MOLECULAR AND CELLULAR BIOLOGY, Apr. 1994, p. 2468–2478
0270-7306/94/$04.00+0

Vol. 14, No. 4

Loss of p53 Protein during Radiation Transformation of Primary Human Mammary Epithelial Cells

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Received 8 September 1993/Returned for modification 11 November 1993/Accepted 24 January 1994

The causative factors leading to breast cancer are largely unknown. Increased incidence of breast cancer following diagnostic or therapeutic radiation suggests that radiation may contribute to mammary oncogenesis. This report describes the in vitro neoplastic transformation of a normal human mammary epithelial cell strain, 76N, by fractionated γ-irradiation at a clinically used dose (30 Gy). The transformed cells (76R-30) were immortal, had reduced growth factor requirements, and produced tumors in nude mice. Remarkably, the 76R-30 cells completely lacked the p53 tumor suppressor protein. Loss of p53 was due to deletion of the gene on one allele and a 26-bp deletion within the third intron on the second allele which resulted in abnormal splicing out of either the third or fourth exon from the mRNA. PCR with a mutation-specific primer showed that intron 3 mutation was present in irradiated cells before selection for immortal phenotype. 76R-30 cells did not exhibit G_1 arrest in response to radiation, indicating a loss of p53-mediated function. Expression of the wild-type p53 gene in 76R-30 cells led to their growth inhibition. Thus, loss of p53 protein appears to have contributed to neoplastic transformation of these cells. This unique model should facilitate analyses of molecular mechanisms of radiation-induced breast cancer and allow identification of p53-regulated cellular genes in breast cells.

Breast cancer is among the leading causes of cancer-related deaths of women in the United States, with approximately 175,000 newly diagnosed cases each year, a quarter of them fatal. However, the molecular mechanisms involved in the initiation and progression of breast cancer are largely unknown. Numerous studies have suggested an important role of the p53 tumor suppressor gene product in the evolution of this disease (9, 37). For example, allelic loss of the short arm of chromosome 17 (17p), which includes p53, and p53 missense mutations are found in a large proportion of breast cancers (9, 33, 37). Women with germ line missense mutations in p53 are at high risk of developing breast cancer (34, 40). Consistent with these clinical observations, our earlier in vitro studies revealed that loss of p53 protein as a result of human papillomavirus (HPV) E6-induced degradation immortalized the primary human mammary epithelial cells (3, 4). The efficiency of immortalization directly correlated with the ability of E6 genes to cause p53 loss (3). Thus, p53 protein appears to play a particularly dominant role in regulating the growth of mammary epithelial cells.

p53 is a nuclear phosphoprotein that is thought to regulate cell cycle progression by modulating transcription and by interacting with cell cycle regulatory proteins (10, 21, 41). Recent in vitro studies have indicated that normal p53 protein is a cell cycle checkpoint determinant that controls the length of G_1 phase to ensure an intact genome (27, 31). Exposure to DNA-damaging agents, such as radiation, leads to an increase in p53 levels followed by G_1 arrest. Cells that lack wild-type p53 protein fail to arrest in G_1 following irradiation, and transfection of wild-type p53 restores this response (27, 28, 31). These results have led to the hypothesis that p53 protein is part of a protective mechanism to prevent propagation of DNA damage. Loss of p53 protein by deletion or mutation may allow accumulation of mutations that lead to aberrations in cell growth control and eventually tumorigenesis. Consistent with such a proposal, epidemiologic studies have shown an increased incidence of breast cancer particularly in younger women who received diagnostic or therapeutic radiation for either breast cancer or other clinical disorders (11, 12, 23).

To directly examine the role of p53 in radiation-induced mammary oncogenesis, we exposed a normal human mammary epithelial cell strain, 76N, with a sequence-documented wild-type p53 (19) to fractionated doses of γ-irradiation using a clinically employed regimen. Exposure to a 30-Gy dose of radiation led to neoplastic transformation of 76N cells. Significantly, these radiation-transformed cells lack p53 protein, fail to arrest in the G_1 phase of the cell cycle after γ-irradiation, and are growth arrested by transfection of the wild-type p53 gene. These results support the idea that p53 protein plays an important role in the homeostasis of mammary epithelial cell growth and loss of p53 may be an essential lesion in their neoplastic transformation.

MATERIALS AND METHODS

Cells and growth media. Derivation of the normal mammary epithelial cell strain 76N from a reduction mammoplasty has been described previously (5). These cells were grown in DFCI-1 medium (5). HPV type 16 (HPV-16) E6-, HPV-6 E6-, and bovine papillomavirus type 1 (BPV-1) E6-immortalized and radiation-transformed cells were grown in a modified version of DFCI-1 medium called D2 medium (3, 4, 6). D2 medium is identical to DFCI-1 medium, except that it lacks fetal calf serum and bovine pituitary extract and contains 0.05% bovine serum albumin as an additional supplement. To obtain clones of the HPV-16 E6-immortalized (16E6R) 76N mammary epithelial cells and radiation-transformed 76R-30

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cells, 500 cells were plated per 100-mm-diameter dish and individual colonies were picked with cloning cylinders.

Radiation treatment of 76N cells. Exponentially growing 76N cells in DFCI-1 medium were irradiated with five 2-Gy fractions of γ-irradiation per week for a total dose of 30 Gy, by using a Mark cesium-137 irradiator at a dose rate of 2 Gy/min. After each 10-Gy increment, the cells were allowed to recover for 3 to 4 days. At each 10-Gy increment, a portion of the culture was seeded in DFCI-1 or D2 medium to select for transformed cells and remaining cells were used for further irradiation.

Anchorage-independent growth. Cells (5×10^4/60-mm dish) were plated in a top layer of 0.3% agar with a bottom layer of 0.5% agar (1:1 in 2× DFCI-1 medium). Cells were examined for clonal growth after 2 weeks.

Growth of radiation-transformed cells in nude mice. BALB/c nude mice were subcutaneously injected in the mammary gland area with 10^7 cells per mouse and examined daily for the appearance of palpable tumors. Excised tumors were used for histopathology and to assess the ability of tumor cells to grow in culture.

Immunoprecipitation analysis of p53 protein. Cells (50 to 70% confluent) in 100-mm-diameter dishes were prestarved in cysteine- and methionine-free α minimal essential medium (α-MEM) for 30 min at 37°C and then labelled with 250 μCi of [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S; NEN) for 3 h at 37°C. Cells were lysed in NETN lysis buffer (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitations were performed as described previously (19), with optimal amounts of control (P3; American Type Culture Collection [ATCC]) (29) or the anti-p53 monoclonal antibodies PAb 122 (ATCC), PAb 1801 (obtained from L. Crawford), PAb 240 (obtained from D. Lane), PAb 1620 (obtained from D. Lane), PAb 421 (obtained from J. DeCaprio), and BP53-12 (Biogenex).

RNA isolation and analysis. Total RNA was isolated from 50 to 70% confluent cell monolayers by the guanidium-isothiocyanate method (17). Northern (RNA) blot hybridizations were carried out by using nylon membranes (Hybond-N; Amersham) with a 1.8-kb p53 cDNA probe, as described previously (44). Poly(A)$^+$ RNA was made with a Poly(A)tract mRNA isolation system (Promega, Madison, Wis.) according to the manufacturer's instructions.

