Adenovirus Type 12 Early Region 1B 54K Protein Significantly Extends the Life Span of Normal Mammalian Cells in Culture

PHILLIP H. GALLIMORE,* PHILIP S. LECANE, SALLY ROBERTS, SUSAN M. ROOKES,
ROGER J. A. GRAND, AND JULIAN PARKHILL

*CRC Institute for Cancer Studies, The Medical School, University of Birmingham, Edgbaston,
Birmingham B15 2TA, United Kingdom*

Received 13 February 1997/Accepted 15 May 1997

The life span of normal human cells in culture is extended by two to four total life spans following retrovirus-mediated transfer of the adenovirus type 12 E1B 54,000-molecular-weight protein (54K protein). This extension of the in vitro growth potential was accomplished without any of the obvious changes in morphology or growth properties that are usually associated with viral transformation. These 54K⁺ cells escape the normal senescence checkpoint (M₁) and show a very extended secondary growth phase. The 54K⁺ human cells eventually enter crisis (M₂), which does not appear to be due to either telomere attrition or the activation of the senescence-associated proteins p21^{Sdi1Cip1Waf1} and p16^{INK4A}. Even in the absence of telomerase activity, high-molecular-weight heterogeneous telomeres are produced and maintained in both 54K⁺ adult dermal fibroblasts and embryo kidney cells, indicating that the 54K protein may interfere with the normal metabolism of telomeric structures during cell division. These findings are discussed with reference to the known ability of the 54K protein to influence p53 function.

Since the original observation that human cells have a finite life span in culture (38, 39), human skin fibroblasts have been used to study the processes regulating normal growth and mortality. Factors which can undermine growth control, such as DNA tumor virus transforming proteins, chemical carcinogens, and ionizing radiation, have all been shown to increase the frequency of escape from mortality (immortalization) (2, 11, 41, 44, 46). The agent which has been most intensively studied and has generated the greatest number of immortalized human cell lines is simian virus 40 (SV40) (reviewed in reference 9). The transforming proteins of SV40, large T antigen (Tag) and small t antigen (tag), are both required for the extension of the normal life span of human cells in culture (18), although the human genome has to sustain additional mutations for immortal clones to develop (21) and for a malignant phenotype to be acquired (62). SV40 transformation extends the culture life span of normal human cells by at least 20 population doublings (PD) (26), but after this, the culture enters a static phase normally referred to as crisis; from these cultures, rarely growing clones emerge which on subsequent isolation are found to be immortal. This, with additional studies, led to the proposal of a scheme in which the normal life span of human cells in culture is referred to as mortality stage 1 (M₁) and the extended life span seen following the introduction of SV40 transforming genes is referred to as mortality stage 2 (M₂) (70).

Cessation of growth of normal mammalian cells in culture has been defined as senescence, and cells in a senescent culture, although still biochemically active, no longer undergo mitosis. Hypotheses presented to explain the senescence process include the stochastic model, whereby the accumulation of abnormal macromolecules over the lifetime of the culture leads to cessation of growth (reviewed in reference 40), and the programmed cell death model, in which senescence is an active process involving changes in the expression of certain genes

(reviewed in references 9 and 27). More recently, it has been shown that during the life span of normal somatic cells the specialized DNA structures at the end of the chromosomes (telomeres) are eroded at each cell division (34). Immortal cells, such as stem cells, tumor cells, and a high proportion of immortal transformed cell lines, express an enzyme, telomerase, that can repair these structures and maintain the length of the telomeres through indefinite cell divisions (15, 16, 37, 47). It has been postulated that telomeres below a critical length may serve as a signal for cells to enter senescence. If this checkpoint is overcome without activation of telomerase, the telomeric sequences will continue to be lost until the ends of the chromosomes are susceptible to damage and the cells will then enter crisis (34). The immortal clones that escape crisis are generally found to have activated telomerase, thereby preventing further telomere loss (17), or to have used another mechanism for telomere maintenance which generates very large telomeres (10).

As previously mentioned, the transforming proteins of the DNA tumor viruses SV40 (25, 26), human papillomaviruses (HPVs) (45, 46), and adenovirus (Ad) (11, 13, 25, 28) have all been shown to alter the growth control of human cells and increase the frequency of escape from the senescence process. To some extent, these viruses have a common mechanism, in that they target tumor suppressor gene products such as p53 and the retinoblastoma family of proteins (50, 74) and nuclear acetylases (4), resulting in the uncoupling of cell cycle regulation. Integration of viral DNA into the host cell genome maintains the expression of the viral transforming genes with the consequent continuous stimulation of cells through the cell cycle. We and others have shown that normal human cells can be transformed and immortalized by the Ad E1 region (13, 25, 28, 68). The E1 region contains two transcription units designated E1A and E1B. The E1A proteins are transcriptional regulators and bind to a number of important cellular proteins involved in growth control (5, 42). The E1B proteins, designated 19K and 54K (19,000 and 54,000 molecular weights, respectively), have been shown to affect processes such as apoptosis (67) and the localization and function of p53 (31, 75).

* Corresponding author. Phone: 121-414 4471. Fax: 121-414 4486.
E-mail: p.h.gallimore@bham.ac.uk.

A

B

C

FIG. 1. Photomicrographs and growth profiles of HSFs. (A) Senescent-phase HSF1 cells. (B) HSF1 cells at 40 PD, the time of AM-Ad12 54K virus infection (the arrow denotes a postmitotic cell as defined in reference 6). (C) HSF1/54K-C fibroblasts. (D) Growth profiles of HSF1 primary cells and HSF1/54K strains A, B, C, and E. Magnifications, $\times 250$.

The E1A proteins produce the morphological changes associated with Ad transformation, and the E1B proteins, although not in themselves morphologically transforming (65), nevertheless contribute to the frequency of transformation and the overall transformed and malignant phenotype (24). The large E1B protein of the nononcogenic Ad type 5 (Ad5 E1B 58K) forms a complex with p53 (60) in transformed cells, and both proteins are sequestered into "dense bodies" outside the nucleus (75). The equivalent protein in the oncogenic Ad12 (E1B 54K) has been shown, like the 58K Ad5 counterpart, to inactivate the transcriptional functions of p53 (31, 72, 73), but only a small proportion of cellular p53 is found in a complex with Ad12 E1B 54K (30). No study of the long-term consequences of the expression of either Ad2/5 or Ad12 E1B proteins in normal mammalian cells has been reported.

In the present study, Ad12 E1B 54K protein has been expressed in primary rodent kidney cells and normal human cells following infection with an amphotropic retrovirus carrying the Ad12 E1B 54K gene. The consequences of the introduction of this viral protein were monitored in terms of growth characteristics, culture life span, and telomere and telomerase status. Rodent cells expressing the 54K protein were genetically unstable, and morphological variants developed with time in tissue culture. Human cells were therefore also used in this study because they rarely spontaneously immortalize and they have tighter growth control than rodent cells (20, 43). Evidence is provided that 54K protein expression in human cells significantly increases the life span and that this occurs in the absence of telomerase activation but with some interference with the normal telomere attrition process. On the basis of these data, we propose that, as 54K abrogates p53 function and p21 is not activated in the senescent 54K⁺ cultures, human cells have an additional senescence pathway which is independent of p21^{Sdi1Cip1Waf1} (57).

