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Review

Cell proliferation, carcinogenesis and diverse mechanisms of telomerase regulation

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Abstract. Replication of linear genomes is incomplete and leaves terminal gaps. Solutions to this 'end replication' problem can be traced back to the prebiotic RNA world: 'fossils' of the presumptive archetypes of telomere structure and of the telomerase enzyme are retained in the terminal structures of some RNA viruses. Telomerase expression in mammals is ubiquitous in embryonic tissues but downregulated in somatic tissues of adults. Exceptions are regenerative tissues and, notably, tumor cells. Telomerase activation is controlled by cellular proliferation, and it is an early step in the development of many tumors. In contrast to mammals, indeterminately growing multicellular organisms, such as fish and crustaceae, maintain telomerase competence in all somatic tissues. In human tumor diagnostics, detection of proliferation markers with monoclonal antibodies is well established, and in this review, the significance of additional telomerase assays is evaluated. Telomerase inhibitors are attractive goals for application in tumor therapy, and telomerase knockout mice have proven that telomere erosion limits the lifespan of cells in vivo. In contrast, telomerase stimulation can be used to expand the potential of cellular proliferation in vitro, with possible applications for transplantation of in vitro expanded human cells, for immortalizing primary human cells as improved tissue models and for the isolation of otherwise intractable products, such as genuine human monoclonal antibodies.

Key words. Polymerase; molecular fossils; rainbow trout; endometrium; inhibitors.

Introduction

Telomere structures and their maintenance are the subject of intense research in very diverse fields. Basic scientists explore the unique telomere structures and properties of the telomerase enzyme, but most excitement was created in biomedical research after the first presumptive linkages to tumorigenesis were proclaimed almost 10 years ago. Experimental tests of this hypothetical claim were the basis for the exponential increase in publications in this field. An overview of pertinent findings in telomere and telomerase research is provided in table 1. This review will focus on the linkage between telomerase expression and cellular proliferation and on potential practical application in tumor diagnostics.

The end replication problem—and how it can be solved

The duplication of all types of linear genomes is hampered by the 'end replication problem'. Obviously, a very precise initation of the 5′-terminal synthesis of the new nucleic acid chain is mandatory to provide an accurate duplication without loss of a single nucleotide

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Table 1. Overview of findings in telomeres and telomerase research.

Year	Comments
1938	Cytologic observation of the 'making of new chromosome ends' after γ-irradiation [23].
	Creation of the term 'telomere' (telos = end; meros = part).
1941	Analyses of 'breakage-fusion-bridge cycles' in maize confirm Muller's observation that breakage-induced chromosome ends are 'sticky', in contrast to native termini [206].
1961	Postulation of the 'Hayflick limit'
	Serial passages of human fibroblasts lead to proliferation stop, after a defined number of cell divisions [66].
1971/1972	Theoretical concept of the 'end replication problem': replication of linear DNA molecules inadvertently results in loss of terminal sequence [9–11].
1978	Analysis of 'extrachromosomal' rDNA in macronuclei of the ciliate *Tetrahymena thermophila*: sequencing of the termini and identification of the tandem repeats 5′-CCCCAA (and 5′-TTGGGG) [12].
1980/1981	Generalized identification of 'telomeric repeats' at chromosome termini in macronuclear DNA of several ciliates, with TTTTGGGG forming a 3′-overhang [18, 39].
1985	First functional characterization of a 'telomerase' enzyme in extracts from *T. thermophila* with properties of a ribonucleoprotein [41].
1986/1988	Longer telomeres in sperm DNA relative to somatic cells [61, 62].
1988/1989	Identification of the telomeric repeat sequence TTAGGG in humans [207] and vertebrates in general [14].
1989	Cloning and sequencing of the RNA component of telomerase from *T. thermophila*; identification of an RNA template segment encoding the telomeric repeats [42].
1989	Characterization of telomerase in human immortalized cell lines: a ribonucleoprotein that synthesizes TTAGGG repeats [56].
1989	Regulation of telomere length: excess of telomeric repeats results in elongated telomeres in yeast [176].
	Recently: similar observation in human cell lines [177].
1990	Mutations in the template segment of the RNA component of *T. thermophila* telomerase result in altered telomere sequences and cellular senescence [208].
1990/1991	Telomere hypothesis of cellular replicative aging and of cancer [64, 65]
	Linking telomere shortening to telomerase absence. Telomere length is the molecular clock and determines the remaining replicative potential of cells. See also Allsopp et al. [209].
	Telomerase activation not only in immortalized cell lines but also in human tumor cells.
1990 (1986)	Model of cellular 'replicative ageing' or 'mitotic clock' exemplified in humans.
	In vitro:
	Continuous telomere shortening during serial passage of (telomerase-negative) fibroblasts: ~50 bp lost per cell division [60].
	In vivo:
	Shorter telomeres in lymphocytes from older individuals: ~30 bp lost per year [59, 63].
	Within the same adult individual: longer telomeres in DNA from germline cells than from somatic cells [61, 62]. Similar data were reported for mice [101].
	Within the same adult individual: shorter telomeres in DNA from carcinoma [59].
1992	Reduced telomere length associated with ageing syndromes:
	Hutchinson-Gilford progeria [209].
	Down's syndrome [210].
1992	Telomerase expression in human immortalized cell lines is linked to 'arrested telomere shortening' in continuously dividing cells [67].
	Telomerase activity, a functional solution to the end replication problem, seems crucial for immortalization of human cells.
1994	Telomeres shorten in telomerase-competent hematopoietic stem cells with increased age [211].
	Consequences for recipients of bone-marrow transplants [186, 187].
1994	Potential linkage of telomerase activation and tumorigenesis.
	First detection of telomerase activity in human ovarian carcinoma [68] and in hematopoietic tumors [212].
1994	Development of the PCR-based 'TRAP assay' for telomerase activity permits detection of telomerase activity in 90% of a wide panel of analyzed human, malignant neoplasia, but not normal somatic tissues [57].
	Telomerase as novel tumor marker with high promise induced worldwide screening of many different tumor entities (recent review by Shay [124]).
	Substantial improvements of the assay protocol were introduced later [91, 105, 132].
1994/1995	Long-term maintenance of human cell lines without telomerase activity [147, 148].
1995	Presence (or absence) of telomerase as significant prognostic marker for brain tumor outcomes [108–111].
	All other tumor entities require evaluation of quantitative telomerase activity (reviewed by Shay [124]).
1995	Biochemical enrichment of the telomerase complex from *Tetrahymena thermophila* results in cloning and sequencing of genes for two protein components p80 and p95 [44].
	However, both protein sequences did not contain the expected polymerase or reverse-transcriptase motives.
1995	Cloning and sequencing of the RNA component of human telomerase [43].
	Expression of antisense RNA in immortalized cell lines results in abolished telomerase activity, telomere shortening and cellular senescence in continuously dividing cells.
1995	First detection of telomerase activity in normal human somatic cells: hematopoietic stem cells and lymphocytes [75–77].
	Many other examples of telomerase expression in regenerative human tissues followed: skin [82, 83], hair follicles [85], intestine [84], endometrium [86–90].
1995	Characterization of the human telomere binding protein factor TRF1 [213].
	This study is followed by the identification of further telomere-associated proteins and functional implications on telomere length regulation [28–30].

Table 1. (Continued)

Year	Comments
1996	Biochemical enrichment of the telomerase complex from *Euplotes aediculatus* [47].
1995/1996	Initial studies on the linkage of telomerase activity, cellular differentiation and proliferation [70]. Differentiation results in irreversible proliferation stop and telomerase inhibition [69–71, 113]. Starvation or contact inhibition results in cessation of reversible proliferation and telomerase inhibition. Further confirmed in subsequent work [91, 95].
1997	Generation of the 'telomerase knockout mouse', which lacks the gene for the RNA component of telomerase [151]. A strain of the mouse *Mus musculus* with exceptionally long telomeres was used, which provided a presumptive buffer against 'replicative senescence'. Mice were viable and could develop tumors. Only later generations displayed very short telomeres, associated with severe growth defects and loss of reproductive ability [152].
1997	Cloning and sequencing of the human homologue of p80 from *T. thermophila* telomerase [45, 46].
1997	Cloning and sequencing of the gene for the protein component p123 of the telomerase from *Euplotes aediculatus* and *est2* (103 kD) from yeast *S. cerevisiae* [48]. This protein is the presumptive catalytic component and displays reverse transcriptase motives.
1997	Cloning and sequencing of the catalytic protein component of human telomerase, later named hTERT [49–52].
1997	In situ reconstitution of active human telomerase enzyme from RNA (hTRT) and catalytic protein (hTERT) components within cellular, translation-active extract (reticulocyte lysate) [54, 55].
1997/1999	Protein phosphorylation is important in controlling telomerase activity: inhibition by protein phosphatase 2A [141], stimulation by protein kinase Calpa [142], Akt protein kinase [143].
1998	Expression of the gene for the catalytic protein component hTERT results in telomerase activity and extension of the life span of human epithelial cells [178, 214–216].
1999	Cloning of the complete gene for the catalytic protein component hTERT, including potential promoter control elements [217–220].
1999	c-Myc activates hTERT transcription [197, 198]. Possible direct linkage to tumorigenesis in skin [199].
1999	Expression of functional mRNA is required but does not always result in expression of telomerase activity [139, 184].
1999	Cloning of mammals: reduced telomere length in somatic cells is not restored by passage through germline [185].
1999	Beyond crisis: genetic catastrophe defines a novel threshold of telomere shortening [221].
1999	DNA telomeres are not stretched out in linear form: telomeric DNA folds back to form 't-loops' [35]. New functions for telomere-binding proteins in forming this structure.

