SPECIAL ARTICLE

Telomeres, Telomerase, and Immortality

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A current hypothesis gaining prominence proposes that activation of the enzyme telomerase is necessary for cells to become immortal, or capable of proliferating indefinitely. The theory suggests that almost all cancer cells must attain immortality for progression to malignant states and, hence, require activation of telomerase. This article reviews the function and formation of telomeres as background to evaluating the "telomere hypothesis." Experiments in support of and experiments that challenge the hypothesis are examined. Possible approaches to telomerase inhibition are discussed. [J Natl Cancer Inst 87:884-894, 1995]

Cancer is believed to be caused by multiple mutations that cumulatively subvert the normal growth controls of a cell. Although different cancers may involve mutations in a particular gene, it appears that individual types of cancer may be distinguished by mutations in characteristic sets of genes. In spite of these distinct etiologies, recent findings support the model that activation of the enzyme telomerase may be important in many malignant tumor cells.

After much anticipation, a collaborative team led by Calvin Harley at Geron Corporation (Menlo Park, CA) and Jerry Shay at The University of Texas, Southwestern Medical Center (Dallas), has shown a provocative correlation between tumor cells and increased activity of the enzyme telomerase (*1*). Using a new, highly sensitive assay for activity of the enzyme, these researchers were able to show that tissue extracts from 90 of 101 distinct tumors representing 12 cancer types and 98 of 100 independent immortalized cell lines (cells capable of growing indefinitely in culture) contain telomerase activity, whereas no enzyme activity could be detected in benign tumors, somatic (non-germline) tissues, and mortal cell lines examined. Such a strong correlation initially suggested that telomerase may play a critical role in the progression or maintenance of the malignant state. The observation that telomerase activity is absent or present at low levels in most somatic tissues, combined with speculation about an obligate role for telomerase in tumorigenesis, has fueled interest in targeting telomerase for anticancer therapy.

What is the relevance of this finding given the landscape of existing telomerase research, and what therapeutic hope does the telomerase-inhibiting strategy offer? To better understand the biological basis of this potential anticancer therapy, this article describes key experiments that have led to our current knowledge of telomeres and telomerase, with special attention to the evolution of the theory that telomerase activation is required for the continued proliferation of tumor cells. Following this survey of the basic research, this article evaluates the existing evidence for the telomerase activation theory, discussing further experiments necessary to validate the theory. Finally, a few approaches to isolating a telomerase inhibitor are described.

Telomeres

Müller (*2*) and McClintock (*3*) in the 1930s and 1940s were the first to recognize that chromosome ends, or telomeres, are essential to maintain chromosomal integrity. Chromosomes with truncated ends are unstable, fusing with other chromosomes or becoming lost upon cell division. More recently, investigators sought to elaborate the molecular basis of the telomere's important properties. Capitalizing on the large number of nuclear chromosomes (and, accordingly, the large number of telomeres) in the ciliate *Tetrahymena*, Blackburn and Gall (*4*) determined that *Tetrahymena* telomeres consist of the short sequence 5′ GGGGTT 3′ repeated in tandem arrays. The number of these repeats is variable at each chromosome end, causing restriction endonuclease fragments that bear telomeric repeats to migrate as diffuse bands in electrophoretic gels (Fig. 1, B).

After identification of the telomeric repeats in *Tetrahymena*, similar repeats were identified in a variety of organisms (*5*). The sequence of the repeating unit was found to vary among different species, but most known repeats are 5-8 base pairs (bp) in length and are rich in G bases on the DNA strand that extends 5′ to 3′ toward the chromosome end. Human telomeres contain the repeat TTAGGG, which may be reiterated in tandem for up to 15 kilobases (kb) (*6-8*). Initial work with ciliates and yeast suggested that "telomeric DNA" associates with specific proteins to form a telomeric nucleoprotein complex (*9*). Binding of the necessary proteins may rely on the sequence of the repeat, as alteration of the telomeric sequence in both human and *Tetrahymena* cells causes formation of incompetent telomeres (*10-12*). Human and other telomeres have been demonstrated to associate

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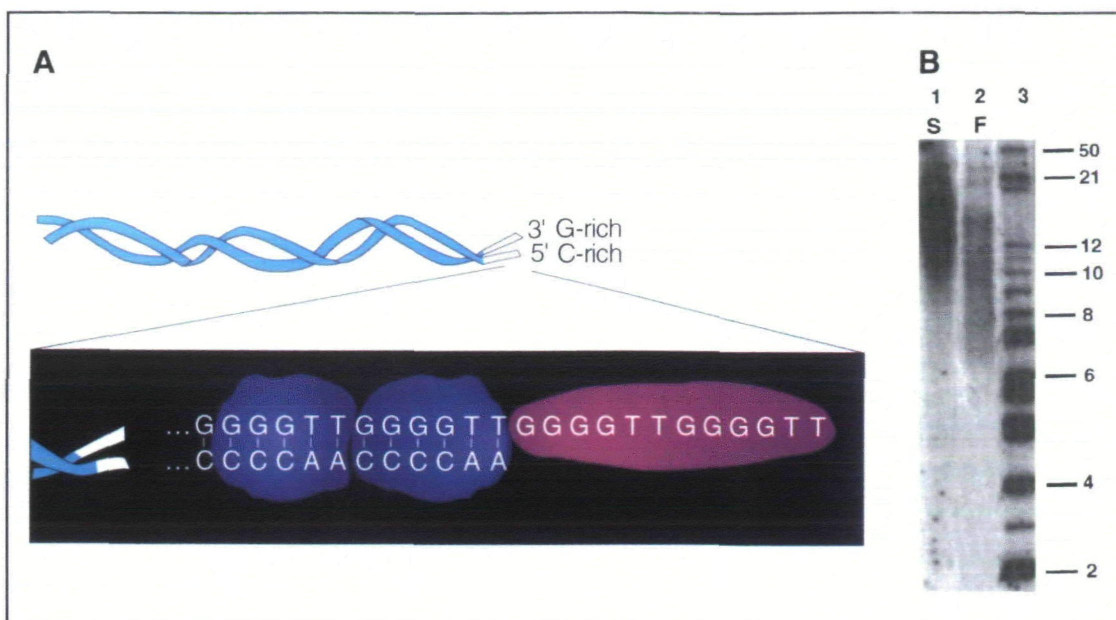

Fig. 1. Structure of a telomere. **A**) Telomere (white section depicted for one end of blue chromosome) is made up of a DNA component and multiple protein components. The DNA consists of a short sequence (for *Tetrahymena*, 5′ GGGGTT 3′) repeated in tandem for many kilobases. The telomeric region is double stranded, except for the extreme 3′ end, where the short single-stranded stretch may assume a characteristic structure (structure not shown). Telomere-binding proteins (in purple and magenta) associate with the double- and single-stranded regions and may be critical for preventing degradation and recombination. **B**) Length of telomeres from sperm (lane 1) and adult skin fibroblasts (lane 2) are visualized by digesting DNA with a restriction enzyme that cuts just proximal to the telomere. After probing the gel with a ^{32}P end-labeled telomere-specific oligonucleotide $(CCCTAA)_3$, the released terminal fragments (telomere restriction fragments [TRF]) appear as a smear in the lane, reflecting TRF lengths that vary in size. The size and position of the molecular weight markers (lane 3) are indicated on the right. The average TRF of sperm is longer than the average TRF from the fibroblasts (gel provided by R. C. Allsopp and C. Harley).

with the nuclear matrix protein fraction, which may include nuclear envelope and other nuclear proteins (*13*). Although the precise molecular structure of any telomere has yet to be elucidated, it appears to carry out at least two essential functions.

