PALA selection at 33°C yielded colonies of ≈200 cells. After selection, the cells were shifted to 39.5°C for 13 days (experiment A) or 3 days (experiment B) in the presence of PALA and were then labeled with BrdUrd for 3 hr. The labeling index of control REF52 cells was 13%. Note that this index is lower for new colonies when PALA is present than for new colonies grown without PALA (see Table 2). % B^+, % of BrdUrd-positive cells per colony.

Table 2. Incorporation of BrdUrd into intermediate and late PALA-resistant colonies shifted to a nonpermissive temperature

		% of BrdUrd-positive cells	
Exp.	Colony	Maintained at 33°C	Shifted to 39.5°C
A	E1	34	0
	E2	24	0
	E3	8	0
	E4	21	6
B	E2	22	5
	E3	18	16
C	L1	30	6
	L2	13	8
	L3	24	7
	L4	35	16

In experiment A, intermediate colonies (≈10^3 cells) selected at 33°C in the presence of PALA were incubated for 3 days without PALA at either 33°C or 39.5°C and then labeled with BrdUrd for 3 hr. In each case, 100–200 cells were scored. In experiment B, colonies were grown at 33°C in the presence of PALA for an additional 10 days (E2) or 26 days (E3) before transfer to PALA-free medium and continuation as in experiment A. In experiment C, late colonies (≈10^7 cells) selected at 33°C in the presence of PALA were assayed as in experiment A.

The growth of clones L1–L4 might be insensitive to incubation at 39.5°C either because the p53 gene has become inactive or because the signal inducing p53 is no longer present. To test for p53 function, clones L1 and L2 were assayed for induction of the p53-responsive gene *waf1* (31) after exposure to γ-radiation. Although *waf1* can be induced in a p53-independent manner by some stimuli (35), induction by γ-radiation is p53-dependent (36, 37). As shown in Fig. 3, when the cells were exposed to γ-radiation, waf1 mRNA was induced to comparable levels in L1, L2, and REF52 cells. These results establish that normal p53 function is maintained in cells containing amplified CAD genes that are no longer arrested at a nonpermissive temperature.

DISCUSSION

In the present study, the availability of wild-type p53 protein in REF52 cells has been manipulated by use of the temperature-sensitive p53-binding protein tsA58. Cells allowed to initiate amplifications of the CAD gene at a permissive temperature in the absence of p53 were shifted to a nonpermissive temperature, where p53 function was restored. Arrest occurred, even in the absence of the selective agent PALA.

FIG. 1. FISH analysis of clone E2. A chromosome pair with six copies of the CAD gene is indicated by an arrowhead. A normal chromosome pair with two copies of CAD can be seen below and to the right of the arrowhead.

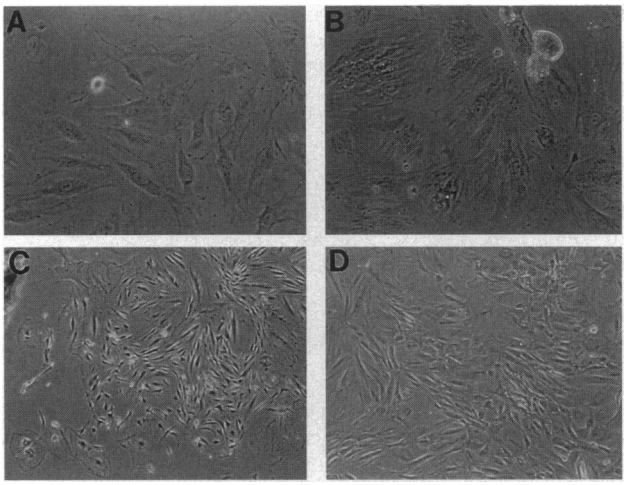

FIG. 2. Morphology of PALA-resistant clones. After selection at 33°C, clone 10 was kept at 33°C with PALA (*A*) or shifted to 39.5°C for 3 days without PALA (*B*); clone 23 was kept at 33°C with PALA (*C*) or shifted to 39.5°C for 16 days with PALA (*D*). (*A* and *B*, ×25; *C* and *D*, ×65.)

When the shift was performed early, the cells were arrested stably, with no evidence of apoptosis, and a shift back to a permissive temperature showed that the arrest was reversible. With increasing time of growth at a permissive temperature, the PALA-resistant cells became progressively more resistant to arrest at a nonpermissive temperature, despite the fact that they still contained functional p53 protein. Yanai and Obinata (38) observed that mouse hepatocyte and kidney tubule cell lines containing a temperature-sensitive mutant of T antigen underwent apoptosis when shifted to 39°C, in contrast to our results with REF52 cells. The differences are likely to be due to differences in factors such as levels of expression of T antigen or the cell types studied.