MATERIALS AND METHODS

Construction of the Ad12 E1B 54K retrovirus and growth of an Ad5-SV40 early-region recombinant virus. The gene encoding the Ad12 E1B 54K protein was excised from pAsc10.3 (12) by *Xho*II digestion and ligated to the parental retroviral vector, pLXSN (55). The backbone structure of the pLXSN vector is based on the Moloney murine leukemia provirus, where expression of the in-

serted DNA is driven by the viral long terminal repeat. Recombinant Ad12 E1B 54K vector DNA (20 μ g) was electroporated into 10^6 cells of the PE501 ecotropic cell line which had been selected with hypoxanthine-aminopterin-thymidine (HAT) prior to use. The cells were serially diluted in the range of 10^{-1} to 10^{-4} and plated onto four 9-cm dishes in growth medium, and G418 (1 mg/ml) was added after 24 h. At 14 days postelectroporation, G418-resistant colonies were picked and expanded, and 54K expression was confirmed by Western blotting. The clones were then assessed for 54K ecotropic virus production on mouse 3T3 cells. Retroviral supernatant (1 ml) harvested from the best 54K⁺ PE 501 ecotropic packaging line was used to infect an 80% confluent dish of the amphotropic packaging cell line PA317 (54), which had been selected with HAT before use. The cells were then split, diluted, and selected as described above. After 14 to 21 days of G418 selection, isolated PA317 colonies of G418-resistant cells were picked and transferred to a 24-well plate, and G418 selection was maintained. Once these cells had been expanded, they were checked for 54K expression by Western blotting. High levels of 54K expression was seen in more than 70% of the PA317 clones. These were subsequently expanded, and supernatants derived from them were subjected to titer determination on exponentially growing A549 cells. Titers between 1×10^3 and 3×10^6 CFU/ml were obtained. The PA317 clone expressing the highest titer of recombinant Ad12 E1B 54K was subsequently expanded, the retroviral supernatant was harvested, Polybrene (6 μ g/ml) was added, and the virus stock was filtered through a 0.45- μ m-pore-size filter and stored at -80°C . This seed stock (AM-Ad12 54K) was then subjected to titer determination with A549 cells, and the absence of replication-competent helper virus was determined by standard procedures. An amphotropic retrovirus encoding the neomycin resistance gene from an internal SV40 early promoter, but with no Ad gene, was isolated (AM-Neo-1) and used as a control virus.

The Ad5-SV40 early-region recombinant virus, designated Ad5.SVR4, has an origin-minus early region of SV40 cloned into an Ad5 E1 deletion vector (kindly supplied by Y. Gluzman). This recombinant virus efficiently delivers the expression of both Tag and tag to mammalian cells. Ad5.SVR4 virus seed stocks were produced and subjected to titer determination with Ad5/293 cells (28).

Tissue culture. Adult human HSF1 and HSF2 skin fibroblasts were provided by Malcolm Taylor, Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom. These adult dermal fibroblasts (HSF) were obtained from healthy male volunteers aged 20 years. To determine the longevity of the HSF cell strains, normal cells were passaged at a 1:3 split ratio and E1B 54K⁺ cells were passaged at a ratio of 1:4. The growth medium was Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, and the cells were passaged with trypsin and EDTA. Human embryo kidney cell cultures were set up as previously described (68). G418 selection was maintained throughout the passaging of 54K⁺ cell lines. BALB/c mouse (BMK cells) and Hooded Lister rat kidney (BRK cells) cells were prepared (24) from 7-day-old animals.

The 54K⁺ human cells are referred to throughout the paper as cell strains and not cell lines because they are not immortal.

Measurement of TRF length by Southern blot analysis. Terminal restriction fragments (TRFs) were studied by a modification of the procedures described previously (33, 37). Chromosomal DNA from the cell strains was prepared by gentle lysis as described previously (59). Chromosomal DNA (1 μ g) was digested with *Hinf*I and *Rsa*I (Boehringer Mannheim Ltd.) and electrophoresed on a 6% agarose gel at 75 V for 14 h. The DNA was capillary blotted onto Hybond N (Amersham International) and hybridized with a telomeric probe in $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 18 h. The blot was washed in $2\times$ SSC and $1\times$ SSC at room temperature. The probe was generated by labelling a 144-bp telomeric repeat DNA cloned in pUC19 with a (CCCTAA)₃ oligonucleotide primer, Klenow fragment of DNA polymerase I (Boehringer Mannheim Ltd.), and [α - ^{32}P]dCTP (Amersham International). The blot was analyzed on a Molecular Dynamics Phosphorimager, and the molecular weight of each TRF band was estimated from the position of the peak of intensity corresponding to that band. We have not attempted to calculate the average TRF length for each track by integration, as has been done previously, because we wished to identify and isolate individual bands within each lane. The telomeric nature of the high-molecular-weight bands in the 54K⁺ cells was confirmed by incubating 1 μ g of intact chromosomal DNA with 1 U of BAL 31 (New England Biolabs) for 0, 30, 60, and 90 min under the conditions recommended by the supplier. The DNA was then repurified and subjected to *Hinf*I and *Rsa*I digestion and Southern hybridization, as described above.

Telomerase assay. Telomerase activity was detected by the telomere repeat amplification protocol (TRAP) (47). A total of 10^6 cells in phosphate-buffered saline were pelleted at $3,000 \times g$ for 6 min and then resuspended in 200 μ l of 10 mM Tris-HCl (pH 7.5)–1 mM MgCl₂–1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride–5 mM β -mercaptoethanol–0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)–10% glycerol. The cells were incubated for 30 min on ice and centrifuged at $16,000 \times g$ for 20 min, and the supernatant was removed to a fresh tube and snap-frozen in liquid nitrogen. A volume of 2 μ l (10^4 cells) or 20 μ l (10^5 cells) was used in the following protocol.

CX primer (CCCTTACCCTTACCCTTACCCTAA) (0.1 μ g) was placed in the bottom of a PCR tube and sealed under a wax pellet (Ampliwax; Perkin-Elmer). Above the pellet was added a reaction mixture containing 0.1 μ g of TS primer (AATCGTCGAGCAGAGTT), 2 or 20 μ l of cell extract, 20 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EDTA,

FIG. 2. Photomicrographs and growth profiles of human HEK cells. (A) HEK cells at the time of AM-Ad12 54K virus infection. (B) HEK/54K-A. (C) HEK/54K-E. (D) HEK/54K-G. (E) Growth profiles of HEK/54K-A, HEK/54K-E, and HEK/54K-G. Magnifications, $\times 225$.

and a low-light-level video camera. The two channels were recorded independently, and pseudo-color images were generated and superimposed with Adobe Photoshop 3.0 software.

Western blotting. Actively growing or senescent cells were rinsed with ice-cold saline and harvested. The cell pellets were solubilized in 9 M urea–50 mM Tris-HCl (pH 7.4)–0.15 M β -mercaptoethanol, and aliquots containing 50 μ g of protein were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose filters, and the Ad12 E1B 54K protein and p53 were detected with antibodies XPH9 and SAPU (a polyclonal sheep anti-human p53 kindly supplied by D. Lane, University of Dundee), respectively (29). p21^{Sdi1Cip1Waf1} and p16^{INK4A} were detected with a rabbit polyclonal serum (Santa Cruz Biotechnology, Inc.).

