

Regulation of Telomerase Activity in Immortal Cell Lines

SHAWN E. HOLT, WOODRING E. WRIGHT, AND JERRY W. SHAY*

*Department of Cell Biology and Neuroscience, University of Texas
Southwestern Medical Center, Dallas, Texas 75235-9039*

Received 23 January 1996/Returned for modification 26 February 1996/Accepted 8 March 1996

Telomerase is a ribonucleoprotein whose activity has been detected in germ line cells, immortal cells, and most cancer cells. Except in stem cells, which have a low level of telomerase activity, its activity is absent from normal somatic tissues. Understanding the regulation of telomerase activity is critical for the development of potential tools for the diagnosis and treatment of cancer. Using the telomeric repeat amplification protocol, we found that immortal, telomerase-positive, pseudodiploid human cells (HT1080 and HL60 cells) sorted by flow cytometry had detectable telomerase activity at each stage of the cell cycle. In contrast, telomerase activity was repressed in quiescent cells. This was true whether quiescence was induced by contact inhibition (NIH 3T3 mouse cells), growth factor removal (bromodeoxyuridine-blocked mouse myoblasts), reexpression of cellular senescence (the reversibly immortalized IDH4 cells), or irreversible cell differentiation (HL60 promyelocytic leukemia cells and C2C12 mouse myoblasts). Taken together, these results indicate that telomerase is active throughout the cell cycle in dividing, immortal cells but that its activity is repressed in cells that exit the cell cycle. This suggests that quiescent stem cells that have the potential to express telomerase may remain unaffected by potential antitelomerase cancer therapies.

Vertebrate telomeres (the ends of chromosomes) contain repeated TTAGGG sequences (22). Telomeric repeats are synthesized by the enzyme telomerase (10; see references 2, 9, and 12 for recent reviews). Telomerase is present in human germ line cells (testis and ovary), cancer cells, and immortal and tumor-derived cell lines, but it has not been detected in normal adult somatic tissues or cultured human diploid cells, with the exception of renewal tissues that contain stem cells (e.g., blood, skin, and intestine) (4, 7, 13–16, 26, 28, 32). In the absence of telomerase, telomeric repeats are lost with each cell division because DNA polymerases cannot replicate the very end of a linear DNA molecule (18, 21, 27). This is thought to be due to the fact that lagging-strand synthesis proceeds as a series of discrete events (i.e., the formation of Okazaki fragments), each requiring an RNA primer. Since there is no DNA beyond the 3' end of the chromosome to which this primer can anneal, DNA polymerase cannot fill in the gap between the final Okazaki fragment and the end of the chromosome (the "end replication problem"). The incompletely replicated telomeres are inherited by daughter cells, and the process repeats itself in subsequent cell divisions, progressively shortening the telomeres as cell divisions increase. Telomere shortening has been proposed as a regulatory mechanism that controls the number of times a cell can divide before undergoing a process known as cellular senescence (1, 6, 11, 33, 34).

Immortal cells, including cancer cells, avoid the progressive loss of telomeric DNA by expressing the enzyme telomerase, an RNA-dependent nucleoprotein enzyme, which is capable of adding back the tracks of short repetitive telomeric sequences to the ends of the chromosome. This addition of TTAGGG repeats compensates for the end replication problem and stabilizes the lengths of telomeres, allowing the cells to divide indefinitely. Using a PCR-based telomerase assay (the telomerase repeat amplification protocol [TRAP] assay), we have

found that approximately 85 to 90% of over 1,000 primary human tumors examined and 100% of tumor-derived cell lines display telomerase activity (13–16, 23; reviewed in reference 26). Benign growths, such as uterine leiomyomas, do not have detectable telomerase activity, so these differences in telomerase activity cannot be explained simply as a difference in cellular proliferation (16, 23).

The association of the end replication problem with DNA replication has led to the assumption that telomerase synthesizes telomeres during the S phase of the cell cycle. In *Saccharomyces cerevisiae*, single-stranded G-rich overhangs appear late in the S phase of the cell cycle, suggesting that late S phase is the time when telomerase is actually active (30). Currently, very little is known about the regulation of telomerase activity in vertebrates. If telomerase synthesizes telomeric repeats only during S phase, a variety of mechanisms may be involved in the regulation of telomerase during the other phases of the cell cycle. For instance, it is possible that telomerase is expressed and active only during S phase when telomeres are replicated, is present during all phases of the cell cycle but is able to add repeats only during S phase, or is continuously synthesizing telomeric DNA throughout the cell cycle.

Stem cells, found mainly in regenerative tissues such as the blood, the skin, and the intestine, may remain in a quiescent or nondividing state for much of their life span and give rise to actively dividing progeny cells, which function to renew tissues. Nondividing stem cells have no need to replicate their telomeres and thus may be capable of down regulating telomerase. Several pieces of evidence suggest that primitive nondividing stem cells may not exhibit telomerase activity. CD34⁺ CD38^{lo} hematopoietic stem cells have very low levels of telomerase activity, while their dividing CD34⁺ CD38⁺ progeny have increased levels of telomerase activity (5, 14). Similarly, although the testis was found to be telomerase positive (19, 24), sperm cells are telomerase negative (32). However, the status of telomerase activity in nondividing stem cells has not yet been specifically examined.

Telomerase inhibitors are being actively considered as potential antitumor agents, with the hope that the lack of telomerase expression in normal somatic cells would result in a

* Corresponding author. Mailing address: Department of Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9039. Phone: (214) 648-3282. Fax: (214) 648-8694.

highly specific treatment with fewer side effects than conventional chemotherapy. An obvious concern is the potential effects of such inhibitors on normal stem cells. The present study was initiated to examine the cell cycle regulation of telomerase activity, both as a function of stages of the cell cycle in actively dividing cells and in a variety of nondividing G_0 states that may represent the physiological equivalent of quiescent stem cells. In this report, we show that telomerase activity is present in the G_1 , S, and G_2/M phases of the cell cycle in immortal cells but is not detected in cells that have exited the cell cycle (G_0). This suggests that nondividing primitive stem cells may be unaffected by antitelomerase cancer therapies.

MATERIALS AND METHODS

Cell culture. All cell types were grown at 37°C in 5% CO_2 in a 4:1 mixture of Dulbecco's modified medium and medium 199 containing 15% iron-supplemented calf serum (HyClone Laboratories, Logan, Utah) and 25 μ g of gentamicin (Sigma Chemical Co., St. Louis, Mo.) per ml, unless otherwise indicated. HL60 promyelocytic cells were induced to differentiate by the addition of 1 μ M all-*trans*-retinoic acid or 1.25% dimethyl sulfoxide (DMSO) for 4 to 10 days. Retinoic acid was prepared as a stock solution in DMSO. When diluted to 1 μ M, the concentration of the solvent (0.125%) had no effect on the differentiation of HL60 cells, as previously described (20). Protein synthesis was inhibited by treating cells with 300 μ g of cycloheximide per ml.