Southern blot analysis. Portions (10 μg) of genomic DNA were digested to completion with restriction enzymes (New England Biolabs), resolved on agarose gels, transferred to Hybond-N nylon membranes, and hybridized with a 1.8-kb p53 cDNA probe, as described previously (6).

PCR and sequencing. The following p53-specific primers were used for PCR (base 1 is the first base of the initiation codon) (32): sense (S), bases −32 to −15 (1-S), 181 to 197 (2-S), 427 to 444 (3-S), 763 to 790 (4-S), and 961 to 978 (5-S); antisense (AS), bases 294 to 277 (1-AS), 495 to 478 (2-AS), 798 to 781 (3-AS), 999 to 982 (4-AS), and 1182 to 1166 (5-AS). The complete primer sequences have previously been published (3, 19). The 5′ PCR primer for specific amplification of the mutated p53 allele (with a 26-bp intron 3 deletion) in 76R-30 cells included nucleotides 8 to 15 fused to nucleotides 42 to 46 of the third intron. The sequence was 5′-cccggatcCAAGGGT TGCTGG-3′ (p53-specific bases are indicated by uppercase letters). The primer sequences incorporated *Bam*HI (sense) or *Eco*RI (antisense) recognition sequences for directional cloning.

To determine the sequence of p53 mRNA, 10 μg of total cellular RNA was reverse transcribed to cDNA. Overlapping PCR products were generated with six sets of primers (1-S/1-AS, 1-S/2-AS, 2-S/2-AS, 2-S/3-AS, 3-S/3-AS, and 4-S/5-AS) and analyzed on a 1.5% agarose gel. PCR products from primer combinations 1-S/1-AS and 1-S/2-AS (see Results) were cloned into M13mp18 and M13mp19 vectors. At least seven single-stranded DNA templates were sequenced for each fragment by using M13-specific primers and Sequenase (U.S. Biochemicals).

To determine the intron 3 sequences, genomic DNA was subjected to PCR with 1-S and 2-AS primers and the amplified fragments were cloned into M13 vectors and sequenced as described above. Specific amplification of mutated intron 3 sequences in 76R-30 cells was carried out with the mutation-specific sense primer (see above) together with 1-AS antisense primer.

Radiation-induced G_1 arrest. Exponentially growing cells in tissue culture flasks were treated with 8 Gy of γ-irradiation from a Mark cesium-137 irradiator and harvested with trypsin-EDTA at various times. Harvested cells were fixed in 70% ethanol for 30 min at 4°C, washed twice with phosphate-buffered saline (PBS), and treated with 10 μg of RNase per ml for 1 h at 37°C. The treated cells were washed once with PBS and stained with propidium iodide (69 μM in 38 mM sodium citrate) for 30 min at room temperature, and the proportion of cells in different phases of the cell cycle was analyzed by FACScan Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) as described previously (18).

Suppression of clonal growth by wild-type p53 transfection. Wild-type p53 (pC53-SN3), mutant p53 (pC53-SCX3), and the vector (pCMV-neo-Bam) were provided by B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, Md. (2). 76R-30 cells were released from culture dishes with trypsin-EDTA and plated in DFCI-1 medium at 10^6 cells per 100-mm-diameter dish 18 h prior to transfection. Linearized plasmid DNA (8 μg) was transfected by calcium phosphate coprecipitation. After 6 h, cells were treated with 15% (vol/vol) glycerol for 4 min and fresh medium was added. At 48 h after transfection, G418 selection (75 μg/ml; GIBCO) was initiated. After about 2 weeks, G418-resistant colonies were visualized by crystal violet staining.

RESULTS

Derivation of radiation-transformed 76R-30 cell line. To examine the possible transforming effect of radiation on normal mammary epithelial cells, we exposed the reduction mammoplasty-derived 76N cell strain (5) to 2-Gy fractionated doses of γ-irradiation for a total of 30 Gy. At each 10-Gy interval, a proportion of radiation-treated cells was plated in either the DFCI-1 or the D2 medium to assess the emergence of immortal cells. DFCI-1 medium supports the growth of both normal and immortal mammary epithelial cells and would not select for the immortal phenotype. In contrast, the defined medium D2 (see Materials and Methods) was anticipated to select for immortal cells, as it supports the long-term growth of HPV-16 E6-immortalized cells but not the normal cells (3, 4, 6). Radiation-treated cultures (10 and 20 Gy) plated in either DFCI-1 or D2 medium continued to grow for three to four passages and then senesced. This was also true for 30-Gy-treated 76N cells cultured in DFCI-1 medium. In contrast, 30-Gy-treated cells cultured in D2 medium showed a gradual loss of cells, as expected, and were maintained without subculture for about a month, at which time morphologically transformed proliferating cells were observed. These cells grew as densely packed polygonal cells, in contrast to loosely arranged spindle-shaped normal parental cells (Fig. 1A). The ability of