RESULTS

Growth properties of Ad12 54K-expressing rodent cells. BMK or BRK cell cultures were infected with either AM-Neo-1 or AM-Ad12 54K at a multiplicity of infection of 2. Cells were selected with G418 (500 μ g/ml) 2 days postinfection (p.i.). In virus-infected cultures, very few cells died within 10 days of starting selection, providing some evidence of an efficient infection. Over the same period, all of the cells on mock-infected control dishes exposed to G418 died. At 3 days after infection with AM-Ad12 54K, immunofluorescence microscopy revealed that between 94 and 97% of the rodent cells were positive for E1B 54K protein expression by using an Ad12 E1B 54K monoclonal antibody (53). As previously reported for the intact E1B region (65), we observed no morphological changes to the rodent kidney cells (they retained their epithelial morphology) infected with AM-Ad12 54K up to 6 weeks p.i. However, in both BMK and BRK cultures infected with AM-Neo-1 virus,

0.05 mM deoxynucleoside triphosphates, 1 μ g of T4 gene 32 protein (Boehringer Mannheim), 0.1 mg of bovine serum albumin per ml, 2 μ Ci of [α -³²P]dCTP, and 2 U of *Taq* polymerase. The assay mixture was incubated at 30°C for 30 min and then transferred to a thermal cycler for 31 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 45 s). Half of the sample was electrophoresed on a 10% polyacrylamide gel (National Diagnostics), which was subsequently dried and autoradiographed.

Indirect immunofluorescence staining. The cells were grown on glass slides in Dulbecco's minimal essential medium–8% fetal calf serum for 24 h and fixed in cold acetone (–20°C) for 3 min. The 54K and p53 proteins were detected with the XPH9 (53) and 1801 (3) murine monoclonal antibodies, respectively. Immune complexes were visualized with anti-immunoglobulin G subclass-specific fluorescein and rhodamine conjugates (Southern Biotechnology Associates). Confocal microscopy was performed with a Zeiss Axiopt fluorescence microscope, and images recorded with the Biovision software package (ImproVision)

FIG. 3. (A) Expression of Ad12 E1B 54K protein and p53 in HSF and HEK cell strains. The cells were harvested and solubilized as described in Materials and Methods, and aliquots (containing 50 μ g of protein) were fractionated by SDS-PAGE and subjected to Western blotting with antibodies raised against the Ad12 E1B 54K protein and p53 as indicated. Lanes: HSF C, control uninfected HSF1; HSF 1 to 8, HSF strains isolated after infection with AM-Ad12 54K (HSFB1/54K-E, PD 40; HSFB1/54K-D, PD 40; HSFB1/54K-A, PD 40; HSFB1/54K-B, PD 40; HSFB1/54K-C, PD 40; HSFB2/54K-D, PD 35; HSFB2/54K-B, PD 35; HSFB2/54K-A, PD 35); HEK C, control uninfected HEK cells; HEK 1 to 3, HEK cell strains isolated after infection with AM-Ad12 54K (HEK/54K-I, PD 30; HEK/54K-B, PD 30; HEK/54K-G, PD 30). (B) Expression of p21^{Sdi1 Cip1 Waf1} and p16^{INK4} in exponentially growing and senescent cells. Western blotting was done as described for panel A and in Materials and Methods, with commercially available antibodies against p16 and p21. Lanes: 1, 3, and 5, exponentially growing HSF 54K⁺ strains B, C, and E, respectively; 2, 4, and 6, 12-week senescent cultures of the same strains; 7 and 8, 70 and 100% senescent cultures (12 weeks senescent) of normal human skin fibroblasts (HSFB1); 9 and 10, exponentially growing and senescent HEK 54K-C cells, respectively; 11, senescent normal HEK cells.

the epithelial cells started to die around 4 to 5 weeks p.i. but colonies of fibroblasts continued to proliferate. These cultures failed to proliferate following the fourth passage, with a split ratio of 1:5. In contrast, there was no evidence of loss of proliferative capacity in AM-Ad12 54K-infected BMK or BRK cultures. Following the third passage of BMK cells expressing the 54K protein, morphologically altered colonies of more tightly packed cells with a high mitotic index became apparent, and they completely took over the culture during the fourth passage. Similar morphological changes occurred in the 54K⁺ BRK cultures at passage 5, and the morphological variants predominated in the culture by passage 7. These findings implied that the presence of the 54K protein extended the life span of rodent cells and that other genetic changes took place during this extended growth phase. Cytogenetic studies revealed that the morphological variants developed due to genetic instability in the 54K⁺ cultures (data not shown). The morphological and genetic changes did not represent malignant events, because no tumors were observed when these 54K⁺ cells were inoculated into athymic nude mice (25a).

Growth properties of Ad12 54K-expressing human cells. To provide a potentially more stable genetic background, HSFs and human fetal epithelial kidney (HEK) cells were infected with AM-Ad12 54K. Before this was carried out, the normal adult human skin fibroblast strain HSFB1 was assessed for its life span in culture. Cultures were passaged on a 1:3 split until they entered the senescence phase of growth. HSF-1 cultures were also infected with AM-Neo-1 to examine their clonogenic

potential. The life span of G418-selected HSFB1 clones was compared to that of continually passaged HSFB1 cultures. The mean number of PD at the time of entry into senescence (Fig. 1A) for HSFB1 G418-resistant clones was 44 (ranging from 38 to 48) compared to 48 (ranging from 42 to 52) for the non-selected HSFB1 cultures. This control experiment showed that there was nothing unusual about the ability of the HSFB1 cell strains to enter the senescence pathway (6, 39, 61, 70).

A total of 2×10^5 HSFB1 cells already at 40 population doublings (Fig. 1B) (note that postmitotic senescent cells, which were large, flat, and multinucleate, were already appearing in these cultures) were infected with 8×10^5 CFU of AM-Ad12 54K per ml. Immunofluorescence microscopy was used to show that in the AM-Ad12 54K-infected cultures, over 90% of the fibroblasts were 54K positive 3 days p.i. Following G418 selection (500 μ g/ml) of AM-Ad12 54K-infected HSFB1 cell cultures for 4 weeks, more than 100 healthy colonies of fibroblasts were observed per 9-cm dish of infected cells. No fibroblast colonies survived in parallel cultures of HSFB1 cells infected with AM-Neo-1. A colony was isolated from each of six independent dishes (designated HSFB1/54K-A to HSFB1/54K-F) of G418-selected AM-Ad12 54K-infected HSFB1 fibroblasts, trypsinized, and then expanded. It was clear from this initial experiment that the presence of the 54K protein had significantly extended the culture life span, in that the life expectancy of these cultures, if unmodified, would have been only a further 8 PD and all of the G418-resistant colonies isolated were expanded up to 4×10^6 cells in the first instance (equivalent to an additional 22 PD). Evidence that these HSF cell strains expressed 54K was provided by immunofluorescence and Western blotting (see below). The six 54K-expressing cell strains were then subcultured 1:4 on a continuous basis to assess longevity. The life span of these 54K⁺ strains, including the initial 40 PD prior to infection, ranged from 120 PD (clone B) to 166 PD (clone C) (Fig. 1D). In addition, two of these 54K⁺ cell strains (B and D) were further subcloned. It was observed that in the subcloning experiment, total number of PD exceeded that seen with the parental strain. For strain B the parental line survived to 120 PD while the subclones survived to 130 PD, and for clone D the parental strain survived to 100 PD while the subclones survived again to 130 PD. We currently have no explanation for this observation. During the expansion of these 54K⁺ human skin fibroblasts, there was no evidence of morphological change (Fig. 1C), acquisition of reduced serum requirement, or anchorage-independent growth (data not shown). That the expression of 54K protein in these normal human cells was responsible for the extended life span was confirmed by using HSFs from another donor, HSFB2. These HSFs showed the same extended life span following the introduction of Ad12 54K (data not shown). When the 54K⁺ HSFs finally enter the M2 senescent growth phase, they are morphologically indistinguishable from normal senescent HSFs, and they also express the senescence-associated β -galactosidase (SA- β -Gal) (19) (data not shown).

