

Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins

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Genomic integrity is maintained by a network of cellular activities that assess the status of the genome at a given point in time, provide signals to proceed with or halt cell cycle progression, and provide for repair of damaged DNA. Mutations in any part of these pathways can have the ultimate effect of disturbing chromosomal integrity. Recent work suggests that p53 performs this integrator function in mammalian cells. Our present study demonstrates that in mortal cells, the expression of E6 and E7 viral oncoproteins of type 16 human papillomavirus each disrupts the integration of these signals by diverged pathways. Cells expressing E6 protein, which binds and degrades the p53 protein, exhibited alterations in cell cycle control when placed in drug and displayed the ability to amplify the CAD gene. The expression of E7, which binds different cellular proteins important for transformation, including Rb, led to a p53-independent alteration in cell cycle control, a widespread cytotoxic response, and polyploidy as a mechanism of drug resistance. These results demonstrate that diverse perturbations of molecular pathways can have different effects on chromosomal integrity.

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Loss of genomic integrity is a hallmark of neoplastic cells and fuels the multistep process of carcinogenesis. Changes in genomic integrity are seen when cells are transformed spontaneously, virally, or with chemicals. In studying the loss of genomic integrity, our previous work has focused on spontaneously transformed cells (both rodent and human) and their ability to exhibit one form of genetic instability, gene amplification. Gene amplification is an increase in gene copy number of a given locus and occurs spontaneously at a high frequency (10^{-6} – 10^{-3}) in preneoplastic and neoplastic cells (Sager et al. 1985; Otto et al. 1989; Tlsty et al. 1989; Jonczyk et al. 1993). In contrast, gene amplification is undetectable in normal cells ($<10^{-9}$) as measured by the ability to become resistant to the drug *N*-[phosphonoacetyl]-*L*-aspartate (PALA) (Tlsty 1990; Wright et al. 1990). Genetic analysis of this property indicates that the ability to amplify is a recessive genetic trait (Tlsty et al. 1992). These data suggest that normal cells have a gene, or set of genes, that suppresses amplification. In our search for possible candidate genes, the p53 gene emerged as an important modulator of gene amplification frequency as well as other genomic alterations.

One method of maintaining genomic integrity involves the action of cell cycle checkpoint genes, which integrate proper sensing of environmental signals and appropriate cellular responses. Data to support this idea come from several sources, most importantly, studies of genes in yeast that control cell cycle progression. Hartwell (Hartwell and Weinert 1989) has demonstrated that some of these genes, called checkpoint genes, coordinate cell cycle progression with cellular signals and allow for the maintenance of chromosomal integrity. In mammalian cells, the p53 protein is part of a pathway that allows cells to arrest their cell cycle progression when conditions for growth are unfavorable, such as the presence of antimetabolites (Livingstone et al. 1992; Yin et al. 1992). One consequence of this action is to prevent the emergence of drug-resistant colonies (Livingstone et al. 1992; Yin et al. 1992). Studies by Kastan and co-workers implicate p53 in a similar activity, the cellular response to γ -radiation (Kastan et al. 1991; Kuerbitz et al. 1992). In this capacity it is suggested that p53 receives information that DNA damage has occurred and mediates, through the possible action of GADD 45, growth arrest after DNA damage in normal cells. Hence, p53 exhibits several functions of a checkpoint gene in mammalian cells and appears to play a key role in maintaining genomic integrity. Further studies demonstrated that some

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human tumorigenic cells showed loss of genomic integrity while containing two wild-type p53 alleles, suggesting that alternate pathways or alternate regulation of p53 can bypass the role of p53. Therefore, mutations in p53 protein are not the sole determinant for the regulation of genomic integrity (Livingstone et al. 1992).

Ideally, to investigate events that promote the acquisition of genomic instability, a model system that starts with a normal cell and disrupts genomic integrity at a defined point was needed. The DNA tumor viruses provided such a system. Our previous studies (Jonczyk et al. 1993) demonstrate that virally transformed cells possess the ability to amplify. In this study we used the human papillomavirus (HPV) type 16 (HPV-16) to determine when, after expression of viral oncoproteins, the cells acquire the ability to amplify genes. The HPVs include numerous genotypes that induce benign genital warts or tumors of the epithelial or fibroepithelial components of skin or mucosa. Numerous clinical, epidemiological, and molecular evidence associates specific viral types with the formation of carcinomas of the genital and oral mucosa (zur Hausen 1991). Some HPV types such as 16, 18, 31, 33, 39 are referred to as "high risk" because of their association with cervical carcinoma. Other types such as 6 and 11 associated with benign lesions are termed "low risk."

Although frequently found in cervical carcinoma cells, the presence of a high risk HPV type is not sufficient to trigger malignant progression. The virus may be present with a long latency and only rarely does progression of genital lesions into carcinomas occur (zur Hausen 1991). Expression of two viral proteins, E6 and E7, is consistently found in the majority of cervical carcinomas and derived cell lines (Schwarz et al. 1985; Smotkin and Wettstein 1986; Baker et al. 1987; Wilczynski et al. 1988). Expression of these two viral proteins is sufficient to efficiently immortalize human keratinocytes and extend the normal life span of human fibroblasts (Hawley-Nelson et al. 1989; Munger et al. 1989a; Watanabe et al. 1989; Dhanwada et al. 1992). The HPV-16 E6 gene product binds and aids in the degradation of p53 (Scheffner et al. 1990; Werness et al. 1990) while the E7 gene product binds a set of proteins that includes hypophosphorylated pRb, p107, p130, and cyclin A (Dyson et al. 1989, 1992; Davies et al. 1993; Tommasino et al. 1993). The mechanistic consequences of these protein couplings on the neoplastic process is under intense investigation.

In this study we examine amplification potential, chromosomal integrity, and cell cycle perturbations in normal human fibroblasts that have been infected with genes that code for high risk HPV-16 and low risk HPV-6 viral oncoproteins. We found that normal human fibroblasts that expressed type 16 E6/E7 together or E6 alone acquired the ability to generate PALA-resistant colonies by CAD gene amplification immediately after viral infection. In contrast, the majority of cells expressing the E7 protein alone underwent a rapid cytotoxic response when placed in drug, with rare PALA-resistant variants acquiring multiple copies of chromosome 2 (aneuploidy) as a mechanism of drug resistance. In each case, these

virally altered cells have clearly acquired genetic instability prior to immortalization, as all of these cells eventually underwent cellular senescence. Normal human fibroblasts carrying type 6 low risk HPV proteins do not disrupt the genomic integrity of NHF. Our data suggest a physiological consequence of these interactions and their importance in viral carcinogenesis; both results relate to the maintenance of genomic integrity.

