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The p53-Mediated G_1 Checkpoint Is Retained in Tumorigenic Rat Embryo Fibroblast Clones Transformed by the Human Papillomavirus Type 16 E7 Gene and EJ-*ras*

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Rat embryo fibroblast clones transformed with the human papillomavirus type 16 E7 gene and the H-*ras* oncogene (ER clones) fall into two groups on the basis of endogenous p53 genotype, wild type or mutant. We have compared these clones with the aim of identifying physiological differences that could be attributed to p53 protein function. We show that all ER clones, regardless of p53 gene status, are tumorigenic and metastatic in severe combined immunodeficiency mice. We demonstrate that only the wild-type p53 protein expressed in ER clones is functional on the basis of its site-specific double-stranded DNA-binding activity and its ability to confer a G_1 delay on cells following treatment with ionizing radiation. These data indicate that disruption of the p53 growth-regulatory pathway is not a prerequisite for the malignant conversion of rat embryo fibroblasts expressing the E7 gene and mutant *ras*. Differences in phenotype that were correlated with loss of p53 protein function included the following: serum-independent growth of ER clones in culture, decreased tumor doubling time in vivo, and increased radioresistance. In addition, we demonstrate the p53-dependent G_1 checkpoint alone does not determine radiosensitivity.

The p53 tumor suppressor gene is frequently a target for recessive mutations in a wide range of human and rodent malignancies. Loss of p53 expression, however, is not a prerequisite for tumor growth. In neuroblastoma and human acute myelogenous leukemia, for example, p53 gene mutations are infrequent; in breast, colon, and lung tumors, in which p53 gene alterations are more common, the incidence of p53 mutation only reaches 50 to 60% (19, 26, 27). In addition, several reports indicate that mutation of the p53 gene is not required for the initiation of tumorigenesis but, rather, represents a late event involved in tumor progression (reviewed in reference 1).

The realization that p53 gene function can be disrupted not only through mutation but also through nongenetic mechanisms including protein-protein interactions (38, 41, 47), protein conformational change (36, 51), or nuclear exclusion (37) has raised the possibility that in tumor cells expressing a wild-type p53 gene, the encoded protein may be nonfunctional. It has also been suggested that the requirement for p53 mutation can be bypassed in certain tumor cells upon the expression of other oncogenes (32). Mutation of genes that lie upstream or downstream of p53 function on the same growth-regulatory pathway may obviate the requirement for p53 mutation.

The loss of wild-type p53 expression in tumor cells appears to provide these cells with a selective growth advantage in vivo. However, no satisfactory explanation exists at the cellular or molecular level to account for the growth advantage experienced by cells that have acquired a mutation in the p53 gene. Two models have been put forward to explain the role of p53 as a tumor suppressor. In one model, p53 participates in the cellular response to DNA damage by delaying cell cycle progression at a G_1 checkpoint (22, 24). Arrest in G_1 is likely the result of p53-dependent induction of the *WAF1/CIP1* gene, which encodes a 21,000-Da protein that inhibits the activity of cyclin-dependent kinases necessary for the G_1-S transition (11, 17, 54). This delay is postulated to allow time for repair of damaged DNA, which otherwise might interfere with accurate DNA replication, and for repair of lesions that might be perpetuated as mutations in cells entering S phase. Loss of the p53-mediated G_1 checkpoint has been shown to be associated with genetic rearrangement and gene amplification in cells treated with the metabolic inhibitor *N*-phosphonacetyl-L-aspartate (PALA) (28, 55).

In a second model, the role of p53 as a tumor suppressor is explained through its involvement in apoptosis (48, 57). In short-term assays, p53 has been shown to initiate apoptosis when it is induced by agents that cause DNA strand breakage, including ionizing radiation and treatment with various chemotherapeutic agents (4, 14, 30, 31). Moreover, oncogenes such as c-*myc* and the E1A gene can induce apoptosis particularly after serum depletion, and this form of apoptosis has also been shown to be p53 dependent (6, 29). p53-dependent apoptosis does not necessarily require cell cycle arrest. In this model, wild-type p53 expression might be expected to diminish cell growth and long-term survival in response to DNA damage. p53 mutation would abrogate oncogene-associated apoptosis and allow survival of oncogene-expressing cells. The observation that cells expressing wild-type p53 are more sensitive to ionizing radiation than cells expressing mutant p53 protein, as measured by clonogenic survival, lends support to this model (3, 25).

Analysis of a series of rat embryo fibroblast (REF) clones established in our laboratory after transformation with the human papillomavirus type 16 (HPV-16) E7 gene and the activated H-*ras* gene (ER clones) provides an opportunity to identify the selective advantage, if any, conferred by p53 mutation. While all of these clones are morphologically transformed and capable of growth in methylcellulose, we previously found that they fell into two distinct classes. Clones of

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one class have a single missense mutation in the endogenous p53 gene and fail to express wild-type p53 RNA or protein. These clones have a reduced serum requirement for growth in methylcellulose, show density-dependent growth in serum-free medium, and display responsiveness to growth factor(s) present in conditioned medium. The growth of these cells involves an autocrine mechanism. In contrast, clones of the second class express wild-type p53 protein and exhibit an absolute dependency on growth factors present in serum at all cell densities tested (42). In this study, we have assessed the functional state of p53 protein in these clones, their tumorigenicity, and their response to ionizing radiation. We demonstrate that a p53-dependent G_1 checkpoint is present in transformed cells expressing wild-type p53 and absent in cells expressing mutant p53 protein, that loss of this checkpoint is not a prerequisite for tumor growth, and that loss of this checkpoint is correlated with increased clonogenic cell survival following ionizing radiation.

MATERIALS AND METHODS

Cell lines. ER clones were used between the 10th and 20th passages in culture; ER clones bearing endogenous mutant p53 alleles (ER12L5, ER10K1, ER11-2, and ER5-2) or an exogenous murine p53 allele in which mutation at codon 193 converts arginine to proline (ERM115) were described previously (42). ERMts clones were transformed with the HPV-16 E7 gene, EJ-H-*ras*, and a temperature-sensitive (*ts*) murine p53 (p53ts) allele in which mutation at codon 135 converts alanine to valine (35). Cells were grown in α minimal essential medium supplemented with 10% fetal calf serum.

Western blot (immunoblot) analysis of HPV-16 E7 protein. Cell extracts were prepared from 10^7 cells and lysed on ice for 30 min with 0.5 ml of radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 20 μg of phenylmethylsulfonyl fluoride per ml, 2 μg of leupeptin per ml, 4 μg of aprotinin per ml, 2 μg of pepstatin per ml, 1.4 mM EDTA, 150 mM NaCl, 1 mM Tris [pH 7.2]). Lysates were cleared by centrifugation for 15 min. Eighty micrograms of total protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper by using a semidry electrophoretic transfer cell (Bio-Rad) for 30 min in a solution containing 75 mM glycine, 10 mM Tris, and 20% methanol. Filters were incubated overnight at 4°C in a solution containing 10 mM Tris (pH 7.4), 0.9% NaCl, 0.05% Tween 20 (TBST), and 10% instant skim milk powder. Filters were then incubated in 40 ml of TBST containing 1:100 HPV-16 E7 mouse monoclonal antibody (Triton Biosciences Inc.) for 60 min at room temperature. Filters were washed three times in a solution of TBST containing 1% milk powder for 5 min. Finally, peroxidase-conjugated sheep anti-mouse immunoglobulin (Sigma) was incubated with the filter and detected by the enhanced chemiluminescence method (Amersham).

