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The Implications of Telomerase Biochemistry for Human Disease

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The replication of linear chromosome termini (telomeres) cannot be completely replicated by conventional DNA polymerases. Telomerase is a special DNA polymerase used by most eukaryotes to solve the telomere end replication problem. Telomerase is necessary for indefinite cell division in most immortal cells, but apparently unnecessary for the normal function of most somatic tissues. Telomerase may play a critical role in some genetic diseases, in regulating the lifespan of normal cells, and in tumorigenesis. This article reviews the structure and reaction mechanism of mammalian telomerase and how it may be exploited to control some human diseases. © **1997 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

TELOMERE FUNCTIONS can be broadly distributed into three areas; chromosome positioning, protection and replication. Studies in several organisms have shown that telomere positioning impacts on the localisation of chromosomes within the nucleus, the pairing of homologous chromosomes in early meiotic nuclear division and the movement of chromosomes during division (reviewed in [1, 2]). The earliest genetic descriptions of telomeres by Muller and associates [3] and McClintock [4] demonstrated the protective function telomeres provide to chromosome termini, and the deleterious affects of double strand breaks are well documented [5]. Telomeres consist of repetitive DNA specific for telomeres (see article in this Special Issue by R.J. Wellinger and D. Sen, pages 735–749) and the proteins which bind them. The protective function of telomeres is believed to be provided by the telomeric chromatin complex. The identity and mechanism of action of those telomeric proteins remains unclear, especially in higher eukaryotes, although significant progress has been made in the identification of ciliate and fungal telomere proteins (reviewed in [6, 7]). The elucidation of DNA polymerases and their properties led Olovnikov [8] and Watson [9] to realise that linear DNAs required special mechanisms to replicate their termini (see below). The identification of specific telomeric DNA sequences (see article by R.J. Wellinger and D. Sen, pages 735–749) and the subsequent identification of an enzyme that added these sequences to chromosome ends [10] elucidated the means commonly employed to solve the end replication problem. The critical enzyme, called telomerase, has an essential RNA component, implicating a primordial origin, and unusual polymerisation properties. This article focuses on the structure and properties of human telomerase and how they may impact human disease.

THE TELOMERE END REPLICATION PROBLEM

The replication of linear DNA cannot be completed by conventional primer- and template-dependent DNA polymerases. If the parent DNA molecule has blunt termini, then one side of the product daughter molecule will not extend to the extreme terminus on the lagging strand once the last RNA primer that was used to prime Okazaki fragment synthesis is removed (Figure 1) [8, 9]. Thus, for blunt ended DNAs, a 5′ terminal gap remains after DNA replication. After replication the lagging strand 5′ end on the daughter chromosome is shorter than on the parent molecule, and after another round of replication this end creates a blunt end chromosome shortened by the length of the unreplicated gap. Left unchecked, this process leads to chromosome shortening and the eventual loss of coding information. However, the chromosomes of mammals may not have blunt end termini since in some lower eukaryotic organisms, where the telomere structures are known, the 3′ strand overhangs the 5′ strand by approximately 12–16 nucleotides [11–13]. Lingner and associates [14] recently pointed out that replication of linear DNAs with terminal 3′ overhangs creates an end replication problem on the leading, rather than the lagging, end of the daughter molecule. If the last RNA primer (usually 8–12 nucleotides [15]) is

