MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 2214–2225 Vol. 16, No. 5
0270-7306/96/$04.00+0

Human Chromosome 3 Mediates Growth Arrest and Suppression of Apoptosis in Microcell Hybrids

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Received 26 May 1995/Returned for modification 17 July 1995/Accepted 16 November 1995

Chemotherapeutic treatment of tumor cells leads either to tumor cell death (usually by apoptosis) or to the formation of drug-resistant subpopulations. Known mechanisms of cancer cell drug resistance include gene amplification and increased expression of drug transporters. On the other hand, normal cells survive many forms of chemotherapy with minimal damage probably because of their capacity for growth arrest and stringent control of apoptosis. Microcell hybrids between B78 (murine melanoma) and HSF5 (normal human fibroblasts) were analyzed to identify a new human chromosomal region involved in the promotion of drug-induced growth arrest and suppression of apoptosis. In these hybrids, the presence of human chromosome 3 was strongly associated with suppression of apoptosis via G_1 and G_2 growth arrest during exposure to the antimetabolite *N*-phosphonoacetyl-L-aspartate (PALA), suggesting that a gene(s) on chromosome 3 serves an antiproliferative role in a drug-responsive growth arrest pathway.

Negative regulation of the cell cycle is an important normal cellular response to environmental stress, such as depletion of growth factors or serum, increased crowding, or administration of DNA-damaging and chemotherapeutic agents (6, 23, 50, 51, 65). Growth restriction is associated with delays in progression of the cell cycle, from G_1 to S and G_2 to M. These delays, or cell cycle checkpoints, reduce the probability of transmission of damaged or inaccurately replicated DNA to daughter cells (1, 69). Upon improvement in the external environment or when the damaged DNA is repaired, normal cells escape the transiently arrested state via positive signal transduction pathways (2, 5, 25). Tumor cells, on the other hand, often lack normal cell cycle checkpoints and, failing to respond appropriately to environmental stress, undergo apoptosis (8, 10, 28). As a result, most tumor cells die readily following chemotherapeutic and radiation treatments. However, some cells may survive to later emerge as drug-resistant tumor cell subpopulations. These resistant cells eventually become a major impediment to permanent remission following initially successful cancer therapy. A number of mechanisms for tumor cell drug resistance and survival have been proposed and studied. For example, tumor cell dormancy, with reentry into the proliferative state upon improvement in cell growth conditions, is a possible mechanism of drug resistance in metastatic cells (20). Alternatively, cancer cells may be able to bypass growth arrest and apoptosis pathways through avoidance of the toxic effects of treatment. Proposed drug survival mechanisms which minimize chemotherapeutic toxic effects and permit continued proliferation include alterations in drug transport and gene amplification (3, 29, 48, 68). Gene amplification is also a marker for genomic instability and is responsible for oncogene overexpression in some advanced tumors (7, 39, 45, 49, 57).

Apoptosis and growth arrest are complex processes involving the action of numerous gene products, many of which have already been identified (12, 18, 19, 21, 37, 40, 43, 51, 61, 72). The p53 tumor suppressor gene is of particular interest, since it appears to play a central role in the activation of apoptosis in transformed cell types while mediating growth arrest in normal cells (24, 30–32, 71). While p53 appears to be essential to the maintenance of genomic stability and efficient activation of growth arrest and apoptosis, additional genes can influence the cell's decision to arrest or die. For example, the retinoblastoma tumor suppressor protein, pRb, was recently linked with p53 in the control of cell growth and apoptosis and was shown to be capable of protecting cells from p53-mediated apoptosis (18). Furthermore, the ability of pRb to protect cells from stress-induced apoptosis was found to be independent of wild-type p53 expression (17). This suggests that the inactivation of genes like *RB*, which have dual functions of both growth and apoptosis suppression, may not only result in decreased growth regulation but also cause increased susceptibility to apoptosis, in a manner similar to oncogene activation (11, 17, 53).

To identify new loci implicated in the induction of drug-induced growth arrest and suppression of apoptosis, we have transferred, by microcell fusion, normal human chromosomes into the mouse melanoma cell line B78 and exposed the resulting hybrids to the antimetabolite and chemotherapeutic agent PALA (*N*-phosphonoacetyl-L-aspartate). A similar strategy was successfully used recently to identify rat chromosomes involved in *trans* repression of glucocorticoid-induced apoptosis in intertypic hybrids (16).

PALA is cytotoxic to immortalized and tumorigenic cells and induces cell death in a p53-independent fashion (30, 71). Usually, only rare, drug-resistant colonies survive PALA exposure, via amplification of the carbamyl phosphate synthase-aspartate transcarbamylase-dihydroorotase (CAD) gene (42, 63). In contrast, normal fibroblasts lack detectable gene amplification and are resistant to PALA-induced cytotoxicity via a p53-mediated growth arrest pathway (30, 60, 62, 70, 71). In a previous report, we showed that mouse × human whole-cell hybrids responded to PALA with reduced apoptosis and morphological characteristics of growth arrest. However, this property was lessened with the loss of human chromosomes during passaging (56). Here we have screened a panel of mouse × human microcell hybrids for their ability to undergo growth

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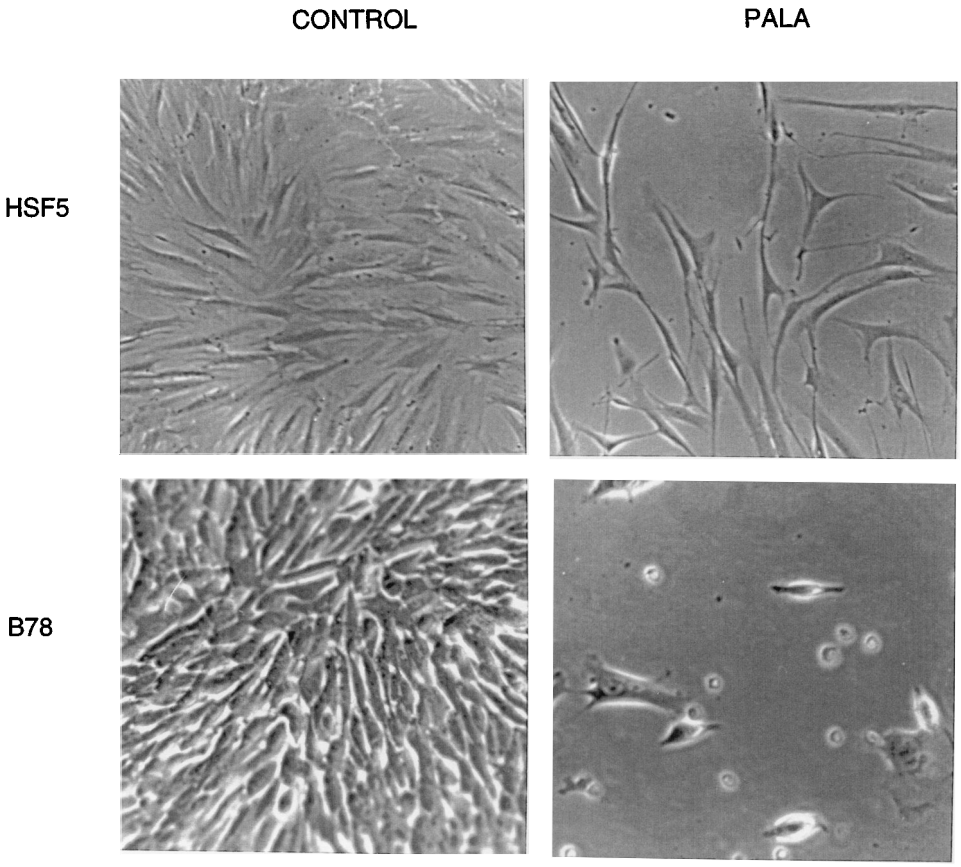

FIG. 1. Effect of PALA on B78 and normal human fibroblasts (HSF5). B78 showed cell shrinkage and detachment, previously shown to be accompanied by nucleosomal degradation (56).

arrest during PALA exposure. The parental mouse cell line B78 responds to continuous PALA exposure with apoptosis within 48 to 72 h (56). This contrasts with low-passage normal fibroblasts, HSF5, which undergo growth arrest in the presence of PALA (Fig. 1). Initially, we analyzed the response of microcell hybrids (B78MC hybrids) generated from B78 and HSF5 to treatment with PALA by qualitative assessment of morphological changes indicative of growth arrest, followed by quantification of viability, growth, and apoptosis. Flow cytometry was used to evaluate cell cycle responses to PALA. Our analyses revealed that the presence of human chromosome 3 was specifically associated with reduced cell death and evidence of growth arrest. Removal of human chromosome 3 by dominant negative selection resulted in increased apoptosis, accompanied by a loss of growth arrest in the presence of PALA. We conclude that a gene (or genes) on human chromosome 3 mediates drug-induced growth arrest and suppression of apoptosis in normal cells. The loss or inactivation of this gene may result in continued cycling during nonproliferative conditions and sensitization of the cells to induction of apoptosis. Alternatively, retention of this gene in cancerous cells may be implicated in tumor cell dormancy and an increased apoptosis threshold.