It has been reported that there are tissue-specific mechanisms involved in the control of the M1 stage of cellular immortalization (61), and with this in mind, secondary cultures of HEK cells were infected with either AM-Neo-1 or AM-Ad12 54K. Although G418-resistant colonies developed in the secondary HEK cultures infected with AM-Neo-1, they could not be subsequently subcloned (a life span of less than 20 PD). However, following AM-Ad12 54K infection, 10 G418-resistant (250 μ g/ml) 54K⁺ HEK colonies that were picked expanded and were shown to have life spans ranging from 60 to 93 PD (Fig. 2E). In agreement with the data derived from the fibroblast experiments, extended growth potential was not ac-

FIG. 4. Localization of p53 and Ad12 E1B 54K proteins. (A) 54K⁺ HSFs (HSFB1/54K-E); (B) 54K⁺ HEK cells (HEK/54K-G). The 54K and p53 proteins were detected with mouse monoclonal antibodies XPH9 and PAb 1801, respectively. Immune complexes were visualized with fluorescein (54K, green)- and rhodamine (p53, red)-conjugated anti-mouse immunoglobulin G subclass-specific antibodies. Images from each fluorochrome were recorded and superimposed; colocalization of the two proteins is indicated in yellow. Although the two proteins are predominantly colocalized, a proportion of each protein is not. (B) The arrow indicates cytoplasmic flecks of 54K not colocalized with p53. (C) Localization of 54K in senescing HSFs and HEK cells. Similar cytoplasmic and nuclear distribution of the 54K proteins was observed in the senescing cells, which were identified by their aberrantly shaped nuclei or multinuclear status. Bar, 20 μ m.

accompanied by morphological changes (Fig. 2B to D). It should be noted that secondary HEK cultures are a mixture of cell types with different epithelial morphologies.

54K⁺ HSF senescent cultures are not irreversibly postmitotic. To examine whether 54K⁺ senescent cultures were irreversibly postmitotic, two of the HSF 54K⁺ cell strains (HSF 54K-C and HSF 54K-E) which had been maintained in 175-cm² flasks (7×10^6 cells in total per cell strain) as senescent cultures for 16 weeks were infected at a multiplicity of infec-

tion of 50 PFU per cell with Ad5.SVR4. This recombinant Ad, containing the SV40 early region in place of Ad E1A and E1B, efficiently delivers expression of both Tag and tag to human cells (25a). Ad5.SVR4-infected cultures were examined daily, under high-power phase-contrast microscopy, for cells in metaphase. Mitoses first became apparent 48 h p.i., and the number of cells in metaphase increased until 6 days p.i., when 12% of the cells in HSF 54K-C and 7% of the cells in HSF 54K-E were mitotic. A high proportion (greater than 80%) of the mitoses

FIG. 5. Analysis of TRF length in control (HSFB1) and 54K-expressing (HSFB1/54K) cell strains at various stages of passage. (A) Sample Southern blots showing *HinfI* and *RsaI* digests of DNA from HSFB1, HSFB1/54K-E, and HSFB1/54K-F hybridized to a telomere probe. Tracks on the right show the BAL 31 digestion of intact HSFB1/54K-E chromosomal DNA. (B) Graph showing the length of each distinct band from telomere blots of HSFB1 (□), HSFB1/54K-A (◆), HSFB1/54K-B (△), HSFB1/54K-C (■), HSFB1/54K-E (◇), and HSFB1/54K-F (○) cells. Lines are plotted through the low- and high-molecular-weight bands of the HSFB1/54K cells and extrapolated past the point of senescence of the control cells.

were abnormal, with large polyploid metaphases and aberrant spindles being the most common features. No evidence of mitosis was observed in parallel mock-infected senescent cultures. At 7 days after infection with Ad5.SVR4, cells started to die and were shed into the tissue culture medium so that by 16 days p.i., approximately 75% of the culture had been depleted; there was no evidence of cell death in the mock-infected senescent cultures. There was a possibility that the cell death seen in the Ad5.SVR4-infected cultures was due to Ad5 replication, either as a result of a low level of wild-type virus being a component of the seed stock or as a result of the E1B protein and the SV40 Tags providing complementing functions for replication. This was ruled out by collecting dying cells and examining them by immunofluorescence for Ad5 structural proteins with a rabbit polyclonal antiserum raised against SDS-disrupted purified virions. No evidence of Ad5 replication was detected, and therefore the reason for the cell attrition following Ad5.SVR4 infection remains unresolved. However, infection of 54K⁺ HSFs in the exponential phase of growth did not induce cell death. The Ad5.SVR4 virus dose used to induce mitosis in the senescent 54K⁺ HSFs would give rise to over 200 transformed foci in cultures of exponentially growing HSFs (25a). No transformed foci were observed in the Ad5.SVR4-infected 54K⁺ senescent cultures 6 weeks p.i., however, mitotic cells were still evident, and there was a limited sign of focal proliferation (four to nine colonies per 175-cm² flask). We therefore conclude that the 54K⁺ senescent HSFs were not irreversibly postmitotic and that expression of SV40-transforming proteins in these cells only leads to limited additional proliferation.

Expression of 54K, p53, p21^{Sdi1Cip1Waf1} and p16^{INK4A} in 54K⁺ human cell lines. We and others (29, 52) have previously shown that expression of Ad12 E1B 54K in both rat and human Ad12 E1-transformed cell lines significantly enhances the expression of p53 and that E1B 54K expression is an essential component of Ad12 tumorigenicity (14, 24). In addition, expression of the 54K protein inhibits p53 transcriptional activity (31, 72, 73). Western blot analysis was carried out on both 54K⁺ HSF and HEK cell strains to examine the levels of p53 and 54K. Figure 3A demonstrates that all the cell strains which express 54K had elevated levels of p53, with a correlation between the levels of expression of the two proteins. For example, lanes 3 and 4, which show the highest level of expression of 54K, also showed the highest level of p53, and conversely, lanes 7 and 8, which show the lowest level of 54K, also consistently expressed lower levels of p53. These data conclusively show that the E1B 54K protein makes a considerable contribution to the high levels of p53 seen in Ad12 E1-transformed human cells. Interestingly, significantly higher levels of 54K protein as well as p53 were observed in the HEK strains than in the HSF strains. Figure 3A shows that the normal endogenous level of p53 was higher in the HEK cells (HEK-lane C) than in HSFB1 fibroblasts (HSF-lane C). It is possible, therefore, that the higher initial levels of p53 in the HEK cells require higher levels of 54K expression to produce the same effect on p53 activity.

