

Reversible Manipulation of Telomerase Expression and Telomere Length

IMPLICATIONS FOR THE IONIZING RADIATION RESPONSE AND REPLICATIVE SENESCENCE OF HUMAN CELLS*

Received for publication, April 18, 2002, and in revised form, May 24, 2002
Published, JBC Papers in Press, May 28, 2002, DOI 10.1074/jbc.M203747200

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Most human cells do not express telomerase and irreversibly arrest proliferation after a finite number of divisions (replicative senescence). Several lines of evidence suggest that replicative senescence is caused by short dysfunctional telomeres, which arise when DNA is replicated in the absence of adequate telomerase activity. We describe a method to reversibly bypass replicative senescence and generate mass cultures that have different average telomere lengths. A retrovirus carrying *hTERT* flanked by excision sites for Cre recombinase rendered normal human fibroblasts telomerase-positive and replicatively immortal. Superinfection with retroviruses carrying wild-type or mutant forms of TIN2, a negative regulator of telomere length, created telomerase-positive, immortal populations with varying average telomere lengths. Subsequent infection with a Cre-expressing retrovirus abolished telomerase activity, creating mortal cells with varying telomere lengths. Using these cell populations, we show that, after *hTERT* excision, cells senesce with shorter telomeres than parental cells. Moreover, long telomeres, but not telomerase, protected cells from the loss of division potential caused by ionizing radiation. Finally, although telomerase-negative cells with short telomeres senesced after fewer doublings than those with long telomeres, telomere length *per se* did not correlate with senescence. Our results support a role for telomere structure, rather than length, in replicative senescence.

Most eukaryotic cells do not divide indefinitely owing to a process termed replicative senescence. Replicative senescence was formally described more than four decades ago for cultures of normal human fibroblasts (1). Since that time, many cell types from many animal species have been shown to undergo replicative senescence, both in culture and *in vivo* (reviewed in Refs. 2 and 3). Recent data suggest that, at least in mammalian cells, replicative senescence is an example of a more general process, termed cellular senescence. Cellular senescence arrests cell proliferation (used here interchangeably with growth) in response to insults that have the potential to cause neoplastic transformation. These and other findings suggest that cel-

lular senescence is important for suppressing tumorigenesis in organisms with renewable tissues (4, 5). Cellular senescence has also been proposed to cause or contribute to aging in selected mammalian tissues (4, 6, 7).

Cellular senescence entails an essentially irreversible arrest of cell growth. In replicative senescence, this growth arrest occurs as a consequence of cell division. The number of divisions that normal cells complete before they senesce depends on the cell type and the species, age, and genotype of the donor (2, 3). This number can be large; for example, >80 population doublings (PD)¹ for fetal or neonatal human fibroblast cultures. In the last decade, it has become clear that human cells sense the number of divisions they have completed through the telomeres, which shorten progressively with each cell cycle owing to the inability of DNA polymerases to replicate the ends of linear chromosomes.

Telomeres are the repetitive DNA sequence and associated proteins that cap the ends of linear chromosomes. Telomeres allow cells to distinguish chromosome ends from double-strand DNA breaks and are essential for chromosome stability (8). Because the DNA replication machinery cannot completely replicate termini, 50–200 bp of telomeric DNA are lost during each S phase. This loss can be prevented by telomerase, the enzyme that adds telomeric DNA to termini *de novo*. Most human cells do not express telomerase, and thus their telomeres shorten with each division (9). Telomere shortening was first demonstrated in cultured human fibroblasts, which completely senesced with an average telomere length of 4–7 kb (reduced from 10–15 kb in the germ line) (10). These findings led to the hypothesis that human cells undergo a senescence growth arrest when their telomeres reach a critically short length (11). This hypothesis was strengthened by subsequent studies showing that ectopic expression of *hTERT*, the rate-limiting and catalytic subunit of telomerase, can prevent telomere erosion and replicative senescence (12, 13). Recent findings have refined, and added complexity to, this hypothesis.

First, telomere-associated proteins have been identified that regulate telomere length indirectly. Some of these proteins appear to alter the telomeric structure and hence the ability of telomerase to access the telomere (14–17). One such protein is TIN2. TIN2 negatively regulates telomere length in a telomerase-dependent fashion but does not act directly on the enzyme (18). Thus, telomerase expression alone may not be sufficient to prevent replicative senescence.

* This work was supported by the Ellison Medical Foundation, by Grant AG17242 from the NIA, National Institutes of Health (to J. C.), and by Grant 7KB-0151 from the University of California Breast Cancer Research Program (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PD, population doublings; IR, ionizing radiation; TRAP, telomerase repeat amplification protocol; TRF, terminal restriction fragment; RT, reverse transcription; CMV, cytomegalovirus; LI, labeling index; HYGRO, hygromycin; HISD, L-histidinol; PURO, puromycin.

Second, short telomeres may be more prone than long telomeres to structural dysfunction, and telomere function, rather than length, may control cellular senescence. Indeed, human fibroblasts that express ectopic telomerase can proliferate indefinitely with decidedly subsenescent telomere lengths (19). Likewise, in telomerase-deficient mice, the shortest telomeres appear to be responsible for the telomere dysfunction that compromises cellular and organismal survival (20). These findings suggest that telomerase can prevent cellular senescence by preferentially capping and acting on the shortest telomeres and that the senescence response is not induced by telomere length but, rather, by a dysfunctional telomere structure (21, 22).

Third, both replicative and cellular senescence induce similar phenotypes, but cellular senescence can occur after very few doublings and in response to stimuli that likely act independently of telomeres (17, 23–26). Some of these stimuli, however, may damage telomeres. For example, human fibroblasts cultured under hyperoxia senesce very rapidly but accumulate single-strand breaks at the telomeres (26). Conversely, telomeres may influence the sensitivity of cells and organisms to DNA-damaging agents such as ionizing radiation (IR). Thus, there was an inverse correlation between telomere length and chromosomal radiosensitivity in the lymphocytes of some breast cancer patients (27), and short telomeres enhanced IR-induced lethality in telomerase-deficient mice (28, 29). In addition, cells from organisms with defects in telomere maintenance are frequently radiosensitive (30–32). Moreover, telomerase has been reported to protect some cells from the lethality that results from severe damage to telomeres (33–35).

The above findings cast doubt on the relevance of telomere length *per se* in signaling the replicative senescence of human cells. They also suggest that telomeres can act as sensors or transducers of DNA damage signals and that telomerase can repair or protect cells from telomeric damage. One caveat to these possibilities is that cell types may differ in their responses to telomere dysfunction and/or DNA damage. Moreover, given the significant differences in telomere biology between rodents and humans (36, 37), it may not be possible to extrapolate findings in rodent cells to human cells.