NIH 3T3 cells become quiescent when kept at confluence. NIH 3T3 cells were maintained at confluence without a change of medium until passaged. Mouse myoblast cells (C2C12) differentiate into myotubes when shifted from 15% bovine calf serum to 2% horse serum. Quiescent myoblasts were obtained by first growing the cells in the presence of 8 μ M bromodeoxyuridine (BrdU)—10 μ M deoxycytidine for 7 days to block differentiation (31) and then incubating them in 2% horse serum in BrdU-containing medium for an additional 2 to 10 days. Myoblast cells (10^5) were harvested for determination of telomerase activity at various time points, with the remaining cells replated to verify viability.

IDH4 cells are cells that were immortalized by using a vector in which the expression of simian virus 40 (SV40) large T antigen is under the control of the mouse mammary tumor virus (MMTV) promoter (33). The MMTV promoter is inducible upon the addition of dexamethasone. In the absence of dexamethasone, T antigen is no longer expressed, and the IDH4 cells become quiescent and reexpress the senescent phenotype (33). Briefly, IDH4 cells were rinsed with medium containing charcoal-stripped serum without dexamethasone three or four times to eliminate residual dexamethasone and then were incubated in charcoal-stripped medium for up to 14 days. Reversal of the senescence state was obtained by feeding the cells normal medium containing dexamethasone.

Assay for telomerase activity. Telomerase activity was measured by the TRAP assay (16, 23) as modified by Wright et al. (35). Briefly, 10^5 cells were pelleted and either lysed immediately or stored at $-80^\circ C$. Cell lysates were clarified by centrifugation at $14,000 \times g$ for 20 min, and the supernatants were removed and flash frozen. Detection of telomerase activity in the extracts from cultured cells was performed in a two-step process: (i) telomerase-mediated extension of an oligonucleotide (TS) and (ii) PCR amplification of the resultant product with the forward (TS) and reverse (CX) primers. An appropriate amount, which is in the linear range of telomerase activity versus the internal standard (generally the equivalent of 100 to 1,000 cells, depending on the cell type), of each of the cell extracts was incubated for 30 min at room temperature with the TS primer in a tube containing the CX primer under a wax barrier. After telomerase synthesized TTAGGG repeats onto the TS primer, the reaction products were amplified by PCR for 30 cycles in the presence of 5 attograms (ag) of an internal TRAP assay standard (ITAS); this was followed by electrophoresis on 10% polyacrylamide gels. The ITAS is a 150-bp DNA fragment derived from the rat myogenin coding sequence and flanked by sequences required for PCR amplification with the TS and CX primers (35). The nonlinearity of the TRAP assay is primarily a result of either *Taq* DNA polymerase inhibitors or an inadequate *Taq* polymerase activity as reaction products build up. Compensation for these effects can be obtained by normalizing the intensity of the telomerase ladder to the intensity of the ITAS. Gels were analyzed and products were quantitated by using the PhosphorImaging system from Molecular Dynamics. Relative telomerase activities were quantitated by taking the ratio of the ITAS to the entire telomerase ladder in each lane and comparing the ratio with that obtained for the untreated cells or the cells at day 0 or h 0.

Protein extracts and immunoblotting. IDH4 protein extracts were prepared by lysis in 2% sodium dodecyl sulfate (SDS)—50 mM Tris-HCl—100 mM NaCl at a concentration of 2.5×10^5 cells per μ l. Protein extracts corresponding to 5×10^4 cells were electrophoresed on an SDS—8% polyacrylamide gel and transferred to nitrocellulose (Amersham). The nitrocellulose was blocked in phosphate-buffered saline (PBS) and 5% nonfat dried milk, subjected to immunoblotting for detection of SV40 T antigen with the monoclonal antibody Ab419, washed with PBS plus 0.1% Tween 20, incubated with alkaline phosphatase-conjugated goat anti-mouse antiserum (Applied Biosystems, Birmingham, Ala.), and washed with

PBS plus 0.1% Tween 20. T-antigen-specific bands were detected by using NitroBlock and disodium 3-(4-methoxyphosphoryl-1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan-4-yl)phenyl phosphate (CSPD) as recommended by the manufacturer (Tropix). Autoradiographic bands were quantitated by densitometry.

Flow cytometry. HL60 and HT1080 cells were rinsed with PBS containing 1% serum to remove residual growth medium. The presence of 1% serum helped to maintain viability. The rinsed cells were resuspended at a concentration of 10^6 cells per ml in PBS containing 1% serum and 1.8 μ g of Hoechst 33342 stain per ml and incubated at 37°C for 30 to 45 min. The cells were kept on ice until sorted into G_1 , S, and G_2/M phases of the cell cycle on the basis of DNA content on a FACScan flow cytometer (Becton Dickinson). HT1080 cells, which contain pseudodiploid and pseudotetraploid populations, were sorted on the basis of DNA content into a pseudodiploid population.

Measurement of DNA synthesis. The rate of DNA synthesis in IDH4 cells was measured by the incorporation of [3H]thymidine. Cells were labeled with 5 μ Ci of [3H]thymidine per ml and 2.65 μ g of fluorodeoxyuridine per ml in medium with or without dexamethasone for 3 h. The cells were washed three times with Dulbecco's PBS and then twice for 10 min each with ice-cold 10% trichloroacetic acid to precipitate radiolabeled DNA. Precipitated cells were lysed in 1 ml of 0.25 N NaOH—1% SDS at room temperature for 10 min. Radioactivity was measured by adding 0.5 ml of lysis mixture to a 10-ml liquid scintillation cocktail and disintegrations per minute were measured with a scintillation counter (Beckman). Disintegrations per minute per cell were calculated to normalize for discrepancies in the cell number in each sample.

RESULTS

Telomerase activity has been detected in a number of human cancers (4, 7, 13–16, 23, 28, 32). Since approximately 90% of all human cancers contain telomerase activity, a determination of the regulatory pathways involved in telomerase regulation will be important for the clinical diagnosis, prognosis, and treatment of cancer. In this report, we first show that telomerase activity is present throughout the cell cycle. Second, using a variety of cell lines from both human and rodent origins, we show that telomerase activity is repressed when cells are growth arrested in either a quiescent state or a differentiated state. Third, we provide evidence for the reversible repression of telomerase activity in quiescent cells.

