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Telomere Dynamics and Genome Instability in Human Cancer

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TELOMERE LOSS AND THE ACQUIRED INSTABILITY OF TUMOR GENOMES

More than 80 years ago, Boveri (1914) proposed that chromosome non-disjunction can cause malignant transformation of human cells. Anecdotes about anomalous behavior of cancer chromosomes have accumulated since. For instance, epidemiologists calculated that some human malignancies evolve through the combined effects of five or six independent mutations. Since the mutation rate in normal human cells is too low to account for all these changes, it appears that cancer cells have acquired the ability to deconstruct their DNA (Nowell 1976). Observations reviewed here suggest that this mutator phenotype may in part be caused by the loss of chromosomal telomeres during tumorigenesis.

To a great degree, the instability of tumor genomes can be blamed on changes in the regulation of the cell cycle and loss of cell cycle checkpoints (Hartwell and Kastan 1994). For instance, the recognition of the tumor suppressor gene p53 as part of both a DNA damage checkpoint and a spindle checkpoint explains why p53⁻ tumors tolerate genotypic changes (Hartwell and Kastan 1994; Cross et al. 1995). But what is the source of the DNA damage in these cells? Some of the mutations are probably inherent mistakes in the normal process of genome maintenance, as suggested by increased genome instability in normal cells from p53^{-/-} mice (Livingstone et al. 1992; Cross et al. 1995). Second, environmental assaults are a likely source of chromosomal lesions.

Third, the genotypic changes in human tumors may betray the malfunction of (repair) genes that normally maintain the integrity of the genome. The latter is certainly true for the non-polyposis form of hereditary colon carcinoma, whose chromosomes are scarred from the lack of either of two repair functions (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Papadopoulos et al. 1994). Similarly, the high tumor incidence in families with xeroderma pigmentosum, Fanconi's

anemia, Bloom's syndrome, and ataxia telangiectasia is likely to be due to a mutator phenotype of altered repair genes.

This review focuses on a fourth factor that could destabilize cancer chromosomes: the status of their telomeres. The programmed telomere shortening in human somatic cells and the resulting collapse in telomere function can explain a significant portion of the genetic instability in tumors. This chapter discusses telomere structure and dynamics in human tumors, the role of telomere shortening in loss of heterozygosity and gene amplification, and the contribution of telomerase to tumor progression.

THE TELOMERIC COMPLEX AT HUMAN CHROMOSOME ENDS

DNA Sequences at Telomeres

Like all vertebrate chromosome ends, human telomeres contain tandem TTAGGG repeats oriented such that the G+T-rich strand extends toward the 3' ends of each chromosome (Moyzis et al. 1988; Brown 1989; Cheng et al. 1989; Cross et al. 1989; Meyne et al. 1989; de Lange et al. 1990). The DNA configuration at the very end is not known. A 3' overhang would be expected from the mechanism of telomere replication and the structure of telomere termini in unicellular organisms (see Henderson, this volume). It will be of interest to determine whether human chromosome ends actually carry such 3' tails and what their role is in telomere function.

Although it has not been possible to determine the complete sequence of a human telomere, it is clear that much of the terminal repeat array is composed of unadulterated T_2AG_3 repeats. Not a single deviation from the T_2AG_3 motif was found in a total of 6 kb sequenced on short telomeric clones (de Lange et al. 1990), suggesting that $(T_2AG_3)_n$ is the predominant telomeric sequence. In contrast, a motley selection of T_2AG_3 -related repeats blends into the first 1 kb at the base of many (and possibly all) human telomeres (see Fig. 1) (Allshire et al. 1989; Brown et al. 1990; Cross et al. 1990). Still further into the chromosome, the T_2AG_3 motif is entirely lost and the telomere-adjacent DNA is dominated by the much larger and highly variable subtelomeric repeats (see Henderson, this volume).

Measurements of Telomere Length

In considering telomere dynamics, it is clearly important to establish the length of the T_2AG_3 repeat array. Two methods have been used to assess telomere length. One method, which is similar to the standard procedure

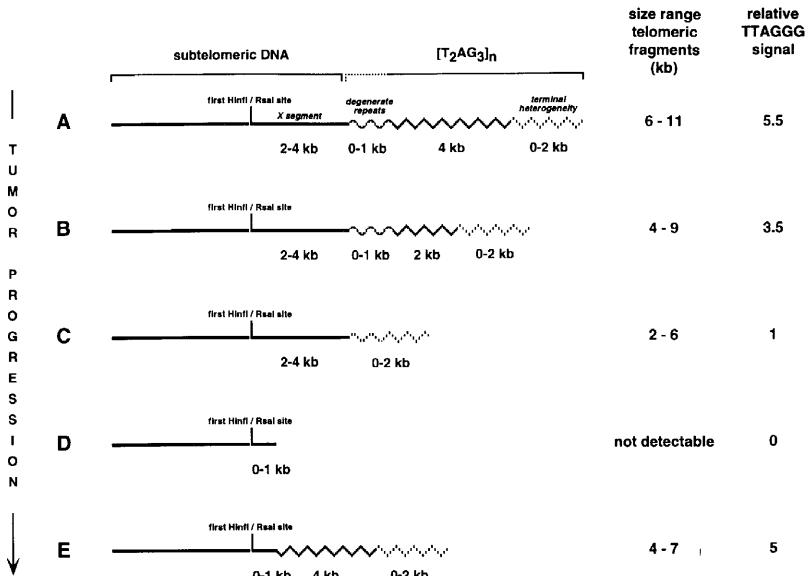


Figure 1 Schematic of the structure of human chromosome ends and possible changes in telomere structure in tumors. (A) Structure of human chromosome ends in normal cells and neoplastic cells in early stages of tumor progression. Two segments are indicated. The most distal segment ($[T_2AG_3]_n$) is composed of several kb of T_2AG_3 repeats as well as ~1 kb of degenerate repeats at the base of the telomere. Both regions anneal to T_2AG_3 repeat probes. The telomere termini display considerable length heterogeneity, as indicated. The second segment indicated represents subtelomeric DNA that does not anneal to T_2AG_3 repeats. The X segment represents subtelomeric DNA that contributes to the length of terminal restriction fragments (e.g., *HinfI*/*RsaI* fragments). The columns to the right list hypothetical numbers for the length and hybridization signal of terminal restriction fragments. (B-D) Chromosome ends in various stages of telomeric decay. (E) Hypothetical structure of chromosome ends after restoration by telomerase. Note that the chromosome ends in E have lost part of their X segment. As a consequence, they display disproportionately high T_2AG_3 repeat signals for their length (e.g., compared to B). See text for detailed discussion.

used to determine the length of yeast telomeres (see Zakian, this volume), employs a subset (about 10 in number) of human chromosome ends that carry the same subtelomeric repetitive element and, as a consequence, have the same physical map (de Lange et al. 1990). The position of the telomeric sequences within this map is known from sequence analysis of cloned DNA. Using this information, telomere length can be

measured fairly accurately in genomic Southern blots. Since there is no reason to expect that this subset of telomeres deviates from the other chromosome ends in the cell, the results can be extrapolated to all telomeres. The validity of the method was demonstrated by removing (with BAL 31 exonuclease) 2 kb from telomeres that were estimated to carry 4 kb of telomeric repeats. As expected, this size reduction was accompanied by a drop in T_2AG_3 repeat signal of about 50% (Saltman et al. 1993).