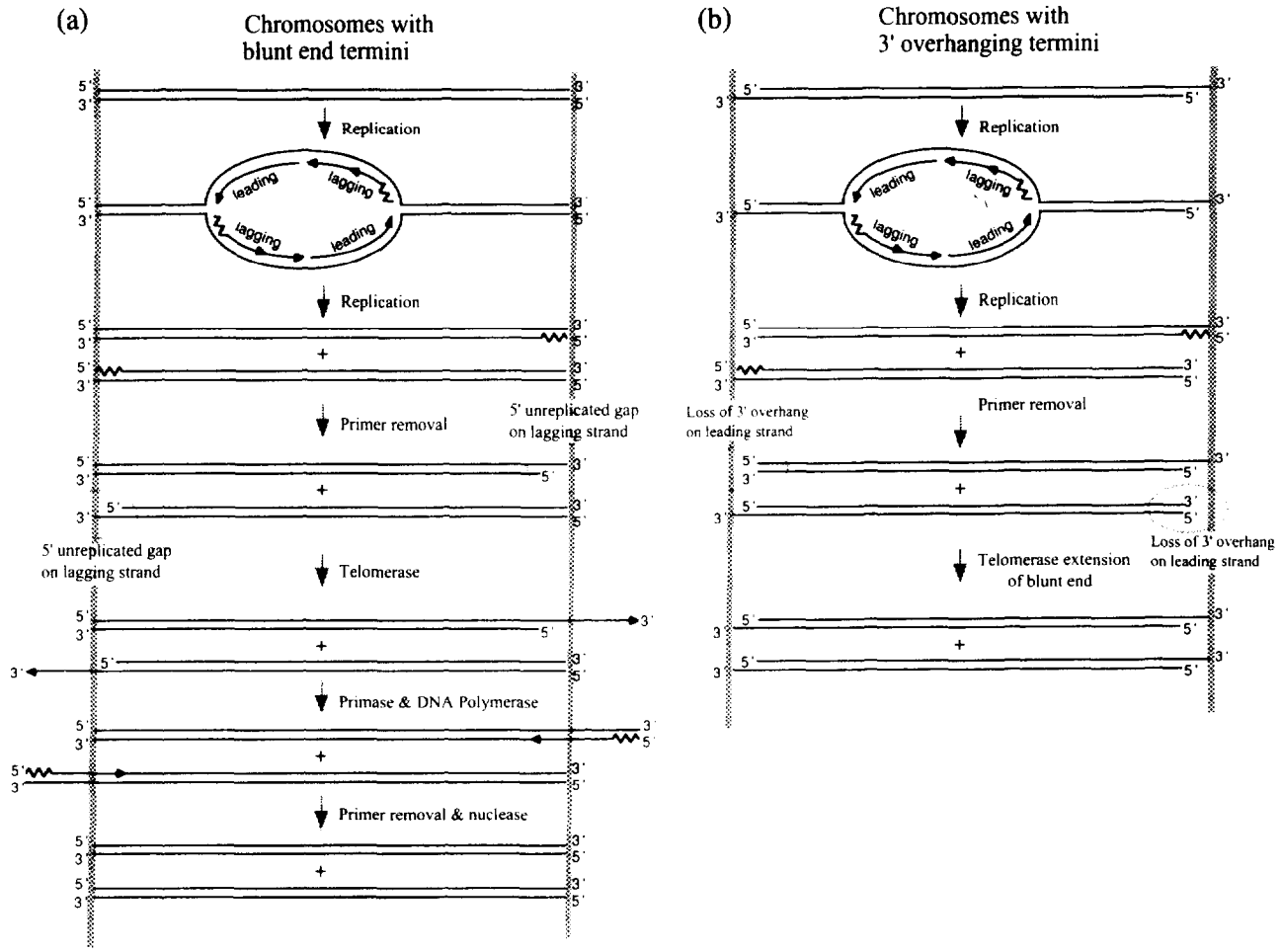

Figure 1. The telomere end replication problem. (a) The consequence of replication of a linear chromosome when it has blunt end termini. After replication a 5′ terminal gap remains on the lagging strand on one end of each daughter chromosome. A proposed scheme for correction of this gap is the action of telomerase to extend the overhanging 3′ terminus, followed by the activity of RNA primase to lay down a RNA primer, extension of the RNA primer by a DNA polymerase, and ligation of the new strand to the downstream strand. This generates a terminus with a 3′ overhang that is longer than the parent chromosome. To generate a blunt end and restore the length to the length of parent chromosome a hypothetical nuclease activity would be required. (b) The consequence of replication of a linear chromosome when it has termini with a 3′ overhang or extension. After replication a blunt end is generated on one side of each daughter chromosome. This terminus is missing the 3′ extension present on the parent chromosome. A potential means of generating the 3′ overhang would be recognition and extension of the 3′ end of blunt end molecule by telomerase. However, telomerase inefficiently recognises blunt end termini (see text).

confined to the 3′ single strand extension, then after replication and primer removal the 3′ extension of the lagging strand end is regenerated with no loss of double strand DNA (Figure 1b). However, on the other end, the leading strand end, a blunt end molecule is generated whose length coincides with the double strand portion of the parent molecule, but is missing the 3′ single strand extension. While no coding information is lost, another round of replication of the now blunt end would leave a 5′ unreplicated gap, eventually leading to DNA loss. Thus, for DNAs with 3′ terminal overhangs there is a loss of the 3′ overhang after DNA replication. Regardless of the terminal structure of a linear chromosome, a means of compensating or correcting the DNA loss must exist so that intact chromosomes can be transmitted to the next generation.

As the solution generally employed by cells to alleviate the end replication problem requires the activity of telomerase, what then are the consequences of a DNA/protein complex with blunt or overhanging DNA terminal structures for telomerase action and recognition? Telomerase recognition of blunt end DNAs is very poor in *Tetrahymena* [16] and *Euplotes* [17], and human telomerase recognises similar substrates with lower efficiency compared to single stranded primers (G.B. Morin, Geron Corporation, California, U.S.A.). Half the replication termini of blunt end chromosomes should be capable of extension by telomerase since they have 3′ extensions (Figure 1a), but this would create a telomere longer than the parent, and to regenerate a parent-like blunt end, nucleolytic trimming needs to be hypothesised (Figure 1a). No telomere specific nuclease activity has yet been observed with the properties needed to regenerate the terminal structure of the parent chromosome. Similarly, telomerase extension of chromosomes with 3′ extensions before replication would also lead to telomeres

longer than the original chromosome. When chromosomes that have 3′ extensions undergo replication first, before telomerase action, then half the termini have a blunt end (Figure 1b). To restore the blund end to the parental 3′ extension with no loss of DNA, then it must be hypothesised that telomerase extends the blunt end DNA (Figure 1b), which has not been efficiently demonstrated *in vitro*. *In vivo*, telomerase recognition of DNAs could be mediated by a protein which aids recognition of blunt DNAs, possibly by partially unwinding the DNA, exposing the 3′ strand for telomerase recognition, and interacting with telomerase to facilitate its binding. Alternatively, an activity could degrade the 5′ strand generating a 3′ extension which telomerase subsequently recognises.

A composite hypothesis that avoids postulating unknown activities is that telomerase preferentially recognises 3′ extensions, but that telomeres consists of a mixture of blunt and overhanging termini. In this case, only a subset of ends would be used by telomerase during any replication cycle, and blunt ends or 5′ overhangs would not be substrates for telomerase. However, after another round or two of replication, these termini are converted to structures telomerase could recognise (Figure 1) and telomere length could be restored as the ends become accessible to telomerase. A prediction of this mechanism is that telomeres would have heterogeneous lengths. The highly heterogeneous lengths of telomeric DNA observed in a wide variety of organisms (see article by R.J. Wellinger and D. Sen, pages 735–749) supports such a stochastic mechanism. Small losses or gains in telomere length should have no functional consequences.