The observations by Müller (*2*) and McClintock (*3*) suggest that telomeres stabilize chromosome ends, protecting them from recombination and end-degrading enzymes. In yeast, it has been shown that removal of the telomere of a nonessential chromosome causes a dramatic loss of that chromosome (*14*). It is unknown how the telomeric structure affords this protection, but it has been suggested that interactions with proteins in the nuclear membrane may effectively shield chromosomal ends from degrading enzymes (*13*).

Another function of chromosomal ends was first recognized after the double-stranded structure of DNA and its semiconservative mode of replication were discovered (*15*). This function addresses a dilemma called the "end replication problem." According to our understanding of DNA replication, the polymerase that copies the strands of DNA prior to each cell division absolutely requires a short "RNA primer" sequence to begin DNA polymerization in the 5′ to 3′ direction. After DNA polymerization, the RNA primers are degraded and replaced by DNA synthesis extending from an upstream primer. As shown in Fig. 2, the primers that are annealed to the extreme 3′ end of each strand cannot be replaced. This replication strategy therefore predicts the progressive shortening of chromosomal DNA at the 3′ ends over multiple cycles of replication—a potentially catastrophic trend. Telomeric repeats could temporarily nullify this trend by providing a cushion of expendable noncoding sequence at the chromosome ends.

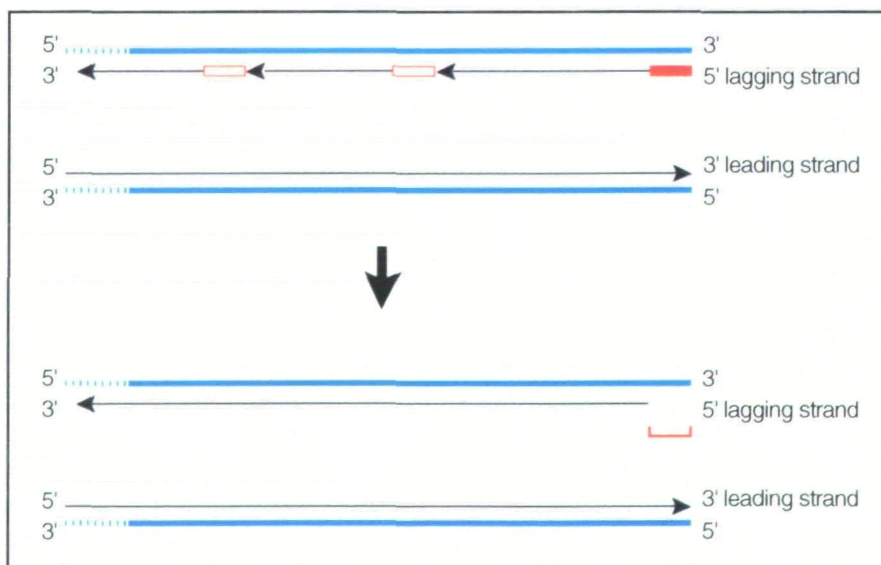

Fig. 2. End replication problem. Original chromosome strands are depicted in blue. As the replication fork proceeds from left to right, the leading strand (black arrow directed rightward) proceeds continuously to replicate one strand of original DNA. The direction of the lagging strand is opposite to the direction of the replication fork and relies on the ligation of Okazaki fragments (leftward arrows), which are primed with short stretches of RNA (red boxes). Most RNA primers are replaced with DNA from an upstream Okazaki fragment, but the terminal RNA primer (solid red box) is never replaced with DNA. Consequently, each round of replication produces daughter chromosomes, which lack the sequences corresponding to the original 3′ ends (red bracket). (The terminal primer actually may not anneal to the extreme 3′ end, contributing to further loss of end sequences.)

Telomerase Adds Telomeric Sequences

Evolutionary conservation of related telomeric repeats among different species affirmed the importance of these ends, but the question of how the repeats are propagated remained unresolved until 1985. Shampay et al. (*16*) had demonstrated that *Tetrahymena* telomeric DNA introduced into yeast became elongated with yeast telomeric sequences. From this result, they proposed that each organism possesses the ability to transfer host-specific terminal sequences onto exogenous DNA. This model was tested directly and verified by Greider and Blackburn (*17*), who, using *Tetrahymena* extracts, detected an activity that added telomeric sequences to single-stranded telomeric DNA oligonucleotide primers. Their experiments elegantly proved that the terminal transferase, or *telomerase*, activity was independent of DNA polymerase-α and of a DNA template but that the activity required an oligonucleotide primer containing repeats of a telomeric sequence. This sequence could be from *Tetrahymena* or yeast. These groundbreaking experiments confirmed the existence of telomerase activity but also unearthed a new crop of mysteries: How could telomerase add sequences de novo, without a template, and why did it require a primer containing telomeric sequences? Further purification of the telomerase activity provided answers to both queries.

Greider and Blackburn (*18*) found that, if extracts containing telomerase activity from *Tetrahymena* are treated with the RNA-degrading enzyme ribonuclease (RNase) A, the extract is no longer competent to elongate primers. Concluding that this result reflected an essential role for RNA, in addition to proteins, in the telomerase complex, they isolated a 159 nucleotide RNA that copurified with telomerase activity. In the original article describing this work, Greider and Blackburn (*18*) theorized a specialized role for the RNA:

> It is tempting to speculate that the RNA component of the telomerase might be involved in determining the sequence of the telomeric repeats that are synthesized and/or the specific primer recognition. If the RNA of telomerase contains the sequence CCCCAA, this sequence could act as an internal guide sequence. . . .

