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Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination

(FLP and Cre recombinases/simian virus 40 large T/replicative cell senescence/telomeres/gene therapy)

KAREN A. WESTERMAN* AND PHILIPPE LEBOULCH*†‡

*Harvard–Massachusetts Institute of Technology Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139; and †Harvard Medical School, Hematology–Oncology Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115

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ABSTRACT **A procedure of reversible immortalization of primary cells was devised by retrovirus-mediated transfer of an oncogene that could be subsequently excised by site-specific recombination. This study focused on the early stages of immortalization: global induction of proliferation and life span extension of cell populations. Comparative analysis of Cre/LoxP and FLP/FRT recombination in this system indicated that only Cre/LoxP operates efficiently in primary cells. Pure populations of cells in which the oncogene is permanently excised were obtained, following differential selection of the cells. Cells reverted to their preimmortalized state, as indicated by changes in growth characteristics and p53 levels, and their fate conformed to the telomere hypothesis of replicative cell senescence. By permitting temporary and controlled expansion of primary cell populations without retaining the transferred oncogene, this strategy may facilitate gene therapy manipulations of cells unresponsive to exogenous growth factors and make practical gene targeting by homologous recombination in somatic cells. The combination of retroviral transfer and site-specific recombination should also extend gene expression studies to situations previously inaccessible to experimentation.**

Various research strategies and clinical applications, such as gene targeting by homologous recombination in somatic cells, have been hampered by difficulties in obtaining populations of primary cells that actively divide while maintaining their stage of differentiation. However, transfer of specific oncogenes can generate cell lines that propagate an intermediate stage of differentiation, a process known as cell immortalization. While only few cells become truly "immortal" following transfer of an immortalizing oncogene, early stages of immortalization involve global induction of proliferation and life span extension of cell populations (1). Notable examples include the following: (*i*) immortal multipotent neural cell lines have been successfully engrafted with appropriate cytoarchitecture in the brain of rodents (refs. 2 and 3; for review see ref. 4) and (*ii*) global, nonleukemogenic amplification of primitive hematopoietic stem cell populations has been recently obtained *in vivo* by retroviral transfer of HOXB4 cDNA (5). Studies with temperature-sensitive forms of oncogenes have also shown that inactivation of the oncoprotein at a nonpermissive temperature allows cells to resume apparently normal differentiation both *in vitro* and *in vivo* (ref. 2; for review see ref. 4), although "senescent-like" degenerative changes and growth arrest upon inactivation of the oncoprotein have been described (6).

Because the use of thermosensitive mutants has many limitations and is obviously inappropriate for clinical applications, we have devised a general gene transfer strategy, referred to as reversible immortalization, which allows temporary expansion of primary cell populations by transfer of an oncogene that can be subsequently excised by site-specific recombination. The present report focuses on the early stages of cell immortalization.

Our strategy required (*i*) efficient gene transfer to primary cells, (*ii*) stable chromosomal integration at single copy, and (*iii*) isolation of cell populations before and after excision of the oncogene. These requirements were met through the design of polycistronic retroviral vectors with dominant positive and negative selectable markers under stringent, alternative translational control. Both the Cre recombinase from *E. coli* (7) and the FLP recombinase from yeast (8) were evaluated in this system, in conjunction with their respective recombination targets, LoxP (7) and FRT (8). These recombinases have been shown previously to operate in mammalian cells and catalyze the excision of chromosomal DNA segments flanked by two recombination targets placed in direct orientation, leaving a single recombination target behind (ref. 7–8). Simian virus 40 large T (SV40T) was used as a prototypical immortalizing gene. We demonstrate the complete elimination of the transferred oncogene following site-specific recombination and differential selection of the cells and provide arguments substantiating phenotypic reversion of the cells to a preimmortalized state, according to growth characteristics and modulations in p53 levels. In addition, we observed that the cumulative number of cell divisions at the time when site-specific recombination is performed determines whether or not the cells undergo immediate growth arrest and degenerative changes, as predicted by the telomere hypothesis of replicative cell senescence.