Cell cycle analysis by flow cytometry. Cells (5.0×10^6 per dish) exposed to ionizing radiation (^{60}Co gamma rays) and nonirradiated controls were incubated 16 h after irradiation with 10 μM bromodeoxyuridine (BrdU) for 2 h, harvested, and washed with 10 ml of immunofluorescence assay (IFA) buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 150 mM NaCl, 4% fetal calf serum, 0.1% NaN_3). Cells were fixed by dropwise addition of 70% ethanol (5 ml; prechilled at −20°C) with gentle mixing on a vortex mixer and incubated on ice for 30 min. Cells were collected by centrifugation at 4°C, resuspended in a solution containing 0.1 N HCl and 0.7% Triton X-100, and incubated on ice for 10 min. Cells were diluted and resuspended in 2 ml of cold phosphate-buffered saline (PBS; minus Mg and Ca), pelleted, resuspended in 0.5 ml of deionized water, and transferred to microtubes containing 16 μl of 0.1 N HCl. Samples were incubated at 97°C for 10 min and immediately transferred to ice for a further 10 min. Cells were washed twice with 1 ml of IFA buffer containing 0.5% Tween 20 and resuspended in a final volume of 1 ml of IFA buffer. Twenty microliters of a fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson) was added to each sample; the samples were incubated on ice for 30 min, washed twice with 1 ml of IFA buffer containing 0.5% Tween 20, resuspended, and incubated for 15 min at 37°C in 0.5 ml of IFA buffer containing 100 U of RNase A per ml. Finally, samples were treated with 50 μg of propidium iodide (Sigma) per ml and incubated for a minimum of 1 h at 4°C. The stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson) with the Lysis II software program.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from cells that were lysed in guanidinium isothiocyanate (33). Northern blot analysis was performed by using Zeta probe membranes (Bio-Rad) as described by the manufacturer. Solutions for prehybridization and hybridization contained 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 2% SDS, 5× Denhardt's solution, 0.1 mg of denatured salmon sperm DNA per ml, and 0.1 mg of poly(rA) (Sigma) per ml. The *WAF1* probe was a 700-bp *Eco*RI fragment of the mouse *WAF1* cDNA clone pCMW35 (provided by Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, Md.). The *GAPDH* probe was a 1,300-bp *Pst*I fragment of rat *GAPDH* cDNA (13). Both probes were labelled by the random primer method (12) using [α-^{32}P]dCTP.

Electrophoretic gel mobility shift assay (EMSA). To prepare nuclear extracts, cells were lysed for 5 min on ice in buffer A (20 mM HEPES [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 4 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml). Cells were scraped off dishes and pelleted. The supernatant was discarded, and the nuclear pellet was resuspended in 3 volumes of modified buffer A containing 500 mM NaCl, incubated on ice for 30 min, and centrifuged for 10 min. The supernatant was retained and frozen at −70°C. The DNA binding reactions were carried out in a final volume of 10 μl containing 1 μl of working buffer (100 mM HEPES [pH 7.4], 70% glycerol, 10 mM $MgCl_2$, 5 mM dithiothreitol), 1 μl of poly(dI-dC) (1 μg/μl; Sigma), 1 μl of nuclear extract, and 1 μl of ^{32}P-labelled double-stranded p53CON oligonucleotide, containing the p53 consensus binding sequence (15) underlined (GGATCCAAGCTT<u>GGACATGCCCGGGCATGTCC</u>CTCGAGGGATCC), with or without 1 μl of monoclonal antibody PAb421 (16). The oligonucleotide was labelled with Klenow DNA polymerase and [α-^{32}P]dCTP in a fill-in reaction after annealing with a short antisense oligonucleotide (GGATCCCTCGAG). Binding reaction mixtures were incubated at room temperature for 25 min, and samples were analyzed on a 4% nondenaturing polyacrylamide gel run at 200 V for 2.5 h. Gels were dried and exposed to X-ray film overnight at room temperature.

Assessment of tumorigenicity and spontaneous metastasis. Tumorigenicity was assessed following injection of 10^5 cells intramuscularly into the hind legs of severe combined immunodeficiency mice. Tumor doubling times were calculated as the time for tumors to increase from 0.5 to 1.0 g in weight during the logarithmic period of tumor growth. Serial measurements of tumor diameters were converted to weight, using a previously established calibration curve (2). Spontaneous lung metastases were scored as macrocolonies visualized on the lung by using a low-power dissecting microscope following fixation in Bouin's solution at necropsy, when primary leg tumors had reached a size of 2.5 g.

Radiation sensitivity of transformed REFs. Cells were washed in PBS, trypsinized, and plated onto 60-mm-diameter plastic tissue culture dishes prior to irradiation. Cells were irradiated 16 to 18 h after plating at cell densities that would yield 25 to 70 surviving colonies at the time of enumeration. Repeated experiments on all clones revealed that the multiplicity index was less than 1.1 at the time of irradiation. Irradiation (0 to 10 Gy) with ^{137}Cs gamma rays was given under aerobic conditions at room temperature. Surviving colonies were fixed and stained 8 to 10 days postirradiation with methylene blue solution containing 50% ethanol. Mean survival curves were obtained from repeated experiments (minimum of four) for each of the clones tested between the 10th to 20th passages in culture. Cell survival data were modelled to the linear quadratic model of cell kill as described previously (3). As the cellular surviving fraction for many of the REF clones was greater than 0.01 at the doses used (maximum of 10 Gy), the slope of the survival curve in the high-dose region and the mean inactivation dose could not be calculated with certainty. Consequently, the mean surviving fraction at 2 Gy (SF_{2Gy}) and the radiation dose required to reduce fractional survival to 0.10 (D_{10}) were calculated for each clone.

Statistical analyses. Parameters of radiosensitivity and tumor growth for all of the transformed clones were compared by using the nonparametric Mann-Whitney test.

RESULTS

Tumorigenicity of ER clones. ER clones were isolated initially as foci of morphologically transformed cells that overgrew a monolayer of normal REFs after gene transfer and subsequently could be established in long-term culture. We have confirmed that all ER clones used in this study express E7 and Ras proteins by radioimmunoassay using monoclonal antibodies against E7 protein and mutant Ras proteins (data not shown). These clones were previously shown to fall into two classes on the basis of p53 genotype, wild type or mutant. We have compared these clones with the aim of identifying physiological differences which might be attributed to wild-type p53 protein function. One difference, described previously, is the ability of clones expressing mutant p53, but not those expressing wild-type p53, to grow in the complete absence of serum. Growth of the cells containing mutant p53 was shown to be cell density dependent and to involve an autocrine pathway (42).

We wished to investigate whether loss of wild-type p53 expression was associated with increased tumorigenic or metastatic potential. Severe combined immunodeficiency mice were

TABLE 1. Tumorigenicity and metastatic ability of ER clones

ER clone	Tumor doubling time (days)	No. of animals with metastases[a]	No. of metastases (mean ± SEM)
Expressing wild-type p53 protein			
ER7-5	2.9	3	3 ± 18
ER8-3	3.4	3	6 ± 3
ER14-5	1.7	3	70 ± 18
ER15-1	3.4	3	18 ± 5
ER16-4	3.7	3	36 ± 15
ER17-1	3.5	3	67 ± 7
ER19-1	1.9	3	7 ± 3
ER414	3.8	3	26 ± 4
Mean	3.0 ± 0.3		29 ± 9
Expressing mutant p53 protein			
ER12L5	1.7	3	52 ± 25
ER10K1	1.7	3	24 ± 9
ER11-2	1.7	2	20 ± 12
ER5-2	1.1	3	3 ± 2
Mean	1.4 ± 0.2		25 ± 10

[a] Spontaneous lung metastases. Each group consisted of three animals with primary tumors.