MATERIALS AND METHODS

Cell lines. The generation of the B78MC hybrids has been described elsewhere (55). These hybrids contain different human chromosomes tagged with a dual selectable marker, tgCMV/HyTK (34). Retention of the tagged chromosome was achieved through culture in medium supplemented with 400 μg of hygromycin per ml. The tagged chromosome was removed by culturing the cells in 100 μM ganciclovir (provided by Syntex) until drug-resistant colonies emerged. Subclones of B78MC166, which had lost the human chromosome 3 (B78MC166-R series), were thus obtained and were confirmed to lack human chromosome content by fluorescence in situ hybridization (FISH) with total human DNA probe and by PCR amplification (see below). B78MC hybrids were grown in Dulbecco's modified Eagle medium-high glucose (Gibco), and the primary human fibroblast cells, HSF5, were grown in minimal essential medium (Gibco). All cells were cultured in 10% fetal bovine serum (Gibco), at 37°C and 5% CO_2. During PALA experiments, dialyzed fetal bovine serum (Gibco) was used. All cell lines were free of mycoplasmas, as determined by Hoechst 33258 staining (4).

PALA assay. The antimetabolite PALA (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) is a specific inhibitor of aspartate transcarbamylase activity of the trifunctional CAD enzyme. Eighteen pools of B78MC microcell hybrids (5×10^4 cells), each containing 10 to 14 individual clones, were initially exposed to 20 μg of PALA per ml. Cell survival was qualitatively assessed by microscopic examination. Hybrid pools, showing evidence of growth arrest (reduced cytotoxicity, with enlarged, flattened single-cell morphology), were selected for further screening. The individual hybrids represented in those pools were treated with PALA, and only hybrids showing better than 50% survival, accompanied by a growth arrest morphology, were selected for further study. B78MC hybrids selected in this manner were analyzed further, including identification of human chromosomal content and PALA-induced responses. Note that hybrids showing colonization during PALA exposure were not selected for further characterization, since these cells were at risk for containing amplified CAD genes.

PALA-responsive 50% lethal dose (LD_{50}) determinations and gene amplification frequencies. LD_{50}s and gene amplification frequencies were determined according to the established protocol (42). Briefly, the LD_{50} (dose allowing 50% clonogenic survival) of each cell line was determined via interpolation of percent cell survival over a dose-response range between 0 and 4 μg/ml (0 and 11.5 μM). For determination of CAD gene amplification frequencies, 10^6 cells were plated in triplicate in 100-mm dishes and incubated in medium supplemented with PALA at a concentration of 9 LD_{50}s. Following 12 to 16 days of incubation, with regular replacement of selection medium, the plates were rinsed and stained, and the number of colonies per dish was counted. In addition, for each experiment,

plating efficiencies were determined by plating 200 cells in duplicate 60-mm dishes in nonselective medium. At 6 days, the dishes were stained and colonies were counted. Plating efficiency was calculated as the number of colonies counted per dish divided by 200. The gene amplification frequency was calculated as the number of PALA-resistant colonies counted per dish divided by the number of cells plated multiplied by the plating efficiency.

Apoptosis and cell cycle analysis. A total of 2.5×10^5 cells were seeded in 25-cm^2 flasks and incubated with or without PALA (13 μg/ml) for up to 6 days. Cells were harvested at 24- or 48-h intervals. The cells and medium were combined, an aliquot (0.5 ml) was taken for trypan blue viability testing and cell counting (by hemacytometer), and the remainder was centrifuged. The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS)-EDTA, fixed with 3 ml of 95% ethanol, and stored at −20°C until analysis. For flow cytometric analysis, the fixed cells were centrifuged and resuspended in 0.25 ml of PBS-EDTA. The cell suspensions (0.25 ml) were stained with propidium iodide (1 ml; Coulter) for 45 min at room temperature and analyzed on an EPICS XL (Coulter) flow cytometer. The raw data were gated to remove doublets from the analyses. The cell cycles were determined by using histograms gated to counts within the cell cycle range and analyzed by Multicycle AV DNA Content cell cycle analysis software (Phoenix Flow Systems). The percent counts representative of apoptotic cells in the PALA-treated samples were determined by quantifying the low fluorescence peak appearing in log-scale histograms (38).

Viable cell counts were determined from trypan blue-negative counts, and total viable counts were determined by calculation of viable counts per milliliter multiplied by volume. The relative plating efficiencies (RPE) were calculated as the viable growth in the presence of PALA divided by the viable growth in the absence of PALA, at a single time point after culture setup.

Fluorescence microscopy was used to examine nuclear changes in PALA-exposed cells following 2 days of growth. Chamber slide cultures of B78, grown in the presence or absence of PALA for 2 days, were fixed with 70% ethanol for 30 min at −20°C. The slides were rinsed with water and stained with Hoechst 33258 (final concentration, 500 ng/ml) for 10 min. The slides were then rinsed with PBS and mounted. The samples were examined with a Zeiss Axiophot fluorescence microscope.

DNA fragmentation analysis was performed as follows. Each cell line was plated in two 150-cm^2 flasks. One flask was treated with 9 LD_{50}s of PALA for 6 days, and the other flask served as a control, harvested at confluency. Cells were harvested and lysed, and detergent-soluble DNA was extracted as described previously (56). Samples were fractionated on a 1% agarose gel stained with ethidium bromide in order to detect nucleosomal degradation.

FISH analysis of hybrids. Human chromosomal content of the hybrids selected in the initial PALA screen was determined by FISH with total human DNA probe (Oncor) and by *Alu*-PCR FISH, as described previously (9, 55).

PCR analysis of human chromosome content. PCR amplification using oligonucleotide primers identifying human microsatellite sequences (sold as MapPairs by Research Genetics) was used to confirm the chromosome content and to establish the presence or absence of individual chromosome 3 loci in the hybrids. High-molecular-weight DNA (50 ng) from B78 (negative control), human cells (positive control), or the hybrids was added to PCR hybridization mixtures containing 10 pmol of each primer, 200 μM deoxyribonucleotide triphosphates (Boehringer Mannheim Canada), PCR buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, and 0.01% gelatin), and 0.75 U of AmpliTaq (Perkin-Elmer Cetus, Applied Biosystems, Canada) in a volume of 25 μl. Samples were incubated in a Perkin-Elmer DNA Thermal Cycler 480 with the following parameters: 94°C for 5 min followed by 35 cycles of successive denaturation (94°C for 30 s), annealing (60 or 55°C for 30 s), and synthesis (72°C for 30 s). A final cycle of 72°C for 5 min ended the amplification program. The amplified products were analyzed by electrophoresis on 2% agarose (NuSieve 3:1; FMC) gels stained with ethidium bromide. To confirm the identity of the human chromosomes present in the hybrids, the following primers were used: chromosome 3, D3S1210 (3p) and D3S1215 (3q); chromosome 4, GABRB1; chromosome 7, D7S523; chromosome 9, D9S146; chromosome 11, D11S929; chromosome 12, D12S43; chromosome 13, D13S131; chromosome 17, D17S957; chromosome 22, D22S273.