MATERIALS AND METHODS

DNA Construction and Cell Culture. Constructions were made using standard techniques (9). Oligonucleotides were synthesized by Research Genetics (Huntsville, AL). Accuracy of DNA construction was verified by sequencing. LXSN retroviral vector (10) was provided by D. Miller (Fred Hutchinson Cancer Research Center, Seattle); hygromycin B (11) phosphotransferase gene was provided by D. Housman (Massachusetts Institute of Technology, Cambridge); herpes simplex virus thymidine kinase gene (11) was provided by M. R. Capecchi (University of Utah, Salt Lake City); U19 SV40T mutant gene (2) was provided by R. D. McKay (Massachusetts Institute of Technology, Cambridge) and G. Almazan (McGill University, Montreal); Cre recombinase gene (7) was provided by D. W. Ow (U.S. Department of Agriculture, Albany, CA); FLP recombinase (8) was provided by Stratagene (pOG44 vector); pBabe retroviral vector (12) was provided by R. Weinberg (Massachusetts Institute of Technology, Cam-

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Abbreviations: BSMC, primary smooth muscle cells from bovine aorta; MDHF, human foreskin cells; MPDL, mean population doubling level; RKC, primary rabbit kidney cells; SV40T, simian virus 40 large T.

‡To whom reprint requests should be addressed.

bridge); pcDNA1 was provided by Invitrogen; and pOPRS-VICAT was provided by Stratagene. Primary bovine smooth muscle cells from bovine aorta (BSMC) were provided by S. Hahn (Massachusetts Institute of Technology, Cambridge); primary rabbit kidney cells (RKC) and human foreskin cells (MDHF) were provided by BioWhittaker; NIH 3T3 cells were provided by American Type Culture Collection; BOSC23 cells (13) were provided by W. Pear and D. Baltimore (Rockefeller University, New York); and Ψcre and Ψcrip (14) cells were provided by R. C. Mulligan (Whitehead Institute and Massachusetts Institute of Technology, Cambridge).

NIH 3T3 cells, BSMC, and Ψcre and Ψcrip cells were grown at 37°C with 5% CO_2/95% air in DMEM-supplemented with 10% heat-inactivated calf serum, 4.5 mg of glucose per ml, 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. For BOSC23 cells and RKC, calf serum was replaced by 10% heat inactivated fetal calf serum. MDHF were grown at 37°C with 5% CO_2/95% air in a 3:1 mixture of DMEM and F12 medium (Sigma) with 10% fetal calf serum, the same supplements as above, and 0.4 μg of hydrocortisone per ml, 5 μg of insulin per ml, 5 μg of transferrin per ml, 10^{-10} M cholera toxin (GIBCO), 1.8×10^4 M adenine, 10 ng/ml epidermal growth factor (GIBCO), and 2×10^{-9} M 3,3',5-triodo-L-thyronine.

Cell Infection, Transfection, and Selection. The packaging cell lines BOSC23, Ψcre, and Ψcrip were grown and transfected as described (refs. 13 and 14). Plasmid DNAs were prepared by the Qiagen (Chatsworth, CA) procedure and transfected in BOSC23 cells using a calcium phosphate procedure (5 Prime → 3 Prime, Inc). Viral supernatants from producers were harvested and filtered as described (refs. 13 and 14). All infections were carried out in the presence of 8 μg of polybrene per ml (Sigma). Viral supernatants from BOSC23 were used to generate stable viral producers following infection and selection of Ψcre and Ψcrip. Virus titers were estimated by infection and selection of NIH 3T3 cells using standard calculations previously described (refs. 13 and 14). Detection of helper viruses was performed by a β-galactosidase mobilization assay as described (refs. 13 and 14). Selection was applied 2 days after infection, with the exception of gancyclovir selection, which was started after completion of G418 selection to avoid the "bystander" effect. Standard concentrations (1×) of selection agents were 320 μg/ml for hygromycin B (Calbiochem), 500 μg (active fraction)/ml for G418 (GIBCO), 5 μM for gancyclovir (Syntex), and 1.5 μg/ml for puromycin (Sigma). Packaging cells, RKC, and NIH 3T3 cells were selected with 1×, MDHF were selected with 0.5×, and BSMC were selected with 2× concentrations.

Southern and Western Blot Analysis and Immunofluorescence. Southern and Western blot analyses were performed using standard techniques (9). For Western blot analyses, SV40T monoclonal antibody (Ab-2 no. DPO2, Oncogene Science) and p53 monoclonal antibody (DO-1 no. SC-126, Santa Cruz Biotechnology) were diluted 100-fold. For immunofluorescence staining, SV40T antibody (2-fold dilution) was revealed with Texas red–antibody conjugate (Oncogene Science), and fluorescein isothiocyanate-labeled phalloidin (Sigma; 100-fold dilution) was used for counterstaining, as described (Oncogene Science).