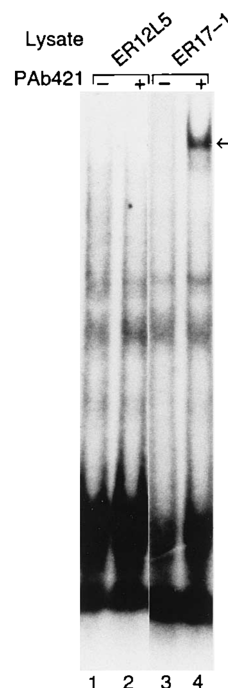

FIG. 1. DNA binding of endogenous wild-type p53 protein expressed in ER clones. EMSAs were performed with nuclear extracts prepared from ER clones expressing mutant p53 protein (ER12L5) or wild-type p53 protein (ER17-1). Binding reactions were performed with a ^{32}P-labelled p53CON oligonucleotide with (+) or without (−) monoclonal antibody PAb421. The position of the supershifted protein-DNA complex is indicated by the arrow.

injected with wild-type p53-expressing or mutant p53-expressing ER clones and monitored for tumors at the sites of injection (Table 1). All of the clones were tumorigenic regardless of p53 gene status. Median tumor doubling times in the ER clones expressing wild-type p53 ranged from 1.7 to 3.8 days, while those in clones expressing mutant p53 ranged from 1.1 to 1.7 days. This difference in tumor doubling time was significant in the Mann-Whitney test ($P < 0.02$). Spontaneous lung metastases were seen with all of the ER clones, and the mean number of lung colonies per animal was not significantly different between ER clones expressing wild-type or mutant p53 protein (Table 1).

Biochemical properties of wild-type p53 protein expressed in ER clones. The finding that the tumorigenic potential of ER clones is independent of p53 gene status raises a question regarding the functional state of the wild-type p53 protein expressed in ER clones. It has been suggested that transformation by other oncogenes may bypass the need for p53 mutation, and it has been demonstrated that the interaction of wild-type p53 protein with cellular or viral proteins can result in the neutralization of the tumor suppressor activity of normal p53 protein. To address this question, we examined two biochemical properties of p53 protein that have been shown to be intimately associated with its ability to act as a tumor suppressor, namely, site-specific DNA binding and transcriptional activation (43).

DNA binding was investigated by using an EMSA. Crude nuclear extracts were prepared from ER cells and incubated with a ^{32}P-labelled p53CON oligonucleotide. Inclusion of the p53-specific monoclonal antibody PAb421 in the binding reaction facilitated visualization of the p53-DNA complex with the EMSA. Binding was evident in reactions containing nuclear extracts prepared from all ER clones expressing wild-type p53 protein but not in reactions containing nuclear extracts prepared from mutant p53-expressing ER clones. Binding reactions for two representative clones are shown in Fig. 1. Competition with an excess of unlabeled p53CON oligonucleotide but not with an equivalent amount of unrelated oligonucleotide having the same nucleotide composition as p53CON confirmed that binding was specific (data not shown).

The *WAF1/CIP1* gene, which encodes a 21,000-Da protein that inhibits the activity of cyclin-dependent kinases, was recently shown to be a target for transcriptional regulation by wild-type p53 protein (11). WAF1/CIP1 is induced in wild-type p53-containing cells by exposure to DNA-damaging agents, including ionizing radiation, but not in mutant p53-containing cells. The *WAF1/CIP1* gene has been proposed to be a critical downstream effector in the p53-specific pathway of growth control in mammalian cells (8, 10, 49). We therefore investigated the level of *WAF1/CIP1* mRNA induction in ER clones treated with ionizing radiation. RNA was isolated from untreated cells and from treated cells 4 and 16 h after treatment with a dose of 6 Gy, and the level of *WAF1/CIP1* mRNA was determined by Northern blotting; *GAPDH* served as a control transcript in these experiments. A representative Northern blot is shown in Fig. 2A. The relative abundance of *WAF1/CIP1* mRNA in cells was estimated by first normalizing to the value of *GAPDH* mRNA in each sample. The data for nine independent ER clones expressing wild-type p53, each tested once or twice, were combined and are summarized in Fig. 2B. These data show an increase in the level of *WAF1/CIP1* mRNA in irradiated normal REFs as well as in irradiated ER clones expressing wild-type p53 protein. Induction was the same at 4 and 16 h after irradiation. In contrast, induction was not seen in two irradiated ER clones lacking a wild-type p53 gene (ER12L5 and ER5-2 cells) or in irradiated REFs transfected with the E7 gene, *ras*, and a dominant negative mutant p53 allele (ERM115 cells). A small increase in the level of *WAF1/CIP1* mRNA was seen in irradiated ER11-2 cells.

Cell cycle analysis in ER cells following ionizing radiation. *WAF1/CIP1* mRNA induction after irradiation correlated with wild-type p53 gene expression in the ER clones. Since p53-

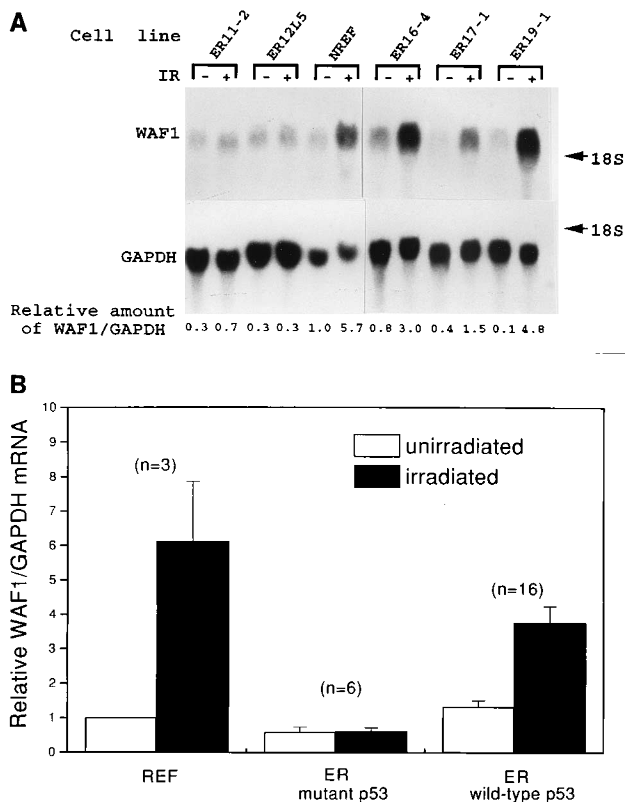

FIG. 2. Induction of *WAF1* mRNA in irradiated cells expressing wild-type p53 protein. (A) Northern blot analysis of *WAF1* mRNA levels in wild-type p53-expressing cells (ER16-4, ER17-1, ER19-1, and normal REFs) and mutant p53 expressing cells (ER11-2 and ER12L5) treated with ionizing radiation. RNA was isolated from cells either left untreated (−) or 4 h after treatment with a dose 6 Gy of ionizing radiation (+). Blots were probed first with a *WAF1* cDNA probe and subsequently with a *GAPDH* probe to normalize for the amount of RNA loaded in each lane. The amount of *WAF1* RNA and *GAPDH* RNA was determined with a Molecular Dynamics PhosphorImager using Multiquant software. (B) The results from panel A and additional experiments in which cells were irradiated with 6 Gy and harvested either at 4 or 16 h after irradiation are summarized in the form of a histogram. Each mean value for the REFs was determined from three experiments, and each mean value for the wild-type p53-expressing ER clones represents an aggregate derived from nine clones, seven of which were tested twice and two of which were tested once. Each mean value for the mutant p53-expressing ER clones represents an aggregate derived from three clones each tested at least once and a single ERM clone expressing exogenous mutant p53 protein tested once.

mediated *WAF1/CIP1* induction is believed to confer G_1 arrest on cells following ionizing radiation, we next examined the ability of irradiated ER cells to enter S phase. This analysis measures a cellular response that is dependent on wild-type p53 protein function; moreover, it represents a late event in the p53-specific pathway of growth control. The inability of irradiated cells to enter S phase would provide a further line of evidence indicating that the endogenous p53 protein is functional and that the p53-specific growth-regulatory pathway is retained in the presence of the HPV-16 E7 gene and mutant *ras*.