Analysis of CAD gene copy number. Southern blots were prepared according to standard procedures. DNA was isolated from cells exposed to 13 μg of PALA per ml for 0, 2, and 4 days (plus 2-day recovery) and digested with *Eco*RI restriction enzyme (Boehringer Mannheim Canada) with the manufacturer's recommended conditions. The digested DNA was ethanol precipitated, dissolved in Tris-EDTA buffer, and quantified by fluorometry (TKO 100 DNA Fluorometer; Hoefer Scientific Instruments). Equal amounts (6.5 μg) of DNA were loaded into each lane of a 0.8% agarose gel and fractionated by electrophoresis. The DNA was transferred to a nylon membrane by alkaline transfer. The hamster CAD cDNA probe is a 6-kb fragment derived from *Hin*dIII-digested pCAD142 (54). The rat albumin cDNA probe is a 1.5-kb fragment derived from *Eco*RI-digested pHDQ835 (66). The probes were radiolabelled via the random primer method, hybridized to the membrane overnight at 65°C, and washed identically (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS] twice for 15 min at room temperature and 0.2× SSC–0.1% SDS twice for 15 min at 42°C). Values for intensity of hybridization to a 2.3-kb murine CAD gene restriction fragment and a 5-kb albumin gene restriction fragment were determined by phosphorimaging (Molecular Dynamics ImageQuantNT; Molecular Dynamics).

Immunoblot analysis of cell lines. Total cellular protein extracts were prepared by lysing untreated cells with protein lysis buffer containing protease inhibitors (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X, 1% deoxycholate, 0.1% SDS, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml) and quantified by Bio-Rad Protein Assay (Bio-Rad). After boiling, 50 μg of cellular protein was fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Following blocking with 0.5% milk–TBST (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20), the blot was washed twice with TBST for 15 min and hybridized for 1 h at room temperature with primary p53 antibody Ab-1 (Oncogene Science), diluted to 10 μg/ml in TBST. The blot was then washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Oncogene Science), diluted to 0.2 μg/ml in TBST. The blot was washed again and autoradiographed by chemiluminescence according to the manufacturer's instructions (Amersham Canada).

Irradiation of cell lines. Twenty-four hours after plating of 250,000 cells per 25-cm^2 flask, cells were exposed to 0 or 5 Gy of ^{137}Cs gamma radiation (Gammacell-40 Self-Contained Irradiator; Nordion International) at a rate of 1.08 Gy/min. The cells were then incubated at 37°C for 24 and 96 h. Flow cytometry was performed for cell cycle analysis as described above.

RESULTS

PALA screening of the B78MC microcell hybrid series. Fusion between human tumorigenic cell lines and normal human fibroblasts results in the dominance of growth arrest and suppression of gene amplification in the presence of the antimetabolite PALA (64). This finding suggests that recessive genes are responsible for the ability of cells to control the cell cycle and that these genes are either deleted or mutated during the oncogenic process. The tumor suppressor gene p53 has been shown to be involved in PALA-induced growth arrest in normal cells, and its loss is sufficient for growth arrest failure during PALA exposure (30, 71). However, other genes may exist, ones which, when mutated or inactivated, have a similar effect on the ability of the cell to respond to chemotoxic drugs with growth arrest.

Preliminary experiments with whole-cell hybrids between murine melanoma cell line B78 and normal skin fibroblasts, HSF5, suggested that several chromosomes, in addition to human chromosome 17 (the locus for p53), were involved in PALA-induced growth arrest (56). To further characterize the PALA-induced growth arrest and to identify which individual human chromosomes are responsible for this response, we used a panel of microcell hybrids constructed from B78 and HSF5 (B78MC hybrids). We qualitatively screened the panel for evidence of growth arrest and suppression of PALA-induced apoptosis.

To efficiently screen over 200 newly generated B78MC hybrids, we combined the hybrids into 18 pools. PALA (20 μg/ml) was cytotoxic to B78 and to all of the B78MC pools tested, with the majority of cells dying by day 4 of drug exposure. However, in 5 of 18 pools tested, nuclear and cytoplasmic enlargement and a flattened morphology were observed in a minority of cells, suggesting the presence of a subpopulation capable of a PALA-induced growth arrest response. Individual hybrids from the five selected pools were subjected to PALA exposure and screened. Seven B78MC hybrids showed evidence of growth arrest and a reduction in the degree of PALA cytotoxicity (estimated at less than 50% cell death by day 6 of PALA exposure). The human chromosome content of these hybrids was then determined.

***Alu*-PCR FISH analysis of microcell hybrids.** The human DNA present in the growth-arrested hybrids was PCR amplified with *Alu* primers, and the products were used as FISH probe on normal human male chromosome spreads (9). Chromosomes painted by this method (*Alu*-PCR FISH) represent

TABLE 1. Cell line responses to short-term PALA treatment

Cell line	Chromosome content (human)	PALA response[a]				
		RPE[b]	G_1/S[c]	G_2/S[c]	Viability (%)[d]	Apoptosis (%)[e]
HSF5	46, XY	0.051	8.88	1.09	74.4	6.7
B78	None	0.016	1.63	0.52	48.9	61.6
B78MC166	3	0.033	5.02[f]	3.3[f]	75.7[f]	28.7[f]
B78MC166-R1	None	0.020	1.66	0.47	64.1	45.5
B78MC166-R2	None	0.017	2.38	2.0	42.2	53.9
B78MC166-R5	None	0.034	1.81	0.81	58.6	36.7
B78MC166-61	μdel3[g]	0.017	2.41	1.07	43.1	59.2
B78MC56	3p, 17q	0.060	4.9[f]	1.68[f]	75.5[f]	24.2[f]
B78MC27	7q, 22q, 17	0.022	2.02	0.97	54.7	51.2
B78MC9	12	0.008	1.53	1.73	52.2	47.7
B78MC108	4	0.018	1.52	0.56	55.5	45.7
B78MC173	9, 11	0.043	1.09	0.54	66.5	40.0
B78MC16	12, 13	0.016	1.02	1.11	56.7	50.3

[a] Data were taken from cells exposed to 13 μg of PALA per ml for 4 days and are means of three independent experiments.
[b] RPE following 4 days of PALA exposure. The RPE is the number of viable cells grown in the presence of 13 μg of PALA per ml divided by the number of viable cells in the absence of PALA.
[c] G_1/S and G_2/S ratios of cell lines determined from cell cycle analysis of flow cytometric histograms.
[d] Percent viability determined by trypan blue exclusion.
[e] Percent apoptosis determined by quantifying apoptotic peak from log-scale flow cytometric histograms (counts in apoptotic range/counts in apoptotic range + counts in cell cycle range).
[f] Data significantly different from B78 ($P < 0.01$) and all other hybrids tested ($P < 0.05$).
[g] μdel3, abnormal human chromosome 3 containing several microdeletions.

the highly retained human elements present in the hybrids. The *Alu*-PCR FISH-positive findings were confirmed by PCR analysis with human chromosome-specific primers. Overall, human chromosomes 3, 4, 9, 11, 12, and 13 were identified as highly retained in the seven hybrids; however, chromosome 3 and chromosome 12 were overrepresented. Four of the seven hybrids contained elements of chromosome 3, while three of the seven contained elements of chromosome 12. In order to rule out a bias in representation of chromosomes 3 and 12 in the B78MC panel, *Alu*-PCR FISH analysis of randomly selected B78MC hybrids was used to establish an estimate of the overall proportion of B78MC hybrids stably containing a chromosome 3 or 12. We estimated that 30 to 50% of randomly selected B78MC hybrids contained human chromosome 12, whereas only 10 to 15% stably retained human chromosome 3. Thus, the increased representation among the PALA-selected hybrids of chromosome 12, but not chromosome 3, could be explained by a bias due to overrepresentation in the unselected B78MC hybrid panel. We therefore hypothesized that chromosome 3 had played a role in the PALA-induced growth arrest phenotype observed in four of the seven hybrids.