Cell cycle component of telomerase. The activities of telomerase in the different stages of the cell cycle were determined in immortal cells separated by flow cytometry on the basis of DNA content into the G_1 , S, and G_2/M phases of the cell cycle. Telomerase-positive, human promyelocytic leukemia cells (HL60 cells) and the HT1080 human fibrosarcoma cells were selected because they are pseudodiploid, which permits good resolution of the stages of the cell cycle on the basis of DNA content. Pseudotetraploid cells, which can accumulate in HT1080 cell populations, were eliminated by using recently sorted pseudodiploid cells (see Materials and Methods). Actively growing cells were stained with Hoechst 33342 stain, sorted on the basis of DNA fluorescence into the G_1 , S, and G_2/M phases of the cell cycle, and subjected to the PCR-based TRAP assay (Fig. 1). The fluorescence-activated cell sorter sorting gates were set to be particularly narrow to minimize the cross-contamination of cells from other phases of the cell cycle (Fig. 1A and B). Telomerase activity was detected at all stages of the cell cycle for both cell types (Fig. 1C).

The TRAP assay has been made semiquantitative (35) by the inclusion of an ITAS. The ITAS is a nontelomeric 150-bp fragment flanked by sequences that allow its amplification with the standard TRAP assay primers, which enables effective quantitation and standardization from sample to sample. Cell lines were tested for linearity on the basis of quantitation with the ITAS and telomerase activity corresponding to the entire 6-bp telomerase ladder. One hundred cell equivalents was found to be in the linear range for each cell line tested (data not shown). Quantitation, based on three separate experiments (data not shown), of the telomerase activity relative to the ITAS indicated that there was no substantial variation of the

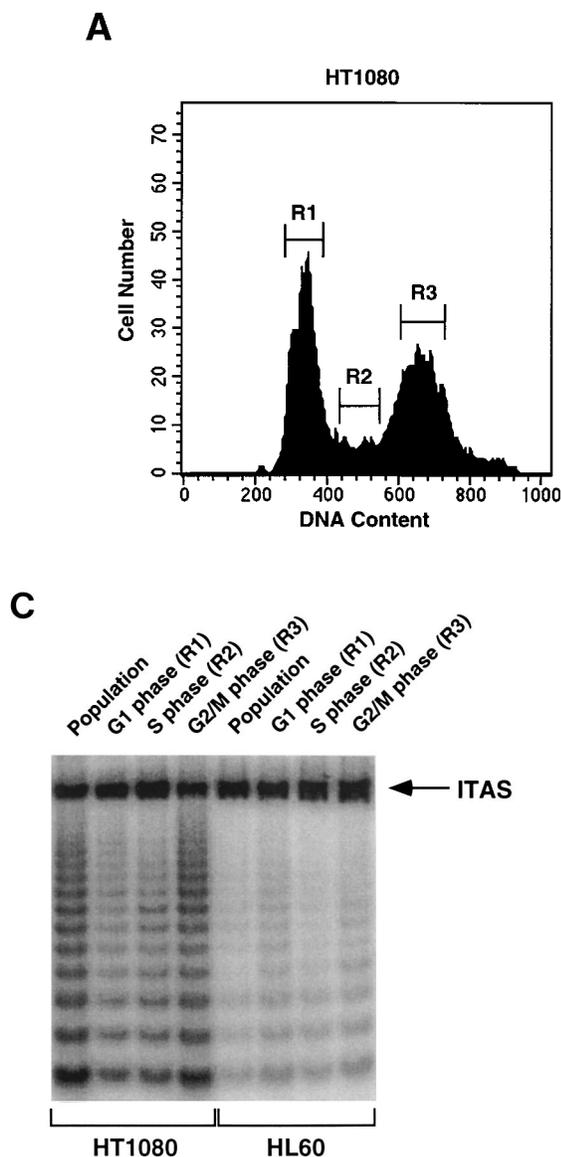


FIG. 1. Telomerase activity in different stages of the cell cycle. (A and B) Profiles of the stained cells and the regions sorted for HT1080 fibrosarcoma cells (A) and HL60 promyelocytic leukemia cells (B). These immortal, telomerase-positive, pseudodiploid human cells were sorted by flow cytometry into three stages of the cell cycle: G₁ (R1), S (R2), and G₂/M (R3). The cells were stained with Hoechst 33342 and sorted on the basis of DNA content. (C) The sorted cells were pelleted and analyzed by the TRAP assay with the ITAS. The PCR-amplified telomerase product was electrophoresed (10% polyacrylamide gel). Telomerase activity is visualized by the characteristic 6-bp ladder. No significant variation in activity as a function of cell cycle stage was seen for either cell type. HL60 cells consistently show a lower extracted-telomerase activity than HT1080 cells.

lack of variation of telomerase activity as a function of stages of the cell cycle.

Differentiation and telomerase activity. Actively dividing immortal or cancer-derived cell lines exhibit telomerase activity, but the status of telomerase in nondividing cells has not been examined. HL60 cells differentiate and become postmitotic after treatment with chemical agents such as all-*trans*-retinoic acid, phorbol 12-myristate 13-acetate, and DMSO (20, 29). The status of telomerase activity in differentiating HL60 cells

levels of extracted telomerase activity during the cell cycle. In addition, we believe that two- to threefold differences in telomerase activity can be detected on the basis of quantitation with the ITAS, especially when the results of three independent experiments are combined.

Cell cycle-regulated proteins typically have a short half-life. The half-life of telomerase activity was determined in cells treated with cycloheximide, a potent inhibitor of protein synthesis. The concentration of cycloheximide was adjusted to inhibit protein synthesis by 95% in less than 6 h while still maintaining cell viability. The viability of cycloheximide-treated cells, assessed by determining the plating efficiency (HT1080 cells) or by trypan blue exclusion (HL60 cells), was approximately 75 to 80% after 36 h of cycloheximide treatment for both cell lines (data not shown). Telomerase activity was approximately 50% of the control level after 24 h of treatment compared with untreated cells (Fig. 2). Telomerase is thus a highly stable protein complex with an activity half-life of about 24 h in these cycloheximide-treated cells. This long half-life is consistent with the

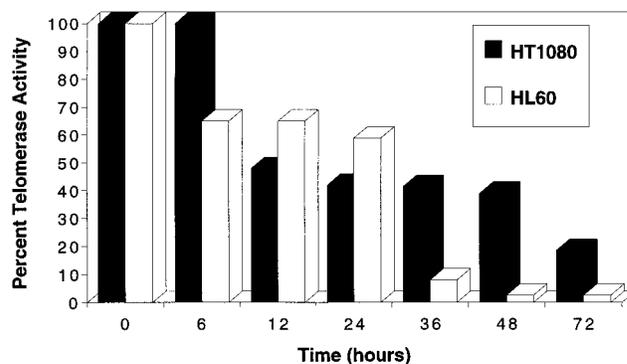


FIG. 2. Half-life of telomerase activity in cycloheximide-treated cells. HT1080 and HL60 cells were treated with a final concentration of 300 μ g of cycloheximide per ml for 72 h. Cells were harvested at the indicated times and tested for telomerase activity by the TRAP assay. Quantitation of telomerase activity in the cycloheximide-treated cells was done by determining the ratio of ITAS to telomerase activity. The subsequent levels of telomerase activity are represented as a fraction of the 0-h activity.

