Telomerase: the reverse transcriptase subunit as a key to enzymatic function and regulation during cell immortalization

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Joachim Lingner has recently joined ISREC as a junior group leader. He is a biochemist and molecular biologist who graduated in Biology II at the Biocenter of the University of Basel. His thesis work, carried out between 1989-92 in the laboratory of Dr. Walter Keller, emphasized on the maturation of messenger RNAs using yeast as a model system. J. Lingner received thereafter a postdoctoral fellowship from the European Molecular Biology Organization (EMBO) to work in the laboratory of Thomas R. Cech at the University of Colorado (1993-1997). There, he studied the structure, mechanism and composition of telomerase in Euplotes (a protozoan) and yeast. He identified the catalytic subunit of telomerase in these organisms which allowed the subsequent isolation of the human homologue. J. Lingner was awarded a START-fellowship to carry out research on the mechanism and regulation of telomerase.

Introduction

The ends of eukaryotic chromosomes are formed by non-nucleosomal DNA-protein complexes called telomeres [1]. Telomeric DNA usually consists of simple repetitive sequences that is GT-rich in the strand that points 5'-3' towards the chromosome end. The length of telomeric tracts varies considerably among species and is typically heterogeneous. Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA with a length of 5,000-15,000 bases.

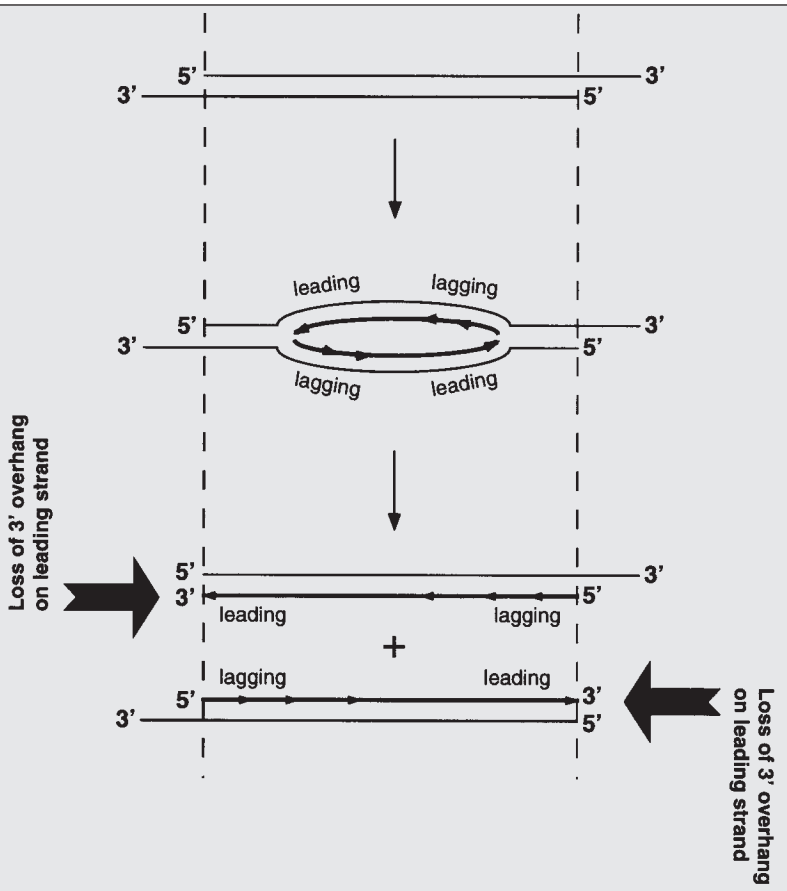

Fig. 1. The DNA end replication problem. Conventional DNA polymerases need a template for the synthesis of the daughter strand. Since telomeric DNA contains 3' terminal extensions the parental molecule (thine lines) cannot provide the template for the synthesis of this 3'overhang. Upon replication by conventional DNA polymerases the daughter molecules (thick line) will lose the 3'overhang on the end synthesized by leading strand DNA replication.

Telomeres serve as protective caps of chromosomes and are the substrates for telomerase, the enzyme which is required for the complete replication of chromosomal ends [2]. The very end of telomeric DNA is single-stranded and forms a 3'overhang. This overhang cannot be replicated by the conventional DNA replication machinery because the parental CA-rich strand is recessed and cannot function as a template [3] (Fig. 1). Telomerase can solve the end replication problem because it is a specialized reverse transcriptase that is independent of preexisting parental DNA templates. Instead, the reverse transcriptase subunit of telomerase uses a short region of its associated RNA moiety as a template for telomeric repeat synthesis (Fig. 2).