To further characterize the effect of human chromosome 3 on PALA-induced apoptosis, several hybrids with known human chromosome content were tested for their ability to undergo growth arrest and survive during PALA exposure. To represent all the chromosomes identified in the preliminary screen (3, 4, 9, 11, 12, and 13), we tested B78MC166, B78MC56, B78MC108, B78MC173, B78MC16, and B78MC9. B78MC27, which contains a whole human chromosome 17 and chromosomes 7 and 22, was also evaluated.

Cell cycle analysis and apoptosis. The relative sensitivities of B78 and the hybrids to short-term PALA exposure were compared by using 13 μg/ml (standardized 9-LD_{50} PALA dose for B78), delivered over an exposure period of 4 days. PALA has been used in the past to measure relative frequencies of gene amplification in a variety of cell lines (30, 42, 47, 62, 64, 71). CAD gene amplification allows clonogenic survival of immortalized and tumorigenic cells during long-term PALA exposure, and it is well established that this is a rare event (42, 63). A 9-LD_{50} PALA dose calibrated to each cell line was used in these studies, to compensate for differences in PALA sensitivities between cell lines. In contrast, our study was designed to evaluate and compare the overall PALA-induced responses of growth arrest and apoptosis in the cell lines, following short-term exposure, irrespective of CAD gene amplification potentials. For this reason, a calibrated 9-LD_{50} PALA protocol was not required.

Preliminary analysis of B78 and the selected microcell hybrids indicated that there were no significant differences in their growth characteristics, or ploidy, which might potentially influence their responses to PALA. The ability of the cell lines to grow during short-term, continuous PALA exposure was evaluated by measuring the RPE of each of the cell lines at a single dose and time point. All of the cell lines tested showed low RPE, indicating growth failure in the presence of PALA (Table 1). PALA-induced growth failure can be due to either growth arrest, as seen in normal fibroblasts, or induction of apoptosis, as seen in B78 (56). In order to differentiate these two processes in each of the hybrid cell lines, we quantified PALA-induced apoptosis by trypan blue staining and flow cytometric log-scale histograms.

B78 showed a distinct apoptotic peak on log-scale histograms by day 4 of PALA exposure, which, when quantified, closely matched trypan blue-positive counts taken from the same cell samples (Fig. 2 and Table 1). HSF5, consisting of low-passage normal human fibroblasts, underwent very little apoptosis during PALA exposure (Fig. 2). However, increased debris present in log-scale histograms and trypan blue positive staining of PALA-exposed HSF5 suggested a reduction in viability (Fig. 2 and Table 1). A limited degree of necrosis may therefore have occurred in the normal fibroblast population during PALA exposure.

As expected from our preliminary screening process, several hybrids showed a reduced level of apoptosis in comparison with B78 (Table 1). The most significant reduction in apoptosis was measured in two hybrids containing human chromosome 3.

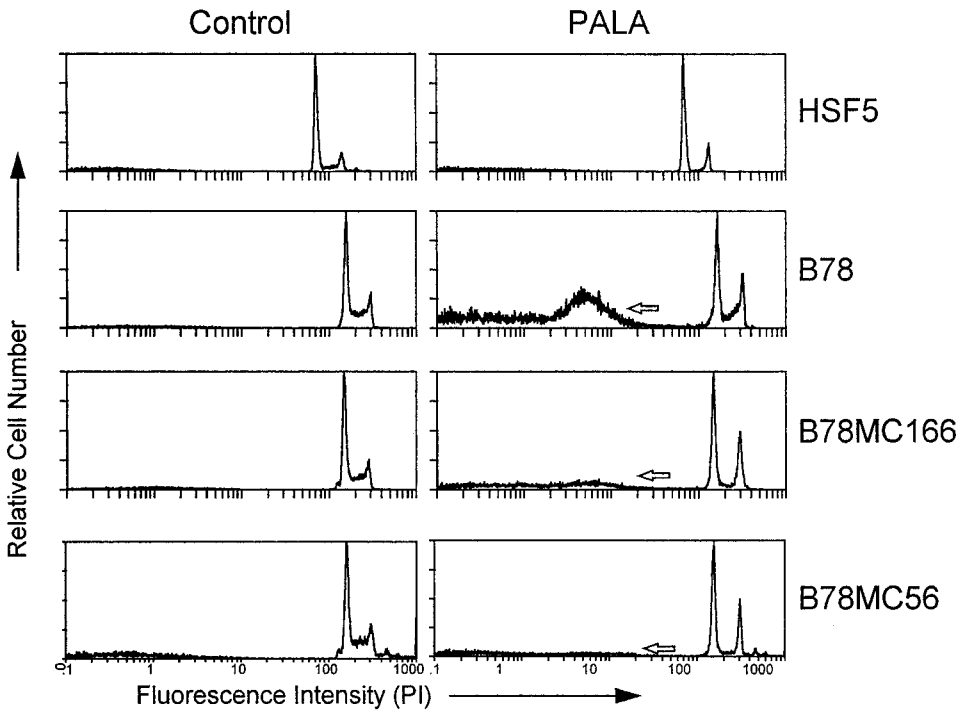

FIG. 2. Comparison of log-scale histograms of propidium iodide-stained cells. Cells were exposed to 13 μg of PALA per ml for 4 days. The low fluorescence peak (open arrows) observed in PALA-exposed cells represented a population of apoptotic cells. Controls were untreated cells in exponential phase.

B78MC166, a monochromosomal hybrid containing a rearranged chromosome 3, and B78MC56, containing the short arm of chromosome 3 and the long arm of chromosome 17, showed significantly lower amounts of PALA-induced apoptosis compared with B78 and the other hybrids tested (Table 1). These two hybrids showed a general increase in debris during PALA exposure, as determined by flow cytometry (Fig. 2), with a poorly distinguishable apoptotic peak. They also showed significantly increased viability in the presence of PALA, compared with B78 and the other hybrids tested, while maintaining poor RPE (Table 1). To determine if cell cycle checkpoints were responsible for loss of growth potential in B78MC166 and B78MC56, we compared the cell cycle responses of the cell lines in the presence of the PALA by using single-parameter flow cytometry to determine the relative numbers of cells in G_1, S, and G_2/M.

During the first 2 days of PALA exposure, flow cytometric histograms of all the cell lines tested revealed accumulation of cells in S phase, a typical cellular response to antipyrimidine drugs such as PALA (Fig. 3). The S-phase delay was transient and ended spontaneously by day 3 of exposure. Accumulation of apoptotic cells began to occur at this time, as detected by trypan blue staining and log-scale flow cytometric profiles. Significant nuclear changes were already evident in some PALA-exposed cells by day 2 of treatment (Fig. 4). At this time, some cells showed nuclear malformation and shrinkage with chromatin condensation, suggestive of apoptosis. Many of the remaining cells showed nuclear enlargement, most likely due to partially completed DNA synthesis and G_2 arrest. In spite of S-phase delay, the PALA-exposed cells proliferated slowly and doubled in number at least once during the first 2 days of treatment, after which viable cell numbers either stabilized or declined (Fig. 5). Thus, the majority of cells passed through at least one cell cycle prior to undergoing either growth arrest or apoptosis in the presence of PALA.

Evaluation of flow cytometry data from day 4 of PALA exposure showed that B78MC166 and B78MC56 had an increase in G_1 and G_2 counts, accompanied by a decrease in S counts, compared with B78 and the other hybrids. This response was similar to that of HSF5 and resulted in G_1/S and G_2/S ratios which were significantly higher than those of B78 and the other hybrids (Table 1). To further analyze the effect of the presence of chromosome 3 on PALA-induced growth arrest, the G_1/S ratios of B78MC166 and B78 were measured over a 6-day PALA exposure period. Both B78 and B78MC166 showed increasing G_1/S ratios over most of the PALA exposure period. However, B78MC166 showed a more rapid increase, which, by day 4 of PALA exposure, was threefold higher than that of B78. The high G_1/S ratio was maintained over the remaining exposure period (Fig. 6). Furthermore, when subjected to increasing doses of PALA, B78MC166 underwent a dose-responsive increase in the G_1/S ratio, indicating a drug-responsive induction of G_1 arrest. In contrast, B78 showed a depressed G_1/S ratio over the same dosage range (Fig. 7). These findings indicate that human chromosome 3 contains a gene(s) which may play a role in growth arrest and apoptosis suppression.