To explore the relationships between telomere length, replicative senescence, and IR sensitivity, we created essentially isogenic human cell populations that have varying average telomere lengths. We started with normal human fibroblasts and used genetically defined manipulations. We show that telomere length does indeed influence replicative senescence but that human fibroblasts can senesce with telomeres that are significantly shorter or longer than expected. Our results support the idea that telomerase acts preferentially on short telomeres and that telomere structure, rather than length, causes replicative senescence. Finally, our results suggest that telomere length, but not telomerase, influences the response of human cells to IR.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Normal human fibroblast strains 82-6 and BJ (HCA2) were obtained from J. Oshima (University of Washington, Seattle) and J. Smith (University of Texas, San Antonio), respectively. Cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum, as described previously (38), until they reached 70–80% confluence, whereupon they were subcultured at $1\text{--}3 \times 10^3/\text{cm}^2$. To determine the fraction of senescent cells, cells were plated at $10^3/\text{cm}^2$ and labeled with [^3H]thymidine for 72 h; the percentage of labeled nuclei was determined by autoradiography and/or the cells were stained for the senescence-associated β -galactosidase, as described previously (38). The labeling index (LI) refers to the percentage of cells that incorporated [^3H]thymidine during the 72-h labeling interval. Cultures were considered completely senescent when the LI reached <10%.

Vectors—Retroviral vectors pLXSN, pLXSH, and pLXSHD were from

D. Miller (Fred Hutchinson Cancer Center, Seattle, WA) (39), and pBABE-PURO was from H. Land (Imperial Cancer Research Fund, London) (40). pCI-NEO-hTERT was from R. Weinberg (Whitehead Institute, Boston, MA) (41), and pLOX and pACN were from K. Thomas (42). pL-TIN2 and pL-TIN2-13 have been described previously (18). To create pLCRESH and pCMV-CRE, the 1.2-kb *PstI-MluI* fragment from pACN was subcloned in the *HpaI* site of pLXSH or *PstI* and *EcoRV* sites of pCMV-1. To create pBloxTSH, the 3.5-kb *EcoRI-SalI* fragment from pCI-NEO-hTERT was first cloned in the *EcoRI* and *SalI* sites of pBABE-PURO, to create pBABE-PURO-hTERT. We then cloned the 1.7-kb *HpaI-HindIII* L-histidinol resistance gene (HISD) from pLXSHD into the 8.2-kb *SalI-HindIII* fragment from pBABE-PURO-hTERT, creating pBTSH. The 5.2-kb *EcoRI-HindIII* fragment from pBTSH was excised, blunt ends were created with Klenow, and the fragment was cloned between the *loxP* sites in pLOX. The resulting plasmid was digested with *EcoRI* and *HindIII*, and the 5.2-kb fragment was subcloned into the *EcoRI-HindIII* sites in pBABE-PURO, creating pBloxTSH. Each cloning step was verified by sequencing across the ligation junctions.

Transfection and Infection of Cells—Fibroblasts were transfected using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. Infectious virus was produced by transient transfection, as described previously (43). Proliferating cells (30–50% confluent) were infected for 6 h on two successive days, with a 16-h interval and medium change between infections. Forty-eight h after the second infection, the cells were selected with the appropriate antibiotics (Sigma Chemical Co.): L-histidinol (50 mM, 7–10 days, using a 2 M stock solution in water, pH 7.0), hygromycin B (100 $\mu\text{g}/\text{ml}$, 5–7 days), puromycin (1 $\mu\text{g}/\text{ml}$, 3–5 days), or G418 (400 $\mu\text{g}/\text{ml}$, 5–7 days). Uninfected control cells did not survive these selection regimens. From the number of infected cells present after selection and the number of mock infected cells present without selection, we estimate that this protocol typically yielded 80–90% infected (antibiotic-resistant) cells.

Analysis of Telomere Length and Telomerase Activity—Genomic DNA was digested with *HinfI* and *RsaI* and analyzed by Southern blotting using a (TTAGGG) $_3$ probe, as described (10, 18). Signals were quantified using a PhosphorImager (Amersham Biosciences) and ImageQuant software. Mean TRF lengths were calculated as described previously (10). Values reported are the average results of at least two independent Southern blots. Telomerase activity was determined by the telomere repeat amplification protocol (TRAP), using a commercial kit (Intergen).

RT-PCR Analysis of Endogenous and Exogenous hTERT Expression—Total RNA was isolated, and the reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (43). The primers were: hTERT sense (2795–2816), 5'-CTG GGT GGC ACG GCT TTT GTT C-3'; hTERT antisense (3478–3499), 5'-GGG CTG CTG GTG TCT GCT CTC G-3'; pLXSHD antisense (1987–2008), 5'-ctc atg gtc cgg gat ctg cct c-3'. PCR conditions were 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min for 35 cycles. Fragments of 705 and 1021 bp were amplified from the endogenous and integrated retroviral hTERT genes, respectively.

Cell Proliferation and Survival Assays—Infected cells were maintained as continually proliferating cultures and passaged for the indicated intervals. Cell number was determined by trypsinizing the monolayer and counting a dilution using an electronic particle counter (Coulter). The population doubling (PD) number was calculated as $\text{PD} = \log(N_f/N_0)/\log 2$, where N_f is the final cell number and N_0 is the number of initially seeded cells. To determine IR sensitivity, proliferating cells were x-irradiated at the indicated doses using a Pantak x-ray generator operating at 320 kV/12 mA and allowed to recover for 4 h before trypsinization. The trypsinized cells ($0.5\text{--}32 \times 10^3$) were replated in duplicate 100-mm dishes and left undisturbed for 13 days. The plates were then stained with crystal violet, and the number of colonies that were >1 mm in diameter were counted.

RESULTS

Retroviral Vectors—We designed a retroviral-based system to allow conditional expression of telomerase and selection for cells that received or lost telomerase (Fig. 1). The system is based on the well-characterized ability of Cre recombinase to site-specifically excise DNA sequences that are flanked by *loxP* sites (44). The starting vector was pBABE-PURO (40), into which we introduced three modifications. First, we cloned into the multiple cloning site the cDNA encoding the catalytic subunit of human telomerase (hTERT) (41). Second, we introduced a histidinol resistance gene (HISD) from pLXSHD (39) between

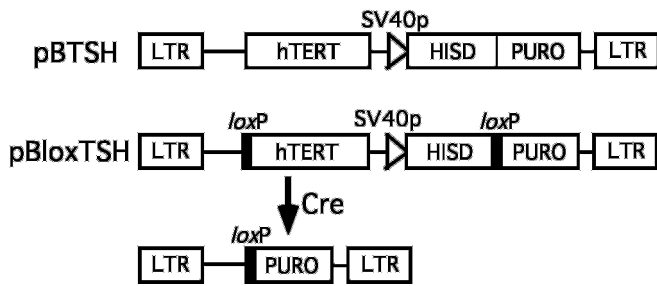


FIG. 1. Schematic representation of the retroviral vectors pBTSH and pBloxTSH before and after Cre-directed recombination. LTR, retroviral promoter; SV40p, SV40 early promoter.

the SV40 early promoter (SV40p) and puromycin resistance gene (PURO). This vector was designated pBTSH. Third, we introduced loxP sites upstream of the hTERT cDNA and between the HISD and PURO genes. This vector was designated pBloxTSH. Thus, pBTSH and pBloxTSH were identical except for the presence of loxP sites. We also used pLXSH (39) to create a retrovirus (pLCRESH) carrying Cre and the hygromycin resistance gene (HYGRO).