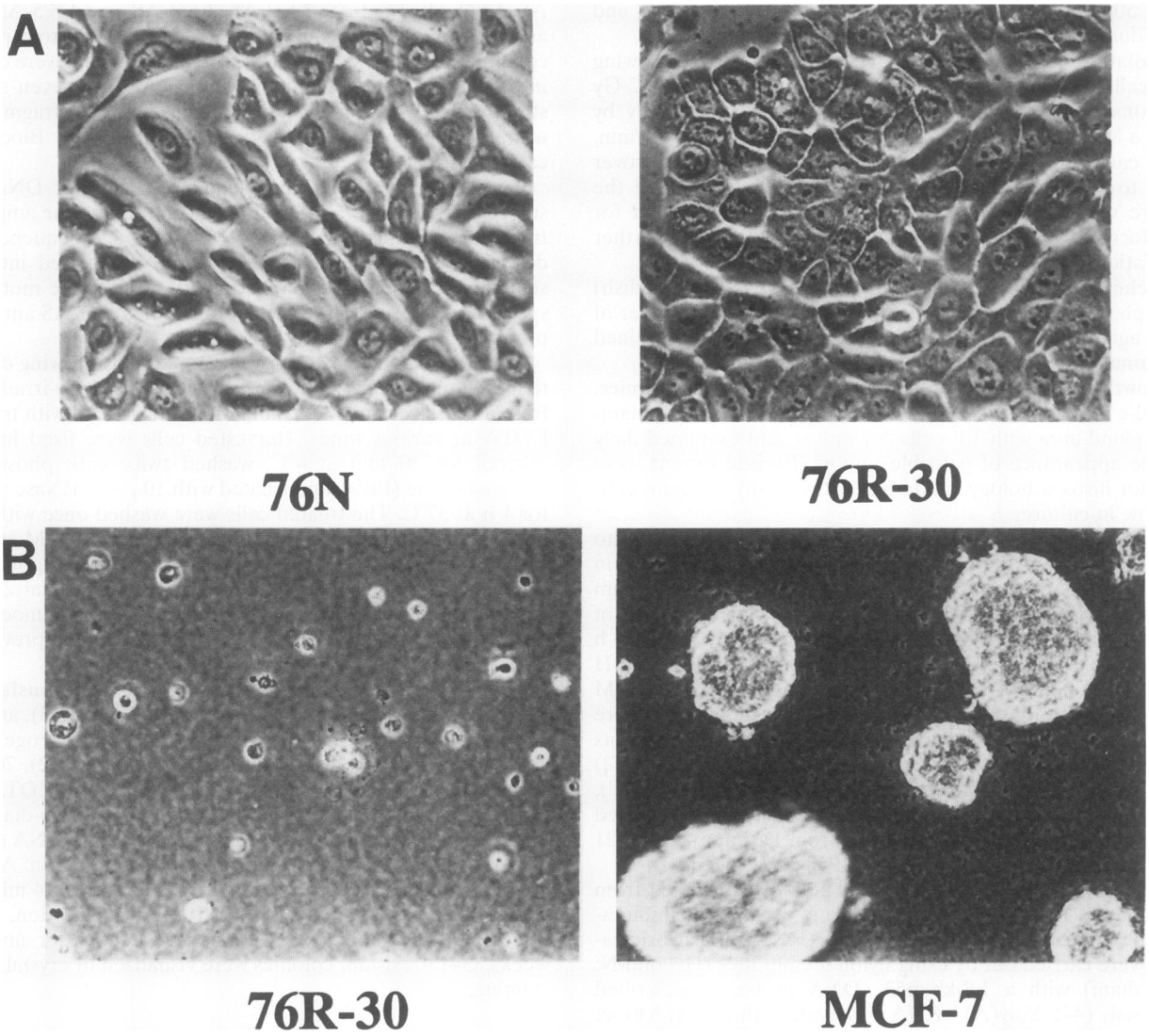

FIG. 1. (A) Morphology of radiation-transformed 76R-30 cells. Note that 76R-30 cells are more often polygonal compared with spindle-shaped 76N cells and grow with higher density. (B) Lack of anchorage-independent growth of 76R-30 cells. Cells (5×10^4/60-mm dish) were plated in soft agar and colonies were photographed after 2 weeks. Note the lack of growth of the 76R-30 cells, whereas macroscopic colonies are easily seen with MCF-7 cells, used as a control.

radiation-transformed cells to grow in D2 medium demonstrated that these cells no longer required the essential growth factors (bovine pituitary extract and fetal calf serum) which are critical for growth of their normal parent cells. In this characteristic, they resemble HPV-immortalized derivatives of 76N cells which are able to grow in D2 medium, as we have shown earlier (3, 4, 6). Radiation-treated cells have grown continuously in D2 medium for more than 80 passages (>800 population doublings) without any signs of senescence. Untransformed 76N cells are unable to survive in D2 medium beyond 3 to 4 passages (3, 4, 6). Together, these results strongly indicated that radiation-transformed cells were immortal. Hereafter, the transformed cells will be referred to as 76R-30.

Anchorage-independent growth. To assess the extent to which 76R-30 cells were transformed, we examined their anchorage-independent growth in soft agar. Anchorage independence has been observed with radiation-transformed human fibroblasts (14, 36). 76R-30 cells did not grow in soft agar, whereas MCF-7, a metastatic breast cancer cell line used as a control, formed large soft-agar colonies (Fig. 1B). Thus, growth of 76R-30 cells is not anchorage independent.

Neoplastic transformation of irradiated cells. To determine whether anchorage-dependent growth of 76R-30 cells reflected their incomplete neoplastic transformation, we examined their ability to grow as xenogeneic transplants in nude mice, which correlates well with advanced malignant behavior of human breast cancer cells (7). For this purpose, we injected 10^7 76R-30 cells per mouse subcutaneously into the mammary fat pad of BALB/c nude mice and determined the size of resulting tumors at various time points. In the first experiment, four of four mice injected had tumors (size at 2 weeks, 0.4 to 0.5 cm); in the second experiment, five of five mice injected had tumors (size at 2 weeks, 0.5 to 1.0 cm). In all injected mice (nine of nine), palpable tumors were observed by 3 days. A portion of each tumor excised after 2 weeks was chopped into small pieces and cultured in DFCI-1 medium, while the other

portion was subjected to histopathological examination. Cell lines could be established from all of the three nude mouse tumors from which it was attempted. Histopathological examination of nude mouse-derived tumors showed morphological characteristics of malignant epithelial tumors with marked squamous differentiations in which the tumor cells showed intra- and extracellular keratin formation (Fig. 2A) and intercellular bridges and abnormal mitoses (Fig. 2B). The bizarre nuclear forms associated with abnormal mitoses with degenerative nuclear changes, i.e., marked pyknosis and karyorrhexis, were also seen (Fig. 2B). Together, these analyses demonstrate that 76R-30 radiation-treated cells have undergone a tumorigenic transformation.

Status of p53 protein in 76R-30 cells. The normal parental cells (76N) of the 76R-30 cell line carry a wild-type p53 (19) capable of mediating their cell cycle arrest upon exposure to radiation (see below). Hence, it was of considerable interest to examine the status of p53 in 76R-30 cells.

To assess p53 protein expression in 76R-30 cells, we carried out immunoprecipitations from NETN lysates of [^{35}S]methionine- and [^{35}S]cysteine-labelled cells using a number of anti-p53 monoclonal antibodies specific for distinct epitopes near the N terminus (BP53-12 [amino acids 1 to 45] and PAb 1801 [amino acids 32 to 79]), middle (PAb 240 [amino acids 156 to 335]), or C terminus (PAb 122 [amino acids 370 to 378]) of the p53 protein (9, 22). As expected, immunoprecipitable p53 was readily detectable in 76N cells with all antibodies. In contrast, none of the antibodies detected the p53 protein in 76R-30 cells carried in D2 medium for >5 passages (Fig. 3A). Similar results were observed with PAb 1620 (against an unmapped conformationally sensitive epitope) and PAb 421 (against C-terminal residues 370 to 378) (data not shown). To assess whether the loss of p53 protein occurred early during radiation transformation or during selection in D2 medium, we also examined p53 protein in 30-Gy radiation-treated cells before these cells were transferred into selective medium D2. A clear decrease in the PAb 122-immunoprecipitable p53 protein was seen even at this early time point when not all untransformed cells (presumably expressing normal p53 protein) were yet eliminated (Fig. 3B). Even when we performed immunoprecipitations using 20-fold more ^{35}S-labelled cell lysates, we were unable to detect any p53 protein in 76R-30 cells (data not shown). No p53 protein was observed even after pulse-chase labelling followed by immunoprecipitation, excluding the possibility that p53 protein might be synthesized but very rapidly degraded (data not shown). Together, these results demonstrate that no detectable p53 protein synthesis occurs in radiation-transformed 76R-30 cells.

p53 RNA expression in 76R-30 cells. To examine whether the lack of p53 protein synthesis in 76R-30 cells was due to a reduction in the steady-state levels of mRNA, we performed Northern blot analysis using a p53-specific cDNA probe. While low levels of p53 RNA were observed at early passages [76R-30(1)], late-passage radiation-immortalized cells [76R-30(2)] showed no detectable RNA (Fig. 4A). In contrast, HPV-6 E6-, HPV-16 E6-, or BPV-1 E6-immortalized 76N cells and other breast cancer cell lines (used as controls) showed a level of p53 RNA comparable with that seen in 76N cells (Fig. 4A and B). To allow detection of even lower levels of p53 mRNA, we examined the presence of p53 transcripts in poly(A)$^+$ RNA derived from 76R-30 cells. This analysis revealed the presence of two p53-specific transcripts of about 2.5 and 2.1 kb, distinct from the 2.6-kb p53 mRNA in 76N cells (Fig. 4C), and raised the possibility of DNA rearrangements and/or splicing errors in p53 transcript. The level of expression of p53 transcript in 76R-30 was at least 10-fold lower than that in 76N cells (Fig. 4C).