Alu-PCR FISH and standard FISH using total human DNA probe (Oncor) revealed that B78MC166 contained a single rearranged human chromosome 3. B78MC56 contained the short arm of chromosome 3 and the long arm of chromosome 17. The PALA-induced growth arrest phenotype observed in B78MC56 was probably due to the presence of the short arm of human chromosome 3, since B78MC27, a hybrid containing chromosome 17, responded to PALA with growth arrest failure and a high percentage of apoptotic cells by day 4 of exposure (Table 1).

Analysis of B78MC166 subclones. The B78MC panel of microcell hybrids is unique among human × rodent hybrids in that each hybrid in the panel contains an individually tagged

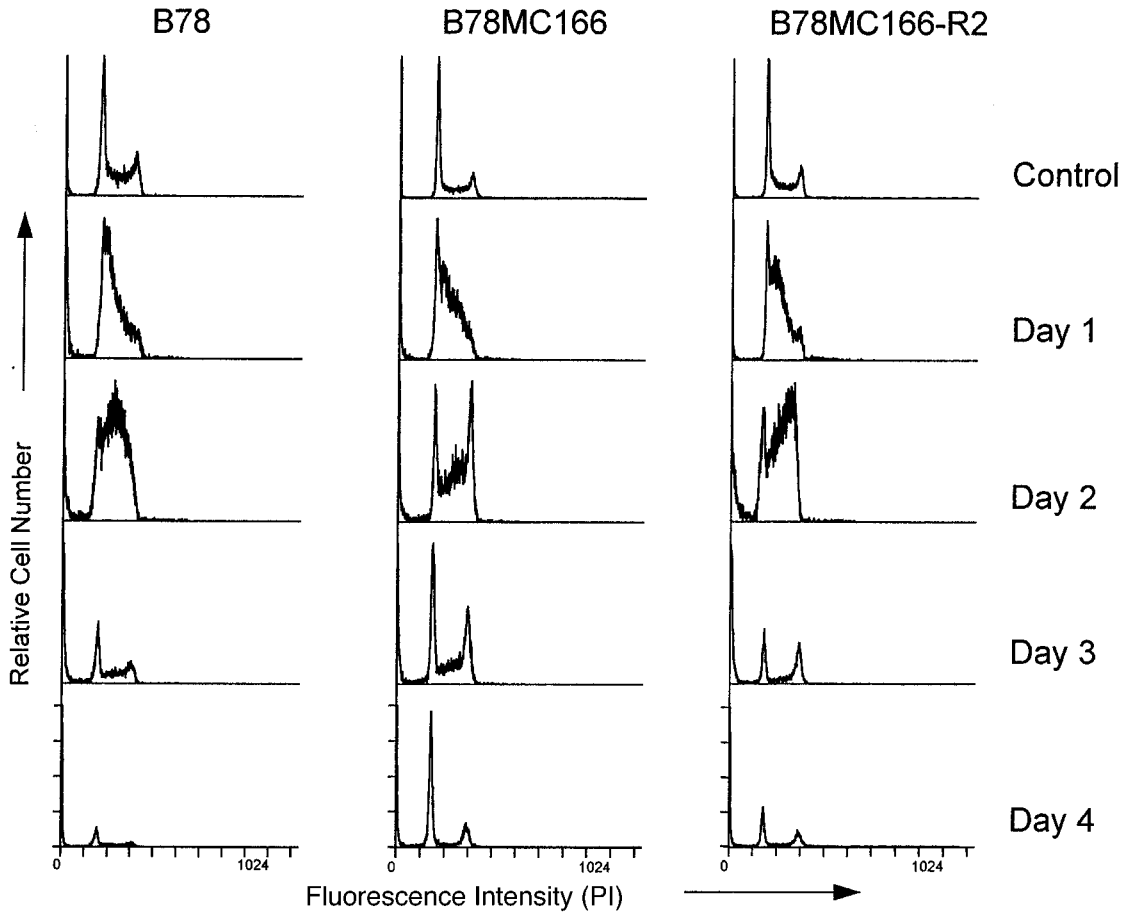

FIG. 3. Linear scale flow cytometric histograms of cells continuously exposed to 13 μg of PALA per ml over a 4-day period. Controls were exponential-phase untreated cells.

human chromosome which can be retained, or removed via dominant positive or negative selection, as desired. This property can be used to test the hypothesis that a particular phenotype is associated with the presence of an introduced, tagged chromosome. If the phenotype in question is due to the presence of the tagged chromosome, as opposed to genetic heterogeneity between clones, one would expect removal of the tagged chromosome to result in loss of the phenotype. In this case, if a gene on chromosome 3 appeared to be responsible for the PALA-induced growth arrest phenotype and associated

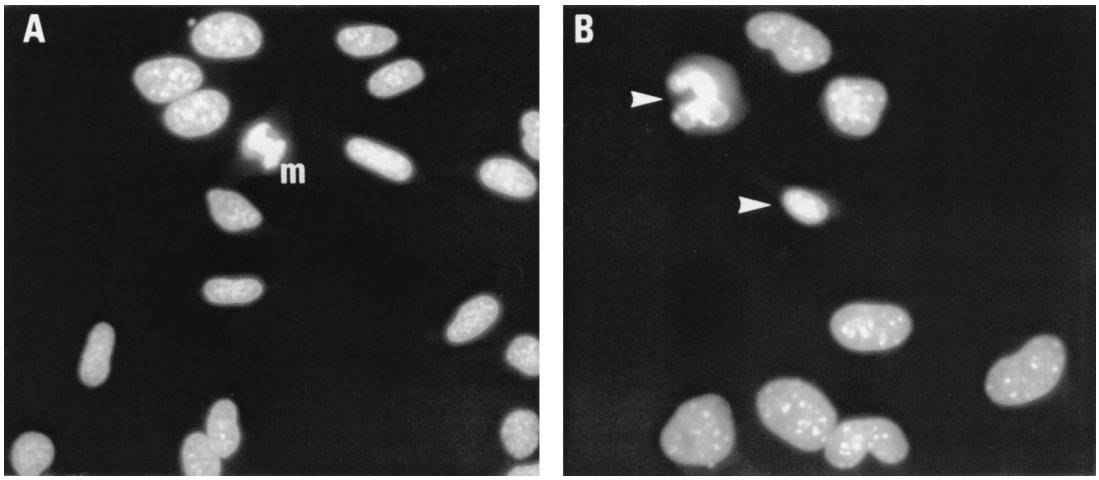

FIG. 4. In situ Hoechst 33258-stained cells. (A) Untreated B78. (B) PALA-exposed B78, following 2 days of treatment. Arrowheads indicate nuclei with apoptotic changes. Malformed, enlarged nuclei are probably cells in S or G_2 delay. m, mitotic figure.

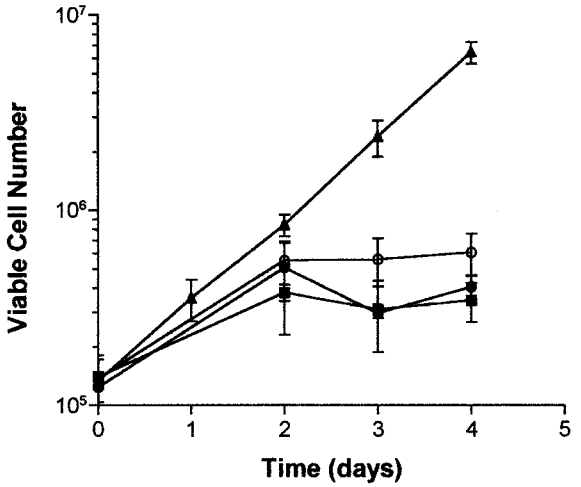

FIG. 5. Viable growth during PALA exposure. Control was mean combined growth in the absence of treatment. Symbols: ■, B78; ○, B78MC166; ●, B78MC166-R2; ▲, control.