(fig. 1A). This has been realized in the replication of some RNA and DNA viruses which carry a covalently linked protein at the 5′-end of their genomic nucleic acid. Examples are poliovirus RNA, adenovirus DNA and bacteriophage ϕ29 DNA [1]. The starter nucleotide is attached to the protein, and the complex is precisely positioned at the very 3′-end of the template. This guarantees the complete replication of the viral genome. If the complete replication of the termini is not guaranteed, a repair mechanism for lost nucleotides provides an alternative. Numerous 'end solutions' have been found in nature, and several are presented here.

Molecular fossils from RNA genomes

RNA genomes route back to the primordial, prebiotic RNA world. One end solution is the restoration of a few lost nucleotides by the precise addition of 3′-terminal nucleotides, independent of any template. Today, an enzyme with this function is still ubiquitous: the transfer RNA (tRNA) nucleotidyl transferase which adds (or repairs) the CCA-end of tRNAs. Interestingly, the RNA genomes of many plant viruses carry tRNA-like 3′-terminal structures. This means that truncated 3′-termini can be regenerated by the tRNA nucleotidyl transferase. Thus, the viral tRNA-like end can be considered as archetype of a 'telomeric structure' with a corresponding repair enzyme or 'telomerase' [2, 3]. This tRNA-like structure itself also functions as the origin of replication for the RNA polymerase (replicase) which copies the viral RNA genomes [4]. Furthermore, its characteristic, 3′-protruding CCA-end is an archetype promoter for all RNA polymerases. Indeed, it is utilized by DNA-dependent RNA polymerases, which are normally strictly dependent on 'bona fide' promoters. This includes polymerases from bacteriophages [5], as well as all eukaryotic RNA polymerases [6].

Another end solution is the transient adoption of a circlelike template in the replication of retroviruses. The reverse transcriptase reaches the 5′-end of the viral RNA, and after template switching it continues the replication at the 3′-end of the viral RNA [7]. Similar events have been observed with RNA polymerases. After completing a full 'round' on their linear template, the template-independent addition of a few more nucleotides leads to a template switch. Since RNA polymerases are capable of strand displacement, they can

perform multiple rounds as in a standard 'rolling circle' replication and synthesize multimeric linear copies [5]. The replication of viroids and virusoids may route back to this model reaction, thus retaining another molecular fossil from the RNA world [8].

Eukaryotic chromosomes

The end replication problem for linear, double-stranded DNAs was anticipated almost 30 years ago [9–11]. A further problem of DNA replication is the incapacity of DNA polymerases for de novo initiation of nucleic acid synthesis; this capacity is limited to RNA polymerases. DNA replication is absolutely dependent on preformed primers. In some viral systems, a protein-linked nucleotide is used (see above), but cellular DNA replication requires short RNA chains as primers [1]. Subsequently, these RNA primers are removed, thus leaving behind an even larger gap (fig. 1B). The ends of eukaryotic chromosomes are provided with two different, but related end solutions. First, their telomeres contain multiple copies of telomeric repeat sequences. This means that they provide a buffer function, and significant truncations can occur without immediate deleterious effects.

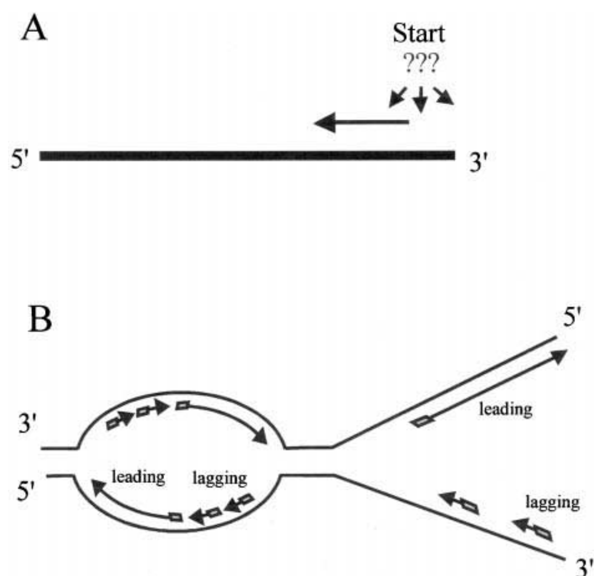

Figure 1. End replication problem. (*A*) In general, terminal nucleotide(s) are lost, because precise start requires special functions, such as protein-linked primers (see text). (*B*) End replication problem with double-stranded DNA. Replication of internal sequences is assured, even in the synthesis of the discontinuous lagging strand (left loop). At termini, only the leading strand reaches the very 5′-end of the template. A large stretch (~50 bp) is lost in the lagging strand, due to imprecise start (as in *A*) and because DNA replication requires RNA primers (hatched blocks). Subsequently, these primers are removed by RNase H, and at the very end, they cannot be replaced.

Second, (lost) telomeric repeats can be added by telomerase.

Telomere structures

In most eukaryotes, telomeres contain double-stranded, short G-rich repeats. The short telomeric repeat sequences were found in unicellular eukaryotes—ciliates [12] and yeasts [13] have been intensively studied. Telomeres exist in multicellular organisms, including all vertebrates [14], plants [15], arthropods, such as the insect *Bombyx mori* (lepidopteran silk moth) [16] and the crustacean *Homarus americanus* (American lobster) [17] (see table 2). Telomere termini are not blunt-ended double-stranded DNA, but rather end with a 3′-protruding, G-rich single-stranded overhang [18, 19, 21, 39, 40]; in humans, these single-stranded tails are up to 200 nucleotides long [20]. In vitro, the G-rich single-stranded segments can fold back to form intramolecular hairpins and tetrads with noncanonical G_{anti}:G_{syn} pairs [21] (fig. 2). The immediate result is a sequestered 3′-end of the chromosomal DNA. This is an attractive explanation for protection against exonucleolytic attack and prevention of the otherwise high recombinogenic activity of DNA termini, which is generally observed with exogenously added linear DNA [22] or at the internally generated ends of broken chromosomes [23]. Functional evidence for these structures in vivo is lacking, although some support was provided by findings that telomerase is inhibited by compounds which either interfere or stabilize tetrads with their noncanonical base pairs [24] (see also below). The structure of native telomeres is complex, and several mammalian telomere binding proteins have been described, such as hnRNP A1 [25], TRF1 [26], TRF2 [27] and tankyrase [28]. TRF1 is directly involved in maintaining telomere length [29], and TRF2 in the functional sequestering of DNA ends [30]. Recent, ingenious analysis of telomere structures by electron microscopy has revealed that telomeres are not simply stretched-out linear structures; rather, they form so-called t loops. This means that the linear, single-stranded end is folded back and tugged into intratelomeric stretches of double-stranded telomeric repeat sequences, presumably mediated by binding of TRF2 at these junctions [35]. These t loops were not only formed in vitro, they were also isolated after in vivo cross-linking [35]. The full molecular details are not yet known, e.g. the possible involvement of G-tetrads.

Exceptional structures were reported for dipteran insects. Their telomeres contain very large repeat elements with >6 kb in *Drosophila* [36] and 340–350 bp in *Chironomus* [37]. Although very different from the standard telomere, they share mechanistic similarities. Also, the maintenance of these retroposons requires RNA

Table 2. Telomeric structures in diverse organisms and detection of telomerase activity. Question marks (??) indicate unavailable data.

Organism	Telomeric repeat sequence	Telomere length	Telomerase activity (in vitro primer elongation)
UNICELLULAR ORGANISMS			
Ciliates			
Holotrichous: *Tetrahymena* *Paramecium*	TTGGGG	< 100 bp [12]	yes [41]
Hypotrichous: *Oxytricha* *Stylonichia* *Euplotes*	TTTTGGGG	< 100 bp [39, 40]	yes [222]
Yeasts			
S. cerevisiae	$TG_{(1-6)}$	< 500 bp [13]	yes [223]
K. lactis	TTTGATTAGGTATG TGGTGTACGGA	< 500 bp [224]	yes [224, 225]
Flagellates			
Trypanosoma *Leishmania*	TTAGGG	strain variations [226, 227] ≈ 2 kb and > 10 kb	yes [228]
Plasmodium	TTYAGGG	≈ 1.5 kb	yes [240]
Algae			
Chlamydomonas	TTTTAGGG	< 500 bp [229]	*??*
MULTICELLULAR ORGANISMS			
Slime molds			
Physarum	TTAGGG	≈ 2 kb [230]	yes [231]
Dictyostelium	$AG_{(1-8)}$ [232]	*??*	*??*
Sponges			
Suberites Geodia	*??*	*??*	yes [233] (proliferation-associated)
Higher Plants			
Arabidopsis	TTTAGGG	≈ 3 kb [15]	yes [103, 104]
Zea mays		≈ 40 kb [234]	(proliferation-associated)
Arthropodes: Insects			
Diptera:	Retroposon		no
Drosophila		repeat size 6–10 kb [36]	
Chironomus		340 bp [37]	
Lepidoptera: *Bombyx mori*	TTAGG	> 10 kb [16]	not detected [202]
Arthropodes: *Crustaceae*			
Lobster	TTAGG [17]	*??*	yes [106] (somatic: ubiquitous)
Vertebrates	TTAGGG in all vertebrates [14]		
Human	TTAGGG	> 10 kb [207]	yes [56, 57] (somatic: tight repression)
Mouse	TTAGGG		yes [101]
Mus musculus		> 50 kb [235]	(somatic: loose repression)
Mus spretus		> 5 kb [236]	
Rainbow trout	TTAGGG	> 20 kb *??* [237]	yes [106] (somatic: very high, ubiquitous)

template-dependent reverse transcription, at least in *Drosophila*, whereas RNA-independent mechanisms are discussed for *Chironomus* and *Anopheles* (reviewed by Pardue [38]).