We expected normal telomerase-negative cells infected with either pBTSH or pBloxTSH to acquire telomerase activity and histidinol resistance. After superinfection with pLCRESH, we expected pBloxTSH-infected cells to acquire hygromycin resistance but lose telomerase activity and histidinol sensitivity, owing to Cre-mediated excision of the hTERT and HISD genes. Simultaneously, these cells should acquire puromycin resistance because the excision brings the PURO gene under the control of the retroviral 5' promoter (long terminal repeat). pBTSH-infected cells, by contrast, were predicted to acquire hygromycin resistance but no other changes after superinfection with pLCRESH (Fig. 1).

We infected normal human fibroblasts with these retroviruses, using a protocol that produced 80–90% infected cells. Thus, the manipulations we describe were applied to mass cultures, obviating the need for single cell cloning.

Reversible Telomerase Expression—We infected early passage (PD 20, LI = 74%) normal human fibroblasts (strain 82-6) with pLXSHD, pBTSH, or pBloxTSH and assessed uninfected and infected populations for their ability to survive and proliferate in the presence of L-histidinol. As expected, uninfected cells were histidinol-sensitive, whereas the infected cells were histidinol-resistant (Table I). We also assessed these populations for telomerase activity, using a telomerase repeat amplification protocol (TRAP). After histidinol selection, cells infected with the hTERT-expressing viruses, either pBloxTSH or pBTSH, showed robust telomerase activity (Table I; see also Fig. 3A below). Uninfected cells, and cells infected with pLXSHD, remained telomerase-negative (Table I).

Uninfected 82-6 fibroblasts typically senesce after 40–45 PD. Cells infected with pLXSHD senesced ~20 PD after infection (Fig. 2 and Table I), consistent with their lack of telomerase activity. Because 82-6 cells were infected at PD 20, this result confirms that our infection protocol does not substantially alter the replicative life span of the cells (Table I) or the mean telomere length at senescence (see below). In contrast to the telomerase-negative cells (uninfected and pLXSHD-infected), cells infected with pBloxTSH or pBTSH had markedly extended replicative life spans, showing no signs of senescence many PD after infection (Fig. 2). To date, these cells have undergone >100 PD and appear to be replicatively immortal.

We superinfected pBloxTSH- and pBTSH-infected cells with pLCRESH or pLXSH (control), both of which conferred hygromycin resistance, as expected (Table I). After selection in hygromycin, the cells were retested for histidinol sensitivity and

telomerase and tested for puromycin sensitivity. As expected, pLXSH did not alter the histidinol or puromycin sensitivity, that is, pBloxTSH- and pBTSH-infected cells remained histidinol-resistant and puromycin-sensitive. In addition, pLCRESH did not alter the histidinol and puromycin response of pBTSH-infected cells. However, pLCRESH completely reversed the antibiotic response of pBloxTSH-infected cells, abolishing resistance to histidinol and conferring puromycin resistance (Table I). Moreover, only pBloxTSH + pLCRESH doubly infected cells lost telomerase activity (Table I and Fig. 3A). This loss appeared to be complete, because lysates from these cells had no detectable TRAP activity, even when a 10-fold greater number of cells was assayed (Fig. 3A). Moreover, loss of telomerase activity resulted in progressive telomere shortening (see Fig. 4). Most important, pBloxTSH + pLCRESH doubly infected cells, but none of the other populations, underwent replicative senescence (Fig. 3B). The cells senesced 42 PD after hygromycin selection (Table I). Thus, after expression and excision of hTERT, cells underwent an additional 20 PD before senescing. Consistent with this additional proliferative potential, the cells senesced with shorter telomeres, compared with those of senescent cells that never expressed hTERT (Fig. 4).

Twenty-one PD after hTERT was excised from pBloxTSH cells by pLCRESH, seven cell clones were isolated. All the clones underwent senescence after an additional 15–18 PD (data not shown), consistent with the results obtained with the mass-infected population.

These results indicate that all the retroviruses conferred the predicted phenotypes on cells. Moreover, they demonstrate that sequential infection with pBloxTSH and pLCRESH can efficiently and reversibly immortalize uncloned populations of normal human fibroblasts.

We observed no deleterious effects owing to stable expression of Cre. However, Cre was reported to be mildly to moderately toxic in some cells (45). We therefore constructed a CRE expression vector (pCMV-CRE) suitable for transient transfection and expression. We then transfected pBloxTSH-infected fibroblasts with control (pCMV-1) or expression (pCMV-CRE) vector DNA, using a protocol by which about 10% of the cells transiently take up and express the transfected DNA. Cultures that received the control vector did not survive puromycin selection, but numerous puromycin-resistant cells grew out of cultures that received pCMV-CRE (Table I). After transfection and puromycin selection, 10^3 cells were replated. In two experiments, these cultures of pooled puromycin-resistant colonies were completely histidinol-sensitive and telomerase-negative, suggesting that transient expression of Cre had efficiently excised the hTERT and HISD genes (Table I). Eventually, these cultures senesced (Table I). Thus, reversible hTERT expression and immortalization was possible after transient expression of Cre.

In one experiment, 10^5 transfected cells were plated. Telomerase activity did not completely disappear, and a few cells remained histidinol-resistant (Table I). Because ectopically expressed hTERT was reported to induce the endogenous hTERT in human mammary epithelial cells (46), we asked whether the residual telomerase was derived from the endogenous hTERT gene. We used RT-PCR and primers to distinguish the retroviral and endogenous hTERT mRNAs (Fig. 3C). The results showed that the retroviral hTERT was expressed but endogenous hTERT was not (Fig. 3C). We surmise that one or a few cells in this population harbored more than one copy of pBloxTSH and that transient Cre expression was insufficient to excise all the copies of hTERT and HISD. Consequently, some cells retained histidinol resistance and hTERT but also became puromycin-resistant (Table I). As expected, these cells

TABLE I
Phenotypes of infected human fibroblasts

82–6 human fibroblasts were either mock infected (–) or infected with the indicated retroviruses. Cells were assessed for sensitivity to L-histidinol (HISD), puromycin (PURO) and hygromycin (HYGRO), telomerase activity (TRAP), and population doubling (PD) level at replicative senescence, as described in Experimental Procedures.

Virus 1	Plasmid ^a or virus 2	HISD ^b	PURO	HYGRO	TRAP	Senescence	PD ^c
pLXSHD		S	S	S	–	+	20–25 20
pBTSH		R	S	S	–	+	
pBloxTSH		R	S	S	+	–	
pBTSH	pLXSH	R	S	R	+	–	42
pBTSH	pLCRESH	R	S	R	+	–	
pBloxTSH	pLXSH	R	S	R	+	–	
pBloxTSH	pLCRESH	S	R	R	–	+	>30 ^{d,e}
pBloxTSH	pCMV-1	R	S	S	+	–	
pBloxTSH	pCMV-CRE	S	R	S	–	+	
pBloxTSH	pCMV-CRE	S/R	R	S	–/+ ^f	– ^f	

^a CMV-based vectors.