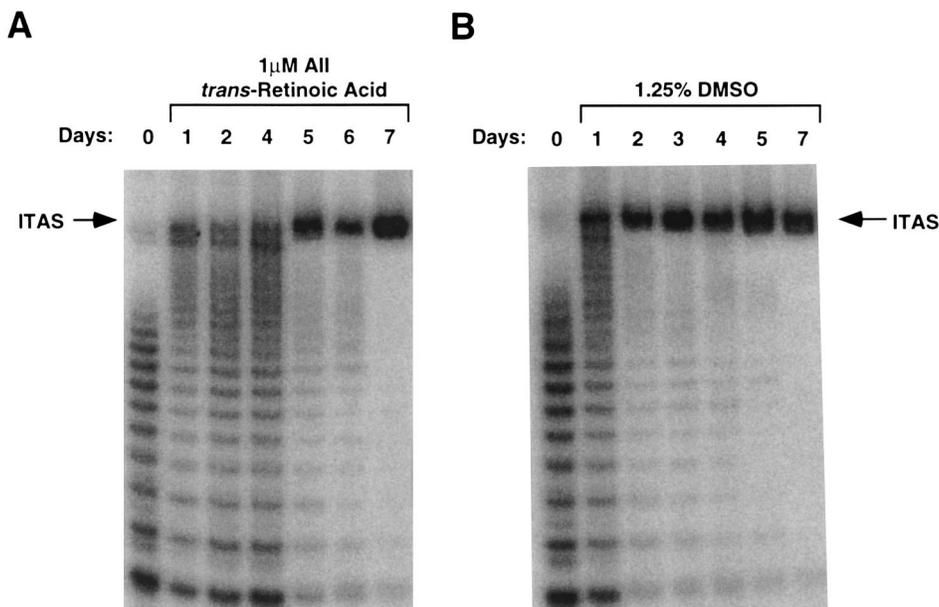


FIG. 3. Telomerase activity in differentiating HL60 cells. (A) HL60 cells, a promyelocytic leukemia cell line, were induced to differentiate by using 1 μ M all-*trans*-retinoic acid. Approximately 90% of the HL60 cells had differentiated by day 4, as visualized by the attachment of the cells to the plate. Cells (both attached and in suspension) were harvested at 24-h intervals. Extracts were subjected to the TRAP assay, including the ITAS, and electrophoresed as described for Fig. 1. (B) HL60 cells were induced to differentiate by the addition of 1.25% DMSO to the medium. Cells were harvested and tested as described for panel A. In both cases, telomerase activity is negligible 5 to 7 days after treatment with either retinoic acid or DMSO.

treated with either 1 μ M retinoic acid or 1.25% DMSO was monitored.

Undifferentiated HL60 cells remain in suspension, but the cells become loosely attached to the culture dish after approximately 3 days of retinoic acid treatment. The viability of these cells with either retinoic acid or DMSO treatment was greater than 80% after 5 to 7 days (data not shown). Minimal cell proliferation was observed after 2 to 3 days of treatment with either agent, when differentiated cells visually appeared to constitute greater than 90% of the cultures.

Telomerase activity declined by more than 1,000-fold in differentiated HL60 cells compared with untreated cells (0 days) (Fig. 3). The kinetics of this decrease are complex and suggest that under some conditions, telomerase can have a half-life considerably shorter than the 24-h half-life observed for the cycloheximide-treated HL60 cells. There appears to be a rapid, approximately fivefold decrease during the first 24 h. For the retinoic acid-treated cells, telomerase activity stabilized for the next 3 days and then again rapidly declined, while the rapid decrease in telomerase activity in DMSO-treated cells continued following the first 24 h. We cannot distinguish whether the very low persisting levels reflect a few undifferentiated or slowly differentiating cells or highly reduced levels in differentiated cells. This decrease in telomerase activity does not reflect a direct effect of retinoic acid on telomerase, since treatment of HT1080 cells with 1 μ M retinoic acid had no effect on the activity of telomerase or the proliferation of the cells (data not shown).

C2C12 mouse myoblasts become postmitotic and fuse to form multinucleated myotubes following growth factor removal (a shift from 15% fetal calf serum to 2% horse serum). Differentiated cultures, in which more than 90% of the nuclei were fused to form myotubes, showed a greater than 90% decrease in telomerase activity (data not shown). Differentiated HL60 promyelocytic leukemia cells and C2C12 mouse myotubes both showed minimal telomerase activity, suggesting

that nondividing cells have a mechanism for the regulation of telomerase activity.

Telomerase activity in quiescent cells. NIH 3T3 cells, a Swiss mouse cell line that is telomerase positive, are contact inhibited and enter a quiescent G_0 state when kept at confluency for 10 to 14 days. NIH 3T3 cells were plated near confluency, became confluent between day 1 and day 2, and were maintained in normal medium with 15% serum at confluency for approximately 28 days. No significant change in telomerase activity was seen just after confluence was reached (after 3 days [data not shown]), yet by 7 days telomerase activity was reduced 5- to 8-fold, and after 13 days it was further reduced to 25- to 30-fold (Fig. 4). These results indicate that cells down regulate telomerase activity when they are quiescent. In contrast to the case for differentiated HL60 cells or C2C12 myotubes, which are permanently postmitotic, quiescence due to contact inhibition is reversible. The quiescent state of NIH 3T3 cells was reversed by enzymatically dissociating the confluent monolayer and passaging the cells. After a recovery period of 3 to 5 days, telomerase activity was near levels seen in normal cells (0 days) (Fig. 4). On the basis of cell counts and a visual assessment of the cultures, cell death and cell division appeared to reach an equilibrium (data not shown), suggesting that the residual activity that remains in the confluent cultures can be partially explained by the presence of a small number of cells that continue to proliferate despite cell-cell contact.