Our data are consistent with the following scenario: the predominant mechanism of CAD amplification in rat REF52

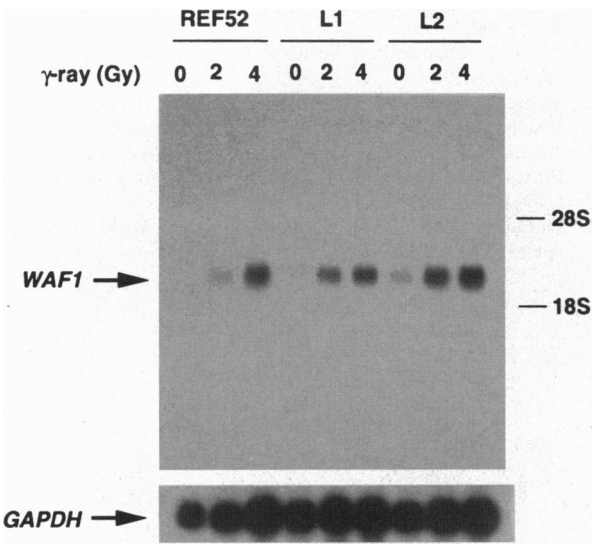

FIG. 3. Induction of waf1 mRNA by γ-radiation of PALA-resistant clones. Clones L1 and L2 and control REF52 cells were studied. Cells were irradiated with 2 or 4 Gy, and total cellular RNA was prepared, fractionated by electrophoresis, and transferred to a membrane. The probes used corresponded to mouse *waf1* and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative to the normalized levels of waf1 mRNA in unirradiated cells, the fold inductions at 2 Gy were 2.3, 3.1, and 2.5 and at 4 Gy were 3.2, 3.2, and 2.6 for REF52, L1, and L2, respectively.

cells is probably similar to the mechanism in other rodent (Syrian and Chinese hamster) cells, involving initial formation of a dicentric chromatid and subsequent BBF cycles (14). The event likely to be regulated in nonpermissive cells is their response to a double-strand break in DNA. If chromosome breakage were the initial amplification event, a nonpermissive cell would probably respond by inducing wild-type p53 protein, which would in turn induce the transcription of genes such as *waf1*, whose products arrest the cell cycle. If this response were sufficiently rapid, the initial dicentric chromatid would not even form, since the cells must replicate the broken chromosome first (14). If telomere-region fusion was the initial event, broken DNA would not appear until after the dicentric chromatid had been ruptured during segregation of the two daughter cells, and amplification might occur before regulation was imposed. In either case, the BBF model of amplification predicts that broken DNA will be produced in every cell division and for many cell generations thereafter, until the cells either lose their dicentric chromosomes (unlikely when there is selection for amplified DNA) or heal the broken ends. In the absence of p53, broken DNA is tolerated and the cells can grow. Gradually, the broken ends are healed, dicentric chromosomes are no longer formed, and the newly amplified DNA evolves to a more stable form. In such cells, with amplified DNA but no broken ends, the presence of functional p53 no longer leads to arrest of cell growth, since amplified DNA *per se* does not provide a regulatory signal. The recent work of Bertoni *et al.* (39) on the structure of amplified DNA in PALA-resistant CHO cells provides an explanation for at least one mechanism of healing. If dicentric chromosomes break within interstitial sequences that contains TTAGGG repeats, these can seed new telomeres.

We believe that p53 is induced in REF52 cells containing broken DNA and that this induction is maintained stably until other events lead to the demise of the arrested cell. If p53 is removed from such cells within a few days, the arrest is reversed and the cells can grow again. The BBF model predicts that a minimum of one double-strand break is present in the arrested cells. If so, this small signal is sufficient to give stable growth arrest. The conclusion that a very small number of double-strand breaks can give stable arrest has also been reached independently by Di Leonardo *et al.* (40) in their study of the response of normal human fibroblasts to γ-radiation. In our work, no exogenous means has been used to break the DNA, removing the potential complication that other processes induced by ionizing radiation (such as the formation of highly reactive chemical species) might contribute to the effects observed. Our data are also consistent with a major conclusion of Di Leonardo *et al.* (40), namely, that p53-mediated arrest in response to DNA damage is essentially irreversible. In our case, the arrested cells do not grow until they are shifted to a temperature at which p53 function is inactivated. The stable arrest of growth observed in both studies indicates that the main role of p53 is not to allow time for repair of DNA damage but rather to prevent the propagation and even the survival of cells that have experienced unrepaired damage.

The ability to arrest at a nonpermissive temperature, retained in most members of 200-cell colonies, is beginning to be lost in the 10^2- and 10^3-cell colonies and is not apparent in the 10^7-cell colonies. This rate of loss is consistent with the initial rapid appearance and slow disappearance of dicentric chromosomes bearing amplified CAD genes in PALA-resistant Syrian hamster cells (18). Dicentric chromosomes were observed in 39% of these cells a few generations after the initial event. However, this percentage is certain to be an underestimate of the number of cells containing dicentric structures since dicentric chromatids, formed earlier in the amplification process, are not distinguished readily from monocentric chromosomes carrying amplified DNA [see Toledo *et al.* (19) for a

full explanation]. When colonies of PALA-resistant Syrian hamster cells were analyzed in the 10^5-cell colonies, dicentric chromosomes were already rare (17).

Alteration of permissivity in the presence of wild-type p53 has been observed in REF52 cells, where cointroduction of cDNAs encoding both adenovirus E1A and mutant ras proteins, neither of which binds to p53, allows the cells to become permissive for CAD gene amplification (7), and also in some human cell lines that have a wild-type functional p53 (5). It is logical to expect that disruption of genes that lie downstream of p53 in pathways that regulate the normal response to broken DNA will also lead to permissivity for amplification via the BBF mechanism. Candidates include *waf1* (31).

The only two stable cell lines we know of that are not permissive for gene amplification are REF52 and a human line that we have created recently by introducing a cDNA encoding wild-type p53 regulated by the normal p53 promoter into p53-negative Li-Fraumeni cells (Munna Agarwal and G.R.S., unpublished observations). Therefore, loss of the normal ability to respond to DNA damage via p53-dependent pathways, through alterations of p53 itself or of downstream effectors, must be a very common event in the establishment of immortal cell lines from senescent normal cells or cell strains. The reasons for this high degree of correlation will be important to discover.

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