Telomere Analysis. Southern blot analysis of terminal restriction fragments was performed as described (1). Genomic DNA was digested with *Hin*fI and *Rsa*I. In each lane, 1.0 μg of digested DNA was separated by electrophoresis on 0.5% agarose gel and hybridized with labeled human telomeric oligonucleotide probe $(CCCTAA)_4$, as described (1).

RESULTS

Strategy and Vector Design. Two polycistronic retroviral vectors, SSR#41 and #69, were first designed (Fig. 1). These vectors derive from LXSN (10), and comprise the following, from 5′ to 3′: (*i*) a long terminal repeat with packaging signal (Ψ+); (*ii*) an initiation codon followed by a recombination target (FRT in SSR#41; LoxP in SSR#69), whose overlapping open reading frame was fused to a hygromycin resistance/herpes simplex virus thymidine kinase fusion gene (11); (*iii*) the encephalomyocarditis virus internal ribosome entry site, which allows internal initiation of translation (17); (iv) the "supertransforming" U19 mutant of SV40T (2), from which the intron was deleted to avoid splicing of the viral transcript and prevent expression of SV40 small t; (*v*) a second recombination target in direct orientation followed in frame by the neomycin resistance (*neo*^{r}) gene, but, importantly, lacking an initiation codon; and (*vi*) another long terminal repeat preceded by its polypurine track. The configuration of these vectors was chosen so that only hygromycin resistance/herpes simplex virus thymidine kinase and SV40T should be expressed in transduced cells in the absence of recombinase. We predicted that, following transient transfection of recombinase-expressing constructs, the intervening DNA segment between the two recombination targets would be excised, with loss of both the hygromycin resistance/herpes simplex virus thymidine kinase fusion gene and the SV40T oncogene. In contrast, the *neo*^{r} gene should become functional by juxtaposition in frame of the initiation codon linked to the 5′ recombination target and the coding region of the *neo*^{r} gene. Thus, only the cells having excised SV40T should become simultaneously G418- and gancyclovir-resistant (Fig. 1). For potential applications to cell types that cannot be maintained *in vitro* long enough for G418 selection after excision of SV40T, we designed another retroviral vector, SSR#109 (Fig. 1). This vector is similar to SSR#69, except that the *neo*^{r} coding region has been replaced with that of the murine heat-stable antigen, since heat-stable antigen and its human homologue, CD24, have been shown previously to allow efficient isolation by fluorescence-activated cell sorting of cells infected with retroviruses expressing them, within a few hours of retroviral infection (15).

Cell Proliferation Is Induced in Transduced Primary Cells. Retroviruses were generated in ecotropic (Ψcre and Bosc23) and amphotropic (Ψcrip) packaging cells, with viral titers measured on NIH 3T3 cells in the range of 10^5 colony-forming units/ml of viral supernatants. No contamination with replication competent retroviruses was detected by provirus mobilization assay. Cell proliferation following infection was assayed on primary cells or cultures of finite life span whose mean population doubling levels (MPDL) could be determined: RKC, BSMC, and MDHF (BioWhittaker). Cells were infected with diluted viral supernatants to obtain only one proviral copy in most infected cells. Following infection with SSR#41 and #69, clones resistant to hygromycin arose, and all these died upon subsequent exposure to gancyclovir or G418. Southern and Western blot analysis of infected cells demonstrated stable proviral transmission and efficient SV40T expression (Fig. 2). Transduced primary cells showed modified growth properties and extended life spans characteristic of SV40T expression (Table 1 and Fig. 3).

Cre Recombinase but Not FLP Recombinase Triggers Efficient Site-Specific Recombination in Transduced Primary Cells. The procedure was assayed with retrovirally transduced NIH 3T3 cells and primary cells upon transient transfection of Cre- and FLP-expression vectors. For primary cells, recombination efficiencies were measured first at low MPDL, so that the bias of replicative cell senescence would not be introduced. Accuracy of the site-specific recombination and purity of the cell populations isolated were tested by Southern and Western blot analysis of G418- and gancyclovir-resistant cells (Fig. 2). Complete extinction of SV40T expression in all cells was confirmed by examining large numbers of cells under double immunofluorescence (Fig. 3). However, drastic differences in