Results

Generation of HPV-16 oncoprotein-infected NHF

Introduction of the viral oncoproteins from HPV-16 into normal human fibroblasts (Materials and methods) was accomplished by use of a recombinant retroviral system. Retroviral constructs contained the gene coding for neomycin resistance alone, or in combination with the open reading frames (ORFs) for HPV-16 E6/E7 together, or each of the viral oncoproteins individually (Fig. 1). Normal human fibroblasts at population doubling (PDL) 33 (neo, E6) and PDL 36 (E6/E7, E7) were infected at high titer with the described retroviral vectors and selected in the neomycin analog G418 sulfate. Similar numbers of G418 sulfate-resistant colonies arose from each retroviral infection. Resistant cells were pooled, resulting in the cell populations NHF neo, NHF16 E6/E7, NHF16 E6, and NHF16 E7. Radioimmunoprecipitation of HPV-16 E6 and E7 proteins in the cell populations demonstrated their expression, with the E6/E7-infected population expressing both viral oncoproteins and the E6 and E7-infected populations expressing the appropriate individual proteins (data not shown; Materials and methods; Halbert et al. 1991). Analysis of p53 and pRb protein by Western blot revealed decreased p53 protein levels in E6-expressing cells and decreased pRb levels in E7-expressing cells (data not shown). Oncogene-expressing cells were examined for the effect of the viral proteins on genomic integrity and the cellular potential to amplify.

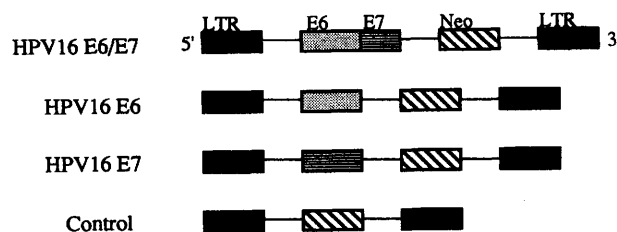


Figure 1. Recombinant retroviral vectors containing HPV-16 sequences. Expression of the HPV oncoproteins in NHF was accomplished by infection of the cells with retroviral vectors. The viral particles contained Moloney murine leukemia virus promoter–enhancer sequences (LTR, solid box), HPV-16 E6/E7 sequences together or separately (E6, stippled box; E7, horizontally striped box), and under control of the SV40 promoter, the neomycin resistance gene (Neo, hatched box). Integration of the viral sequences into the NHF genome allowed stable expression of HPV protein(s) and conferred resistance to the neomycin analog G-418 sulfate in the infected cell and progeny cells. (Diagram modified from Halbert et al. 1991).

Characterization of chromosome complements in HPV-infected NHF

Chromosome complements were examined in the uninfected and HPV-infected NHF cells (Table 1). The karyotypic stability of the five cell populations was examined during their extended propagation in culture, using samples taken 20 PDLs after the viral oncogene-expressing populations were selected as well as prior to their senescence in culture. Normal diploid karyotypes were observed in the parental NHF cells at both early passage and at late passage close to senescence. At early passage after infection with the indicated oncoprotein-coding genes, the NHF neo, NHF16 E6, and NHF16 E7 cells produced karyotypes that were indistinguishable from the parental NHF cells (Table 1). All three had a basic normal karyotype with no rearrangements or telomeric association. The karyotypes of the NHF16 E6/E7 cells at early passage were found to have telomeric association in 8% of the cells and rearrangements in 6% of the cells. The telomeric association figures seen were simple, each consisting of two recognizable chromosomes attached at their telomeres with no noticeable loss or gain of chromatin at the junctions. When the karyotypes of the E6/E7- and E6-expressing cells were examined late in passage, they were found to have numerous genomic rearrangements. Few of the cells were diploid, and most contained multiple rearrangements, telomeric associations, and gross aneuploidy (T. Tlsty, in prep.). In contrast to the E6-expressing cells, the karyotypes of the E7-expressing cells at late passage were completely diploid.

Characterization of growth control in HPV-infected NHF

Cell morphology, PDL time, mortality, and behavior at confluence were examined in the uninfected and HPV-

16-infected NHF cells (Table 2). Morphologically, the HPV protein-expressing cells were indistinguishable from each other. Although similar to the uninfected NHF, all three of the HPV-infected cell populations were smaller and less extended than the NHF parental cells (Fig. 2). The PDL time of each cell population was determined. The parental NHF and NHF neo-containing cells had similar PDL times when grown in standard media. The NHF cells that expressed both viral oncoproteins demonstrated a slightly longer doubling time (Table 2). Past studies have indicated that individually, the E6 or E7 proteins of HPV-16 do not significantly influence the life span nor immortalize NHF, but together, the proteins can efficiently extend the life span of NHF compared with that of normal controls (Watanabe et al. 1989). In agreement with these studies, we found the E6- and E7-expressing cells senesced between 78 and 88 PDL, whereas in the E6/E7-expressing cells an extension of life span of ~20 PDL compared with NHF was noted (Table 2). Immortalization of HPV oncoprotein-expressing fibroblasts is rare or negligible (Shay et al. 1993) and was not observed in this set of experiments.

The oncoprotein-expressing cells demonstrated rather striking responses to growth conditions at confluence. As normal diploid fibroblasts such as NHF become confluent, they become tightly packed in a monolayer and cease proliferation. This contact inhibition results in a cessation of progression through the cell cycle with the cells arrested in a G_0 state indefinitely. The cells containing E6/E7 together or E6 alone demonstrated a response that was similar to each other but slightly different than that exhibited by parental NHF. The cells formed a monolayer, and for the most part, arrested in growth, but infrequent mitotic figures could be observed even several days after the cells achieved confluence (Fig. 2D,F). The cells could be held in this state for long periods of time (months). Interestingly, the E7-expressing cells demonstrated an entirely different response after

Table 1. Karyotypic characterization of NHF and HPV protein-expressing fibroblasts

Cell population	Diploid ^a (46 ± 2)	Tetraploid (92 ± 2)	Aneuploid	Telomeric association ^b	Translocation rearrangement
Early passage populations					
NHF	100	0	0	0	0
NHF neo (pd 40)	94	6	0	0	0
NHF16 E6/E7 (pd 46)	96	0	4	8	6
NHF16 E6 (pd 56)	100	0	0	0	0
NHF16 E7 (pd 50)	96	4	0	0	0
PALA-resistant subclones					
NHF16 E6/E7 C1	86	0	14	34	80
NHF16 E6/ E7 C3	74	2	24	48	72
NHF16 E6 C1	48	6	46	66	86
NHF16 E6 C4	78	2	20	54	86

A minimum of 50 metaphase spreads was examined from each cell population. The numbers indicate the percentage of cells in each category.

^aInfrequently, in normal cells there is a random loss of a single chromosome or the observation of a tetraploid cell. These deviations are found in <10% of normal cells. Our observations are in keeping with those reported for normal human fibroblasts and lymphocytes in the literature (Aurias 1993).

^bTelomeric association is defined as end-to-end fusion of chromosomes.

