inates contamination by microbes, which is an important criterion for maintaining food industry standards. Fermentative growth of naturally heterotrophic microalgae has resulted in dry biomass accumulation to 100 g/liter (*3*, *27*), which is 10 to 50 times the yields obtained by using light-dependent culture systems. Fermentation-based systems can reduce production costs of microalgae by an order of magnitude relative to that incurred by photosynthesis-based production; cost reduction analyses factor in expenses for both fixed-carbon supplementation and equipment operation (*28*). Commercial benefits of fermentation-based systems result from increased biomass, productivity, harvesting efficiency, and reduced losses from contamination. The ability to grow microalgae heterotrophically increases the feasibility for developing a large range of new algal products.

Marine ecosystems also depend on diatoms, which contribute substantially to the reduction of inorganic carbon in marine habitats. Such a contribution may increase substantially as the ecology of oceanic environments is altered (*29*–*32*). The exploitation of diatoms that can be genetically manipulated and that can grow heterotrophically will facilitate the use of mutants to augment our understanding of both photosynthesis and other metabolic pathways that are essential for competing in marine ecosystems.

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15. After 4 weeks in the dark, the transformants that grew were restreaked and maintained on 1.0% glucose. Liquid cultures were grown with 1.0% glucose at 20°C on an orbital shaker. All characterized transformants were generated from independent particle bombardments. Cells were grown at 20°C with continuous illumination at 75 μmol photons m^{-2} s^{-1} in Provasoli's enriched seawater medium with 10× the nitrogen and phosphorus by using Instant Ocean artificial seawater, at 0.5× concentration. Glucose was maintained between 5 and 10 g/liter. Growth rates were determined in 250-ml flasks (50 ml of media) with silicon foam closures. Daily samples measured cell numbers and nutrients. Flasks were stirred at 100 rpm. Fermentations were done in a 2-liter Applikont vessel by using an agitation rate of 100 rpm, dissolved oxygen was maintained at >20% saturation.
16. Cells in logarithmic phase growth were harvested, washed two times, and resuspended in fresh medium. Assays were initiated by adding unlabeled glucose and [D-^{14}C]glucose to 0.05 μCi/ml; the cells were maintained in the light. Samples were removed at 0, 2, 5, 10, and 15 min after the addition of labeled glucose. The cells were collected by filtration, washed with medium containing 1.0% unlabeled glucose, and transferred to scintillation vials.
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18. The cells were broken by using a MinibeadBeater by two cycles at full speed on ice. Cell membranes were pelleted by centrifugation at 100,000*g* for 30 min, solubilized in 2.0% SDS, resolved on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes.
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Telomere Position Effect in Human Cells

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In yeast, telomere position effect (TPE) results in the reversible silencing of genes near telomeres. Here we demonstrate the presence of TPE in human cells. HeLa clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones. The dependence of TPE on telomere length provides a mechanism for the modification of gene expression throughout the replicative life-span of human cells.

Most normal human cells lack the enzyme telomerase, which maintains telomeres, and as a consequence, telomeres shorten with each division until the cells reach replicative senescence (the Hayflick limit). This growth arrest is mediated by p53 and has been suggested to be the result of a DNA damage response to telomeres that have become too short (*1*–*3*). No mechanism has been demonstrated in vertebrates that can account for differences between young and old (but not yet senescent) cells. In *Saccharomyces cerevisiae*, telomere position effect (TPE) can result in the reversible silencing of a gene near a telomere by a mechanism that depends both on telomere length and on the distance to the gene (*4*–*6*). Because telomeres in most human cells shorten with age, TPE would provide a mechanism to incrementally alter phenotype with increasing cellular age (*7*). However, previous efforts to identify TPE in mammalian cells have not been successful (*8*–*10*). We demonstrate here the presence of TPE in human cells and that the strength of the silencing effect is dependent on telomere length.