In human, telomerase is inactive in most somatic cells. Thus, telomere shortening is occurring with successive cell divisions which is thought to serve as a counting mechanism that prevents unlimited proliferation of human somatic tissues [4, 5]. Conversely, telomerase can be detected in the germ line, and in immortalized cells

and tumors, and such cell populations maintain telomere length. These observations have led to a model which proposes that the process of telomere shortening limits the replicative potential of most human cell lineages, and that cancer cells have to overcome this limit by activating telomerase expression in the course of tumor progression.

The isolation and identification of telomerase components has been crucial to the understanding of telomerase structure and mechanism. In addition, it has allowed to identify the protein target which is regulated during cell immortalization. This is setting the stage to dissect the role and mechanism of telomerase activation during cell immortalization and tumorigenesis. These recent advances are discussed here.

Telomerase reverse transcriptase

Telomerase is a ribonucleoprotein particle (RNP) that uses its stably associated RNA moiety as the template for synthesis of telomeric repeats (Fig. 2). Numerous telomerase RNA's have been isolated which vary considerably in size and sequence. The telomerase RNA secondary structure is conserved among ciliated protozoan [6, 7] but the structures of the yeast and mammalian RNA's are yet to be reported. Functions of this RNA other than providing the template remain ill defined.

How is reverse transcription of the RNA template catalyzed?
To address this question, enzymatically active telomerase was purified from the ciliated protozoan *Euplotes aediculatus* [8]. This organism was chosen as starting material for the purification, because it provides a much richer source for telomerase than for example human cells. This is due to the unusual high number of chromosomes and therefore telomeres in *Euplotes* macronuclei [9]. Two protein subunits, p123 and p43, were identified.

The gene sequence of the larger subunit, p123, revealed homology to the yeast *EST2* gene [10]. The latter was one of four genes identified genetically, in a screen for mutants that have an *e*ver *s*horter *t*elomeres (*est*) phenotype [11]. The *est* phenotype is the phenotype expected for a telomerase mutant. It is characterized by continuous telomere shortening and cessation of growth or senescence after 50-100 generations, which is caused by incomplete chromosome end replication. Significantly, both p123 and Est2p contained reverse transcriptase (RT) motifs in their C-terminal part (Fig. 2B). The N-terminal half of both polypeptides is very basic. Sequence similarity is low but detectable throughout the proteins.

Fig. 2A

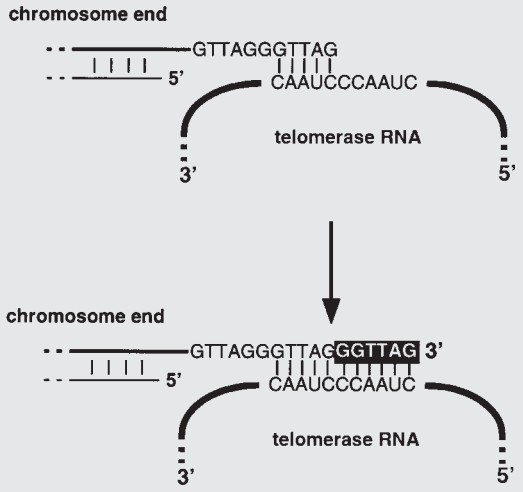

Fig. 2B

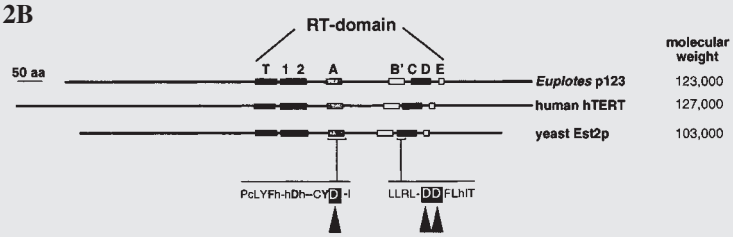

Fig. 2. Telomere DNA synthesis by telomerase. Telomerase consists of essential protein and RNA subunits. (A) A small region of the RNA serves as a template for telomeric DNA repeat synthesis. The chromosome 3'end is annealing to the RNA template. The telomerase reverse transcriptase subunit than uses the RNA as a template to synthesize a new telomeric repeat at the chromosome 3'terminus. (B) The telomerase reverse transcriptase subunits of *Euplotes* human and yeast contain sequence features of retroviral RT's. The aspartates (D) in motifs A and C are crucial for function.

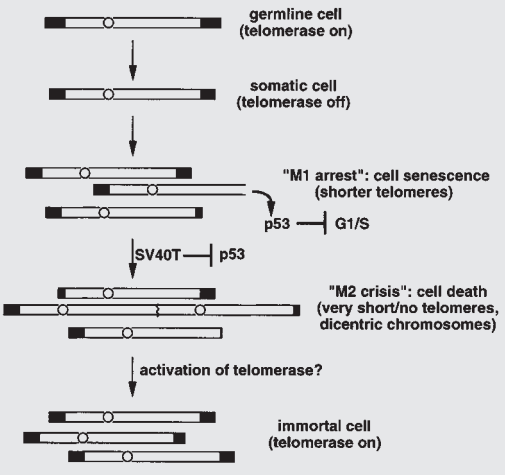

Fig. 3. Model for role of telomere loss and telomerase activation during immortalization of human cells (see text for details).