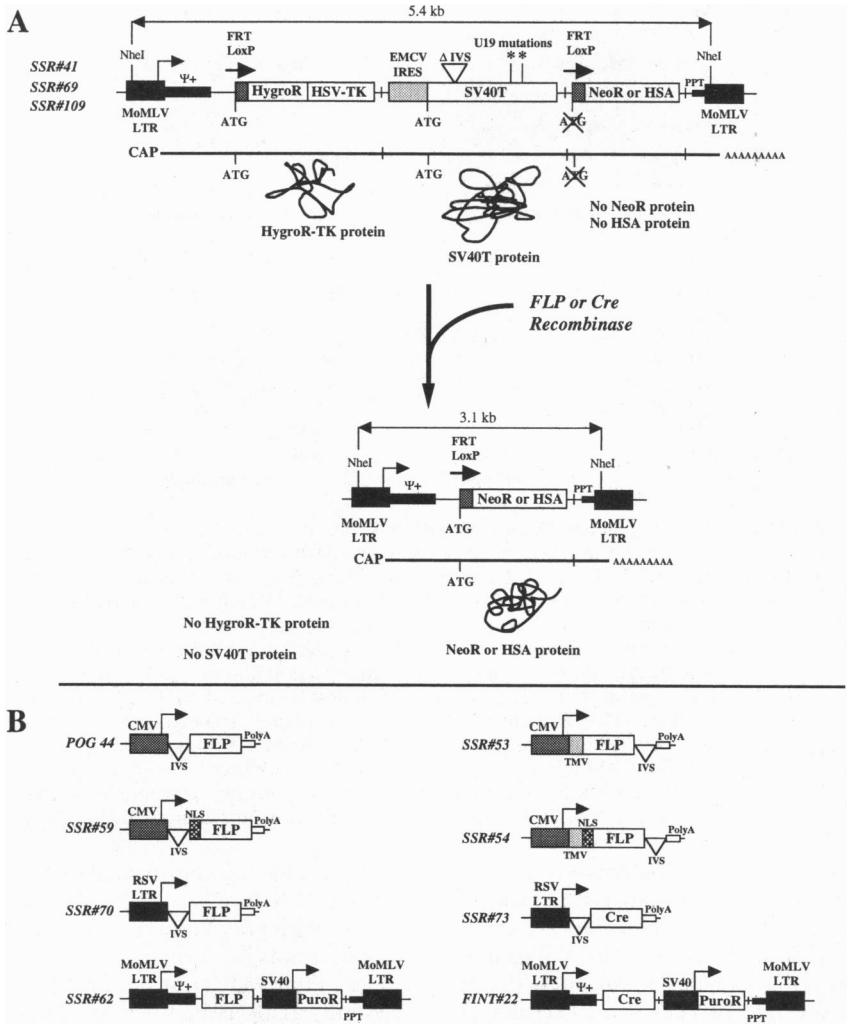

FIG. 1. (*A*) Schematic drawings of polycistronic proviral structures for SSR# 41, 69, and 109, before (*upper*) and after (*lower*) site-specific recombination. (*B*) Schematic drawings of POG#44 (5) (Stratagene) and the various Cre and FLP expression vectors constructed. CMV, human cytomegalovirus promoter; EMCV IRES, encephalomyocarditis virus internal ribosome entry site; HSA, heat stable antigen; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; PuroR, puromycin resistance gene; SV40, SV40 early promoter; RSV, Rous Sarcoma virus; PolyA, polyadenylation signal; IVS, synthetic intron; NLS, nuclear localization signal; and TMV, tobacco mosaic virus leader (translational enhancer) (23).

recombination efficiencies between Cre/LoxP and FLP/FRT were observed. FLP was much less efficient than Cre in NIH 3T3 cells, and only Cre-mediated recombination could be observed in primary cells (Table 1). In an effort to improve FLP-mediated recombination, several expression vectors were constructed in pcDNA1 and pOPRSVICAT, with various enhancer/promoters, intron locations, 5′ leaders, and addition of a nuclear localization signal (Fig. 1). None of these modifications was able to increase the efficiency of FLP/FRT recombination. To compare further the intrinsic recombination efficiencies of Cre/LoxP and FLP/FRT in this set-up, SSR#69- or #41-infected cells were superinfected with retroviral vectors deriving from pBabe (12) and transferring either the Cre or the FLP recombinase genes along with a gene cassette conferring resistance to puromycin (SSR#62 and FINT#22 vectors in Fig. 1). Virtually all (80–100%) FINT#22-infected cells (Cre recombinase) surviving puromycin selection had excised the SV40T gene, whereas no recombination could be detected with the SSR#62 virus (FLP recombinase) (Table 1).