Southern blot analysis of the p53 gene in 76R-30 cells. To examine the status of the p53 gene in 76R-30 cells, we carried out Southern blot analyses of their genomic DNA digested with *Ban*II, *Bgl*II, *Eco*RI, or *Pvu*II enzyme using a p53-specific cDNA probe which encompasses exons 1 through 11 and includes the entire coding region and the 5′ and 3′ untranslated regions (nucleotides −130 to 1671) (1). The expected p53-specific DNA fragments were observed in all restriction enzyme digests of DNA isolated from the parental cell strain 76N with a sequence-documented wild-type p53 (Fig. 5A, lane a in each digest). As we have shown before, 76N cells show a polymorphism at the p53 locus which is detectable with *Bgl*II enzyme (19). The 76R-30 cell line (Fig. 5A, lanes b) showed p53-specific bands identical to those observed with 76N cells, with the exception that the polymorphic *Bgl*II fragment seen in 76N cells was lacking. This result suggested that one p53 allele was deleted in 76R-30 cells; consistent with this conclusion, the intensity of p53-hybridizing bands in 76R-30 DNA was reduced (to roughly half) compared with that in 76N DNA (Fig. 5A). The p53 sequence analysis (see below) directly demonstrates the absence of p53 transcript corresponding to this p53 allele, further supporting the possibility that one p53 allele in 76R-30 cells is deleted. However, it remained possible that the presence of a single p53 allele in 76R-30 cells may be due to the presence of two separate populations in the 76N parental cell strain which had not been formally cloned (presumably, one population may have lacked the p53 allele with *Bgl*II polymorphism). Since 76N cells could not be cloned (as they senesce under these conditions), we derived clonal derivatives of 76N cells immortalized by the HPV-16 E6 gene in a retroviral vector. *Bgl*II-digested DNA from six individual clones was examined by Southern blot analysis with a p53-specific probe as described above. Both p53 alleles were present in each subclone (Fig. 5B). Thus, the absence of a *Bgl*II-specific polymorphic fragment in 76R-30 cells is likely to be due to deletion of one p53 allele. Lack of any additional p53 hybridizing fragments, together with the presence of normal-sized fragments, indicated that no gross rearrangements occurred in the second p53 allele.

p53 transcripts in 76R-30 cells have spliced out either exon 3 or exon 4. To further examine the nature of two abnormal-sized p53 transcripts of 2.5 and 2.1 kb in 76R-30 cells, we generated five overlapping PCR fragments covering the region from 32 bp upstream of the initiator methionine to the termination codon, as described in Materials and Methods. Compared with 76N parent cells (Fig. 6A, lanes a) which contain wild-type p53 gene (19), the PCR products generated from 76R-30 cells (lanes b) with two primer combinations (1-S/1-AS and 1-S/2-AS) were distinct (Fig. 6A). The PCR products of altered sizes were found to be p53 specific by Southern analysis with a p53-specific cDNA probe (data not shown). The smaller PCR product derived from 76R-30 cDNA with the 1-S/1-AS primer combination was consistent with the absence of the 22-bp exon 3, resulting in a 321-bp product, compared with the expected 343-bp product in 76N cells. The prominent PCR product from 76R-30 cells with the 1-S/2-AS primer combination was about 265 bp instead of the 544 bp observed in the control RNA, consistent with the absence of exon 4 (279 bp) in the transcript. Other primer combinations resulted in the synthesis of PCR products of expected size. Together, these analyses suggested that p53 transcripts observed in 76R-30 cells may lack exons three or four, as a result of abnormal splicing.

To examine whether the p53-specific PCR products in

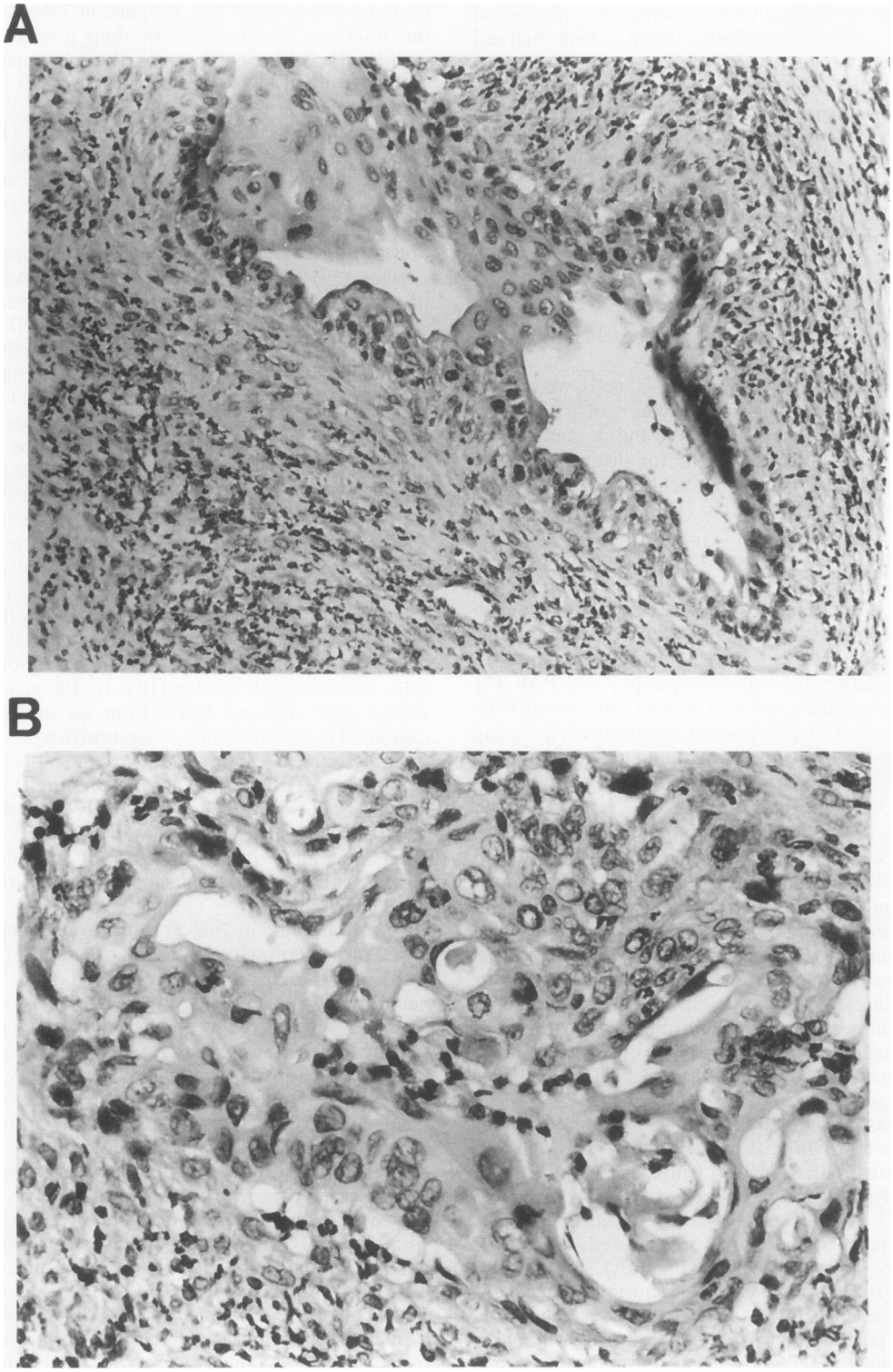

FIG. 2. Histopathological features of 76R-30 cells grown as transplanted tumors in nude mice. The tumors were fixed in 10% formalin and processed for routine histologic examination. Note that tumors show a marked squamous differentiation with keratin formation (A); abnormal mitoses and degenerative nuclear changes with marked pyknosis and karyorrhexis are prominent (B).

