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# Human telomerase reverse transcriptase-immortalized MRC-5 and HCA2 human fibroblasts are fully permissive for human cytomegalovirus

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**MRC-5 cells are a well-characterized human diploid fibroblast cell line approved for vaccine production and favoured for the routine propagation of human cytomegalovirus (HCMV). Ectopic expression of telomerase in fibroblasts is capable of overcoming replicative senescence induced by telomere shortening. Following delivery of the hTERT gene to MRC-5 cells using a retrovirus vector three clones were generated that (i) expressed functional telomerase activity, (ii) exhibited telomere extension and (iii) were sustained for > 100 population doublings. Immortalized MRC-5-hTERT and also HCA2-hTERT human fibroblasts were both fully permissive for HCMV as determined by plaque assay, studies of virus growth kinetics and measurement of virus yields. Furthermore, telomerase-immortalized HCA2 cells proved capable of supporting the stable maintenance of an EBV-based episomal vector with efficient transgene expression when driven by the HCMV immediate early promoter. An indicator cell line suitable for the efficient detection of HCMV infection was also generated using an episome containing a reporter gene (*lacZ*) under the control of the HCMV β-2.7 early promoter. Telomerase immortalization of human fibroblasts will thus facilitate the growth and detection of HCMV and also the generation of helper cell lines for the propagation of HCMV deletion mutants. Immortalization of fibroblasts by telomerase does not affect cell morphology or growth characteristics. The MRC-5-hTERT clones may therefore be suitable for additional applications in virology, cell biology, vaccine production and biotechnology.**

## Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus associated with lifelong persistence in its host. It is an important pathogen, being a significant cause of infectious mononucleosis, a major virus cause of congenital malformation and responsible for life-threatening disease in immunosuppressed or immuno-compromised individuals. *In vivo*, HCMV is capable of replicating in a wide range of cell types including fibroblasts, smooth muscle cells, endothelial cells, hepatocytes and tissue macrophages (Plachter *et al.*, 1996; Sinzger *et al.*, 1995). $CD34^+$ haematopoietic progenitors have been implicated as a site of virus latency, with re-activation being detected following differentiation to monocytes and virus release associated with a macrophage population expressing dendritic cell markers (Fish *et al.*, 1995; Soderberg-Naucler *et al.*, 1997). In cell culture, however, fully permissive HCMV replication associated with high titre virus production has been restricted almost exclusively to primary human fibroblasts.

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The finite replicative lifespan of primary human fibroblasts imposes restrictions on HCMV research. Adequate supplies of fibroblasts can be difficult for laboratories to sustain. As many laboratories acquire their cells from non-standard sources, comparing data can be problematical and there is an increased danger that adventitious agents may be introduced if continually refreshed short-term cultures are used, typically human foreskin fibroblasts. Fibroblasts also change significantly as they accrue population doublings. Productive *in vitro* HCMV infection has been demonstrated in a range of other cell types (e.g. endothelial cells, epithelial cells, trophoblasts and monocyte-derived macrophages), but replication tends to be slow, yields of infectious virus poor and the virus may have to be especially adapted for the target cell (Hart & Norval, 1981; Knowles, 1976; Fish *et al.*, 1995, 1998; Halwachs-

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Baumann *et al.*, 1998; Sinzger *et al.*, 1999). HCMV is capable of replicating in certain immortalized human cells. The pluripotent human embryonal carcinoma cell line Tera-2 is permissive, but only following retinoic-acid-induction of cellular differentiation (Gönczöl *et al.*, 1984, 1985). The U373 MG astrocytoma cell line is naturally permissive and extensively employed in research (Koval *et al.*, 1991). Human fibroblast lines immortalized by the human papillomavirus (HPV) type 16 E6 and E7 oncogenes can support the production of high titre virus. Such cells have proved invaluable for the complementation of HCMV deletion mutants (Compton, 1993; Greaves & Mocarski, 1998), but exhibit an atypical cellular morphology and the expression of HPV oncogenes can be expected to interfere with HCMV gene function assays.

MRC-5 cells are a human diploid fibroblast cell line first isolated in 1966 from normal lung tissue of a 14 week old foetus (Jacobs *et al.*, 1970). MRC-5 cells support efficient HCMV replication (Oram *et al.*, 1982), are the cell of choice for HCMV culture and detection (Boeckh *et al.*, 1991; Gregory & Menegus, 1983; Mazeron *et al.*, 1992), and have been used as a standard for over 30 years in basic research and vaccine production (Jacobs *et al.*, 1970). Although MRC-5 cells are capable of up to 46 population doublings, their limited lifespan has resulted in low passage stocks becoming increasingly difficult to source.

The limited lifespan of MRC-5 cells in culture is due to the onset of replicative or cellular senescence. Cells that have entered replicative senescence usually reside in $G_1$ phase and fail to enter S phase after the addition of growth factors. The phenotype of senescent cells differs in terms of gene activation and repression, cell morphology and possibly also in their capacity to support virus replication (Faragher & Kipling, 1998). In fibroblasts senescence is caused by erosion of chromosomal telomeres.