The sequence of the 159 nucleotide RNA remarkably fulfilled these predictions. In the third of the trio of articles by Greider and Blackburn that provided the basis of our understanding of telomerase, the authors (*19*) cloned the single gene encoding this RNA and identified the region from positions 43 to 51 within the RNA as having the sequence 5′ CAACCCCAA 3′ (Fig. 3). They showed that preincubation of telomerase with an antisense deoxyoligonucleotide complementary to this region (i.e., containing the sequence 5′ TTGGGGTTG 3′) interfered with primer elongation activity, while DNA oligomers directed to other regions of the RNA had no effect. When the RNA/DNA duplex-degrading enzyme RNase H was added together with the antisense oligonucleotide, the telomerase RNA was cleaved in the region to which the oligonucleotide had hybridized. In sum, these results strongly implicated the CAACCCCAA sequence as the template for repeat synthesis. This conclusion was later confirmed by site-specific mutations in this region, which yielded telomerases that now synthesized repeats containing the corresponding change in the cell (*10*). These results also showed

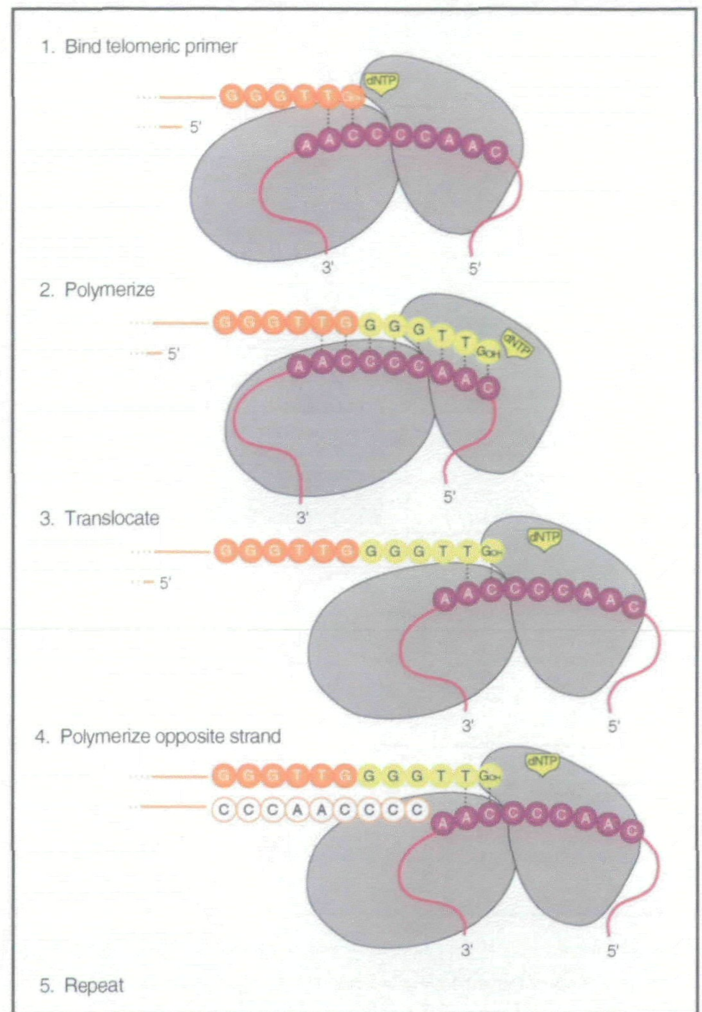

Fig. 3. Mechanism of telomere addition. Chromosomal DNA end is shown in orange, telomerase RNA is indicated in purple, and telomerase proteins are shown in gray. Step 1: Chromosomal DNA binds part of the template region in telomerase RNA. Step 2: Telomerase RNA provides the template for elongating the 3′ end of the chromosome (newly synthesized sequence in yellow). Step 3: The chromosome is translocated and repositioned to repeat the polymerization step. Step 4: The complementary strand is synthesized (orange outlined circles), presumably by a standard DNA polymerase. Step 5: The process is reiterated to add multiple copies of the telomeric repeat sequence.

that telomerase was responsible for telomere synthesis in the cell as well as in the test tube.

Greider and Blackburn (*19*) recognized that the template RNA contains approximately one and one-half times the complement of the GGGGTT repeat and speculated that the surplus sequence enables telomerase to hybridize to an existing telomeric repeat via several bases and to extend the repeat using the remaining bases as a template (Fig. 3). This model would explain the need for a primer containing telomeric sequences. These authors further suggested that the observation that heterologous telomeric repeats from other species successfully prime the *Tetrahymena* telomerase reaction reflects a conserved secondary structure formed by the repeats that can be recognized by the enzyme. Although some strong evidence now exists in support of both contentions, these issues are still being pursued.

It was thus found that telomerase is a ribonucleoprotein (RNP) complex that utilizes a region in the enzyme's RNA

component as a template for repeat synthesis. In other words, telomeric repeats are added de novo to the 3′ end of chromosomes via an RNA-dependent DNA polymerase (i.e., a reverse transcriptase) mechanism. It is speculated that a standard DNA polymerase completes the duplex telomere by synthesizing the DNA strand complementary to the telomerase-generated region. Telomerase RNAs from many single-cell organisms have now been characterized, and each contains a potential template sequence complementary to its respective telomeric repeat.

Isolation of the protein components of the telomerase enzyme has proved more elusive. At last, the prolonged drought in the field of telomerase proteins may be over, as Greider et al. (*20*) recently described the purification and cloning of two polypeptide constituents of *Tetrahymena* telomerase.

Human Telomerase

The discovery of telomerase and its RNA dependence was a watershed in the understanding of telomere biology. This work fostered a renewed interest in the function of chromosome ends, leading to inquiries into the role of telomeres and telomerase in human cells. While initial attempts to find telomerase activity in human tissues encountered considerable difficulties, Morin (*8*) ultimately identified the activity in immortalized HeLa cells. This telomerase activity is RNase A sensitive, suggesting that, like the *Tetrahymena* telomerase, it is an RNP. At the time of Morin's discovery, the implications of finding telomerase in an immortalized cell line were not widely appreciated.

Now, with the latest reports of telomerase activity in malignant and immortalized cells and the reported cloning of the human telomerase RNA sequence (*21*), this field may be poised for another breakthrough. The assertion, first put forth by Harley, Futcher, and Greider (*22*) in 1990, is that telomerase activation is a necessary step toward immortalization. Immortalization has since been proposed to be a necessary step in the pathogenesis of tumor malignancy (*23-25*). With the steady accumulation of data consistent with this proposal, the theory is gaining acceptance. Does this signal a major paradigm shift, or are these conclusions premature? The next section explains the experiments and observations that directed the development of this hypothesis of telomerase's role in cancer.

Telomere as a Mitotic Clock?