It is clear from the above discussion that the terminal structure of the telomere is critical for determining the enzymes and mechanisms used for telomere addition and maintenance. In humans the terminal structure is unknown. In hypotrichous ciliates the evidence shows that 3′ extensions are universal [11, 12], but the experimental evidence showing 3′ extensions in the holotrichous ciliate *Tetrahymena* [13], and in yeast [18] and the slime mold *Didymium* [13], just demonstrate the presence of terminal single stranded G-rich telomeric DNA, but does not indicate if the 3′ extensions are universally present on all telomeres. In general, extrapolation of results from the ciliates to higher eukaryotes must be done cautiously as ciliates have a unique nuclear architecture employing two nuclei; the micronucleus, which is used only in the sexual cycle, and the macronucleus, from which all vegetative gene expression occurs [19]. The chromosomes of the macronucleus are fragments of the large micronuclear chromosomes amplified many times. The hypotrich macronucleus is even more unique since each of its millions of mini-chromosomes have identical telomeres with a very short and defined telomeric DNA region and 3′ extension [11, 12], while the telomeric DNA of other organisms, including the holotrichous ciliates, have highly heterogeneous lengths and are much longer [1, 2]. Thus, the hypotrichs may have developed some unique systems for the function and maintenance of their telomeres. The timing of telomere replication versus chromosomes replication, whether variant forms of telomerase exist, if telomerase displays cell-cycle dependency, the structure of the *in vivo* telomerase substrate, and the role of telomere proteins in these processes remain to be illuminated, particularly in higher eukaryotes, and will be important in understanding the normal and disease biology of telomeres in humans.

TELOMERASE ACTIVITY

The basic function of telomerase is to extend the 3′ end of telomeres by *de novo* synthesis of the G-rich telomeric repeat DNA. It can be considered an RNA dependent DNA polymerase, though a highly specialised one, as it synthesises only one sequence and does not use an exogenous RNA template. The conventional telomerase assay for telomerase is similar to a DNA polymerase assay, i.e. incubate nuclear extracts containing telomerase with a single-stranded DNA primer representing the telomeric repeat sequence, e.g. $(TTAGGG)_4$ for vertebrates, and deoxynucleotide triphosphates, one radiolabelled, then observe additional repeat addition to the primer on DNA sequencing-type gels by autoradiography [20, 21]. This telomerase assay, while highly informative about the properties of telomerase reaction, is not particularly sensitive. For instance, it is not useful for analysing the level of telomerase activity in typical tumour samples. A highly sensitive telomerase assay, called TRAP (Telomeric Repeat Amplification Protocol), has been developed by Kim and associates [22]. After a short incubation using relatively standard telomerase conditions, this procedure amplifies the telomerase products by PCR using a primer complementary to the synthesised DNA and the telomerase primer, which is a non-telomeric DNA sequence that is efficiently recognised by telomerase. The TRAP assay is highly sensitive and has been extensively used to assay telomerase activity in tumour samples.

TELOMERASE RNA

Like many other core functions of the nucleus that involve manipulation of nucleic acids, telomerase is a ribonucleoprotein particle (RNP). The necessity of the telomerase RNA moiety was established by the sensitivity of telomerase products to pre-treatment of telomerase preparations with ribonucleases [20, 21]. The heat lability and chromatographic properties of telomerase are also consistent with it being an RNP. Convincing proof came with the identification of an RNA that copurifies with the *Tetrahymena* telomerase activity [23]. Subsequent sequence modification of the putative template region caused the synthesis of different telomeric repeats [24]. Similar experiments have now been performed for the telomerase RNAs of *S. cerevisiae* [25], *K. lactis* [26] and humans [27]. Genetic manipulation of the yeast telomerase RNA gene further established the necessity of the RNA component.

The study of conserved sequences in RNAs from ribonucleoproteins provides models for RNA secondary structures, protein binding sites, and RNP function. The telomerase RNAs from over 20 ciliates [23, 28–34] as well as fungi [25, 26], humans and mice [27, 35] have been identified and sequenced. The telomerase RNAs vary widely in length from 150 to 200 nucleotides in the ciliates to approximately 450 in mammals and approximately 1300 nucleotides in *S. cerevisia* and *K. lactis*, yet they all contain a putative template specific for the repeat sequence of each species. Analysis shows the telomerase RNAs have a primary sequence and size which are poorly conserved. There is little sequence conservation between the holotrichous and hypotrichous ciliates and to the yeast and higher eukaryotic

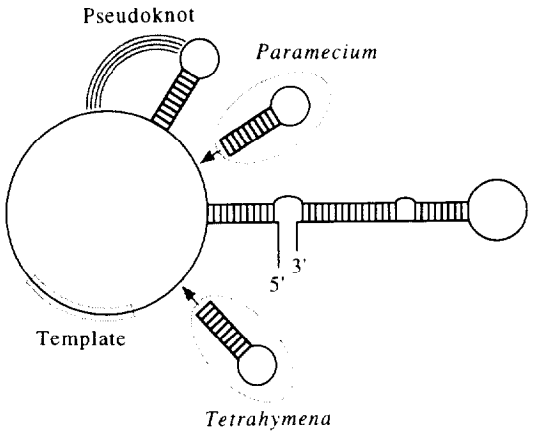

Figure 2. The conserved secondary structure of the telomerase RNA based on ciliate telomerase RNA sequences [29–31, 33, 34]. The template region sequence is complementary to the telomeric repeat sequence of the organism, it is used to direct addition of the appropriate deoxynucleotide. The pseudoknot is where nucleotides in the loop can also be base-pair to a region in the central loop. The circled regions are stem-loops found only in the *Tetrahymena* or *Paramecium* telomerase RNAs.

sequences. However, the large numbers of ciliate sequences has facilitated secondary predictions based on sequence alignment and compensatory base changes [29–31, 33, 34]. Accessibility to ribonuclease and base modification reagents largely supports the proposed secondary structures [31, 33]. Unfortunately, secondary structure analysis of the fungal and mammalian telomerase RNAs is difficult because of their larger size, lack of sequence conservation and small sample size. The observation of poor sequence conservation but conserved structure is a common paradigm for small nuclear RNAs (snRNAs) of many different functions. The common structure motifs of the telomerase RNA (Figure 2) are a long stem-loop which brings the 5′ and 3′ ends into close proximity, a smaller stem loop which has the potential to form a pseudo-knot and a conserved location near the 5′ end for the template sequence. Methylation experiments show the template region of the *Tetrahymena* telomerase RNP is accessible *in vivo* [33]. *Paramecium* and *Tetrahymena* species have an additional small stem-loop, though in different locations (Figure 2).