Telomeres protect the natural ends of linear chromosomes (Kipling, 1995) and are composed of arrays of $(TTAGGG)_n$ complexed with proteins such as hTRF1 (van Steensel & de Lange, 1997), hTRF2 (van Steensel *et al.*, 1998), tankyrase (Smith *et al.*, 1998), hRap1 (Li *et al.*, 2000), TIN2 (Kim *et al.*, 1999), the Mre11 complex (Zhu *et al.*, 2000) and others arranged into a T loop structure (Griffith *et al.*, 1999). Conventional DNA polymerases cannot fully duplicate the terminus of a linear molecule leading to an inexorable loss of terminal DNA with repeated cell division. The loss of telomeric DNA is in the order of 50–200 bp per division in somatic cells such as fibroblasts and telomere length decreases to a threshold of about 5 kb (including subtelomeric regions) in senescent cells.

Certain cell types such as stem cells and those of the germ line overcome the problem of telomere shortening by the action of telomerase. Mammalian telomerase synthesizes TTAGGG repeats *de novo* on to chromosome ends. Telomerase acts as a reverse transcriptase as the enzyme is associated with an RNA template encoding the telomeric repeat sequence. The telomerase RNA hTERC (or hTR) is expressed in most cell types. Therefore ectopic expression of the human telomerase reverse transcriptase gene (hTERT) alone is usually sufficient to restore telomerase activity (Weinrich *et al.*, 1997). Introduction of hTERT alone into fibroblasts restores telomerase activity, induces telomere extension and allows cells to avoid senescence and proliferate indefinitely (Bodnar *et al.*, 1998). Of great importance to the work described here is that immortalization of fibroblasts by telomerase does not confer changes associated with malignancy. Cells remain karyotypically normal, become quiescent at high density and under conditions of serum starvation, fail to grow in soft agar, fail to induce tumours *in vivo* and cell cycle checkpoints remain intact (Jiang *et al.*, 1999; Morales *et al.*, 1999). The cells retain the morphology of younger cells and do not express a $\beta$-galactosidase activity associated with senescent cells.

In order to facilitate the continued use of MRC-5 cells as a standard in the laboratory we exploited this recent alternative approach to cell immortalization by reactivating telomerase activity in these cells. Previously, we described the immortalization of HCA2 normal diploid fibroblasts and three fibroblast cultures taken from individuals with the progeroid Werner syndrome (Wyllie *et al.*, 2000). In this study, we demonstrate that MRC-5 cells can also be immortalized using hTERT and that these fibroblasts support efficient HCMV replication. hTERT-immortalized fibroblasts were also found to be capable of maintaining an Epstein–Barr virus-based episomal vector.

## Methods

**Cells and viruses.** MRC-5 human diploid fibroblasts passage 16 and human foetal foreskin fibroblast (HFFFs) were received from ECACC (European Collection of Cell Cultures, Porton Down, UK). HCA2 fibroblasts, HCA2-hTERT (Wyllie *et al.*, 2000) and the HPV-immortalized Ihf-2 cells (Greaves & Mocarski, 1998) have been described previously. 293 cells (Graham *et al.*, 1977) were used as a telomerase-positive control. All cells were cultured in Eagle's minimum essential medium supplemented with Earle's salts containing 10% (v/v) foetal calf serum, $1\times10^5$ IU/l penicillin, 100 mg/l streptomycin and 2 mM glutamine. HCMV infections were performed using strain AD169 or the recombinant RCMV288. RCMV288 is based on HCMV strain AD169 but has a copy of EGFP (enhanced green fluorescent protein) inserted in one copy of the HCMV long repeat under the control of the HCMV $\beta$-2.7 early promoter (between nucleotides 4576 to 2154 with respect to the strain AD169 genomic sequence). This insertion does not incapacitate the virus but provides a convenient reporter system for monitoring infection in live cells. All HCMV stocks were produced in HFFF cells.

**Immortalization of MRC-5 cells.** The cloning of the hTERT cDNA (Geron Corporation) into the retrovirus vector pBABE-puro to generate pBABE-hTERT has been described (Wyllie *et al.*, 2000). Both plasmids were first transfected into the ΩE cell line (Morgenstern & Land, 1990) and stable ecotropic retrovirus-producing cell lines were generated. Infection of ΨCRIP cells (Danos & Mulligan, 1988) with the ecotropic retrovirus followed by puromycin selection (2·5 µg/ml) generated a stable amphotropic retrovirus-producing cell line. MRC-5 cells were seeded in 60 mm dishes and infected with either pBABE-puro

control or pBABE-hTERT retrovirus supernatants derived from ΨCRIP producer cells. Cells were passed into new 100 mm dishes 2 days after infection at 1/2, 1/10, 1/50, 1/250 and 1/500 fold dilutions and the following day puromycin (1 μg/ml) selection was applied. In dishes seeded at low density after infection, colonies became apparent and were isolated by trypsinization within cloning rings and passage in 12-well dishes. One MRC-5 puro (clone 1) and three MRC-5-hTERT clones (clones 2, 3 and 4) were further characterized.