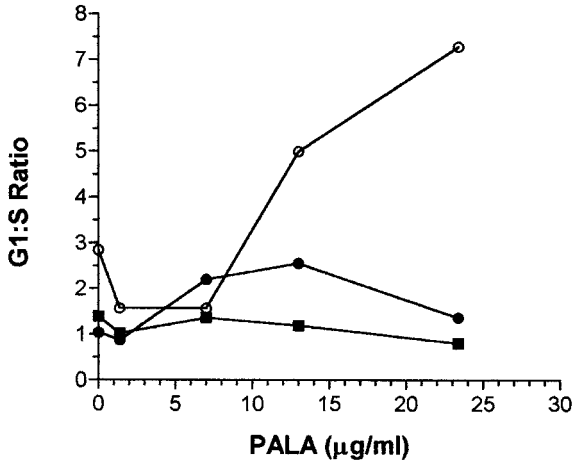

FIG. 7. G_1/S ratios at day 4 of PALA exposure, over a wide dosage range. The G_1/S ratios of unexposed cells were obtained at confluency (day 4). Symbols: ■, B78; ○, B78MC166; ●, B78MC166-R2.

suppression of apoptosis, the subsequent loss of the gene through removal of the tagged chromosome would then be expected to result in a return to the PALA-induced apoptotic phenotype and growth arrest failure. Using ganciclovir, we selected against the retention of human chromosome 3 in B78MC166 and isolated several ganciclovir-resistant subclones (B78MC166-R series). PCR amplification and FISH using total human DNA probe showed that these subclones lacked human chromosome 3 (not shown). We tested the PALA response of several ganciclovir-selected subclones and determined that they showed reduced viability compared with B78MC166 and an increase in apoptosis accompanied by low G_1/S ratios during continuous PALA exposure (Table 1). We further evaluated the ability of one of the subclones, B78MC166-R2, to respond to PALA with growth arrest. When subjected to PALA treatment, B78MC166-R2 showed a B78-like response, with G_1/S ratios failing to increase over time and over a wide dosage range (Fig. 6 and 7). These findings demonstrate that the presence of human chromosome 3 in B78MC166 was indeed responsible for inducing growth arrest in response to PALA and suppressing apoptosis.

At higher passages (>passage 8), B78MC166 became more sensitive to PALA-induced apoptosis compared with early passage (Fig. 8). We hypothesized that the reduction in apoptosis suppression in response to PALA in this hybrid at late passage was due to a lack of linkage between the chromosomal element responsible for the growth arrest phenotype and the selectable marker. This was supported by the fact that the chromosome 3 in B78MC166 was rearranged. As chromosome breakage is common during microcell fusion, we reasoned that chromosomal fragmentation occurring during the microcell fusion process could result in subsequent loss of acentric fragments during passaging. If an acentric fragment contained the gene providing the growth arrest phenotype, it could segregate from the tagged chromosome and either be reincorporated into the hybrid genome or be lost from the cells. This would then explain the observed reduction in PALA-induced growth arrest phenotype of late-passage B78MC166. To test this, we sub-

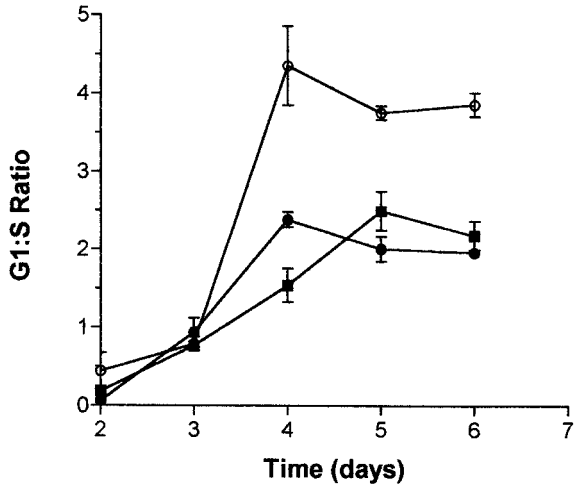

FIG. 6. G_1/S ratio responses of cell lines during continuous PALA exposure (13 μg/ml). The graph begins at day 2 of PALA exposure, following release from S-phase delay. Symbols: ■, B78; ○, B78MC166; ●, B78MC166-R2.

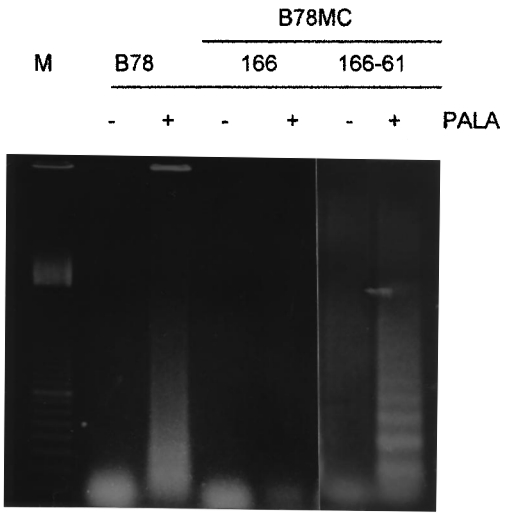

FIG. 8. Nucleosomal degradation effect of PALA on cells. Cells were grown in the absence (−) or presence (+) of PALA (9 LD_{50}s) for 6 days. M, 100-bp ladder. Low-molecular-weight band is digested remnant RNA.

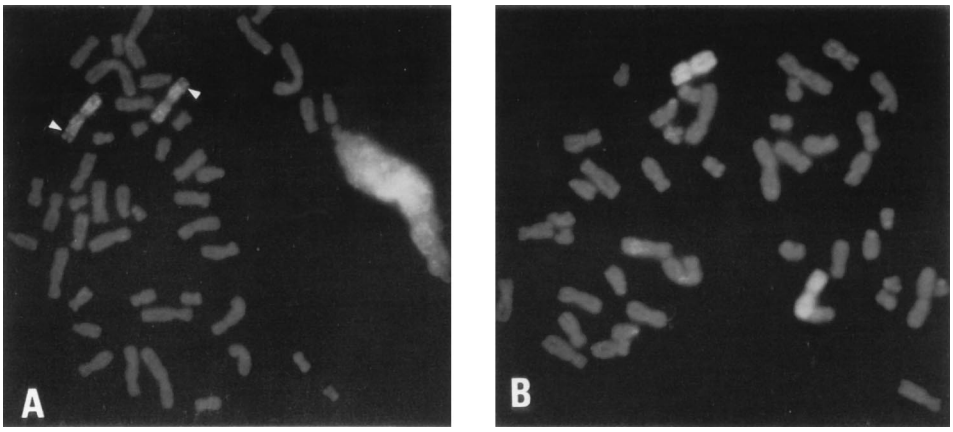

FIG. 9. *Alu*-PCR FISH of chromosome 3-containing hybrids, performed on normal male human metaphase spreads. (A) Probe derived from B78MC166-61, a revertant subclone of B78MC166. (B) Probe derived from B78MC166. The arrowheads in panel A indicate the largest deletion detected, at 3q26-q28.

cloned B78MC166 and analyzed this new set of hybrids (B78MC166-*n* series) for loss of the growth arrest phenotype. Subclone B78MC166-61 showed an increase in PALA-induced apoptosis compared with its B78MC166 parent, with an associated low G_1/S ratio at day 4 of exposure (Table 1). *Alu*-PCR FISH showed that, while B78MC166 *Alu*-derived probe uniformly painted human chromosome 3, the *Alu* probe from B78MC166-61 revealed gaps in the fluorescence signal on both arms of chromosome 3 (Fig. 9). Some gaps appeared to localize to chromosome 3 fragile sites. PCR amplification using chromosome 3-specific primers identified some loci which were absent in B78MC166-61, including D3S1276 at 3p13, D3S1302 at 3q12, and a large region between 3q26 and 3q28 bracketed by D3S1564 and SST. These findings may explain the reversion of B78MC166-61 to the B78 PALA-induced apoptotic phenotype and suggest that the gene in question is located within one of the microdeleted regions of chromosome 3.