Table 2. Growth properties and frequency of PALA resistance

Cell name	PDL ^a time (hr)	Total PDL	PE ^b (%)	PALA ^c LD ₅₀ (μM)	PALA (9 × LD ₅₀)	PALA resistance frequency (9 × LD ₅₀)
NHF	20 ± 0.52	72	33 ± 0.05	13 ± 1	117	(<1 × 10 ⁻⁷)
NHF neo	22 ± 2.9	70	30 ± 0.18	12 ± 1	108	(<1 × 10 ⁻⁷)
NHF16 E6E7	26 ± 3.2	98	21 ± 0.07	19 ± 9	171	3.8 × 10 ⁻⁵ ± 2.7 × 10 ⁻⁵
NHF16 E6	19 ± 1.4	88	32 ± 0.17	24 ± 4	216	1.8 × 10 ⁻⁵ ± 2.3 × 10 ⁻⁵
NHF16 E7	22 ± 1.5	78	31 ± 0.01	11 ± 3	99	4.3 × 10 ⁻⁶ ± 2.6 × 10 ⁻⁶

^a(PDL) Population doubling.

^b(PE) Plating efficiency.

^c(LD₅₀) Concentration of PALA that permits 50% survival.

reaching confluence; they died (Fig. 2H). The cells formed a tight monolayer as they approached confluence, and mitotics could be detected, similar to those seen with the E6-expressing cells. However, after ~3 days at confluence these cells first became thin, elongated, and eventually rounded and refractile as they lifted from the plate and died, as measured by trypan blue uptake. Within 48 hr the plate was completely clear of adherent cells.

Cellular response to metabolic inhibitor

To analyze amplification potential in these cells, we incubated them in the drug PALA under the standard conditions of the clonogenic assay (see Materials and methods). The normal human fibroblasts and those expressing the viral oncoproteins demonstrated dramatic differences in both the cellular response to the drug and the generation of PALA-resistant colonies. Uninfected NHF cells and neo-containing NHF exhibited a response to PALA that we have found is typical of normal human fibroblasts. The cells remained on the plate and failed to increase their cell number. There was no observable outgrowth of PALA-resistant colonies. Hence, uninfected NHF cells, as described previously (Tlsty 1990; Wright et al. 1990; Livingstone et al. 1992), and those containing the neo control lacked a detectable frequency of CAD gene amplification. When E6/E7- and E6-expressing cells were incubated in PALA, the vast majority gradually enlarged and sloughed from the plate over a period of 1–2 weeks, resulting in clearing of cells from the background. Infrequent, nondividing cells remained in the background while actively growing PALA-resistant colonies emerged during the selection process. Those cell populations containing the E6/E7 proteins together or the E6 protein alone generated PALA-resistant colonies at a frequency of 3.8×10^{-5} and 1.8×10^{-5} , respectively (Table 2; Fig. 3).

In contrast, when E7-expressing cells were incubated in the presence of PALA, there was a distinctly different response to the drug, which resulted in the clearing of cells from the plate within ~6 days. The E7-expressing cells became thin, elongated, and refractile, and after 3 days in PALA they began demonstrating a condensed, rounded morphology as they loosened from the substrate

of the tissue culture plate. This morphological change is identical to that seen when these cells are held at confluence and is reminiscent of an apoptotic sequence of events that is initiated in certain cells under adverse conditions. The characteristic nuclear degradation ladder that occurs during apoptosis was not detected by gel electrophoresis (data not shown). At the termination of the PALA selection with the E7-expressing cells (~6 weeks later), there was no background of nondividing cells. The plate was cleared of cells except for the rare PALA-resistant colonies. Cells that expressed E7 alone generated PALA-resistant colonies at a frequency that was 10-fold lower than that observed for the E6/E7-expressing cells (Fig. 3). The frequencies of the generation of PALA-resistant colonies obtained with the viral oncoprotein-expressing cells are within the range of those observed previously in preneoplastic and neoplastic cell lines (10^{-6} to 10^{-3}) (Sager et al. 1985; Otto et al. 1989; Tlsty et al. 1989; Jonczyk et al. 1993).

Whereas the drug resistance curves for the three HPV-16-expressing cell populations revealed similar frequencies of generation of PALA-resistant colonies (Fig. 3), there were differences in the amount of time required for the formation of the visible colonies. The average time for isolation of NHF-16 E6/E7 PALA-resistant subclones was 3 weeks, compared with 4 and 6 weeks for the E6 and E7 PALA-resistant clones, respectively. The proliferative capacity of isolated subclones also differed, predictably as a direct reflection of the effect the HPV proteins on NHF life span in culture. The NHF16 E6/E7 PALA-resistant subclones grew very well. Ten E6/E7 PALA-resistant clones were chosen for further study and were found to senesce at ~86 PDL. PALA-resistant cells expressing only the E6 protein divided for fewer PDL than the subclones expressing both the viral oncoproteins. When expanded in culture they senesced at ~PDL 76. The NHF-16 E7 drug-resistant subclones had a diminished proliferative capacity when compared with the E6-expressing subclones. The population ceased growth at ~PDL 66. In the first set of experiments they ceased cell division before they could be expanded and examined for CAD gene copy number. We reinfected NHF cells at an earlier passage in subsequent experiments (passage 9) and were able to isolate PALA-resistant subclones for analysis of CAD gene copy number. These subclones