Molecular characterization of the telomerase enzyme

As a reference guide for this chapter, the presently known subunits and their functions are listed in table 3. Direct studies of the enzyme from higher eukaryotes were impossible due to its scarcity, estimated at only about 100 copies per cell [34]. All molecular studies were restricted to ciliates, the prime organisms for telomerase studies due to their unique genome organization. In brief, they have two types of nuclei, a small transcriptionally inactive micronucleus and a large macronucleus which contains the entire transcriptionally active genome. The DNA of the micronucleus is organized in chromosomes, whereas the micronucleus contains thousands of subchromosomal DNA molecules ranging in size from 400 to 20,000 base pairs. The macronucleus is formed de novo from a diploid micronucleus after sexual conjugation. Formation of the macronucleus involves polytenization of micronuclear chromosomes. During this period, intragenic DNA segments (termed internal eliminated sequences, IESs) are removed, and flanking DNAs are rejoined. This is followed by chromosome fragmentation to generate the much smaller macronuclear DNA molecules,

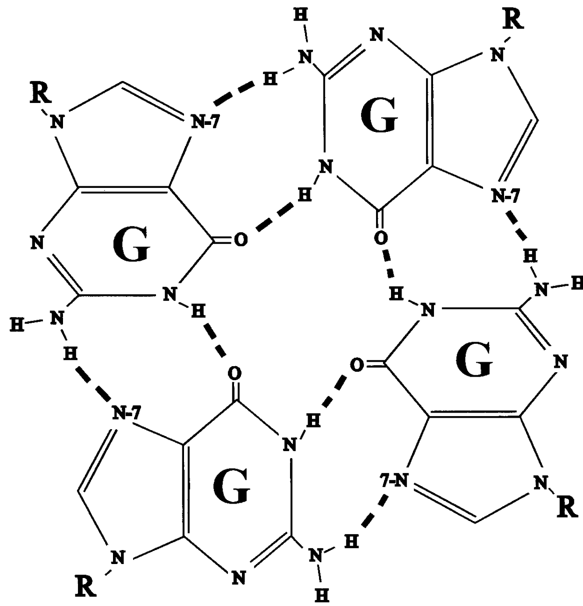

Figure 2. Structure of G-quartets. In the G-rich telomeric sequences, these noncanonical G:G pairs can form and can combine to quartets. This structure requires nitrogen at position 7 (indicated as N-7) which is absent in 7-deaza-G.

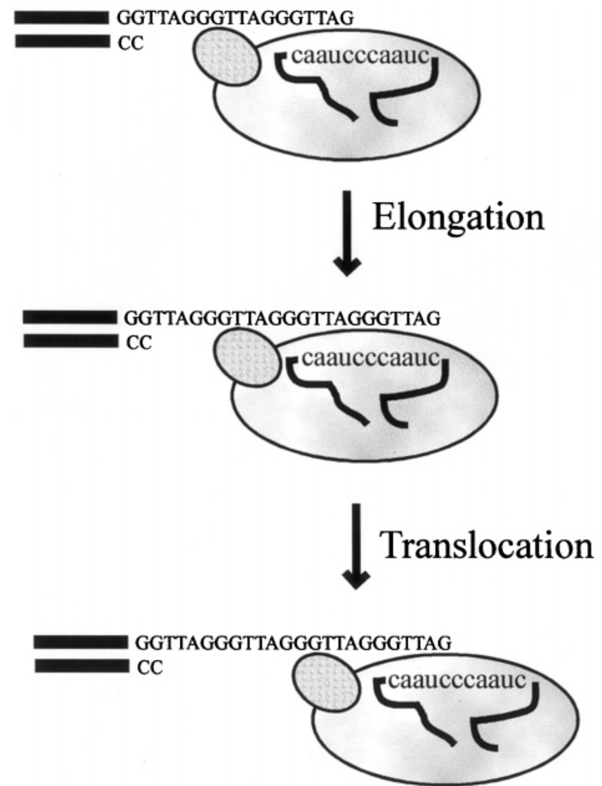

Figure 3. Schematic action of telomerase. Telomeres contain double-stranded DNA (solid bars) with long single-stranded 3′-extensions (capitals). The ribonucleoprotein enzyme telomerase elongates these stretches in a stepwise manner. It contains an RNA subunit with the indicated template region (small letters). A second template binding domain, the 'anchor site' is indicated as a separate subunit. The molecular entity is p95 in *Tetrahymena* [204] and p130 in *Euplotes*, an as yet not further characterized protein [205]. Although some data suggest an anchor site in human telomerase [58], the functional subunit has not yet been identified in a metazoan telomerase complex.

and telomeric repeat sequences are added. Finally, the macronucleus contains $>20{,}000$ different DNA sequences, and each is present in multiple (average of 1000) copies [12, 18, 39]. Obviously, this necessitates an extremely high telomerase activity, about 1000-fold higher than in human cell lines [34]. This made it possible to discover the enzyme telomerase and detect an essential RNA component, thus characterizing telomerase as a ribonucleoprotein (RNP) enzyme [41]. The first succcess on the molecular level was the identification and sequencing of the RNA component from *Tetrahymena* telomerase [42]. This unique enzyme can be regarded as a specialized reverse transcriptase which includes its own template: a short stretch of the enzyme's RNA component encodes the telomeric repeat sequence. Several years later, the RNA subunit from human telomerase was sequenced [43]. With its template for telomeric repeats, telomerase is shown schematically in figure 3. Again, it lasted several years, until the first

Table 3. Known components of human telomerase and their homologs in ciliates. Homologs do not exist for all species (??), or data are not available.

Function	Organism		
	Ciliates		Human
	Tetrahymena	*Euplotes*	
Template RNA	159 nt [42]	190 nt [238]	hTR 450 nt [43]
RNA-binding protein	p80 [44]	??	hTEP1 290 kDa [45, 46]
ssDNA-binding protein	p95 [44]	p130 [205]	??
Catalytic subunit: reverse transcriptase	p133 [53]	p123 [48]	hTERT 127 kDa [49–52]
Unknown	??	p43 [47]	??

gene sequences of protein subunits, p80 and p95 from *Tetrahymena* were known [44]. These hallmark results spurred search for human homologs, which succeeded in the complementary DNA (cDNA) sequence of hTEP1 [45, 46], the homolog for p80.

However, sequence comparisons did not reveal one of them as a presumptive polymerase subunit. Again, studies of telomerase from a ciliate could fill this gap. Purification of the enzyme from the ciliate *Euplotes* resulted in the identification of two associated protein bands in SDS gels, p123 and p43 [47]. Fortuitously, the further hunt could be combined with an independent genetic approach in the yeast *Saccharomyces cerevisiae*. The prey were p123 from *Euplotes* and *est2* (encoding a 103-kDa protein) from yeast, containing the long sought for sequence motives of a reverse transcriptase enzyme [48]. Another homolog search ensued, yielding gene sequences for the catalytic subunits hTERT from human [49–52] and also p133 from *Tetrahymena* telomerase [53].

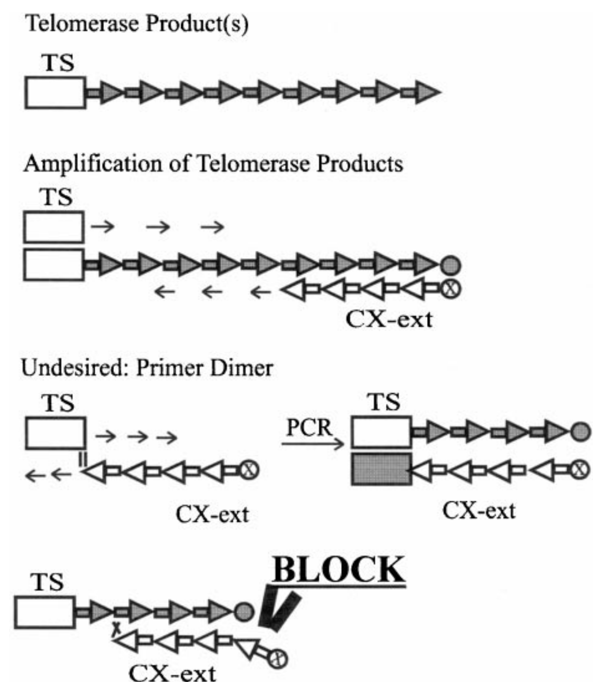

Figure 4. Schematic presentation of the TRAP assay [57], modified according to Krupp et al. [105]. Primer TS with a nontelomeric sequence (box) is a substrate for telomerase which adds telomeric repeats (arrows). The very small amount of the resulting products is amplified by PCR with excess primer TS and a reverse primer CX-ext which is complementary to the added repeats and in the extended version [105] contains an extra 5′-terminal sequence (encircled X). Subsequently, the amplified products are analyzed by gel electrophoresis and detected by staining or by fluorescence if a fluorescent-labeled primer is used. False positive results were a serious problem, and they derive from the undesired but unavoidable primer dimers (bottom section). Larger products (as with authentic telomerase activity) result from primer slippage, if the reverse primer anneals out of register (bottom line). With the extra sequence at CX-ext, mismatched bases block the extension and amplification of these erroneous products; for full details see [105].

The minimal elements of a core human telomerase enzyme were suggested from in vitro reconstitution experiments. Transcripts from the RNA component hTR were combined in situ with ongoing in vitro translation of the catalytic subunit hTERT in rabbit reticulocyte lysate [54, 55]. In this experiment, the involvement of rabbit homologs for hTEP1 (and other as yet unknown human telomerase components) is uncertain, and the search goes on. Recently, the molecular chaperones p23 and Hsp90 were identified as accessory components of the telomerase complex [244]. But a homolog for p95, the telomeric DNA-binding 'anchor' subunit from *Tetrahymena* was not yet identified. The protein p43, a potential small subunit from *Euplotes*, awaits characterization of its function and of its homologs in other species. The direct characterization of the human telomerase complex (or from other higher eukaryotes) is a formidable challenge, and the possibility of accessory components with regulatory functions is barely explored.