ER clones expressing endogenous wild-type or mutant p53 protein were treated with 6 Gy of ionizing radiation. Sixteen hours after treatment, cells were incubated in BrdU for 2 h. Cell proliferation was assessed by DNA content (propidium iodide staining) and incorporation of BrdU (using an FITC-conjugated antibody for BrdU) by flow cytometry (Fig. 3), and changes in the proportion of cells in S phase and in the G_1:S

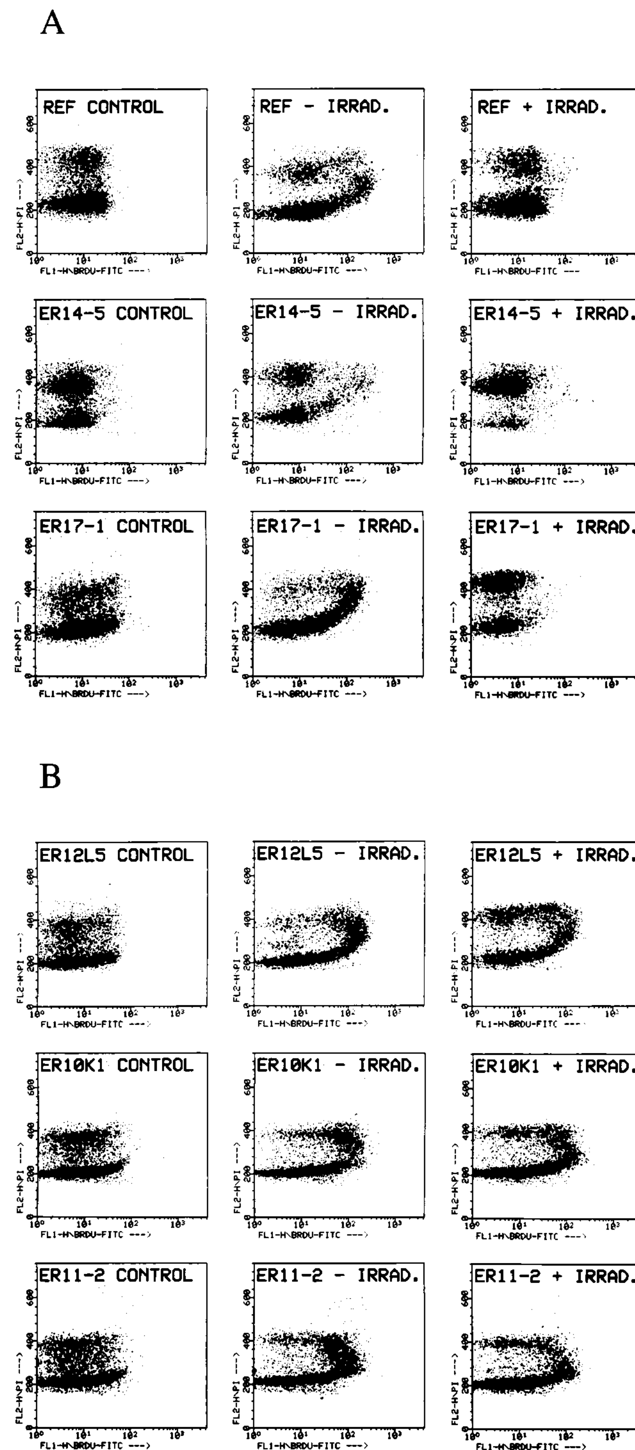

FIG. 3. Cell cycle changes in ER clones after exposure to ionizing radiation (6 Gy) as a function of p53 gene status. Flow cytometric dot plots display simultaneously the analysis of S-phase cells, as determined after a 2-h pulse with BrdU at 16 h after irradiation on the *x* axis and DNA content, as determined by staining with propidium iodide, on the *y* axis. In panel A are shown results for early-passage REFs and two ER clones expressing wild-type p53 protein. In panel B are shown results for three independent clones expressing endogenous mutant p53 protein. The control for each cell line represents unirradiated cells that were not pulsed with BrdU but were, nevertheless, stained for replicative DNA synthesis with an FITC-conjugated anti-BrdU antibody and for DNA content with propidium iodide.

TABLE 2. Cell cycle progression[a] following exposure to ionizing radiation

Cells	G1:S (0 Gy)	G1:S (6 Gy)	G1:S+/ G1:S−	%S−/ %S+
ER clones expressing wild-type p53				
REFs	2.0	13.6	6.8	5.3
	3.9	10.2	2.6	2.4
ER17-1	4.0	>82.8	>20.7	>32.4
ER7-5	16.6	>143	>8.6	>8.6
ER414	2.1	5.6	2.7	2.9
ER19-1	3.5	9.6	2.7	3.7
ER511	13.0	41.2	3.2	4.8
ER8-3	2.6	6.2	2.4	2.7
ER14-5	2.7	5.8	2.1	4.0
ER clones expressing mutant p53				
ER5-2	4.6	3.7	0.8	1.0
	2.7	3.7	1.4	1.5
ER11-2	1.7	1.8	1.1	1.1
	6.8	12.7	1.9	1.7
ER10K1	1.5	1.1	0.7	0.9
	7.8	10.5	1.3	1.4
ER12L5	2.1	2.8	1.3	1.6
	1.3	1.5	1.2	1.3
ERM clones				
ERM115				
At 37°C	1.0	1.3	1.3	1.3
At 32°C	1.9	2.1	1.1	1.2
ERMts-2				
At 37°C	0.8	1.4	1.8	1.5
At 32°C	4.8	82.9	17.3	17.7

[a] Cell proliferation was assessed by dual-parameter flow cytometry on the basis of DNA content (propidium iodide staining) and incorporation of BrdU during a 2-h labelling period (using an FITC-conjugated anti-BrdU antibody). S− refers to untreated cells, and S+ refers to cells treated with a dose of 6 Gy of ionizing radiation and labelled with BrdU at 16 h after irradiation. Identification and quantitation of the BrdU-positive (S-phase) cells was based on the level of antibody binding compared with control cells that were not irradiated and were not labelled with BrdU but were exposed to the FITC-conjugated anti-BrdU antibody. When the number of irradiated cells staining with the anti-BrdU antibody was low and not significantly different from the background level of staining seen in the control group (0.5% of all cells), then the decrease in the S-phase fraction and the increase in the G_1:S ratio after irradiation were calculated by using the background level and represent minimum estimates.

ratio after irradiation were determined (Table 2). An increase in the G_1:S ratio after irradiation provides a good indicator of G_1 delay. It is necessary to compare this ratio since γ-irradiated cells arrest in G_2 and this block alone can lead to decreases in the proportion of cells in G_1 and S phase. Seven independently derived ER clones expressing wild-type p53 protein were examined, and each showed a decrease in the proportion of cells incorporating BrdU during the 2-h pulse following irradiation. Dot plots representing cell cycle progression are shown in Fig. 3A for two of the clones and for untransfected normal REFs as a control. In contrast, four ER clones expressing endogenous mutant p53 protein and one ERM clone expressing a dominant negative mutant p53 allele in addition to the E7 gene and *ras* continued to synthesize DNA after γ irradiation. Representative dot plots for three of these clones are shown in Fig. 3B. The G_1:S ratio increased and the S-phase fraction decreased after irradiation of wild-type p53-expressing ER clones. These changes were greater than in mutant p53-expressing ER clones (Table 2). Wild-type p53 expressing clone ER8-3 was tested at a higher radiation dose and showed a more complete arrest in G_1 at 9 Gy than at 6 Gy. Mutant p53-expressing ER clones continued to enter S phase even at this higher radiation dose (data not shown).