We seeded de novo telomere formation in (telomerase-positive) HeLa cells by introducing a linear plasmid containing a luciferase reporter adjacent to 1.6 kb of telomere repeats (Web fig. 1) (*11*). Integration of a repeat-containing plasmid can result in breakage of the chromosome, followed by extension of the plasmid telomeric sequences by telomerase and loss of the distal chromosome fragment (*12*). Clones with a telomeric reporter were identified by Southern blotting of purified telomeres (Fig. 1A and Web fig. 2) (*11*) and confirmed by in situ hybridization (Fig. 1B). The mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequence) was estimated from Southern blots to be between 1.5 and 2 kb. Control clones were generated by transfection of an other-

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wise identical linearized construct that lacked telomere repeats. As expected with plasmid transfections, there was a high degree of variation within each group. The clones with a telomeric reporter nonetheless expressed luciferase at a 10 times lower average level than did the clones with an internal integration site (Fig. 2A).

We next sought to demonstrate that the lower expression levels in telomeric clones were the result of heterochromatin formation rather than of damage to the transgene or the presence of a mixed population of clones. Heterochromatin in mammalian cells is normally dependent on histone deacetylation. We therefore investigated whether treatment with trichostatin A (TSA), a highly specific inhibitor of histone deacetylases (*13*), could eliminate the telomeric silencing effect we had observed. Telomeric and internal clones were treated with TSA. After treatment, both sets of clones expressed the reporter at an enhanced level, representing a 2.6 ± 0.4–fold increase for the internal clones and a 51 ± 37–fold increase for the telomeric clones (Fig. 2B). The initial difference in the level of luciferase expression is thus histone deacetylase–dependent. Enhancement of transgene expression by histone deacetylase inhibitors has been noted previously (*14*). Luciferase expression returned to pre-experiment levels within 72 hours after withdrawal of the TSA (*15*). Although the TSA dose used in these experiments is somewhat cytotoxic, the toxicity did not play a role in increasing luciferase expression, because nonspecific treatment with toxic doses of hygromycin led to a moderate decrease in luciferase activity (*15*).

We next extended telomeres in order to establish the length dependence of the observed silencing effect. Increasing the telomerase activity of HeLa cells by infection with a human telomerase reverse transcriptase (hTERT)–encoding retrovirus causes them to elongate their telomeres (Fig. 3A), as has been observed in several other cell lines (*16*). We observed an additional 2- to 10-fold decrease in luciferase activity after telomeric clones were infected with a telomerase-containing retrovirus, as compared to control, vector-only infections (Fig. 3B). This change was not observed in clones with an internal luciferase reporter. These results demonstrate that this effect shares some similarities with yeast TPE and provides a mechanism by which the expression of subtelomeric human genes could increase with replicative age.

The strongest evidence against the existence of mammalian TPE comes from a comparison of mRNA levels for a telomeric *neo* gene in subclones of SV40-transformed human fibroblasts with varying telomere lengths (*8*). This cell line uses the ALT (alternative lengthening of telomeres) pathway to maintain its telomeres, a phenotype that involves altered telomere biology and a substantial increase in total