Setting the stage for functional telomerase analyses

The pioneering studies with ciliates required a direct primer elongation assay, in which the enzyme telomerase adds telomeric repeats on a DNA primer in the presence of α-^{32}P-GTP. The products were detected by gel electrophoresis and autoradiography. This direct assay was also successfully applied to characterize telomerase from an easily available human source, from immortalized cell lines [56].

The stage for large-scale analysis of telomerase was set with the invention of the polymerase chain reaction (PCR)-based TRAP assay for telomerase [57] (fig. 4). The development of this assay format was not straightforward. In the standard PCR approach, the amplified sequence is enclosed by two primers which hybridize specifically to terminal sequences at both ends. However, this is impossible with telomerase products. They contain only repetitive DNA sequences: primers with telomeric repeat sequences are elongated with further repeats. At least one unique primer sequence was possible after Morin observed that several primers with nontelomeric sequences are efficiently elongated by human telomerase [58]. Only the very sensitive TRAP assay permitted the detection of telomerase in crude lysates from cells and tumor biopsies, and spurred extensive, ongoing activities in cancer and telomerase research. Problems and their solutions which hampered application of this first assay format will be discussed

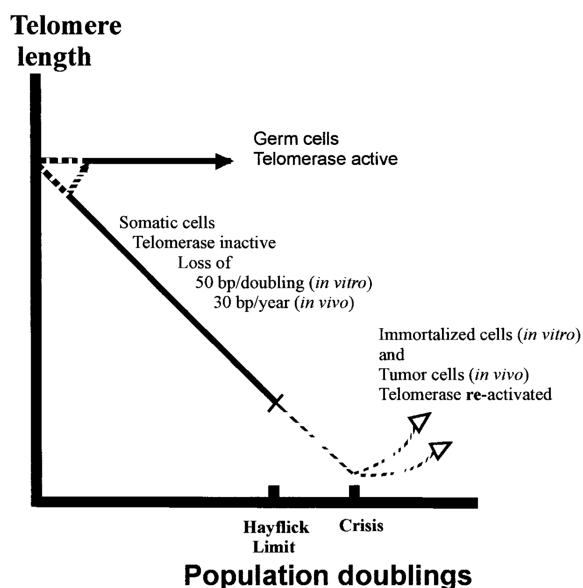

Figure 5. Graphic presentation of the initial telomere/telomerase hypothesis [64] as exemplified by Harley [65]. The model applies primarily to human development. The broken lines indicate unknown status in embryonic tissues. Germ cells are telomerase-active, sufficient to maintain the long telomeres. All somatic cells were expected to be telomerase-negative, resulting in the indicated loss of telomeric DNA. If a critical telomere length is reached for one or for several chromosomes within a cell, a checkpoint signal stops cell division (Hayflick limit, also termed M1). This block can be bypassed if growth control is lost by transformation, and continued cell division leads to almost complete elimination of telomeres. Chromosome fusions and genomic aberrations become frequent after the Hayflick limit and lethal if crisis (also termed M2) is reached. Further cell division requires reactivation of telomerase to maintain telomere length at sufficiently high levels. This behavior was observed in culturing primary cells in vitro, with rare events of established immortalized cell lines. A similar series of events was postulated in carcinogesis and immortalization of tumor cells (which were postulated to be telomerase-positive).

below. Until 1994, the number of publications related to telomerase research was < 30 per year; afterwards the number doubled each year, with > 500 in 1998.

Functional linkage of telomerase with cellular ageing and with carcinogenesis

In 1990, the available experimental analysis of telomere length (called TRF, terminal restriction fragment) in human tissues had revealed (i) a gradual telomere length shortening in lymphocytes with increased age of the donor [59, 60]; (ii) within the same individual, longer telomere lengths in germline than in somatic cells [61–63] and (iii) shorter telomere lengths in tumor biopsies than in adjacent normal tissue [59, 63]. These results prompted the hypothesis for the role of telomere length as a 'mitotic clock' in cellular ageing and in carcinogenesis [64, 65] (fig. 5), providing a mechanistic model for the Hayflick limit [66]: proliferation of telomerase-negative cells results in telomere shortening or 'replicative ageing', leading to cellular senescence and death [60]. In contrast, immortalized human cell lines express telomerase activity, which arrests telomere shortening and resets the mitotic clock [67]. A consequent extension was the postulation that telomerase is also active in indefinitely growing human carcinoma cells [64, 65]. In the model for carcinogenesis, mutational events result in loss of proliferation control, and rapid proliferation of telomerase-negative tumor-progenitor cells leads to replicative ageing and shortened telomeres. In a later stage, telomerase is reactivated to rather high levels, and thus stabilizes the previously shortened telomeres [64, 65].

Only in 1994 did this hypothesis for carcinogenesis receive experimental support when telomerase was detected in tumor biopsies, with ovarian carcinoma [68] and malignant hematopoietic cells [212] as the first examples. Subsequently, the very difficult direct assay for detection of telomerase in tissue lysates was replaced by the PCR-based TRAP assay (see above). This dramatically extended the range of clinical samples analyzed. Already the initial publication of the TRAP assay included a wide panel of human tumor biopsies as well as normal human somatic tissue samples. Almost all the tumors analyzed (90%) were telomerase-positive, whereas normal references were negative [57].

As a model for differentiated cells in telomerase-negative somatic tissues, differentiation effects were analyzed in several cell lines. Promyelocytic HL60 cells can be induced to differentiate to granulocyte-like cells and become postmitotic by treatment with chemicals such as all-*trans*-retinoic acid or dimethyl sulfoxide (DMSO). Similarly, sodium butyrate can induce differentiation of the erythroid leukemia cell line K-562. In these studies, differentiation and proliferation arrest were coupled. These events resulted in rapid, drastic and irreversible telomerase inhibition [69–71]. It is important to note that this decline of telomerase activity was much faster than the observed half-life (24 h) after shutdown of protein synthesis with cycloheximide [70]. In consequence, not only enzyme replenishing is inhibited, but also a mechanism for rapid distruction of telomerase is activated.

After obtaining more detailed results of telomerase expression in normal tissues, the initial model for carcinogenesis required revisions. It postulated a conversion from differentiated, telomerase-negative cells to telomerase-positive tumor cells. The first reports suggested a very low abundance of telomerase-positive cells (if any) in normal somatic tissues. This argued against the possibility that the clonal expansion of telomerase-positive cells could lead to the establishment of tumors, which requires the combination of several very

infrequent steps. It also seemed plausible that tumor cells with significantly shortened telomeres originated from telomerase-negative cells. Telomere erosion would result from a transient phase of cellular proliferation in the absence of telomerase [72]. However, as already evidenced in hematopoietic stem cells in older individials, even 'the presence of telomerase does not necessarily imply stable and thus unchanging telomere lengths' [73]. The carcinogenesis concept was challenged, and a revised version was postulated by Greaves [74] (fig. 6), at least valid in the genesis of leukemia. Greaves's model was based on the more recent findings that hematopoietic progenitor cells as well as lymphocytes in peripheral blood were found to be 'telomerase-competent' [75–77]. This means, if they are nondividing or quiescent, they are telomerase-negative, and they become telomerase-positive in response to stimulated proliferation [77–81]. These rather abundant telomerase-competent blood cells present a reasonable potential for carcinogenesis, and their phenotypes resemble more closely 'dedifferentiated' leukemia tumor cells.

This situation is not restricted to blood, since cells in all tissue sections with a high turnover must possess a high proliferation potential, and ensuing research revealed that these cells have retained their telomerase competence. Apart from hematopoietic stem cells and