Cells Revert to a Preimmortalized State Following Elimination of SV40T. We finally assessed the fate of the primary cells that had undergone the reversible immortalization procedure. Growth characteristics of the cells appeared to return to those of a preimmortalized state (Table 1). In addition, we evaluated the reversibility of molecular events immediately downstream of SV40T by monitoring steady-state levels of p53 protein, since p53 is believed to be stabilized in an inactive form upon interaction with SV40T (18). We found that p53 levels increased many-fold in SV40T-expressing cells, as compared with nonimmortalized cells, and that they returned to normal after Cre-mediated excision of SV40T (Fig. 2).

Excision of SV40T Gene in the M1–M2 Interval Leads to Replicative Cell Senescence. We also observed a striking correlation between the cumulative number of cell divisions at the time when site-specific recombination was performed and whether or not the cells underwent immediate growth arrest. This phenomenon was best explained by the theory of replicative cell senescence (ref. 1; for review see ref. 19). This theory predicts that somatic cells go through a finite number of divisions before they reach a crisis and stop dividing (M1 arrest). M1 arrest can be bypassed by transfer of an immortalizing oncogene, and cells continue to divide for several divisions before dying (M2 crisis), although a few clones may survive the M2 crisis through spontaneously acquired mutations. It has been proposed that M1 may be triggered by progressive telomere decay, which may lead to p53-dependent G1 arrest. Cells bypassing M1 arrest by expression

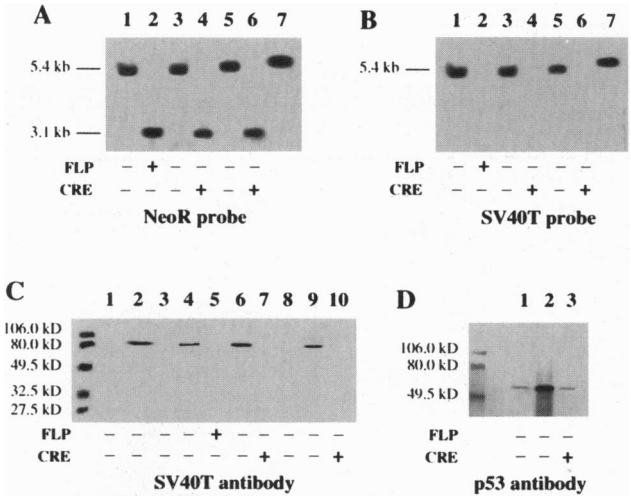

FIG. 2. (*A*) and (*B*) Southern blot analysis of genomic DNA from infected cells, before and after site-specific recombination and appropriate selection. Genomic DNA was cut with *Sac*I, which cuts in each long terminal repeat. Hybridization was performed with *neo*^{r} probe in (*A*) and SV40T probe in (*B*). Lanes 1 and 2, SSR#41-infected, NIH 3T3 cells, before and after FLP expression, respectively; lanes 3 and 4, SSR#69-infected, NIH 3T3 cells, before and after Cre expression, respectively; lanes 5 and 6, SSR#69-infected, MDHF, before and after Cre expression, respectively; and lane 7, *Sac*I-digested SSR#69 plasmid as size control. Cells in lanes 1, 3, and 5 were selected with hygromycin, and cells in lanes 2, 4, and 6 were subsequently selected with G418 following recombination. (*C*) Western blot analysis for SV40T expression in infected cells (antibody from Oncogene Science), before and after site-specific recombination and appropriate selection. Lanes 1 and 3, Noninfected NIH 3T3 cells; lane 2, NIH 3T3 cells infected with control retroviral vector pZipSV40T (1); lanes 4 and 5, SSR#41-infected NIH 3T3 cells, before and after FLP expression, respectively; lanes 6 and 7, SSR#69-infected NIH 3T3 cells, before and after Cre expression, respectively; lane 8, noninfected MDHF; lanes 9 and 10, SSR#69-infected MDHF, before and after Cre expression, respectively; Cells in lanes 4, 6, and 9 were selected with hygromycin, and cells in lanes 5, 7, and 10 were subsequently selected with G418 following recombination. (*D*) Western blot analysis for p53 expression in infected MDHF (antibody from Transduction Laboratories), before and after site-specific recombination and appropriate selection procedures. Lane 1, noninfected MDHF; lane 2, SSR#69-infected, hygromycin-selected, MDHF; and lane 3, same cells as in lane 2 but following Cre expression and G418 selection.