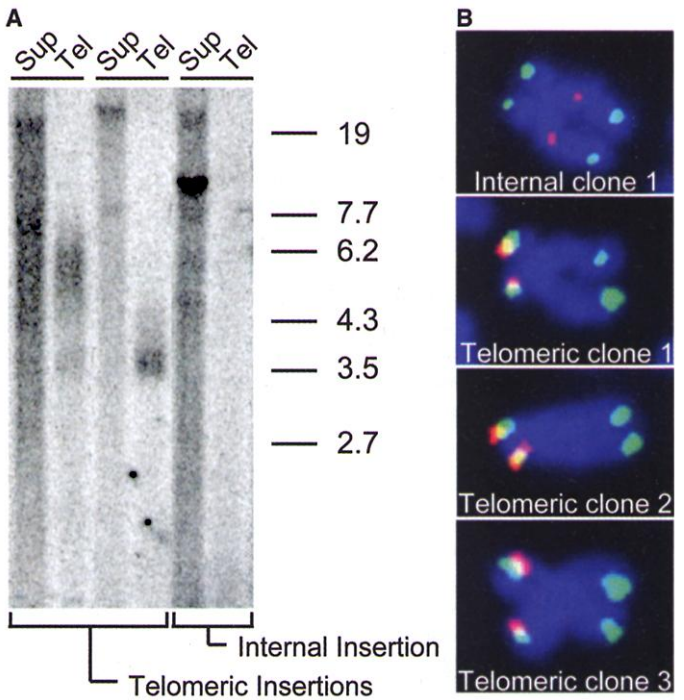

Fig. 1. Identification of telomeric clones by Southern blotting and in situ hybridization. (**A**) Genomic DNA was digested with Stu I, leaving the luciferase gene attached to the plasmid telomere sequences. Telomeres were then separated from bulk genomic DNA as described previously (*23*). Both the telomere fraction and the supernatant were separated on a 0.7% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, Hercules, California), and probed with luciferase sequences. Telomeric luciferase genes appear as a smear in the telomere fraction because of the heterogeneous lengths of the attached telomeres, whereas internally integrated genes appear as a discrete band in the supernatant fraction. Multiple integrations were noted in several of the internal control clones; however, the average was less than two (*11*). Markers shown are from λ DNA digested with *Sty* I (in kilobases). (**B**) Cells were fixed and probed simultaneously with the luciferase plasmid labeled with Spectrum Orange (Vysis, Downers Grove, Illinois), shown in red, and a fluorescein isothiocyanate–labeled oligonucleotide N3′-P5′ phosphoramidate probe complementary to telomere sequences $[(CCCTAA)_3]$, shown in green. 4′,6′-diamidino-2-phenylindole staining is shown in blue. The top panel shows a clone with an internal integration site; the lower panels demonstrate the colocalization of the telomere and luciferase signals in three independent telomeric clones.

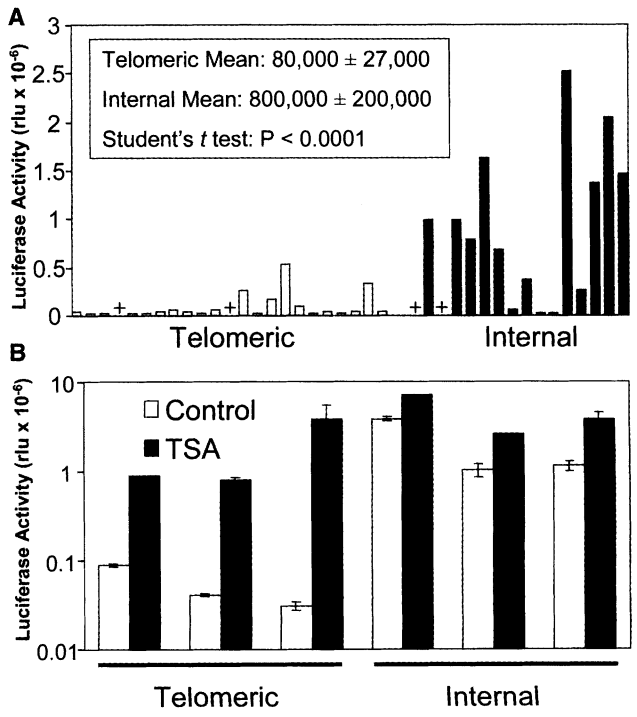

Fig. 2. Telomeric clones show a 10 times lower level of luciferase activity that is restored by a histone deacetylase inhibitor. (**A**) Puromycin-resistant clones were screened with a Luciferase Assay System (Promega, Madison, Wisconsin) on an Optocomp I luminometer (MGM Instruments, Hamden, Connecticut). The results for 23 telomeric and 15 internal integrations are shown. The plus signs indicate clones with a level of expression too low to be visible on this scale. (**B**) Silencing is relieved by the histone deacetylase inhibitor TSA. Three telomeric and three internal clones were treated with TSA (200 ng/ml) (Sigma, St. Louis, Missouri) for 24 hours. The medium was replaced, and the cells were incubated for an additional 24 hours before collection for luciferase assays. Note the switch to a logarithmic scale. rlu, relative light units.