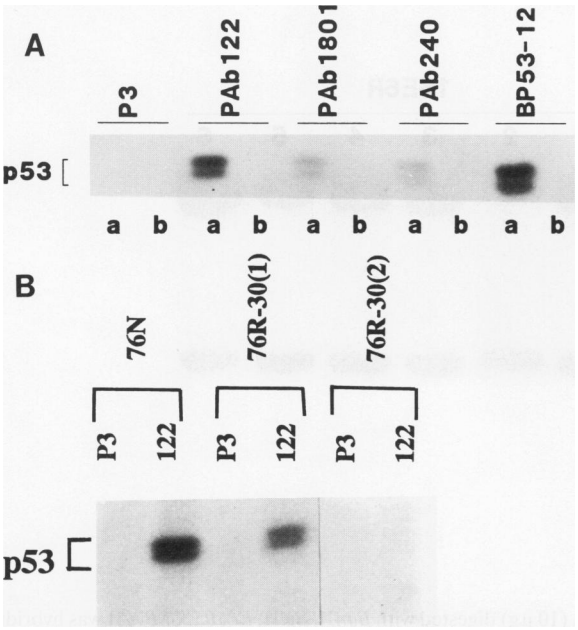

FIG. 3. Immunoprecipitation analysis of p53 protein in normal and radiation-transformed cells. (A) NETN lysates of metabolically labelled 76N (lanes a) or 76R-30 (lanes b) cells were immunoprecipitated with the indicated negative control (P3) or anti-p53 monoclonal antibody and analyzed by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis. The position of p53 protein is indicated. Note that while normal mammary epithelial cells (76N) express easily detectable p53 protein, no p53 protein is detected in late-passage 76R-30 cells. Longer exposures showed the same results. (B) Analysis of p53 protein in early- [76R-30(1)] and late-passage [76R-30(2)] radiation-treated 76N cells using PAb 122 monoclonal antibody (122); P3, negative control. Note that early-passage [76R-30(1)] cells show a decreased level of p53 protein [compare 76N and 76R-30(1)].

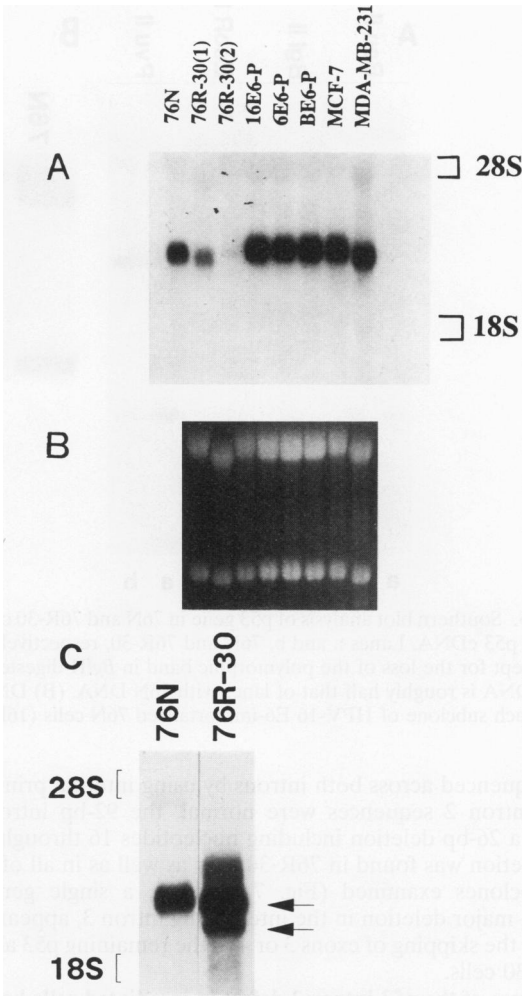

FIG. 4. Northern blot analysis of p53 mRNA expression in normal and radiation-treated cells. (A) Total cellular RNA (10 μg) was resolved on a 1.5% agarose–formaldehyde gel, transferred to the nylon membrane, and hybridized with a 1.8-kb human p53 cDNA. Locations of the ribosomal RNAs (28S, 4,850 bp; and 18S, 1,740 bp) are indicated. Note that fully established 76R-30 cells have no detectable p53 RNA [76R-30(2)], whereas normal parent (76N), E6-immortalized (16E6-P, 6E6-P, and BE6-P), and breast cancer (MCF-7 and MDA-MB-231) cells have similar levels of RNA. (B) Ethidium bromide staining of the gel in panel A shows roughly equal loading. (C) Poly(A)$^+$ RNA from 76R-30 (6 μg) or total RNA from 76N cells (10 μg) was hybridized with p53 cDNA probe. Note the presence of two transcripts of 2.5 and 2.1 kb (indicated by arrows) in 76R-30 cells, compared with one transcript of 2.6 kb in 76N cells. 28 and 18S rRNAs are indicated.

76R-30 represented abnormal splicing, we cloned these PCR products into M13 vectors and determined their nucleotide sequences. As shown in Fig. 6B, seven of seven M13 clones obtained from the 321-bp PCR product (1-S/1-AS primer combination) completely lacked exon 3 sequences (Fig. 6B, panel A). Exons 2 and 4 were present in their entirety and fused to each other at the normal splice junction. Similarly, six of seven M13 clones representing the 265-bp PCR product (obtained with the 1-S/2-AS primer combination) showed a direct splicing of exon 3 to 5 with complete loss of exon 4 sequences (Fig. 6B, panel B), while one clone showed normal sequences. These results directly demonstrated the existence of abnormally spliced p53 mRNA transcripts, which accounted for nearly all of the expressed p53 mRNA in 76R-30 cells. Together with a more than 10-fold reduction in steady-state mRNA levels (see above), this abnormal splicing would be sufficient to account for essentially complete loss of p53 protein synthesis. Notably, the sequence analysis showed the absence of transcripts with codon 72 polymorphism in exon 4 that correspond to the p53 allele detected in Southern blots as a polymorphic *Bgl*II fragment (data not shown). This finding confirms the deletion of this p53 allele.