The Impetus: Telomerase Is Inactive in Normal Somatic Cells

If telomeres are fundamental to chromosomal stability, why would activation of telomerase be associated with aberrant tumor cells? This question broaches the puzzle of telomerase activity in human cells, a riddle that has, until recently, been obscured by the difficulty in measuring telomerase activity in human tissues. Unlike the ciliate *Tetrahymena*, which contains up to 40 000 telomeres per macronucleus and a correspondingly robust telomerase activity, human cells with the normal 46 chromosomes (and 92 telomeres) appear to contain dramatically lower levels of telomerase activity. Furthermore, primary tissue samples are not available in the large quantities possible when culturing ciliates.

The telomerase detection problem was initially addressed indirectly by measuring changes in the average telomere length of chromosomes in various tissue types and inferring telomerase activity by monitoring changes in this length. A battery of such studies suggested that telomerase activity is regulated in a cell type-specific manner: Sperm cells were found to contain long telomeres (10-14 kb) whose length does not appear to shorten with increased age (*26*). Telomeres from these germline cells are consistently several kilobases longer than telomeres of somatic cells (peripheral blood cells or fibroblasts) from the same individual (*13,27,28*). In vivo, these somatic cell telomeres were calculated to shrink by about 15-40 bp per year (*26,29*). These results suggest that telomerase sustains and replenishes telomeric sequences specifically in germ cells (sperm and oocytes) but is inactive in most somatic tissues, allowing the gradual depletion of the chromosomes' terminal sequences. These predictions have since been validated by direct testing of telomerase activity in various tissue types.

Shrinking Telomeres Reflect Mitotic Age: the Model and Evidence

Publishing in the early 1970s, the Soviet scientist A. M. Olovnikov (*15*) was the first to suggest that gradual loss of chromosome ends could lead to an exit from the cell cycle. Spurred by mounting experimental support for this theory, Harley et al. (*22*) introduced a revised theory in 1990; they proposed that the shrinking telomere may be the cell's measure of the mitotic age of the organism. Specifically, this hypothesis posits that, as chromosomes are incompletely replicated with each division, terminal sequences are inevitably lost to the point where they no longer protect the chromosome end from recombination and degradation (Fig. 4, A). Such a phenomenon could represent a molecular equivalent of aging, with the end point being an exit from the cell cycle and senescence. It should be noted that mortality by senescence, or the loss of the cell's ability to propagate, is to be distinguished from apoptosis, the activation of a cell death program. Although senescence could precede the activation of programmed cell death, such a relationship has yet to be established.

The strongest evidence for telomere involvement in senescence is the finding by Allsopp et al. (*26*) that telomere lengths are predictive of the replicative capacity of fibroblasts in culture. These authors collected cells from donors aged zero (fetal tissues) to 93 years and established primary cultures from these samples. By measuring telomere length early in the establishment of the culture and determining the life span of each culture, they found that the age of the donor did not correspond well to fibroblast telomere length and did not correlate strongly with the proliferative ability of the cells. Instead, the number of divisions that the cells could undergo in culture was directly proportional to the initial length of their telomeres when placed in culture. Allsopp et al. further determined that cell samples from individuals with the premature aging disease Hutchinson-Gilford progeria contained telomeres with a significantly shorter average length than telomeres from normal donors of the same age. In culture, the cells from progeria patients exhibited reduced proliferative ability. Although these experiments do not demonstrate cause, the correlation between telomere length and

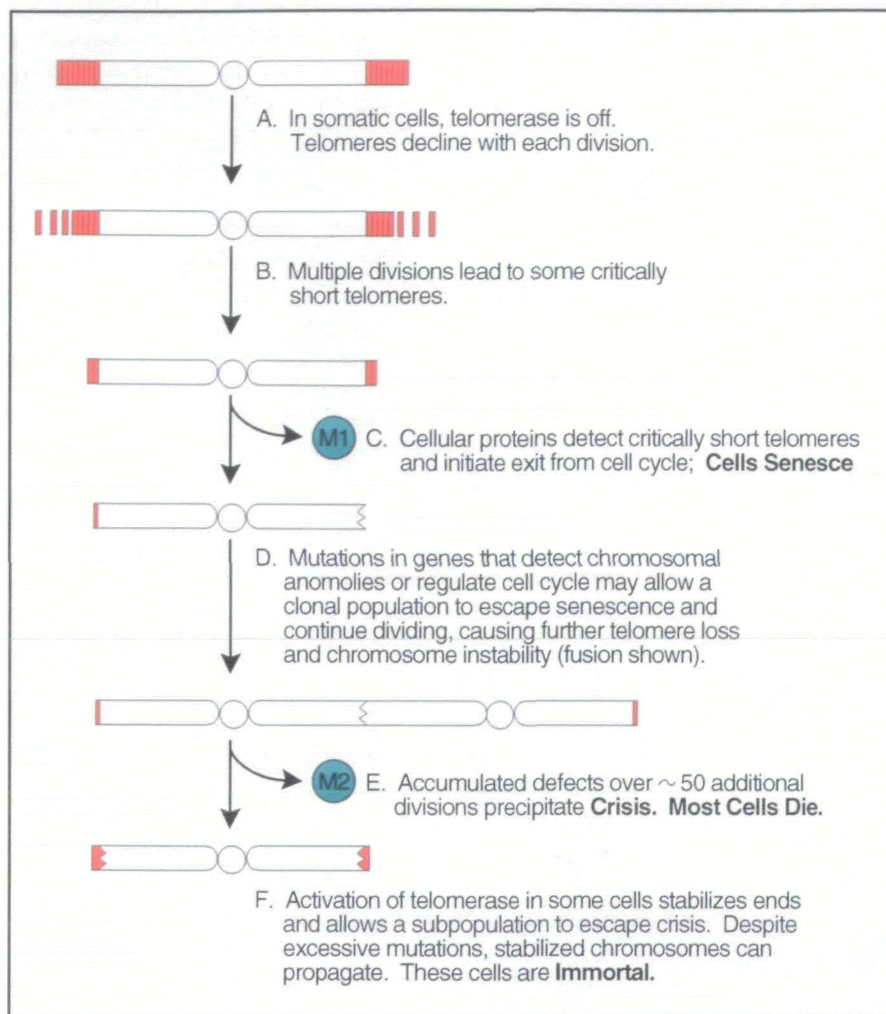

Fig. 4. Model for telomere role in aging and immortality.

the number of doublings before senescence supports the idea that telomeres may gauge and limit the number of divisions that a cell undergoes.