In addition, we performed mixing experiments with the NIH 3T3 samples at 0 days (telomerase active) and 21 days (telomerase repressed) to determine whether a telomerase repressor was induced. Equal amounts of each extract were incubated together for 30 min prior to use in the TRAP assay. No decrease in telomerase activity was seen after mixing of the telomerase-repressed sample with that containing telomerase activity. While this *in vitro* mixing result does not eliminate the possibility of a telomerase repressor, it does suggest that if telomerase repression is the pathway taken by cells destined to

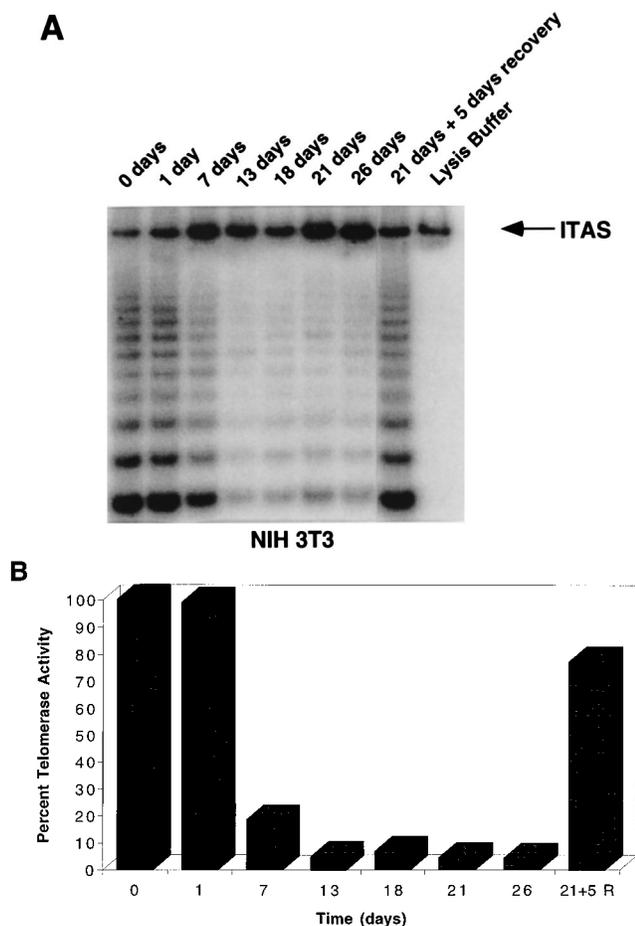


FIG. 4. Disappearance of telomerase activity in quiescent NIH 3T3 cells. (A) NIH 3T3 cells are a telomerase-positive Swiss mouse cell line that can be growth arrested by contact inhibition. Cells were plated at a high density and allowed to grow to confluency. At various times, cells were harvested, lysed, and subjected to the TRAP assay as described for Fig. 1. A representative gel is shown, indicating a decrease in telomerase activity after 7 to 13 days. Confluent cells were passaged at day 21 and subjected to the TRAP assay 5 days later, showing that the disappearance of telomerase activity in these cells is a reversible phenomenon. (B) Quantitation of the telomerase activity in quiescent cells reveals that activity was reduced approximately 30-fold compared with that in cells harvested in log-phase growth (0 days). 21+5 R, 21 days plus 5 days of recovery.

undergo differentiation or quiescence, it is likely that telomerase repression may be due to transcriptional regulation rather than to a direct repressor-telomerase interaction.

Myogenesis is inhibited following the incorporation of BrdU by dividing myoblasts. This permitted us to dissociate the quiescence induced by growth factor removal from differentiation into myotubes. C2C12 myoblasts were grown in $8 \mu\text{M}$ BrdU for approximately five cell divisions before being shifted to medium containing 2% horse serum. The cultures became mostly quiescent, and although some mitotic figures continued to be seen, growth rates were reduced at least four- to fivefold. Staining with antimyosin antibodies, which will stain only differentiated myotubes, indicated that only a few percent of the nuclei were able to escape the inhibitory effects of BrdU and differentiate. Telomerase activity declined approximately 10-fold in these cultures (Fig. 5). The background level of telomerase can be attributed to cells that remain unaffected by the shift to 2% horse serum and continue to proliferate. Although the down regulation of telomerase in differentiated myotubes

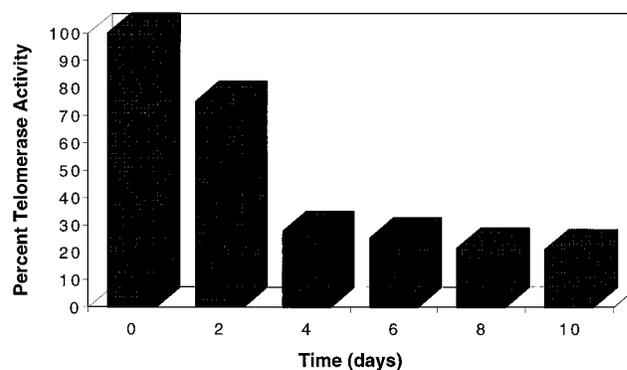


FIG. 5. Disappearance of telomerase activity in quiescent undifferentiated mouse myoblasts. Mouse C2C12 myoblasts can be induced to differentiate when deprived of normal growth factors (15% fetal calf serum) and fed 2% horse serum. However, after the myoblasts have incorporated BrdU, myoblast differentiation is inhibited and most of the cells become quiescent. Myoblasts were incubated in BrdU for 3 days prior to incubation in 2% horse serum (10 days). The cells were counted, harvested for TRAP assay, and replated to verify viability. Quiescent undifferentiated myoblasts show a 5- to 10-fold decrease in telomerase activity. The residual telomerase activity seen after 10 days is consistent with the lack of complete quiescence in these cultures.

may reflect a specific response as part of the myogenic program, the decrease in telomerase activity in quiescent myoblasts suggests that it could also reflect a general response to a nondividing G_0 state.

Reversible senescence and DNA synthesis. The two-stage model of cellular senescence postulates the existence of two mechanisms that limit the proliferation of normal diploid cells. Cellular senescence is induced when telomere shortening induces the mortality stage 1 (M1) mechanism. This mechanism involves the activation of the tumor suppressor proteins p53 and a pRB-like protein (33). Viral oncoproteins, such as SV40 large T antigen, that can sequester and functionally inactivate these tumor suppressor proteins allow cells to overcome M1, leading to an extended life span. Telomere shortening continues until mortality stage 2 (M2) is induced. During M2, cells undergo a period of crisis which may be the result of terminally short telomeres. Only a rare event, not directly related to the action of the viral oncoprotein, allows cells to escape M2 and reactivate telomerase in order to restore and stabilize telomere length (34). IDH4 cells are an immortal, telomerase-positive lung fibroblast cell line that expresses SV40 large T antigen under the control of the MMTV promoter (33). The MMTV promoter requires the addition of dexamethasone to the culture medium for expression of T antigen. If dexamethasone is removed from the medium, T-antigen expression is down regulated, the normal M1 functions of p53 and the pRB-like protein resume, and the cells reexpress the senescent phenotype (33).