■ **Detection of telomerase activity.** Telomerase present in whole cell extracts was detected using the telomeric repeat amplification protocol (TRAP assay) essentially as described by Kim *et al*. (1994). Cells were harvested, washed in PBS then once in 10 mM HEPES–KOH pH 7·5, 1·5 mM $MgCl_2$, 1 mM KCl, 1 mM dithiothreitol before being lysed for 30 min by resuspension in 10 mM Tris–HCl pH 8·3, 1·5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0·5% CHAPS, 5 mM 2-mercapto-ethanol, 1 mM PMSF, 5000 cells per μl lysis buffer. Lysates were subjected to centrifugation at 100000 ***g*** for 30 min and the supernatant was retained and snap-frozen. The TRAP assay is a two stage protocol in which telomerase adds TTAGGG repeats to a primer. In the second stage extension products are detected by PCR. Cell extract (3000 cell equivalents) was added to 50 μl of a buffer containing 20 mM Tris–HCl pH 8·3, 1·5 mM $MgCl_2$, 63 mM KCl, 0·005% Tween 20, 1 mM EGTA, 50 μM dCTP, 50 μM TTP, 50 μM dGTP, 50 μM dATP, 0·1 mg/ml acetylated BSA, 1 μg T4 gene 32 protein and 100 ng TS primer (5′ AATCCGTCGAGCAGAGTT 3′) and incubated for 30 min at 30 °C in a thermal cycler. The temperature was then raised to 94 °C to destroy the telomerase activity and maintained while 2·5 U Taq polymerase, 100 ng CX primer (5′ CCCTTACCCTTACCCTTACCCTAA 3′) and $0·5 \times 10^{-18}$ g of ITAS (150 bp internal standard) were added. The samples were then subjected to 31 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s) and extension (72 °C, 90 s), then held at 4 °C. Negative controls were duplicate samples where the extract was heat denatured at 85 °C for 10 min prior to addition to the reaction. The 293 cell line provided the telomerase-positive control. Reaction products were separated on non-denaturing 10% polyacrylamide gels and visualized by Sybr Gold staining and fluorimaging on a STORM system using blue fluorescence mode (AP Biotech).

■ **Telomere length determination.** Incubation of genomic DNA with restriction endonucleases leaves a terminal restriction fragment (TRF) resistant to enzymatic digestion containing telomeric and sub-telomeric DNA. Separation of these on gels and hybridization with a TTAGGG-specific probe produces a smear representing a distribution of telomeric sequences of all the chromosomes from a population of cells. TRF length was determined by digesting 1 μg genomic DNA with *Hin*fI and *Rsa*I, followed by electrophoresis in 0·5% agarose gel. DNA was denatured by gel immersion in 1·5 M NaCl, 0·5 M NaOH (15 min) then neutralized with 1·5 M NaCl, 0·5 M Tris pH 8 (10 min). Gels were dried under a vacuum for 1 h at room temperature and 30 min at 50 °C. Dried gels were hybridized in 25 ml 5 × SSC, 0·5 mM sodium pyrophosphate, 10 mM $Na_2HPO_4$, 5 × Denhardt's solution. An oligonucleotide DNA probe 5′ $(CCCTAA)_3$ 3′ (500 ng), end-labelled using [$\gamma$-$^{32}$P] ATP and T4 polynucleotide kinase, was hybridized overnight at 37 °C with the dried gel, which was then washed extensively in 0·1 × SSC prior to phosphorimaging (STORM).

■ **Evaluation of virus growth of immortalized cells.** To measure plaquing efficiency confluent cells in 6-well plates were infected in triplicate with tenfold dilutions of an RCMV288 virus stock for 90 min in a rocking incubator. Cells were then washed with PBS, fresh medium was added and 10 days post-infection (p.i.) fluorescent green plaques (i.e. expressing EGFP) were enumerated using an inverted fluorescence microscope (Leica DMIRBE).

To monitor the rate of virus replication MRC-5 and MRC-5-hTERT clone 3 cultures were infected in duplicate with RCMV288 (m.o.i. of 0·1) for 90 min. At 3, 6, 9, 12 and 15 days p.i. tissue culture supernatant was harvested. The virus titre was then determined by plaque assay on HFFFs as above.