Senescent cultures of HEK and HSFB1 54K⁺ cells were examined by both Western blotting (data not shown) and immunofluorescence (Fig. 4C) and shown by both techniques to be expressing 54K protein. Therefore, the entry of these cells into a senescence phase was not simply due to the loss of 54K expression. There was also no evidence of a change in p53 levels or of increased expression of the senescence-induced cell cycle-dependent kinase inhibitory protein p21^{Sdi1Cip1Waf1} or the up-regulation of another known cell cycle kinase inhibitor, p16^{INK4A} (Fig. 3B). Figure 3B shows the results of Western

FIG. 6. Analysis of TRF length in control (HEK) and 54K-expressing (HEK/54K) cell strains at various stages of passage. (A) Sample Southern blot showing *HinI* and *RsaI* digests of DNA from HEK/54K-E and HEK/54K-G hybridized to a telomere probe. The high-molecular-weight bands discussed in the text that appear and disappear during passage are marked with arrowheads. Tracks to the right of the figure show the BAL 31 digestion of intact HEK/54K-E chromosomal DNA. (B) Graph showing the length of each distinct band from telomere blots of HEK ($\square$), HEK/54K-E ($\blacksquare$), and HEK/54K-G ($\triangle$) cells. For comparison, a line has been plotted through the HEK points showing a hypothetical loss of telomere length of ~ 44 bp/generation, as seen in the HSF1 cells.

blotting studies obtained for three 54K⁺ HSF cell strains growing exponentially and 12-week senescent cultures, one 54K⁺ HEK cell strain growing exponentially, and a 12-week senescent culture, normal HSF cultures entering the senescence phase (70% senescent), and 12-week senescent cultures of normal HEKs and HSFs. p16^{INK4A} expression was observed only in the very senescent normal cells, whereas p21^{Sdi1Cip1Waf1} was also easily detected in a normal HSF culture entering the senescence phase. p21^{Sdi1Cip1Waf1} could also be detected by overexposure of the Western blot in all 54K⁺ cell strains, in both senescent and exponentially growing cultures. However, the level of expression was at least 50-fold lower than that seen in normal HSFs entering senescence (Fig. 3B, lane 7). Because 100% of the cells in the 54K⁺ senescent cultures were fully senescent, as judged by SA- β -Gal expression, we conclude that neither p21 nor p16 participates in the entry of 54K⁺ cells into the senescence pathway or maintains the senescence phenotype.

Cellular localization of p53 and 54K in 54K⁺ HSF and HEK cell lines. To determine the cellular distribution of both p53 and 54K proteins in the 54K-expressing cell strains, we used confocal microscopy and performed dual immunofluorescence staining with anti-p53 and anti-54K monoclonal antibodies (PAb 1801 and XPH9, respectively). In 100% of the HSF and HEK cells, the 54K and p53 proteins were localized to the nucleus and there was additional staining in the cytoplasm of some cells. Cytoplasmic p53 was always colocalized with 54K;

54K was observed as multiple flecks (Fig. 4A and B). However, cytoplasmic dense bodies containing only 54K protein were more frequently observed than those with both p53 and 54K. The nuclear distribution of both proteins was variable, being both diffuse and associated with nuclear aggregates (Fig. 4A and B). In contrast to the distribution of p53 in the cytoplasm, there was good evidence that not all of the p53 and 54K colocalized in the nucleus and that this was true for dispersed and aggregated forms of these proteins (Fig. 4A and B).

Telomere length and telomerase activity in 54K⁺ HSF and HEK cell lines. Evidence has been presented that the senescence pathway in human cells is activated when telomeres have been eroded to a critical level (34, 37) and that telomerase is activated in most human cells immortalized by DNA tumor viruses (10). Studies were therefore undertaken to examine the status of the telomeres and telomerase in 54K⁺ human cell strains. The TRF length was measured for each strain at various stages of growth until senescence by probing Southern blots with a telomere-specific probe. Sample Southern blots for primary and 54K⁺ HSF and HEK cell strains are shown in Fig. 5A and 6A. The average molecular mass of each band detected on all the telomere blots is shown graphically in Fig. 5B and 6B.

The low-passage uninfected control HSF cells showed a characteristic smear of TRF DNA from approximately 19 to 4.5 kb, with an average molecular size of 9.5 kb (Fig. 5A). During passage in culture, the bulk TRFs in the 54K⁺ HSF

cells decrease in size by ≈ 44 bp per generation, in agreement with previous estimates (33, 76). After prolonged passage, some discrete higher-molecular-mass bands start to appear, and these also decrease in size after further passage. The 54K⁺ HSF strains E and F (Fig. 5A) again have the bulk of the TRF DNA in a lower-molecular-mass smear (Fig. 5A), but there are also one or more discrete high-molecular-weight bands of telomeric DNA. Some of these bands correspond approximately to the bands seen in the control HSF cells at 40 PD, the point at which the 54K-expressing construct was introduced into the cell line. However, in contrast to the control cells, these TRFs did not alter significantly during the lifetime of the cells, even after 154 PD just prior to senescence. Other 54K⁺ HSF cell strains showed bands with various molecular sizes ranging from ≈ 4 to ≈ 16 kb (Fig. 5B), which either remained at the same molecular size (e.g., HSF/54K-F) during passage (Fig. 5A) or demonstrated the appearance and disappearance of bands with differing molecular sizes during passage (e.g., HSF/54K-C) (Fig. 5B), suggesting that recombination events were occurring in the telomeric DNA.

The primary HEK cells also showed a characteristic continuous smear of DNA centered around 11 kb (Fig. 6B). These cells are obviously younger than the adult HSF cells and hence had longer telomeres. After the introduction of the Ad12 54K gene, the TRF DNA split into several discrete bands, ranging from sharp high-molecular-mass bands of up to 17 kb to diffuse low-molecular-mass smears of ≈ 6 to 7 kb (Fig. 6A). The molecular mass of most of these bands decreased slowly during the lifetime of the cells, with an apparent overall value of ≈ 21 bp/generation, slower than that seen in normal cells. In addition to this overall decrease of all the bands, novel high-molecular-mass bands appeared and disappeared during passage (Fig. 6). For example, a band of ≈ 16 kb appeared between PD 39 and 41 of HEK/54K-E, and a strong band of ≈ 12 kb disappeared between PD 61 and 75 of HEK/54K-G (both bands are marked with arrowheads). That the high-molecular-mass bands seen in the 54K⁺ cell strains are telomeric was demonstrated by incubation of the intact chromosomal DNA with BAL 31, a double-strand exonuclease. All of the high-molecular-mass bands (Fig. 5A and 6A) reduced in size, at a rate proportional to their length, on digestion with BAL 31. The low-molecular-mass bands at the bottom of the gels (Fig. 5A and 6A) are internal or subtelomeric sequences and are protected from BAL 31 digestion; therefore, they retain their original molecular mass following exposure to the exonuclease (the apparent small decrease in the size of these bands in Fig. 5A is an artifact of the gel).

All of the uninfected and 54K-expressing human cell strains were assayed for telomerase activity (Fig. 7) by a PCR-based assay (47). Ad5/293 is an Ad5 DNA-immortalized HEK cell line (28), which has previously been shown to contain telomerase activity (10, 15). A ladder indicating telomerase activity is clearly visible when 10^4 293 cells are used (the activity was also detected with 10^3 293 cells [data not shown]). However, telomerase activity could not be detected in 10^5 cells of any of the 54K-expressing cell strains, at low or high passage levels. Telomerase activity was also not observed in the noninfected HSFBI or HEK parental cultures (data not shown). That the lack of detectable telomerase activity is not due to a telomerase inhibitor in the cell extracts was demonstrated by including 10^4 293 cells in each of the other cell extracts and repeating the assay. No loss of telomerase activity was seen under these conditions (data not shown).

FIG. 7. The 54K-expressing cell strains do not display telomerase activity. Telomerase activity was assayed by the TRAP protocol with 10^4 immortal 293 cells as a positive control. Assays of 10^5 cells from various 54K-expressing HEK and HSFBI cell strains at one or two different passage levels are also shown.

DISCUSSION

It has been known for many years that normal human cells have a finite life span (38, 39) and are genetically more stable than rodent cells (20, 43). However, most mammalian cells enter a postmitotic phase, termed senescence, following multiple passaging in tissue culture. There is now good evidence that this process also occurs in somatic cells *in vivo* (19) and is therefore likely to be a natural checkpoint to cell growth that must be overcome before the onset of immortalization *in vitro* and possibly malignancy *in vivo*.