To examine whether the two abnormally spliced p53 transcripts in 76R-30 cells were present in separate cell populations or within each individual cell, we derived subclones of this cell line and carried out PCR as described above. As shown in Fig. 6C, all nine 76R-30 subclones showed both PCR products that correspond to the skipping of exon 3 or 4; sequence analysis of the PCR products confirmed their identity. Thus, both abnormal p53 mRNA transcripts are present in each individual cell of the 76R-30 cell line, suggesting that splicing out of exons 3 or 4 may arise from a related defect.

p53 genetic defect in 76R-30 cells. Lack of either exon 3 or exon 4 in p53 transcripts arising from a single p53 allele indicated an exon-skipping mechanism presumably due to abnormal structure of the intron between the two exons. To examine this possibility, we obtained PCR products from genomic DNA of 76R-30 cells or its subclones using 1-S and 1-AS primers. The PCR products included introns 2 and 3 and

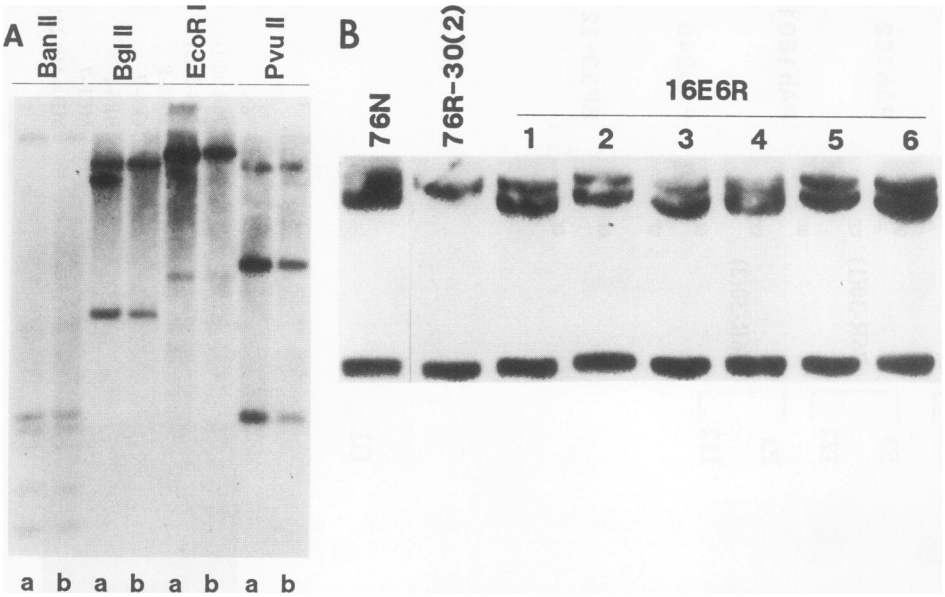

FIG. 5. Southern blot analysis of p53 gene in 76N and 76R-30 cells. (A) DNA (10 μg) digested with *Ban*II, *Bgl*II, *Eco*RI, or *Pvu*II was hybridized to 1.8-kb p53 cDNA. Lanes a and b, 76N and 76R-30, respectively. Note that the patterns of p53-hybridizing bands are similar in all the pairs of lanes except for the loss of the polymorphic band in *Bgl*II-digested 76R-30 DNA compared with 76N DNA. The intensity of bands in lanes with 76R-30 DNA is roughly half that of lanes with 76N DNA. (B) DNA was digested with *Bgl*II and hybridized to p53 cDNA. Note that 76N cells as well as each subclone of HPV-16 E6-immortalized 76N cells (16E6R) show the *Bgl*II polymorphism whereas 76R-30(2) cells lack this fragment.

were sequenced across both introns by using internal primers. While intron 2 sequences were normal, the 92-bp intron 3 showed a 26-bp deletion including nucleotides 16 through 41. This deletion was found in 76R-30 cells as well as in all of the five subclones examined (Fig. 7A). Thus, a single genetic lesion, a major deletion in the intervening intron 3, appears to result in the skipping of exons 3 or 4 in the remaining p53 allele in 76R-30 cells.

Presence of the p53 intron 3 defect in irradiated cells before selection. To determine whether the p53 defect defined above arose early during radiation-induced transformation, we designed a sense PCR primer across the intron 3 deletion (see Materials and Methods) and used it in conjunction with an antisense 1-AS primer to carry out PCR from genomic DNA. The expected PCR product of 273 bp was observed in genomic DNA derived from late-passage 76R-30(2) cells (Fig. 7B). In contrast, no specific product was observed in DNA derived from 76N cells, confirming the specificity of the PCR primers. Notably, a specific PCR product of 273 bp was also observed in DNA derived from 76R-30(1) cells, which represent an early passage before selection in D2 medium (Fig. 7B). Sequence analysis demonstrated that the specific product obtained from 76R-30(1) cells indeed represented the partial intron 3 deletion identical to that seen in established 76R-30 cells. Thus, cells with p53 intron 3 deletion arose relatively early following radiation treatment of 76N cells and were selected for continued growth in D2 medium, which only supports growth of immortal cells.

Radiation-induced G_1 arrest is lost in 76R-30 cells as compared with 76N cells. While the analyses described above clearly showed a lack of p53 protein expression selectively in 76R-30 cells, we wished to directly examine the loss of p53-mediated function in these cells. As shown previously, expression of a functional wild-type p53 is obligatory for G_1 arrest of cells after γ-irradiation (27, 28, 31). We therefore treated 76N and 76R-30 cells with γ-irradiation and examined their cell cycle distribution using propidium iodide staining. As expected, a time-dependent G_1 arrest was observed in 76N cells (data not shown). The G_1/S ratio in untreated 76N cells was 3.1, whereas that in 8-Gy γ-irradiated cells after 36 h was 31 (Fig. 8). In sharp contrast to the response of 76N cells, 76R-30 cells failed to show G_1 arrest after γ-irradiation. However, there was significant accumulation of cells in G_2/M. Thus, radiation-induced G_1 cell cycle arrest fails to occur in 76R-30 cells, indicating an essentially complete loss of p53-mediated function, while p53-independent G_2 arrest remained intact, as expected.

Wild-type p53 transfection inhibits growth of 76R-30 cells. Several studies have shown that transfection of wild-type p53 into human cell lines that have either deleted the p53 gene or carry a nonfunctional mutant p53 gene causes a growth arrest (2, 16, 25). In contrast, p53 transfection into cells that express a wild-type p53 reportedly produces no growth suppression (16). To assess the effect of exogenous p53 on cell growth, we transfected the 76R-30 cells (5×10^5 per 100-mm dish; five dishes for each transfection) with wild-type or mutant p53 cDNA or with vector alone (control). The growth of transfectants was measured by counting the number of colonies after 2 weeks of G418 selection.

As seen in Table 1 (average of two experiments), 56 colonies were obtained with vector-transfected cells. In contrast, wild-type p53-transfected cells gave rise to 13 colonies (a 4-fold reduction). The number of colonies obtained from 76R-30 cells transfected with a mutant p53 cDNA (V143A) (2) was comparable with that obtained with vector transfectants (41 versus 56). These results are consistent with a lack of functional p53 protein expression in 76R-30 cells and support a role for p53 loss in their transformed behavior.