TELOMERASE PROTEIN COMPOSITION

There are several potential roles of proteins in the telomerase RNP: RNA–protein contacts could provide for RNP assembly and structure, proteins may form the binding sites for the DNA and dNTP substrates, and they could provide the catalytic groups for the polymerase activity. Collins and associates [36] have identified 95 and 80 kD proteins in the *Tetrahymena* telomerase, and Lingner and colleagues [17] have identified 123 and 43 kD proteins in the *Euplotes* telomerase RNP. Crosslinking experiments showed the p95 and p123 subunits of *Tetrahymena* and *Euplotes*, respectively, bound the DNA primer. Similar experiments showed that the *Tetrahymena* p80 subunit contacts the telomerase RNA. The *Tetrahymena* 95 kD protein has weak identity to RNA-dependent RNA polymerase motifs consistent with the catalytic function of telomerase. *EST1* is a yeast mutant defective in telomere maintenance [37, 38], but whether it is a component of yeast telomerase is unclear, as several recently developed biochemical assays for *S. cerevisiae* telomerase conflict on the necessity of the EST1 protein for telomerase activity [39–42].

The larger sizes of the fungal and mammalian telomerase RNAs, and by analogy to splicing RNPs [43], predict more than two proteins constitute the telomerase RNA in these organisms. In yeast, the U1 and U2 snRNAs are very largely compared to other organisms [44–46], but outside of an evolutionarily conserved core region much of the RNA is dispensable [47, 48]. In humans, some RNA regions may also be unneeded for the basic catalytic activity of telomerase, suggesting the critical catalytic functions of mammalian telomerase may reside on a minimal number of protein subunits. However, other telomerase proteins may provide regulatory, stabilising, compartmentalisation, or telomere binding functions critical to the biological function of the enzyme. Identification of human telomerase proteins could be accomplished by traditional chromatographic procedures, by affinity chromatography protocols exploiting the telomerase RNA, or by utilising evolutionary conserved regions in the ciliate telomerase proteins to identify human homologues. Regardless, the identification of human telomerase proteins will be critical to understanding fully the biochemical and biological roles of telomerase in humans.

GENERAL REACTION CHEMICAL

Telomerase has many common features to the reactions of DNA polymerases and reverse transcriptases. For instance, it recognises and extends the 3′ hydroxyl termini of a primer/template duplex. Telomerase will also extend primers with circularly permuted telomeric sequences by adding the next appropriate nucleotide [10, 20, 21]. This implies telomerase adds one nucleotide at a time and that the DNA template has both recognition and templating roles. Experiments which change the telomerase template sequence specify the synthesis of an appropriately altered telomerase product [24] demonstrating the templating role of telomerase RNA. A simplified model of the telomerase reaction involves annealing of the primer to the template, template-directed nucleotide addition and translocation (Figure 3). Translocation occurs in conventional polymerase complexes by advancing along the template molecule, but in telomerase translocation allows the repetitive utilisation of the same region. This is analogous to the template-slippage mechanism of error introduction in other polymerase reactions and illustrates that the conformational gymnastics of telomerase may not be totally unique. Other unusual properties of telomerase (below) suggest the general telomerase reaction mechanism (Figure 3), while useful as a model, is simplistic and masks the complexities of the telomerase reaction.

NON-DISCRIMINATORY SUBSTRATE RECOGNITION BY TELOMERASE

The annealing of a primer or DNA substrate to telomerase necessitates the primer be single-stranded. On some chromosome termini in ciliates and yeast, the G-rich strand protrudes over the 5′ end making it a convenient substrate for telomerase [11–13]. However, it is unknown if this terminal structure is evolutionary conserved and if it is found at all chromosome termini. In ciliates, which process their

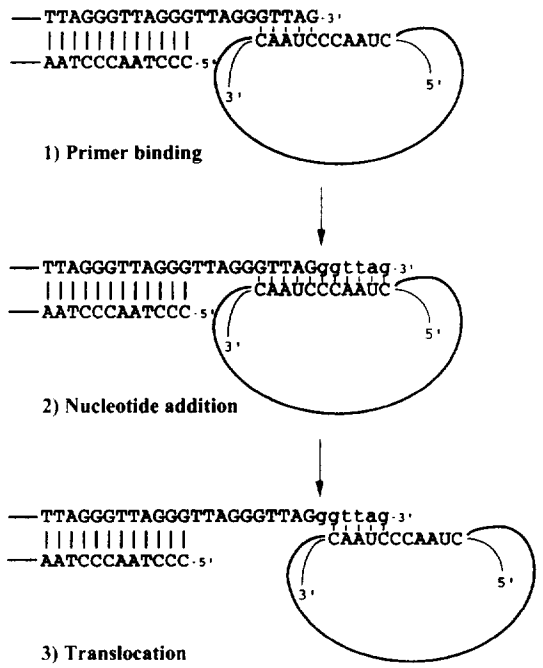

Figure 3. The general telomerase reaction mechanism.

chromosomes by cleaving them into smaller chromosome-like fragments [19], the addition of telomeres to the new termini cannot be explained by the recognition properties of telomerase as currently understood. Telomerase addition to these substrates may involve proteins which render the terminal DNA accessible to telomerase or mediate telomerase binding and recognition to otherwise unsuitable termini. Further knowledge of the telomeric nucleoprotein complex and telomerase recognition properties will be required to understand this problem.