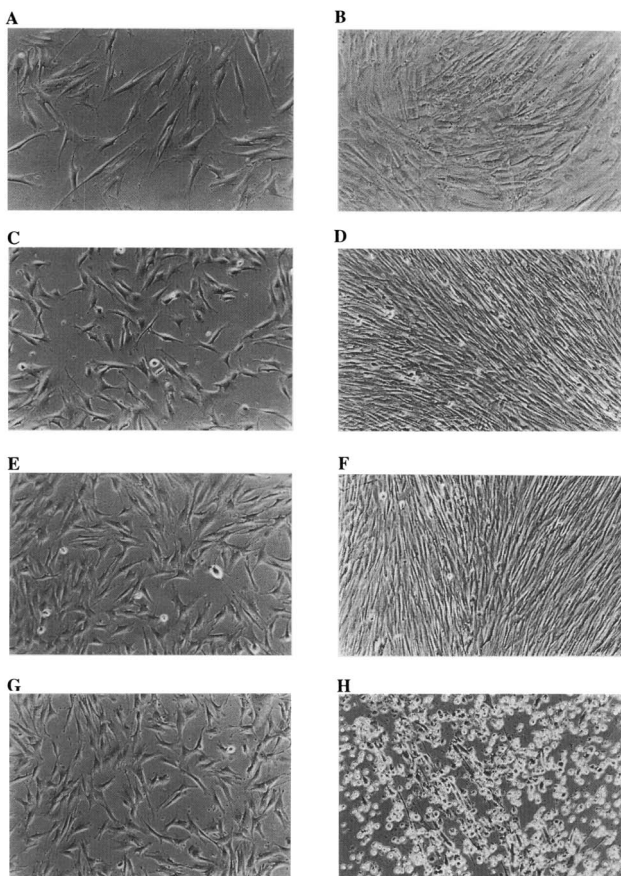


Figure 2. Morphology of normal and HPV-16 oncoprotein-expressing fibroblasts in the absence of PALA. Phase-contrast photographs of cells at subconfluence and confluence, grown in the absence of PALA, are shown. Cells were plated at 2×10^5 cells per 60-cm petri dish and grown until they attained confluence. Cells were photographed at subconfluence after 3 days in culture and after 6 days at confluence. At subconfluence, the HPV protein-expressing cells were indistinguishable from each other [NHF16 E6/E7 (C); NHF16 E6 (E); NHF16 E7 (G)] but distinct from the control cell populations [NHF (A); NHFneo (not shown)]. NHF cells expressing the HPV proteins were smaller in size and had less extended processes compared with the parental cells. At confluence, control cell populations [NHF (B); NHFneo (not shown)] were a tight monolayer of nondividing, viable cells. In contrast, NHF cells expressing both the E6 and E7 proteins or only the E6 protein continued to proliferate after reaching confluence as indicated by the presence of mitotic cells [NHF16E6/E7 (D); NHF16E6 (F)]. Massive cell death occurred in the E7-expressing NHF after 2–3 days at confluence. Death was verified by cellular incorporation of the dye trypan blue. Magnification, 63X.

ceased growth soon after isolation as well. In summary, all PALA-resistant clones were mortal.

As a control, NHF cells individually expressing low risk HPV-6 E6 and E7 proteins were assayed for PALA-resistant colony formation. Studies of human cell transformation have shown that HPV-6 does not efficiently immortalize primary, human epithelial cells [Schlegel et

al. 1988; Pecoraro et al. 1989; Halbert et al. 1992]. There is no evidence for cellular degradation of p53 by HPV-6 E6 [Scheffner et al. 1990; Werness et al. 1990; Crook et al. 1991], and the affinity of HPV-6 E7 protein for cellular retinoblastoma protein (pRb) is ~ 10 -fold lower than the affinity of HPV-16 E7 for pRb [Munger et al. 1989b; Barbosa et al. 1990; Gage et al. 1990; data not shown]. In the presence of PALA, the NHF expressing the HPV-6 E6 and E7 proteins exhibited a phenotype typical of uninfected normal human fibroblasts. The cells enlarged, remained on the plate, and failed to proliferate. There was no cytotoxic response nor PALA-resistant colony formation observed. These results suggested that the diminished ability of the type 6 viral proteins to efficiently bind specific cellular proteins (such as p53, pRb, etc.) when compared with the type 16 oncoproteins correlated with the lack of ability to generate drug-resistant variants at a detectable frequency.

Mechanism of drug resistance

Several PALA-resistant subclones from each of the viral oncogene-expressing cell populations were analyzed for amplification of the CAD gene. Fluorescent in situ hybridization (FISH) analysis of the subclones verified intrachromosomal CAD gene amplification as the mechanism of resistance to PALA in the E6/E7- and E6-expressing cells. In all cases, the amplified copies were clustered with no distinct patterns observable. Representative hybridizations are seen in Figure 4, A and B. Further karyotypic analysis of two PALA-resistant subclones from each of the cell populations indicated a variety of chromosome rearrangements and telomeric

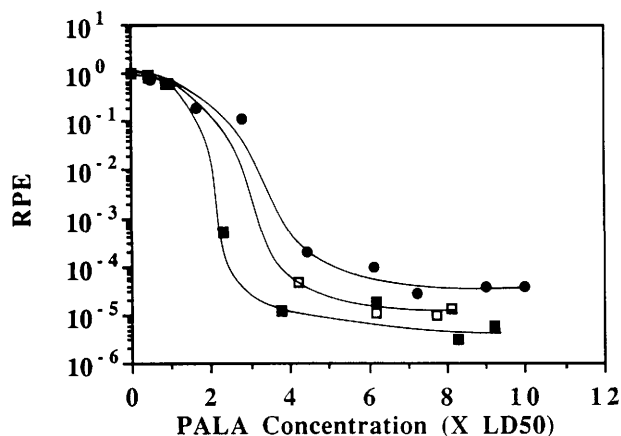


Figure 3. Determination of PALA resistance. Cells were plated at appropriate densities and placed in PALA concentrations indicated. When drug-resistant colony size exceeded 50 cells, colonies were fixed, stained, and counted. Relative plating efficiency (RPE) is the ratio of the percentage of cells surviving in PALA to the percentage of cells surviving in the absence of PALA. PALA resistance frequency was determined by the RPE value at $9 \times LD_{50}$. The curves are an average of at least four determinations. (●) NHF-16 E6/E7; (□) NHF-16 E6; (■) NHF-16 E7.