■ **Episomal vectors.** All EBV-based episomes were based on p220.2 (kindly provided by B. Sugden, University of Wisconsin, USA), which contains *ori*P, the EBNA-1 gene, the hygromycin selectable marker and a polylinker cloning site (Akrigg *et al*., 1991). The episome pAL357 encodes GFP under the control of the HCMV major immediate early promoter whilst the episome pAL105 contains *lacZ* under the control of the CMV *β*-2.7 early promoter. HCA2-hTERT cells seeded into a 60 mm dish were infected with a replication-deficient adenovirus RAd114 (m.o.i. of 30) (G. W. G. Wilkinson & N. Blake, unpublished data; Blake *et al*., 1997). Twenty-four hours later cells were transfected with the episome using Effectene (Qiagen) and stable transfectants selected using hygromycin (30 μg/ml). Prior infection with RAd114, an adenovirus expressing the EBV EBNA-1, improved the transfection efficiency achieved using EBV episomal vectors by seven- to tenfold (data not shown).

## Results

### Telomerase-immortalized MRC-5 cells

An amphotropic retrovirus encoding hTERT and an appropriate vector control encoding only the selectable marker were generated and used to transduce low passage MRC-5 cells as described in Methods. Passage number is an inexact definition that does not take into account the absolute numbers of cells carried over each time or their viability. A more accurate measure of a culture's age is the number of population doublings (pd) and this is therefore used here, calculated by counting cells after trypsinization and determining the percentage of viable cells re-seeded. Two days after cells were transduced by the recombinant retroviruses, they were seeded to tissue culture dishes at various densities and puromycin selection was applied. The cells seeded at high density rapidly formed monolayers and were subcultured into flasks. These populations, designated MRC-5 puro-mixed and MRC-5-hTERT-mixed, contain cells that have integrated the retroviral cassette into different areas of their genomes. MRC-5-hTERT cell lines were also cloned from single cells. In dishes seeded at low density after infection, colonies were harvested using cloning rings. A single clone was expanded from vector-transduced MRC-5 cells (MRC-5 puro clone 1) while simultaneously three independent clones were generated from hTERT-transduced cells (MRC-5-hTERT clones 2, 3 and 4). As a basis for further characterization of cell lines it was assumed that obtaining 1 million cells from a single cell required 20 population doublings (i.e. $2^{20} = 1·05 \times 10^6$).

### Assays of telomerase activity

In the presence of an appropriate primer, telomerase in whole cell extracts can promote the synthesis of TTAGGG repeats that can then be detected following PCR amplification in the TRAP assay. An extract is considered telomerase-positive if a DNA ladder of 6 bp periodicity is present and no

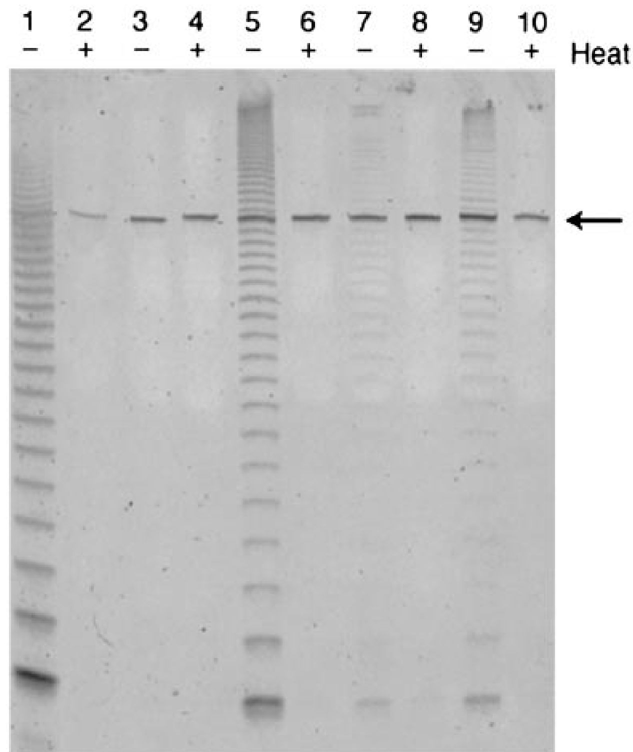


Fig. 1. Analysis of telomerase activity in MRC-5 subclones in a TRAP assay. Lanes 1 and 2, 293 cells; 3 and 4, MRC-5 pBABE puro clone 1 analysed at 26 pd after retrovirus transduction; 5 and 6, MRC-5-hTERT clone 2 (pd = 30); 7 and 8, MRC-5-hTERT clone 3 (pd = 24); 9 and 10, MRC-5-hTERT clone 4 (pd = 24). The arrow indicates the position of the internal telomerase amplification standard that provides a control for PCR.