The integrity of the wild-type p53-dependent G_1 checkpoint was confirmed with REF clones transfected with the E7 gene, *ras*, and either a mutant p53ts allele (ERMts clones) or a non-*ts* mutant p53 allele (ERM clones). The p53 protein expressed by the p53ts allele has been shown to behave as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C. Both types of cells grew at 37°C and at 32°C in medium supplemented with 10% serum, albeit more slowly at the lower temperature (Fig. 4). The ability of ERMts cells to tolerate p53ts expression and to grow at 32°C was expected in light of the fact that certain ER clones express high levels of endogenous, stable, wild-type p53 protein (42). In addition, Vousden et al. (52) have reported that REFs coexpressing p53ts and activated Ras rapidly stop proliferating at 32°C and that expression of HPV-16 E7 overcomes this growth arrest. One of the ER clones expressing an exogenous mutant p53ts allele (ERMts-2) and a control ER clone expressing an exogenous non-*ts* mutant p53 allele (ERM115) were grown at 37 or 32°C overnight, irradiated (6 Gy), and incubated for a further 16 h at the original starting temperature prior to labelling with BrdU and cell cycle analysis by flow cytometry (Fig. 4). ERM115 cells cultured at 37 or 32°C continued to enter S phase after irradiation, with little difference in the G_1:S ratio after irradiation. In contrast, ERMts-2 cells continued to incorporate BrdU at 37°C after irradiation but not at 32°C, the temperature at which the expressed p53ts protein behaves like wild-type p53 protein. At 32°C, the G_1:S ratio of ERMts-2 cells increased 17-fold after irradiation (Table 2).

Radiation sensitivity of ER clones expressing wild-type or mutant p53 protein. In the experiments described above, we have demonstrated that ER clones expressing wild-type p53 protein accumulate in G_1 and in G_2/M following treatment with ionizing radiation, while ER cells expressing mutant p53 protein continue to synthesize DNA. It has been suggested that the G_1-S and the G_2-M transitions serve as checkpoints at which cells delay cell cycle progression in order to maintain genomic integrity and to facilitate repair of DNA damage. To assess the effect that loss of wild-type p53 expression and, hence, loss of the p53-mediated G_1 checkpoint might have on long-term survival of cells following exposure to ionizing radiation, we used a clonogenic assay to compare the relative radiosensitivities of ER clones expressing wild-type or mutant p53 protein. The mean radiation survival curves for all the wild-type p53-expressing ER clones and for all the mutant p53-expressing ER clones are shown in Fig. 5A and B, respectively. Plots of SF_{2Gy} and D_{10} for individual ER clones expressing wild-type or mutant p53 are shown in Fig. 6. Examination of these data indicated that the SF_{2Gy} values for ER clones expressing endogenous mutated p53 alleles were significantly higher ($P < 0.02$) than the SF_{2Gy} values of ER clones expressing wild-type p53. The D_{10} values for the mutant p53-expressing ER clones were also significantly higher ($P < 0.009$) than the D_{10} values for wild-type p53-expressing ER clones. ER clones expressing wild-type or mutant p53 protein exhibited markedly increased radioresistance relative to REF controls or *ras*-transfected REFs, as determined from SF_{2Gy} ($P < 0.006$) and D_{10} ($P < 0.003$) value comparisons (3).

We draw two conclusions from these data. First, ER clones expressing wild-type p53 protein are more radiosensitive than ER clones expressing endogenous mutant p53 protein. Hence, the presence of a p53-mediated G_1 checkpoint in ER cells does not confer enhanced survival in response to ionizing radiation. Second, the variability in the radiation survival curves reflected by variable SF_{2Gy} and D_{10} values for the wild-type p53 expressing ER clones, all of which have a G_1 checkpoint as measured by cell cycle analysis after irradiation, indicates that the p53-

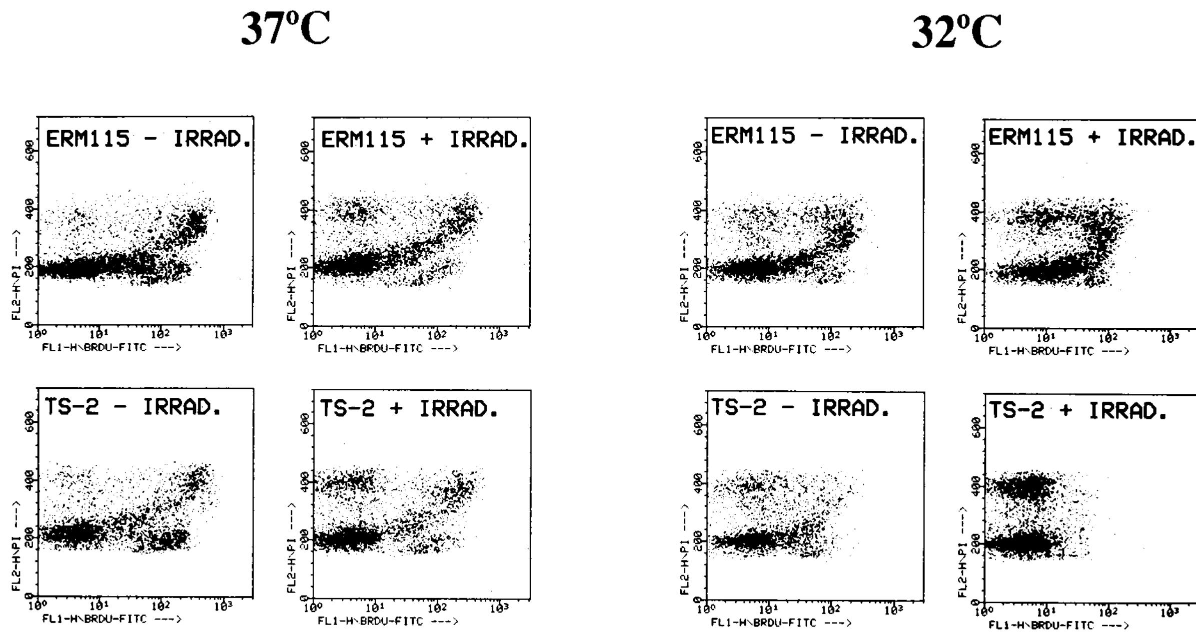

FIG. 4. Cell cycle changes in ERM clones expressing a mutant p53ts allele (ERMts-2) and a non-*ts* mutant p53 allele (ERM115). Cells were irradiated with a dose of 6 Gy and maintained at 37 or 32°C for 16 h prior to labelling with BrdU for 2 h and analysis by flow cytometry as described in the legend to Fig. 3.

dependent G_1 checkpoint alone does not determine radiosensitivity and that additional factors must be involved.