The template region of the telomerase RNA is reverse transcribed giving rise to telomeric DNA repeats (Fig. 2). Therefore, Greider and Blackburn which discovered telomerase more than 10 years ago [12] had formulated that telomerase is a reverse transcriptase in a functional sense [13]. The presence of an RT-domain in one of its subunits suggested that this is also true in a structural sense. As in most other polymerases, the active site of reverse transcriptases is present in a cleft whose structure is often compared to a half-open right hand with fingers, palm and thumb [14]. Seven short sequence motifs are usually conserved among reverse transcriptases [15]. Most notable are motifs A and C which contain one and two very critical aspartate residues, respectively (Fig. 2B). They are part of the palm subdomain and crucial for catalysis, coordinating the active-site Mg^{2+} ions.

These telomerase proteins being new members of the RT family was a testable hypothesis, since it was known from the biochemical and structural analysis of HIV1-RT which of the conserved residues should be critical for function. When any of the three putative active site aspartates was mutated to alanine in *EST2*, telomerase function was abolished completely (Fig. 2B) [10]. The observed *est*-phenotype was indistinguishable from that observed with a complete knock-out of the gene. Furthermore, *in vitro* telomerase activity was completely absent in extracts derived from strains carrying these point mutations. On the other hand, mutation of aspartates outside of the identified motifs had no or less severe effects. These results provided compelling evidence that p123/Est2p provides the reverse transcriptase-like active site of telomerase. These findings were confirmed by Counter et al. [16] who identified the *EST2* gene independently in an elegant genetic screen.

The *Euplotes* catalytic subunit gene led to the identification of the homologous genes from S*chizosaccharomyces pombe* and human [17], [18]. With examples now identified in protozoa, fungi and mammals, it appears that the telomerase reverse transcriptase subunit is universal among telomerases. The other telomerase RT's share the sequence features of p123/Est2p with sizes between 103-127 kDa, a basic N-terminal region and an RT-domain more C-terminal (Fig. 2B). Other conserved characteristics of the telomerase RT family include a telomerase-specific motif T immediately preceding the RT-domain and an unusually large distance between motifs A and B'. Both of these regions may be part of the «finger» domain which is presumed to interact with the template RNA. How this may contribute to the specialized reaction mechanism of telomerase remains to be established.

Evolutionary implications

The availability of several telomerase RT-sequences has stimulated proposals regarding the evolutionary relationship of the telomerase proteins to other RT's. Reverse transcriptases can be divided into two major branches, those encoded by retroviruses and long terminal repeat (LTR) retroposons and those encoded by non-LTR retroposons and group II introns [15]. Telomerase RT's are more similar to the non-LTR retroposon branch. However, they form a novel subfamily with very distinct features. Since with the exception of telomerase, reverse transcriptases are encoded by parasitic genetic elements, it becomes an intriguing question if retroposon-encoded RT's evolved from telomerase or *vice versa*. It would be amazing if retroelements, which constitute more than 30% of the human genome [19], evolved from telomerase reverse transcriptase. Alternatively, telomerase RT and retroelement-encoded RT's may be descendants of a common ancestor. The first reverse transcriptase may have evolved during the transition from an RNA world to current DNA-based information systems.

Telomerase and cell immortalization

In man telomerase activity is regulated during development [4, 5]. It can be detected in cells of the germ line where it is required to maintain telomere length during passage from one generation to the next. In most somatic cells, however, telomerase activity is low or undetectable [20, 21]. Because telomeres shrink with age in somatic cells, younger individuals have longer telomeres than old donors [22].

More than 30 years ago, Hayflick and Moorhead [23] first described the limited replicative capacity of normal human fibroblasts in culture as a manifestation of cellular senescence. These cells stop dividing at a senescence stage called M1 (Fig. 3). Remarkably, there is a good correlation between the number of divisions the fibroblasts execute before they senesce and their initial telomere length [22]. Senescent cells do not divide but are still metabolically active. One of the factors required for M1 arrest is p53, a protein that recognizes damaged DNA. It is unclear what cellular signal triggers M1 arrest. It could be that p53 recognizes short telomeres. The average telomere length at M1 is approximately 5 kb but individual telomeres may be significantly shorter. M1 arrest can be bypassed by a variety of agents. Oncogenes such as SV40 large T, E1A or Myc will allow fibroblasts to progress beyond M1. Such cells continue to divide for several generations before encountering a second crisis, called M2 (Fig. 3). In contrast to senescence at M1 most cells die at M2. While progressing towards M2, cells continue to lose telomeric DNA. The average telomere length drops to 2-4 kb and some chromosomes may have lost telomeric DNA altogether. Degradation of uncapped chromosome ends may eventually affect essential genes. Probably more importantly, unprotected chromosome ends form dicentric chromosomes through end-to-end fusion. The frequency of dicentric chromosomes rises sharply in cells approaching M2.
Nondisjunction of dicentrics results in chromosome losses many of which are lethal. Cells that escape from M2 are immortal, maintain telomere length and express active telomerase. They arise at a very low frequency, probably as a result of mutations in unknown cellular genes. Some of these mutations presumably affect the regulation of telomerase.