DISCUSSION

Malignant transformation of human cells is thought to be a consequence of cumulative genetic lesions which inactivate (tumor suppressor genes) or activate (oncogenes) genes whose

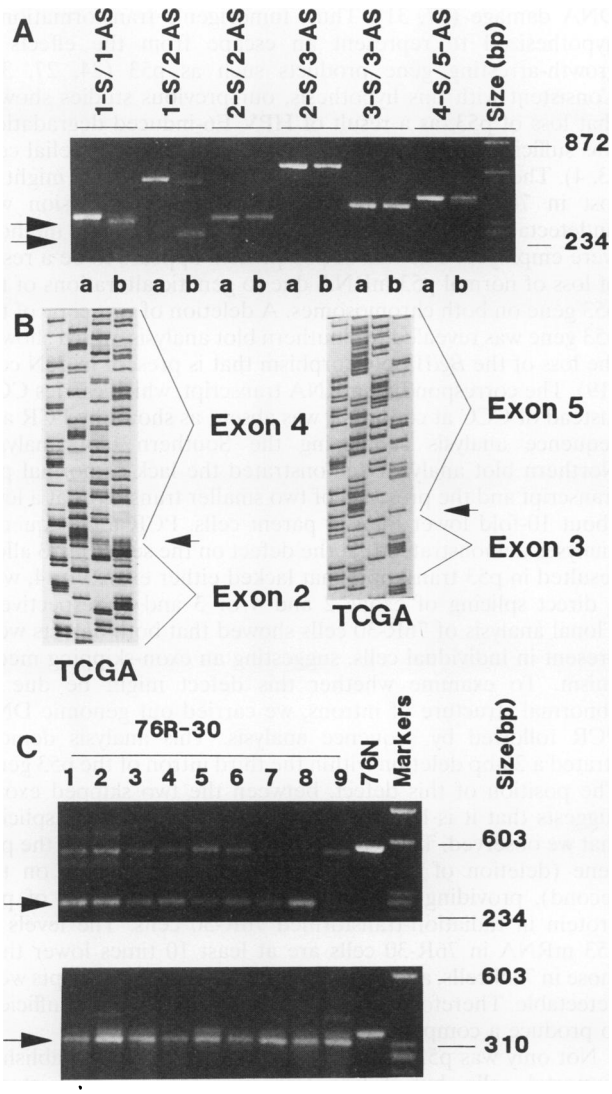

FIG. 6. PCR and sequence analyses of p53 transcripts expressed in 76R-30 cells. (A) Six sets of primers were used for PCR from 76N (lanes a) and 76R-30 (lanes b) cDNA. Note that all primer combinations amplified PCR products from 76R-30 cDNA; however, the products obtained with primer combinations 1-S/1-AS and 1-S/2-AS were of abnormal size (indicated by arrows) compared with those obtained from 76N cells. Sizes (in base pairs) are shown at the right. (B) PCR products obtained with primer combinations 1-S/1-AS and 1-S/2-AS were cloned into M13 vectors and sequenced. Nucleotide sequences across exons 2 and 4 (left panel) and exons 3 and 5 (right panel) from the 76R-30 cell line are shown. Arrows indicate the loss of exon 3 or exon 4 in 76R-30 cells. (C) cDNA from nine subclones of 76R-30 cells or from 76N cells was used to amplify the PCR product with primer combinations of 1-S/1-AS (bottom panel) and 1-S/2-AS (top panel). Note that all 76R-30 subclones show both abnormal size products with each primer combination (indicated by arrows), unlike parent 76N cells.

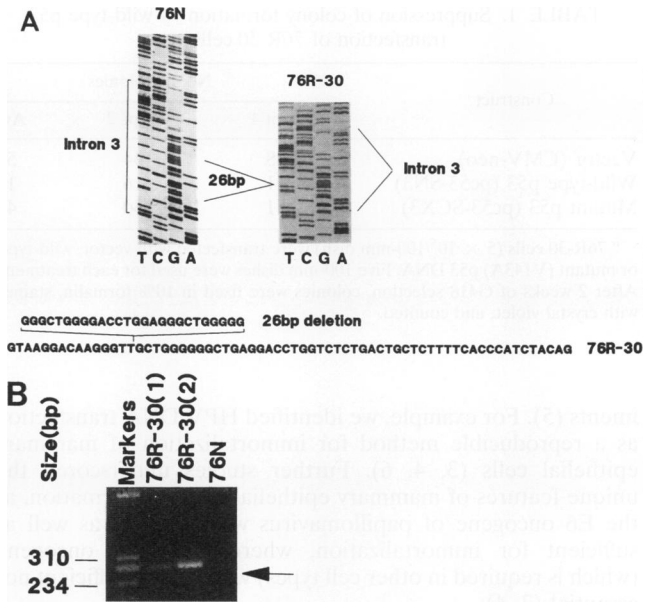

FIG. 7. p53 genetic defect in 76R-30 cells. (A) PCR products obtained from genomic DNA of 76N and 76R-30 cells with the primer combination 1-S/1-AS were cloned into M13 vectors and sequenced. Nucleotide sequences across intron 3 are shown. Note that the 76R-30 p53 gene had a 26-bp deletion in intron 3. (B) Genomic DNA PCR of 76N, 76R-30(1), and 76R-30(2) cells with a mutation-specific sense primer (across the intron 3 deletion; see Materials and Methods) and an antisense 1-AS primer. The arrow points to the specific PCR product of 273 bp obtained in both early and late passages of 76R-30 cells and absent in 76N cells. Sequence analysis (panel A, bottom) confirmed the 26-bp intron 3 deletion in this PCR product.

products tightly regulate cell proliferation and life span (39). The vast majority of human cancers are epithelial in origin, yet tumorigenesis has been typically studied in fibroblasts, usually of rodent origin. This trend reflects the ease of establishment of rodent fibroblasts and their susceptibility to transformation by chemical carcinogens, radiation, and tumor viruses or their oncogenes. In contrast, transformation of cultured human cells has been rare (20, 38). This is in part due to difficulties in establishment and growth of normal cell strains and to the relative resistance of human cells to tumorigenic agents used for rodent cells.

To establish models of human epithelial cancer, we have focused on breast epithelial cells, as breast cancer is extremely common and large amounts of normal (from reduction mammoplasties or mastectomies) and tumor tissues (from mastectomies) are available for study. Earlier, we developed a medium, DFCI-1, which allowed us to establish normal mammary epithelial cells suitable for in vitro transformation exper-

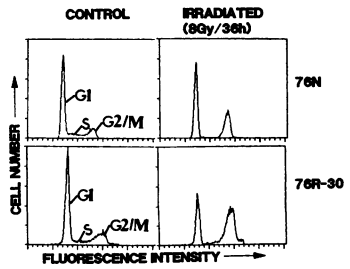

FIG. 8. Radiation-induced G_1 arrest in 76N and 76R-30 cells. Cells (2×10^6 per 75-cm^2 flask were plated in DFCI-1 medium. After 48 h, cells were treated with 8 Gy of γ-irradiation, and they were processed for cell cycle analysis at 36 h. Different phases of cell cycles (G_1, S, G_2/M) are shown. Note that normal 76N cells show predominant G_1 arrest whereas 76R-30 cells show a decrease in G_1 phase and a large increase in the G_2/M phase.

TABLE 1. Suppression of colony formation by wild-type p53 transfection of 76R-30 cells[a]

Construct	No. of colonies		
	Expt 1	Expt 2	Avg
Vector (CMV-neo)	68	44	56
Wild-type p53 (pc53-SN3)	11	16	13
Mutant p53 (pc53-SCX3)	51	30	41

[a] 76R-30 cells (5×10^5/100-mm dish) were transfected with vector, wild-type, or mutant (V143A) p53 DNA. Five 100-mm dishes were used for each treatment. After 2 weeks of G418 selection, colonies were fixed in 10% formalin, stained with crystal violet, and counted.

iments (5). For example, we identified HPV DNA transfection as a reproducible method for immortalization of mammary epithelial cells (3, 4, 6). Further studies underscored the unique features of mammary epithelial cell transformation, as the E6 oncogene of papillomavirus was essential as well as sufficient for immortalization, whereas the E7 oncogene (which is required in other cell types) was neither sufficient nor essential (3, 4).