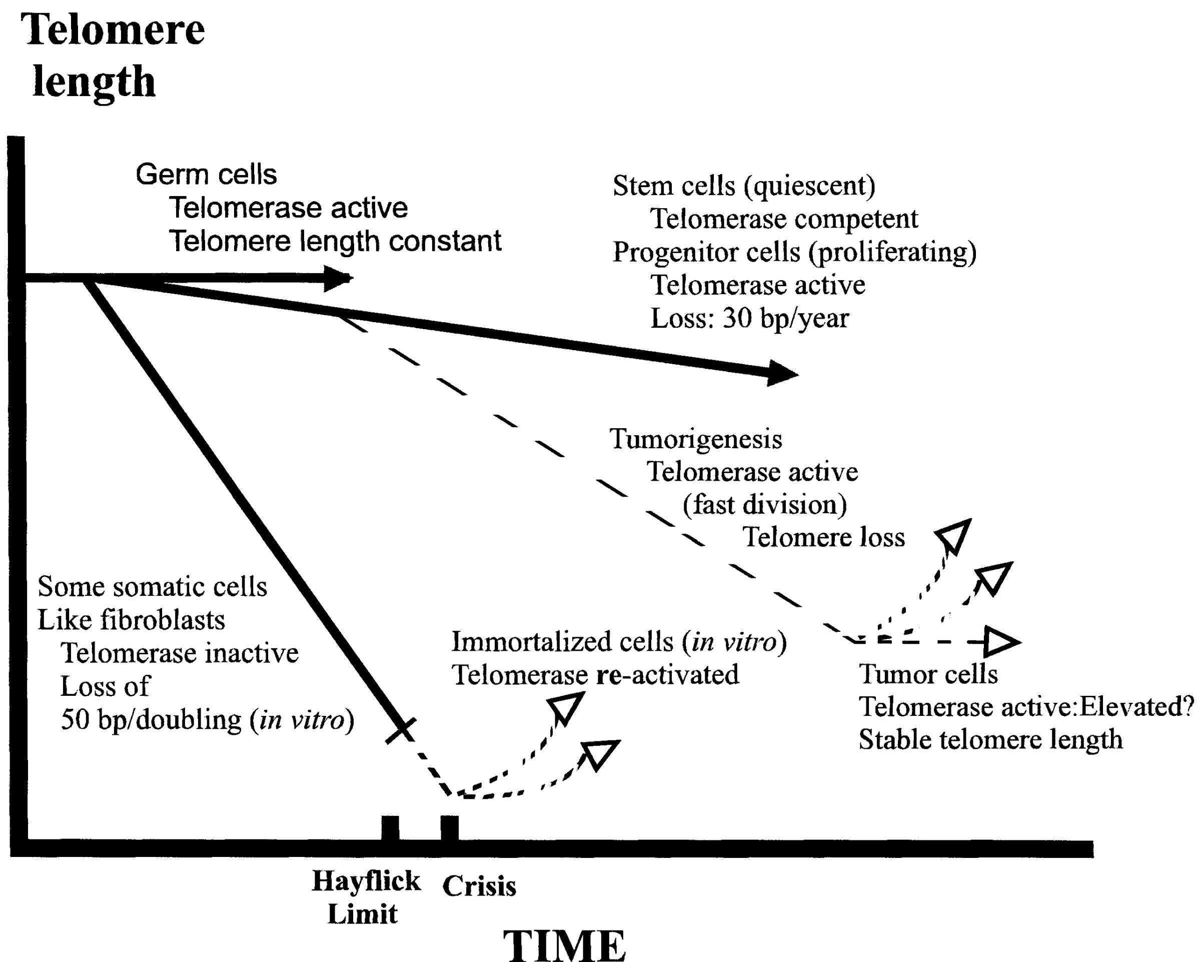

Figure 6. Revised telomere/telomerase hypothesis, based on discussions by Greaves [74], Shay and Wright [72], Shay et al. [73] and others. In embryonic tissues, telomerase activity had been detected [97], now shown as solid line. Two features remain unchanged: germ cells are telomerase-active, sufficient to maintain the long telomeres. Telomerase-negative cells were observed with in vitro cultures of some somatic cell types, e.g. fibroblasts. However, cells in regenerative tissue sections in vivo remain telomerase-competent: if quiescent (stem cells), they are telomerase-negative; proliferating progenitor cells (including lymphocytes in peripheral blood) are telomerase-positive, but this is insufficient to prevent telomere erosion. In tumorigenesis, telomerase-positive cells have lost growth control, and during their fast expansion, telomere loss speeds up. At a threshold (variable for cell type and tumor entity?), telomere length is stabilized, possibly by elevated telomerase levels. Another postulation may be added for indeterminately growing animals: proliferating cells in all somatic tissues have high telomerase activity, as shown for lobster [17] and trout [106], and no telomere erosion is expected.

lymphocytes [75–77], this includes skin keratinocytes [82, 83], intestinal crypt cells [84] and proliferating entities in hair follicles [85] and in endometrium [86–90]. These findings are remarkable, considering the serious practical limitations. In mixed samples, the TRAP assay is rather insensitive; only 1 equivalent of highly proliferating, telomerase-positive tumor cells could be detected in 300 equivalents of telomerase-negative fibroblasts [91]. Even with claimed sensitivities of 1 in 10,000 [57], detection of the presumptive telomerase-competent stem cells remains problematic because only proliferating cells display telomerase activity. Telomerase-competent stem cells are nonproliferating, with proliferation and telomerase activity restricted to their immediate derivatives. The now well documented, widespread presence of telomerase-competent cells makes them reasonable targets for initial establishment of tumors in general, not only restricted to leukemia [83, 91].

It remains to be shown how telomere erosion is stopped. The already present telomerase activity in regenerating somatic tissues seems insufficient to maintain long telomeres, but a telomerase increase in tumor cells may be unnecessary because there is a positive feedback mechanism which should favor the elongation of short telomeres [29]. As another possibility, a significant increase in telomerase activity may be required to maintain the short telomeres or to lengthen the already critically shortened telomeres and keep their increased length constant. Depending on the actual mechanism, high telomerase levels reflect either a high fraction of fast proliferating tumor cells in the tumor biopsy or significantly elevated telomerase activity in the individual tumor cell. The second alternative gets some support from recent findings that high telomerase activity in tumor samples (endometrioid adenocarcinoma) correlated with faster-dividing cells (a very short G1 phase in the cell cycle) (see below).

Telomerase activity in telomerase-competent cells is linked to cellular proliferation

Most tumor biopsies as well as in vitro proliferating tumor-derived, immortalized cell lines have been reported as telomerase-positive [57]. With the bona fide telomerase-competent cell lines, careful monitoring of proliferation and its relation to telomerase activity should provide valuable insights into telomerase regulation in vivo. This is of prime importance in the evaluation of telomerase activity as a novel tumor marker and of potential side effects of telomerase inhibitors. Apart from morphological examinations, the established grading of tumors relies on determination of the fraction of actively proliferating cells in tumor biopsies, in general based on the immunohistochemical detection of proliferation markers with monoclonal antibodies. A widely accepted marker is Ki-67 [92, 93] and monoclonal antibodies for its detection, such as Ki-S5 [94].

The following section summarizes reports about correlation of proliferation and telomerase activity in vitro and in vivo with (1) immortalized human cell lines in vitro, (2) human blood lymphocytes and epithelial cells in vitro, (3) human tissue sections in vivo and, (4) whole, indeterminately growing organisms.

(1) Effects of starvation (serum depletion) on the proliferation reduction of human tumor cell lines were analyzed by monitoring cellular proliferation with Ki-S5. After 4 days in the absence of serum, a significant proliferation reduction (from >90 to 70% Ki-S5-positive) ensued which was reelevated upon readdition of serum. The changes in telomerase activity were qualitatively similar and reversible as well, but quantitatively much more pronounced (minimum at $\sim 20\%$ of the initial value) [91]. Holt et al. have reduced growth by serum depletion or by contact inhibition and monitored proliferation by cellular DNA content. They reported similar dynamics in telomerase activity of murine [70] as well as of human cell lines [95].

(2) Studies with in vitro proliferation of normal human cells and tissues were already closer to in vivo situations. T-lymphocytes from peripheral blood have only low telomerase activity [75–77], and some somatic epithelial tissue samples (uroepithelial, breast, prostate) are completely negative [96]. However, telomerase activity drastically increased upon in vitro stimulation of cellular proliferation [77–81, 96].

(3) Several detailed in vivo studies (with human samples) were performed with proliferating, normal somatic tissue sections. Telomerase activity in human skin was potentially localized in the epidermis (at least in foreskin from newborns) [82]. Mechanical and enzymatic dissection of the skin corroborated these findings and assigned the source of telomerase to the basal layer of the epidermis, But it remained unclear if telomerase activity was derived from stem cells or from keratinocytes, their proliferating derivatives [83]. In hair follicles, telomerase activity was clearly concentrated in mitotically active segments, but not within sections with quiescent stem cells [85]. An example of continuous changes between low and high cellular proliferation is the cyclic, hormonally controlled renewal of the functional layer of the human female endometrium. Several groups detected telomerase activity in proliferative phases of the endometrium, and the suspected source was stem cells or their immediate derivatives in the basal layer [86–89]. More recent data confined the major telomerase activity to the highly proliferating functional layer and not to the less-proliferating basal layer where the presumptive quiesecent stem cells reside [90]. Quantitative changes in telomerase activity were

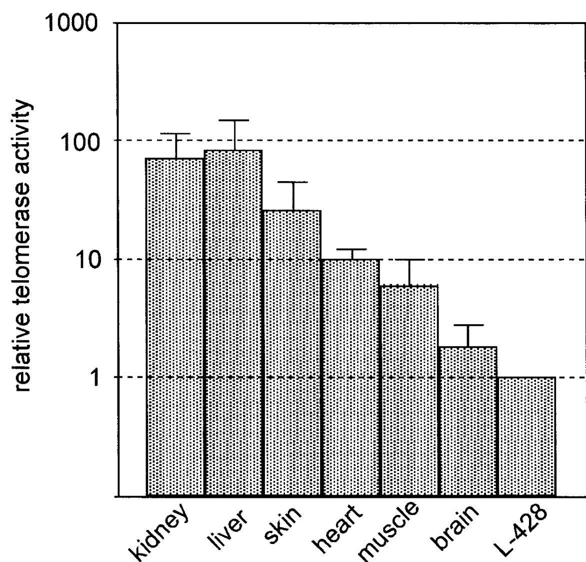

Figure 7. High levels of telomerase activity in somatic tissues from indeterminately growing rainbow trout (*Oncorhynchus mykiss*). Relative levels of telomerase activity are shown and compared with the immortalized human tumor cell line L428. Data shown are for fish with a weight of about 400 g [106]. Please note the logarithmic scale.

parallel to changes in the expression of the established proliferation marker Ki-67 and of a novel marker which is expressed only in actively cycling cells, monitored with the monoclonal antibody Ki-S2 [90] (see also below). In conclusion, quiescent cells are always telomerase-negative, and cellular proliferation is a prerequisite for activation of the enzyme in telomerase-competent cells.

(4) Initially, telomerase research was focused on unicellular organisms, ciliates and yeasts. These organisms have no defined growth phase, and individuals do not die, but live on after cell division. In sharp contrast are mammals, the other well-studied group. Mammals grow only in their youth, but adults have no net length increase and live on only in their progeny, derived from specialized germline cells. Most differentiated cells are postmitotic, and a high proliferation potential is required only in stem cells and their immediate derivatives, which remain telomerase-competent (see above). This expression pattern is closely followed in humans. Only samples from human embryonic organs are telomerase-positive, whereas telomerase is undetectable from the neonatal period onward [97]. Some liver samples from patients with hepatitis and liver cirrhosis were telomerase-positive, which may indicate a less tight shutoff in this organ [98, 99]. Mice are different from humans, with the very large telomeres in *Mus musculus* (see table 2) and with a comparable, but less stringent downregulation of telomerase which remains readily detectable in liver, kidney and spleen of adult mice, but is absent in brain [100–102].