In a second approach to measure telomere length, human DNA is digested with frequently cutting restriction endonucleases, such as *HinfI* and *RsaI*, and the length of the resulting telomeric fragments is measured in genomic blots (Allshire et al. 1988). This procedure relies on the fact that telomeric repeats lack recognition sequences for most type II restriction endonucleases (de Lange et al. 1990). However, any telomere-adjacent sequence without such sites also persists uncleaved, adding to the length and heterogeneity of the telomeric fragments (see Fig. 1). The size of this subtelomeric segment on terminal *HinfI/RsaI* fragments was deduced from the comparison of the T_2AG_3 repeat signals in cells with telomeres of different length. For instance, to explain that DNA with telomeric fragments of 8 kb has twofold more T_2AG_3 repeat signal than a sample in which the telomeric fragments average around 6 kb, it is assumed that the terminal fragments in both DNAs contain a nontelomeric segment (referred to as X) of approximately 4 kb (Levy et al. 1992). Similar calculations in another study pointed to an X segment of around 2.5 kb (Counter et al. 1992), and an independent analysis of the sequence composition of human telomeres indicated that telomeric fragments contain 3–4 kb of DNA that does not anneal to T_2AG_3 repeat probes (Allshire et al. 1989).

These estimates for X are not confirmed by sequence analysis of cloned telomere-adjacent regions. In most of the cloned chromosome ends, *HinfI* and other restriction sites occur within 1 kb of the telomeric repeat array, suggesting very short X segments (Brown et al. 1990; Cross et al. 1990; de Lange et al. 1990). Perhaps telomeres with longer X regions have eluded cloning. Another possibility is that some subtelomeric sequences are not digested due to modification of the restriction endonuclease recognition sites. For instance, cytosine methylation, which is abundant in subtelomeric DNA (de Lange et al. 1990), prevents the cleavage of GANTC sites by *HinfI* (McClelland et al. 1994). Since the level of subtelomeric methylation may well vary in different cell types (and during tumorigenesis), such changes could potentially influence the apparent length of terminal restriction fragments and distort telomere dynamics. Clearly, it is preferable to measure terminal restric-

tion fragments using enzymes that are not sensitive to MeC in or near their recognition sequence (for instance, *TaqI*, *RsaI*, and *MboI*) (McClelland et al. 1994).

With regard to the effect of telomere loss on cell viability and chromosome behavior, it is pertinent to consider the length of the shortest telomere(s) in a cell. None of the current methods addresses this issue. Future improvements in quantitative in situ hybridization techniques, perhaps coupled with improved chromatin spreading methods (for instance, the DERVISH technique, [Parra and Windle 1993]), may reveal the actual length of the telomeric repeat arrays at individual chromosome ends.

***cis*-Acting Requirements for Telomere Function**

Several lines of evidence indicate that T_2AG_3 repeats are the only sequence required for telomere function in somatic human cells. For instance, strings of T_2AG_3 repeats (but no other sequences) are conserved at all vertebrate telomeres, and the addition of this sequence has healed broken chromosomes in several α -thalassemia patients (Meyne et al. 1989; Wilkie et al. 1990; Lamb et al. 1993; Wilkie 1993). However, a number of questions regarding the *cis*-acting requirements for human telomeres remain unanswered. First, it is unclear how long the T_2AG_3 repeat array needs to be. Although the longest human telomeres seem to contain as much as 20–30 kb of $(\text{T}_2\text{AG}_3)_n$ (de Lange et al. 1990; Hastie et al. 1990; Saltman et al. 1993), many cell lines have no more than an average of a few kilobases of this sequence per chromosome end (de Lange et al. 1990; Counter et al. 1992; Saltman et al. 1993), and individual telomeres in transformed cell lines can be shorter still (Barnett et al. 1993; Hanish et al. 1994; Murnane et al. 1994). The interpretation of these data is confounded by two issues. First, the shortest human telomeres documented to date occur in transformed cells, where the requirements for telomere function may well be less stringent. A second issue is related to the extreme length heterogeneity of human telomeres (Cooke and Smith 1986; Wilkie et al. 1990; Barnett et al. 1993; Murnane and Yu 1993; Hanish et al. 1994; Murnane et al. 1994), which makes it likely that every cell carries a few telomeres that are substantially shorter than the rest. Thus, the average length of human telomeres may not be informative about the minimal requirements for telomeric DNA at individual ends.

A second question is whether other sequences can substitute for the T_2AG_3 repeats. For instance, it is not known whether the T_2AG_3 -related motifs that are clustered at the base of many human telomeres could ful-

fill telomere function. Some information about this issue was gleaned from transfection of telomeric DNA into human tissue culture cells (Hanish et al. 1994). Remarkably, most stable transfectants isolated from such experiments carry a truncated chromosome with the introduced telomeric DNA at its broken end. The fragmented chromosomes are stable, apparently because the transfected T_2AG_3 repeats seed a new functional telomere (see Cooke, this volume). In this setting, telomere seeds require at least 0.5–0.8 kb of telomeric DNA (Farr et al. 1991; Barnett et al. 1993; Hanish et al. 1994), and telomere formation displays a critical dependence on the T_2AG_3 sequence (Hanish et al. 1994). Although newly formed telomeres were observed after transfection of T_2AG_3 telomeric seeds, stretches of closely related repeat motifs from other eukaryotes failed to seed new telomeres (Hanish et al. 1994). These results indicate stringent sequence requirements for telomere formation and predict that functional significance is primarily limited to the arrays of precise T_2AG_3 repeats in the most distal segment of human telomeres.