DISCUSSION

ER clones fall into two groups on the basis of endogenous p53 genotype, wild type or mutant. We have compared these clones with the aim of identifying physiological differences that could be attributed to p53 protein function. Previously, we showed a strong association between loss of wild-type p53 expression and serum-independent growth of ER cells in culture (42). The role of p53 protein is likely to be at the level of regulating apoptosis. We have recently observed that ER clones expressing wild-type p53 undergo apoptosis when deprived of serum, as measured by the presence of internucleosomal DNA fragmentation on agarose gels, while ER clones expressing mutant p53 protein continue to grow without evidence of apoptosis (data not shown). Loss of wild-type p53 may abrogate the apoptotic response that normally occurs in transformed cells deprived of serum. A similar explanation was

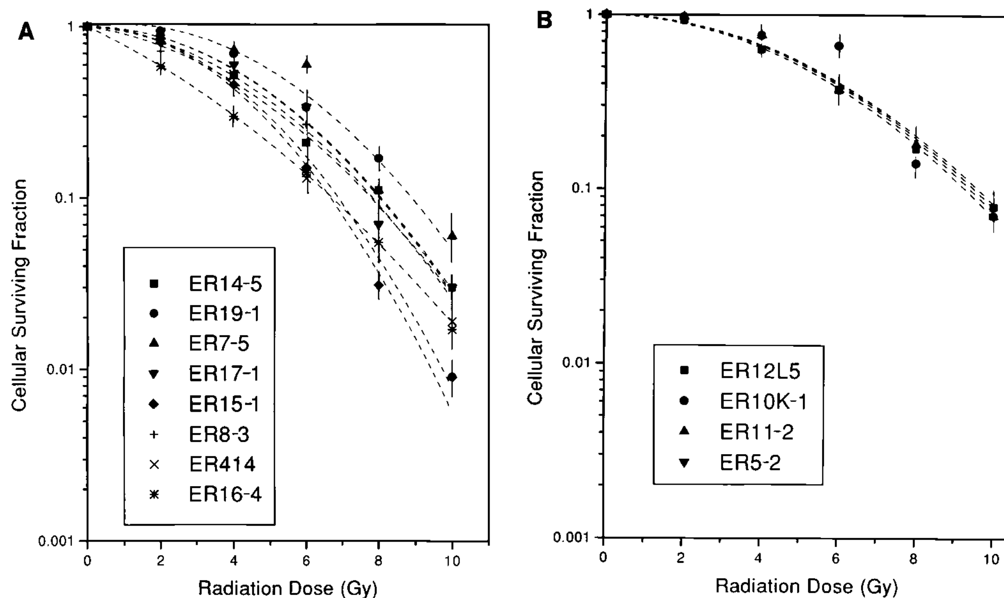

FIG. 5. Radiation survival curves for ER clones expressing either endogenous wild-type p53 protein (A) or endogenous mutant p53 protein (B). Each datum point represents the mean clonogenic survival of the cell line at the radiation dose indicated, determined in at least four independent experiments. Curves were fitted to the survival data by the linear quadratic model of cell kill. Error bars represent the standard error of the mean.

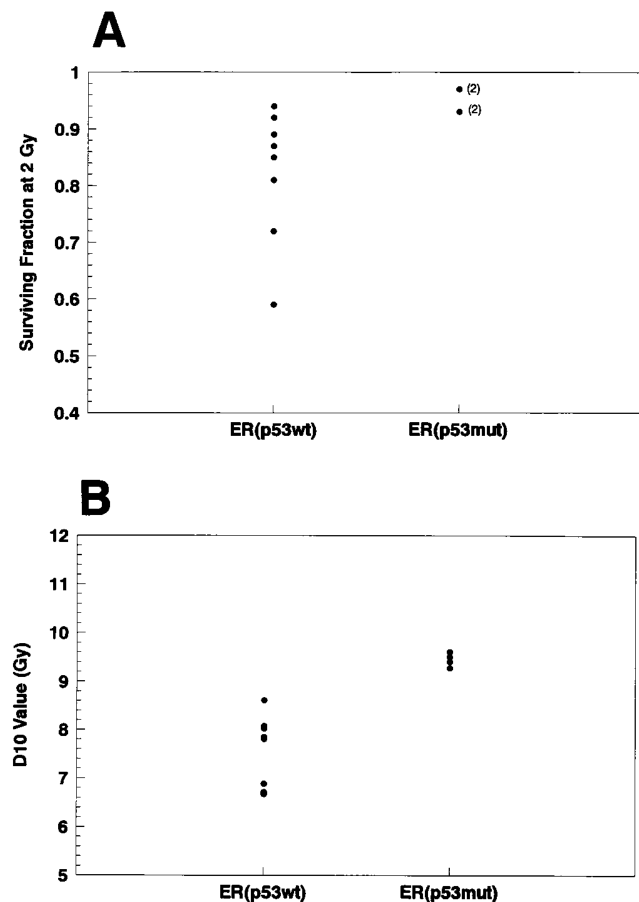

FIG. 6. Mean SF_{2Gy} values (A) and D_{10} values (B) for ER clones expressing either endogenous wild-type p53 protein [ER(p53wt)] or endogenous mutant p53 protein [ER(p53mut)]. The SF_{2Gy} and D_{10} values were obtained from the survival curves shown in Fig. 5.

proposed by Lowe et al. (29), who observed that tumorigenic cells coexpressing EJ-*ras* and E1A were susceptible to p53-mediated apoptosis upon serum withdrawal.

We demonstrate that all ER clones, regardless of p53 genotype, are tumorigenic and metastatic in severe combined immunodeficiency mice. This is expected in light of the numerous reports identifying wild-type p53 alleles in malignant human tumors. Analysis of tumor growth rates in vivo revealed that tumors derived from ER clones lacking wild-type p53 expression grew significantly faster than tumors derived from ER clones expressing wild-type p53 protein. In a separate study, Lowe et al. (29) found that the latency of tumors derived from p53-negative mouse embryo fibroblasts transformed with the adenovirus E1A and *ras* oncogenes was shorter than that of tumors derived from normal fibroblasts transformed with the E1A gene plus *ras*. These findings support the idea that loss of wild-type p53 expression, while not necessary for tumor growth, provides tumor cells with a selective growth advantage that is displayed both in cell culture (serum-independent growth) and in vivo (decreased tumor doubling time and shortened latency). The fact that all ER clones, irrespective of p53 genotype, had acquired the ability for spontaneous metastasis indicates that expression of the HPV-16 E7 gene and activated *ras* is sufficient to promote this phenotype.

In this study, we have shown that the wild-type p53 protein expressed in ER clones is functional on the basis of site-specific double-stranded DNA binding activity. Moreover, two cellular responses to DNA-damaging agents that have been shown to be mediated by wild-type p53 protein, namely, induction of the *WAF1/CIP1* gene and transient arrest of cells in the G_1 phase of the cell cycle, are retained in ER clones expressing wild-type p53 protein and absent in ER clones expressing mutant p53 protein. We conclude that at least one activity of p53, namely, its participation in the control of the G_1-S transition, remains unperturbed in malignant REF clones expressing wild-type p53 protein. In another study, two human tumorigenic cell lines, U2-OS (derived from an osteosarcoma) and RKO (derived from colorectal carcinoma), were similarly found to contain only wild-type p53 alleles and to arrest in G_1 following γ irradiation (22, 23). Together, these findings demonstrate that disruption of the p53 control pathway (either by p53 mutation or p53 binding to other proteins or by other mechanisms that affect upstream or downstream components of the pathway) is not a requirement for tumorigenesis.