A completely different group of multicellular organisms displays indeterminate growth, that is continuous growth throughout life. Some examples are plants, micelles of fungi, as well as some animals, including species of molluscs, arthropods, amphibia, reptiles and fish. This continuous growth requires the maintenance of a high proliferation potential in all somatic tissues. Possibly, concomitantly required telomere maintenance is based on ubiquitous telomerase expression in all proliferating tissues.

This expectation was fulfilled in several examples. For the analysis of plant telomerase it was necessary to modify the TRAP assay. The reverse primer was changed to accommodate the plant telomeric repeat sequence TTTAGGG. The assay revealed that mitotically active meristematic tissue and cultured cells in vitro have high telomerase activity, whereas nondividing cells from leaves and axillary buds are telomerase-negative [103, 104].

As an example from the animal kingdom, the American lobster (*Homarus americanus*) was analyzed. The telomeric repeat sequence was unknown, and the TRAP assay was used, as developed for the vertebrate sequence TTAGGG [105]. The resulting, PCR-amplified telomerase products were cloned and sequenced. This revealed the repeat sequence TTAGG, already known from another arthropod, the insect *Bombyx mori* (see table 2). In the indeterminately growing lobster, cells in all tissues must proliferate, and rather high telomerase activities were detected in all samples from (with decreasing activity) hepatopancreas, heart, skin and muscle [17].

Interesting data were obtained from a vertebrate, the rainbow trout (*Oncorhynchus mykiss*). Since telomeric repeat sequences are conserved for all vertebrates, the TRAP assay permitted a direct, quantitative comparison with human cell lines. Regardless of the age or weight of the fish (4 g up to 2000 g), samples from all organs contained extremely high telomerase activities. The highest values were found in liver and kidney, more than 100-fold higher than in optimally proliferating human tumor cell lines [106] (fig. 7). The fraction of proliferating cells in thin sections from fish tissue can be determined with a monoclonal antibody for PCNA, the proliferating cell nuclear antigen [107]. Confirming the linkage, different proliferation levels were correlated with relative telomerase levels (fig. 8).

Repression of telomerase activity

In contrast to the telomerase-competent cells described above, telomerase incompetent cells are always telomerase-negative. For example, rapidly growing primary human fibroblasts are telomerase-negative, and continuous telomere erosion limits the number of population doublings [60, 209]. This limited proliferation capacity could be overcome by enforced expression of the catalytic component hTERT in transformed fibroblasts [178]. Although mechanisms of telomerase repression are poorly understood, there are reports of telomerase repressor gene(s) on chromosome 3p [241–243].

Potential practical applications: I. Tumor diagnostics

Early on, telomerase activity was regarded as a novel marker which is unique to tumor cells. With proliferating germ cells as the only exception, telomerase should be absent in all normal somatic tissues and in benign lesions, but universally present in all tumor entities.
This expectation still holds only for a single entity, brain tumors [108–112]. Most if not all normal brain cells in mammals are postmitotic, and no telomerase expression occurs. Fortunately, benign neoplastic lesions were also found telomerase-negative, and a high correlation was observed between telomerase activity, malignancy and recurrence of tumors. In a study with 30 patients with meningioma [110], 25 patients without detectable telomerase activity had no relapse, whereas 5 patients with telomerase-positive samples had recurrence of cancer. In another study with 105 patients with neuroblastoma [112], the absence of telomerase activity was associated with spontaneous regression, whereas high activity (23 patients) correlated with poor prognosis.
Results with other tumor entities are not so clear-cut, since normal reference tissues are also telomerase-positive. Nevertheless, if different quantitative levels of telomerase activity are compared, high levels indicate poor prognosis. This was reported for acute leukemia [113, 114] and for breast cancer [115–117]. Increasing telomerase levels were correlated with skin tumor progression [82, 118, 119]. Initial reports of a similar correlation in gastric cancer [120, 121] were questioned later [122]. More details are available in recent reviews by Urquidi et al. [123], Shay [124], McKenzi [125], in special issues of the *European Journal of Cancer* [31] and of *Biochemistry* (Moscow) [32], and in several books [33, 34].
Further evaluation of telomerase activity as a novel tumor marker should be based on the comparison with other established markers in tumor diagnostics, such as determination of the fraction of actively proliferating cells in tumor biopsies. Immunohistochemical detection of proliferation markers is accomplished with monoclonal antibodies, such as Ki-S5 for the widely accepted marker Ki-67 [92–94]. This marker protein is not expressed in quiescent cells (cell cycle phase G0) but present in proliferating cells (all phases of the cell cycle: G1, S, G2 and M). A shortcoming of this proliferation marker is the inclusion of G1, with its highly variable

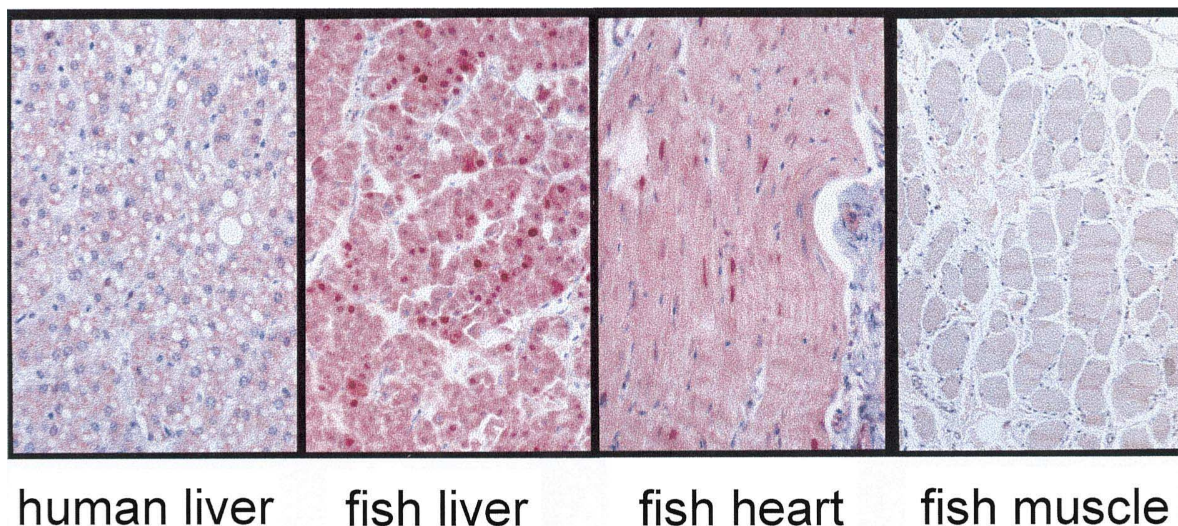

Figure 8. Immunohistchemical detection of proliferation in different tissues of rainbow trout, with monoclonal antibody against PCNA, the proliferating cell nuclear antigen. Stained nuclei are abundant in fish liver and heart, rare in fish muscle and absent in a sample from human liver, which is shown for comparison.

duration. Recently, a monoclonal antibody, Ki-S2, was described, which detects a novel, more specific marker that is expressed only in phases S, G2 and M [126]. With this antibody, the actively cycling cell fraction is demarcated.

In a recent study with 53 patients with uterine endometrioid adenocarcinoma, both proliferation markers and quantitative telomerase levels were evaluated (G. Bonatz et al., unpublished observations). A general correlation of increased proliferation (Ki-S5 and Ki-S2) with higher telomerase activity was observed, but a stronger association with the Ki-S2/Ki-S5 ratio. These results indicate that increased telomerase activity in endometrioid adenocarcinoma is not only associated with an increased number of cycling cells but is also more elevated in cells with a shorter G1 phase and thus with a shorter duration of the cell cycle. Although both, high telomerase activity (not telomerase positivity per se) and the associated high Ki-S2/Ki-S5 ratio, correlated well with histologic evaluations (as approved by the International Federation of Gynecology and Obstetrics (FIGO), namely FIGO stage and FIGO grade) and tumor recurrence (5 of 53 patients), quantitative relations should also be considered. The Ki-S2/Ki-S5 ratios differed at most fourfold, between 0.2 and 0.8, whereas telomerase levels varied more than 100-fold. Considering the recent suggestion that telomerase activity is not a unique tumor marker but 'only' another marker for cellular proliferation [96], these results indicate that telomerase activity as a prognostic tumor marker has at least a superior diagnostic resolution. In addition to telomere maintenance, the possible involvement of telomerase in DNA repair could make it even more informative in predicting chemoresistance (see below).

Due to the high sensitivity of the TRAP assay it can be performed with a small number of cells. This assay was shown to be superior to cytologic examination of pancreatic duct cells [128] or peritoneal fluid from patients with ovarian carcinoma [129; reviewed by McKenzie in 125]. This could increase the value of minimal-invasive sampling procedures in the early evaluation of suspected cancer or detection of minimal residual disease. Repeated monitoring would be reasonable for following the antiproliferative effect of tumor therapies and of subsequent, potential tumor recurrence. The feasibility of the approach was shown with a model system, radiocuring of tumors in nude mice [130]. Potential practical applications are exfoliated urinary cells from urological neoplasias [131], fine-needle aspirates from breast cancer patients [117] or hysteroscopy in endometrioid carcinoma, which requires consideration of the hormonal status of the patient [90]. See also the extensive compilation in the review by McKenzie et al. [125].