^b S, sensitive; R, resistant.

^c Number of PD, after the last infection, at which senescence occurred.

^d Approximation. PD can only be estimated because transfection efficiency is low (<10%).

^e Two out of three transfection experiments.

^f One out of three transfection experiments.

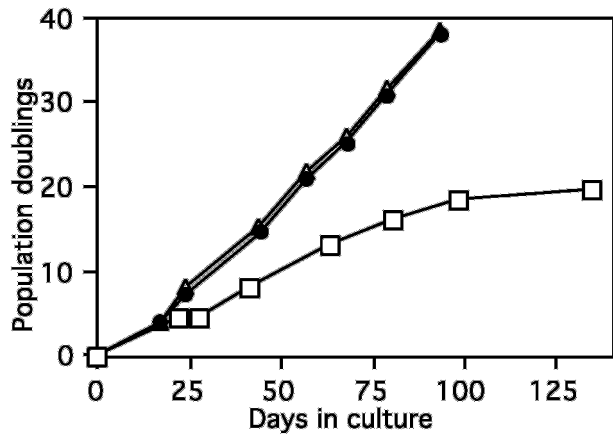


FIG. 2. Growth of 82-6 normal human fibroblasts infected with a control virus (pLXSHD) (□), the hTERT-expressing viruses pBTSH (△), or pBloxTSH (●).

eventually overgrew the culture and did not senesce (Table I). This was the only experiment in which we generated puromycin-resistant cells that retained histidinol resistance and telomerase activity, suggesting that incomplete excision by Cre is relatively rare when the recombinase is transiently expressed.

Human Fibroblasts with Different Telomere Lengths—To analyze the effects of pBTSH and pBloxTSH on telomere length, we used Southern blotting to analyze the sizes of the terminal restriction fragments (TRF) (Fig. 4). Within 9 PD, both viruses cause a slight increase in the mean TRF length, from 5.7 kb to 6.4 and 7 kb for pBloxTSH and pBTSH, respectively. We then used TIN2, a human telomere-associated protein that negatively regulates telomere length (18), to generate immortal cells with varying telomere lengths. TIN2 overexpression has been shown to modestly shorten telomeres, whereas dominant mutants such as TIN2–13 elongate them, both in a telomerase-dependent fashion (18).

We superinfected pBloxTSH-infected cells with a control virus (pLXSN) or viruses that express either wild-type (pL-TIN2) or dominant mutant (pL-TIN2–13) TIN2 proteins. These viruses carry a neomycin resistance gene (*NEO*), rendering infected cells resistant to the antibiotic G418. Their ability to produce the expected proteins has been described (18). After

G418 selection, the cells were propagated for 41–43 PD, after which TRF lengths were determined (Fig. 4). Control (pLXSN) cells had a mean TRF length of 5.7 kb (Fig. 4), compared with the 6.4-kb TRF of the parental (pBloxTSH-infected) cells (Fig. 4). Cells that received pL-TIN2 and overexpress wild-type TIN2 had slightly shorter TRFs (Fig. 4), averaging 5 kb. By contrast, cells that received pL-TIN2–13 and express the dominant mutant TIN2–13 had markedly longer TRFs (Fig. 4), averaging 9 kb. These findings confirm that functional TIN2 proteins were expressed and show that pBloxTSH can be used in conjunction with TIN2 proteins to alter telomere length.

Effects of Telomere Length on Replicative Life Span—To obtain mortal equivalents of the TIN2-expressing cells and determine the effect of telomere length on replicative life span, we infected the cells with control (pLXSH) or Cre-expressing (pLCRESH) retroviruses. Control-infected cells retained robust telomerase activity, whereas Cre expression completely abolished telomerase activity (Table II and Fig. 5A). Moreover, Cre rendered the cells puromycin-resistant and histidinol-sensitive (Table II). This result confirms that retrovirally delivered Cre efficiently excises *hTERT* and *HISD* from pBloxTSH in normal human fibroblasts and indicates that TIN2 proteins do not interfere with the excision of *hTERT*.

None of the telomerase-positive cells underwent replicative senescence, regardless of the presence of wild-type or mutant TIN2 proteins (Fig. 5B). These cells (infected with pBloxTSH, pLXSH, and either pLXSN, pL-TIN2, or pL-TIN2–13) have now undergone 99–103 PD and appear to be immortal. Interestingly, cells that overexpress wild-type TIN2 grew slightly more slowly than control (LXSN) cells or cells that express TIN2–13 (Fig. 5B). Nonetheless, they continue to proliferate without signs of senescence.

In contrast to the telomerase-positive cells, all cells that received the Cre-expressing retrovirus, and hence lost telomerase activity, underwent replicative senescence. However, the presence or absence of TIN2 proteins prior to *hTERT* excision strongly influenced the kinetics of senescence. Cells that received control virus (LXSN) senesced 37 PD after loss of *hTERT* (Table II and Fig. 5B). By contrast, cells that received wild-type TIN2 senesced 22 PD after losing *hTERT* (Table II and Fig. 5B), consistent with their slightly shorter telomeres. Conversely, cells that received TIN2–13 underwent more than