Evaluation of gene amplification frequencies in the chromosome 3-containing hybrids. Long-term exposure of immortalized and tumorigenic cells to high doses of PALA results in the emergence of drug-resistant colonies containing amplified CAD genes (71). A calibrated PALA dose of 9 LD_{50}s is used to permit accurate comparisons of gene amplification potential between cell lines and types. To rule out the possibility that the hybrids had increased gene amplification potential, compared with B78, PALA clonogenic assays were performed (Table 2). B78, HSF5, and several hybrids were tested and showed PALA-responsive LD_{50}s which were within the range reported in a variety of human and rodent cell types (62). B78MC166 and B78MC56, as well as HSF5, were found to have LD_{50}s which were roughly double that of B78. However, several B78MC166-derived cell lines which lacked the apoptosis suppression phenotype also showed similarly high LD_{50}s in comparison with B78 (Table 2). Thus, relative PALA sensitivities, by LD_{50} determinations, were not consistently predictive of the ability to suppress PALA-induced apoptosis. All of the hybrids tested showed gene amplification frequencies which were lower than that of B78, indicating that the CAD gene amplification potential of the hybrids was noncontributory to the growth arrest and apoptosis responses in the majority of cells.

Southern blot analysis was used to further confirm that the CAD gene was unamplified in the PALA-exposed cells of B78MC166 and B78MC56 compared with B78 and the B78MC166-derived PALA-sensitive cell lines (Fig. 10). The hybridization intensity of the CAD probe for each hybrid (0 days of PALA treatment) was approximately equal to that of B78 when quantified by phosphorimaging, with hybridization to the albumin probe as a loading control. This indicated that the overall CAD gene copy number of each of the hybrids was similar to that of B78, prior to PALA treatment. Quantification of the hybridization signals following 2 and 4 days of treatment showed a trend for the CAD signal to increase slightly over the exposure period; however, B78MC166 and B78MC56 showed no evidence of an increased tendency to

TABLE 2. Cell line characteristics and gene amplification frequencies

Cell line (ploidy)[a]	PE[b] (%)	CGT[c] (h)	PALA LD_{50} (μg/ml)	Gene amplification[d]
B78 (4n)	48.8 ± 5.7	14.5 ± 1.4	1.43 ± 0.07	$8 \times 10^{-5} \pm 1 \times 10^{-5}$
HSF5 (2n)	30.5 ± 4.5	18.8 ± 0.3	2.46 ± 0.15	ND[e]
B78MC166 (4n)	59.9 ± 8.6	14.0 ± 0.5	2.82 ± 0.11	$5 \times 10^{-6} \pm 1 \times 10^{-6}$
B78MC166-R2 (4n)	49.1 ± 3.0	15.2 ± 1.9	1.86 ± 0.19	$1 \times 10^{-5} \pm 1 \times 10^{-6}$
B78MC166-R1 (4n)	64.7 ± 4.5	14.1 ± 1.0	2.73 ± 0.03	$1 \times 10^{-5} \pm 1 \times 10^{-5}$
B78MC166-61 (4n)	59.2 ± 12.1	14.5 ± 0.8	2.54 ± 0.14	$2 \times 10^{-6} \pm 1 \times 10^{-6}$
B78MC56 (4n)	51.0 ± 10.0	15.4 ± 0.6	2.75 ± 0.38	$1 \times 10^{-5} \pm 1 \times 10^{-5}$

[a] B78 and the hybrids were determined cytogenetically to be pseudotetraploid. HSF5 is normal diploid.
[b] PE, plating efficiency.
[c] CGT, cell generation time, determined according to the method of Leibovitz et al. (27).
[d] Frequency of colony formation determined by clonogenic assay.
[e] ND, not determined.

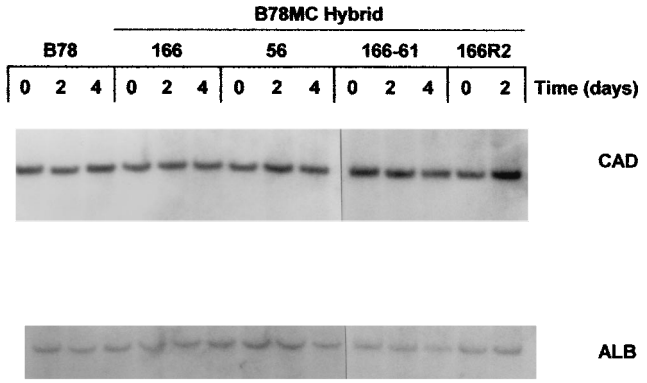

FIG. 10. Southern analysis of PALA-exposed cell lines. DNA was isolated from cells exposed to 13 μg of PALA per ml for 0, 2, or 4 days; *Eco*RI digested; and hybridized with a hamster CAD probe (CAD). Relative hybridization intensities were determined with an *Eco*RI rat albumin probe (ALB) as a loading control. The locus for human albumin is chromosome 4q11-q13.

amplify the CAD gene during short-term PALA exposure, compared with B78 and the other hybrids tested.

Determination of p53 protein status in B78 and hybrids. We suspected that B78 contained nonfunctional p53 protein. Tumorigenic cell lines with nonfunctional or mutant p53 often show high cellular p53 protein levels, because of altered conformation and stabilization (44, 67). This contrasts with nontransformed, wild-type p53-expressing cell lines, which, when analyzed by immunoblotting, show very low or undetectable levels of p53 protein, because of a very short half-life (15, 21, 33, 41). To determine if p53 protein was stabilized in B78 and the hybrids, total cellular protein was extracted from the cell lines and analyzed by immunoblotting with a p53 monoclonal antibody, Ab-1, which reacts with both mutant and wild-type forms of p53 protein (Fig. 11). Untreated B78 and B78MC166-derived hybrids were found to contain high levels of p53 protein, compared with normal human and murine cell lines (NH and NM, Fig. 11). This suggested that B78-derived cells contained inactivated p53 protein.

Tumorigenic and transformed cell lines which lack wild-type p53 protein activity are usually radioresistant and fail to undergo G_1 arrest, because of failure of apoptosis (21, 26, 32, 36, 46). Since radiation-induced G_1 arrest and apoptosis are under the control of wild-type p53 protein, we tested the ability of B78 and B78MC166 to undergo either G_1 arrest or apoptosis following ionizing radiation. The cells were exposed to 5 Gy of gamma radiation and, following 24 h or 96 h recovery, were harvested and analyzed by flow cytometry (96 h shown, Fig. 12). Both cell lines showed poor G_1 and G_2 checkpoint responses and continued to proliferate following irradiation. Viability, as determined by trypan blue exclusion, 96 h after irradiation, was 95 and 92% for B78 and B78MC166, respectively. Furthermore, log-scale histograms of the irradiated cells showed little accumulation of debris and lacked a detectable apoptotic peak (Fig. 12). These findings are consistent with cells possessing mutant or nonfunctional p53 protein. These findings further suggest that ionizing radiation does not induce chromosome 3-mediated growth arrest in these cells.

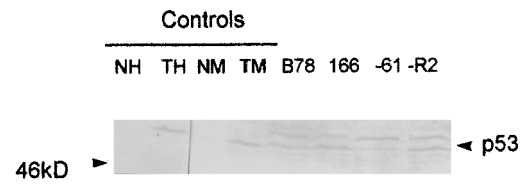

FIG. 11. Immunoblot of cell lines to detect stabilized p53 protein in B78 and B78MC166 hybrids. Controls: NH, normal human cell line HSF5; TH, human tumorigenic cell line DU-145; NM, normal murine cell line NIH 3T3; TM, tumorigenic murine cell line P19.

DISCUSSION

In this study, microcell hybrids were used to show that the presence of human chromosome 3 is associated with a PALA-induced growth arrest phenotype accompanied by reduced apoptosis. Removal of chromosome 3, or microdeletions of specific regions of chromosome 3 from the hybrid, resulted in loss of the PALA-induced growth arrest and increased levels of apoptosis.