Stabilization of Telomeres and Cellular Immortality: the Model and Evidence

A compelling extension of the theory that marginal telomeres induce cell mortality is the suggestion that reactivation of telomerase may enable cells to prolong their life span; i.e., the immortal character of tumor cells may rely on the aberrant reactivation of the telomerase enzyme. While the measurement of telomeres in sperm and fibroblasts gave support to the hypothesized role of telomeres in cellular mortality, telomere lengths in tumor tissues appeared to contradict the idea that telomerase activation is necessary for immortality. Telomeres from a variety of tumor types were found to be notably shorter than those in normal tissues, implying that telomerase may be active at a low level in somatic cells and *decline* in tumor tissues (*29-36*). The issue was made more perplexing by the demonstration that immortalized HeLa cells derived from a uterine cervical carcinoma contain telomerase activity as well as exceptionally long telomeres (*8,31*). But is telomere length an accurate reflection of telomerase activity? Is telomerase actually active in somatic cells, and does this activity change in tumor cells?

Partial resolution of these issues came from the examination of an in vitro model for cell immortalization (Fig. 4). Human cells in culture have been demonstrated to undergo a finite number of divisions before they senesce (*37*). This first mortality stage (M1) can be averted with mutations in the tumor suppressor genes p53 or Rb, indicating that the proteins encoded by these genes normally act to mediate exit from the cell cycle (*38*). Likewise, M1 can be avoided by the addition of specific viral DNA sequences capable of overriding the cellular growth control signals (*24,39,40*). In either case, survivors of M1 may undergo an additional 50 divisions. Eventually, these aberrant cells reach a second mortality stage (M2), also known as *crisis*, which is punctuated by another surge of cell death. While most transformed cells do not survive M2, a subpopulation may escape from this crisis point at a low frequency, giving rise to "immortal" cells, which have unlimited proliferative potential. Whether the immortalized state is a representative model for cancer remains a matter of debate. Specifically at issue is whether the cells of a tumor can progress to the malignant state without gaining the ability to divide indefinitely. In the midst of this controversy, this in vitro system has at least served to distinguish telomere stabilization from the other events that may be involved in carcinogenesis.

Counter et al. (*23*) followed telomere length and telomerase activity through the stages of immortalization for a primary cell

line transformed with simian virus 40 or adenovirus DNA. They observed that telomere attrition continued through M2, often coinciding with the appearance of abnormal or fused chromosomes, but that the exceedingly short telomeres became stable upon immortalization. These authors were further able to assay telomerase activity in extracts from these cells and determined that telomere stabilization during immortalization corresponds to the onset of telomerase activity. These experiments resolved the conundrum of short telomeres existing in the presence of active telomerase: Telomerase could be activated after dramatic telomere loss, acting to stabilize the degraded ends. Though persuasive, this in vitro evidence fell short of demonstrating telomerase activity in malignant cells in vivo.

Measuring Telomerase in Primary Tissues

The standard method of measuring the terminal transferase activity of telomerase was initially to assay the ability of the cell extract to add telomeric repeats to the 3′ end of a synthetic deoxyoligonucleotide primer (*8,18*). The incorporation of radioactively labeled nucleotides could be observed by separating the reaction products on a polyacrylamide gel and exposing the gel to autoradiographic film. Because telomerase appears to pause after synthesis of each set of six nucleotides, products of a reaction yield a typical telomerase pattern of bands spaced at six nucleotide intervals. Because of the low levels of telomerase in mammalian cells, interfering activities, and limited quantities of tumor samples, detecting telomerase activity by this method proved difficult in primary tumor samples. Two groups nonetheless managed to use this technique to demonstrate active telomerase in extracts of ovarian epithelial carcinoma (*41*) and malignant hematopoietic carcinoma cells (*42*), lending support to the theory that reactivation of telomerase accompanies immortalization.

Further evidence for this association was obtained by Kim et al. (*1*) through two technical achievements that substantially improved the sensitivity of the telomerase assay. The first advance optimized extract preparation, allowing efficient telomerase extraction from a smaller starting quantity of cells via detergent-mediated cell lysis. (The old method employed hypotonic lysis.) The second, most significant, breakthrough allowed exponential amplification of the products generated in a telomerase reaction (Fig. 5). This amplification was accomplished by using the products of a telomerase-catalyzed primer extension reaction as templates for a polymerase chain reaction (PCR). Through successive rounds of oligonucleotide-primed DNA synthesis and strand separation, the telomerase reaction products were amplified many times over, enhancing the sensitivity of the conventional assay 10^4-fold.

This sensitized assay, termed the TRAP (telomere repeat amplification protocol) assay, enabled large-scale testing for telomerase activity in a collection of 101 tumor biopsy specimens and 100 immortal cell populations as well as in their normal counterparts. Consistent with previous reports, 98 of the 100 immortal lines exhibited telomerase activity. But most compelling was the telomerase activity found in 90 of 101 actual tumor tissues, representing 12 distinct tumor types. No telomerase activity was seen in 22 mortal cultured cell populations and 50 normal somatic tissues tested. Benign proliferating fi-