Telomere-associated Proteins

Human telomeres have a large (several kb) domain of unusual, diffuse chromatin suggestive of an altered nucleosomal organization (Tommerup et al. 1994; Lejnine et al. 1995). This telomere-specific chromatin may be related to the large complexes (telosomes) detected at chromosome ends in unicellular organisms (see Fang and Cech, this volume). An important challenge will be to characterize the proteins in the mammalian telomeric complex (for review, see de Lange 1996).

A preliminary survey of the protein/DNA interactions at mammalian telomeres has uncovered several leads. Human telomeres cofractionate with the nuclear matrix (de Lange 1992), suggesting that this subnuclear fraction contains telomere-binding factors. A candidate telomeric DNA-binding factor, referred to as T_2AG_3 repeat-binding factor or TRF (Zhong et al. 1992), is expressed in somatic mammalian cells, including primary and transformed human cells. On the basis of its *in vitro* DNA-binding activity, TRF would be expected to bind along the length of the double-stranded part of the telomere. Results obtained with transfection-mediated chromosome fragmentation suggest that TRF binds telomeric DNA *in vivo* (Hanish et al. 1994). Specifically, the sequence required for efficient telomere seeds matches the TRF recognition site as determined by *in vitro* binding studies. The recent cloning of the gene for TRF should allow further assessment of the role of this factor at human telomeres (T. de Lange et al., unpubl.).

In several ciliated protozoa, the 3' telomeric overhang of G-rich

repeats is stably bound by a protein complex (see Fang and Cech, this volume). Biochemical and genetic data hint that similar telomere terminus-specific proteins may be expressed in holotrichous ciliates, in yeast, and in *Xenopus* (Cardenas et al. 1993; Sheng et al. 1995; Wiley and Zakian 1995). Although these observations fuel expectations that mammalian telomeres also carry a terminal protein, searches for such a single-stranded binding activity in mammalian cells have so far identified hnRNP proteins with a remarkable specificity for T₂AG₃ strings (McKay and Cooke 1992a,b; Ishikawa et al. 1993).

TELOMERE SHORTENING

The Decline of Somatic Telomeres

In cells of the male germ line, in placenta, and in fetal cells, human chromosomes carry 10–20 kb of telomeric T₂AG₃ repeats at their termini (Allshire et al. 1988; Cross et al. 1989, 1990; de Lange et al. 1990; Hastie et al. 1990). At an unknown point during somatic development, this telomeric stretch begins to wither with successive cell divisions, resulting in a marked size reduction of telomeric restriction fragments in adult somatic cells as compared to germ-line material from the same donor (Cooke and Smith 1986). Although enzymatic degradation of telomere termini has not been excluded, human telomeric decline is generally blamed on a shortcoming of the chromosomal replication strategy. It has been extensively argued that chromosome termini are destined to dwindle because the exact duplication of a DNA end would require activities that are alien to cellular DNA polymerases: They would either have to initiate replication without a primer or add nucleotides to a 5' end (see Greider, this volume). In principle, this end-replication problem can be negated by any reaction that elongates chromosome ends (for instance, by recombination, terminal sequence transfer, slippage during replication, and transposition). In human cells, however, telomerase appears directly responsible for the maintenance of telomeric DNA.

Human telomerase can be readily demonstrated in hypotonic lysates of established cell lines (e.g., HeLa cervical carcinoma cells and 293 adenovirus-transformed embryonic kidney cells [Morin 1989; Counter et al. 1992; Prowse et al. 1993]). However, in normal human cells, telomerase is much more difficult to detect (Counter et al. 1992, 1994a,b; Kim et al. 1994; Broccoli et al. 1995). On the basis of these results, telomeric decline is attributed to repression of telomerase in somatic cells (Hastie and Allshire 1989; de Lange et al. 1990; Harley et al. 1990; Hastie et al. 1990; Counter et al. 1992). It is further speculated that germ-line cells express telomerase, explaining the longer sperr telomeres of

older men (Allsopp et al. 1992), the persistence of T₂AG₃ repeats over human generations, and the fact that the telomeric sequence has not changed over 400 Myrs of vertebrate evolution (Meyne et al. 1989). In agreement, high levels of telomerase activity have recently been found in extracts of human testes and ovaries (Kim et al. 1994).

The decline of somatic human telomeres has been documented in three ways. For perhaps a dozen individuals, it was shown that their sperm telomeres are several kilobases longer than telomeres in peripheral blood leukocytes (Cooke and Smith 1986; Allshire et al. 1988; de Lange et al. 1990). Second, telomere shortening was witnessed directly during growth of primary human cells in vitro. In this setting, the telomeres of fibroblasts, embryonic kidney cells, bone marrow, T- and B-lymphocytes, mammary epithelial cells, and cervical cells shorten by 30–120 bp per population doubling (Harley et al. 1990; Counter et al. 1992; Shay et al. 1993; Vaziri et al. 1993, 1994; Klingelutz et al. 1994). Finally, telomeres in peripheral blood leukocytes, colon mucosa, and skin fibroblasts were shown to be shorter in older donors (Harley et al. 1990; Hastie et al. 1990; Lindsey et al. 1991; Allsopp et al. 1992; Vaziri et al. 1993). The rate at which these telomeres appear to decline is about 20–40 bp per year. Perhaps this modest rate of telomeric shortening reflects a limited number of yearly divisions or low levels of telomerase activity in these tissues.

Taken together, these studies suggest that telomeric decline is a general phenomenon in the human soma. It should be stressed, however, that it is not known exactly how much telomeric DNA is lost per cell division and whether this rate is variable in different cell types or during development. Furthermore, little is known about the regulation of telomere dynamics in human cells. If the situation in yeast is any indication (see Zakian, this volume), it is likely that multiple factors (in addition to telomerase) contribute to the regulation of telomere length.

The Decline of Tumor Telomeres

Like telomeres in normal somatic cells, telomeres of primary human tumors appear to undergo progressive sequence loss at some stage in their development (illustrated in Fig. 1). This process is evident from the fact that tumor telomeres are generally shorter than the telomeres in neighboring normal cells. To evaluate changes in tumor telomeres, comparison to the presumed precursor of the malignancy is important because possible differences in replicative history invalidate the use of other tissues as a control. Furthermore, because of the large variation in telomere length in the human population, the corresponding normal tissue should be from the same individual.