Detection of telomerase: experimental considerations

The initial format of TRAP, the PCR-based telomerase assay, had some problematic features which were resolved later: (i) Variable amounts of PCR inhibitors in tumor samples can be identified by the coamplification of an internal amplification standard, ITAS, and more reliable quantitative comparisons were possible [91, 132]. (ii) Telomerase products contain only one unique primer sequence; the reverse primer still consists of repetitive DNA, and false positive results were obtained due to template slippage on PCR-derived primer dimers. The introduction of extended reverse primers eliminated this problem, and a simplified protocol was possible (fig. 4) [105, 133]. (iii) Different tumor entities contain substantial and highly variable RNase activities, which are destructive to the ribonucleoprotein enzyme telomerase [134–136]. With a sensitive and quantitative, fluorescence-based RNase assay it was shown that the suggested proteinous RNase inhibitor RNasin [135] provides only limited protection, which was improved by including the more universal inhibitors VRC (vanadyl ribonucleotide complex) and ATA (aurintricarboxylic acid) [136]. Several of these improvements have been implemented in commercially available kits from Oncor/Appligene (gel assay) or Boehringer/Roche-Diagnostics (high throughput enzyme-linked immunosorbent assay (ELISA) format, but lacking the normalizing option of a coamplified ITAS). However, these assays require the analysis of fresh or flash-frozen (liquid nitrogen) samples, and handling is not without experimental challenges. Furthermore, they are limited to the analysis of lysates, and in situ analysis which preserves cell morphology would be highly preferred. Although some success with an in situ TRAP assay was reported [137], quantitative comparisons are—at least—problematic.

A possible alternative is the direct detection of enzyme subunits. Cloning efforts yielded the genes for the RNA component [43] and a telomerase-associated protein, the human homolog of p80 from *Tetrahymena thermophila* telomerase [45, 46]. However, both components are not strictly linked to telomerase activity and not suitable for quantitative comparisons. More recently, the cDNA of hTERT, the catalytic protein component, has been characterized, and initial data have shown a good correlation of messenger RNA (mRNA) levels with telomerase activity [49–52]. This linkage was questioned in subsequent studies, which have shown that several splicing variants [52] are functionally defect [138]. But even considering these results, fully functional hTERT mRNA is expressed in lymphocytes, irrespective of telomerase levels [139], and in normal, telomerase-negative tissues, such as human brain, heart, liver, prostate, ovary and primary

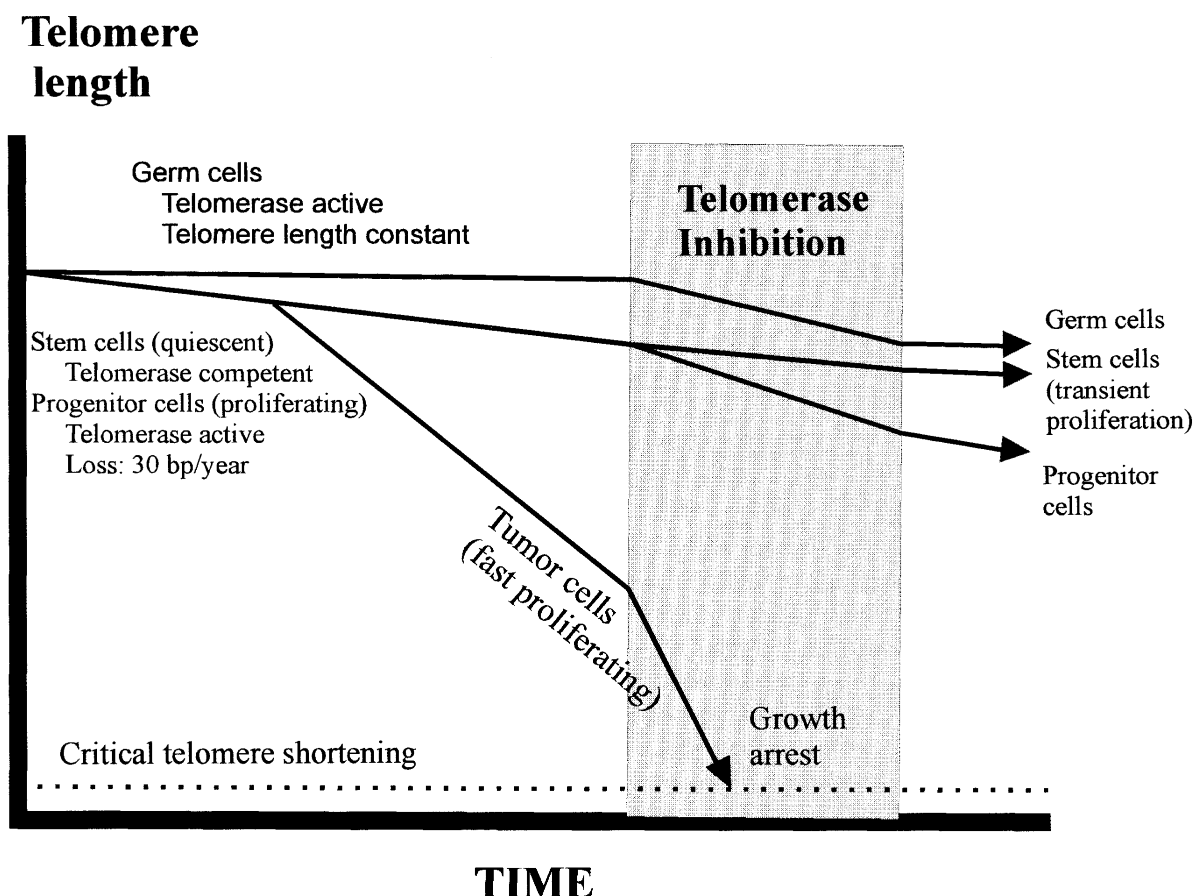

Figure 9. Envisioned effects of tumor therapy with telomerase inhibitors. Fast-proliferating germ cells (sperm cells) would suffer, whereas transiently activated stem cells would remain largely unaffected. They would also soon replace the affected progenitor cells, and derived somatic tissues. Tumor cells with already shorter telomeres would shorten even faster, leading to irreversible growth arrest and cell death.

fibroblasts [140]. These data suggest posttranscriptional control, and further complications arose from findings that protein phosphorylation stimulates telomerase activity [141–143]. Large-scale sampling with immunohistochemical detection of the catalytic protein subunit as the preferred format may help in resolving these issues. However, despite some success with polyclonal antibodies [144], no highly specific monoclonal antibodies for reliable analysis of paraffin-embedded thin sections are yet available. With these presently unresolved issues, direct detection of telomerase activity with the TRAP assay remains the only reliable quantitative method for the detection of this marker enzyme.

Potential practical applications: II. Telomerase inhibitors in tumor therapy

Therapy with telomerase inhibitors should be less deleterious than conventional chemotherapy with its immediate attack on all proliferating cells. Effects of telomerase inhibition are delayed since they require a critical shortening of telomeres before growth arrest and cell death occur. Increased specificity can be expected because the reservoir of telomerase-competent but quiescent stem cells is unaffected. Furthermore, the actively proliferating progenitor cells are also much less vulnerable than tumor cells, which have very short telomeres and thus a reduced proliferative life span. Promising candidates are colorectal [59] and ovarian carcinoma [68], where samples from many patients displayed telomere lengths with only half the value of normal reference tissue (reviewed by Bacchetti in [145]). A suggested therapy regime [124] could combine conventional steps to reduce tumor mass, followed by a recovery period to permit transient high levels of stem cell proliferation (replacement of damaged regenerating tissues) and their return to quiescence. The final antitelomerase treatment would hit actively proliferating

tumor cells and limit their proliferative capacity, thus preventing tumor relapse (fig. 9). As anticipated, a severe side effect of systemic application of telomerase inhibitors would be the erosion of telomeres in the continuously fast-proliferating sperm cells. This damage may be irreversible, as anticipated from results with the cloned sheep Dolly [185], where passage of somatic cells through the germline did not restore the original telomere length. Thus, at least in males, localized treatments seem mandatory.

For effective antitelomerase treatment, the absence of telomerase must seriously limit the cellular proliferative capacity. Initial data with antisense constructs against the human telomerase RNA component support this notion [43]. Furthermore, only very few cells may escape this limitation with an alternative mechanism for telomere maintenance. A recombination-based telomerase-independent mechanism is well documented for unicellular yeast [146], and also several in vitro experiments with human cell lines suggest the existence of an alternative mechanism of telomere lengthening (ALT) [147–150]. On the other hand, telomerase-negative mice [151] with critically shortened telomeres (their initial, extreme length required more than five generations) displayed severe defects in regenerative tissues [152], suggesting that escape from telomerase-dependent telomere maintenance is not frequent enough to prevent these in vivo defects.

The list of already known telomerase inhibitors includes normal cytostatic drugs that also act on cellular polymerases, e.g. nucleotide analogs [153–156], cisplatin [157] and tea catechins [158, 159]. The advent of antisense technology enables target-specific design of antisense oligonucleotides, combined with nuclease stabilization by 2′-*O*-methyl-ribose [160], or phosphorothioates [161, 162], or by including a catalytic ribozyme motif [163, 164]. A completely altered backbone is provided with peptide nucleic acids, PNAs [150, 165], and initial problems with cellular delivery may have been solved [166]. Although the promiscuous protein-binding properties of phosphorothioate-modified oligonucleotides is a determinant of their telomerase inhibition [162] and thus limits the target specificity, some data of their promising in vivo effects in nude mice models have been reported [161].

The unique G:G interaction in telomere G-quartet structures seems another good target. Telomerase was inhibited both by structural interference with the nucleoside analog 7-deaza-guanosine [167, 168] and by conditions which stabilize the quartet structure [24]. Especially promising are low molecular weight inhibitors with quartet stabilizing effects, such as alterperylenol [169, 170], amidoanthracene-diones [171] or dicarbocyanine [172]. Also, unbiased screening programs show promise in identifying synthetic moieties such as benzo[b]thiophenes [173] or natural compounds, e.g. a polysaccharide from the marine microalga *Gymnodinium* [174]. In the near future, the powerful approach of modern combinatorial chemistry [175] will be applied to promising, albeit still unsatisfactory lead structures, and this may yield compounds for practical tests of the ever hopeful telomerase inhibitor therapy.