A key observation that led to the telomerase hypothesis of telomerase replication and the eventual identication of the enzyme was linear plasmids possessing *Tetrahymena* $(TTGGGG)_n$ termini functioned in yeast in that they were modified by addition of yeast specific TG_{1-3} telomeric repeats [49, 50]. This suggested the presence of an enzyme capable of telomere synthesis. Telomerase was then identified in *Tetrahymena* using an assay designed to detect the putative activity [10]. Later biochemical characterisation of telomerase enzymes from many different species showed they can recognise telomeric repeat sequences of highly varied sequences, yet synthesise only the repeat specified by the RNA template [20, 21, 51, 52]. A hypothesis to explain this ability was that the G-rich telomeric DNAs possessed a common secondary structure recognised by telomerase [20, 53]. Single-stranded G-rich DNAs do form structures termed 'G-quartets' stabilised by inter- or intra-strand G-G base-pairing among four DNA strands [54, 55]. However, *in vitro* experiments showed buffer conditions that promoted G-quartet formation and attenuated utilisation of the substrate by telomerase [56]. Other experiments on primers too short to form secondary structures with sequences or modifications hindering secondary structure formation have also discredited this hypothesis [21, 57–59].

Primer recognition involves two processes: recognition of the 3′ terminal sequence so that the correct nucleotides are added, and interactions with the RNP which energetically stabilise primer binding. Primer/template base-pairing could provide both the specificity and stabilisation for substrate binding, and while other arguments (see below) suggest other DNA/telomerase interactions help stabilise primer binding, other primer data show template base-pairing plays a key recognition role. Oligonucleotides with the general structure 5′-$(N)_{4-20}$TAG-3′ or 5′-$(N)_{4-20}$AG-3′ are used by human telomerase while fully degenerate primers are not (G.B. Morin, Geron Corporation, California, U.S.A.), indicating that cognate termini are involed in recognition. Telomerase primers with substitutions of non-pairing nucleotides near the 3′ terminus reduce telomerase recognition, indicating that the loss of base-pairs destabilises primer interaction [57]. Furthermore, telomerase recognises primers which can base-pair with the template in multiple registers by favouring those pairings which maximise base-pairing [57]. Termini with as few as two base pairs in the terminal four are recognised by telomerase [57]. These indicate that 3′ terminal recognition is primarily mediated by template/primer base-pairing, but they also show that telomerase is quite non-discriminatory. As discussed below, this property can have severe consequences *in vivo*.

UNDERSTANDING THE TELOMERASE TEMPLATE

The telomerase RNA template region can be divided into two domains, a templating domain and a binding domain (Figure 4). One full telomeric repeat functions as a template specifying nucleotide addition and another partial repeat is used to rebind the 3′ end after translocation. The binding domain in *Tetrahymena* is three nucleotides long [20, 24] while in humans it can be as long as five nucleotides [27]. Clearly with some DNA primers, for instance, primers ending with ...GGG-3′, the templating domain is used to bind the primer initially. Conversely, can nucleotides in the bind-

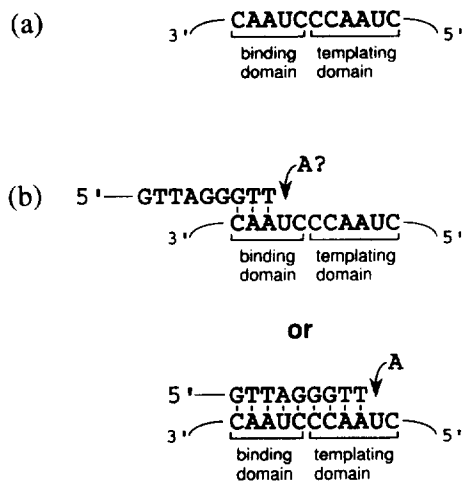

Figure 4. The two regions of the telomerase template region. (a) The binding domain binds the 3′ terminus of the DNA primer. The templating domain specifies the addition of the appropriate deoxynucleotides. (b) This primer can anneal to the RNA template region in two registers. Annealing to the binding domain may not be productive, since this region of the RNA might not direct the incorporation of deoxynucleotides, possibly because of separation from the catalytic residues.

ing domain also be used to template nucleotide addition? In a series of experiments where the *Tetrahymena* RNA template nucleotides have been systematically changed, the functions of the nucleotides in binding and templating domains have begun to be delineated [24, 60–63]. These experiments have shown that nucleotides at the 3′ end of the binding domain are used for binding the 3′ terminus only, while nucleotides at the 5′ end of the binding domain can be both used to pair with the terminus and to template nucleotide addition. In some cases binding of the primer 3′ terminus can be accomplished with as few as two base pairs. The binding energy of this interaction is minimal, insufficient for stabilising primer binding, illustrating that other telomerase/primer contacts are necessary to stabilise primer binding. A surprising finding of some mutations in the template domain are their distal affects. Mutations of template nucleotides specify the addition of their complementary Watson–Crick nucleotide, but some changes also cause premature dissociation, nucleotide misincorporation at distal sites, or poly-G polymerisation *in vitro* [62, 63]. This suggests these residues directly or indirectly influence catalytic moieties in the RNP and critically influence catalysis beyond their templating function.