The decline of tumor telomeres was initially observed in Wilms' tumor, breast, and colon carcinoma (de Lange et al. 1990; Hastie et al. 1990). More recently, telomere loss has been documented in most major human tumor types (Table 1). In the majority of cases, the tumor telomeres have lost 3–5 kb. If tumor telomeres shorten as fast as most normal somatic telomeres (50–100 bp/pd), loss of 3–5 kb of telomeric DNA suggests that on the order of 30–100 population doublings have occurred during the clonal expansion of the originally transformed cell. This replicative age is not unexpected. Most human tumors must go through at least 30 doublings to become sufficiently large for clinical detection (1 gram or cm^3), and a tumor of 1 kilogram requires at least 10 more divisions. In fact, the preclinical growth period may far exceed the number of divisions estimated from the tumor mass because high rates of cell death (up to 80% of cell growth) temper tumor expansion.

There are some large tumors, however, with unexpectedly long telomeres. For instance, Mehle et al. (1994) noted several large (diameter ≥ 5 cm) renal carcinomas that appear to have lost only 1.5 kb of telomeric DNA. Similarly, Hastie et al. found long telomeres in two colon carcinomas that must have gone through at least 30 divisions, based on their size (Hastie et al. 1990; R.C. Allshire, pers. comm.). One interpretation is that these tumor telomeres initially declined but were restored (by telomerase, see below) at a preclinical stage (this process is illustrated in Fig. 1). Because of this effect, telomere length is probably not a good indicator of the replicative history of a tumor. Mehle et al. (1994) also suggested that there may be substantial intratumor variation in telomere length, and this could further confound any relationship between telomere loss and tumor age.

EVIDENCE FOR LOSS OF TELOMERE FUNCTION IN TUMORS

Critically Shortened Tumor Telomeres

Many primary and metastatic human tumors appear to have extremely short telomeres. Although it is not possible to determine the length of the remaining T_2AG_3 repeat stretch accurately, the average length of the T_2AG_3 repeat arrays in some tumors is only 1–2 kb per chromosome end. Because of the size heterogeneity of telomeric arrays, many ends must have considerably less T_2AG_3 repeat DNA. Whether these tumor telomeres are on the verge of functional breakdown is hard to assess because the minimal *cis*-acting requirements for human telomeres are not well defined.

More definitive arguments for the loss of telomere function in tumors could come from a demonstration of chromosome ends that lack T_2AG_3

Table 1 Telomere shortening in human tumors

Tumor/ control tissue	Frequency of telomere reduction	Telomeric shortening range (mean)	Reference
Wilms' tumor/normal kidney	2/2	4-5 kb (4.5 kb)	de Lange et al. (1990)
Breast carcinoma/normal breast	1/1	2 kb	de Lange et al. (1990)
Colon carcinoma/normal mucosa	19/20	1-7 kb (4 kb)	Hastie et al. (1990)
Colon adenoma/normal mucosa	3/3	3-5 kb (3.5 kb)	Hastie et al. (1990)
Endometrial carcinoma/ normal uterine epithelium	8/11	1.3-10.4 kb (4.3 kb)	Smith and Yeh (1992)
ALL, AML, CGL diagnosis/remission	4/6	1.5-5.0 kb (3.2 kb)	Adamson et al. (1992)
AML/refractory anemia	3/16	6.0-6.3 kb (6.2 kb)	Ohyashiki et al. (1994)
Renal cell carcinoma/ normal renal cortex	10/10	0.4-2.5 kb	Mehle et al. (1994)
Ovarian carcinoma ascites nonadherent/adherent cells	8/8	2-4.5 kb (3 kb)	Counter et al. (1994a)
Lung carcinoma/normal lung	12/45	2.5-7.5 kb (5.7 kb)	Shirotani et al. (1994)
Breast carcinoma/normal breast	18/22	not reported (1.6 kb)	Odagiri et al. (1994)

repeats altogether. Although such fully spent telomeres have not been documented yet, indirect evidence indicates that telomere-deficient termini may well have appeared transiently during the outgrowth of some tumors. Mehle et al. (1994) observed several renal carcinomas in which the terminal restriction fragments had shortened without a concomitant drop in the T_2AG_3 repeat signal. This discrepancy could be explained if the terminal decay in these tumors had reached into the subtelomeric sequences followed by telomere restoration by telomerase. As a result, the new telomeric fragments would contain shorter X segments and display a disproportionately high T_2AG_3 repeat signal for their length (illustrated in Fig. 1). To demonstrate the occurrence of telomere-deficient chromosome ends, it will be necessary to look for loss of telomere-adjacent sequences in human tumors. However, such uncapped ends may not be easy to demonstrate because they may be short-lived, rare, and possibly confined to early stages in tumorigenesis when telomerase levels are still low (see Fig. 2).

Telomere Associations

One function of the telomeric complex is to prevent the end-to-end fusion of chromosomes. However, the behavior of certain cancer chromosomes indicates that their telomeres fail to provide this function (Hastie and Allshire 1989). Fitzgerald and Morris (1984) were the first to notice an unusual kind of dicentric chromosome in two leukemias. These dicentrics appeared to consist of two intact chromosomes that are fused at their termini; they are referred to as telomere associations (TAs). TA-dicentrics have now been observed in a wide variety of tumors (see Table 2). Since the chromosomes involved in telomere associations do not display a tumor-specific pattern (Table 2), it is unlikely that these TA-dicentrics confer a selective growth advantage on the cells. Rather, it seems that the tendency of these tumor chromosomes to fuse at their termini betrays a malfunction in (some of) the telomeres in these cells.

Why these telomeres fuse is not known. Telomere associations had previously been noted in SV40-transformed kidney cells and in senescent fibroblasts (Yerganian et al. 1962; Benn 1976). Although this was not checked directly, it is likely that in both cases the telomeres had been shortened extensively. In more recent examples of TAs, the correlation with short telomere length is quite clear (Counter et al. 1992, 1994b; Saltman et al. 1993). Although short telomeres are more prone to display associations, other aspects of telomeric complex (such as telomeric proteins or the structure of the telomere terminus) and the physiology of the cell (e.g., DNA repair functions) are likely to contribute as well (Salt-