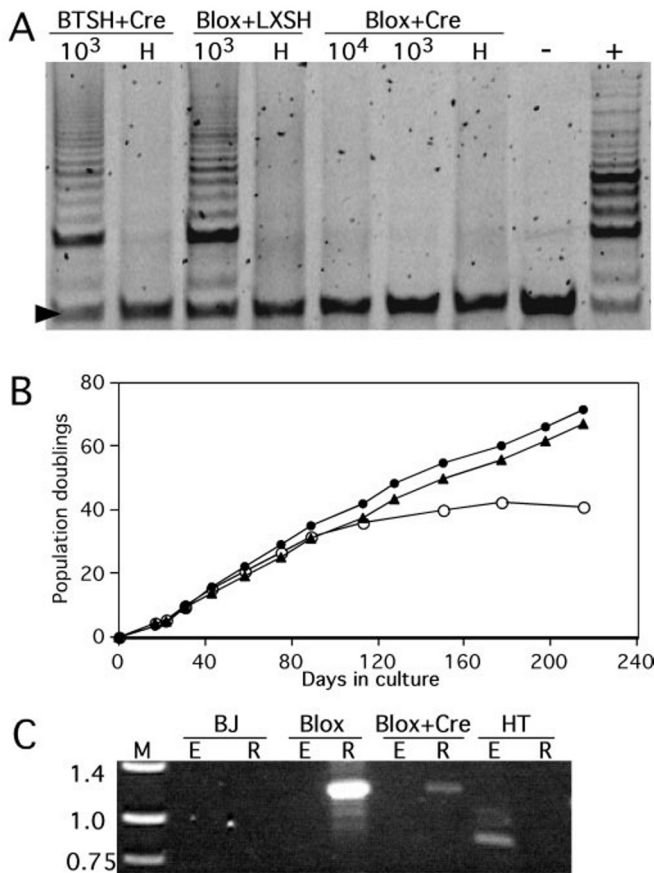


FIG. 3. *A*, telomerase activity in 82-6 cells infected with pBTSH (*BTSH*) or pBloxTSH (*Blox*), superinfected with either pLXSH (*LXSH*) or pLCRESH (*Cre*). Cell extracts from 10³ or 10⁴ cells were analyzed by TRAP assay, as described under "Experimental Procedures." Controls are heat-inactivated extracts from 10⁴ cells (*H*), a positive reaction (synthetic telomerase product) (+), and a cell free reaction (-). The arrowhead indicates the internal standard. *B*, growth of pBloxTSH (○) or pBTSH (▲)-expressing cells superinfected with pLCRESH. Also shown are pBloxTSH-expressing cells infected with pLXSH (control) virus (●). *C*, detection by RT-PCR of hTERT mRNA from the endogenous (*E*) or pBloxTSH retroviral (*R*) gene. See text for details. RNA was analyzed from normal (telomerase-negative) BJ fibroblasts (*BJ*, negative control), 82-6 fibroblasts infected with pBloxTSH before (*Blox*) and after (*Blox+Cre*) transfection with pCMV-CRE and puromycin selection, and telomerase-positive HT1080 human fibrosarcoma cells (*HT*, positive control). *M*, molecular size markers, indicated in kilobases.

twice the PD completed by control cells, senescing after 84 PD (Table II and Fig. 5*B*), consistent with their longer telomeres (Fig. 4). Interestingly, as discussed below, these cells senesced with longer than expected telomeres.

These results demonstrate, in essentially isogenic mortal human cells, that replicative life span is strongly influenced by telomere length, confirming similar conclusions derived from human tumor cells (47) and human fibroblasts from different donors (48).

Effects of TIN2 on Telomere Length—As noted, pBloxTSH-infected cells had an initial mean TRF length of 6.4 kb (Fig. 4). After superinfection with a control LXSN virus, the TRF shortened slightly over 43 PD to 5.7 kb (Fig. 4). Overexpression of wild-type or mutant TIN2 proteins for 42 PD altered the mean TRF to 5 and 9 kb, respectively (Fig. 4). We followed these cells after infection with pLCRESH or pLXSH.

Cells that received control viruses (pLXSN and pLXSH) showed slight TRF shortening, from 5.7 to 5.3 kb over 103 PD (Fig. 6). Thus, the telomerase delivered by pBloxTSH produced an initial modest elongation of telomeres, after which te-

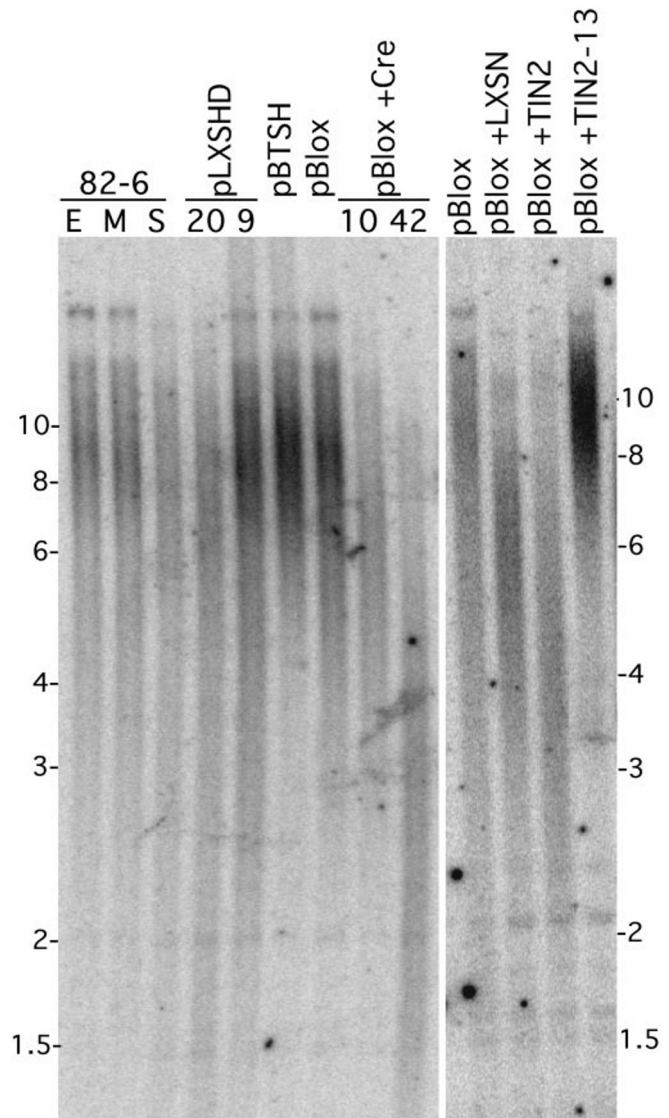


FIG. 4. **TRF length of 82-6 cells infected with various retroviruses.** TRF length was determined by Southern blotting as described under "Experimental Procedures." Cells were assayed at early passage (*E*), middle passage, before the first infection (*M*), and senescence (*S*). Changes in TRF length were observed after infection with pLXSHD, pBTSH, or pBloxTSH before (*pBlox*) or after infection with pLCRESH (*Cre*), pLXSN, pL-TIN2, or pLTIN2-13. Numbers indicate the PD after the last infection (the order of infections is shown in Tables I and II). Molecular size markers are shown in kilobases.

lomeres slowly shortened slightly, but there were no major changes in telomere length over many ensuing PD. In contrast, cells that received wild-type TIN2-overexpressing virus (L-TIN2) and pLXSH showed gradual telomere shortening (Fig. 6*B*), despite remaining telomerase-positive (Fig. 5*A*). After 99 PD, these cells had an average telomere length of 3.4 kb. Nonetheless, they continue to proliferate, albeit slightly more slowly than control cells (Fig. 5*B*), consistent with the idea that telomerase can cap and act preferentially on short telomeres (19, 21, 49). Conversely, cells that received the mutant TIN2-expressing virus (L-TIN2-13) and pLXSH showed telomere elongation, which appeared to stabilize at an average of 8.4 kb after 102 PD (Fig. 6, *A* and *B*). Cultures that received the TIN2-13 mutant initially (PD 11) displayed a bimodal distribution of telomere lengths, with some TRFs having an estimated average length of 8 kb and others having an average length of >13 kb (Fig. 6*A*). At later passages (PD 102), however,

TABLE II
Phenotypes of 82-6-infected cells

See legend to Table 1 for details.

Virus 1	Virus 2	Virus 3	HISD ^a	PURO	HYGRO	NEO	TRAP	Senescence	PD ^b
pBloxTSH	pLXSN	pLXSH	R	S	R	R	+	—	37
pBloxTSH	pLXSN	pLCRESH	S	R	R	R	—	+	
pBloxTSH	pL-TIN2	pLXSH	R	S	R	R	+	—	22
pBloxTSH	pL-TIN2	pLCRESH	S	R	R	R	—	+	
pBloxTSH	pL-TIN2-13	pLXSH	R	S	R	R	+	—	84
pBloxTSH	pL-TIN2-13	pLCRESH	S	R	R	R	—	+	

^a S, sensitive; R, resistant.
^b Number of PD, after the last infection, at which senescence occurred.