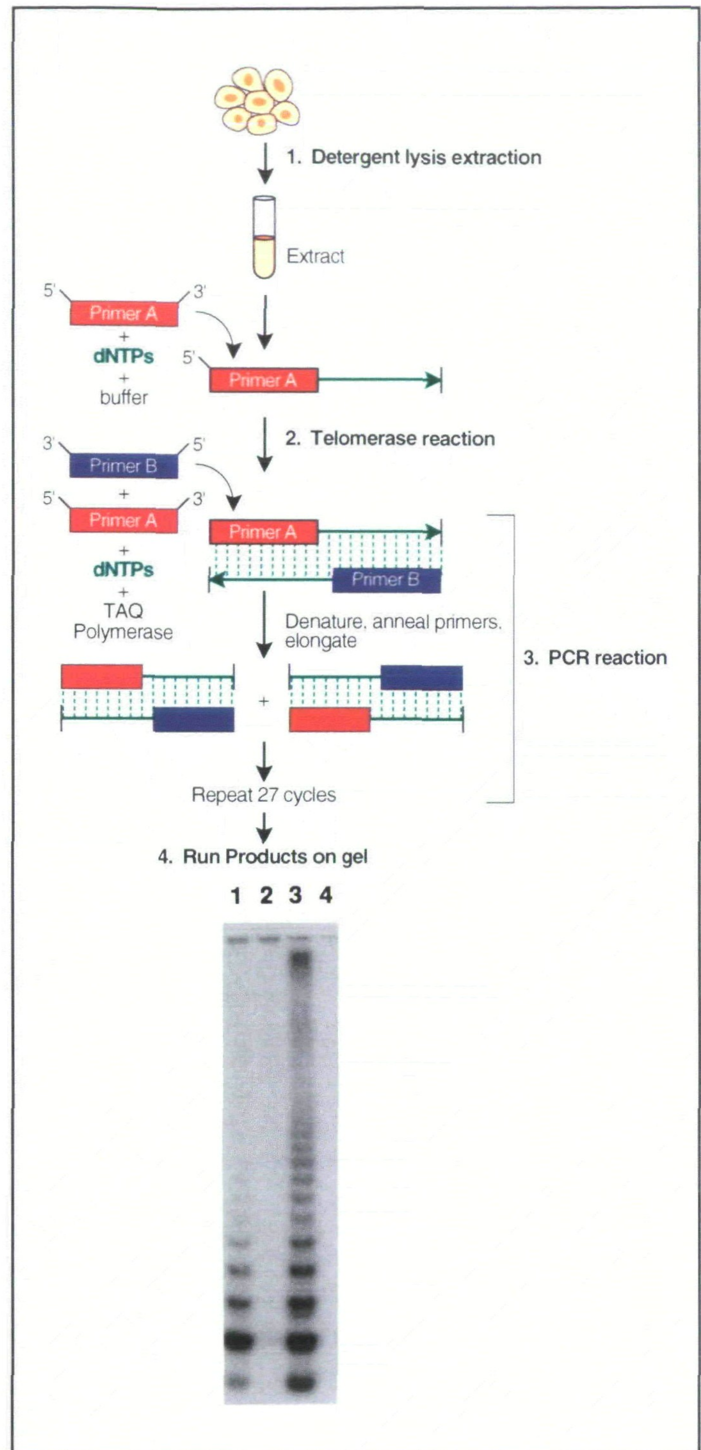

Fig. 5. TRAP assay for telomerase activity. Step 1: Extract is prepared from primary tissues or established cell lines by detergent lysis or the previously established hypotonic lysis method. Step 2: Telomerase in the extract elongates a primer (Primer A, in red) using added deoxynucleotide triphosphates (dNTPs). Since telomerase is a processive enzyme, the products of this reaction will be heterogeneous in length (only one length shown). Step 3: Each product of the telomerase reaction is amplified by PCR: Primer B (in blue), which contains sequences complementary to the telomerase repeat, anneals with the telomerase reaction products; TAQ polymerase extends Primer B to synthesize duplex versions of each telomerase product; these double-stranded fragments are denatured, annealed to primers again, and duplicated. This step is repeated for 27 cycles. Step 4: The amplified products are run in a polyacrylamide gel, where the fragments of heterogeneous length create a "ladder" pattern of bands (gel provided by J. Shay). Gel shows results of TRAP assay using the following tissue samples: lane 1, squamous cell carcinoma; lane 2, normal tissue adjacent to squamous cell carcinoma; lane 3, endometrial carcinoma; and lane 4, normal endometrium adjacent to endometrial carcinoma.

broid tissues also tested negative, while telomerase activity was at last demonstrated for normal ovaries and testes (presumably as a result of activity in the germ cells). Corroborating the previous reports (*41,42*) of telomerase activity in ovarian and hematopoietic carcinomas, this analysis established a strong correlation between cancerous tissues, immortal cell lines, and activation of telomerase. Telomere lengths in the immortal lines varied from less than 4 kb in some lines to greater than 10 kb in others, confirming that, in immortalized cells, telomere length does not accurately reflect telomerase activity. Since the original report of the TRAP assay (*1*), more than 400 independent tumor samples have been tested for telomerase activity using this assay (*43,44*). The recent data confirm that a high percentage (84.8%) of malignant tumors exhibit telomerase activity. These findings are summarized in Table 1.

Authors' Interpretations

Kim et al. (*1*) proposed that the finding that telomerase is active in a wide array of immortal human cell lines strengthens the assertion that this enzyme may be necessary for achieving the immortal state. The additional connection between telomerase reactivation and malignant tumor tissues led these authors to speculate that malignant tumor cells are immortal and, consequently, rely on telomerase. They proposed that early stage tumors are akin to transformed cells in culture and may similarly lose telomeric DNA until a crisis point is reached. (In vitro, the crisis is M2.) Only by stabilizing the telomeres (through the induction of telomerase) can these cells propagate indefinitely, developing and perpetuating their malignant characteristics. From this theory follows the prediction that disruption of telomerase activity would impede the ability of a tumor to grow indefinitely. Since reproductive cells are the only cell population shown to normally have high levels of telomerase, therapy aimed at this enzyme would be expected to have few adverse effects in other cells.

Other Considerations

The predictions of Kim et al. (*1*) have fueled enthusiasm for inhibition of telomerase as a means to combat cancer. As more attention is turned to this strategy, this is a timely juncture to take a closer look at the body of existing work on telomerase and its relation to cancer. Critical examination of the data points to three important issues that warrant consideration when pursuing the telomerase inhibition anticancer strategy: 1) The link between telomerase and cancer is still only correlative; 2) even if causal relationships can be established and telomere stabilization enables survival through the crisis stage (M2), telomerase-independent mechanisms for extending telomeric DNA may thwart the effectiveness of antitelomerase drugs; and 3) in some tumors, this approach may be limited by timing and specificity constraints.

Table 1. Published telomerase activity in human tissues—May 1995

Site or type of tumor: investigators (ref. No.), y	Normal, adjacent to tumor, or benign tissues*	Tumor tissues* (%)
Lung: Hiyama et al. (*44*), 1995	3/68 adjacent	109/136 (80.1)
Breast: Kim et al. (*1*), 1994	0/8 normal 2/20 adjacent	19/24 (79.0)
Prostate:		
Kim et al. (*1*), 1994	0/8 normal	23/27 (85.1)
Sommerfield et al. (*55*), 1995	1/10 benign prostatic hyperplasia	
Colon:	0/24 adjacent	
Kim et al. (*1*), 1994	0/20 benign polyps	22/23 (95.6)
Chadeneau et al. (*56*), 1995	0/1 adenoma	
Liver: Kim et al. (*1*), 1994		1/1 (100)
Ovarian: Counter et al. (*41*), 1994	0/8 normal ascites	7/7 (100)
Renal: Mehle et al. (*57*), 1995	0/55 adjacent	40/55 (72.7)
Neuroblastoma:		
Kim et al. (*1*), 1994	0/13 adjacent	94/100 (94.2)
Hiyama et al. (*43*), 1995	0/4 ganglioneuroma	
Hematological:†		
Lymphoma: Kim et al. (*1*), 1994		5/5
CLL: Kim et al. (*1*), 1994		2/2 (91.3)
ALL: Kim et al. (*1*), 1994		14/16
Brain: Kim et al. (*1*), 1994		6/8 (75)
Miscellaneous: Wilms', head and neck, rhabdomyosarcoma, leiomyosarcoma: Kim et al. (*1*), 1994	6/16 adjacent head and neck 2/6 adjacent Wilms' tumor 0/10 normal myometrium 0/11 leiomyoma (fibroids) 0/50 postmortem tissues	24/26 (92.3)
Total	14/332 = 4.2%	365/430 (84.8)

*Values = number of telomerase-positive samples/total number of samples.
†CLL = chronic lymphocytic leukemia; ALL = acute lymphocytic leukemia.