In this study, we examined the possible tumorigenic transformation of normal human mammary epithelial cells in response to γ-irradiation, which is commonly used for experimental transformation of rodent fibroblasts (13). Furthermore, a number of epidemiologic studies have reported increased incidence of breast cancers among women exposed to large doses of diagnostic or therapeutic radiation, suggesting a higher susceptibility of breast epithelial cells to radiation (11, 12, 23). Thus, the rationale for use of γ-irradiation to transform a mammary epithelial cell appeared to be reasonable. Indeed, exposure of 76N normal mammary epithelial cells to 30 Gy of radiation by a fractionated radiation protocol (2 Gy/day) employed clinically to treat Hodgkin's lymphoma led to outgrowth of cells with a transformed morphology (76R-30). Growth of these cells in a defined medium, D2, established their lack of requirement for a number of undefined growth factors which are critical for growth of normal mammary epithelial cells in vitro. Upon continuous culture, the radiation-transformed 76R-30 cells showed no signs of senescence and hence were immortal. Further analysis showed that 76R-30 cells could grow as tumors in nude mice, establishing directly that these cells had undergone tumorigenic transformation as a consequence of irradiation. To our knowledge, there are only three reports of radiation transformation of primary human cells, two with fibroblasts (14, 36) and one using keratinocytes (42). In the first study, radiation-transformed human fibroblasts formed foci in vitro and formed tumors in nude mice (14); in the second study, fibroblasts became immortal and underwent further spontaneous transformation after continuous culture (35, 36). The third study reported that radiation-treated keratinocytes became immortal but not tumorigenic (42). Thus, 76R-30, together with its normal parent cell strain (76N), represents the first model of radiation-induced tumorigenic transformation of human mammary epithelial cells. We have also established immortal but nontumorigenic derivatives of 76N cells by papillomavirus E6 gene transfection (3, 4, 6). Together, these cell lines provide a system to examine the genetic alterations that accompany the progression of mammary epithelial cells to full malignancy.

Recently, a majority of human breast cancers have been shown to carry deletions or missense mutations of the p53 gene (9, 37), whose normal protein product is an essential mediator of growth arrest in response to γ-irradiation or other forms of DNA damage (27, 31). Thus, tumorigenic transformation is hypothesized to represent an escape from the effects of growth-arresting gene products such as p53 (24, 27, 31). Consistent with this hypothesis, our previous studies showed that loss of p53, as a result of HPV E6-induced degradation, was sufficient to immortalize human mammary epithelial cells (3, 4). Therefore, it appeared likely that p53 function might be lost in 76R-30 cells. Indeed, p53 protein expression was undetectable even when extremely sensitive detection methods were employed. The loss of p53 protein appears to be a result of loss of normal p53 mRNA due to genetic alterations of the p53 gene on both chromosomes. A deletion of one copy of the p53 gene was revealed by Southern blot analysis, which showed the loss of the *Bgl*II polymorphism that is present in 76N cells (19). The corresponding mRNA transcript, which carries CGC instead of CCC at codon 72, was absent as shown by PCR and sequence analysis confirming the Southern blot analysis. Northern blot analyses demonstrated the lack of normal p53 transcript and the presence of two smaller transcripts at a level about 10-fold lower than in parent cells. PCR and sequence analyses demonstrated that the defect on the second p53 allele resulted in p53 transcripts that lacked either exon 3 or 4, with a direct splicing of exons 2 and 4 or 3 and 5, respectively. Clonal analysis of 76R-30 cells showed that both defects were present in individual cells, suggesting an exon-skipping mechanism. To examine whether this defect might be due to abnormal structure of introns, we carried out genomic DNA PCR followed by sequence analysis. This analysis demonstrated a 26-bp deletion within the third intron of the p53 gene. The position of this defect, between the two skipped exons, suggests that it is likely to account for the abnormal splicing that we observed. Thus, we have defined two defects in the p53 gene (deletion of one allele and abnormal splicing on the second), providing a direct explanation for the loss of p53 protein in radiation-transformed 76R-30 cells. The levels of p53 mRNA in 76R-30 cells are at least 10 times lower than those in 76N cells, and only abnormally spliced transcripts were detectable. Therefore, these aberrations appear to be sufficient to produce a complete loss of p53 protein synthesis.

Not only was p53 protein expression lost in the established immortal cells, but it was also decreased at early stages [76R-30(1)] when the culture contained a large population of normal-appearing cells. While abnormal p53 transcripts could not be detected in 76R-30(1) cells by Northern blotting or regular PCR, PCR analysis using a primer specific for the p53 intron 3 deletion revealed that this genetic defect was present at this early stage. Although the status of the other p53 allele (deleted in established 76R-30 cells) at early passage has not been determined, these results are consistent with the early occurrence of p53 mutations upon radiation treatment and their selection during immortalization. Indeed, selection of γ-irradiated cells in D2 medium resulted in the disappearance of untransformed cells and, concomitantly, a complete loss of p53. Thus, loss of p53 appears to be a relatively early lesion in radiation-transformed cells. The loss of p53 protein was functionally significant, as revealed by the inability of 76R-30 cells to exhibit p53-mediated functions such as radiation-induced G_1 cell cycle arrest. These results suggest an important role of p53 loss in 76R-30 radiation transformation. Our finding that reintroduction of normal but not mutant p53 cDNA into 76R-30 led to their growth suppression is consistent with this proposal.

The unique role of p53 loss in radiation transformation of 76R-30 cells is further underscored by the lack of obvious abnormalities in the RNA expression of a number of growth-related genes such as the transforming growth factor α and β,

epidermal growth factor receptor, *mdm-2*, *jun*, H-*ras*, N-*ras*, *fos*, *Rb*, and *erbB2* genes (data not shown). Moreover, sequencing of H-*ras* PCR products from 76R-30 cDNA revealed no mutation at codon 12, 13, or 61, the known hot spots for mutation in the H-*ras* gene (8, 15). The status of p53 protein in other radiation-transformed human cells established by others has not been reported (14, 36, 42). However, a high incidence of p53 mutations has been observed in murine UV- and γ-irradiation-induced tumors (26, 30). Thus, loss of p53 function may be essential for transformation induced by radiation and other DNA-damaging agents, consistent with the recent proposals based on the role of p53 protein as a cell cycle checkpoint determinant (24).

The radiation transformation model established here should be useful in delineating other genetic lesions that are critical in the establishment of a tumorigenic state in breast cancer and perhaps in other epithelial cancers. For example, subtractive hybridization between these cells and either normal 76N cells or their HPV E6-immortalized (nontumorigenic) derivatives should help identify the genes that are transcriptionally up- or down-regulated during tumorigenesis. However, the most direct use of the 76R-30 cell system will be in the identification of cellular genes whose transcription is positively or negatively regulated by p53. The p53 protein has now been established as a transcriptional regulator (43), but the cellular target genes regulated by p53 in a physiological context remain unknown. The availability of genetically identical normal parent cells with a sequence-documented wild-type p53 gene expressed at physiological levels should facilitate such analyses.

ACKNOWLEDGMENTS

We thank B. Vogelstein for providing p53 constructs; D. Lane, L. Crawford, and J. DeCaprio for generous gifts of p53 antibodies; Hamid Band for suggestions in certain experiments and critical reading of the manuscript; and L. Shimkerich for photography of tumor specimens.

This work was supported in part by NIH grant R29 CA56803-01 to V.B.

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