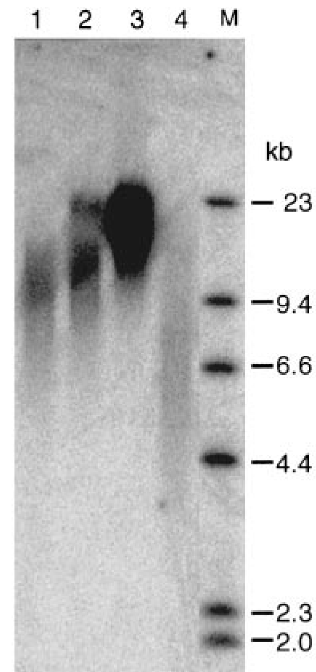


Fig. 2. Terminal restriction fragment analysis of hTERT-immortalized MRC-5 cells. Lanes 1, 2 and 3, MRC-5-hTERT clones 2, 3, 4 respectively; lane 4, MRC-5 bulk culture at pd 18. The molecular mass standard (M) is end-labelled *Hin*dIII-digested λ DNA. The presence of a diffuse hybridization band, mainly between the 9·4–23 kb markers, provides evidence of expanded telomeres in hTERT-transduced clones.

corresponding signal is present in the heat-treated control (Fig. 1). An internal standard (ITAS) is included to exclude false negatives due to the presence of *Taq* DNA polymerase inhibitors present in some cell extracts. The 293 cells provide a telomerase-positive control. Preliminary experiments established that untreated MRC-5 cells and MRC-5 puro-mixed were telomerase-negative while extracts prepared from MRC-5-hTERT-mixed cultures were telomerase-positive (data not shown). Individual MRC-5 subclones were also tested for telomerase activity. MRC-5 puro clone 1 remained telomerase-negative while MRC-5-hTERT clones 2, 3 and 4 all became telomerase-positive, indicating sustained expression of hTERT (Fig. 1).

The effect of hTERT expression on telomeres in the MRC-5-hTERT clones was investigated by hybridizing a telomere-specific oligonucleotide against restriction endonuclease-cleaved genomic DNA in a TRF assay. In MRC-5-hTERT clones 2, 3 and 4, but not in MRC-5 puro clone 1, the telomeres have been extended by the action of telomerase (Fig. 2).

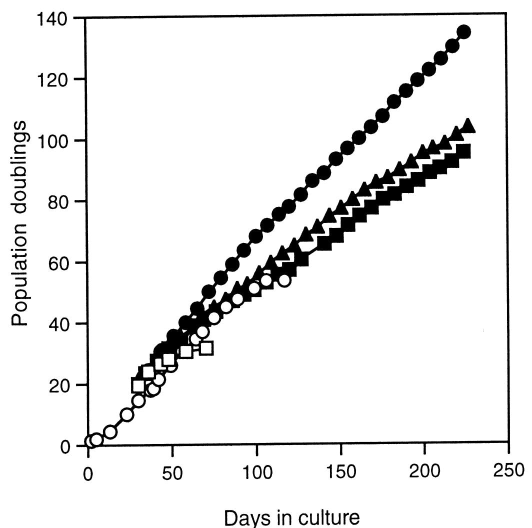


Fig. 3. Cumulative population doubling versus time for MRC-5 bulk culture (○), MRC-5 pBABE puro clone 1 (□) and the three characterized MRC-5-hTERT clones, MRC-5-hTERT2 (●), MRC-5-hTERT3 (▲) and MRC-5-hTERT4 (■). pd 0 is defined as the time of retrovirus infection.

## Measurement of lifespan extension

The definitive test of whether the MRC-5-hTERT clones were immortalized was determined by counting the number of population doublings the clones would undertake. MRC-5 bulk cultures and MRC-5 puro clone 1 gradually exhibited evidence of senescence based on the kinetics of growth and morphological changes associated with senescent cells (Fig. 3

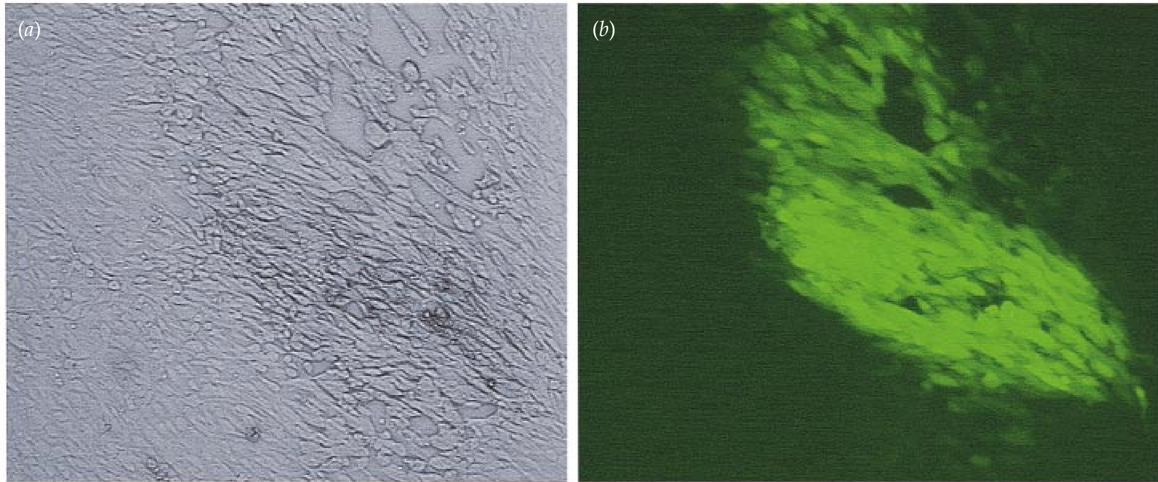


**Fig. 4.** Plaque produced by infection of hTERT-immortalized MRC-5 cells with RCMV288. (*a*) Bright field; (*b*) GFP fluorescence.