The G_1/S ratios were significantly higher in PALA-exposed B78MC166 (which contained chromosome 3) and B78MC56 (which contained the short arm of chromosome 3) as opposed to B78 and the other hybrids. Increased G_1/S ratios in the chromosome 3-containing hybrids were associated with significantly reduced levels of apoptosis. These findings suggest a G_1 checkpoint linked to a gene on human chromosome 3. The G_2/S ratio was also significantly higher in PALA-exposed B78MC166 and B78MC56 compared with B78 ($P < 0.01$), and removal of chromosome 3 from B78MC166 resulted in a significant reduction in the G_2/S ratio (B78MC166-R hybrids, Table 1). However, because the G_2/S ratio remained relatively high in B78MC166-R2 compared with B78 and the other hybrids, it is not clear whether the G_2 arrest appearing during PALA treatment was directly due to the presence of chromosome 3. Extended G_2 arrest is thought to contribute to cell survival following genotoxic exposure (35). In yeasts, the failure of G_2 arrest, due to checkpoint rad mutations, leads to cell death and genomic instability following irradiation damage (1, 69). The presence of a strong G_1/S ratio in B78MC166 and B78MC56 in response to PALA may be due to the action of a similar mammalian checkpoint gene, acting to induce growth arrest in G_1, with possible downstream effects in G_2.

To explain the differences in the PALA-induced responses of B78 and the chromosome 3-containing hybrids, we hypothesize that chromosome 3 contains a growth regulatory gene which also inhibits apoptosis. Because of cell-to-cell variations, only a proportion of cells in the chromosome 3-containing hybrids may actually carry a functional copy of this gene. During PALA exposure, these cells may be able to avoid the apoptotic process by arresting in G_1 following initial S phase delay. The ability to maintain a G_1 arrest may result in increased survival compared with B78 and the other hybrids. Cells which fail to observe the G_1 checkpoint during PALA exposure may be destined to die via apoptosis. This would explain the observation of high day 4 G_1/S ratios in the viable PALA-exposed chromosome 3-containing hybrid cells, since nonarrested cells would be selected for apoptosis. Some cycling cells may also survive via other drug resistance mechanisms, such as CAD gene amplification or increased CAD gene copy number by chromosomal rearrangement (47). CAD gene amplification was shown to occur rarely in these hybrids, and their gene amplification potential was calculated to be less than that of B78 (Table 2). Gene amplification would not, therefore, be expected to emerge as a drug resistance mechanism in a significant proportion of the population of viable cells during the 4-day PALA exposure period. Southern blot analysis of DNA from treated and untreated cells confirmed that the CAD gene copy number was not appreciably different or was not increased in B78MC166 and B78MC56 during short-term

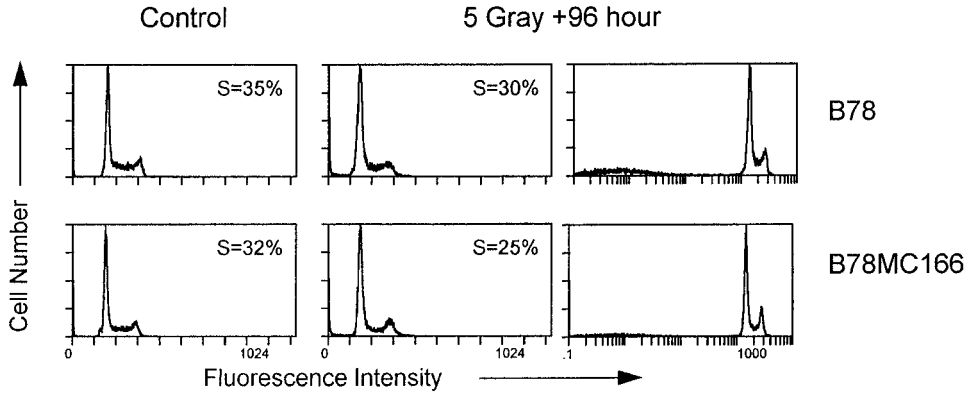

FIG. 12. Linear and log-scale flow cytometric histograms of B78 and B78MC166, 96 h after gamma radiation (5 Gy). S is percentage of cells in S phase. Controls were growing untreated cells.

PALA exposure, compared with B78 and the other hybrids (Fig. 10).

Indirect evidence supports the view that CAD gene amplification was not responsible for the PALA-responsive phenotypes of B78MC166 and B78MC56. For example, it should be noted that *Alu*-PCR FISH failed to reveal any human chromosome 2 elements (the human locus of the CAD gene) in the selected hybrids (B78MC166 shown, Fig. 9). Furthermore, if additional CAD genes were responsible for the reduced apoptosis observed in B78MC166, removal of human chromosome 3 would not have resulted in loss of the PALA-responsive phenotype of this hybrid. Reduced PALA-induced cell death was not accompanied by cellular proliferation, signalling classical drug resistance, in B78MC166 and B78MC56. Moreover, the differences in apoptosis and G_1/S ratios could not be explained by differences in rates of proliferation, since the hybrids tested had cell generation times which were not significantly different from that of B78 (Table 2). This and the high G_1/S and G_2/S ratios of B78MC166 and B78MC56 during PALA exposure support the view that continuous short-term PALA exposure resulted in selection for survival of growth-arrested cells in chromosome 3-containing hybrids, rather than survival through drug resistance by gene amplification.

Interestingly, *Alu*-PCR FISH revealed several human chromosome 3 breakpoints in B78MC166-61 which appear to be in proximity to established cytogenetic markers for chemically induced chromosome 3 breaks. One of these fragile sites, FRA3B, is closely linked to at least one candidate tumor suppressor gene locus, at 3p14.2 (59). Since the other growth-arrested hybrid, B78MC56, contained only the p arm of chromosome 3, the microdeleted regions of the short arm of chromosome 3 are the candidate regions for the gene acting in PALA-induced growth arrest and suppression of apoptosis.

It was determined that B78 lacked wild-type p53 protein. This resulted in a poor G_1 response to both PALA and ionizing radiation but did not prevent PALA-induced apoptosis. Although the presence of wild-type p53 appears to be required for efficient activation of growth arrest and/or apoptosis, cells which lack functional wild-type p53 have been shown to be capable of undergoing radiation- and drug-induced apoptosis, albeit at higher doses (22, 28, 52, 58). This suggests that the apoptosis threshold of a particular cell is dependent upon its genetic makeup (13, 14).

Interestingly, the presence of human chromosome 17, in hybrid B78MC27, failed to result in either increased sensitivity to PALA or induction of G_1 arrest (Table 1). It is possible that, in B78MC27, human p53, under control of its natural promoter, mediated growth arrest inefficiently in the murine cells or was inactivated either by mutation or by interaction with mutant murine p53 protein. Alternatively, p53 may be incapable of promoting drug-induced growth arrest in highly tumorigenic cells or may only be effective in growth suppression in cooperation with other growth suppressor genes. These genes, acting as comediators of growth arrest during toxic conditions, may be required for efficient activation of growth suppression. It is likely that such growth regulators would be selected against during tumor progression. If some growth regulators are also survival genes, acting to suppress apoptosis, their inactivation may, as is the case with oncogene activation, result in both deregulated growth and increased apoptosis. The chromosome 3-mediated growth arrest revealed in our experiments may represent part of a complex growth arrest pathway which operates in normal cells to suppress apoptosis and control the cell cycle during toxic conditions. Genes in this pathway, if intact in some tumor cells, may provide a mechanism for tumor cell drug resistance by raising the apoptosis threshold in the absence of p53. Alternatively, these genes could control proliferation during suboptimal conditions, leading to dormancy and thereby preventing apoptosis. An understanding of the molecular basis for differences in the responses of normal cells versus tumor cells to cancer therapy is of utmost importance. Identification of genes affecting tumor cell sensitization and resistance to apoptosis will lead to new approaches to cancer therapy (13). The eventual isolation of a survival gene implicated in suppression of drug-induced apoptosis may thus help further the understanding of the mechanisms of cell death in anticancer therapy as well as contribute to the study of cell cycle control in normal and neoplastic cells.

ACKNOWLEDGMENTS

We thank G. R. Stark for providing the hamster CAD probe, L. Filion for helpful advice, and H. Gourdeau and M. Tenniswood for critical comments on the manuscript. We also thank J. Bell, D. Blakey, and A. MacKenzie for valuable discussions.

M.D.S. was supported by a Cancer Research Society studentship and the Ontario Graduate Studies studentship. This research was funded by the Cancer Research Society.

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