IDH4 cells were cultured in the absence of dexamethasone for 14 days, and then dexamethasone was added to the medium. Cells were harvested and tested for (i) telomerase activity (Fig. 6A), (ii) expression of SV40 T antigen (Fig. 6B), and (iii) DNA synthesis (Fig. 6C). All three activities declined in parallel to minimal levels after approximately 7 days in steroid-free medium. Increased levels of both T antigen and telomerase activity could be detected within 3 h after the addition of dexamethasone. However, the onset of the first S phase, experimentally determined by the measurement of DNA synthesis, was approximately 9 h later than either the reexpression of T antigen or the detection of telomerase activity (Fig. 6). Telomerase activity increased progressively during the first 48 h

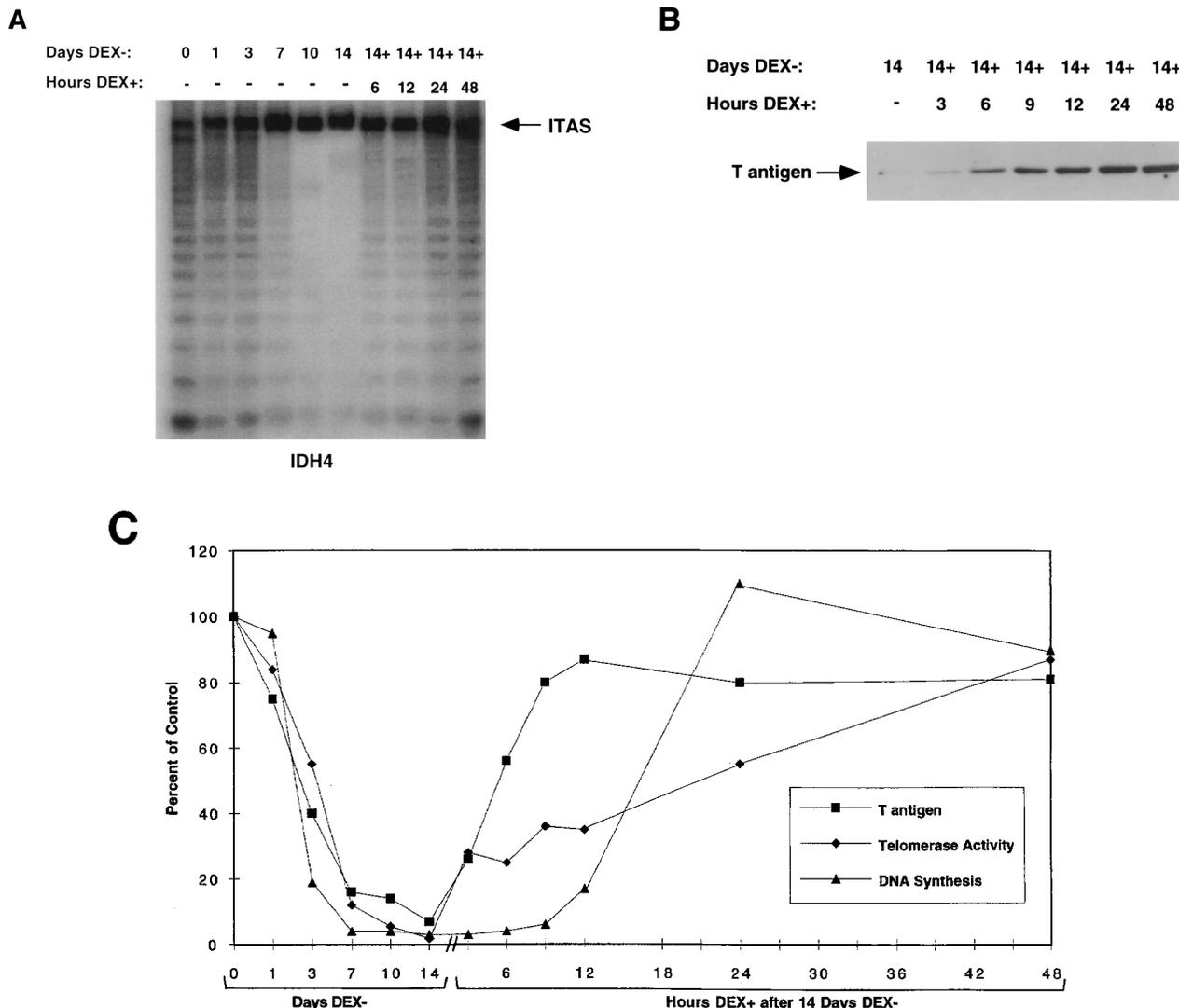


FIG. 6. Reversible repression of telomerase activity in IDH4 cells. IMR90 human diploid lung fibroblasts were immortalized by using a dexamethasone-inducible SV40 T-antigen expression system, giving rise to the cell line IDH4 (33). In the presence of T antigen (DEX+), the M1 mechanism of cellular senescence is blocked and the cells divide. If the expression of T antigen is deinduced (DEX-), the action of the M1 mechanism causes the cells to reexpress the senescent phenotype and stop dividing. Telomerase activity (A), T-antigen levels (B), and DNA synthesis (C) were measured during 14 days of dexamethasone deprivation and the first 2 days following readdition of the steroid.

following steroid replenishment and showed no increases specifically associated with the onset of or progression through S phase. This indicates that extracted telomerase activity reappears in G₁ as cells exit G₀ and does not correlate with DNA synthesis.

DISCUSSION

The regulation of telomere length in immortal cells involves the action of telomerase, an RNA-dependent DNA polymerase responsible for synthesizing telomeric repeats on the ends of chromosomes. Telomerase activity has been detected in 85 to 90% of all human cancers (4, 7, 13-16, 23, 28, 32). One concern about the potential side effects of antitelomerase cancer chemotherapies is the effect on stem cells in regenerative tissues, which may express telomerase. The experiments described here demonstrate that although telomerase activity is detectable throughout the cell cycle, telomerase activity is

repressed as cells exit the cell cycle (G₀) via either differentiation or reversible quiescence (Fig. 7). Our results with the HL60 cell line directly confirm those of Sharma et al. (25), in that terminal differentiation of leukemia cells inhibits the activity of telomerase. However, in the present studies, we have shown that telomerase-positive cells that exit the cell cycle, via either differentiation or reversible quiescence, have the ability to repress telomerase activity. The finding that telomerase-positive cells repress the activity of telomerase when shifted to a non-dividing state (Table 1) is significant in that therapies designed to inhibit telomerase in malignant cells may not affect reversibly quiescent stem cells in regenerative tissues.