of SV40T continue to shorten their telomeres but go unchecked, possibly because p53 is rendered inactive. Survival beyond M2 for immortal cell lines appears to require spontaneous telomerase activation, which stabilizes chromosomes ends. To determine if this model is consistent with our observation, we studied the fate of cells after excision of SV40T, in function of their MPDL and telomere contents at the time of expression of the Cre recombinase. We found that cells having approached or reached the M1–M2 interval (Table 1) before expressing the recombinase led to very few clones with very limited expansion capability. When recombination was performed several divisions before cells would reach their M1 point, many clones arose in the Cre/LoxP system, as described above. Telomere decay appeared to parallel MPDL increment, as estimated by the average size of the terminal restriction fragment, whether or not cells

Table 1. Growth properties of cells and recombination frequencies

	Measured lifespans, MPDL			Frequency of FLP recombination, % (no. colonies)		Frequency of Cre recombination, % (no. of colonies)	
Cells	M1	M2*	Immortalization†	Transient expression‡	FLP Retrovirus§	Transient expression‡	Cre Retrovirus§
NIH 3T3	NA	NA	NA	0.05-0.5 (50–500)	~0 (0–3)	10–30 (1×10^4–3×10^4)	80–100 (10^5)
MDHF	≈40	≈60	+	0 (0)	0 (0)	1–5 (1×10^2-5×10^2)	80–100 (10^4)
BSMC	≈52	≈97	+	0 (0)	0 (0)	8–20 (8×10^2–2×10^3)	80–100 (10^4)
RKC	≈12	≈44	+	ND	ND	ND	ND

NIH 3T3 and primary cells were infected with diluted SSR#41 or SSR#69 retroviral supernatants, selected with hygromycin, and pooled (over 10^3 independent clones per pool). These pools were subsequently submitted to the recombination procedure by transient transfection or superinfection (number of cells per pool per transfection/superinfection experiment: 10^5 for NIH3T3 cells and 10^4 for MDHF and BSMC. Data were collected from at least four independent replicates. MPDL increments were estimated by cell counting, and recombination efficiencies in primary cells were measured before cells reached their expected M1 point. NA, not applicable; ND, not determined.

*M2 represents the measured cumulative lifespan extension in SV40T-expressing cells.

†Immortalization criteria include life span extension and increase in doubling times and plating efficiencies.

‡Cell pools were transiently transfected with SSR#1 (FLP) or SSR#73 (Cre) using cationic liposomes (Transfectam, Promega); recombination efficiencies were estimated by counting the proportion of cells that became G418 resistant (G418 selection was applied 2 days following transfection).

§Cell pools were superinfected with FLP or Cre-retroviruses (SSR#62 and FINT#22, respectively), using normalized viral titers (10^4/ml on NIH3T3 cells); superinfected cells were subsequently selected with puromycin. Puromycin-resistant cell pools were then selected with G418; recombination efficiencies within the puromycin resistant population were estimated by counting the proportion of cells that became G418-resistant.