Telomerase in human tumors

Support for a critical role of telomerase expression in tumorigenesis came from a comparison of telomerase activity levels in normal somatic tissues and in numerous tumors [20, 21]. Telomerase activity is absent in most somatic cells. An exception to this are germ line cells which do contain active telomerase. Conversely to somatic cells, close to 100% of tumors were found to be telomerase positive [21]. This indicated that telomerase reactivation may be an essential step in carcinogenesis, even though alternative mechanisms to stabilize telomeres may exist. Evidence for such mechanisms came from the work of Bryan et al. [24] who have investigated telomeres from *in vitro* immortalized cell lines. They found that some lines were telomerase deficient but maintained telomeres presumably through a mechanism that involves recombination between different telomeres.

Telomerase reverse transcriptase mRNA-levels are regulated

The mechanism of telomerase activation during cell immortalization had been elusive until recently. Telomerase consists of essential protein subunits and an RNA moiety. Activity might be regulated by changes in the synthesis, the stability or the activity of any of the components, or by control of their intracellular localization. There is no good correlation between telomerase RNA levels and activity in different tissues, indicating that telomerase activity is not controlled through the level of this RNA [25, 26, 17]. Nor do the levels of mRNA of a proposed telomerase protein subunit called TP1 [27] or TLP1 [28] correlate with activity [17].

The human catalytic telomerase protein subunit gene is called hTERT (human telomerase reverse transcriptase). hTERT mRNA levels correlate very well with telomerase activity in different cells [17, 18]. No hTERT mRNA could be detected in six different telomerase negative somatic tissues, whereas it was readily detectable in six immortal, telomerase positive human cell lines (melanoma LOX IMVI, leukemia U251, lung carcinoma NCI H23, colon adenocarcinoma SW620, breast tumor MCF7, and kidney carcinoma 293). This indicated that telomerase activity is limited by the level of hTERT mRNA and that upregulation of hTERT mRNA levels may be crucial for telomerase activation and escape from the M2 crisis.

Overexpression of telomerase reverse transcriptase immortalizes normal human cells

Most compelling evidence for a causative relationship of telomere shortening and cellular senescence was provided most recently [29]. The identification of the human telomerase reverse transcripase subunit (hTERT) as a regulated component [17] allowed to test its role during cellular senescence. When hTERT was overexpressed in primary human cells (which are normally telomerase-negative), activity was reconstituted and telomere length was stabilized [30]. This indicates that of all the components that are required for telomerase activity, only the telomerase reverse transcriptase subunit hTERT was missing in the examined cells (retinal epithelial cells, foreskin fibroblasts, vascular endothelial cells). When the replicative potential of the hTERT transfected and therefore telomerase positive cells was examined during proliferation, the result was spectacular. Whereas the telomerase negative control cells senesced and stopped dividing after 50-60 generations, the telomerase positive cells continued to divide vigorously. They had overcome the limited replicative potential of normal somatic cells through stabilization of their telomere length. Their karyotype remained normal [29]. Therefore, telomere shortening is causing cellular senescence. This barrier of unlimited proliferation in normal human cells appears to provide a powerful tumor suppressor system.

Future prospects

The causative relationship of telomerase activation and cell immortalization make it a prime target for cancer therapy and thus an important subject of future research. The reverse transcriptase-like catalytic subunit will be studied structurally and mechanistically. A better understanding of the telomerase mechanism should help to design and identify drugs that inhibit this enzyme in human tumors.

The regulation of human telomerase reverse transcriptase occurs on the mRNA level. It will be important to differentiate a regulation via mRNA stability, transcription or processing. The transacting factors that mediate this regulation are not known. There identification will be crucial to understand the molecular events that lead to telomerase activation in tumors.

Finally, as mentioned earlier at least one other mechanism than telomerase exists to stabilize telomere length in immortal human cell lines. This mechanism which presumably involves recombination between telomeres, might take over once telomerase is out of commission. Therefore, if telomeres are the Achilles heel of tumors, a successful anti-cancer therapy might require inhibition of telomerase and alternative telomere maintenance pathways at the same time.

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