Recent studies have shown that the Ad large E1B protein inhibits the transcriptional transactivation functions of the tumor suppressor protein p53 (31, 63, 72, 73) and that this is most likely to be a crucial component of Ad transformation. Efficient transformation of both rodent and human cells by Ad DNA requires E1B to cooperate with E1A to produce cells with unlimited growth (13, 24). In addition, we have reported that Ad12 mutants, defective for 54K expression, have a dramatically reduced capacity to transform rat cells and induce tumors (14). However, it has previously been reported that expression of the whole E1B transcription unit in rodent cells has little impact on cell morphology and growth behavior (65), although it should be noted that this investigation did not examine the long-term consequences of E1B protein expression. In the present study, we have shown that the Ad12 E1B 54K protein can significantly increase the life span of both rodent and human cells in culture. Initially, the morphology of 54K⁺ rat and mouse kidney cells was normal, but after a limited number of tissue culture passages, morphological variants with some features of partially transformed cells (more tightly packed with a high mitotic index) outgrew the "normal-appearing" cells. These 54K⁺ rodent cells were cytogenetically abnormal but nontumorigenic (25a). In contrast, 54K⁺ human dermal fibroblasts and kidney epithelial cells did not sponta-

neously change morphology or alter their in vitro growth properties during the extended M_1 growth phase. This may be explained by the observation that no cytogenetic instability was observed in any of the human $54K^+$ cell strains until they reached a presenescence phase of growth when polyploidy and aneuploidy predominated and the presence of dicentric chromosomes and chromosome fragments became evident (25a). When the $54K^+$ HSFs enter the M_2 stage of growth, they are indistinguishable from normal senescent cells in that the cultures remain postmitotic and viable for months, contain a high proportion of flat giant or multinucleate cells, and express SA- β -Gal (19).

Although our study demonstrates that human cells expressing the Ad12 E1B $54K$ protein bypass the M_1 stage of cellular senescence and have a considerably extended life span, they nevertheless still enter the M_2 senescence phase (crisis), from which they do not escape. The signal for entry into senescence is not due to the loss of $54K$ expression (Fig. 4C). Also we found no evidence that the late entry into M_2 was due to the up-regulation of $p21^{Sdi1Cip1Waf1}$ (22, 35, 57) induced by p53-dependent (22) or -independent mechanisms or to up-regulation of $p16^{INK4A}$ (Fig. 3B). In contrast, it has been reported that SV40-transformed human fibroblasts show increased levels of $p21^{Sdi1Cip1Waf1}$ mRNA as these cells progress to the M_2 senescence phase; however no evidence of changes in p21 protein levels were presented (64). If the p21 protein level does increase in SV40 transformed cells entering the M_2 phase of growth, there is a clear difference between this and our findings with senescent $54K^+$ cells.

Studies carried out on human cells with the transforming proteins of HPVs (48, 61) indicated that the E6 protein, which targets p53 for ubiquitin-mediated degradation, extends the life span of human epithelial cells but not to the same degree as E1B $54K$ does (48, 61). The difference observed between the life span extension produced by HPV16 E6 and that produced by $54K$ may be because $54K$ stabilizes p53 rather than causing p53 to be rapidly degraded. Of interest was the observation that senescent $54K^+$ fibroblasts can be stimulated back into mitosis by the expression of SV40 Tag and tag. This shows that when $54K^+$ cells enter the senescence pathway, it is not due to loss of gene functions regulating mitosis. In the future, we intend to carry out experiments on $54K^+$ cells prior to their entry into the M_2 phase to see whether Tag or tag can individually further extend the life span of the $54K^+$ cells.

The confocal microscopy studies (Fig. 4A and B) support our previous biochemical analysis (30–32) showing that Ad12 $54K$ and p53 colocalize in the nucleus and cytoplasm of $54K^+$ cells, although there is a significant proportion of each protein that is not physically associated with the other (30). However, we (31, 32) and others (63) have shown that the presence of the E1B protein stabilizes p53 and inhibits p53 transcriptional activity, thus implying that $54K$ can inhibit p53 transcriptional activation without binding all of the p53 molecules present in the cell. The present study clearly demonstrates that stabilization of p53 and therefore overexpression can be accomplished by the $54K$ protein alone, and we can conclude that the presence of the $54K$ protein in E1 transformed human and rat cells contributes significantly to the high level of p53 seen in these cells (29). Thus far, the $54K$ protein has not been observed to bind to or to have any effect on cellular proteins other than p53, but it can act as a potent transcriptional repressor when recruited to a promoter via its interaction with p53 (72, 73). The extended life span observed in our studies is therefore most probably due to abrogation of p53 function. Support for this comes from the experiments with HPV16 E6 and SV40 and from recent studies in which it was shown that transfection

of a p53 dominant-negative mutant into normal human cells extends their life span in culture (7), although not to the degree reported here.

It has recently been suggested that one function of p53 may be to detect critical-length telomeres as a form of DNA damage and initiate the senescence pathway (71). The same group has provided evidence, obtained with HSFs, that transcriptional activation by p53 plays a direct role in activation of the senescence phase of growth (8). This implies that ablation of p53 function (in our case by the expression of Ad12 $54K$) is one route to permit human cells to bypass the normal M_1 check point.

The behavior of the TRFs in the HEK cell lines expressing $54K$ correlates to some extent with previously reported results; these cells do not appear to express telomerase, and the bulk of the telomeric DNA apparently reduces in size, although at a lower rate than that described for normal cells (33). However, long TRFs appear and disappear during passage in a manner reminiscent of the rapid acquisition of telomeric DNA on a single chromosome in cells which do not express telomerase (56). This rapid change in telomere length was postulated to be due to nonreciprocal recombination between telomeres, as has been demonstrated to occur in *Saccharomyces cerevisiae* (66). It is intriguing that the $54K^+$ HSF cell strains E and F, which have apparently stable telomeres, do not appear to express telomerase. Although most immortal cell lines have stable telomeres and express telomerase (10, 17, 47), some immortalized lines lack telomerase activity, and in every case these cell lines had very large (≤ 50 -kb) and heterologous TRFs (10). The Southern blots in Fig. 5 and 6 show only the overall TRF length on all the chromosomes, and it is possible that this masks underlying changes similar to those that occur in the $54K$ -expressing HEK cells and in the other $54K^+$ HSF cell strains. The same mechanism may therefore be responsible for the maintenance of telomere length in these HSF cell strains. Interestingly, unlike the situation with $54K^+$ cells, it has recently been reported that a proportion of human epithelial cells expressing the HPV16 E6 gene product show low levels of telomerase activation (49). However, the activation of telomerase was not in itself sufficient to produce immortalization.

It has been suggested that entry into crisis is due to erosion of telomeres to a critical length and that escape from crisis is possible only when cells have activated telomerase (34, 61). The cell strains described in the present study do not appear to have reached this critical limit when they enter senescence, and, indeed, in the $54K^+$ HSFs telomere attrition is considerably reduced, such that the telomeres do not appear to reduce in size at all. It therefore seems unlikely that the signal for late entry into the senescence pathway seen with the $54K^+$ cells derives from the length of the telomeres, although it is possible that a small proportion of the telomeres in such a heterogeneous population are of a length that will signal entry into senescence. This could be due to loss of functional sequences adjacent to telomeres at the chromosome ends or to the loss of chromosomes with the development of aneuploidy.