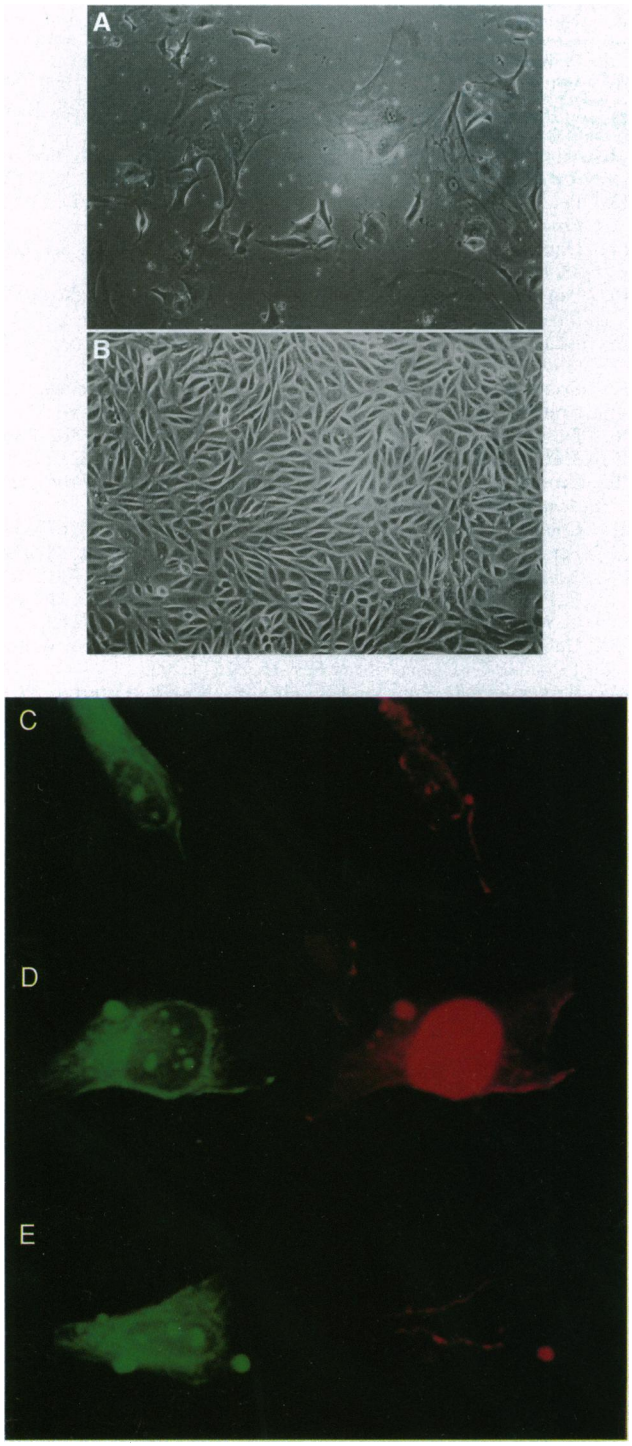

FIG. 3. (*A*) Nonimmortalized primary RKC grow slowly with low plating efficiency and short life span (≈12 MPDL). (B) SSR#41-infected RKC grow rapidly as islands or cords of epithelial cells and acquire extended life span (≈44 MPDL). (*C–E*) Double immunofluorescence of MDHF stained with fluorescein isothiocyanate-phalloidin (green), which binds actin specifically, together with monoclonal antibody anti-SV40T revealed with Texas red–antibody conjugate (red). Green and red fluorescence of single cells are shown separately on left and right parts of the picture, respectively. (*C*) Nonimmortalized MDHF; the dull red fluorescence indicates the nonspecific background level. (*D*) SSR#69-infected MDHF; SV40T expression is revealed by intense staining of the cell nucleus. (*E*) Same as in *D* but following Cre expression; the red fluorescence returns to background levels as seen in *C*.

were previously transduced with SV40T-expressing retrovirus (Fig. 4).

DISCUSSION

The Cre/LoxP reversible immortalization procedure may facilitate investigations of cell differentiation, oncogenesis, cell cycle, and senescence by allowing controlled cell proliferation and accurate on/off studies of various oncogenes in primary cells. For instance, the present report contributes with another recent evidence (20) to implicate directly p53 in the enforcement of the M1 checkpoint. We also anticipate the use of retroviral vectors to extend greatly the versatility of site-specific recombination to control gene expression in mammalian cells. We have shown that retroviruses can provide highly efficient chromosomal integration at single copy of genes flanked by LoxP sites, even in primary cells, and that virtually 100% recombination efficiency could be subsequently obtained using Cre-retroviruses. Our side-by-side comparison of FLP and Cre recombinases also demonstrates that Cre is highly superior to FLP in mammalian cells. Our experiments also indicate the existence of a time constraint, so that the various steps of the reversible immortalization procedure must be performed before the M1 point is reached to avoid premature senescence of the cells. As a corollary, our results support the current formulation of the telomere hypothesis of replicative cell senescence. Alternatively, it may be advantageous to excise the oncogene in truly immortal cell lines after M2, when telomeres have been spontanously stabilized. However, we favor the first situation to preserve genome integrity, since formation of dicentric chromosomes occurs at high frequency during the M1–M2 interval (ref. 1; for review see ref. 19). This time constraint might not exist with certain cell types, such as hematopoietic cells, which may constitutively express telomerase activity (21), or with specific combinations of oncogenes (20). Since several telomerase components have been recently cloned (refs. 22 and 23), transient expression of telomerase activity might also allow in the future the resetting of the mitotic clock during the Cre/LoxP reversible immortalization procedure to delay replicative senescence of the cells. Because the stringent positive and negative selection yielded highly pure cell populations having permanently excised the transferred oncogene, this method may also prove especially adapted for the safety concerns of cell and gene therapies. Taking advantage of the cell expansion phase to perform gene targeting by homologous recombination may make genetic diseases amenable to correction rather than gene addition.