and data not shown). The bulk culture is a mix of cells whose individual members arrive at the senescence threshold at different times. The observation that MRC-5 puro clone 1 senesced before the bulk population of uninfected MRC-5 implies that the cell from which this clone was derived had relatively limited replicative potential. The MRC-5-hTERT subclones 2, 3 and 4 have been propagated through over 100+ population doublings while maintaining normal growth kinetics and cellular morphology. All three MRC-5-hTERT clones have continued to proliferate far beyond the normal senescence threshold and by these criteria we view them as immortal.

**Table 1.** Comparison of HCMV titres

| Cell line | Virus titre (p.f.u./ml) |
|---|---|
| HCA2 | $3{\cdot}33 \times 10^7$ |
| HCA2-hTERT | $1{\cdot}33 \times 10^7$ |
| Ihf-2 | $1{\cdot}2 \times 10^6$ |
| HFFF p18 | $9{\cdot}33 \times 10^7$ |
| MRC-5 p26 | $8{\cdot}33 \times 10^7$ |
| MRC-5-hTERT clone 2 | $7 \times 10^7$ |
| MRC-5-hTERT clone 3 | $5{\cdot}67 \times 10^7$ |
| MRC-5-hTERT clone 4 | $6{\cdot}33 \times 10^7$ |

## HCMV replication in hTERT-immortalized human diploid fibroblasts

Investigations into the capacity of HCMV to replicate in telomerase-immortalized fibroblasts were facilitated by using a virus encoding EGFP. RCMV288 is derived from HCMV strain AD169 and contains the EGFP gene inserted in one copy of the HCMV long repeat under the control of the HCMV *β*-2.7 early promoter. This insertion deletes one of two copies of the *β*-2.7 structural gene in strain AD169 but does not incapacitate the virus; indeed HCMV clinical isolates appear to contain only a single copy of this gene (Cha *et al*., 1996; B. P. McSharry & G. W. G. Wilkinson, unpublished data). Infection of MRC-5-hTERT clones 2, 3 and 4 with RCMV288 induced typical HCMV plaque production (an example is shown in Fig. 4). Telomerase-immortalized MRC-5 cells are therefore permissive for HCMV.

The relative susceptibility of hTERT-immortalized and non-immortalized MRC-5 and HCA2 fibroblasts was investigated by the simultaneous titration of a standard stock of RCMV288 in a plaque assay (Table 1). hTERT-immortalization of HCA2 and MRC-5 human fibroblasts did not affect the plaquing efficiency of HCMV, although slightly lower virus titres were recorded in both HCA2/HCA2-hTERT cells (Table 1). A greater than tenfold reduction in virus titre was obtained in the Ihf-2 cells, which are immortalized by HPV-16 E6 and E7. The small differences in RCMV288 titres observed between HFFFs, MRC-5 cells and MRC-5-hTERT cloned lines (Table 1) were not consistent in repeat experiments and are attributed to normal experimental variation. Even with high passage MRC-5-hTERT lines, HCMV titres were similar in HFFFs, MRC-5 cells and MRC-5-hTERT cells (not shown).

The rate of HCMV replication in MRC-5 and MRC-5-hTERT clone 3 cells was compared by infecting parallel cultures with RCMV288 (m.o.i. of 0·1) and assaying for virus in the supernatant at regular intervals. An increase in virus production was detected at day 6 p.i., with highest levels at days 9 and 12, then a small decrease followed at day 15 as the monolayer began to disintegrate (Fig. 5*a*). The kinetics of HCMV infection in MRC-5 and the telomerase-immortalized MRC-5 were identical. Neither the rate of infection nor the peak rate of virus production was significantly affected. The