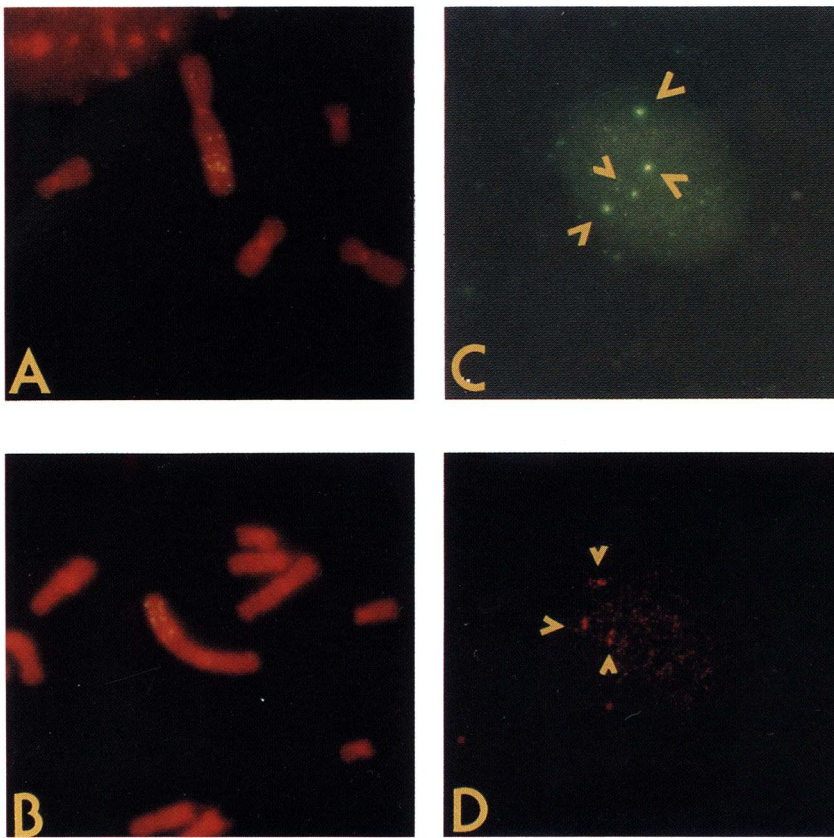


Figure 4. Fluorescence in situ hybridization of HPV-16 protein-expressing PALA-resistant clones. Metaphase spreads from PALA-resistant subclones NHF16 E6/E7, clone 120-1 (A), and NHF16 E6, clone 180-4 (B), were hybridized to a human CAD gene probe to locate CAD gene sequences as described previously (Tlsty et al. 1992). The normal loci for the CAD gene is on chromosome 2. CAD gene amplification in the subclones was manifested as multiple copies of the gene in tandem arrays on a single chromosome arm. The PALA-resistant NHF16 E7 cells ceased proliferation when subcloned. Metaphase spreads were not obtained. Instead, interphase cells were analyzed for CAD gene and chromosome 2 centromere sequence copy number using different colored fluorochromes to determine whether the mode of drug resistance was gene amplification or multiple copies of chromosome 2. Equivalent CAD gene copies and centromere 2 sequences were observed in most clones, although in some clones centromere 2 sequences were more abundant than the CAD gene. Positive signals in the interphase cells are indicated by open arrowheads. (C) NHF16 E7, clone 108-2D centromere 2; (D) CAD gene

associations (Table 1), similar to that seen in the unselected populations after extended passage in culture. Analysis of PALA-resistant subclones from the E7-expressing population was difficult because of their limited proliferative capacity and the difficulty in obtaining adequate metaphase spreads. Although complete karyotypic analysis was not possible, several samples were noted to have excessive numbers of chromosomes (<100). As an alternative to metaphase analysis, two-color FISH analysis was performed on interphase preparations using probes specific for human chromosome 2 centromeric sequences and for the human CAD gene. Most often, multiple copies of centromeric chromosome 2 and of the CAD gene (four to seven copies) were detected indicating chromosome 2 aneuploidy as the primary mechanism of resistance to PALA (Fig. 4C,D). Hybridization with sequences specific for chromosome 6 also demonstrated multiple signals suggesting possible polyploidy (data not shown).

Perturbed cell cycle progression

Our previous studies had shown that normal human fibroblasts arrested in the G_1 and G_2 phases of the cell cycle when incubated with PALA (Livingstone et al. 1992). Figure 5, A–G, shows the cell cycle distribution of normal cells at various times after placement in drug (0, 1, 2, 3, 4, 5, 6 days). Hours after exposure to the meta-

bolic inhibitor PALA, an exponentially growing population of normal, diploid cells accumulated the majority of the cells in the S phase of the cell cycle. This increased S fraction gradually decreased until, at day 2, there is a complete absence of cells in the S phase indicating an arrest in cell cycle progression. The population was distributed between the G_1 and G_2 phases of the cell cycle for the duration of the selection. This temporal distribution of cells within each phase of the cell cycle is presented as a scatter plot (data directly from the flow cytometer) in Figure 5, A–G, and as a compilation over time in Figure 5H. This graphic representation of the relative fractions of the population in G_1 , S, or G_2 /M phase is helpful for comparing the response of normal cells in drug with that of the oncoprotein-expressing cells in drug. The status of multiple checkpoint functions can be monitored at one time. The white area on the graph in Figure 5H represents the percent of cells in the S-phase fraction, whereas the lower gray area represents those in G_1 , and the upper dark area represents those in G_2 .

Deviations from this pattern were seen in the NHF cells expressing HPV-16 viral oncoproteins. In this assay, the various cell populations are pulsed with BrdU prior to harvesting. Only the cells that are actively incorporating nucleotides for synthesis of DNA during that period of time will appear in the S-phase fraction. Figure 6A demonstrates that after 2 days of incubation in PALA,

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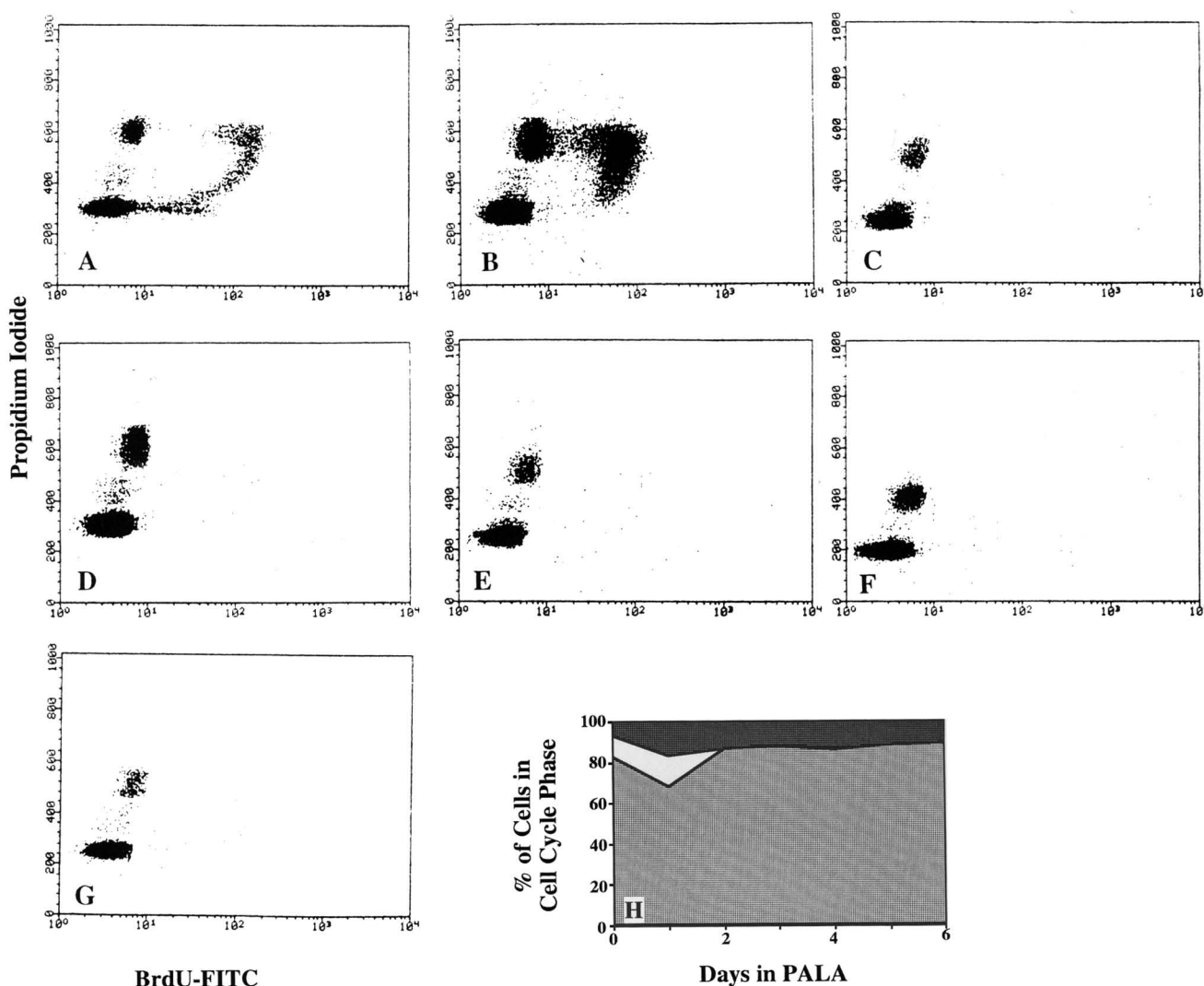