To date, the only reported effects of the large E1B proteins in human cells are the blocking of wild-type p53 activity and the regulation of the levels and transport of late viral and cellular mRNAs during permissive viral infection (1, 58). The data presented here indicate that the presence of $54K$ in some way interferes with telomere metabolism. If this is due solely to $54K$ ablation of p53 transcriptional activity, it is possible that one of the functions of p53 is to prevent, directly or indirectly, aberrant or nonreciprocal recombination between telomeres during normal cell replication. Very high molecular weight

heterogeneous and rapidly changing telomeres have previously been seen in the absence of telomerase activity in a proportion of SV40- or HPV16-immortalized human cells and immortal Li-Fraumeni cells. These cell lines do not have functional p53, and these aberrant telomeres may be generated by a similar mechanism, as in the 54K⁺ cell strains reported here. Such instability of telomeric DNA may explain one of the effects of the loss of wild-type p53 activity, i.e., the genomic instability and/or chromosome loss that is observed in the absence of functional p53 (36, 51). In support of this hypothesis, it has recently been shown that there is an inhibitory effect of p53 on homologous recombination of SV40 chromosomes (69). In addition, p53 has been implicated in the regulation of centrosome duplication (23), and therefore the transcriptional regulatory functions of p53 may not be uniquely important to cell cycle control and life span regulation.

Evidence has accumulated that the life span of cells in culture can be extended by interfering with p53 and/or Rb functions (9, 61) and that senescence may be the result of increased expression of p21^{Sdi1Cip1Waf1} or p16^{INK4A}. The finding that there was no increased expression of either of these cell cycle-dependent kinase inhibitors in the senescent 54K⁺ human cells suggests that human cells have additional senescence pathways which are independent of p16 and p21.

Finally, Ad12 54K protein may have functions apart from p53 inactivation, and we are currently investigating this possibility.

ACKNOWLEDGMENTS

We are extremely grateful to the Cancer Research Campaign for supporting this research. P.H.G. is a Gibb Fellow of the Cancer Research Campaign.

We thank Susan Williams for the photography.

REFERENCES

- Babiss, L. E., H. S. Ginsberg, and J. E. Darnell. 1985. Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular RNA translation and transport. *Mol. Cell. Biol.* 5:2552-2558.
- Bai, L., K. Mihara, Y. Kondo, M. Honma, and M. Namba. 1993. Immortalization of normal human fibroblasts by treatment with 4-nitroquinoline 1-oxide. *Int. J. Cancer* 53:451-456.
- Banks, L., G. Matlashewski, and L. Crawford. 1986. Isolation of human p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur. J. Biochem.* 159:529-534.
- Bannister, A. J., and T. Kouzarides. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* 384:641-643.
- Bayley, S. T., and J. S. Mymryk. 1994. Adenovirus E1A proteins and transformation. *Int. J. Oncol.* 5:425-444.
- Bayreuther, K., H. P. Rodemann, R. Hommel, K. Dittmann, M. Albiez, and P. I. Francz. 1988. Human skin fibroblasts *in vitro* differentiate along a terminal cell lineage. *Proc. Natl. Acad. Sci. USA* 85:5112-5116.
- Bond, J. A., F. S. Wyllie, and D. Wynford-Thomas. 1994. Escape from senescence in human diploid fibroblasts induced directly by mutant p53. *Oncogene* 9:1885-1889.
- Bond, J. A., M. Haughton, J. Blaydes, V. Gire, D. Wynford-Thomas, and F. S. Wyllie. 1996. Evidence that transcriptional activation of p53 plays a direct role in the induction of cellular senescence. *Oncogene* 13:2097-2104.
- Bryan, T. M., and R. R. Reddel. 1994. SV40-induced immortalization of human cells. *Crit. Rev. Oncog.* 5:331-357.
- Bryan, T. M., A. Englezou, J. Gupta, S. Bacchetti, and R. R. Reddel. 1995. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.* 14:4240-4248.
- Byrd, P. J., K. W. Brown, and P. H. Gallimore. 1982. Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA. *Nature* 298:69-71.
- Byrd, P. J., W. Chia, P. W. J. Rigby, and P. H. Gallimore. 1982. Cloning of DNA fragments from the left end of the adenovirus type 12 genome: transformation by cloned early region 1. *J. Gen. Virol.* 60:279-293.
- Byrd, P. J., R. J. A. Grand, and P. H. Gallimore. 1988. Differential transformation of primary human embryo retinal cells by adenovirus E1 regions and combinations of E1A + *ras*. *Oncogene* 2:477-484.
- Byrd, P. J., R. J. A. Grand, D. Breiding, J. F. Williams, and P. H. Gallimore. 1988. Host range mutants of adenovirus type 12 E1 defective for lytic infection, transformation and oncogenicity. *Virology* 163:155-165.
- Counter, C. M., A. A. Avilon, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* 11:1921-1929.
- Counter, C. M., H. W. Hirte, S. Bacchetti, and C. B. Harley. 1994. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA* 91:2900-2904.
- Counter, C. M., F. M. Botelho, P. Wang, C. B. Harley, and S. Bacchetti. 1994. Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes. *J. Virol.* 68:3410-3414.
- deRonde, A., C. J. A. Sol, A. van Strien, J. ter Schegget, and J. van der Noorda. 1989. The SV40 small t antigen is essential for the morphological transformation of human fibroblasts. *Virology* 171:260-263.
- Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, M. Peacocke, and J. Campisi. 1995. A biomarker that identifies senescent human cells in culture and in ageing skin *in vivo*. *Proc. Natl. Acad. Sci. USA* 92:9363-9367.
- Di Paolo, J. A. 1983. Relative difficulties in transforming human and animal cells *in vitro*. *JNCI* 70:3-8.
- Duncan, E. L., N. J. Whitaker, E. L. Moy, and R. R. Reddel. 1993. Assignment of SV40-immortalized cells to more than one complementation group for immortalization. *Exp. Cell Res.* 205:337-344.
- El-Deiry, W. T., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumour suppression. *Cell* 75:817-825.
- Fukasawa, K., T. Choi, R. Kuriyama, S. Rulong, and G. F. Vande Woude. 1996. Abnormal centrosome amplification in the absence of p53. *Science* 271:1744-1747.
- Gallimore, P. H., P. J. Byrd, J. L. Whittaker, and R. J. A. Grand. 1985. Properties of rat cells transformed by DNA plasmids containing adenovirus type 12 E1 DNA or specific fragments of the E1 region: comparison of transforming frequencies. *Cancer Res.* 45:2670-2680.
- Gallimore, P. H., R. J. A. Grand, and P. J. Byrd. 1986. Transformation of human embryo retinoblasts with simian virus 40, adenovirus and *ras* oncogenes. *Anticancer Res.* 6:499-508.
- Gallimore, P. H. Unpublished results.
- Girardi, A. J., F. C. Jensen, and H. Koprowski. 1965. SV40 induced transformation of human diploid cells: crisis and recovery. *J. Cell. Comp. Physiol.* 65:69-84.
- Goldstein, S. 1990. Replicative senescence: the human fibroblast comes of age. *Science* 249:1129-1133.
- Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 65:59-72.
- Grand, R. J. A., P. S. Lecane, S. Roberts, M. L. Grant, D. P. Lane, L. S. Young, C. W. Dawson, and P. H. Gallimore. 1993. Over-expression of wild type p53 and c-Myc in human fetal cells transformed with adenovirus early region 1. *Virology* 193:579-591.
- Grand, R. J. A., M. L. Grant, and P. H. Gallimore. 1994. Enhanced expression of p53 in human cells infected with mutant adenoviruses. *Virology* 203:229-240.
- Grand, R. J. A., P. S. Lecane, D. Owen, M. L. Grant, S. Roberts, A. J. Levine, and P. H. Gallimore. 1995. The high levels of p53 present in adenovirus early region 1-transformed cells do not cause up-regulation of MDM2 expression. *Virology* 210:323-334.
- Grand, R. J. A., D. Owen, S. M. Rookes, and P. H. Gallimore. 1996. Control of p53 expression by adenovirus 12 early region 1A and early region 1B 54K proteins. *Virology* 218:23-43.
- Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* 345:458-460.
- Harley, C. B. 1991. Telomere loss: mitotic clock or genetic time bomb? *Mutat. Res.* 256:271-282.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 CDK interacting protein CIP1 is a potent inhibitor of G1 cyclin dependent kinases. *Cell* 75:805-816.
- Harvey, M., M. J. McArthur, C. A. Montgomery, J. S. Butel, A. Bradley, and L. A. Donehower. 1993. Spontaneous and carcinogen-induced tumorigenesis in p53 deficient mice. *Nat. Genet.* 5:225-229.
- Hastie, N. D., M. Dempster, M. G. Dunlop, A. M. Thompson, D. K. Green, and R. C. Allshire. 1990. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 346:866-868.
- Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid strains. *Exp. Cell Res.* 25:585-621.
- Hayflick, L. 1965. The limited *in vitro* lifetime of human diploid cell strain. *Exp. Cell Res.* 37:614-636.
- Holliday, R. 1990. Minireview. The limited proliferation of cultured human diploid cells: regulation or senescence? *Gerontology* 36:1-6.
- Huschtscha, L. I., and R. Holliday. 1983. Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. *J. Cell Sci.* 63:77-79.