Correlation Versus Cause

The distinction between causality and correlation is vital when choosing a focus for therapeutic attack. Although experiments have not disproved the model specifying an obligatory role of telomerase in malignant tumor formation, the model has not been rigorously tested. Two tenets of the model rely almost entirely on correlative data. First, the model poses that the crisis in M2 is caused solely by erosion of the telomere. While it has been shown that some cultured human cells at this stage have, on average, short telomeres and a high frequency of abnormal chromosomes, it has yet to be established that loss of telomeric sequences triggers the chromosomal instability. Some immortal tissues even have exceedingly long telomeres, arguing against the supposition that crisis occurs as a consequence of the diminution of terminal sequences (*1,31*). If the chromosomal aberrations observed during crisis are not the result of unstable ends, then emergence from crisis may have no dependence on telomerase activation. To date, the best evidence that shortened telomeres induce chromosome instability exists for yeast (*14*). In yeast, however, the isolation of a mutant that uncouples telomere length and senescence suggests that telomere length does not dictate the life span of the cell (*45*).

The second instance where the data show correlation, not necessarily cause, is the demonstration of telomerase activity in an array of human tumor and immortalized cells. Hiyama et al. (*43*) have expanded on this work to show that telomerase activity is at higher levels in the more severe grades of neuroblastoma examined. Furthermore, they showed that, in a subclass of advanced neuroblastoma called 4s, telomerase activity is not detected. This complementing correlation is particularly intriguing, since 4s metastases are sometimes observed to undergo spontaneous remission. Despite these provocative correlations, a causative relationship remains to be demonstrated. These observations could, for example, be explained if the process of immortalization activates telomerase secondarily. In such a case, activation may commonly be seen when a cell is immortalized, but telomerase activity would not necessarily be required for immortalization.

The need for establishing cause is emphasized by the existence of some data that appear to contradict an obligate role for telomerase in immortalization. For example, in some immortalized and tumor cell lines, telomerase activity has not been detectable, even with the extremely sensitive TRAP assay (*1,25,43,44,46*). While it remains possible that the assay may fail to detect a low or transient telomerase activity for technical reasons, these cells may be evidence of a telomerase-independent pathway to immortalization. Alternatively, some metastatic tumors simply may not require immortalization (*43,44*).

The length of the average mouse telomere poses another apparent challenge to the telomerase activation theory. Mouse telomeres are typically between 40 kb and 50 kb in length and do not appear to decay at a faster rate than human telomeres (*47,48*), yet the advantage of up to 10-fold more telomeric DNA does not give murine cells a corresponding increase in life span. If senescence in mice is actually caused by telomere erosion, it may be that dangerously short telomeres exist within the population whose average telomere length is 40-50 kb. These critically short telomeres could still induce chromosomal instability. Alternatively, it has been suggested that the 40-50 kb measured for mouse telomeres may include sequences that are similar to but distinct from actual telomeric repeats. However, if no "critically short" telomeres exist within the population and telomeric repeats do constitute 40-50 kb of sequence, the mouse example would be evidence that telomere length does not endow cells with increased life span.

Telomerase-Independent Telomere Addition

Even if telomerase activation is required to achieve the immortalized state, experiments in the yeast *Kluyveromyces lactis* and one immortal human cell line suggest that inhibition of telomerase may not prevent the stabilization of telomeres via another mechanism. Indeed, while most *K. lactis* cells gradually lose terminal sequences and senesce when mutated for the gene encoding its telomerase RNA, such selective pressure leads to the emergence of a subpopulation that appears capable of rapidly adding long stretches of telomeric repeats to the chromosomal termini (*49*). In contrast to the rather gradual telomeric repeat addition seen with telomerase-mediated elongation, these terminal sequences appear to undergo abrupt, dramatic changes in length.

A recent report by Murnane et al. (*46*) described a comparable telomerase-independent mechanism in an immortalized human cell line that does not appear to contain detectable telomerase activity. Rather than looking at average telomere lengths, as has been done in all previous studies, Murnane et al. followed telomeres of single chromosomes and found that they undergo rapid changes in length. Though little else is known about these telomerase-independent mechanisms, their mere existence indicates that cells may evolve ways to circumvent telomerase inhibition in order to stabilize chromosomes. If tumor cells can usurp this mechanism to stabilize the chromosomes of cells in crisis, telomerase-blocking anticancer agents might have minimal effect.

Timing and Specificity

Telomere lengths vary broadly in the tumor and immortalized cells that have been examined. In the absence of telomerase activity, these telomeres would no longer be stabilized, but they also would not be rapidly degraded. Before encountering crisis, these cells would experience the normal telomere attrition that accompanies continued rounds of cell division. Thus, in tumors initiated by cells with extremely long telomeres, telomerase inhibitors may potentially require an exceedingly long time to be effective.

The initial observation (*1,13,27,28*) that telomerase is normally expressed only in sperm and oocytes has led to speculation that side effects of blocking its activity would be limited to these germ cells. But recent reports (*44,50,51*) suggest that hematopoietic stem cells may also have low levels of telomerase activity. Although the long telomeres found in these cells may be protective if their telomerase activity is disrupted, the evidence of activity in these vital cells presents the possibility that drugs targeting telomerase activity may have greater adverse side effects than originally anticipated.

It Still Might Work

Having elaborated the pitfalls, it is important to reiterate that telomerase remains a strong candidate for cancer therapeutics. Expanding this area of research could rapidly overcome the experimental difficulties that have thus far prohibited the demonstration of cause. That is, if telomerase inhibitors prevent tumor progression, this would in itself be a demonstration of the causal role of telomerase. Furthermore, even though telomerase expression may not be the exclusive mode to stabilize telomeres in the cell, it may be consequential enough to slow the proliferation of tumor cells if inhibited. Thus, this approach may be effective if used in combination with existing therapeutic agents or treatments.

If telomerase activation is proved to be required for continuous cell proliferation, detection of telomerase may be a useful indicator of the capacity of a primary tumor to metastasize. More extensive studies are required to investigate whether telomerase is activated early enough to be a useful diagnostic tool and to determine if detection of active telomerase is a sufficient prognosticator of later malignancy. Should these relationships be established, the sensitized telomerase assay and improved methods of identifying and extracting early stage tumors will serve to potentiate this application.

A Target for Cancer Therapy: Strategies for Telomerase Inhibition

How can inhibitors of telomerase be found? Several general approaches might be taken. A classical approach to drug discovery is to test a battery of candidate small molecules for their effects on telomerase activity. This brute force approach may not immediately identify an inhibitor that is specific for the telomerase enzyme alone, but it may discover classes of inhibitors that, through further chemical modification, may prove to be optimal therapeutic drugs.