Telomerase activity has been observed during both the S and M phases of the cell cycle in *Xenopus* oocytes (19), suggesting that telomerase activity was not restricted exclusively to the S phase. Here we have shown that the level of extracted telomerase activity from human cells does not vary significantly during the cell cycle. The fact that extracted telomerase activity does

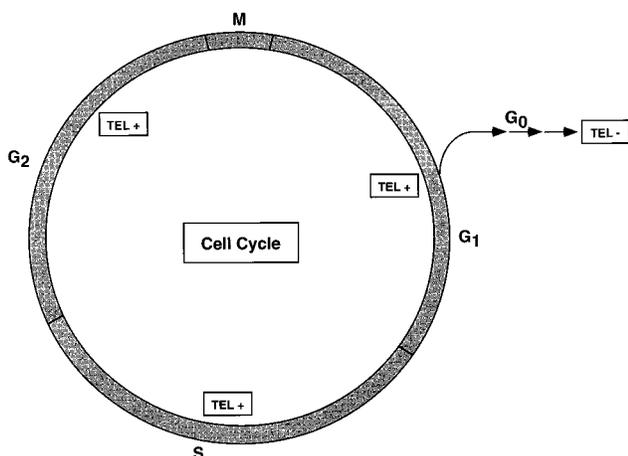


FIG. 7. Model for the repression of telomerase activity in cells that exit the cell cycle. Telomerase-positive (TEL+) immortal cells express telomerase activity at each stage of the cell cycle. As cells exit the cell cycle (G_0) and enter a state of nondivision via quiescence, senescence, or differentiation, telomerase activity is dramatically decreased until it becomes virtually undetectable. Quiescent cells are able to reenter the cell cycle and reexpress telomerase activity.

not vary with the cell cycle does not imply that telomeric repeats are being continuously added to the ends of the chromosomes at each stage of the cell cycle. These ends may be sequestered by telomere-binding proteins or other mechanisms so that they are inaccessible to telomerase except when coupled to DNA replication (8, 36). It is also possible that telomerase may be complexed with cell cycle regulatory factors that inhibit its intracellular activity outside S phase and that dissociate during cell lysis and extraction of telomerase. Assuming that intracellular telomerase activity is in fact coupled to DNA replication as in *S. cerevisiae*, it is unlikely that post-translational modifications (e.g., phosphorylation) are the primary mechanism for controlling its function, since such covalent changes would be likely to persist in the extracted enzyme. The long half-life, greater than a single round of the cell cycle, is consistent with the presence of telomerase activity at each stage of the cell cycle. It is also possible that the expression of telomerase is constitutive and is not limited to a single stage of the cell cycle.

The activity of telomerase must be tightly regulated if a telomerase-positive cell is to maintain a stable telomere length. A cell that remains quiescent for long periods of time would thus require a very efficient mechanism for preventing telomerase from extending the telomeres in the absence of DNA replication. The present results show that under these conditions a mechanism different from that used during the cell cycle is used, since extracted telomerase activity disappears

during quiescence but is constitutive during the cell cycle. The distinction between a decrease due to the repression of telomerase protein synthesis and that due to inactivation of the enzyme complex must await the availability of probes for the protein components of telomerase.

In addition to the status of telomerase activity in nondividing, quiescent cells, our results suggest the presence of complex mechanisms for down regulating telomerase activity. Both HL60 promyelocytic leukemia cells and HT 1080 fibrosarcoma cells showed a similar long half-life of approximately 24 h for telomerase activity when protein synthesis was blocked with cycloheximide. However, the rate of disappearance of activity was much faster in HL60 cells stimulated to differentiate by retinoic acid or DMSO, and in fact the details of the kinetics with these two treatments differed. Although cycloheximide treatment has been found to alter the half-lives of some short-lived proteins (3, 17), the above-mentioned observation suggests the possibility that the disappearance of telomerase activity during the differentiation of HL60 cells may result from an accelerated degradation of telomerase rather than simply from a cessation of synthesis of new enzyme.

The possibility that the activity of telomerase is highly regulated inside the cell and that this regulation is lost upon extraction could explain the puzzling observation of different apparent levels of telomerase activity in certain tumors. Some small-cell lung carcinomas appear to have 1,000 times as much telomerase activity as other tumors (15) or cell lines in culture. The relatively homogeneous histology of many of the tumor samples and particularly the comparison with pure populations of tumor cell cultures make it very unlikely that this result reflects different numbers of telomerase-positive cells rather than different levels of telomerase per cell. If this 1,000-fold level of telomerase were active intracellularly, it would be difficult to understand how these cells would avoid elongating their telomeres to extreme lengths. However, the ability to regulate the activity of the telomerase enzyme would resolve this problem.

IDH4 cells, which contain an inducible SV40 T antigen, are reversibly senescent; that is, the M1 mechanism of cellular senescence (inhibition of cell proliferation) is reintroduced in these cells when the expression of T antigen is turned off. The functions of the p53 and pRB-like proteins, which are at least partially responsible for the antiproliferative aspects of the M1 mechanism (senescence), are active in the absence of T antigen. When T antigen is induced in senescent IDH4 cells, telomerase activity is detected prior to the beginning of the initial S phase, indicating that telomerase can be activated or synthesized either during exit from G_0 or during the G_1 phase of the cell cycle.

The lack of considerable variation in extracted telomerase activity with the cell cycle, its disappearance in G_0 , and the

TABLE 1. Summary of the cell lines used and telomerase activities in different growth states

Cell line	Description	Telomerase activity
IDH4	Immortal, reversibly senescent human lung fibroblasts expressing an inducible T antigen	Repressed in senescence, reexpressed prior to S phase
HL60	Immortal, pseudodiploid human promyelocytic line that differentiates in response to retinoic acid or DMSO	Invariant with the cell cycle, repressed with differentiation
HT1080	Immortal, pseudodiploid human fibrosarcoma line	Invariant with the cell cycle
C2C12	Mouse myoblasts that differentiate in low serum concentrations	Repressed with differentiation, repressed with quiescence
NIH 3T3	Swiss mouse embryo fibroblasts exhibiting contact inhibition of cell division	Repressed with contact inhibition

variability of its apparent half-life in HL60 promyelocytic cells and HT1080 fibrosarcoma cells treated with cycloheximide versus those stimulated to differentiate all suggest that telomerase is a highly regulated enzyme subject to multiple levels of control. Understanding the mechanisms underlying this regulation will be important as inhibitors of telomerase are discovered and attempts are made to use them as cancer chemotherapeutic agents. The demonstration that telomerase-positive cells become telomerase negative when the cells are arrested in G₀ suggests that the most primitive quiescent stem cells may escape the direct consequences of antitelomerase drugs. Combined chemotherapeutic strategies may take these observations into consideration and in the future may be designed to include conventional therapies to reduce tumor mass, a recovery period to permit primitive stem cells (recruited to replace damaged regenerating tissues) to return to quiescence, and then antitelomerase treatments to limit the proliferative capacity of surviving tumor cells.