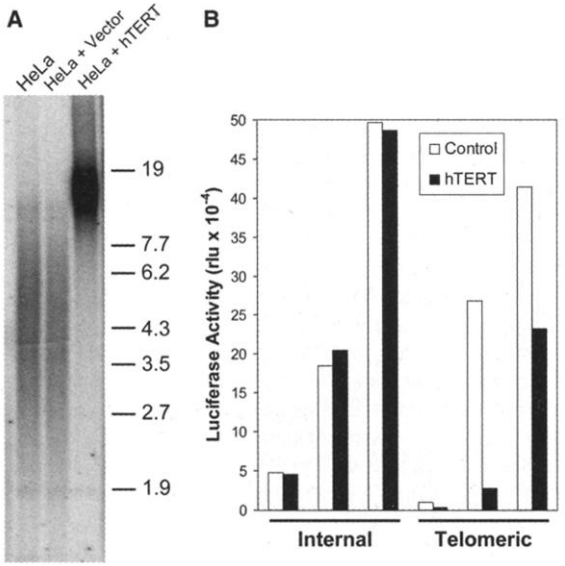

Fig. 3. Silencing in telomeric clones is enhanced by an increase in telomere length. (**A**) Infection of HeLa cells with an hTERT-encoding retrovirus causes telomere elongation, as demonstrated by terminal restriction fragment analysis. Mean telomere length increased from approximately 5 kb to almost 14 kb. Genomic DNA was digested with six restriction enzymes to degrade nonrepetitive sequences. Samples were then separated on a 0.7% agarose gel and probed with an oligonucleotide complementary to telomere repeats. Markers shown are λ *Sty* (in kilobases). (**B**) Telomeric clones infected with hTERT express 2 to 10 times lower levels of luciferase activity as compared to control, vector-only infections. Internal clones having comparable initial values retain full expression of the luciferase reporter after infection with hTERT.

telomeric DNA (*17*). It is possible that the extra telomeric sequences in ALT cells are titrating out factors essential for TPE, as has been observed in yeast (*18*), so that ALT cells might not exhibit TPE. Another report may have failed to identify TPE, because the healed telomere appears to have been extremely short and/or because it was located >50 kb from the nearest gene that could be examined (*9*). In at least one case, data consistent with a very mild mammalian TPE have been described (*19*), and the insertion of telomere repeats into an intron of the *APRT* gene of Chinese hamster cells was shown to cause a twofold reduction in the mRNA level (*20*).

A number of proteins have been reported to change in expression level as a function of the replicative age of the cell (*21*, *22*). The existence of TPE in mammalian cells raises the possibility that some presenescent changes could be "programmed" by the progressive shortening of telomeres with ongoing cell division, leading to altered patterns of gene expression that might affect both cell and organ function. It will be important to identify endogenous genes whose expression is influenced by telomere length in order to determine whether TPE actually influences the physiology of aging or cancer.

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Requirement of *CHROMOMETHYLASE3* for Maintenance of CpXpG Methylation

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Epigenetic silenced alleles of the *Arabidopsis SUPERMAN* locus (the *clark kent* alleles) are associated with dense hypermethylation at noncanonical cytosines (CpXpG and asymmetric sites, where X = A, T, C, or G). A genetic screen for suppressors of a hypermethylated *clark kent* mutant identified nine loss-of-function alleles of *CHROMOMETHYLASE3* (*CMT3*), a novel cytosine methyltransferase homolog. These *cmt3* mutants display a wild-type morphology but exhibit decreased CpXpG methylation of the *SUP* gene and of other sequences throughout the genome. They also show reactivated expression of endogenous retrotransposon sequences. These results show that a non-CpG DNA methyltransferase is responsible for maintaining epigenetic gene silencing.

Cytosine methylation plays a major role in determining the epigenetic expression state of eukaryotic genes. This methylation is most often found at the symmetrical dinucleotide CG (or CpG sites). CpG methylation is maintained by the well-studied DNMT1 subfamily of methyltransferases, which includes *Arabidopsis* MET1 (*1–3*). Methylation at sites other than CpG is also found in many organisms (*4*), but the mechanism by which this methylation is maintained is poorly understood. *Arabidopsis* can tolerate major disruptions in DNA methylation (*2, 3, 5*), making it useful for genetic analysis of methylation pattern-

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