Limiting the template to a small region of the telomerase RNA means barriers to copying the RNA beyond this region must exist. Products of human telomerase have primarily ...TTAG-3′ termini [21]. This occurs by the cessation of continuous polymerisation when the 5′ end of the template is reached. This could occur by physically blocking extension by a RNA/protein or RNA/RNA structure or by physical separation from the active site residues catalysing polymerisation. Furthermore, while human, *Tetrahymena* and *Euplotes* telomerase products terminate with the first G residue [20, 21, 30], the products of *Oxytricha* and *Stylonichia* telomerases terminate with the last T residue (...TTTT-3′) [30] suggesting that there is no intrinsic stability or function associated with a G residue terminus.

The potential length of the DNA/RNA duplex formed when polymerisation reaches the end of the template is 9–11 nucleotides long, long enough so that melting this duplex would be energy-dependent. Similar RNA/RNA duplexes formed in pre-mRNA splicing are unwound by spliceosome-associated helicase activities. These activities typically require ATP hydrolysis. The *in vitro* telomerase reaction has no ATP or GTP hydrolysis requirement [10, 20, 21], but hydrolysis of the dNTP cofactors could serve this purpose. A contrasting hypothesis is that the helix is unwound at one end as it is being formed at the other [29, 64]. Unwinding occurs in several discrete low energy steps in this model. The necessary energy for this process could come from the strain associated with the formation of a duplex within the active site [29, 64]. The formation of energetically stable G–G paired secondary structures in the released product could favour helix unwinding by reducing the energy differential between the released and template bound DNA products [28].

TELOMERASE HAS TWO PRIMER BINDING SITES

Human and *Tetrahymena* telomerase are processive, meaning they are very efficient at multiple repeat addition to a single primer. However, the simple view of the telomerase reaction (Figure 3) indicates contact between the DNA and the enzyme is lost during the translocation step, thus a means of rebinding the released DNA is necessary for a processive reaction. Recognition and alignment of the 3′ end of DNA primers by telomerase is mediated by base-pairing to the RNA template, but telomerase better utilises long primers (10–12 nucleotides) compared to shorter primers (4–6 nucleotides) with identical 3′ termini [57, 65], indicating contacts beside the primer/template pairing exist. Base composition 10–15 nucleotides upstream of the 3′ terminus also affects the human telomerase reaction. Primers with a G-poor region upstream are less processive than primers with a G-rich region upstream, even though they have identical lengths and 3′ termini [57]. These data and related experiments on the *Tetrahymena* enzyme have suggested telomerase has two distinct sites of primer interaction [21, 57, 58, 65]. The second site, termed the upstream site (Figure 5), or the anchor or lagging site [63, 66], binds the primer approximately 8–15 nucleotides upstream of the 3′ end and may provide a significant fraction of the overall binding energy. By coordinating DNA binding between the upstream site and the template site, the processivity of telomerase can be explained (Figure 5). Simply maintaining a DNA/telomerase interaction at the upstream site after the 3′ end is released from the template (Figure 5, step 3) increases the probability of rebinding the 3′ terminus in the binding region of the template (Figure 5, step 4B) and achieving a processive reaction. Product release occurs when dissociation at the upstream site occurs before the 3′ end is rebound (Figure 5, Step 4A). A similar two-site mechanism is believed responsible for the processive nature of the RNA polymerase reaction [67, 68].

In the two-site model the ratio of DNA release at the upstream site to DNA reannealing at the template site determines the processivity of the reaction (Figure 5, Step 4). This model cannot explain how the upstream base composition of primers affects processivity once a few repeats are added to the primer and that region has dissociated from the enzyme [57]. The released 3′ terminus has a ...TAG-3′ end in every polymerisation cycle, thus rebinding of the ...TAG-3′ end should be equivalent in every cycle. Thus product release and differential processivity is dependent on the interaction at the upstream site. If two-site translocation occurs (Figure 5, Step 5A), then after one or two rounds of polymerisation the upstream site is occupied by only TTAGGG sequences and product release and processivity should be equivalent regardless of the starting primer sequence. The differential processivity problem can be solved by simply not exchanging the DNA in the upstream site [57] and looping-out the newly synthesised DNA (Figure 5, Step 5B), thus translocation occurs only in one site; the template site. By remaining bound throughout the reaction the initial sequences occupying the upstream site can affect product release at each subsequent step. A primer with a G-rich upstream region would have a stronger interaction with the upstream site and a low probability of release, thus rebinding of the 3′ end and a processive reaction is favoured, while if the interaction at the upstream site is weak (G-poor sequences) then the probability of primer release before 3′ end rebinding is high and the reaction will be more distributive. Further characterisation of the base preferences and translocation mechanism at the upstream site will be necessary to test and confirm these models.