The involvement of p53 in regulating a G_1 checkpoint was also demonstrated in studies of cells exposed to metabolic inhibitors (28, 55). Cells expressing wild-type p53 arrested in G_1 when exposed to the uridine biosynthesis inhibitor PALA and failed to demonstrate PALA-selected *CAD* gene amplification. The latter provides a measure of genomic integrity. Cells lacking wild-type p53 expression or expressing mutant p53 protein failed to arrest when placed in drug and inappropriately entered S phase. These cells displayed *CAD* gene amplification at high frequency. Hence, increased genetic instability appears to be one consequence of loss of p53-mediated G_1 checkpoint function. Interestingly, the wild-type p53-expressing, human tumorigenic cell lines U2-OS and RKO were also shown to be capable of amplifying the endogenous *CAD* gene upon exposure to PALA (28). Thus, alternate pathways that bypass the role of p53 and leave a p53-mediated G_1 checkpoint intact likely exist and control genomic integrity and tumorigenic potential.

In contrast to our findings, a number of recent reports demonstrate that the p53-dependent G_1 cell cycle arrest can be abrogated by the HPV-16 E7 protein through its interaction with the retinoblastoma susceptibility gene product (pRb) and perhaps pRb-related proteins. White et al. (53) have reported that expression of the HPV-16 E7 gene in normal human fibroblasts relieved the p53-dependent G_1 checkpoint arrest in response to treatment with PALA. Human keratinocytes or human MCF-7 cells expressing E7 failed to arrest in G_1 after treatment with actinomycin D (7, 18a). Similarly, human RKO cells expressing E7 failed to arrest in G_1 after treatment with ionizing radiation (49). The E7 protein binds different cellular proteins, including pRb (9). This interaction has been shown to release the E2F transcription factor, which is maintained in an inactive state while bound to pRb. Inactivation of pRb, either by mutation or by binding to E7, or normally through phosphorylation events catalyzed by cyclin-dependent kinases, disrupts binding to E2F and is required for cells to transit from G_1 to S phase of the cell cycle. The product of the p53-inducible gene *WAF1/CIP1* has been shown to act as a general inhibitor of cyclin-dependent kinases. Hence, *WAF1/CIP1* protein may participate in cell cycle regulation through the inhibition of cyclin-cdk complex-mediated phosphorylation of pRb. pRb may, therefore, be placed downstream of p53 on the same control pathway (8, 10, 49). Inactivation of pRb or pRb-related proteins through their interaction with E7 may obviate the need to inactivate p53.

In our work, however, ER clones maintained the p53-dependent G_1 checkpoint in response to γ irradiation as long as endogenous wild-type p53 was expressed. The increase in the

G_1:S ratio of the ER clones expressing wild-type p53 ranged from 2.1 to >20 after irradiation, while the increase in the G_1:S ratio of ER clones expressing mutant p53 ranged from 0.7 to 1.9. There is no overlap between these two sets of values. We cannot say with certainty that E7 and/or activated Ras have not had an effect on the p53-mediated G_1 checkpoint. The increase in the G_1:S ratio of normal REFs after irradiation was 2.6 in one experiment and 6.8 in a second experiment. Three of the ER clones (ER414, ER19-1, and ER511) showed changes in the G_1:S ratio after irradiation that fell within the range seen for REFs. Two clones (ER17-1 and ER7-5) showed evidence of a stronger block in G_1 after irradiation on the basis of their G_1:S ratios, and two clones (ER8-3 and ER14-5) showed a weaker block in G_1. The reasons for this variability in response to irradiation are not understood; it does not seem to be related to the level of *WAF1/CIP1* induction.

In assessing these divergent findings, it is pertinent to note that the G_1 arrest response is dependent on radiation dose. Radiation doses of up to 9 Gy were used in this study to demonstrate p53-dependent G_1 arrest. In the study of Slebos et al. (49), a lower dose of 4 Gy was used, which may have been insufficient to detect a G_1 delay in human cells expressing E7. It may also be pertinent to consider the role of EJ-*ras* expression, as well as the possibility that rat cells may respond differently to E7 expression than human cells. It is possible, moreover, that E7-mediated transformation occurs through a different mechanism in rat cells compared with human cells or that different control pathways need to be disrupted in human and rat cells in order to escape senescence. E7 alone, for example, has been shown to immortalize primary rat cells in culture (21), whereas both HPV E6 and E7 are required for efficient immortalization of human primary epithelial cells (18, 39). Transformation by simian virus 40, adenoviruses, and HPVs has demonstrated that inactivation of p53 alone or pRb alone is insufficient and that at least both of these proteins need to be neutralized during the process of virus-mediated cellular transformation. In HPV-negative cervical tumor cell lines, both the pRb gene and the p53 gene have been found to contain mutations (5, 46). Our studies and the lessons learned from the DNA tumor viruses indicate that a linear model in which p53-dependent G_1 arrest involves pRb-related proteins may be too simplistic. Irrespective of these considerations, we are left with the observation that the p53-mediated G_1 checkpoint is present in rat cells transformed by the E7 gene and activated *ras*, whereas it is abrogated in a variety of human cells expressing E7.

We have addressed the question of whether loss of the p53-dependent checkpoint influences sensitivity to γ irradiation in our series of ER clones. Collectively, the four ER clones expressing mutant p53 protein and missing the p53-dependent G_1 checkpoint were more radioresistant than the group of ER clones expressing wild-type p53 protein on the basis of D_{10} and SF_{2Gy} values. This finding is consistent with previous studies also using clonogenic assays in which p53 mutations increase resistance to ionizing radiation (3, 25). Studies using thymocytes from p53-null mice also showed increased radioresistance compared with thymocytes from normal mice on the basis of membrane permeability assays to measure viability (4, 31). Increased radioresistance was also seen in a series of Burkitt's lymphoma cell lines that expressed mutated p53 and fully abrogated G_1 delay (40).

Viewed individually, ER clones expressing wild-type p53 protein and retaining a G_1 checkpoint displayed variability in radiosensitivity. Clone ER7-5, for example, is the most radioresistant within this group and approaches the radioresistance of the ER clones expressing mutant p53. Hence, the p53-dependent G_1 checkpoint alone does not determine radiosensitivity, and additional factors must be involved. Slichenmyer et al. (50) also did not find any association between G_1 checkpoint and radiosensitivity. In their study, loss of the G_1 checkpoint did not lead to significant changes in radiosensitivity. Clone ER7-5 provides an opportunity to investigate factors involved in radiosensitivity. One possibility we have considered is deregulated c-*myc* expression, which has been shown to occur in baby rat kidney cells transformed with the E7 gene and *ras* (44). Moreover, McKenna et al. (34) have shown that REFs transformed with v-*myc* and mutant H-*ras* are radioresistant. However, we have observed that the level of c-*myc* RNA expression in ER7-5 cells is similar to that in radiosensitive REFs (data not shown).

In cells exposed to DNA-damaging agents, the resulting increase in p53 protein level has been associated with G_1 growth arrest or cell death through apoptosis. However, the connection between these two p53-related responses is uncertain. In some types of cells, p53-dependent apoptosis is preceded by G_1 growth arrest (20, 45), while in other cells, it is not (29, 56). For example, in normal thymocytes, radiation-induced cell death occurs by apoptosis, and the radioresistance displayed by p53-negative thymocytes results from loss of the apoptotic response (4, 31). An apoptosis-based explanation for the increased radioresistance of ER clones expressing mutant p53 is attractive because impairment of the apoptotic response likely explains the ability of these cells to survive in culture upon serum deprivation. However, we have found no evidence that radiation-induced cell death in ER clones occurs through apoptosis. Why p53 promotes G_1 growth arrest in some cells and apoptosis in others remains to be determined.