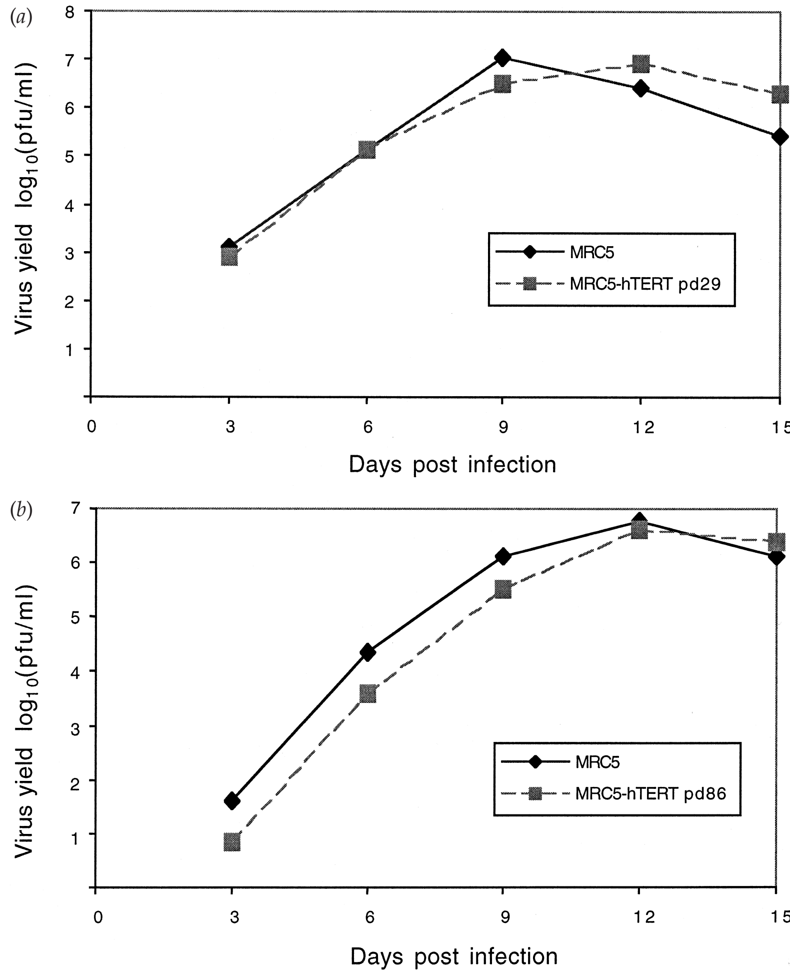


Fig. 5. Rate of HCMV (RCMV288) replication in MRC-5 and MRC-5-hTERT cells. (*a*) MRC-5-hTERT clones at pd 29 and (*b*) MRC-5-hTERT clones at pd 86.

experiment was subsequently repeated with a high passage culture of MRC-5-hTERT clone 3 cells. Although the rate of infection was slightly slower in the MRC-5-hTERT clone, the peak level of virus production was similar (Fig. 5 *b*).

## Maintenance of EBV-based episomes in hTERT-immortalized fibroblasts

Episomes based on the EBV latent origin of replication (*ori*P) are simple, efficient eukaryotic vectors providing for high level expression of transgenes. We wished to investigate whether telomerase-immortalized human fibroblasts were capable of sustaining episomal maintenance. In the first instance an episome encoding EGFP under the control of the HCMV major immediate early promoter (pAL357) was used to optimize conditions for episomal delivery. EBNA-1 encoded by the vector binds directly to *ori*P to promote vector replication and segregation to daughter cells. Prior infection with a replication-deficient adenovirus encoding EBNA-1 (RAd114) increased the efficiency of transient EGFP expression seven- to tenfold following pAL357 transfection and also the frequency with which stable cell lines were generated. The genome of the replication-deficient adenovirus recombinant does not encode a specific mechanism for long-term maintenance and is thus normally lost as the target cell divides. An example of an HCA2-TERT continuous cell line expressing GFP generated following hygromycin-selection of pAL357-transfected cell lines is illustrated in Fig. 6.

Fig. 6. Maintenance of episomes in immortalized fibroblasts (HCA2 cells). Expression of EGFP is readily detected in cells stably transfected with the episome pAL357 although levels are variable.

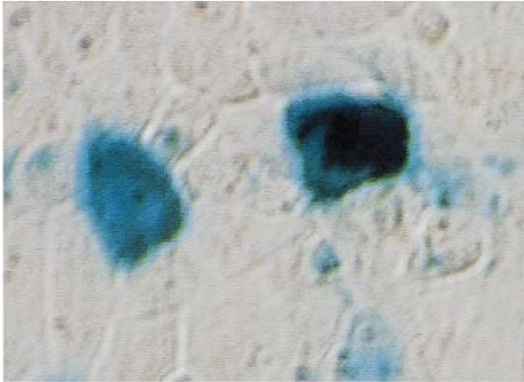

Fig. 7. Detection of HCMV infection using a reporter gene stably maintained in immortalized fibroblasts (HCA2 cells). HCA2 cells stably transfected with pAL105 (an episomal vector containing *lacZ* under the control of the HCMV β-2.7 promoter) were infected with HCMV and stained with the chromogenic substrate X-Gal. Infected cells stain blue due to the activated expression of the *lacZ* reporter gene.

### Generation of an HCMV-indicator cell line

The β-2.7 gene accounts for the majority of HCMV early phase transcription and remains the most abundantly expressed gene through the late phase of infection. The episome pAL105 encodes LacZ cloned under the control of the β-2.7 early promoter. HCA2-TERT cells were transfected with pAL105 and stable cell lines generated following hygromycin selection. Cells harbouring the pAL105 episome did not exhibit detectable levels of constitutive LacZ expression. However, transcription from the β-2.7 promoter was activated following HCMV infection, with infected cells readily identified as staining blue with the chromogenic substrate for β-galactosidase, X-Gal (Fig. 7). This indicator cell line provides a sensitive assay for HCMV infection.