Figure 5. Flow cytometric analysis of NHF cell population in PALA. Cells were labeled with BrdU, fixed, and stained with anti-BrdU-FITC and propidium iodide, and analyzed using a Becton Dickinson FACScan instrument. The x-axis represents cells undergoing DNA synthesis. Cells at the *right* have more BrdU incorporated and are therefore in the S phase of the cell cycle. The y-axis represents DNA content as measured by propidium iodide staining. The upper population has a G₂ phase DNA content, and the lower population has a G₁ phase DNA content. (A) NHF with no PALA exposure; (B–G) NHF days 1–6 in PALA. The cell cycle data were compiled and presented in the form of an area graph, providing a characteristic cell cycle pattern that could be conveniently compared among the cells (H). Each graph indicates the percentage of cells in each phase of the cell cycle (ordinate) as a function of time (axis; 0, 1, 2, 3, 4, 5, and 6 days in PALA). The G₂, S, and G₁ phases of the cell cycle are represented by the *top*, *middle*, and *bottom* areas of the graph, respectively. There was no significant portion of the parental NHF cells in S phase after 2 days in PALA.

none of the control cells are entering or actively participating in the S phase of the cell cycle. This is represented by a lack of white area in the graph beyond 2 days. In contrast, at a similar point in time, those cells expressing HPV-16 E6 and E7 demonstrate that a large fraction of the cells are incorporating BrdU (Fig. 6B). These cells are distributed throughout the S-phase arc (seen in Fig. 5A) indicating that cells are actively synthesizing DNA days after the normal cells have ceased. Expression of the HPV-16 E6 or E7 individually (Figure 6C,D) also relieves checkpoint arrest but in reproducibly distinct patterns (compare S-phase fractions at day 2). Finally,

analysis of cells expressing low risk HPV-6 proteins showed a complete arrest when the cells were incubated in PALA (Fig. 6E,F). These cells continue to respond to negative growth signals.

Discussion

In this study we demonstrated that distinct alterations in growth control circuitry can lead to different types of genomic instability. We began with cell populations that were isogenic and, by expression of HPV-16 E6 and E7 oncoproteins, contained disruptions in specific circuits

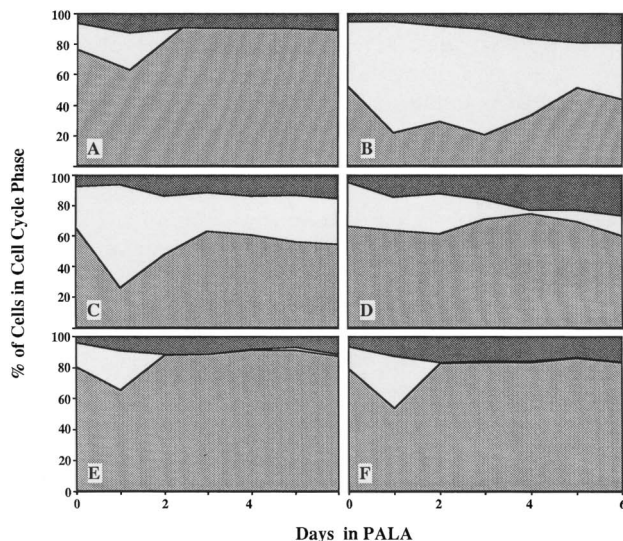


Figure 6. Flow cytometric analysis of NHF retrovirus-infected cell populations in PALA. Cells were treated and data are displayed as in Fig. 5. There was no significant portion of cells in S phase after 2 days in PALA for cells expressing only the neomycin resistance gene and cells expressing the nononcogenic HPV-6 E6 or HPV-6 E7 proteins. All of the HPV-16 protein-expressing cells continue to cycle in the presence of PALA, as indicated by a percentage of cells in the S phase throughout PALA exposure. (A) NHF neo; (B) NHF16 E6/E7; (C) NHF16 E6; (D) NHF16 E7; (E) NHF6 E6; (F) NHF6 E7.

that allowed cells to respond to altered growth conditions. It is important to note that the differential response of the cells depended on their need to respond to outside stimuli. The two stimuli described here are the negative growth signals that cells produced as they attained confluence or when the media was deficient in nutrients (in this case, nucleotide precursors). In the absence of either of these signals, each of the cell populations grew in an exponential fashion with no overt difficulties.

When exposed to the metabolic inhibitor PALA, normal human fibroblasts arrest in the G_1 and G_2 phases of the cell cycle. Each of the virally altered cell populations failed to arrest under these conditions and continued to cycle. While the majority of these cells died, a few generated drug-resistant colonies. In the cells where p53 function was compromised, cells with E6/E7 or E6 protein only, the majority of cycling cells died gradually, leaving the PALA-resistant colonies to expand. The altered karyotypes of these drug-resistant clones manifested CAD gene amplification in addition to other genomic changes. Rare PALA-resistant colonies arising from a background of gradually dying cells is the typical observation in most of the immortalized and tumorigenic cell lines that we have assayed to date. A plausible scenario begins to emerge as to how p53 participates in the generation of the rearrangements that fuel neoplasia. Cells suffer either endogenous or exogenous damage to their DNA, which brings about breakage of their genetic

material. Broken chromosomes are usually detected in a normal cell, and cell cycle progression is arrested while the damage is repaired or the cell is removed. In cells that have mutated parts of the circuitry that sense, repair, or respond to damage in DNA, cell cycle progression fails to arrest. Broken chromosomes can replicate and fuse leading to the formation of dicentric chromosomes (Trask and Hamlin 1989; Toledo et al. 1992). The formation of a dicentric chromosome and the subsequent fusion-bridge-breakage cycle originally described by McClintock >40 years ago (McClintock 1939, 1942), generates duplications, deletions, and other abnormalities. These dicentric intermediates are seen quite often in cells with amplified genes (Smith et al. 1990; Windle et al. 1991; Toledo et al. 1992; Ma et al. 1993). These types of rearrangements activate oncogenes and inactivate tumor suppressor genes.