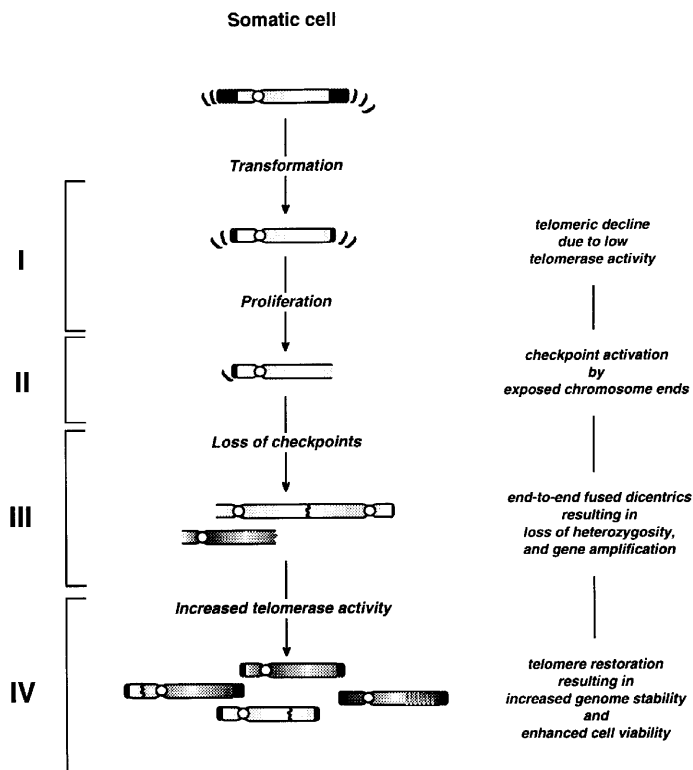


Figure 2 Speculative model for telomere dynamics in human tumors. Normal somatic cells and transformed cells in phase I show low levels of telomerase and their telomeres decline with each division. As a result, one or more chromosome ends lose telomeric function (phase II). The exposed chromosome ends can activate a DNA damage checkpoint and lead to cell cycle arrest and/or apoptosis. In cells that have lost such checkpoints, uncapped chromosome ends may lead to genome instability (phase III). Chromosome end fusion forms dicentric chromosomes, which can contribute to loss of heterozygosity and gene amplification. In phase IV, telomerase is activated, leading to restoration of telomere function. The restored telomeres may increase cell viability through stabilization of the tumor karyotype. Note that there is no direct relationship between phases I–IV and clinical stages of tumor progression.

man et al. 1993). In this context it is also important to bear in mind that it has not been demonstrated that TA-dicentrics are formed by ligation of their DNA termini.

A number of features of the TA-dicentrics make them fairly difficult to detect. First, they are easily missed because there are usually only a few TAs per metaphase, and often more than half of the metaphases of one specimen contain no TA-dicentrics at all (see Table 2). Second, because they are usually not clonal, many TA-dicentrics are only represented once and therefore are hard to identify with certainty. Third, TA-dicentrics may be expected to occur primarily in the earlier stages of tumor development before telomerase activation has restored telomere function (see Fig. 2). According to these considerations, the frequency of TA-dicentrics in human cancer may be grossly underestimated. The fact that TA-dicentrics have already been seen in some of the major human cancers suggests that their contribution to genetic instability could be substantial and pervasive (see below).

TELOMERE SHORTENING AS A TUMOR SUPPRESSOR MECHANISM

It has been argued that the ability of primary human cells to proceed through mitoses may eventually be limited by telomere shortening (see Harley, this volume). The proposal is that uncapped chromosome ends exposed by telomeric decline could activate a DNA damage checkpoint and halt cellular proliferation (see Fig. 2). Obviously, such a mechanism would curb the clonal expansion of somatic cells and constitute a means to limit tumor growth.

If the shortening of somatic telomeres serves to suppress tumor outgrowth, it is probably only useful to long-lived large animals, such as humans, whales, certain turtles, and elephants. Short-lived, small animals may not profit from a mechanism that only suppresses cell growth after a considerable number of divisions; they would need a timing mechanism with a shorter telomeric "fuse" or a faster-running clock. Either of these solutions may have the disadvantage of limiting normal tissue growth and regeneration too readily. It is noteworthy that in laboratory strains of *Mus musculus* both the rate of spontaneous immortalization and telomere length are greatly increased compared to humans (Newbold et al. 1982; Kipling and Cooke 1990; Starling et al. 1990). However, in other mice this correlation may not hold, because in-vitro-grown *Mus spretus* fibroblasts lose their telomeres yet display high rates of immortalization (Prowse and Greider 1995).

Another question raised by the tumor suppressor model is whether evolving human tumors still have the ability to perceive the loss of their telomeres. A case in point are p53-deficient cells, which fail to detect DNA breaks and aberrant spindles. Such cells may also be oblivious to

Table 2 Telomere associations (TAs) in human tumors

Tumor type (tumors with TAs/ total)	% Metaphase with TAs ^a	TAs per cell ^b	Recurrent/ sporadic TAs ^c	Frequently involved telomeres	Nondisjunction; breakage; TA of sister chromatids	References
<i>Hematological</i>						
B-PLL (1/1)	17	1-4	4/8	19p,22p	-	Fitzgerald and Morris (1984)
B-PLL (1/1)	3-11	1-2	4/11	17q,19p	nondisjunction	Howell et al. (1993)
B-ALL (1/1)	22	1	18/0	(marker)	-	Fitzgerald and Morris (1984)
B-cell foll. lymph. (1/1)	4-28	n.d.	n.d.	1,5,12,17	TA of sister chromatids	Saltman et al. (1989)
Large cell lymph. (1/1)	35	1-2	2/22	1p,3p,7p,7q,8q	nondisjunction	Saltman et al. (1993)
pre-T-ALL (1/1)	26	1	2/9	4q,11q	-	Morgan et al. (1986)
<i>Renal</i>						
oncocytoma (1/2)	50	1-2	22/19	19q,20q,22p	nondisjunction	Kovacs et al. (1987)
renal cell carc. (1/18)	50	1-2	4/15	17q,18q	-	Kovacs et al. (1987)
Wilms' tumor (1/1)	88	1-12	22/58	7p,12q,14p,17p,20q	TA of sister chromatids	Fett-Conte et al. (1993)
Wilms' tumor (1/1)	n.d.	n.d.	25/0	7q,9q,11p,14p	TA of sister chromatids	Sawyer et al. (1994)
CNS glioblast. multiforme (1/1)	70	n.d.	n.d.	4p,7p	breakage	Sawyer et al. (1992)