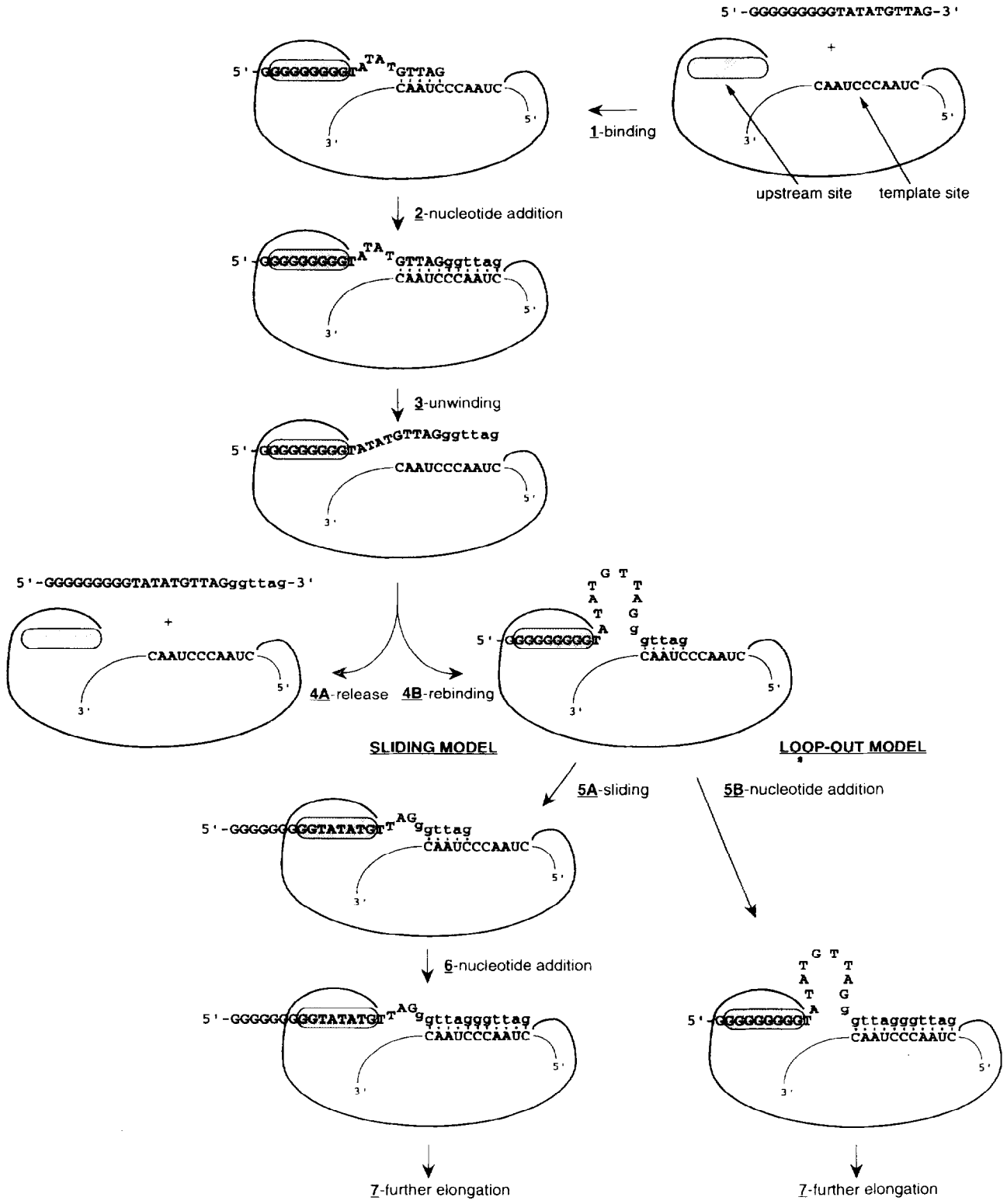

Figure 5. The two-site model for the telomerase reaction mechanism. Step 1: Binding of the DNA primer to the two sites on telomerase, note that this primer has a G-rich upstream region. Step 2: Addition of nucleotides to the 3′ terminus of the primer. Step 3: Unwinding of the DNA/template duplex, note there is no dissociation at the upstream site. Step 4: Decision between product release or translocation. Product is released (step 4A) when dissociation occurs at the upstream site before the 3′ terminus of the DNA re-anneals to the binding region of the RNA template. Translocation occurs (step 4B) when the DNA re-anneals before dissociation at the upstream site. Step 5: Two possible paths for continuation of the reaction. Step 5A: Sliding or two-site translocation model. The upstream site can rebind the DNA six nucleotides further downstream, followed by another round of nucleotide addition (Step 6). Step 5B: Loop-out or one-site translocation model. The DNA occupying the upstream site is not released, the new DNA remains looped out between the upstream and template sites, and nucleotide addition occurs at the 3′ end. Note that in the sliding model the G-rich region of the primer is removed from the upstream site, while in the loop-out model the G-rich region remains bound to the upstream site. The two models differ in what DNA occupies the upstream site, this could affect the processivity of the telomerase reaction (see text). Step 7: Return to Step 2 for continuation of the previous steps.

TELOMERASE REGULATION

Telomerase activity can be regulated at the transcriptional or assembly level and at the enzymatic level. Cellular regulation at the expression level has been well documented by the strong prevalence of telomerase activity in immortal cell lines and cancer cells compared to mortal cells and normal tissues. However, direct regulation of enzymatic activity must also exist as immortal cell lines have a constant mean telomeric repeat length and distribution, even over many cell doublings, yet have high levels of active telomerase. There must be a balance of telomere lengthening and shortening activities to maintain a constant mean length. Other experiments show that the telomeres of chromosomes formed by telomerase-associated chromosome fragmentation, or telomere seeding, are initially short but will achieve the length and distribution of the endogenous chromosomes after many cell doublings [69]. Experiments with altered telomerase RNA templates in *Tetrehymena* show that only 1 2 mutant telomeric repeats are added at one time [24], suggesting telomerase activity is distributive *in vivo*, while the *Tetrahymena* (and the human) enzymes are very processive *in vitro* [70, 71]. These data indicate a means of sensing telomere length exists and that direct regulation of telomerase activity exists.