ACKNOWLEDGMENTS

We acknowledge Paul H. Pompa, Anthony Martinez, and Patricia McChesney for excellent technical assistance and Lauren S. Gollahon and Mieczyslaw A. Piatyszek for helpful discussions.

This work was supported by research grants from the National Institutes of Health (AG07992), the Geron Corporation, The Susan Komen Breast Cancer Foundation, Cap Cure, and the Texas Advanced Technology Program (003660-041).

W.E.W. and J.W.S. contributed equally to this work.

REFERENCES

- Allsopp, R. C., H. Vaziri, C. Patterson, S. Goldstein, E. V. Younglai, A. B. Futcher, C. W. Greider, and C. B. Harley. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**: 10114–10118.
- Bacchetti, S., and C. M. Counter. 1995. Telomeres and telomerase in human cancer. *Int. J. Oncol.* **7**:423–432.
- Boccaccio, C., G. Gaudino, G. Gambarotta, F. Galimi, and P. M. Comoglio. 1994. Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *J. Biol. Chem.* **269**:12846–12851.
- Broccoli, D., J. W. Young, and T. de Lange. 1995. Telomerase activity in normal and malignant hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **92**: 9082–9086.
- Chiu, C.-P., W. Dragowska, N. W. Kim, H. Vaziri, J. Yui, T. E. Thomas, C. B. Harley, and P. M. Landsorp. 1996. Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells* **14**:239–248.
- Counter, C. M., A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**:1921–1929.
- Counter, C. M., H. W. Hirte, S. Bacchetti, and C. B. Harley. 1994. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA* **91**:2900–2904.
- de Lange, T. 1992. Human telomeres are attached to the nuclear matrix. *EMBO J.* **11**:717–724.
- Greider, C. W. 1994. Mammalian telomere dynamics: healing, fragmentation shortening and stabilization. *Curr. Opin. Genet. Dev.* **4**:203–211.
- Greider, C. W., and E. H. Blackburn. 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature (London)* **344**:331–337.
- Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. Telomeres shorten during aging. *Nature (London)* **345**:458–460.
- Harley, C. B., and B. Villeponteau. 1995. Telomeres and telomerase in aging and cancer. *Curr. Opin. Genet. Dev.* **5**:249–255.
- Hiyama, E., K. Hiyama, T. Yokoyama, Y. Matsuura, M. A. Piatyszek, and J. W. Shay. 1995. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat. Med.* **1**:249–255.
- Hiyama, K., Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M. A. Piatyszek, J. W. Shay, S. Ishoika, and M. Yamakido. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.* **155**: 3711–3715.
- Hiyama, K., E. Hiyama, S. Ishoika, M. Yamakido, K. Inai, A. F. Gazdar, M. A. Piatyszek, and J. W. Shay. 1995. Telomerase activity in small-cell and non-small-cell lung cancers. *J. Natl. Cancer Inst.* **87**:895–902.
- Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. C. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**:2011–2015.
- Kioussi, C., and P. Gruss. 1994. Differential induction of Pax genes by NGF and BDNF in cerebellar primary cultures. *J. Cell Biol.* **125**:417–425.
- Lundblad, V., and J. W. Szostak. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* **57**:633–643.
- Mantell, L. L., and C. W. Greider. 1994. Telomerase activity in germline and embryonic cells of *Xenopus*. *EMBO J.* **13**:3211–3217.
- McCarthy, J. V., R. S. Fernandes, and T. G. Gotter. 1994. Increased resistance to apoptosis associated with HL-60 myeloid differentiation status. *Anticancer Res.* **14**:2063–2074.
- McEachern, M. J., and E. H. Blackburn. 1995. Runaway telomere elongation caused by telomerase RNA gene mutations. *Science* **376**:403–409.
- Moyzis, R. K., J. M. Buckingham, S. Cram, M. Dani, L. L. Deaven, M. D. Jones, J. Meyne, R. L. Ratliff, and J.-R. Wu. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA* **85**:6622–6626.
- Piatyszek, M. A., N. W. Kim, S. L. Weinrich, K. Hiyama, E. Hiyama, W. E. Wright, and J. W. Shay. 1995. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.* **17**:1–15.
- Prowse, K. R., and C. W. Greider. 1995. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci. USA* **92**:4818–4822.
- Sharma, H. W., J. A. Sokolowski, J. R. Perez, J. Y. Maltese, A. C. Sartorelli, C. A. Stein, G. Nichols, Z. Khaled, N. T. Telang, and R. Narayanan. 1995. Differentiation of immortal cells inhibits telomerase activity. *Proc. Natl. Acad. Sci. USA* **92**:12343–12346.
- Shay, J. W. 1995. Aging and cancer: are telomeres and telomerase the connection? *Mol. Med. Today* **1**:378–384.
- Singer, M. S., and D. E. Gottschling. 1994. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**:404–409.
- Tahara, H., T. Nakanishi, M. Kitamoto, R. Nakashio, J. W. Shay, E. Tahara, G. Kajiyama, and T. Ide. 1995. Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res.* **55**:2734–2736.
- Watts, R. G. 1995. Role of gelsolin in the formation and organization of triton-soluble F-actin during myeloid differentiation of HL-60 cells. *Blood* **85**:2212–2221.
- Wellinger, R. J., A. J. Wolf, and V. A. Zakian. 1993. *Saccharomyces* telomeres acquire single-strand TG₁₋₃ tails late in S phase. *Cell* **72**:51–60.
- Wright, W. E. 1982. The BrdU content of DNA is decreased during reversal of inhibition of myogenesis by deoxycytidine. *Somatic Cell Genet.* **8**:547–555.
- Wright, W. E., M. A. Piatyszek, W. E. Rainey, W. Byrd, and J. W. Shay. 1996. Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18**:173–179.
- Wright, W. E., and J. W. Shay. 1992. The two-stage mechanism controlling cellular senescence and immortalization. *Exp. Gerontol.* **27**:383–389.
- Wright, W. E., and J. W. Shay. 1992. Telomere positional effects and the regulation of cellular senescence. *Trends Genet.* **8**:193–197.
- Wright, W. E., J. W. Shay, and M. W. Piatyszek. 1995. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res.* **23**:3794–3795.
- Zhong, Z., L. Shiue, S. Kaplan, and T. de Lange. 1992. A mammalian factor that binds telomeric TTAGGG repeats in vitro. *Mol. Cell. Biol.* **12**:4834–4843.