cerebellar astrocyt. (1/1)	24	1	29/4	12q,12p,15p	-	Sawyer et al. (1993)
cerebellar astrocyt. (1/1)	80	1	20/0	18p, 21p	-	Harrison et al. (1994)
<i>Other</i>						
pleom. fibr. histiocyt. (1/1)	90	1-6	20/21	6p,11p,16q,20q,21p	TA sister chr., nondisjunction	Mandahl et al. (1985)
gastric fibr. histiocyt. (1/1)	27	1	0/4	12p	-	Seruca et al. (1992)
cardiac myxoma (3/6)	17-25	1	0/16	2q,12p,22q	TA sister chr., breakage	Dewald et al. (1987)
cardiac myxoma (1/1)	43	1	6/3	13p,15p	-	Richkind et al. (1994)
giant cell bone tumor (6/6)	3-37	1-3	2/40	5p,11p,15p,19q,20q	nondisjunction	Schwartz et al. (1989)
laryng. sq. cell. carc. (2/2)	84	n.d.	n.d.	3,5	-	Pathak et al. (1988)
squamous cell carc. (1/1)	13	n.d.	n.d.	1p,12q,18p,22p	TA sister chr., breakage	Aledo et al. (1989)
uterine leiomyoma (1/1)	50	n.d.	n.d.	n.d.	-	Nilbert et al. (1989)
uterine leiomyoma (2/5)	4,20	1-2	30/2	1q,12p,12q,14q	TA sister chr., nondisjunction	Pandis et al. (1990)
ovarian carc. (15/20)	10-38	1-3	0/98	8p,6p,10p,11q	nondisjunction	Mrozek and Limon (1992)
melanoma cell line (1/1)	9	1	n.d.	n.d.	n.d.	Saltman et al. (1993)

n.d. indicates not determined.

^aWhere more than 2 cases were analyzed, the range of values is given.

^bRanges of number of TAs per metaphase.

^cNumber of recurrent TAs observed in two or more metaphases/number of unique TAs present in a single metaphase.

the occasional loss of a telomere or the presence of TA-dicentrics. However, inactivation of p53 seems to be a late step in tumorigenesis (Fearon and Vogelstein 1990; Prives 1993), and if this holds for other DNA damage checkpoints as well, loss of telomere function may well check tumors early in their development. Clearly, it will be important to establish whether transformed cells generally run out of telomeric DNA before their DNA damage checkpoints are eliminated. In fact, it could be argued that telomere loss and the resulting aberrant chromosomes constitute a main driving force in the selection of cells that are p53^{-/-} (Wynford-Thomas et al. 1995).

CONTRIBUTIONS OF TELOMERE LOSS TO GENETIC INSTABILITY IN CANCER

Tumor karyotypes display three main types of alterations: loss of genetic material (loss of heterozygosity [LOH]), gene amplification, and chromosomal rearrangements. In theory, each of these changes could result from loss of telomere function in tumor cells. As discussed in detail below, LOH and gene amplification can be explained directly from the aberrant behavior of the TA-dicentrics that arise in tumor cells with short telomeres.

Loss of Heterozygosity

The loss of tumor suppressor genes is an important factor in human tumorigenesis (for review, see Knudson 1993). Dicentric chromosomes can promote LOH if their two centromeres engage opposite spindles (see Fig. 3A). The result is an anaphase bridge that can be resolved by severing the spindle apparatus, leading to nondisjunction, or by physical breakage of the chromatid. In either case, one of the daughter cells may end up lacking certain sequences.

In considering LOH, it is pertinent to ask whether TA-dicentrics actually follow the scheme outlined in Figure 3. The cytogenetic data summarized in Table 2 suggest that this is the case. First, TA-dicentrics appear to be unstable. By and large, each dicentric is only seen in one or a few cells, indicating a high rate of loss. With regard to LOH, it is important to ask whether unstable dicentrics are actually lost through nondisjunction and breakage. Direct evidence for nondisjunction of TA-dicentrics has been reported in a number of tumors. In these cases, nondisjunction was conspicuous from the presence of two copies of a TA-dicentric in one metaphase (Table 2). Furthermore, in several cases, TA-dicentrics were observed to break at nontelomeric sites (Table 2).

A second hint that TA-dicentrics are lost through nondisjunction and breakage comes from the fact that they preferentially involve associations between the telomeres of p arms or involve small chromosomes (see Table 2). As a result, the cen-cen distance in most of the dicentrics is short. Such a selection would be expected if the TA-dicentrics are primarily lost due to their attachment to opposite spindles. A dicentric with closely positioned centromeres is less likely to attach to opposite spindles, is less likely to break in the chromatid, and may therefore often segregate normally to both daughter cells (as depicted in the left half of Fig. 3A).

The cytogenetic data on TA-dicentrics thus indicate that they frequently undergo nondisjunction and breakage, two processes that could contribute to LOH. A highly suggestive scenario of how fusion-bridge-breakage cycles of TA-dicentrics may lead to LOH at tumor suppressor loci is presented by a Wilms' tumor with frequent TAs involving 11p (Sawyer et al. 1994). These dicentrics appear unstable, and the karyotype displays a number of subclones with breakpoints in the short arm of chromosome 11. The lost material of 11p includes 11p15 in some subclones and 11p13 in others, indicating that the breakage of the 11p dicentrics may result in loss of tumor suppressor genes on 11p15 and 11p13 (for review of tumor suppressor genes, see Knudson 1993).

On the basis of data from other eukaryotes, one might anticipate that the loss of telomere function in tumors results in LOH through exonucleolytic degradation of proterminal genes (Lundblad and Szostak 1989; Kramer and Haber 1993; Sandell and Zakian 1993). However, for human chromosomes it is not known whether unprotected termini are vulnerable to exonucleolytic attack, as they are in yeast, or merely wither away passively with DNA replication. Without active degradation, the rate of gene loss may not be significant because the terminal sequence attrition would first consume many kilobase pairs of subtelomeric regions in which genes have not yet been found.

Gene Amplification

As originally noted by McClintock, broken chromosome ends have a tendency to fuse and form dicentrics made up of end-to-end joined sister chromatids (see Fig. 3) (McClintock 1941, 1942). If such dicentrics repeatedly progress through fusion-bridge-breakage cycles, they can accumulate multiple copies of the same gene (Cowell and Miller 1983). Unlike gene amplification through overreplication, the terminal fusion of sister chromatids readily explains the gigantic inverted duplications and the dicentric chromosomes observed in early stages of amplification