At present, expectations may be optimistic, but there are also contradictory findings. The occurrence of the alternative mechanism of telomere lengthening, ALT, has been reported for clinical tumors in vivo [245]. This mechanism could occur more frequently in genetically destabilized tumors upon antitelomerase treatment, and thus tumors would become resistant to telomerase inhibition.

Furthermore, effects of complete telomerase suppression at the organism level were studied in the knockout mouse model (homozygous mTR −/− with a deleted telomerase RNA gene) (reviewed in [246]). No immediate effects were evident; even tumor development was unaffected. This was not unexpected, since this mouse strain had very long telomeres, thus providing a buffer against telomere erosion in the absence of telomerase activity. Telomere length declined over successive generations, ultimately (G 6 animals), resulting in cellular defects anticipated for telomere attrition; reduced proliferative capacity and apoptotic cell death were evident in defective spermatogenesis and hematopoiesis [152].

However, in contrast to expectations, these mice had not lost the ability to develop tumors, they even had a slightly increased frequency of spontaneous tumor development, compared with wild-type control mice [247]. These findings are one anticipated consequence of telomere loss, namely an increase in chromosome fusion and in general genomic instability. But in normal cells, these effects would be less evident because critically shortened telomeres would trigger checkpoint functions that lead to cell death by apoptosis. It appears that elimination of these functions is an early step in the development of these tumor cells. In this setting, telomere loss and chromosomal damage are tolerated and thus actually drive tumor development. The lesson from these findings is that telomerase inhibition could be mutagenic in tumor cells, especially if checkpoints were deleted, as in p53 mutants [221]. On the other hand, increased mutation rates are a common side effect of most cancer therapeutics; and in a clinical setting, a transient therapy with telomerase inhibitors would be envisioned. In this case, growth limitation of already established tumors is intended, and achieving this goal may outweigh a slight increase in spontaneous tumor development.

Potential practical applications: III. Elongated life spans of cells

Two different experimental approaches are outlined which may offer this potential. Preliminary reports with yeast [176] and recently with human cell lines [177] suggest that telomere elongation and significantly extended cellular proliferation capacity can be achieved with a relatively simple treatment with unmodified phosphodiester oligonucleotides of the sequence $(TTAGGG)_2$ or variants thereof [177]. A more demanding option is the introduction and expression of the gene for the catalytic telomerase component. This genomic manipulation induced telomerase activity in previously telomerase-negative cells and resulted in a drastically expanded life span of human epithelial cells [178]. So far no changes were found which would suggest that these 'immortalized' cells have gained carcinogenic potential [179, 180]. Results from more rigorous tests are still lacking [181], and according to the model (figs 5 and 6) it was shown that enforced expression of telomerase in cooperation with other genetic changes (oncogene activation and tumor suppressor inactivation) can create human cancer cells [248].
Furthermore, a severe technical problem became evident with the findings that for certain cell types, telomerase gene expression and even established telomerase activity (as determined with the in vitro TRAP assay) is not sufficient for cellular immortalization [182–184]. Resetting the mitotic clock seems a more demanding challenge than anticipated. Even the passage of somatic nuclei with shortened telomeres through the germline was insufficient for restoring full-length telomeres. This can be concluded from reported 'aged telomere ends' in the sheep Dolly that were derived from in vitro expanded somatic cells [185].
If technical problems were solved, a useful application could be based on very early observations. The proliferation potential of blood cells gradually decreases (erosion of telomeres) with increasing age of the donor [59, 63]. This suggests that telomerase activity per se in hematopoietic stem cells is insufficient to maintain stable telomere lengths [73]. An immediate outcome is a potential problem in bone-marrow transplants, which require a massive expansion (proliferation) of stem cells. Even allogeneic transplants display 0.4-kb shorter telomeres, which is equivalent to a median of 15 years' ageing [186, 187]. This problem is more pronounced with a young recipient and an old donor. But at least there is an age window with minor effects. Rapid telomere length decline in leukocytes was restricted to children until age 4, then followed by an almost constant plateau phase until mid-20s and a gradual attrition later in life [188, 189]. In certain situations it may be advantageous to elongate telomeres ex vivo before transplantation. The technical advances in transplantation of lab-grown tissues or even complete organs [190, 191] represent a similar challenge, since they include a step with expansion of somatic cells.
A mere in vitro application could be the immortalization of 'primary' human cells for better tissue culture models, maybe useful for the isolation of products which are intractable or completely shut off in conventional cell lines. A very rewarding challenge is the replacement of the complex hybridoma technology with the chance to produce purely human monoclonal antibodies from immortalized B lymphocytes.

Unsolved problems

Is the mitotic clock relevant for normal cellular senescence in vivo and for organismic ageing?

This is a matter of debate [181, 192–195]. The pathogenesis with severe defects in highly proliferative tissues of telomerase-negative mice [152] is an artificial system. In normal organisms, other ageing mechanisms have prime importance and may have fatal consequences before telomere-dependent proliferative limitations can become important. They are evident in ageing of mice with 'unlimiting' telomere lengths or in signals from the reproductive system that influence the life span of the nematode *Caenorhabditis elegans* [196]. Probably, the slow rates of senescence in indeterminately growing metazoa are not an immediate consequence of, but they clearly require the maintenance of high cellular proliferation potential brought about by, high telomerase activity [106].

What regulates telomerase activity?

Proliferation of telomerase-incompetent cells occurs in the absence of telomerase expression, and in the absence of alternative mechanisms for telomere maintenance, their replicative proliferation capacity is limited [60, 209]. Reports on telomerase repressor gene(s) [241–243] could lead to their exploitation as tumor suppressors.
Telomerase expression by transcriptional activation of the catalytic subunit hTERT by c-Myc is well documented [197, 198] and possibly involved in development of skin tumors [199]. Knowledge of the hTERT promoter structure may reveal more of a regulatory network. The initially suggested tight linkage of telomerase activity and expression of the catalytic component hTERT [49–52, 139] did not hold in further analyses of tissue samples, at least not at the level of expressed mRNA, even if inactivated splicing variants are considered [140]. In spite of these limitations, transcriptional regulation of the hTERT gene is still defined as a major, although not an absolute, determinant of the enzyme activity. Clearly, additional posttranscriptional and

postsplicing steps are involved, and activation by phosphorylation with kinase Cα [142] and/or Akt protein kinase [143] is even beyond translational control. However, regulatory effects of phosphorylation seem only quantitative (no on/off switch), and quantitative correlation of hTERT protein levels and telomerase activity is poorly documented for normal telomerase-positive and -negative tissues. In addition to the recently identified molecular chaperones p23 and Hsp90 [244], further accessory or regulatory components of the telomerase complex may exist. Clearly, telomere-binding proteins are important in controlling the length and access of telomerase to the chromosomal telomeres [29], but we still know very little about the linkage of telomerase detected in vitro and its function in vivo. The extremely high telomerase activity in fish liver is compelling and not understood. The signalling pathway which links cellular proliferation to telomerase activity remains enigmatic.

Immunohistochemical detection of telomerase components in paraffin-embedded thin sections

At present the specificity of polyclonal antibody preparations is limited [144], and even with fresh tissue samples, the preferred and reliable cellular localization of hTERT by immunohistochemical analysis is elusive. Highly specific monoclonal antibodies for analyzing paraffin-embedded thin sections would be the ideal tool for clinical studies and would allow a broad range of retrospective analysis of samples from patients with known pathogenesis. These studies may help to distinguish whether tumor samples with high telomerase activity are enriched in telomerase positive tumor cells or whether telomerase levels are elevated in individual tumor cells.

Telomerase functions other than telomere maintenance

In many examples, high telomerase activity correlated with frequent recurrence of disease in cancer patients. These observations were not only linked to high proliferation rates but also to failure of and resistance to chemotherapy [113, 200]. Several cytostatic drugs cause chromosome breaks and in the resistance mechanism, telomerase may have a function in DNA repair. At least in yeast, the same proteins are shared in repair of double-strand breaks and in telomere maintenance [201]. Understanding the detailed interplay of both functions could advance prediction and fighting of chemoresistance.

What prevents erosion and dysfunction of telomeres?

Telomerase activity per se (at least determined with the in vitro assay) is definitely not sufficient [182–184]. It is known that the human telomeric protein TRF1 is involved in the control of telomere length [29], and for protists it has been shown that mutations in the telomerase RNA template region can result in telomere shortening, as observed in *Tetrahymena* [208] or in runaway replication of very large telomeres in yeast [239]. Even long telomeres cannot prevent chromosome fusion if they lack short single-stranded extensions, which are required for TRF2 interaction [30]. In human carcinogenesis or chromosomal aberrations, nothing is known about the involvement of mutants in human genes for telomere-associated proteins or for components of the telomerase complex.

How many different mechanisms for telomere maintenance?

Presently known alternatives include the following: (i) standard telomeres with short repeats are maintained by the ribonucleoprotein enzyme telomerase; (ii) recombination as a rare rescue function in yeast, possibly also in humans, (iii) in the maintenance of long retroposons in *Drosophila*, reverse transcriptase and RNA template are separate entities. The insect *Bombyx mori* is exceptional with short repeats, but attempts failed to identify a primer-dependent telomerase in larvae, cell lines, pupae or imagines [202, (W. Klapper et al., unpublished observations)]. Is *Bombyx* an example for recombination as the major pathway? Taking a different route and a lesson from the fossil analog tRNA nucleotidyl transferase, a telomerase protein enzyme which can add short sequence repeats and does not require a nucleic acid template seems feasible. In which organisms are chromosomal ends maintained by such an archetypal telomerase?

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