Whereas the elimination of checkpoint function of p53 could have accounted for the tolerance of broken chromosomes and the emergence of cells with amplified DNA sequences in the E6-expressing cells, the E7-expressing cells also generated PALA-resistant colonies. Here, the cellular and genomic response was totally different. The cells expressing only the E7 protein exhibited massive, immediate cell death after ~3 days in PALA, with very rare resistant colonies arising on a cleared background (2×10^{-6} to 6×10^{-6}). These drug-resistant cells contained increased copies of CAD gene sequences, along with increased copies of chromosome 2 centromeric sequences, indicating that aneuploid cells had been generated. How expression of the E7 oncoproteins influences the formation of aneuploid cells is not known at the present time. A previous report indicated that HPV-16 E7 can induce cytogenetic abnormalities in immortalized keratinocytes (Hashida and Yasumoto 1991). As demonstrated in Figure 6D, these cells failed to arrest in cell cycle progression when placed in PALA even though they contained a wild-type p53 gene. This p53-independent abrogation of cellular arrest, in some manner yet to be determined, leads to chromosomal consequences that are clearly distinct from those generated in cells that have eliminated p53. One may speculate that E7, which is known to bind cyclin A as well as cellular kinases (Dyson et al. 1992; Davies et al. 1993; Tommasino et al. 1993), disrupts cyclin homeostasis and leads to elimination of G_2 or relaxation of G_2/M checkpoints. Because aneuploidy is one of the most common genomic aberrations seen in tumor cells, this mechanism deserves further investigation. Studies with polyoma T antigen indicate that the expression of this gene in NHF cells also produces drug-resistant colonies, but the spectrum of genomic changes differs from those seen with HPV-16 E6/E7 (Schaefer et al. 1993). These results demonstrate that diverse perturbations of molecular pathways can have different effects on chromosomal architecture, and although there are functional similarities in some DNA tumor virus proteins (Vousden and Jat 1989; Werness et al. 1990), there are also important differences among them.

These experiments demonstrated that expression of

viral oncoproteins lead to the immediate acquisition of the ability to amplify the CAD gene sequences. These cells relinquished their control of genomic integrity prior to immortalization, clearly indicating that this change is not necessary. Because previous studies with Syrian hamster epithelial cells identified immortal cells that lack the ability to amplify (sup⁺ cells) (Jonczyk et al. 1993), and this study documented mortal cells that have the ability to amplify, it is clear that immortalization of mammalian cells is neither necessary nor sufficient for amplification to occur. Our observation of amplification in these mortal cells also addressed our initial question as to when the virally infected cells acquired the ability to amplify or undergo genomic rearrangements. This change must occur immediately with expression of the E6/E7 genes.

Strikingly, the biology of cervical carcinomas points to the expression of E6 and E7 as important in tumor progression. The high risk viruses are episomal in benign lesions, and in this state, the product of the HPV E2 ORF generates a protein that suppresses transcription of the E6 and E7 genes. Little protein is made. In carcinomas, the high risk viruses are usually found integrated into the host genome (Durst et al. 1985; Pater and Pater 1985). This integration event is accompanied by a change in transcriptional activity and pattern. Reproducibly, this integration results in the disruption of the coding region between the E1 and E2 genes and consequently allows for the more efficient expression of E6 and E7 transcription and subsequent protein production (Romanczuk and Howley 1992). Whereas in the episomal state, the entire spectrum of viral proteins that are used for replication, capsid formation, and so on, are expressed, in the integrated state, the E6 and E7 viral oncogenic products become the dominant transcripts. Our data demonstrate that expression of these oncoprotein products and their concomitant functional inactivation of cellular proteins results in genomic instability. The generation of chromosomal rearrangements ostensibly allows progression of the malignant state.

In addition to mediating growth arrest, p53 is implicated in another process that may serve to protect the organism from a defective genome, the triggering of apoptosis, an active death process (Debbas and White 1993; Yonish-Rouach et al. 1993). Our observations with HPV-16 E6/E7 expression in human cells parallel recent work with adenovirus E1A/E1B in a striking manner, suggesting further functional analogies between the various viral oncoproteins. The E6 and E1B oncoproteins are functional homologs while the E7 and E1A oncoproteins share DNA sequence and functional homology. Cells expressing E1A alone show a rapid loss of viability immediately upon achieving saturation density. Those cells expressing both E1A and E1B do not demonstrate this rapid cytotoxic response (Rao et al. 1992). Transformation of rodent cells by the adenovirus oncoproteins E1A and E1B requires two events, E1A-dependent induction of proliferation and E1B-dependent suppression of apoptosis. The sole expression of E1A results in efficient initiation of focus formation, but the cells cannot sustain

proliferation. They degenerate and die. Cell death is mediated through an up-regulation of p53 that results in the production of a cytotoxic and DNA degradation phenotype. If p53 is functionally removed from these cells, as when E1B is coexpressed, the apoptotic pathway can no longer be induced. The E1A/E1B-expressing cells can now produce transformed foci with a high frequency that have a good probability of immortalizing. In our system the sole expression of the E7 gene in challenged cells resulted in a cytotoxic response and the formation of drug-resistant colonies that cannot sustain proliferation. If p53 is functionally removed from these cells, as when E6 is coexpressed, the cytotoxic response is no longer induced and the drug-resistant colonies grow in a healthy manner. In each case, a negative growth signal (confluence in the rodent cells and nutrient deprivation or confluence in the human fibroblasts) elicited a physiological response that is similar in each cell type and dependent on the genotype of the cell.

Finally, the studies presented here may provide the biological basis for the differences between high risk and low risk HPV types in terms of neoplastic transformation. Both the high risk and low risk viruses cause hyperproliferation in cells, yet only the high risk viruses produce cellular variants that can escape differentiation, immortalize, and progress to malignancy (zur Hausen 1991). The high risk viruses are known to be much more efficient in binding and degrading p53 than their low risk counterparts (Werness et al. 1990; Scheffner et al. 1990; Crook et al. 1991). Likewise, the high risk E7 protein binds pRB with a 10-fold greater affinity than the low risk type (Munger et al. 1989a). We propose that one of the basic differences between these two types of viruses is their maintenance of genomic integrity. Two pieces of data demonstrated that cells infected with low risk virus maintained their genomic integrity while those expressing the high risk virus did not. First, no PALA-resistant colonies were generated when cells infected with HPV-6 E6 or E7 were challenged with drug. The frequency was at least two orders of magnitude lower than measured with HPV-16. Second, the cells expressing the low risk HPV-6 E6 and E7 genes can arrest in cell cycle progression when exposed to PALA or confluence, demonstrating that these cells sense negative growth signals and respond in an appropriate manner; they arrest. In contrast, the biology of the cells containing the high risk virus pointed to the involvement of E6 and E7 in the loss of genomic integrity and in tumor progression.