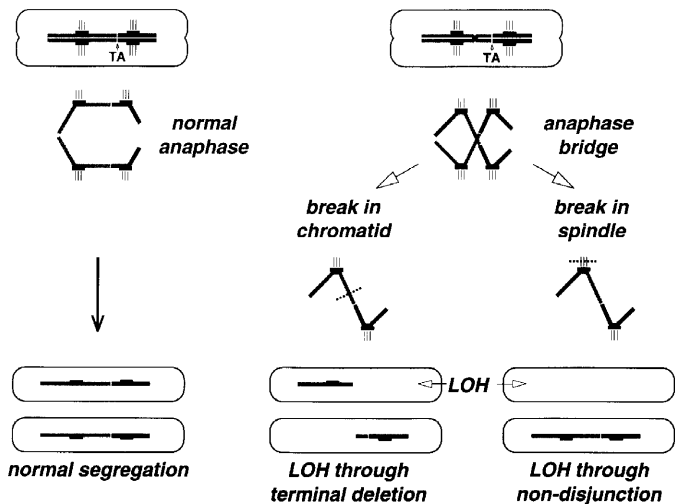
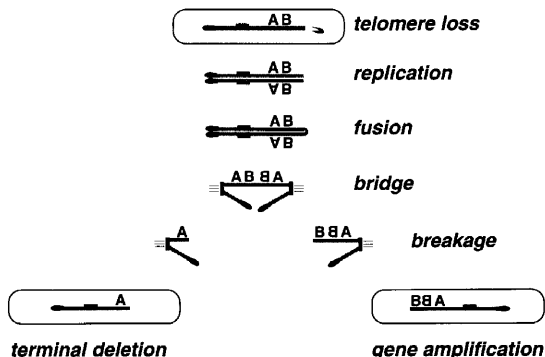
A**B**

Figure 3 LOH and gene amplification through telomere associations. **A** depicts how telomere associations (TAs) may lead to LOH. Two cells are shown with dicentric chromosomes formed through TAs. Such TA-dicentrics may segregate normally (cell on the left) or form an anaphase bridge if the centromeres engage opposite spindles (cell on the right). If the anaphase bridge is resolved by breakage of a chromatid, LOH can result through a terminal deletion in one chromosome. If resolution involves breakage of the spindle, LOH can result from loss of a whole chromosome. In the daughter cells derived from the right-hand mother cell, the segregation of only one of the two chromatids is shown for simplicity. **B** depicts how TAs of sister chromatids can result in fusion-bridge-breakage cycles that lead to gene amplification. Note that both daughter cells carry a chromosome with one uncapped end that is likely to enter additional rounds of fusion-bridge-breakage cycles. See sections on LOH and gene amplification for detailed discussion.

(Smith et al. 1992; Ma et al. 1993; for review, see Stark 1993). Further rounds of fusion-bridge-breakage could build up the complex pattern of subsumed inverted repeats that are typical of amplified loci.

Clearly, the sticky broken ends that initiate gene amplification could arise from loss of telomere function through telomeric decline. Associations of sister chromatids, which could constitute early steps toward gene amplification, have been observed in a number of tumors (Table 2). In addition, the dicentrics containing other (non-sister) telomere associations may undergo breakage and form secondary broken ends that could initiate the fusion-bridge-breakage cycles of gene amplification. If the generation of unprotected DNA ends is a rate-limiting step in gene amplification, the status of tumor telomeres and the level of telomerase may well constitute major determinants of the frequency with which gene amplification occurs.

TELOMERASE ACTIVITY IN HUMAN TUMORS

Although the mutator phenotype associated with telomere loss may propel the clonal evolution of progressively altered tumor cells, high rates of chromosome loss and gene rearrangements could also impede proliferation of transformed cells (Boveri 1914). In agreement with this view, cells that have been transformed with SV40 large T or other viral agents that target p53 and Rb eventually enter a growth crisis in which cell divisions are balanced with cell death (Counter et al. 1992). This final crisis occurs when the telomeres are critically shortened and genome instability is rampant (Counter et al. 1992). With time, the cultures occasionally yield immortalized clonal cell lines. Almost invariably, these cell lines express high levels of telomerase, they have restored telomeres, and their genome is (partially) stabilized (Counter et al. 1992, 1994b; Klingelhutz et al. 1994). These *in vitro* models for cellular immortalization suggest that functional restoration of telomeres may be yet another hurdle in the progression of human tumors.

Perhaps the best indication that tumor cells are indeed affected by the status of their telomeres comes from the observation that telomerase activity is often increased in human tumors. High telomerase activity was first demonstrated in the ascites of metastatic ovarian carcinoma. Counter et al. (1994a) were able to demonstrate the activity directly in S100 extracts using the "conventional" telomerase assay (see Greider, this volume) in which *in vitro* telomerase products are detected directly through the incorporation of labeled guanine in T₂AG₃ ladders (Table 3). Normal ovarian epithelial cells tested in parallel were negative.

A major advance was made by Kim et al. (1994), who developed a

Table 3 Telomerase activity in human tumors

Tumor	Frequency of detection (telomerase positive/total)	Reference
<i>Conventional assay</i>		
ovarian carcinoma ascites	7/7	Counter et al. (1994a)
acute leukemias	0/9	Nilsson et al. (1994)
CLL	1/2	Nilsson et al. (1994)
lymphomas	6/7	Nilsson et al. (1994)
myeloma	1/1	Nilsson et al. (1994)
<i>TRAP assay</i>		
ovarian carcinoma in situ	15/23	D. Broccoli et al. (unpubl.)
acute leukemias	21/23	Kim et al. (1994); Counter et al. (1995);
acute leukemias	(5/5) ^a	Broccoli et al. (unpubl.)
CLL	2/2	Kim et al. (1994)
CLL	(17/18) ^a	Counter et al. (1995)
CML	(11/11) ^a	Broccoli et al. (1995)
MDS	5/5	Counter et al. (1995)
lymphoma	5/5	Kim et al. (1994)
breast carcinoma in situ	28/38	Kim et al. (1994); D. Broccoli et al. (unpubl.)
prostate (PIN3 and adenocarcinoma)	5/7	Kim et al. (1994)
leiomyosarcoma	3/3	Kim et al. (1994)
colon cancer	8/8	Kim et al. (1994)
squamous cell carcinoma	14/16	Kim et al. (1994)
small cell lung cancer	4/4	Kim et al. (1994)
brain tumors	6/8	Kim et al. (1994)
Wilms' tumor	6/6	Kim et al. (1994)
neuroblastoma (stages I-IV)	71/71	Hiyama et al. (1995)
neuroblastoma (stage IV-S)	5/8	Hiyama et al. (1995)

^aThe telomerase activity in these tumors was not significantly higher than in normal peripheral blood and bone marrow lymphocytes.

much more sensitive assay in which in vitro telomerase products are amplified and labeled by PCR. This so-called TRAP (telomeric repeat amplification protocol; see Greider, this volume) assay can be used to detect telomerase in detergent extracts of frozen tissues, including tumor biopsies. With this approach, telomerase activity has been demonstrated in most human tumors (Table 3), including major human cancers such as lung, colon, breast, and prostate carcinomas and in most leukemias and lymphomas. For each tumor type, telomerase is detected in at least 60% of the cases. This percentage must be a lower estimate because of experi-

mental inactivation of the enzyme during processing of the specimens. Thus, high telomerase activity may well turn out to be a nearly universal marker for a large subset of human tumors.