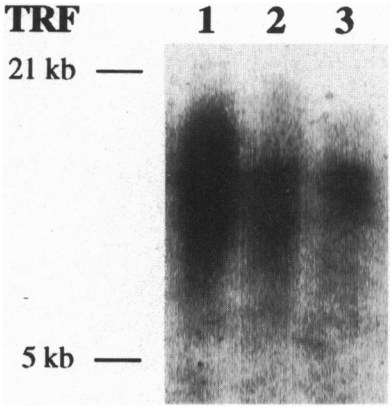

FIG. 4. Telomere shortening as MPDL increases, as shown by Southern blot analysis of terminal restriction fragments. Lane 1, nonimmortalized MDHF at MPDL 17; lane 2, nonimmortalized MDHF at MPDL 26; and lane 3, SSR#69-infected (at MPDL 9), hygromycin-selected, MDHF at MPDL 26. Intensity and mean length of the terminal restriction fragment (TRF) smear decrease as MPDL increases, in both nonimmortalized and immortalized cells.

Note Added in Proof. Bergemann *et al.* (24) have also obtained cre/Lox-mediated excision of DNA-sequences from integrated retroviral vectors in another context.

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1. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. & Bacchetti, S. (1992) *EMBO J.* **11,** 1921–1929.
2. Renfranz, P. J., Cunningham, M. G. & McKay, R. D. G. (1991) *Cell* **66,** 713–729.
3. Snyder, E. Y., Deitcher, D. L., Walsh, C., Arnold-Aldea, S., Hartwieg, E. A. & Cepko, C. L. (1992) *Cell* **68,** 33–51.
4. Geller, H. M., Quinones-Jenab, V., Poltorak, M. & Freed, W. J. (1991) *J. Cell Biochem.* **45,** 279–283.
5. Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M. & Humphries, R. K. (1995) *Genes Dev.* **9,** 1753–1765.
6. Mignotte, B., Larcher, J. C., Zheng, D. Q., Esnault, C., Coulaud, D. & Feunteun, J. (1990) *Oncogene* **5,** 1529–1533.
7. Sauer, B. & Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5166–5170.
8. O'Gorman, S., Fox, D. T. & Wahl, G. M. (1991) *Science* **251,** 1351–1355.
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
10. Miller, A. D. & Rosman, G. J. (1989) *Biotechniques* **7,** 980–990.
11. Lupton, S. D., Brunton, L. L., Kalberg, V. A. & Overell, R. W. (1991) *Mol. Cell. Biol.* **11,** 3374–3378.
12. Morgenstern, J. P. & Land, H. (1990) *Nucleic Acids Res.* **18,** 3587–3596.
13. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8392–8396.
14. Danos, O. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6460–6464.
15. Pawliuk, R., Kay, R., Lansdorp, P. & Humphries, R. K. (1994) *Blood* **84,** 2868–2877.
16. Adam, M. A., Ramesh, N., Miller, A. D. & Osborne, W. R. (1991) *J. Virol.* **65,** 4985–4990.
17. Coulier, F., Imbert, J., Albert, J., Jeunet, E., Lawrence, J. J., Crawford, L. & Birg, F. (1985) *EMBO J.* **4,** 3413–3418.
18. De Lange, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2882–2885.
19. Metz, T., Harris, A. W. & Adams, J. M. (1995) *Cell* **82,** 29–36.
20. Broccoli, D., Young, J. W. & De Lange, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9082–9086.
21. Collins, K., Kobayashi, R. & Greider, C. W. (1995) *Cell* **81,** 677–686.
22. Feng, J., Funk, W. D., Wang, S.-S., Weinrich, S. L., Avilion, A. A., Chiu, C.-P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W. & Villeponteau, B. (1995) *Science* **269,** 1236–1241.
23. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. (1987) *Nucleic Acids Res.* **15,** 3257–3273.
24. Bergemann, J., Kuhlcke, K., Fehse, B., Ratz, I., Ostertag, W. & Lother, H. (1995) *Nucleic Acids Res.* **23,** 4451–4456.