Materials and methods

Cell populations and culture conditions

All cells were grown in α minimal essential medium minus nucleosides and deoxyribonucleosides, but containing glutamine and 10% dialyzed fetal bovine serum (JRH Biosciences). Primary, human foreskin fibroblasts were obtained from Dr. M. Cordiero-Stone (University of North Carolina at Chapel Hill). At PDL 33 or 36, cells in log phase growth were infected with retroviral vectors containing only the neomycin resistance gene (vector LXSNI), vectors containing the neomycin gene and HPV-

16 ORFs for the E6 and/or E7 proteins (LXSN16E6E7, LXSN16E6, or LXSN16E7), or vectors containing the neomycin resistance sequences and HPV-6 E6 or E7 ORFs (LXSN6E6 and LXSN6E7). The vectors were a gift from Dr. Denise Galloway (Fred Hutchinson Cancer Center, Seattle, WA) and have been described previously (Halbert et al. 1991).

Retroviral infections

Retroviruses containing the various vectors were produced by the murine cell line PA317. The procedure for retroviral infection entailed the following. NHF cells were incubated for 8–10 hr in filtered, polybrene-supplemented medium from the PA317 cells containing the appropriate retrovirus. The NHF cells were trypsinized and seeded at varying densities in medium containing 500 mg/ml of G418 sulfate (Geneticin, GIBCO-BRL Laboratories). G418-resistant colonies were visible in ~1 week and were pooled for each cell population. Cell populations were designated NHF neo, NHF16 E6/E7, NHF16 E6, NHF16 E7, NHF 6E6, and NHF 6E7.

PDL time determination

PDL time for each cell population was determined by plating $\sim 5 \times 10^4$ cells in each of ten 100-mm petri dishes. Cell counts from two dishes were averaged daily, beginning 24 hr postplating, and plotted as a function of time. Total PDLs were determined by plating a known cell number and counting cells at each passage until senescence.

Karyotypic analysis

The procedure used to obtain G-banded chromosomes was described previously (Livingstone et al. 1992). Each cell population was analyzed in the absence of PALA. PALA-resistant NHF16 E6E7 and NHF16 E6 subclones were also analyzed.

Clonogenic assay

The assay is used to determine gene amplification frequency and has been described previously (Otto et al. 1989). Briefly, cells were grown in the presence of the drug PALA, which specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme involved in de novo pyrimidine synthesis. PALA selects for cells with CAD gene amplification. Cells were plated in 100-mm dishes at densities of 2×10^2 to 2×10^4 cells and in 150-mm dishes at densities of 2×10^5 cells. PALA-resistant colonies of 50 cells or greater were detectable after 2–6 weeks and were fixed, stained, and counted. Plating efficiency, the ratio of the number of colonies obtained without PALA selection to the number of cells seeded (100% survival), and LD_{50} , the concentration of PALA that allows 50% survival, were determined for each cell population so that direct comparisons among the cells could be made. Amplification frequency was expressed as the number of PALA-resistant colonies at $9 \times LD_{50}$ relative to the plating efficiency of the cell population. PALA was obtained from the Drug Evaluation Branch of the National Cancer Institute.

FISH

PALA-resistant subclones ($9 \times LD_{50}$ and above) were analyzed for CAD gene amplification by FISH as described previously (Tlsty et al. 1992). The human CAD probe was a kind gift from Dr. J. Davidson (University of Kentucky, Lexington). Probe for

centromeric sequences of chromosome 2 was purchased from Oncor.

Cell cycle analysis

Cells were plated in regular growth medium or medium containing PALA at $9 \times LD_{50}$ for the respective cell strain. A modification of the cell cycle analysis used by Kastan and co-workers was used (Kastan et al. 1991). On days 0–6, BrdU was added to a log phase population of cells at a concentration of 10 mM, and after 4–5 hr the cells were trypsinized and counted, and then fixed using 1.5 ml of cold phosphate-buffered saline and 3 ml of cold 95% EtOH per 10^6 cells. The fixed cells were processed as follows for propidium iodide staining. Two million fixed cells were centrifuged for 5 min at 1200 rpm using a Sorvall HT1000B rotor at 4°C, and the pellet was resuspended with vortexing in 3 ml of 0.08% pepsin. After a 20-min incubation at 37°C, the nuclei were centrifuged as above and resuspended in 1.5 ml of 2N HCl while vortexing. After 20 min at 37°C, 3 ml of 0.1 M sodium borate was added to neutralize the nuclei and they were centrifuged for 5 min. The pellet was resuspended in 2 ml of IFA (10 mM HEPES at pH 7.4, 150 mM NaCl, 4% serum, 0.1% sodium azide) with 0.5% Tween 20 and centrifuged as above. The nuclear pellet was resuspended in 100 μ l of a 1:5 dilution of anti-BrdU fluorescein isothiocyanate-conjugated antibody (Becton Dickinson) in IFA. After a 30-min incubation on ice in the dark, 2 ml of IFA with 0.5% Tween 20 was added and the tubes were centrifuged for 5 min. The nuclei were resuspended in 500 μ l of IFA, RNase A (Sigma) was added to 5 μ g/ml, propidium iodide (Aldrich Chemical Co.) was added to 50 μ g/ml, and incubation proceeded at 37°C for 15 min. The nuclei were kept in the dark, on ice for at least 15 min. Cell cycle analysis was then performed using a Becton Dickinson FACScan instrument.

Immunoprecipitation of viral and cellular proteins

Cells (2×10^6) were incubated in methionine and cysteine-free Dulbecco's modified Eagle medium (DMEM), for 1 hr at 37°C and were then labeled for 3 hr with 80 μ Ci/ml of Tran³⁵S-label (70% L-[³⁵S]methionine and L-[³⁵S]cysteine, ICN. Incubation in 800 μ l of lysis buffer (20 mM Tris at pH 7.4, 250 mM NaCl, 0.5% NP-40, 1 mM EDTA, 50 μ g/ml of leupeptin, 30 μ g/ml of aprotinin) for 10 min at 4°C was followed by centrifugation at 14,000 rpm for 30 min to remove cellular debris. Lysates were cleared by absorption with Sansorbin (Calbiochem) for 30 min on ice and microcentrifuged for 15 min. Equivalent trichloroacetic acid precipitable counts (5×10^7) of radioactive protein were immunoprecipitated with protein A-Sepharose coated with mouse anti-HPV-18/16E6 (Oncogene Science), anti-HPV-16E7 (Denise Galloway), anti-human p53 protein (p53 Ab-5, Oncogene Science), or anti-retinoblastoma protein antibody (RB Ab-1, Oncogene Science) for 2 hr. Immune complexes were washed four times in wash buffer (20 mM Tris at pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.1% SDS), twice in PBS, released from the beads by heating to 95°C, and separated on 12% SDS-polyacrylamide gels. Gels were fixed, treated with Amplify (Amersham), dried, and exposed to x-ray film.

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