To what extent is telomerase a tumor-specific enzyme? TRAP assays failed to show telomerase activity in a variety of normal adult somatic tissues, although testes, ovary, placenta, and fetal cells scored positive (Kim et al. 1994). Telomerase was also not detected in several benign lesions and in in-vitro-grown (pre-crisis) fibroblasts, indicating that telomerase in tumors is not simply a reflection of the proliferative state of the cells (Kim et al. 1994). However, recent data demonstrate telomerase activity in bone marrow, peripheral blood leukocytes, and fractions enriched for peripheral granulocytes, T-cells, and monocyte/B-cells (Broccoli et al. 1995; Counter et al. 1995).

Since hematopoietic cells display telomere shortening in vitro and with aging in vivo, their telomerase activity is somewhat puzzling. One explanation is that the telomerase activity in these cells is simply insufficient to keep up with replication-dependent telomere attrition. Another possibility is that the dynamics of telomeres not only is dependent on the expression level of telomerase, but also involves other aspects of the telomeric complex. Telomere length regulation by factors other than telomerase has been documented extensively in budding yeast (see Zakian, this volume). Recent analysis of telomere dynamics in *Mus spretus* has demonstrated telomerase in somatic tissues (Prowse and Greider 1995), and somatic expression of telomerase has been shown in *Xenopus laevis* (Mantell and Greider 1994). Thus, telomerase expression in normal somatic cells may be a general phenomenon in vertebrates.

Because PCR-based assays are notoriously difficult to quantitate, the exact differences in expression level of telomerase in normal versus malignant cells have not been established rigorously. Future experiments using reagents to detect telomerase RNA and protein should clarify the activation level of telomerase in tumors. The cloning of telomerase genes should also reveal whether the high telomerase activity in tumors is due to mutation of a telomerase (regulatory) gene or whether the activity is increased through other routes.

AN OVERVIEW OF TELOMERE DYNAMICS IN TUMORS

The data summarized here indicate that telomere dynamics in human tumors are rather complex. The model in Figure 2 aims to provide a conceptual framework for these events and describes a speculative scenario in which tumor telomeres progress through four distinct phases. In the first phase, the telomeres are in decline but still sufficiently long to

engage telomeric proteins and protect chromosome ends. In phase II, loss of telomere function occurs on one or a few chromosome ends. The resulting unprotected termini are expected to activate DNA damage checkpoints, arrest cell proliferation, and/or induce apoptosis. Cells that break through this barricade are likely to be deficient in the checkpoint(s) that detects uncapped chromosome ends and dicentric chromosomes. Such cells may continue to lose telomeric DNA and enter a period of increased genome instability with high rates of LOH and gene amplification (phase III). The harmful effects of generalized telomere loss may eventually provide a selective advantage to cells that have restored telomere function through the increased expression of telomerase. In the resulting growth phase (phase IV), tumor telomeres are functionally restored (although not necessarily long), chromosome stability is improved, and cell viability is increased. Most tumors may not be clinically detectable until they reach phase IV.

PERSPECTIVE

One of the challenges ahead is to achieve a better understanding of how loss of telomeric repeats inhibits cellular proliferation and promotes genome instability. To a great degree, progress will depend on our understanding of telomere function in normal human cells. We need to know more about the structure and components of the telomeric complex, the replication of telomeres, the mechanisms by which telomeres prevent chromosomal catastrophes, and the fate of chromosomes that have lost their protective caps. In addition, we need to understand the cell cycle checkpoints that might detect telomere loss and the resulting abnormal chromosomes (such as dicentric chromosomes).

Two stigmata of cancer genomes, LOH and gene amplification, can be attributed (in part) to deteriorating tumor telomeres. Of course, these hints need to be corroborated, but one would also like to know what other genomic malformations are caused by failing chromosome ends. Are translocations more frequent in cells that suffer telomere deficiency? Are chromosome ends without telomeric caps prone to exonucleolytic attack? To what degree are such events dependent on the checkpoint status of the cells? In the near future, model systems should become available that allow manipulation of telomere function in mammalian cells. Answers to some of these questions will then be imminent.

Somewhat unexpectedly, elevated telomerase activity is widespread in human cancer. Its prevalence suggests that the increase in telomerase activity occurs at a preclinical stage in tumorigenesis. One possibility is that most tumors go through many more divisions than their accumulated

mass suggests, depleting their telomeres at a preclinical stage and requiring telomerase-mediated telomere restoration before becoming symptomatic. It is also possible that telomerase benefits budding tumors in ways we have not discovered yet. Telomere restoration may be an eventual (and perhaps welcome) side effect of a more acute demand for telomerase deregulation early on in tumor development. Perhaps certain changes in telomerase can modify the cell cycle progression or alter damaged DNA. Clearly, it will be crucial to know whether telomerase only functions to restore tumor telomeres or whether the enzyme is in other ways important to transformed cells.

The possibility has been raised that inhibitors of telomerase could serve as cytotoxic agents in cancer therapy (Harley et al. 1990; Counter et al. 1992). This idea warrants thorough testing. In addition to the usual dilemmas associated with new drug targets (Gibbs and Oliff 1994), in the case of anti-telomerase agents some novel complications may arise. For one thing, tumors with long (restored) telomeres may display a considerable phenotypic lag during treatment. In addition, we need to know how gradual loss of telomeric DNA would affect a fully developed tumor. If telomere shortening is not acutely lethal in this setting, tumors may experience an unwelcome period of increased genome instability before they regress. A third issue is the possibility raised by observations in yeast and in *Drosophila* (see Zakian; Pardue, both this volume) that chromosome ends can be maintained without telomerase.

The attraction of anti-telomerase agents is the promise that their side effects may be limited. Prima facie, the fact that their telomeres shorten suggests normal human cells have no immediate need for telomerase. Indeed, telomerase levels in normal tissue are much lower than in most tumors, but additional functions for (small amounts of) telomerase are not excluded. Low levels of telomerase may be required to modify telomere termini, or the enzyme may be a permanent component of the telomeric complex. In each instance, telomerase may not keep up with terminal sequence loss with DNA replication yet fulfill an important function in normal cells. Therefore, it will be important to scrutinize normal cells for expression of telomerase once sensitive molecular probes become available. In addition, future genetic (knock-out) experiments are expected to reveal whether interference with telomerase is harmless in somatic mammalian cells.

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