There are two basic means of controlling telomerase activity. In one mechanism telomerase adds repeats indiscriminately. To maintain telomere length in this scenario there must be a means of sensing telomere length and actively removing repeats beyond those necessary to replace the repeats lost during division. A leading model for monitoring telomere length is the protection of a defined amount of telomeric DNA by a set level of a telomere binding protein; this would place a limit on the amount of telomeric DNA protected from degrading activities. Thus, excess repeats added by telomerase would be exposed to nuclease action and removed. If the telomere has become too short, free telomere binding protein would protect any repeats added by telomerase until an equilibrium becomes established. A second and opposing mechanism is that telomerase only adds repeats to a subset of chromosomes that are too short. This scenario requires tight regulation of telomerase activity. This could be accomplished by limiting the availability of the telomerase substrate, perhaps by a telomere binding protein that prevents telomerase binding, or by attenuation of telomerase activity directly. The arrival of the DNA replication complex at the telomere during S phase could restrict telomerase substrate availability to a small window of the cell cycle due to removal of a telomere end binding protein that otherwise blocks telomerase access. Conformational changes in telomerase structure or the binding of a factor that blocks substrate recognition by binding the upstream or catalytic sites are two possibilities for direct telomerase regulation. There is no strong evidence for either mechanism except that overly long telomeric DNAs exist in some developmental stages in ciliates [72] and on newly replicated chromosomes in yeast [18] which are subsequently trimmed to a smaller size. The true mechanism may employ both models, as telomere length control must exist and it would be detrimental to the cell to have telomerase indiscriminately active throughout the cell cycle.

CHROMOSOME HEALING BY TELOMERASE CAN CAUSE GENETIC DISEASE

Promiscuous recognition of double strand breaks by telomerase *in vivo* could lead to the formation of a new telomere. The outcome of such an event would be stabilisation of a terminal chromosome deletion and the loss of all gene function distal to the break. The absence of telomerase activity in somatic tissues [22] limits this pathway in most tissues, but the high activity of telomerase in the germ-line [22] makes this route to disease possible. Several cases of α-thalassaemia caused by terminal deletions [73–75] could have arisen by illegitimate recognition of double-strand breaks by telomerase in the germ-line [57, 75]. Examination of the sequences at the sites of telomere healing and extensive experiments on the utilisation of one of those sites by telomerase *in vitro* show that telomerase can recognise the healing sites in 5 of the 6 cases of α-thalassaemia [57, 75]. Telomerase-mediated chromosome healing is also believed responsible for the formation of new telomeres in a process where new telomeres are formed experimentally. In telomere-associated chromosome fragmentation, cells are transfected with linear DNA possessing $(TTAGGG)_n$ DNA at one terminus. Single-crossover recombination events apparently incorporate the DNA and seed the formation of a new telomere [76–78]. In experiments where small portions of the non-TTAGGG DNA were included distal to the TTAGGG block on the linear DNA used to seed the new telomeres, there was TTAGGG addition to the non-TTAGGG sites [69]. However, the sites of addition were compatible with the recognition properties of telomerase in that minimal duplexes with the RNA template were possible [57–69]. Another detailed study of telomere-associated chromosome fragmentation [79] showed a tight requirement for large blocks of TTAGGG sequences; very little healing was observed with even minimal deviation from the TTAGGG sequence. The sequence requirements mirror exactly the sequence requirements for the binding of a known human binding protein called TRF [80]. The study suggests binding of TRF to a new telomere seed may be necessary for the eventual stabilisation of the telomere [79]. However, the healing events of the α-thalassaemia cases do not have internal TTAGGG or TTAGGG-like sequences in the vicinity of the healing site. A plausible pathway for healing is the addition of sufficient repeats by telomerase for the binding of TRF then the TTAGGG/TRF complex stabilises the event and stimulates its conversion to a new telomere. The efficiency of germ-line chromosome healing of double-strand breaks by telomerase may then hinge on the number of repeats initially added by telomerase. Other examples of contiguous gene syndromes may also be caused by loss of multiple loci on a chromosome tip by telomerase-mediated chromosome healing [81, 82] suggesting this means of disease formation may not be limited to the tip of chromosome 16p and the α-globin loci. A better understanding of the *in vivo* substrates for telomerase and the conditions necessary for multiple repeat addition will be necessary to improve the understanding of this newly recognised route to human genetic diseases.

CONCLUSION: TELOMERASE AS A DRUG TARGET

The non-expression of telomerase activity in somatic issues, its nearly universal activation in all types of cancers,

and its potential role in the regulation of cellular ageing [83–88] makes it a very important target for drug intervention. The side-effects of specific telomerase inhibition may be low compared to other cancer drugs because normal cells do not require or possess telomerase activity. Several nucleotide analogues inhibit telomerase [89] but these are likely to affect other polymerase activities in the cell. While inhibition of the telomerase polymerase activity is a logical target, there are other aspects of telomerase and its biology that could provide a more unique intervention target.

The RNA component of telomerase could be targeted by antisense or ribozyme therapies, but these technologies have not been fully realised at this time. That telomerase is an RNP and a polymerase provides unique aspects for intervention. Besides polymerase inhibition, three categories can be broadly defined: (1) disruption of telomerase RNP assembly and transport. RNP assembly typically involves snRNA transcription in the nucleus, RNA modification and processing, export to the cytoplasm for RNA/protein complex assembly, and transport back to the nucleus. Targeting specific RNA/protein contacts required for these steps could lead to loss of telomerase assembly and activity. (2) Inhibition of DNA substrate binding. Telomerase has two sites where the DNA substrate binds and these sites manifest some sequence selectivity. Blocking substrate binding with molecules that compete for these sites will reduce telomerase activity. (3) Telomerase regulation. The activity of telomerase within the cell appears to be controlled. This is accomplished by controlling its access to its natural substrates, by controlling the length of repeats added by telomerase, or both. Identification of the proteins with which telomerase interacts and the systems which regulate telomerase could also open new avenues for clinical control of telomerase activity.

Telomerase has the biological and biochemical characterisation to become an important target for treatment of virtually all cancers and many ageing related disorders. Fulfilling this potential will benefit from further understanding of telomerase structure and function and its biology so that new means of affecting telomerase activity, assembly and regulation can be achieved.

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