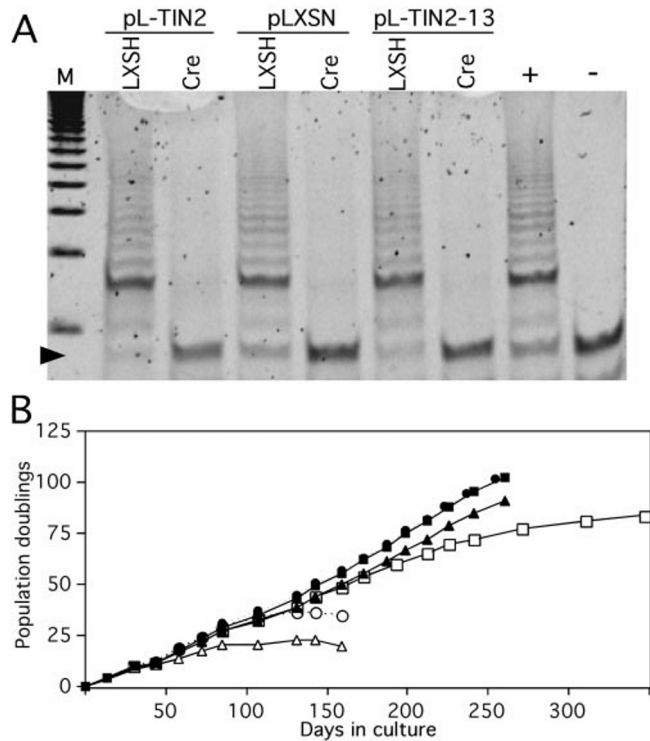


FIG. 5. A, TRAP assay showing loss of telomerase activity after pBloxTSH-expressing cells are superinfected with control (pLXSN) or TIN2-expressing (pL-TIN2, pL-TIN2-13) retroviruses then infected with control (LXSH) or Cre-expressing (pLCRESH) viruses. Extracts were prepared from 10^3 cells. M, 20-bp ladder. The arrowhead, +, and - controls are described in the legend to Fig. 3A. B, growth of 82-6 + pBloxTSH cells after manipulation of telomere lengths by expression of TIN2 proteins, and superinfection with Cre-expressing (pLCRESH) or control (pLXSH) viruses. Open symbols indicate telomerase-negative cells, closed symbols indicate telomerase-positive cells: pL-TIN2 + pLXSH (▲); pLXSN + pLXSH (●); pL-TIN2-13 + pLXSH (■); pL-TIN2 + pLCRESH (△); pLXSN + pLCRESH (○); pL-TIN2-13 + pLCRESH (□).

the longer telomere signals had largely disappeared (data not shown), suggesting that extremely long telomeres may be disadvantageous to cells.

Telomere Lengths at Senescence—We next followed telomere length in control, TIN2, and TIN2-13-expressing cells after excision of *hTERT*. Loss of telomerase activity caused all the cell populations to progressively lose telomeric sequences. Control cells lost telomeric DNA over about 37 PD, senescing with a mean TRF of 3.1 kb (Table II and Fig. 6), similar to the 42 PD and 3.2-kb TRF of senescent cells infected with only pBloxTSH and pLCRESH (Table I and Fig. 4). Thus, control cells senesced with telomeres that were, on average, shorter than those of unmodified cells. This finding indicates that transient expression of telomerase reduces the average telomere length at

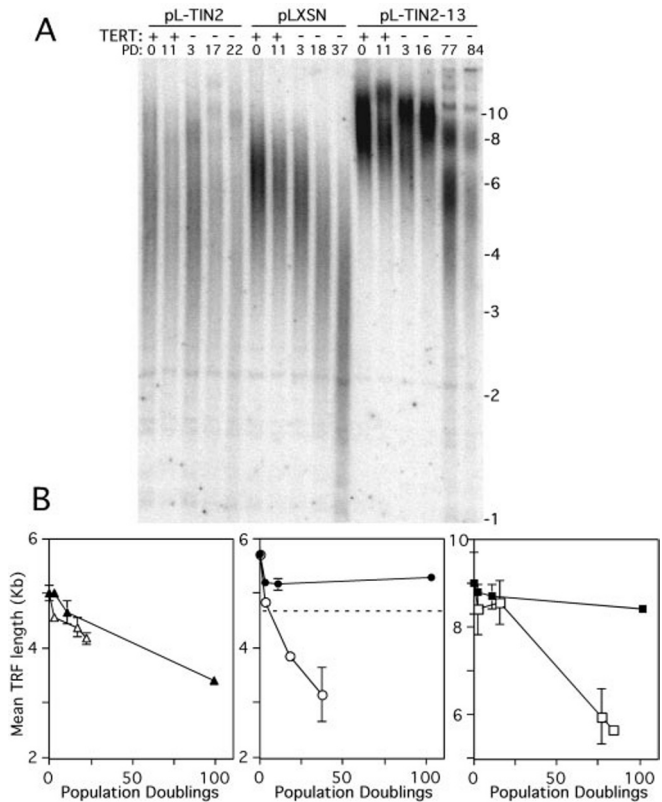


FIG. 6. A, TRF length of 82-6 + pBloxTSH fibroblasts, infected with pLXSN, pL-TIN2, or pL-TIN2-13. Cells were analyzed at the indicated PD just before (PD = 0) or after infection with pLXSH (TERT+) or pLCRESH (TERT-). TERT- cells senesced at PD 22, 37, and 84. Molecular size markers are indicated in kilobases. B, change in mean TRF length with PD. Open symbols indicate telomerase-negative cells, closed symbols indicate telomerase-positive cells: pL-TIN2 + pLXSH (▲); pLXSN + pLXSH (●); pL-TIN2-13 + pLXSH (■); pL-TIN2 + pLCRESH (△); pLXSN + pLCRESH (○); pL-TIN2-13 + pLCRESH (□). Error bars show the \pm S.D. of at least two independent experiments. Some S.D.s are smaller than the size of the symbol. The horizontal dashed line indicates the mean TRF length with which unmodified cells senesced.

which cells senesce, supporting the idea that telomerase can cap short telomeres.

Surprisingly, cells that overexpressed wild-type TIN2 senesced with an average TRF of 4.2 kb, longer than the length with which control cells senesced. These cells also senesced more quickly than control cells (Table II and Fig. 6, A and B). Thus, overexpression of wild-type TIN2 increased the telomere length at which cells senesced after removal of telomerase. Nonetheless, in the presence of telomerase and TIN2 overexpression, cells proliferated with a mean TRF of 3.4 kb (Fig. 6B). These findings are consistent with the idea that TIN2 limits

telomerase from accessing the telomere, thereby decreasing its efficiency at capping the shortest telomeres.