42. Jones, N. C. 1992. The multifunctional products of the adenovirus E1A gene, p. 87–113. In W. Doerfler and P. Bohm (ed.), *Malignant transformation by DNA viruses*. VCH, Weinheim, Germany.
43. Kaighn, M. E., R. F. Camalier, F. Bertolero, and U. Saffiotti. 1988. Spontaneous establishment and characterisation of mouse keratinocyte cell lines in serum-free medium. *In Vitro Cell. Dev. Biol.* **24**:845–854.
44. Kano, Y., and J. B. Little. 1985. Mechanisms of human cell neoplastic transformation: relationship of specific abnormal clone formation to prolonged lifespan in X-irradiated human diploid fibroblasts. *Int. J. Cancer* **36**:407–413.
45. Kaur, P., and J. K. McDougall. 1988. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *J. Virol.* **62**:1917–1924.
46. Kaur, P., and J. K. McDougall. 1989. HPV18 immortalization of human keratinocytes. *Virology* **173**:302–310.
47. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. C. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**:2011–2015.
48. Klingelutz, A. J., S. A. Barber, P. P. Smith, K. Dyer, and J. K. McDougall. 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. *Mol. Cell. Biol.* **14**:961–969.
49. Klingelutz, A. J., S. A. Foster, and J. K. McDougall. 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**:79–82.
50. Lane, D. P., and S. Benchimol. 1990. p53: oncogene or anti-oncogene? *Genes Dev.* **4**:1–8.
51. Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty. 1992. Altered cell-cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70**:923–935.
52. Mak, I., S. Mak, and S. Benchimol. 1988. Expression of the cellular p53 protein in cells transformed by adenovirus 12 and viral DNA fragments. *Virology* **163**:201–204.
53. Merriek, R. M., R. J. A. Grand, J. C. Brown, and P. H. Gallimore. 1991. The use of β -galactosidase fusion proteins encoding the early region 1 transforming proteins of adenovirus type 12 to examine the humoral response in tumour-bearing animals. *J. Gen. Virol.* **72**:955–960.
54. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895–2902.
55. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7**:980–990.
56. Murnane, J. P., L. Sabatier, B. A. Marder, and W. F. Morgan. 1994. Telomere dynamics in an immortal human cell line. *EMBO J.* **13**:4953–4962.
57. Noda, A., Y. Ning, S. F. Venable, O. M. Pereira-Smith, and J. R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211**:90–98.
58. Pilder, S., M. Moore, J. Logan, and T. Shenk. 1986. The adenovirus E1B-55K transforming polypeptide modulates transport and cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* **6**:470–476.
59. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
60. Sarnow, P., Y. Shih Ho, J. Williams, and A. J. Levine. 1982. Adenovirus E1B-58kD tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kD cellular protein in transformed cells. *Cell* **28**:387–394.
61. Shay, J. W., W. E. Wright, D. Brasiskyte, and B. A. Van Der Haegen. 1993. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human epithelial cells but not in human fibroblasts. *Oncogene* **8**:1407–1413.
62. Stacey, M., P. H. Gallimore, C. M. McConville, and A. M. R. Taylor. 1990. Rearrangement of the same chromosome regions in different SV40 transformed human skin keratinocyte lines is associated with tumorigenicity. *Oncogene* **5**:727–739.
63. Steegenga, W. T., T. van Laar, A. Shvarts, C. Terleth, A. J. van der Eb, and A. G. Jochemsen. 1995. Distinct modulation of p53 activity in transcription and cell-cycle regulation by the large (54kDa) and small (21kDa) adenovirus E1B proteins. *Virology* **212**:543–554.
64. Tahara, H., E. Sato, A. Noda, and T. Ide. 1995. Increase in expression levels of *p21^{sd1/cip1/waf1}* with increasing division age in both normal and SV40-transformed cells. *Oncogene* **10**:835–840.
65. van den Elsen, P., A. Houweling, and A. J. van der Eb. 1983. Expression of region E1B of human adenoviruses in the absence of region E1A is not sufficient for complete transformation. *Virology* **128**:377–390.
66. Wang, S.-S., and V. A. Zakian. 1990. Telomere-telomere recombination provides an express pathway for telomere acquisition. *Nature* **345**:456–458.
67. White, E., R. Cipriani, P. Sabbatini, and A. Denton. 1991. Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. *J. Virol.* **65**:2968–2978.
68. Whittaker, J. L., P. J. Byrd, R. J. A. Grand, and P. H. Gallimore. 1984. Isolation and characterization of four adenovirus type 12-transformed human embryo kidney cell lines. *Mol. Cell. Biol.* **4**:110–116.
69. Wiesmüller, L., J. Cammenga, and W. W. Deppert. 1996. In vivo assay of p53 function in homologous recombination between simian virus 40 chromosomes. *J. Virol.* **70**:737–744.
70. Wright, W. E., O. M. Pereira-Smith, and J. W. Shay. 1989. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.* **9**:3088–3092.
71. Wynford-Thomas, D., J. A. Bond, F. S. Wyllie, and C. J. Jones. 1995. Does telomere shortening drive selection for p53 mutation in human cancer? *Mol. Carcinog.* **12**:119–123.
72. Yew, P. R., and A. J. Berk. 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* **357**:82–85.
73. Yew, P. R., X. Liu, and A. J. Berk. 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. *Genes Dev.* **8**:190–202.
74. Zacksenhaus, E., R. Bremner, Z. Jiang, R. M. Gill, M. Muncaster, M. Sopta, R. A. Phillips, and B. L. Gallie. 1993. Unravelling the function of the retinoblastoma gene. *Adv. Cancer Res.* **61**:115–141.
75. Zantema, A., P. I. Schrier, A. Davis-Olivier, T. van Laar, R. T. M. J. Vaessen, and A. J. van der Eb. 1985. Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells. *Mol. Cell. Biol.* **5**:3084–3091.
76. Zevy, M. Z., R. C. Allsopp, A. B. Futcher, C. W. Greider, and C. B. Harley. 1992. Telomere end-replication problem and cell aging. *J. Mol. Biol.* **225**:951–960.