## Discussion

Following delivery of the hTERT gene to MRC-5 cells using a retrovirus vector, expression of functional telomerase was demonstrated in three independent clones by a TRAP assay. Expression of the exogenous gene produced a substantial increase in telomere length in each of the three MRC-5-hTERT clones, enabling them to avoid replicative senescence normally associated with cell ageing. The MRC-5-hTERT clones continued to proliferate in excess of 100 population doublings, without any apparent change in cell morphology or growth rate, and are therefore considered to be immortal.

HCMV replication in both MRC-5 cells and the less characterized HCA2 diploid fibroblasts was not significantly affected by hTERT immortalization as determined by both plaque assay and measurements of virus growth rate. The generation of immortalized permissive human fibroblast lines will facilitate HCMV research. Human fibroblasts are slow-growing cells with a finite lifespan and problems are frequently encountered in generating and sustaining sufficient cell numbers for routine studies. Furthermore, bulk fibroblast cultures constitute a mixed population in which senescent cells appear at an early stage and the proportion increases steadily with population doubling as a consequence of telomere shortening. hTERT-immortalized fibroblasts will provide an unconstrained supply of cells together with a uniform population resistant to the effect of cell ageing.

Studies of HCMV gene function can now be performed in immortalized cells that support full productive HCMV infection and yet are not transformed by viral oncogenes. The generation of HCMV mutants is an arduous process, particularly when the gene being targeted proves to be essential for virus replication. A major aim in this study was to develop an effective cell system for the rescue of HCMV deletion mutants. Continuous cell lines generated by marker-selection following DNA transfection or by using retrovirus vectors tend to be associated with modest levels of a recombinant gene expression following chromosomal integration of the transgene. However, the generation of HCMV mutants in abundantly expressed essential genes can be expected to require high level expression of the complementing function for efficient rescue. The capacity of hTERT-immortalized cells to support the maintenance of an EBV-based episomal vector system was therefore investigated. Since the transfection efficiency was higher in HCA2-hTERT cells than in MRC-5-hTERT cells, they were preferred in these studies. Prior expression of EBNA-1 in cells is known to enhance the efficiency of transient expression and the generation of cell lines using episomal vectors (Langle-Rouault *et al.*, 1998) and this observation was confirmed in these studies. However, the use of a replication-deficient adenovirus recombinant encoding EBNA-1 to facilitate the generation of episomal cell lines is novel. Stable HCA2-hTERT cell lines containing an episome encoding EGFP were readily generated, demonstrating that hTERT-immortalized human fibroblasts were capable of efficiently maintaining an EBV-based episomal vector.

In a further development cell lines were generated using a second episome encoding LacZ under the control of the extremely strong, inducible HCMV β-2.7 early promoter. In uninfected cells, no significant LacZ expression could be detected, but after HCMV infection cells expressed high levels of β-galactosidase. This cell line provides an efficient and sensitive means to monitor active HCMV infection, to perform virus titrations and potentially detect viable virus in clinical samples. Similar cell lines could in principle be readily generated using alternative reporter genes. By substituting the appropriate HCMV gene for the reporter gene, this system can also be used to generate complementing cell lines. Furthermore, by using the inducible β-2.7 early promoter it should also be possible to construct helper cell lines capable of expressing cytotoxic gene products.

More generally, MRC-5 cells support the replication of a wide range of viruses other than HCMV and have proved an invaluable reagent in cell biology research. In particular, MRC-

5 cells are both approved and extensively used for vaccine production (e.g. polio, hepatitis A, rabies, varicella-zoster virus, HCMV). The gradual degeneration of MRC-5 cell stocks with time, passage number and expanded usage has implications for future vaccine development and production. hTERT-immortalized MRC-5 cells could be considered as a safe alternative for the production of viral vaccines. It may also prove feasible to immortalize MRC-5 cells with hTERT by plasmid transfection, dissociating the gene from a proviral element may be considered to enhance safety for pharmaceutical applications. Since fibroblast immortalization by telomerase does not result in changes to cell morphology and growth characteristics associated with malignant transformation, MRC-5-hTERT cells would provide a more homogeneous, reliable and unlimited supply of cells. MRC-5 cells are a non-transformed diploid fibroblast essentially free of adventitious agents and as such are potentially suitable for the production of other therapeutic reagents. With the expansion of recombinant DNA technology, an increasing number of therapeutic products and gene therapy vectors are being produced by eukaryotic cell culture. MRC-5-hTERT cells could also prove useful in the production of recombinant gene products.

**Note added in proof.** Since submission of this article the following related paper has been published which describes replication of HCMV in life-extended human fibroblasts expressing the catalytic subunit of human telomerase: W. A. Bresnahan, G. E. Hultman & T. Shenk (2000). Replication of wild-type and mutant human cytomegalovirus in life-extended diploid fibroblasts. *Journal of Virology* **74**, 10816–10818.

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