Even more striking, cells that expressed TIN2-13 senesced with an average TRF of 5.7 kb (Fig. 6, A and B). These cells underwent many more PD before senescence than control or wild-type TIN2-expressing cells (Table II and Fig. 5B), consistent with their longer telomeres, but they senesced with telomeres that were longer than any of the other cell populations. Of particular interest, the TRFs from senescent TIN2-13-expressing cells showed considerable size heterogeneity, with some signals as high as 8.7 kb and others as low as 5.3 kb (Fig. 6A). Nonetheless, signals of <5 kb, characteristic of the telomeres of senescent control cells, were not detected (however, the presence of rare short telomeres cannot be ruled out). These findings are consistent with the hypothesis that TIN2-13 promotes an open telomeric structure (18).

Together, these results support the idea (19, 20) that average telomere length *per se* is not a critical determinant of replicative senescence. They also support the idea that it is the telomeric structure that is critical in determining the senescence response of human cells. However, it is also possible that the long replicative life span of TIN2-13-expressing cells causes accumulated damage at the telomeres, which could also explain their relatively long telomeres at senescence.

Effects of Telomere Length and Telomerase on IR Response—To explore the relationship between telomere length, DNA damage, and the senescence response, we serially passaged normal human fibroblasts (strain BJ/HCA2) to obtain cells with different telomere lengths owing to telomere attrition. Cells at PD 20, 35, 69, and 71 (labeling indices (LI) 96, 86, 53, and 44%, respectively) were x-irradiated at doses (0–7 Gy) known to cause a senescence arrest in normal human fibroblasts (50, 51). The cells were assessed for ability to form visible clones. The results showed that IR sensitivity increased with increasing PD (Fig. 7A), suggesting that unmodified normal human fibroblasts with shorter telomeres are more sensitive to IR than those with longer telomeres.

To test this idea more critically, we used the reversibly immortalized human fibroblasts with different telomere lengths described above. The cells were made telomerase-positive (infected with pBloxTSH), allowed to acquire telomeres of different lengths (infected with TIN2 or TIN2-13-expressing viruses), and then were made telomerase-negative (infected with pLCRESH) or allowed to remain telomerase-positive (infected with pLXSH). Telomerase-positive cells with varying telomere lengths were x-irradiated (5 Gy) or not, and the number of cells in the mass cultures was followed over 4 days (Fig. 7B). Non-irradiated cells grew exponentially, whereas irradiated cells showed only minimal cell proliferation over this interval. Telomerase-negative cells behaved similarly (not shown). This result confirms that, at this dose, IR does not induce apoptosis but, rather, arrests the growth of normal human fibroblasts (50, 51). Moreover, they show that telomerase does not prevent the IR-induced growth arrest. The same cells were tested for proliferative potential in clonogenic assays (Fig. 7C). Proliferative potential declined with increasing IR dose. Moreover, cells with shorter telomeres (expressing wild-type TIN2) were more sensitive than control cells. However, cells with longer telomeres (expressing TIN2-13) were as sensitive as control cells. Thus, short telomeres render cells IR-sensitive, but longer than average telomeres do not render cells IR-resistant.

Similar results were obtained when cells were rendered telomerase-negative (infected with pLCRESH). Control and TIN2-13-expressing cells were similarly sensitive to IR, whereas cells expressing wild-type TIN2 were more sensitive

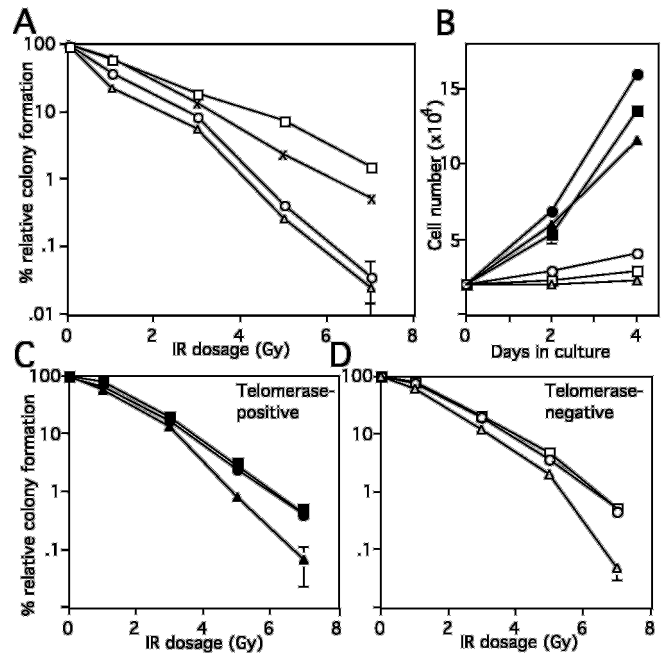


FIG. 7. A, resistance of BJ fibroblasts to IR during passage in culture. Cells were plated, x-irradiated at the indicated doses, allowed to recover, then replated and scored for colony formation, as described under "Experimental Procedures." Symbols correspond to: PD 20, LI 96% (\square); PD 35, LI 86% (\times); PD 69, LI 53% (\circ); PD 71, LI 44% (\triangle). B, growth of non-irradiated or irradiated cells having different telomere lengths. Cells were infected with pBloxTSH and control or TIN2-expressing viruses. Non-irradiated cells are indicated by filled symbols; irradiated (5 Gy) cells are indicated by open symbols. Shown are pL-TIN2 (triangles)-, pLXSN (circles)-, and pL-TIN2-13 (squares)-infected cells. These telomerase-positive cells were infected with the control virus pLXSH, but very similar results were obtained with telomerase-negative cells, infected with pLCRESH. C and D, increase in sensitivity of fibroblasts with short telomeres after exposure to x-rays. The percent relative colony formation was calculated as follows: number of colonies formed by irradiated cells divided by the number of colonies formed by non-irradiated control cells, times 100. Symbols correspond to: pL-TIN2 + pLXSH (\blacktriangle); pLXSN + pLXSH (\bullet); pL-TIN2-13 + pLXSH (\blacksquare); pL-TIN2 + pLXSH (\blacktriangle); pLXSN + pLCRESH (\circ); pL-TIN2-13 + pLCRESH (\square). C, telomerase-positive cells. D, telomerase-negative cells. Each experiment was repeated three times with similar results. Error bars indicate \pm S.D., which in most cases is smaller than the symbols.

(Fig. 7D). Strikingly, the IR-dose response of telomerase-negative cells was indistinguishable from the dose response of telomerase-positive cells. Thus, telomerase did not affect the IR sensitivity of these cells (Fig. 7, compare C and D). Short telomeres, but not telomerase, also sensitized cells to the radiomimetic bleomycin (3–30 μ g/ml, administered to normal BJ, telomerase-positive 82-6, and telomerase-negative 82-6 fibroblasts, each assayed with different telomere lengths; data not shown).

These data suggest that, in these essentially isogenic human fibroblasts, short telomeres sensitize, but long telomeres do not necessarily protect, cells from the IR-induced growth arrest. Moreover, telomerase does not influence IR sensitivity. We note, however, that the presence of overexpressed or mutant TIN2 proteins may independently influence the IR response.