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REFERENCES

1. **Blondal, J. A., and S. Benchimol.** 1994. The role of p53 in tumour progression. Semin. Cancer Biol. **5:**177–186.
2. **Bristow, R. G., and R. P. Hill.** 1990. Comparison between in vitro radiosensitivity and in vivo radioresponse of murine tumor cell lines. Part II. In vivo radioresponse following fractionated treatment and in vitro/in vivo correlations. Int. J. Radiat. Oncol. Biol. Phys. **18:**331–345.
3. **Bristow, R. G., A. Jang, P. Peacock, S. Chung, S. Benchimol, and R. P. Hill.** 1994. Mutant p53 increases radioresistance in rat embryo fibroblasts simultaneously transfected with HPV16-E7 and/or activated H-ras. Oncogene **9:**1527–1536.
4. **Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper, and A. H. Wyllie.** 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature (London) **362:**849–852.
5. **Crook, T., D. Wrede, and K. H. Vousden.** 1991. p53 point mutation in HPV negative human cervical carcinoma cell lines. Oncogene **6:**873–875.
6. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
7. **Demers, G. W., S. A. Foster, C. L. Halbert, and D. A. Galloway.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. USA **91:**4382–4386.
8. **Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge, and S. I. Reed.** 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell **76:**1013–1023.
9. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–936.
10. **El-Deiry, W. F., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, K. G.**

Wiman, W. E. Mercer, M. B. Kastan, K. W. Kohn, S. J. Elledge, K. W. Kinzler, and B. Vogelstein. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. **54:**1169–1174.
11. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
12. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132:**6–13.
13. **Fort, P., L. Marty, M. Piechaczyk, S. El Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13:**1431–1442.
14. **Fritsche, M., C. Haessler, and G. Brandner.** 1993. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene **8:**307–318.
15. **Funk, W. D., D. T. Pak, R. H. Karas, W. E. Wright, and J. W. Shay.** 1992. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. **12:**2866–2871.
16. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39:** 861–869.
17. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
18. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8:**3905–3910.
18a. **Hickman, E. S., S. M. Picksley, and K. H. Vousden.** 1994. Cells expressing HPV 16 E7 continue cell cycle progression following DNA damage induced p53 activation. Oncogene **9:**2177–2182.
19. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
20. **Johnson, P., S. Chung, and S. Benchimol.** 1993. Growth suppression of Friend virus-transformed erythroleukemia cells by p53 protein is accompanied by hemoglobin production and is sensitive to erythropoietin. Mol. Cell. Biol. **13:**1456–1463.
21. **Kanda, T., S. Watanabe, and K. Yoshike.** 1988. Immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. Virology **165:**321–325.
22. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig.** 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
23. **Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan.** 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA **89:**7491–7495.
24. **Lane, D. P.** 1992. p53, guardian of the genome. Nature (London) **358:**15–16.
25. **Lee, J. M., and A. Bernstein.** 1993. p53 mutations increase resistance to ionizing radiation. Proc. Natl. Acad. Sci. USA **90:**5742–5746.
26. **Levine, A. J.** 1993. The tumor suppressor genes. Annu. Rev. Biochem. **62:**623–651.
27. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumour suppressor gene. Nature (London) **351:**453–456.
28. **Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty.** 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70:**923–935.
29. **Lowe, S. W., T. Jacks, D. E. Housman, and H. E. Ruley.** 1994. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. USA **91:**2026–2030.
30. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
31. **Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks.** 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (London) **362:**847–849.
32. **Lu, X., S. H. Park, T. C. Thompson, and D. P. Lane.** 1992. ras-induced hyperplasia occurs with mutation of p53, but activated ras and myc together can induce carcinoma without p53 mutation. Cell **70:**153–161.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **McKenna, W. G., M. C. Weiss, B. Endlich, C. C. Ling, V. J. Bakanauskas, M. L. Kelsten, and R. J. Muschel.** 1990. Synergistic effect of the v-myc oncogene with H-ras on radioresistance. Cancer Res. **50:**97–102.
35. **Michalovitz, D., O. Halevy, and M. Oren.** 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell **62:**671–680.
36. **Milner, J.** 1991. A conformation hypothesis for the suppressor and promoter functions of p53 in cell growth control and in cancer. Proc. R. Soc. Lond. B **245:**139–145.
37. **Moll, U. M., G. Riou, and A. J. Levine.** 1992. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc. Natl. Acad. Sci. USA **89:**7262–7266.
38. **Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine.** 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell **69:**1237–1245.
39. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
40. **O'Connor, P. M., J. Jackman, D. Jondle, K. Bhatia, I. Magrath, and K. W. Kohn.** 1993. Role of the p53 tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. Cancer Res. **53:**4776–4780.
41. **Oliner, J. D., K. W. Kinzler, P. S. Meltzer, D. L. George, and B. Vogelstein.** 1992. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature (London) **358:**80–83.
42. **Peacock, J. W., and S. Benchimol.** 1994. Mutation of the endogenous p53 gene in cells transformed by HPV-16 E7 and EJ c-ras confers a growth advantage involving an autocrine mechanism. EMBO J. **13:**1084–1092.
43. **Pietenpol, J. A., T. Tokino, S. Thiagalingam, W. F. El-Deiry, K. W. Kinzler, and B. Vogelstein.** 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc. Natl. Acad. Sci. USA **91:**1998–2002.
44. **Pim, D., and L. Banks.** 1991. Loss of HPV-16 E7 dependence in cells transformed by HPV-16 E7 plus EJ-ras correlates with increased c-myc expression. Oncogene **6:**589–594.
45. **Ryan, J. J., R. Danish, C. A. Gottlieb, and M. F. Clarke.** 1993. Cell cycle analysis of p53-induced cell death in murine erythroleukemia cells. Mol. Cell. Biol. **13:**711–719.
46. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA **88:**5523–5527.
47. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
48. **Shaw, P., R. Bovey, S. Tardy, R. Sahli, B. Sordat, and J. Costa.** 1992. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA **89:**4495–4499.
49. **Slebos, R. J. C., M. H. Lee, B. S. Plunkett, T. D. Kessis, B. O. Williams, T. Jacks, L. Hedrick, M. B. Kastan, and K. R. Cho.** 1994. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc. Natl. Acad. Sci. USA **91:**5320–5324.
50. **Slichenmyer, W. J., W. G. Nelson, R. J. Slebos, and M. B. Kastan.** 1993. Loss of a p53-associated G1 checkpoint does not increase cell survival following DNA damage. Cancer Res. **53:**4164–4168.
51. **Ullrich, S. J., W. E. Mercer, and E. Appella.** 1992. Human wild-type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells. Oncogene **7:**1635–1643.
52. **Vousden, K. H., B. Vojtesek, C. Fisher, and D. Lane.** 1993. HPV-16 E7 or adenovirus E1A can overcome the growth arrest of cells immortalized with a temperature-sensitive p53. Oncogene **8:**1697–1702.
53. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. Genes Dev. **8:**666–677.
54. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. Nature (London) **366:** 701–704.
55. **Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl.** 1992. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell **70:**937–948.
56. **Yonish-Rouach, E., D. Grunwald, S. Wilder, A. Kimchi, E. May, J.-J. Lawrence, P. May, and M. Oren.** 1993. p53-mediated cell death: relationship to cell cycle control. Mol. Cell. Biol. **13:**1415–1423.
57. **Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren.** 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature (London) **352:**345–347.