A more focused approach to inhibiting telomerase employs genetic therapy. Although effective use of this strategy as a cancer treatment awaits the development of a method of expressing the desired DNA in all tumor cells, the extensive knowledge of telomerase gained from studies in ciliates and yeast presents an exciting opportunity to pursue targeted genetic approaches. For example, the essential function of the RNA in the telomerase complex has been demonstrated in *Tetrahymena*. In addition to showing that a segment of the RNA serves as the template for telomeric sequences, manipulation of the RNA sequence has generated telomerase mutants that behave enzymatically like some mutants in protein components of DNA polymerases (Blackburn EH: personal communication). This observation raises the intriguing possibility that the RNA may itself possess enzymatic activity in the telomerase complex and reiterates the RNA as a reasonable target for inhibition.

The strategy taken by Greider and Blackburn (*19*) for obstructing telomerase RNA activity through an antisense oligonucleotide targeted to the template region is one approach that could be applied to the human telomerase RNA (Fig. 6, A). Another inhibition strategy with a precedent in basic research is the generation of mutant telomerase RNAs (Fig. 6, B). When RNA containing mutations in the template region is expressed at

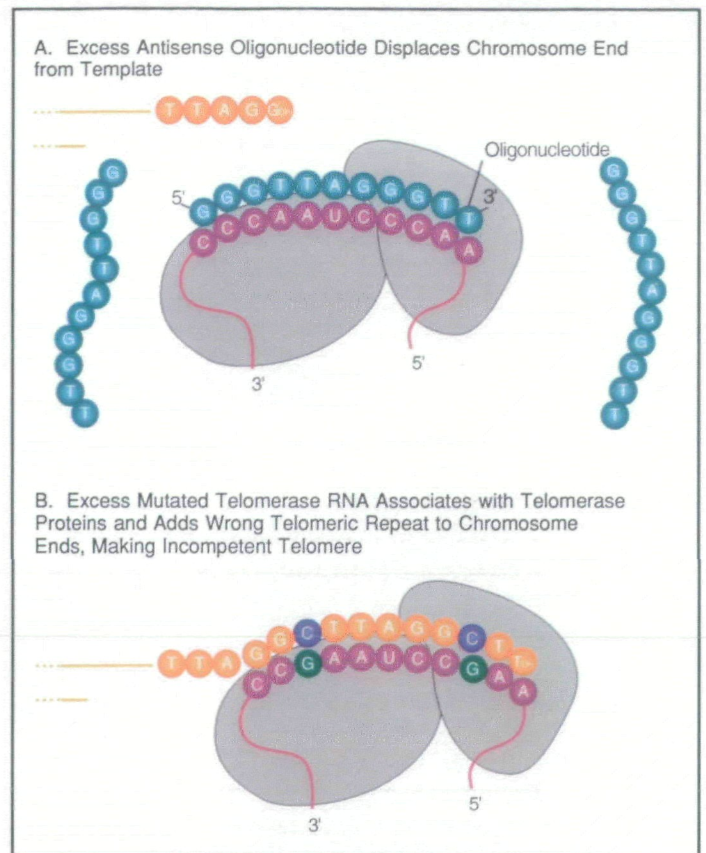

Fig. 6. Genetic strategies for telomerase inhibition. **A**) Antisense strategy. Expression of high levels of a DNA oligonucleotide (turquoise) whose sequence is complementary to the sequence in the telomerase template may displace the chromosome end from the template, thereby preventing addition of telomeric sequences to the chromosome. **B**) Mutated telomerase RNA. Expression of high levels of mutated telomerase RNA (green circles among purple) may cause the preferential assembly of telomerase enzymes that contain the mutated RNA. These telomerases may synthesize telomeric repeats that contain mutations (violet circles among orange), producing telomeres that are incompetent to bind telomere-associated proteins.

high levels in normal *Tetrahymena* cells, the mutated RNA competes with endogenous wild-type RNA for the telomerase proteins. Because the mutated RNA is expressed at many times the quantity of the wild-type RNA, most of the telomerase complexes formed contain the manipulated RNA. These impaired telomerases attach the wrong sequence to chromosome termini, resulting in telomeres that fail to stabilize chromosomes and that consequently induce senescence in the ciliates (*10*). In spite of the current limitations in gene therapy, these two targeted approaches may be the most direct methods to test in vitro whether telomerase is required for immortality and malignancy. For example, if telomerase is inhibited in already immortal cells, will these cells revert to crisis and die?

The protein components of telomerase would present another viable target for inhibition if the human telomerase proteins could be identified. Progress toward this end may be accelerated if sequence elements from telomerase proteins in other organisms are conserved in the human counterpart. Proteins from *Tetrahymena* telomerase have now been partially purified and characterized (*20*). With the identification last fall of telomerase RNA from the yeast *Saccharomyces cerevisiae* (*52*), genetic

isolation of proteins that interact with this RNA is widely anticipated.

It is noted that telomerase RNAs from different species conspicuously lack primary sequence conservation (*53*). Instead, it is the conserved secondary structure, resulting from base pairing between compatible residues within the RNA, that appears to be important for its conserved activity across species. It is not yet known whether or how much the proteins that associate with telomerase RNA are conserved. Nonetheless, while it was not possible to isolate mammalian telomerase RNA via homology to other telomerase RNAs, nucleotide sequence homology may be effective in identifying genes encoding telomerase proteins.

It may also be possible to identify the biological repressor of telomerase. Cell hybridization studies have shown that fusion of a telomerase-expressing cell with a cell lacking telomerase activity yields a hybrid cell that has a limited lifespan (*54*). Such a dominant effect suggests that somatic cells may express an inhibitor or a repressor of telomerase activity. The mechanism and target of this putative repressor could eventually be utilized in approaches to telomerase inhibition.

Unanswered Questions

Intriguing possibilities are raised by the prospect that most cancers rely on activating the enzyme telomerase. Even if this were true of only some cancers, drugs aimed at telomerase inhibition could provide a therapy with relatively limited side effects. But these hopes rest on two still unresolved issues: Do shrinking telomeres cause senescence, and does activation of telomerase stabilize the telomeres of cells that have escaped cell cycle controls, allowing them to continue dividing indefinitely? The causal relationships in question could be tested by manipulating telomere lengths or telomerase activity. Key information could also come from determining how and when telomerase is activated in malignant cells. For instance, does the regulation of the RNA or the proteins determine activity? Are the RNA and proteins regulated at the level of transcription or post-transcriptionally? At what tumor stage is telomerase activated?

The exciting new developments in this field, including the cloning of mammalian telomerase RNA, isolation and cloning of *Tetrahymena* telomerase components, and the TRAP assay, have equipped researchers with invaluable tools to address these questions. With regard to these long-awaited answers, the end may finally be in sight.

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