DISCUSSION

We describe a system to generate normal human cells having different telomere lengths and reversible expression of telomerase. It is based on the use of three well-characterized proteins: 1) hTERT, the catalytic component of human telomerase, which is not expressed by most human cells and immortalizes human cells types when expressed (12, 13); 2) Cre recombinase, which excises DNA between loxP recognition sequences with

high specificity (44); and 3) TIN2, a human telomere-associated protein, which modulates telomere length in the presence of telomerase (18). We used retroviruses to deliver genes with high efficiency to proliferating cells. This feature obviated the need for single cell cloning and is an advantage when working with cells that have a finite replicative life span. Finally, the system features positive selection for cells that have lost hTERT after Cre-mediated excision, thereby eliminating cells in which Cre fails to act. We used this system to create immortal (telomerase-positive) and mortal (telomerase-negative) human fibroblast populations with different telomere lengths and determined the effects of telomere length on replicative senescence and the response to IR.

Our strategy for conditional telomerase expression, flanking *hTERT* by *loxP* sites, resembles that of Steinert *et al.* (52). Our vector, however, conferred histidinol resistance prior to Cre-induced excision and puromycin resistance after excision. Because telomerase confers a strong growth advantage, positive selection for cells that lose hTERT eliminated the need to isolate telomerase-negative clones. Constitutive Cre expression consistently eliminated the *hTERT* and *HISD* genes, evidenced by the loss of detectable telomerase activity and histidinol resistance, and eventual senescence of the population.

Cells that expressed hTERT transiently, owing to Cre-mediated excision, senesced with shorter telomeres than parental cells (mean TRFs, 3.1–3.2 *versus* 4.7–4.8 kb). Thus, short term expression of telomerase allowed human cells to undergo more PD than control cells before reaching replicative senescence. This result supports the idea that transient expression of telomerase could be used to treat chronic high turnover diseases such as liver cirrhosis (53).

Constitutive Cre expression did not affect the growth of human fibroblasts (82-6 and, not shown, BJ/HCA2), although it has been reported to be toxic to mouse fibroblasts (45). Thus, short term Cre expression may be desirable in some instances. One possibility for achieving this is to use self-excising Cre-encoding viruses (54). Another is to use transient expression. Transient transfection completely eliminated the *hTERT* and *HISD* genes in two of three experiments. In one experiment, however, transient Cre expression generated a small number of puromycin- plus histidinol-resistant, telomerase-positive cells. These cells may have harbored multiple copies of the pBloxTSH virus, and Cre expression may have been too transient to excise all the copies. Whatever the case, ectopic hTERT expression did not induce the resident hTERT gene in normal human fibroblasts, as reported by others (52), although this was not the case in p16-negative human mammary epithelial cells (46). Human fibroblasts and mammary epithelial cells may respond differently to ectopic hTERT expression. However, it is also possible that the genomic instability that occurs in p16-negative mammary epithelial cells (55) contributed to the reactivation of the endogenous *hTERT* gene.

Telomere length has been reported to affect the radiation sensitivity of cells and organisms. For example, late generation mTR^{-/-} mice were more sensitive than wild-type or early generation mTR^{-/-} mice to the lethal effects of IR (28). Likewise, fibroblasts from late generation mTR^{-/-} mice were hypersensitive to growth inhibition by IR (29), and a cross-sectional study showed that shorter telomeres predisposed human lymphocytes to IR-induced chromosome aberrations (27). Our system allowed us to determine the radiation response of essentially isogenic human cell populations. Our results support the idea that short telomeres sensitize cells to IR. However, our results also showed that longer telomeres do not confer resistance to IR. These findings argue against telomeric sequences being uniform sensors of DNA damage. Rather, they

support the idea that telomeres are targets that can sensitize cells to IR. Short telomeres may be particularly sensitive to DNA-damaging agents, because they are likely to be less stable than long telomeres (21). Hence, short telomeres may be more susceptible to adopting a dysfunctional conformation when damaged. Alternatively or in addition, telomeres might influence the efficiency of repair at non-telomeric genomic sites after IR. We shortened or elongated telomeres by expressing wild-type or dominant-negative forms of TIN2. These proteins very likely alter the telomeric structure (18). It is unlikely that the differences in IR sensitivity are due to the TIN2 proteins, because unmodified human fibroblasts displayed increasing sensitivity to IR with serial passage, which causes progressive telomere shortening (Fig. 7A).

Telomerase had little effect on the radiosensitivity of human cells with similar telomere lengths. These results are consistent with the response of mTR^{-/-} mice, where sensitivity to IR was not seen in early generation mice, despite the absence of telomerase (28). In contrast, telomerase was reported to confer resistance to apoptosis caused by DNA damaging in rodent neurons and human fibroblasts (33–35). One possibility to reconcile these apparently contrasting findings is that hTERT may not have the same effect on telomere length in all cells. After hTERT expression, telomeres quickly equilibrate to an average length that depends on the cell type. In some cases, the telomeres elongate (12, 13, 52), but in other cases they shorten (56) or show only minor length changes (57).² Thus, telomerase may influence the IR response indirectly by altering telomere length. From our results, it appears that telomerase does not act to repair telomeric damage or at least the repair is insufficient to prevent the growth arrest caused by IR. Given that most somatic human cells do not express telomerase, mechanisms other than telomerase must repair damage to telomeres in these cells.

By creating untransformed human cells with varying telomere lengths, we confirmed that replicative life span is proportional to telomere length (48, 52). We also found that experimental lengthening of the telomeres, at least within the limits achieved here, did not compromise cell proliferation or cause any obvious change in phenotype. Thus, reversible hTERT expression might be useful when the preparation of large numbers of cells for therapeutic purposes is limited by replicative senescence, but constitutive expression of telomerase is not desirable owing to the risk of transformation (46).

Finally, cells in which telomere length was manipulated by TIN2 proteins underwent replicative senescence with telomere lengths that were substantially longer than expected. This was especially true for cells in which the telomeres were elongated by the dominant-negative TIN2-13 protein. These findings suggest that TIN2 proteins, either overexpression of the wild-type protein or expression of the mutant, alter the telomeric structure, thereby altering the length set point at which a dysfunctional conformation is favored. However, it is also possible that, upon losing hTERT, cells arrest growth by mechanisms that are independent or only partially dependent on telomere length. For example, the cells may arrest growth owing to changes in chromatin organization, which has been suggested to play a role in inducing the senescent phenotype (58, 59). This possibility would, of course, suggest that telomerase plays a role in ensuring a presenescent chromatin organization. Telomeres are known to regulate chromatin in yeast (60), and recent evidence suggests this may also occur in human cells (61).

Acknowledgments—We gratefully acknowledge Drs. Junko Oshima and James Smith for providing the human fibroblast strains and Dusty

² M. A. Rubio, S.-H. Kim, and J. Campisi, unpublished results.

Miller, Hartmut Land, Robert Weinberg, and Kirk Thomas for providing vectors.

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